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# Improvement of fungal cellulolytic and xylanolytic enzymes production by new formulation of culture medium using wastes

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***Abstract***— *The use of the I-Optimal mixture design technique of agro-industrial residues in cultivation submerged at 28°C for 15 days with Trichoderma reesei QM 9414, complemented with nutrients, was used to optimize the mixture for the production of fibrolytic enzymes. The results demonstrated that the use of 100% (m/v) of brewer's spent grain was promising for the production of total cellulases (0.42 FPU/mL) and xylanase (39.60 U/mL), as well as the use of 33.3% citrus pulp and 66.7% brewer's spent grain for the production of xylanase (40.2 U/mL). The combination of 16.67% wheat bran, 16.67% citrus pulp, and 66.7% brewer's spent grain was the most promising for the production of endoglucanase (2.03 U/mL), exoglucanase (3.20 U/mL), and β-glycosidase (0.12 U/mL). The study on the demand for minerals, sucrose, and yeast extract (as a vitamin and amino acid source) revealed that 0.1% yeast extract, 0.11% dibasic potassium phosphate, 0.0028% zinc, and 1% of sucrose in 12 days of culture were sufficient to maximize the production of cellulases, increasing by 2.38 times (1.0 FPU/mL) compared to the initial culture (0.42 FPU/mL). Cellulolytic production remained the same with the use of 0.01% tween 80 in citrus pulp (0.40 FPU/mL) compared to that obtained in the design with a brewer's spent grain without tween 80, however it reduced substantially (from 15 to 9 days) the cultivation time. On the other hand, the use of tween 80 dramatically inhibited the fungal production of xylanases (2.96 U/mL). The best combination of salts was combined with tween 80 to obtain 1.12 FPU / mL in 9 days of fermentation. An enzymatic hydrolysis of cassava bagasse was carried out by combining cellulases and amylases, reaching 48 g / L of reducing sugar. Thus, this work shows that by studying the influence of residues, kind of salts and concentration of tween 80, a more efficient and economical bioprocess was possible to obtain, as well as the association between fibrolytic enzymes.*

***Keywords***— ***Agro-industrial residues, cellulases, submerged fermentation, Trichoderma reesei QM 9414, xylanase.***

## I. INTRODUCTION

According to the United Nations data (2019), it is estimated that the world population will reach 9.7 billion by 2050. Such a population growth induces an increase in the consumption of natural resources and, in turn, an increase in the production of agro-industrial waste. The reuse of these residues is an option to reduce pollution of the environment and to add value to such a waste.

Nowadays, the main agro-industrial residues are wheat bran, sugar cane straw, cassava bagasse, citrus pulp, and brewer's spent grain, just to mention a few examples. The brewer's spent grain is also known as spent grain or malt bagasse. Beer production generates 20 kg of brewer's

spent grain for every 100 liters produced. (Aboukila *et al.*, 2018). Considering the annual production of 14 billion liters of beer, a generation of 2.8 million tons of brewer's spent grain can be estimated annually (Cervbrasil 2016). Brewer's spent grains are considered as a fibrous material containing about 19-30% protein, 12-25% of cellulose, 20-25% of hemicellulose, and 12-28% of lignin. The nutritional composition varies according to the process used by the brewery and the origin of the products (Lynch *et al.*, 2016). Regarding the orange pomace, also called the citrus pulp, a fibrous residue composed of peel, seed, and pulp, which representing about 50% of the discarded fruit. The use of these residues is an alternative to add value to the fiber-rich material, making the bioprocess more viable in terms of production costs (Cypriano *et al.*, 2017). The lignocellulosic residues have a rich biochemical composition and a high potential for energy generation, in addition to not interfering with food production (Magalhães *et al.*, 2019). Such residues are an option for the production of second-generation ethanol. The use of orange pomace has shown to be promising in the production of xylanolytic and cellulolytic enzymes (Cypriano *et al.*, 2017). The residues, brewer's spent grain and citrus pulp, are also used as food for dairy cattle because they have high nutritional value, good digestibility, and degradability (Ikram *et al.*, 2017).

Brazil, together with the United States, represent the countries that invest the most in bioethanol production, both accounting for 85% of the world production (Azhar *et al.*, 2017; Spyridon *et al.*, 2016). Investments in second-generation ethanol are growing, as well as the studies to optimize the process of production. However, even though the production of second-generation ethanol is very promising, it is still limited by the high cost of producing cellulolytic enzymes. These productions consist of an enzymatic complex that includes exoglucanases, endoglucanases, and β-glycosidases. The synergistic action of cellulases degrades cellulose polymers present in plant material to release fermentable sugars that can be converted into ethanol (Devi and Kumar 2012).

Several companies are investing in this segment as Danisco / DuPont (Denmark), Basf (Germany), Genencor (USA), Roche (Switzerland), Novozymes (*Denmark*), among others (Li *et al.*, 2012). In particular, cellulases due to their broad spectrum of application encompass the second place regarding industrial interest, being directly related to the viability of second-generation ethanol production. The cost of the enzyme can reach 48% of the minimum sale price of cellulosic ethanol (Liu *et al.*, 2016). The reduction of the production costs is an alternative to support the future development of biorefineries and viable production of second-generation ethanol, which depends not only on the large quantities of lignocellulosic residues generated annually, but also on the production costs of cellulolytic enzymes (Rastegari *et al.*, 2019). In addition to the production of second-generation ethanol, there are other interesting applications that use enzymes such as processes in the textile industry, juice clarification, glucose syrup production, among others. Thus, this work aims to develop an economic bioprocess in order to produce fibrolytic enzymes (cellulase and xylanase enzymes) from the use of agro-industrial wastes.

## II. MATERIAL AND METHOD

In order to optimize a bioprocess for the production of cellulase and xylanase enzymes, studies were carried out in five stages: (i) preparation of the inoculum of the microorganism, (ii) preparation of an experimental design to study fermentations, (iii) fermentation stage, (iv) extraction stage and (v) enzymatic analyzes. The IBM SPSS software was used to perform statistical analyzes. Significance level of 0.05%.

### 2.1 Inoculum preparation

The inoculum of the microorganism culture was prepared using *Trichoderma reesei* QM 9414 strain in petri dishes containing 30 mL of PDA medium (39 g/L - Potato Dextrose Agar - Difco, Detroit, USA) incubated for five days (120 hours) at 28 °C in order to produce spores. The inoculum consisted of transferring a 10 mm diameter circle of PDA containing the fungus *T. reesei* QM 9414. The culture was maintained in mineral oil and PDA medium for preservation. Before each fermentation, two replications were carried out in the PDA medium to activate the fungus.

### 2.2 Experimental design applied to optimize the mixture of agro-industrial residues used in the culture medium of *T. reesei*

Agricultural residues, citrus pulp (Citrovita Agroindustry, Catanduva-SP), wheat bran (Moinho Nacional, Assis - SP) and brewer's spent grain (Cervejaria Malta, Assis - SP) were used in order to select the most suitable combination of substrates for the production of cellulases and xylanases. The residues were previously crushed in an industrial crusher (SPL-048, Spolu, Itajobi, Brazil) and then sieved so that the particle size selected for the experiment (between 1 mm and 2.8 mm) reducing the variations caused by lack of homogeneity of the substrates and facilitating the access of microorganisms to nutrients. Then, an I-Optimal mix design was designed with a sufficient number of experimental combinations to fit a model to determine the influence of the proportions of three substrates on the production of enzymes. The

modeling and statistical optimization were performed using Design-Expert software version 10 (Design-Expert®, StatEase Inc. Minneapolis, USA) with a 5% significance level, with response variables being the enzymatic activities of xylanases (U/mL), total cellulase (FPU/mL), endoglucanase (U/mL), exoglucanase (U/mL) and β-glycosidase (U/mL).

2.3 Cultivation tests with *T. reesei* QM 9414

The fermentation media were prepared in a 250 mL Erlenmeyer containing 100 mL of water and combinations of substrates according to the experimental design of item 2.2. A set of salts, sucrose and yeast extract, previously studied in the laboratory, were used to supplement the submerged fermentation medium (formulation: 0.11% K2HPO4, 0.1% (NH4)2SO4, 0.0017% MgSO4.7H2O, 0.0028% ZnSO4.7H2O, 0.1% MAP (NH4H2PO4), 0.06% KCl and supplemented with 1% sucrose and 0.1% yeast extract) (Campioni *et al.*, 2019). Then, the media were sterilized at 121 °C for 20 minutes with subsequent inoculation of the microorganism according to item 2.1. Cultivations took place in a shaker at 28 °C, 150 rpm for 360h (15 days). After the fermentation period, the enzymatic extract was obtained through filtration on filter paper to remove unfermented substrates and mycelium from the fungus. The extract (60 to 70 ml) was centrifuged at 5000 rpm (956xG) for 10 minutes (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany) and stored in a freezer at -18 °C during the analysis process.

