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# Wheat Milling by-Products: an Alternative to Produce Amylolytic Enzymes by Mushrooms Strains

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**HIGHLIGHTS**

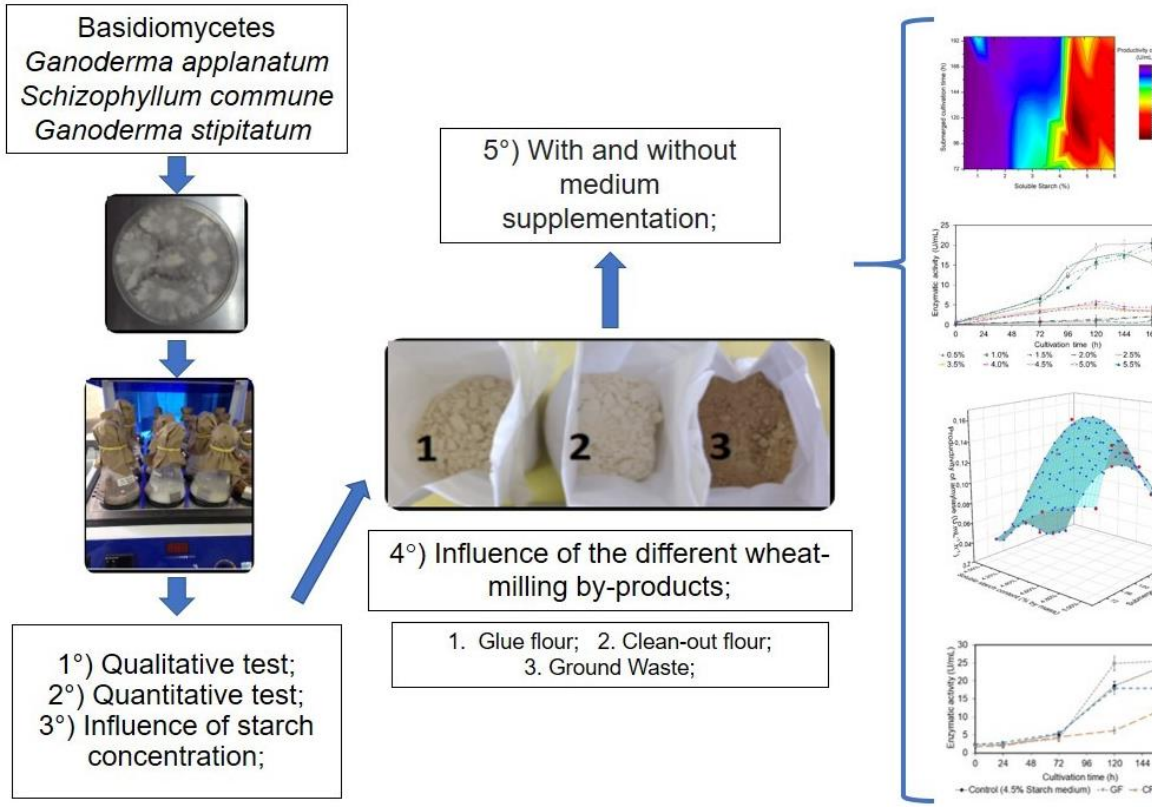
- One of the first works on the amylase production by *Ganoderma applanatum*;
- Production of α-amylase in the wheat milling by-product without supplementation;
- This strain showed potential to produce amylase in several wheat milling by-products;
- Amylase production costs 42-fold less with by-product than synthetic medium;