2.4 Effect of new formulations of Tween 80, salts and surfactants on the production of xylanases and cellulases

Additional studies to the experimental design of mixtures were carried out with Tween 80 and with the nutrients (salts, sucrose and yeast extract), in order to observe the influence on enzymatic production. Tween 80 was used in concentrations of 0.01%, 0.05% and 0.1% for the cultivation carried out with 10% of brewer's spent grain, salts, sucrose and yeast extract according to the standard formulation. Tests with Tween 80 were also performed with 3% citrus pulp. Cultures were performed at 28 °C, 150 rpm in 15 days, with samples taken during this period. Analyzes of enzymatic activity were performed after cultivation.

The study of the influence of salts, sucrose and yeast extract were carried out in individual cultures, with each specific nutrient together with the brewer's spent grain to perform the cultivation at 28 °C, 50 rpm for 15 days. Samples were taken throughout the cultivation. The eight nutrients were tested at this stage. The best conditions of tween 80 and salts were associated with the carbon source in order to maximize the enzymes production in batch fermentations.

2.5 Enzymatic assays

The activity of total cellulase (EC 3.2.1.4), endoglucanase (EC 3.2.1.4.) and exoglucanase (E.C. 3.2.1.91) of the obtained extracts were determined by the absorbance measurement at 540 nm for the estimation of reducing sugars (especially glucose) released in the hydrolysis of cellulose, carboxymethylcellulose (CMC) and avicel (microcrystalline cellulose) respectively by the aforementioned enzymes, as described by Ghose (1987). The reducing sugar released was quantified by the Miller method (1959). 1 unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar, per minute, under the test conditions, using a standard glucose curve.

Xylanolytic activity (EC 3.2.1.8.) was carried out with 0.5% Birchwood xylan substrate (Sigma Aldrich) in acetate buffer pH 5.0 as described by Bailey *et al.*, (1992). The 3,5-dinitrosalicylic acid method (Miller 1959) was used to quantify reducing sugar released from xylan. 1 unit of enzymatic activity is defined as the amount of enzymes required to release 1 μmol of reducing sugar, per minute, under the test conditions, using a standard xylose curve.

β-glucosidase activity (*E.C.* 3.2.1.21) was performed using p-nitrophenyl β-D-glucopyranoside (PNPG) as a substrate, which is hydrolyzed by the enzyme in p-nitrophenyl and D-glucose as described by Grover et al, (1977). 1 unit of enzyme activity is defined as the amount of enzyme needed to form one μmol of p-nitrophenyl per minute under the conditions described. The activities developed in this work are represented in the diagram of Fig 1.

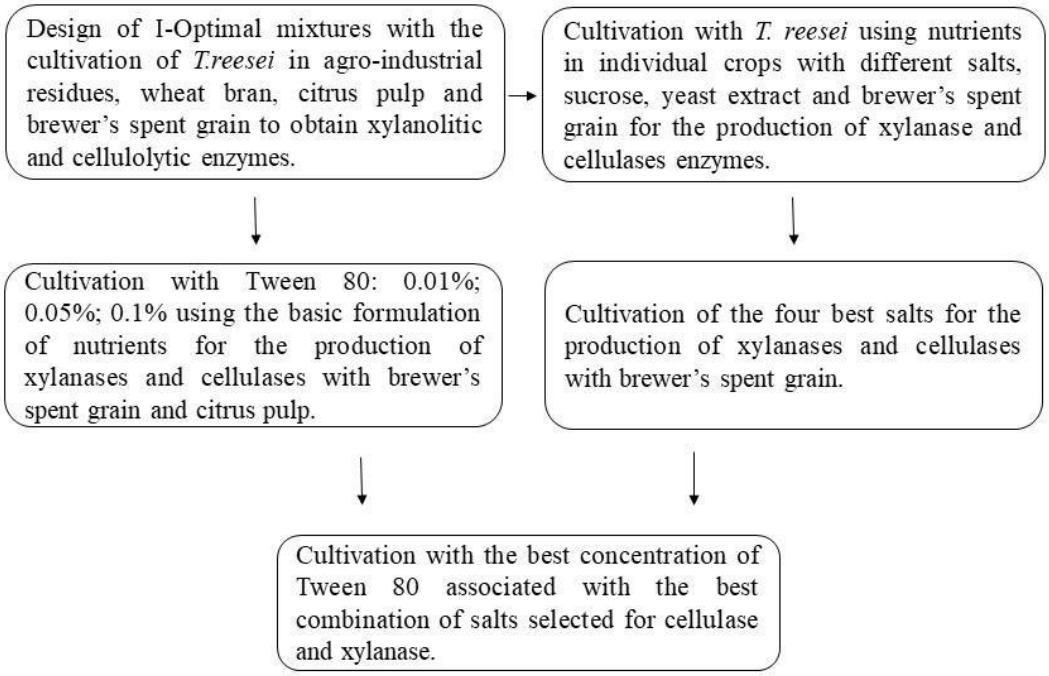


*Fig. 1. Summary of the experimental steps to optimize the enzyme activities studied.*

2.6. Enzymatic hydrolysis

Enzymatic hydrolysis was performed with 10% (w/v) cassava bagasse in pH 4.5 citrate buffer at 50 °C for 24 hours. The samples were tested with and without previous gelatinization (20 min at 80 °C). The enzymes used were amylase (15 U/mL) and cellulase (15 FPU/mL) added at time zero and time ten hours. At the end of hydrolysis, the enzymes were denatured (5 min at 100 °C) followed by quantification of the reducing sugar (DNS) released. Amylolytic enzymes were produced with *Rhizopus oligosporus* (CCT 3762) according to Escaramboni and Oliva-Neto (2016).

## III. RESULTS AND DISCUSSION

3.1. Experimental design of agro-industrial waste mix for optimization of enzyme production

Table 1 presents the experimental matrix of the composition of the fermentation media as well as the results of the output variables for each enzyme studied.

*Table 1. Experimental matrix and observed responses of agro-industrial waste mix to optimize the production of xylanases and cellulases.*

| Samples | Variable Factors | | | Xylanase | Endo-glucanase | Exo-glucanase | β-glycosidase | Total Cellulase |
|---|---|---|---|---|---|---|---|---|
| | A % | B % | C % | U/mL | U/mL | U/mL | U/mL | FPU/ml |
| **1** | 0.00 | 66.67 | 33.33 | 14.228 | 0.050 | 2.381 | 0.049 | 0.230 |
| **2** | 0.00 | 100.0 | 0.00 | 1.796 | 0.025 | 0.900 | 0.013 | 0.096 |
| **3** | 66.67 | 33.33 | 0.00 | 12.712 | 0.030 | 1.777 | 0.036 | 0.235 |
| **4** | 66.67 | 0.00 | 33.33 | 28.690 | 0.377 | 2.218 | 0.078 | 0.360 |
| **5** | 0.00 | 0.00 | 100.00 | **39.607** | 1.127 | 2.634 | 0.077 | **0.427** |
| **6** | 16.67 | 16.67 | 66.70 | 30.707 | **2.030** | **3.202** | **0.126** | 0.395 |
| **7** | 33.33 | 66.67 | 0.00 | 7.419 | 0.010 | 1.294 | 0.016 | 0.000 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **8** | 66.67 | 16.67 | 16.70 | 20.328 | 0.438 | 2.452 | 0.070 | 0.234 |
| **9** | 100.0 | 0.00 | 0.00 | 19.693 | 0.701 | 2.888 | 0.059 | 0.271 |
| **10** | 33.33 | 0.00 | 66.70 | 36.318 | 1.092 | 2.381 | 0.100 | 0.400 |
| **11** | 16.67 | 66.67 | 16.70 | 10.426 | 0.060 | 1.275 | 0.034 | 0.133 |
| **12** | 100.0 | 0.00 | 0.00 | 21.673 | 1.432 | 2.599 | 0.063 | 0.315 |
| **13** | 0.00 | 33.33 | 66.70 | **40.206** | 1.412 | 2.563 | 0.106 | 0.387 |
| **14** | 0.00 | 100.00 | 0.00 | 2.664 | 0.025 | 1.346 | 0.014 | 0.070 |
| **15** | 66.67 | 33.33 | 0.00 | 7.431 | 0.035 | 1.701 | 0.023 | 0.205 |
| **16** | 0.00 | 0.00 | 100.00 | 33.213 | 0.955 | 2.523 | 0.068 | 0.341 |
| **17** | 33.33 | 33.33 | 33.33 | 23.275 | 0.742 | 2.340 | 0.076 | 0.334 |

A – Wheat bran; B – Citrus pulp; C – Brewer's spent grain

As noted in Table 1, responses to enzyme activities varied at different numerical intervals. Generally, when the ratio between the maximum value and the minimum value is greater than 10, a transformation of the response data is necessary for better adjustments of the model. Transformations in these data were performed according to the need and the mathematical modeling for each enzyme was performed prioritizing the models that maximized the values of "Adjusted $R^2$" and the "Predicted $R^2$".

Table 2 presents a summary of the transformations used, the adjusted model that best suited each answer, as well as the $R^2$ statistical data and precision analysis of the modified models, which considers only the significant terms.

*Table 2. Statistical data of the models obtained for each enzyme activity studied (U/mL).*

| Enzyme (U/mL) | Transformation | Adjusted model | $R^2$ | Precision | F - Value | p-value |
|---|---|---|---|---|---|---|
| Xylanase | square root | quadratic | 0.9689 | 31.04 | 118.78 | <0.0001* |
| Endoglucanase | square root | cubic | 0.9758 | 16.30 | 31.30 | <0.0001* |
| Exoglucanase | square root | special quartic | 0.9580 | 17.13 | 29.36 | <0.0001* |
| β-glycosidase | cubic root | cubic | 0.9527 | 20.29 | 44.36 | <0.0001* |
| Total cellulase | cubic root | cubic | 0.9622 | 14.10 | 16.93 | <0.0002* |

*Significant in 5% level

The final equations in terms of the real factors only for the significant terms of each modified model are also presented (1, 2, 3, 4 e 5):

$$\sqrt{Xylanase\ \left(\frac{U}{mL}\right)} = 4.51A + 1.40B + 6.39C + 5.03BC \quad (1)$$

$$3\sqrt{Endoglucanase\ \left(\frac{U}{mL}\right)} = 1.01A + 0.16B + 1.02C - 2.03AB - 0.79AC + 0.49BC + 11.52ABC - 2.59AC(A - C) - 4.74BC(B - C) \quad (2)$$

$$Exoglucanase\ \left(\frac{U}{mL}\right) = 2.74A + 1.15B + 2.56C - 1.85AB - 1.38AC + 2.77BC - 52.57AB^2C + 74.99ABC^2 \quad (3)$$

$$\beta - glycosidase\ \left(\frac{U}{mL}\right) = 0.23A + 0.10B + 0.26C + 0.25AC + 0.42BC - 0.44\ BC(B - C) \quad (4)$$

$$Total\ cellulase\ \left(\frac{U}{mL}\right) = 0.23A + 0.07B + 0.29C + 0.04AB + 0.57AC + 0.48BC + 0.74AB(A - B) - 0.79AC(A - C) \quad (5)$$

The precision analysis presented in Table 2 measures the signal-to-noise ratio, it compares the range of values predicted at the design points with the average forecast error. Ratios greater than 4 are desired and indicates adequate accuracy of the model. Therefore, the equations found can be used to navigate the design space of all optimization experiments. P-values less than 0.0500 indicates that the terms of the model are significant. In this case, the equation in terms of real factors can be used to make predictions about the response for certain levels of each factor. As noted, component C (brewer's spent grain) and the combination BC (citrus pulp/brewer's spent grain) have the greatest impact on the production of the xylanase enzyme.

Trace Chart and 3D Surface graphics were used to compare the effects of all components in the design space (Fig 2 and Fig 3). Through them, it is possible to visualize how sensitive the response to the deviation of the formulation is close to the reference mixture for each model. The x-axis of the plot is in coded units and shows the position relative to the coded scale (0 to 1 for mixtures) and 3D surface located on the right side for the production of xylanase, endoglucanase, exoglucanase, β-glycosidase, and total cellulase.

From Fig 2 and 3, it is possible to observe that the brewer's spent grain (letter C) is good for the highest enzymatic production, presenting a small inhibition of the production of endoglucanase, exoglucanase, β-glycosidase, and total cellulase when in large quantities (trace and 3D graphics). The citrus pulp (letter B) is a good residue for the production of all enzymes when used in small quantities. However, enzyme production is inhibited by increasing the concentration of citrus pulp. Wheat bran (letter A) was the residue that least influenced the enzyme production, demonstrating stability in production regardless of the residue concentration for the enzymes: xylanase, β-glucosidase exoglucanase, and total cellulase demonstrating greater oscillation only in the production of endoglucanase.

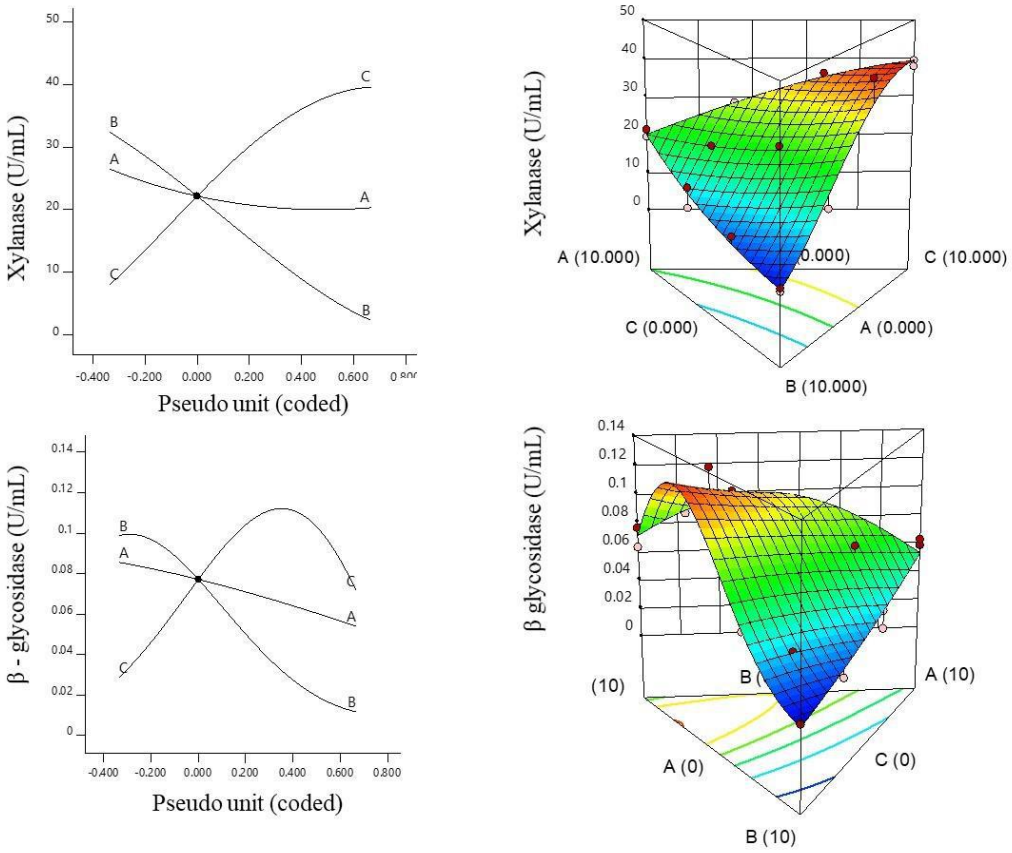


*Fig. 2 Optimization of the production of xylanases and β-glycosidase using T. reesei QM 9414 by mixing agro-industrial residues wheat bran (A), citrus pulp (B) and brewer's spent grain (C) in the culture medium in submerged cultivation at 28 °C and 150 rpm for 15 days. The 3D surface graphs are representing the values obtained min max.*