**Abstract:** The goal of this study was to evaluate the α-amylases production by basidiomycetes in submerged cultivation (SmC), using wheat milling by-products as substrate. Enzymatic activity was evaluated to select the best strain, the ideal concentration, the by-product and the influence of supplementation in the culture medium. The best producer was chosen among three strains, *Ganoderma applanatum* (MR-56), *Schizophyllum commune* (MR-01), and *Ganoderma stipitatum (*MR-72). All of them were cultivated under the same conditions. MR-56 was selected for an initial screening activity of 5.10±0.31 U/mL in 192h. The *Ganoderma applanatum* strain was cultured in a medium with various starch concentrations to determine which concentration resulted in the highest α-amylase activity. After 120h of cultivation, activity in the medium containing 4.5% (w/v) had increased from 0.40±0.03 U/mL to 20.70±0.9 U/mL. Subsequently, the wheat milling by-products [Glue Flour (GF), Clean-Out Flour (CF4) Flour, and Low-grade Flour (LGF)] were evaluated in aqueous suspension with and without nutritional supplementation. It was shown that GF and LGF are potential starch sources which do not require nutritional supplementation, exhibiting increases in α-amylase activity of 31.79 U/mL and 30.98 U/mL, respectively. This is the first report involving the application of *Ganoderma applanatum* in SmC for α-amylase production using synthetic and wheat milling by-products. The results also demonstrated that wheat milling by-products may also be used without supplementation for

cell growth and enzyme production, contributing to the development of more sustainable forms of enzyme production through the innovative use of edible organisms with medicinal properties.

**Keywords:** α-amylases; Submerged cultivation; Grain by-products; Flour; Basidiomycetes.

**GRAPHICAL ABSTRACT**




## INTRODUCTION

The term “fungi” refers to a diverse group of microscopic and macroscopic organisms that are valuable sources of natural products and bioactive compounds. These include enzymes, pigments, alkaloids, flavors, and aromas, and components that brings benefits to human health, such as potassium, vitamin D, riboflavin, selenium, proteins, and niacin. These compounds are also applied in the treatment of diseases and as biofertilizers [1]. Mushrooms (basidiomycetes), which belong to the Fungi Kingdom, are consumed today for their pleasant taste and the health benefits they provide due to their bioactive compounds [2]. Mushrooms are also considered a valuable resource for extracellular enzymatic biosynthesis [3,4]. Despite these benefits, their potential application has not been widely studied, especially regarding the broad range of possibilities for their use as new enzyme sources [3,5]. Few studies have been published regarding the use of basidiomycetes for amylase production, but none have been published involving amylase production by *Ganoderma applanatum* in SmC using synthetic and wheat mill by-products as substrate.

According to the Food and Agriculture Organization of the United Nations (FAO), wheat is the second most consumed food product in the world [6]; in the 2022/2023 harvest, total global wheat production and consumption was approximately 775 million tons [7]. According to the Brazilian Wheat Industry Association (ABITRIGO), flour was the most consumed wheat grain product in 2021, with an estimated national consumption of 9.9 million tons [8], as a result of the various uses of this product in the production of breads, cakes, biscuits, pasta and other foods, as well as its use in glue production and in the pharmaceutical industry [6,8,9]. The wheat milling process generates several by-products, including GF, CF4, and LGF. These by-products are underutilized in industrial processes and have a low cost per ton, making them viable for use in enzyme production.

The global enzymes market is expected to grow by 9.2% from 2017 to 2026, achieving revenues of $ 5.28 billion by 2026, an increase of $ 2.89 billion when compared to $ 2.39 billion in 2017. The consumer

profile has been changing over the last several years in conjunction with increasing interest in industrialized and functional foods having better nutritional values. This is a trend that has stimulated market growth [10]. Amylases are among the most representative enzymes in the global enzymes market, which are used in brewing and baking, as well as in textile, paper, chemical, pharmaceutical, and biofuel production [11–15].

The importance of this enzyme group, specially the α-amylase, largely used in baking and brewery industries, represent a market which may reach US$ 320.1 million by 2024 [16]. The synthesis of this enzyme is influenced by various physical-chemical and environmental parameters. These include the nature and specificity of the substrate, culture medium pH, initial starch concentration in batch experiments, cofactors, and activating compounds. These parameters must be controlled in order to achieve high concentrations of enzyme. Another important factor is the type of culture (solid or liquid). Therefore, evaluating bioprocess conditions and parameters is a crucial step in order to improve enzyme synthesis [17]. The preferred method, due to easier control and handling, is SmC (submerged cultivation) is the most frequently process used by enzyme industries [18,19].