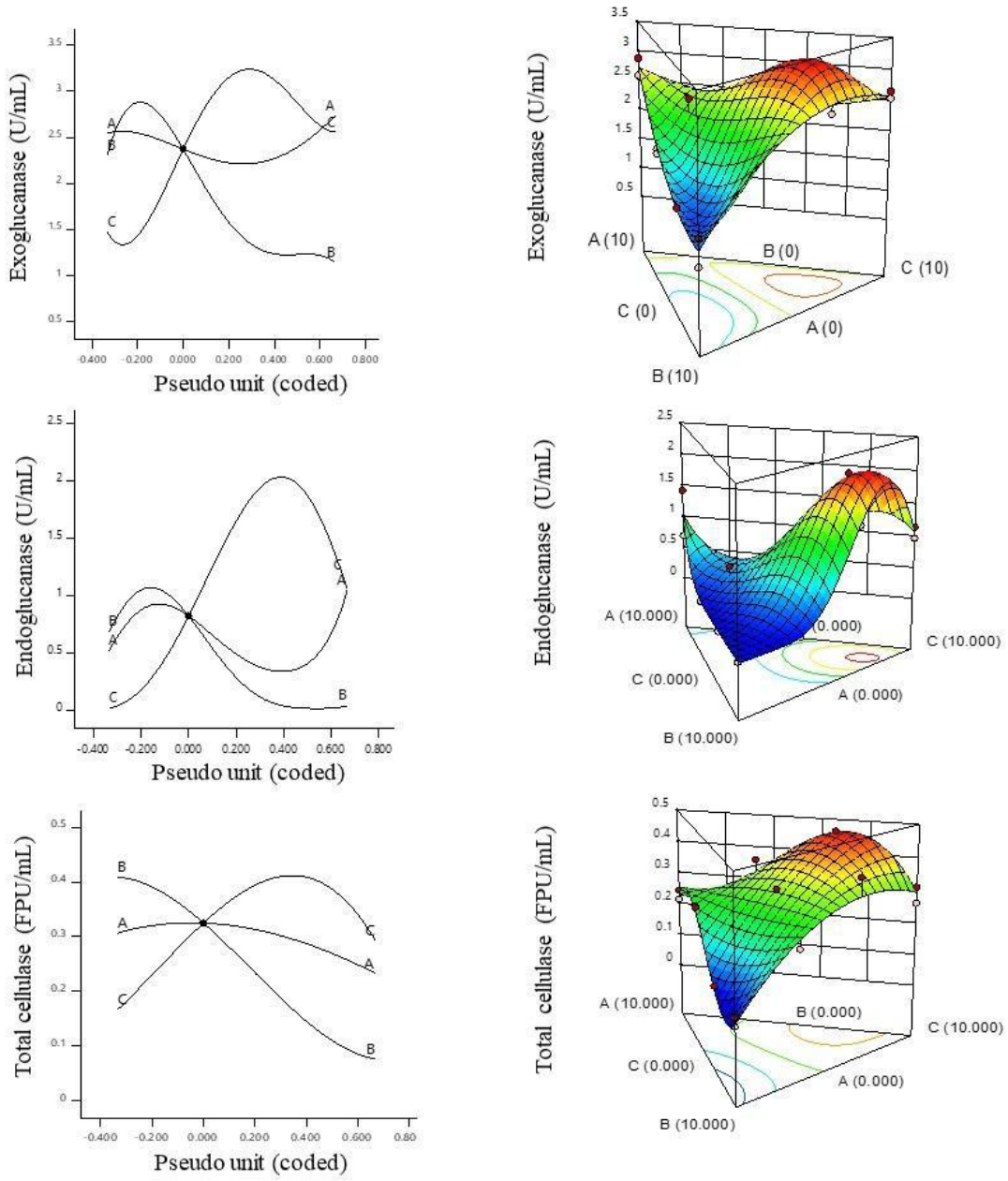


*Fig. 3. Optimization of the production of exoglucanase, endoglucanase and total cellulase of T. reesei QM 9414 by mixing agro-industrial residues wheat bran (A), citrus pulp (B) and brewer's spent grain (C) in the culture medium in cultivation submerged at 28 ° C and 150 rpm for 15 days. The 3D surface graphs are representing the values obtained min max.*

3.2. Validation of the models obtained through the experimental design of the mixture for the different agro-industrial residues

The validation of the studied models was performed using 6.0% bran, 13.30% citrus pulp, and 79.70% brewer's spent grain as suggested by the program. The verification showed that the model presents an adequate adjustment regarding the production of xylanase and exoglucanase that are within the confidence interval, the other enzymes showed a significant error as foreseen in the model. Table 3 presents the results of the validation of the models obtained by the program.

*Table 3. Validation of the models obtained through the experimental design of mixtures for enzymatic activities.*

| Replies (U/mL) | Predicted averages | Predicted median | Observed | Standard deviation | 95% low | 95% high |
|---|---|---|---|---|---|---|
| Xylanase | 37.712 | 37.602 | **38.012** | 4.066 | **28.648** | **47.772** |
| Endoglucanase | 2.015 | 2.002 | 0.120 | 0.315 | 1.226 | 2.968 |
| Exoglucanase | 2.971 | 2.971 | **2.548** | 0.176 | **2.518** | **3.425** |
| β- glycosidase | 0.111 | 0.111 | 0.000 | 0.009 | 0.079 | 0.147 |
| Total cellulase | 0.037 | 0.037 | 0.260 | 0.003 | 0.276 | 0.476 |

It was observed through the graphs of traces the great potential of the citrus pulp when used in small quantities and of the brewer's spent grain when in greater proportion. From these results, we sought to use different strategies, such as the use of surfactants, and to unravel the influence of mineral salts, sucrose and yeast extract for residues with greater production potential, in order to increase enzyme production and reduce cultivation time.

3.3. Tween 80 for enzymatic optimization

The search for increased enzyme production is mostly aimed at favoring the best conditions for the development of the microorganism such as the supply of substrates, moisture, pH, supplementation of the medium with salts, yeast extract, among other conditions. Tween is a non-ionic synthetic surfactant that is proving relevant in helping to increase enzyme secretion by microorganisms. Table 4 presents some works in which the authors used the surfactant tween 80 to increase enzyme production.

*Table 4. Production of enzymes in the literature using tween 80.*

| References | Tween 80 concentration studied | Microorganism | Enzymes studied | Best |
|---|---|---|---|---|
| Reese e Maguire, 1961 | 0.05 a 0.2% | *Trichoderma viride QM6a, T. viride QM2940* | Cellulase, β-glycosidase | 0.1% |
| Long e Knapp, 1991 | 0.06 g/L | *Coprinus cinereus* | Cellulases e xylanase | 0.06 g/L |
| Domingues *et al.*, 2000 | 0.5 g / L | *Trichoderma reesei Rut C-30* | Cellulases | 0.5 g /L |
| Zeng *et al.*, 2006 | 0.05% e 0.15% | *Penicillium simplicissimum* | CMCase e xylanase | 0.15% e 0.05% |
| Liu *et al.*, 2006 | 0.05% e 0.15% | *Trichoderma viride* | Cellulase e xylanase | 0.15% |
| Tangnu *et al.*,1981 | 0.01. 0.02 e 0.1% | *Trichoderma reesei Rut C-30* | Cellulase, β-glycosidase | 0.02% |

The metabolic mechanism involved by using Tween 80 is not yet fully understood. Studies have shown greater effectiveness of tween 80 compared to other types of tween 60, 40, and 20 (Reese and Manguire 1969; Liu *et al.*, 2006). Surfactants were studied in different concentrations, showing inhibition in large quantities. The concentrations selected for the study were 0.1%, 0.05% and 0.01%. Fig 4 shows the influence of using tween for cultivations with citrus pulp for 15 days. The culture with the surfactant in 0.01% demonstrated an acceleration in the enzymatic production taking 9 days to reach values of 0.4 FPU/mL ± 0.06 (2.4 FPU/g) for cellulase. Xylanase production reached its maximum of 2.9 U/mL (17.4 U/g) in 12 days with 0.05% tween 80. The xylanolytic production was low compared to the previous optimization performed without tween 80 (40.2 U/mL), indicating that tween 80 was not favorable for xylanolytic enzymes.

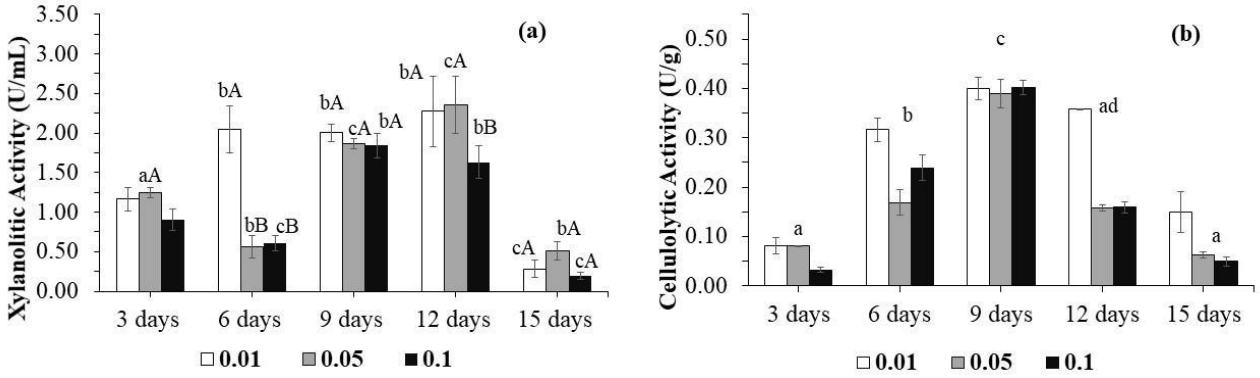


*Fig. 4. Cultivation with different concentrations of tween 80 for total cellulase (a) and xylanase (b) activity using T. reesei and citrus pulp in submerged culture at 28 ° C and 150 rpm for up to 15 days. Xylanase: Different letters indicate significant differences between groups according to the Sidak test for different comparisons. Lower case letters indicate comparisons between days of the same concentration. Capital letters indicate comparisons between the different concentrations of Tweeen for each day. Cellulase: No significant difference was observed between the independent variables, only for the variable "days". The Tukey test was used with a significance level of 0.05%.*

Long and Knapp (1991) using 0.006% tween did not observe an effect on xylanases when compared to the control, a strategy that was not effective for optimizing xylanases in the present work. Tangnu *et al.*, (1981) using 0.02% tween 80 obtained with *T. viride* QM 9414 5.1 FPU/mL, whereas with *T. reesei* Rut C-30 reached from 7.3 to 14.4 FPU/mL, demonstrating the capacity of *T. reesei*. The authors Liu *et al.*, (2006) used 0.05% and 0.15% of tween 80 and rhamnolipids. The percentages of enzymatic degradation among them were similar, considering only tween 80, the best concentration was 0.15%, with a degradation rate of 14.4% for cellulose and 7.3% for hemicellulose, higher than the control without surfactant, justifying the increased production of cellulases. Finally, Tween 80 cultivation using brewer's spent grain inhibited the production of cellulases and xylanases, with all results very close to zero.

### 3.4. Influence of mineral salts, sucrose and yeast extract on the formulation of *T. reesei* culture medium for the production of cellulases and xylanases enzymes

The production of xylanase using cultures with each mineral salt, yeast extract, and sucrose in addition to the brewer's spent grain residue is shown in Fig 5. Cultivations with these nutrients demonstrated the importance of each one for enzymatic production. There was no statistical difference between days 9, 12 and 15 of fermentation and six of the nine salts showed no statistical difference. The following additives were chosen as the best nutrients for xylanase production: ZnSO4 0.0028% (21.8 U/mL), MgSO4 0.0017% (20.4 U/mL), (NH4)2SO4 0.1% (20.1 U/mL) and yeast extract 0.1% (21.4 U/mL). The results were three times superior to the control without salt when compared with zinc and magnesium.

Fig 6 shows the influence of nutrients supplementation for the production of total cellulase.

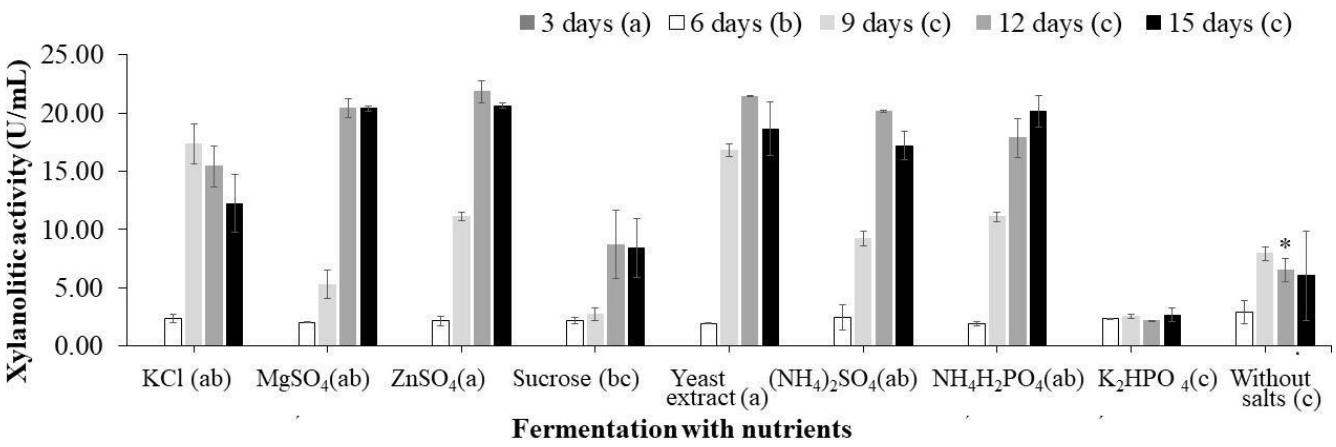


*Fig. 5. Submerged cultivation of T. reesei with salts, yeast extract and sucrose at different times to produce xylanases at 28 ° C and 150 rpm for up to 15 days. The Kruskal-Wallis test was used with a significance level of 0.05%. Different letters indicate significant differences between groups.*

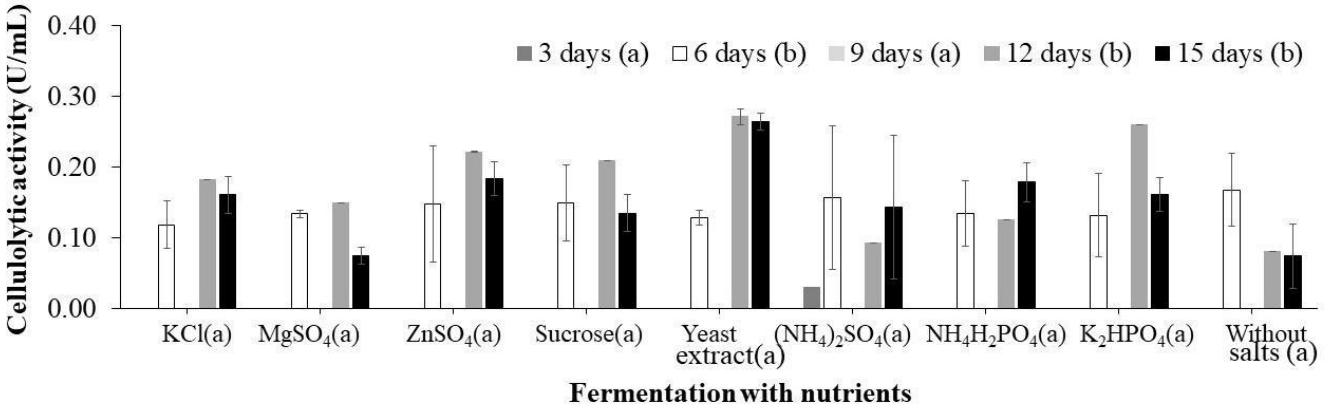


*Fig. 6. Submerged cultivation of T. reesei with salts, yeast extract and sucrose at different times to produce cellulases at 28 °C and 150 rpm for up to 15 days. The Kruskal-Wallis test was used with a significance level of 0.05%. Different letters indicate significant differences between groups.*

There was no statistical difference between the different salts or between days 6, 12 and 15 of fermentation. The nutrients chosen as the best salts for total cellulase were yeast extract 0.1% (0.27 FPU/mL), K2HPO4 0.11% (0.25 FPU/mL), ZnSO4 0.0028% (0.22 FPU/mL) and sucrose 1% (0.20 FPU/mL). The influence of nutrients was no statistical difference for exoglucanases. Days 12 and 15 were the best fermentation days. Endoglucanases showed no statistical difference between the salts and the best days 12 and 15 of fermentation. The salts ammonium sulfate (0.85 U/mL), potassium phosphate (1.0 U/mL), magnesium sulfate (0.87 U/mL), monobasic ammonium phosphate (0.85 U/mL) and yeast extract (0.78 U/ml) were selected as the best salts. The production of exoglucanase and endoglucanase are represented in Fig 7 and Fig 8. Narasimha *et al.,* (2006) observed 0.52 U/mL for endoglucanase using yeast extract, a result lower than that obtained in the current study. The authors found greater results with the use of 0.82 U/mL urea as a source of nitrogen for endoglucanase.