The goal of this work was to evaluate α-amylase production by different strains of basidiomycetes in order to select the best α-amylase producer, as well as to evaluate the use of a synthetic medium containing soluble corn starch P.A. in SmC for amylase production, as compared to the use another culture medium (composed of solid by-products from wheat milling). Thus, this study also aimed to evaluate α-amylase production by various strains of basidiomycetes using wheat milling by-products as substrate.

## MATERIAL AND METHODS

### Strains

Three basidiomycete strains, obtained from the Bioprocesses Engineering and Biotechnology Department Culture Collection of the Federal University of Paraná (UFPR, Curitiba, Brazil), were evaluated for α-amylase production: *Ganoderma applanatum* (MR-56), *Schizophyllum commune* (MR-01), and *Ganoderma stipitatum* (MR-72). These species are found mostly in the Atlantic Forest of Brazil. The strains were kept at 4 °C in Potato Dextrose Agar (PDA). Periodic cultivations (PDA medium, incubation at 30 °C for 5 days) were performed on the strains every 3 months in order to maintain laboratory viability.

### Qualitative selection of the strains

The growth medium was inoculated with 5 pieces of fungus mycelium diameter of 5 mm. The starch agar medium had the following composition (mg/mL): 10 soluble corn starch P.A., 2.0 $KH_2PO_4$, 1.4 $(NH_4)_2SO_4$, 0.3 $CaCl_2$, 0.3 $MgSO_4.7H_2O$, 0.3 Urea, and 20 agar, previously sterilized at 121 °C for 15 min. This was a modified medium described by Aguiar and coauthors [20]. The cultures were then incubated at 30 °C for 5 days, and their growth evaluated by monitoring and measuring their starch degradation halo every 24h. The iodine-iodide reagent (composed of KI 30 mg/mL and $I_2$ 3 mg/mL) was used to confirm starch degradation on the inoculated plates.

### Quantitative selection of strains

The SmC was carried out using the starch medium described above. Three mycelial blocks (5x5 mm) from the periodic cultivations in the PDA medium were used as inoculum in 50 mL of the starch medium. The cultures were then incubated for 5 days at 30 °C under agitation at 120 rpm. Samples were collected at 0h, and subsequently every 24h in order to determine enzyme activity. Samples were centrifuged (8.300 g for 30 min), and the supernatant containing the crude enzyme extract was analyzed.

### Enzymatic assays

The α-amylase activity was evaluated using a modified form of the method described by Anto and coauthors [19], based on the Fuwa method [21]. First, 0.5 mL of 1% (w/v) soluble corn starch P.A. solution, prepared in 0.1 M sodium phosphate buffer (pH 6.5), was incubated for 5 min at 55 °C. The enzyme was then diluted in 0.1 M sodium phosphate buffer (pH 6.5), and 0.5 mL was added to each test tube. The tubes were then incubated at 55 °C for 20 min, and the enzymatic reaction was stopped by adding 1 mL of 3.5-dinitrosalicylic (DNS). The reducing sugar produced was evaluated using the DNS method [22, 23, 24], and absorbance was measured at 540 nm. The α-amylase activity was expressed as the amount of enzyme needed to release 1 micromole of reducing sugar, the standard curve was performed with glucose, per minute per mL.