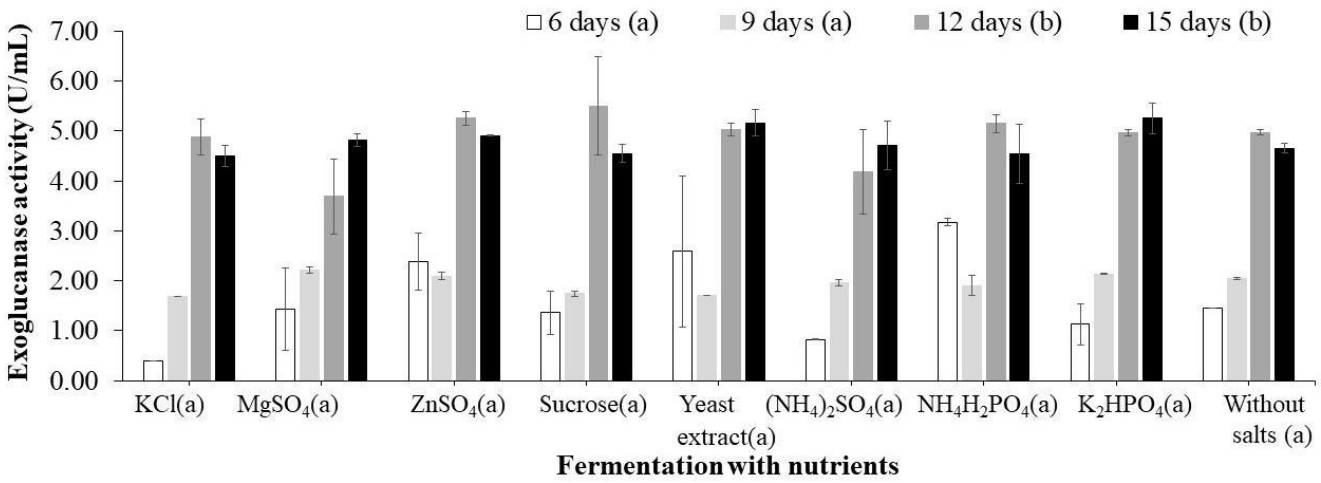


*Fig. 7. Submerged cultivation of T. reesei with salts, yeast extract and sucrose at different times to produce exoglucanase at 28 °C and 150 rpm for up to 15 days. The Kruskal-Wallis test was used with a significance level of 0.05%. Different letters indicate significant differences between groups.*

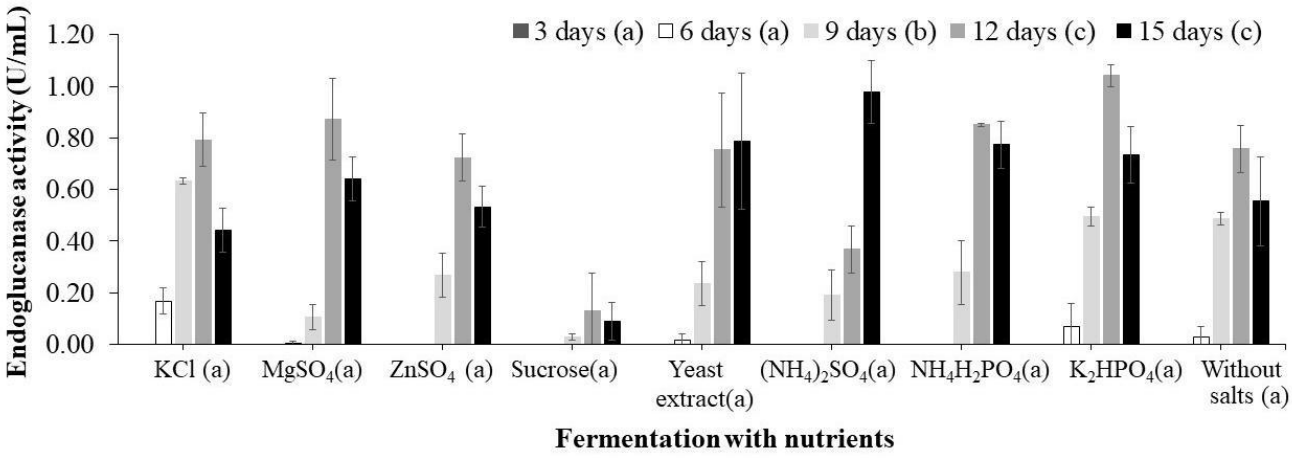


*Fig. 8. Submerged cultivation of T. reesei with salts, yeast extract and sucrose at different times for the production of endoglucanase at 28 °C and 150 rpm for up to 15 days. The Kruskal-Wallis test was used with a significance level of 0.05%. Different letters indicate significant differences between groups.*

Literature reports indicate increased enzyme production when the media are supplemented with simple carbohydrates, such as sucrose (Delabona *et al.*, 2012). Sucrose has the potential to optimize the production of cellulases, proving to be an inducer of cellulase gene expression (Silva *et al.*, 2018). Chandra *et al.*, (2007) observed a rapid increase in biomass due to the addition of sucrose, the sugar that is easily absorbed together with greater enzyme secretion due to the presence of cellulose. Silva *et al.*, (2018) using citrus pulp and sucrose reached 0.85 ± 0.07 FPU/mL in 10 days of submerged culture, increasing production by 48.1% due to the presence of sucrose compared to the culture without this sugar. The authors also found higher values when using a tank-type bioreactor with 1.76 ± 0.00 FPU/mL using *T. reesei*. Delabona *et al.*, (2012) with *Trichoderma harzianum* obtained 1.21 FPU/mL using pretreated bagasse, dignified with NaOH, and sucrose in a stirred tank bioreactor. However, research also reports that a high concentration of

sugars can inhibit fungal metabolism and consequently enzyme production (Rodrigues- Zúñiga *et al.*, 2011).

Yeast extract appears as a source of nitrogen and vitamins for cultivation. Narasimha *et al.*, (2006) demonstrated the differences in the addition of urea (1.68 FPU/mL), peptone (1.21 FPU/mL), sodium nitrate (1.12 FPU/mL) and yeast extract (0.52 FPU/mL) in the production of cellulolytic enzymes by *A. niger*, the yeast extract being one of the least efficient. Ammonium sulfate was also promising in terms of the production of xylanases in the current research.

The use of essential metal ions such as magnesium, zinc, iron, manganese, among others, is related to the activation of fungal metabolism, proving to be essential for the optimization of enzymatic production (Vale *et al.*, 2011). Microorganisms require different concentrations of minerals showing toxicity when they are in quantities greater than tolerated, inhibiting their growth (Fomina and Karl 2003). Zinc, according to Babich and Stotzky, (1978) is linked to the integrity of ribosomes, biological membranes, and fungal growth. The production of cellulases proved to be efficient regarding the use of the mineral zinc, but not magnesium, in contrast to the xylanase that used the two minerals for enzymatic production.

Rodríguez-Zúñiga et al. (2011) compared four different fermentation media and found that the best enzyme activity inducer was the modified Mandels & Weber medium, with addition of carboxymethylcellulose obtaining 0.4 U/g for total cellulase and 13 U/g for endoglucanase. Mandels & Weber medium contains urea, peptone, yeast extract, ammonium sulfate, and other minerals. Cultivation using sucrose was not efficient, as well as the Czapeck Dox medium composed of carboxymethylcellulose, sodium nitrate, sucrose, and other minerals. Among the nitrogen sources studied, the current study found yeast extract as the most promising for the optimization of total cellulase, in addition to sucrose, in the concentrations used to which they were shown to be favorable.

### 3.5. Kinetic study of the production of xylanases and cellulases with the combination of the four best mineral salts and combination with Tween 80

The study with nutrients allowed the selection of four different substances based on the statistics or on the value of the enzyme activity observed if the salts did not show statistical difference. From this, the four best nutrients for the production of xylanases (BX) were selected, which were ZnSO4 (0.0028%), MgSO4 (0.0017%), (NH4)2SO4 (0.1%) and yeast extract (0.1%); and the best ones for the production of cellulases (BC), which were yeast extract (0.1%), K2HPO4 (0.11%), ZnSO4 (0.0028%) and sucrose (1%). These were the nutrients that most influenced the biosynthesis of cellulases and xylanases in the previous stage of the research. The new cultivation with the best nutrients provided promising results in terms of increased enzyme production in a shorter cultivation time.

Fig 9 shows the production of xylanase (a) and total cellulase (b) for each set of chosen supplements. The best results for cellulase were in 12 days of fermentation. There was no statistical difference between BX and BC. The cellulases reached 1 FPU/mL (6 FPU/g) with the best nutrients for cellulase (BC), as for the best nutrients for xylanase (BX) the production of cellulases was also optimized reaching values close to the BC, but with greater standard error (0.91 U/ml ± 0.15). The xylanolytic enzymes reached maximum values of 2.9 U/mL (17.4 U/g) in just 3 days with BX. Cultures with BX were not efficient compared to the standard culture, with all components (40.2 U/mL). The individual study of nutrients provides a basis for the needs of the microorganism, but it does not allow observing interactions between nutrients for the metabolism of the fungus, so it can be said that the results were promising. Other combinations can be studied in the future and even their proportions to optimize production.