### Influence of starch concentration on α-amylase production

After the qualitative and quantitative selection of the basidiomycetes, α-amylase production was carried out by evaluating different concentrations of starch. Culture media containing different concentrations of starch (0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0 and 6.5%) were also composed of 2.0 mg/mL $KH_2PO_4$, 1.4 mg/mL $(NH_4)_2SO_4$, 0.3 mg/mL $CaCl_2$, 0.3 mg/mL $MgSO_4.7H_2O$, and 0.3 mg/mL urea [20]. The cultures were incubated at 30 °C for 5 days under agitation at 120 rpm. Samples were collected at 0h, and subsequently every 24 h for analysis of enzymatic activity.

### Preparation of culture medium and inoculum

The best starch concentration in the medium was selected, after which three wheat milling by-products (which are not feasible for bread, pasta, and biscuit production) provided by a wheat mill (Curitiba-Paraná, Brazil) were used as substrate, as described in Table 1.

A solid-liquid medium was prepared using 4.5% (w/v) soluble corn starch for each respective by-product in water, always equivalent to the amount necessary for achieving the desired concentration, 5.6 mg/mL by-product (GF), 5.5 mg/mL CF4 and 10.23 mg/mL LGF. The by-products were autoclaved, and the liquid fraction was used to allow for the fluidity and dissolution of oxygen in the medium. These media were used for the SmC, together with a control experiment (a medium with 4.5% (w/v) soluble starch). The culture medium was prepared in the first test without salt supplementation. A new test was then performed, both with and without salt supplementation.

The salts used for supplementation were as follows (mg/mL): $KH_2PO_4$ (2.0), $(NH_4)_2SO_4$ (1.4), $CaCl_2$ (0.3), $MgSO_4.7H_2O$ (0.3), and urea (0.3). All culture mediums are described in Table 1.

**Table 1.** Description of the culture media used in the experiments evaluating by-products of the wheat milling.

| Tests | Wheat milling by-products | Description | Characteristics of the medium |
|---|---|---|---|
| 1 | Soluble starch medium (control) | 4.5% (w/v) starch | Soluble corn starch P.A. |
| 2 | GF | The flour used in the adhesive manufacturing industry | 13.24% moisture, 0.85% ashes, 15.18% proteins, 78.98% starch, 35.14 mg/100g $Ca^{2+}$, 149.14 mg/100g P, 43.33 mg/100g $Mg^{2+}$, 182.24 mg/100g $K^+$ and 27.42 mg/100 g $Na^+$ [23] |
| 3 | CF4 | “Clean-out” Flour (a residual flour of silos, pipes, and floor) | 13.48% moisture, 0.92% ashes, 14.57% proteins, 81.07% starch, 50.75 mg/100g $Ca^{2+}$, 156.79 mg/100g P, 46.83 mg/100 g $Mg^{2+}$, 219.28 mg/100g $K^+$ and 252.84 mg/100 g $Na^+$ [23] |
| 4 | LGF | The by-product of ground wheat flour | 12.98% moisture, 5.02% ashes, 15.80% proteins, 43.95% starch, 122.00 mg/100g $Ca^{2+}$, 379.50 mg/100g P, 153.60 mg/100g $Mg^{2+}$, 727.39 mg/100g $K^+$ and 31.88 k mg/100g $Na^+$ [23] |
| 5 | GF + salts | The flour used in the adhesive manufacturing industry | GF + ($KH_2PO_4$ (2.0) mg/mL, $(NH_4)_2SO_4$ (1.4) mg/mL, $CaCl_2$ (0.3) g/L, $MgSO_4.7H_2O$ (0.3) mg/mL and urea (0.3) mg/mL) |
| 6 | CF4 + salts | “Clean-out” Flour (a residual flour of silos, pipes, and floor) | CF4 + ($KH_2PO_4$ (2.0) mg/mL, $(NH_4)_2SO_4$ (1.4) mg/mL, $CaCl_2$ (0.3) g/L, $MgSO_4.7H_2O$ (0.3) mg/mL and urea (0.3) mg/mL) |
| 7 | LGF+ salts | The by-product of ground wheat milling | LGF + ($KH_2PO_4$ (2.0) mg/mL, $(NH_4)_2SO_4$ (1.4) mg/mL, $CaCl_2$ (0.3) g/L, $MgSO_4.7H_2O$ (0.3) mg/mL and urea (0.3) mg/mL) |

Three mycelial blocks (5x5mm) from the periodic cultures of *Ganoderma applanatum* MR-56 in the PDA medium were used as inoculum in 50 mL of the medium. Cultures were incubated for 5 days at 30°C under agitation at 120 rpm. Samples were collected at 0, 72, 120, and 168 h for further analysis of the enzymatic assays, as described above. Enzyme productivity was calculated according to Equation 1.

$$Y_{Ptotal} = \frac{P_f - P_i}{t_t} \quad (1)$$

Where $Y_{Ptotal}$ is total α-amylase productivity (U/mL/h), $P_i$ and $P_f$ are initial and final product concentrations (mg/mL), and $t_t$ is total cultivation time (h).

### Statistical analysis

The data were analyzed using analysis of variance (ANOVA), followed by a multiple comparison test. The quality of fit was determined based on the regression coefficient ($R^2$) and the adjusted $R^2$. It was also used a nonlinear surface fit in the data acquired by means of the OriginPro 8.5 software. The direct search method in the Engineering Equation Solver® software was used for multivariate optimization.

## RESULTS

### Qualitative selection of mushroom strains

The strains *Ganoderma applanatum* (MR-56), *Schizophyllum commune* (MR-01), and *Ganoderma stipitatum* (MR-72) were evaluated for growth capacity in a medium containing a single source of carbon. Radial growth was observed, expressed in terms of the difference between the colonies diameter (cm) of the colony at different incubation times in the starch agar medium. *Schizophyllum commune* MR-01 exhibited lower growth, with a variation in diameter (ΔØ) of 3.3 cm after 5 days of incubation at 30ºC on plates (Table 2). Among the 3 strains tested, *Ganoderma stipitatum* (MR-72) exhibited intermediate growth, with a ΔØ of 4.3 cm. *Ganoderma applanatum* MR-56 showed the highest radial growth, with a ΔØ of 6.0 cm.

**Table 2.** The colonies diameter of mushrooms *Schizophyllum commune* MR-01, *Ganoderma stipitatum* MR-72, and *Ganoderma applanatum* MR-56 at 1, 4, and 5 days of radial growth on the surface of 1% starch agar medium at 30 °C.

| Basidiomycete | Diameter Ø (cm) | | | | Growth increased | Growth** | Relative growth*** |
|---|---|---|---|---|---|---|---|
| | 0 day | 1st day | 4th day | 5th day | ΔØ | | % |
| MR-01 | 0.5 | 0.7 | 2.8 | 4.2 | 3.7 | + | 56.9 |
| MR-72 | 0.5 | 0.7 | 3.0 | 5.2 | 4.7 | ++ | 72.3 |
| MR-56 | 0.5 | 0.8 | 4.6 | **7.0** | 6.5 | +++ | 100.0 |

Ø = diameter; * ΔØ = change in the diameter of the colonies (calculated by the difference between the diameters at the final time (5th day of incubation minus the diameter at the initial time (0 days); ** Growth sign (+, ++, +++), the + sign represents the lowest growth of the colonies; the ++ signal represents intermediate growth; +++ represents the highest growth. *** Relative growth (%) is the diameter of the growth of a specific fungus to growth of the highest growing fungus (MR-56).

Growth increase was calculated according to the difference between the diameters measured on the 1st and 5th days of growth. Relative growth of 100% was obtained for the highest observed growth value, achieved by the *G. applanatum* MR-56 strain. The second highest relative growth (72.6%) was observed for *G. stipitatum* MR-72, followed by the *S. commune* MR-01 strain (56.5%).

### Quantitative selection of mushroom strains

Enzymatic quantification was carried out in SmC for *Ganoderma applanatum* (MR-56), *Schizophyllum commune* (MR-01), and *Ganoderma stipitatum* (MR-72). Table 3 shows α-amylase production for each strains.