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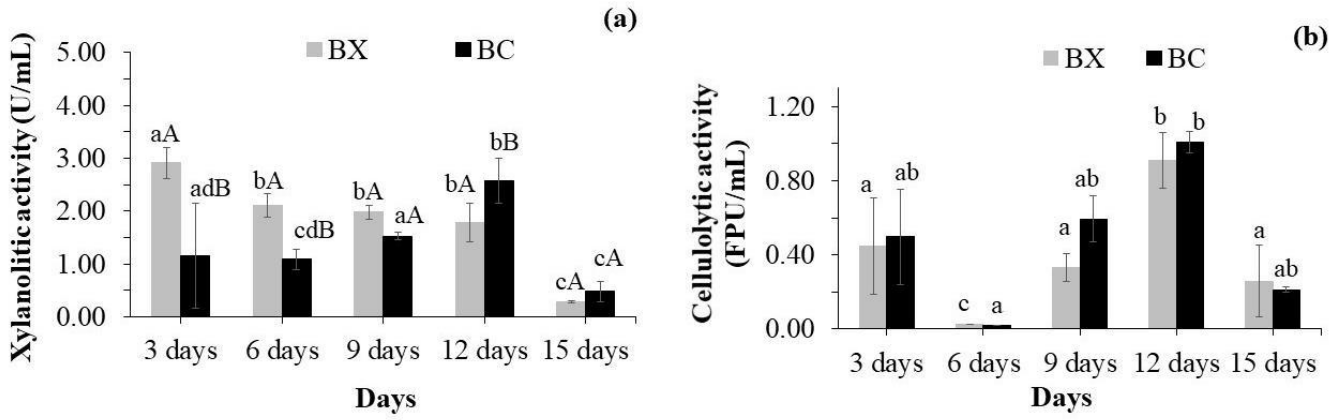


*Fig. 9. Enzymatic activity obtained with the combination of brewer's spent grain and the best salts compositions for xylanase (a) and cellulase (b) using T. reesei in submerged culture at 28 °C and 150 rpm for up to 15 days. The Tukey test was used with a significance level of 0.05%. Different letters indicate significant differences between groups. For Xylanase significant differences were observed between samples (BX and BC) and days. Lower case letters indicate differences between the days of the same sample and capital letters indicate differences between samples on the same day. For cellulase BX and BC were compared individually with variable "Days" because no significant differences were observed between samples (BX and BC). BX: best nutrients for xylanase: $ZnSO_4$ (0.0028%), $MgSO_4$ (0.0017%), $(NH_4)_2SO_4$ (0.1%) and yeast extract (0.1%); BC: Best nutrients for cellulase: yeast extract (0.1%), $K_2HPO_4$ (0.11%), $ZnSO_4$ (0.0028%) and sucrose (1%).*

The best concentration of tween 80 and the best selected salts were combined in order to observe the effect on cellulase and xylanase enzymatic activity (Fig 10). The combination, Tween 0.01% associated with MX, allowed to reach values of 1.12 FPU/mL in 9 days of fermentation, no statistical difference was observed between 9 and 12 days (1.32 FPU/mL). The use of Tween 80 proved to be more promising when combining with the best salt for xylanase (MX) compared to the best salt for cellulase (MC). The production of xylanase that was previously inhibited by the use of Tween, reached 26.35 U/mL in 12 days, there was no statistical difference between MX and MC on day 12. The xylanolytic activity found in this step did not exceed the results obtained through the experimental design (40.2 U/mL).

A summary of the best results found in each activity developed is shown in Table 5.

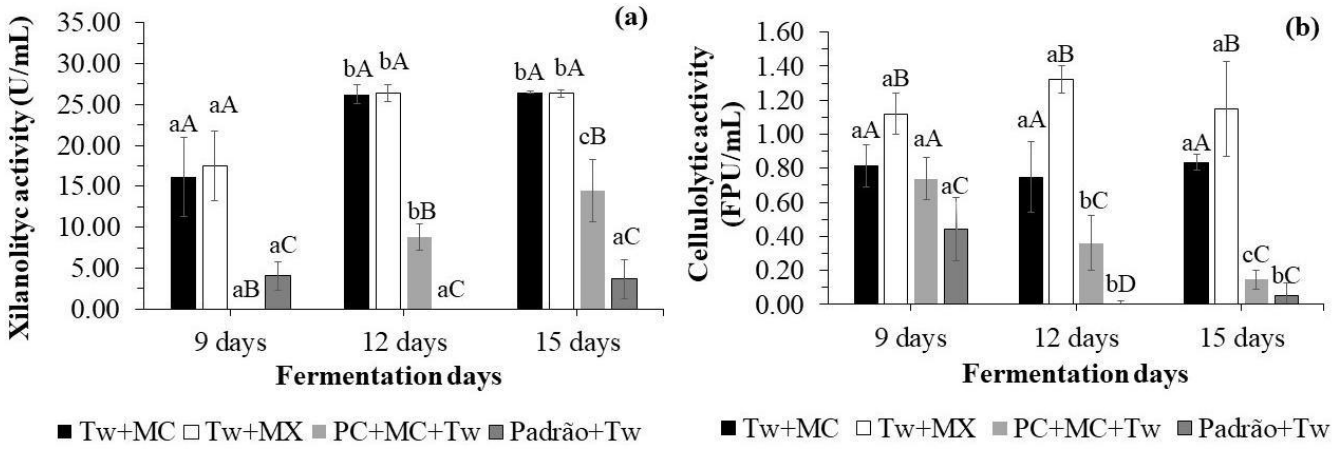


*Fig.10. Enzymatic activity obtained with the combination of the best salts compositions for xylanase and cellulase using T. reesei with the best concentration of tween 80 in submerged culture at 28 °C and 150 rpm for up to 15 days. Tw+MC: brewer's spent grain+0,01% of Tween+ best salts for cellulase. Tw+ MX: brewer's spent grain+0,01% of Tween+ best salts for xylanase. PC+MC+Tw: citrus pulp+ best salts for cellulase+0,01% of Tween 80. Padrão+Tw: brewer's spent grain+ initial set of eight salts+0,01% of Tween 80. The Tukey test was used with a significance level of 0.05%. Different letters indicate significant differences between groups. Lower case letters indicate comparisons between days of the same sample. Capital letters indicate comparisons between the different samples for each day.*

*Table 5. Enzyme activity was obtained at each stage of the study.*

| Stage | FE days | TC (FPU/mL) | Endo (U/mL) | Exo (U/mL) | β -glyco (U/mL) | Xylan (U/mL) |
|---|---|---|---|---|---|---|
| 1) Design with mixed wastes | 15 | 0.42 | 2.03 | 3.202 | 0.126 | 40.20 |
| 2) The best combination of salts, sucrose and yeast extract | 12 | 1 | - | - | - | - |
| 3) Presence of Tween 0.01% | 9 | 0.40 | - | - | - | - |
| 4) Tween 0,01% and MX | 9 | 1.12 | - | - | - | - |
| 5) Tween 0,01% and MX | 12 | 1.32 | | | | |

FE Days: Fermentation / Cultivation Days. TC: Total Cellulase. Endo: Endoglucanase. Exo: Exoglucanase. β-glyco: β glycosidase. Xylan: Xylanase. 1-Design: for TC: 100% of brewer's spent grain; for Endo, Exo and β-glyco: 16.67% bran and citrus pulp and 66.7% brewer's spent grain; for Xilan: 33.33% citrus pulp and 66.7% brewer's spent grain. The composition described for the design was complemented with a set of salts, yeast extract and sucrose. 2- Best combination of salts for cellulase: 0.1% yeast extract, 0.11% dibasic potassium phosphate, 0.0028% zinc and 1% sucrose + 100% brewer's spent grain. 3- Presence of 0.01% Tween: 33% of brewer's spent grain + 0.01% of tween, salts, yeast extract, and sucrose. 4- Tween 0,01% and MX: 100% of of brewer's spent grain.

The importance of understanding the needs of the microorganism used, as well as the potential of the chosen carbon source, is emphasized, in order to follow more optimistic paths regarding the optimization of enzymatic production. Considering the matrix provided by the design, the greatest cellulolytic production was found using a proportion of 10 g brewer's spent grain, 0 g of citrus pulp and 0 g of wheat bran obtaining a value of 0.42 FPU/ml (2.52 FPU/g, considering 60 mL of extract). This production was optimized during the study with tween 80, which reached 0.4 FPU/mL of cellulolytic activity in 9 days of fermentation, 6 days less compared to the culture performed for the design. The third step aimed at the best combination of nutrients for the production of xylanases

and cellulases surpassed the results obtained previously, reaching 1 FPU/mL of cellulolytic activity in 12 days. The best results found for the concentration of Tween 80 and combination of salts were tested together and allowed to reach 1.12 FPU/mL in 9 days of fermentation.