**Table 3.** Results of enzymatic activity (EA) (U/mL) and productivity (P) (U/mL.h) of the quantitative assay under SmC during 72, 144, 168, and 192h aiming for basidiomycete strains selection with the highest production capacity of α-amylases.

| Strain | 72 h | | 144 h | | 168 h | | 192 h | |
|---|---|---|---|---|---|---|---|---|
| | A | P | A | P | A | P | A | P |
| MR-56 | $0.72\pm0.18^{b}$ | 0.010 | $1.67\pm0.28^{a}$ | 0.012 | $3.30\pm0.34^{a}$ | 0.020 | **$5.10\pm0.31^{a}$** | 0.027 |
| MR-01 | $0.94\pm0.11^{a}$ | 0.013 | $1.60\pm0.34^{a}$ | 0.011 | $2.00\pm0.41^{b}$ | 0.012 | $2.80\pm0.42^{b}$ | 0.015 |
| MR-72 | $0.60\pm0.09^{c}$ | 0.008 | $0.79\pm0.1^{b}$ | 0.005 | $0.94\pm0.21^{c}$ | 0.005 | $1.10\pm0.13^{c}$ | 0.006 |

A= Amylase activity (U/mL); P= productivity (U/mL.h); Different letters on the same column means significant difference ($p<0.05$).

Among the evaluated strains, MR-56 exhibited the best enzyme production for both quantitative (Table 3) and qualitative (Table 2) tests. The highest α-amylase enzyme activity was 5.10±0.31 U/mL, with productivity of 0.027 U/mL/h, achieved on the 192 h (Table 3). The MR-01 strain exhibited the second-best result, 2.80±0.42 U/mL with 0.015 U/mL/h of productivity, also on the 192 h. The lowest enzymatic activity was observed for MR-72 (1.10±0.13 U/mL), which achieved a yield of 0.006 U/mL/h on the 192 h (8th day) of

fermentation. The enzymatic activity observed for MR-01 was 45% higher than that observed for MR-56 and 78% higher than that observed for MR-72. A reduction in enzyme production (data not shown) was observed after 192h of cultivation.

### Analysis of the influence of starch concentration on α-amylase production

*Ganoderma applanatum* (MR-56) was evaluated for enzyme production in different starch corn medium P.A. concentrations. It was possible to evaluate enzyme productivity as a function of starch concentration over cultivation (Figure 1). The highest amylase productivity was achieved in 4.5% (w/v) starch concentration. Most of the concentrations evaluated exhibited peak enzymatic activity at 120 h of fermentation. So, it is recommended that the process may be stopped in this time for minimizing costs. The maximum productivity achieved using this starch concentration (4.5% w/v) was 0.162 U/mL/h at 120 h of cultivation.