The research by Campioni *et al.,* (2020) demonstrated by means of a D-optimal experimental design with three agro-industrial residues cane straw, citrus pulp and wheat bran that cane straw is the most influential residue among the three. The design reached the production of 90 U ml of xylanase and 0.5 U/ml of cellulase using 100% cane straw with *T. reesei*. The study by Rodríguez-Zúñiga *et al.,* (2011) using *Aspergillus niger* obtained a maximum of 0.4U/g of cellulase with wheat bran in the solid state after 72 hours, a study that used a little starchy carbon source cultivation time and solid state cultivation reaching a value below that found in the current work. Campioni *et al.,* (2019) obtained 0.3 FPU/mL of total cellulase in 6 days of submerged cultivation with sugar cane straw using *T. reesei* QM 9414, which is also lower compared to the present study, but worked with a shorter cultivation time. On the other hand, Saini *et al.,* (2015) with an isolated strain in the country recognized as *Penicillium oxalicum*, obtained 1.2 FPU/mL in 8 days of submerged cultivation with wheat bran, reaching a value three times higher than that found at this stage of the work and four times greater than Campioni *et al.,* (2019) who also used wheat bran, showing the greater potential of *Penicillium oxalicum* compared to *T. reesei* in wheat bran. Florêncio *et al.,* (2016) obtained 0.7 FPU/g with sugarcane bagasse in solid cultivation for 24 hours, later changed to liquid cultivation (48h) with the *T. reesei* fungus, demonstrating an interesting strategy due to the association between reduced cultivation time and high enzyme activity, but not surpassing current work.

The xylanolytic enzymes showed greater production capacity for the experimental design using 6.66 g of brewer's spent grain, 3.33 g of citrus pulp, and 0 g of wheat bran reaching 40.2 U/mL or 241.23 U/g (402 U/L). The production with 10g of brewer's spent grain was also high (39.6 U/mL). The results of cultivation with tween 80 did not exceed the control (without tween) regarding the biosynthesis of xylanases by the fungus, on the contrary, they proved inhibition of this biosynthesis in the presence of this surfactant during cultivation. The best result with tween 80 for xylanase was 2.96 U/mL using 0.01% tween 80, much lower than that obtained in the design. The combination of selected nutrients such as BX and BC did not favor the production of xylanases.

Regarding the production of xylanases, Gottschalk *et al.,* (2010) produced 0.15 U/mL with *T. reesei* in 5 days of cultivation, while with the fungus *Aspergillus* awamori in 7 days of cultivation they obtained 0.025 U/mL, lower capacity that the optimization of the current study. Campioni *et al.,* (2019) reached 60 U/mL of xylanase using *T. reesei* in 6 days of submerged fermentation with sugar cane straw. Gottschalk *et al.,* (2010) used less cultivation time and obtained values below the current work, in contrast to Campioni *et al.,* (2019) which surpassed the results of the current research in productivity and yield.

The maximum production of endoglucanase in the mix design was found in the proportions of 6.67 g of brewer's spent grain, 1.66 g of citrus pulp, and 1.66 g of wheat bran, obtaining 2.03 U/mL (12,1 U/g). Rodríguez-Zúñiga *et al.,* (2011) found 21.0 U/g for endoglucanase using wheat bran in solid cultivation for 72 hours, values close to the current optimization, already Florêncio et al. (2016) obtained a production of 1.6 U/mL for endoglucanase with *T. reesei* in submerged cultivation with sugarcane bagasse, but it did not exceed the yield studied in this work. Castro *et al.,* (2010) obtained 0.55 U/mL of endoglucanase after 97 hours of cultivation in pyruvate dehydrogenase complex with *Trichoderma harzianum* 30 °C at 200 rpm, lower than that obtained in this work.

The production of exoglucanase in the design was higher using 6.67 g of brewer's spent grain, 1.66 g of citrus pulp, and 1.66 g of wheat bran reaching 3.20 U/mL (19.2 U/g). Silva *et al.,* (2018) reached values of 3.14 U/mL for endoglucanase, 1.25 U/mL for exoglucanase (citrus pulp substrate) and 93.08 U/mL for xylanase (sugarcane bagasse with alkaline pre-treatment), showing a lower value when compared to exoglucanase production, but higher when it comes to endoglucanase and xylanase production.

The production of β-glycosidase in the design of mixtures was higher with proportions of 6.67 g of brewer's spent grain, 1.66 g of citrus pulp, and 1.66 g of wheat bran reaching 0.12 U/mL (0.72 U/mL). Gottschalk *et al.,* (2010) reached 0.15 U/mL with the fungus *T. reesei* in 5 days and with A. awamori 0.018 U/mL in 7 days of cultivation. The authors demonstrated the potential of *T. reesei* in the production of β-glycosidase and found a value close to that of the current study.

From the optimization carried out it was possible to conclude that the fungus *T. reesei* can be used for the production of fibrolytic enzymes with the studied residues, however other strains and other residues are also standing out, mainly regarding the production of total cellulase (FPU) and xylanase, in addition to indicating the capacity of tween 80 for the production of xylanases and the

importance of salts for optimizing enzymatic production, as noted with the increase in total cellulase activity.

3.6 Enzymatic hydrolysis

Thinking of optimizing the production of reducing sugar (glucose syrup), an enzymatic hydrolysis was carried out associating cellulases with amylases. Enzymatic hydrolysis was performed with cassava bagasse (10% w / v) gelatinized or not, combining amylases and cellulases added in zero or ten hours, to obtain a glucose syrup. The highest concentration of reducing sugar was observed in previously gelatinized cassava bagasse (48.6 g / L). There were no significant differences in glucose concentration when adding amylase in zero or ten hours (Fig. 11). Shi et al. (2014) hydrolyzed the cassava bagasse (10% w / v) with more than one type of enzyme, including amylases and cellulases, reaching 44.3 g / L of reducing sugars in 48 hours of reaction, achieving lower concentration of reducing sugar in a longer time of hydrolysis. Chen et al. (2015) using cassava bagasse (10% w / v) carried out enzymatic hydrolysis with commercial cellulase (A1500; 0.2 mL / g) reaching 46.2 g / L of reducing sugar in 48 hours of reaction, a value similar to the present study, however, with a longer reaction time. Gonçalves (2016) observed greater hydrolysis over 10 hours of reaction, stabilizing after this period. Gonçalves (2016) when using amylases and cellulases in 0 and 10 hours of hydrolysis of cassava bagasse obtained 46.1 g / L of reducing sugar in 24 hours, similar to the present study. The synergistic action of the enzymes allowed to reach values similar to those observed in the literature.

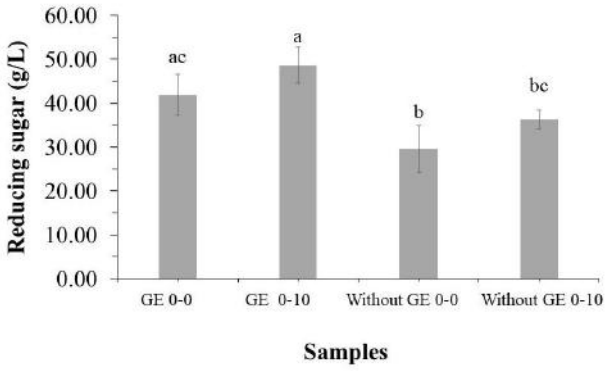


*Fig.11. Enzymatic hydrolysis with cassava bagasse (10% w/ v), pH 4.5 at 50 ° C for 24 hours. Enzymes: 15 U / ml amylase and 15 FPU / ml cellulase. GE 0-0: Gelatinization +amylase and cellulase added at time zero. GE 0-10: Gelatinization+ cellulase added at time zero and amylase at time 10 hours. Without GE 0-0: Without gelatinization + amylase and cellulase added at time zero. Without GE 0-10: Without gelatinization+ cellulase added at time zero and amylase at time 10 hours. The Tukey test was used with a significance level of 0.05%. Different letters indicate significant differences between groups*

Table 2 presents a summary of the transformations used, the adjusted model that best suited each answer, as well as the $R^2$ statistical data and precision analysis of the modified models, which considers only the significant terms. The final equations in terms of the real factors only for the significant terms of each modified model are also presented (1, 2, 3, 4 e 5).

## IV. CONCLUSION

The use of the I-Optimal mixture design technique of agro-industrial residues in submerged fermentation with *T. reesei* QM 9414, showed that the use of spent grain is promising for the production of cellulases and xylanase, as well as the use of citrus pulp. The study on the demand for minerals and yeast extract (as a vitamin and amino acid source) reveals that is possible to reduce the time of cultivation and increase the enzymatic production as we saw with the cellulase production that increased by 2.38 times. The use of 0.01% tween 80 in the culture medium drastically inhibited the fungal production of xylanases and increased total cellulase productivity, substantially reducing the cultivation time. Through enzymatic hydrolysis we observe the importance of synergistic use of enzymes, reaching significant values of reducing sugar.

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