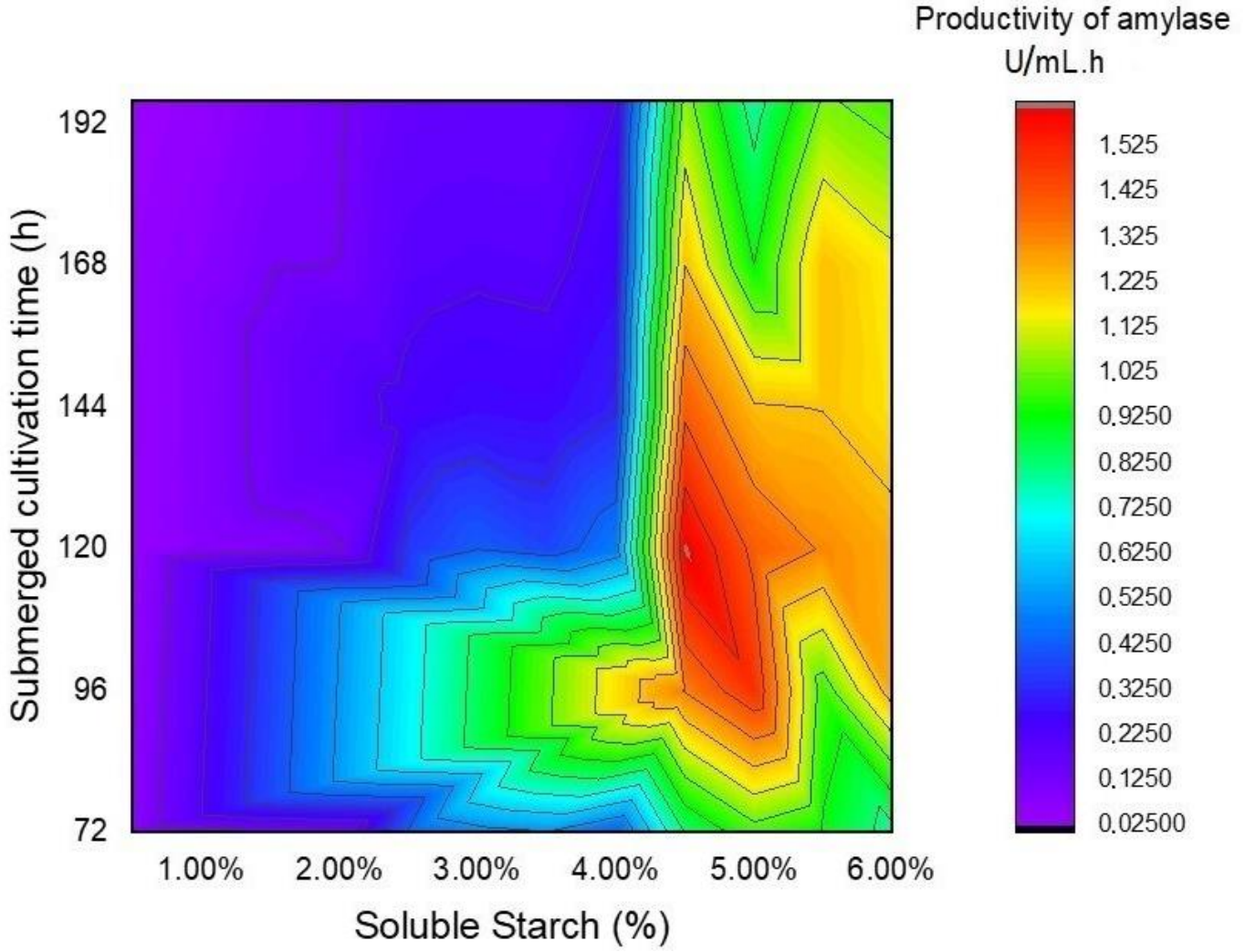



**Figure 1.** α-amylase production by MR-56 strains during SmC using soluble starch medium as the sole carbon source, under constant stirring conditions at 120 rpm, 30 °C for 8 days (192h).

In general, the α-amylase activity increased over the 8 days of cultivation as the starch concentrations increased. However, a limit concentration of 5.5% was observed for the increase in enzymatic activity (Figure 2). However, the results less pronounced with lower starch concentrations (0.5 to 2.0%), whose activities were between 0.08 U/mL and 2.34 U/mL.

The kinetics with starch concentrations between 2.5% (w/v) and 4.0% (w/v) exhibited enzymatic activities with values between 2.34 U/mL to 5.75 U/mL after 120 h of cultivation. *Ganoderma applanatum* (MR-56) exhibited the best α-amylase activity at a starch concentration of 4.5% (w/v), with an increase from 0.39 U/mL (0h) to 20.70 U/mL (192 h) and productivity of 0.11 U/mL/h. At this concentration, maximum productivity was 0.16 U/mL/h (Figure 2) with an activity of 19.44 U/mL after 120h of cultivation.

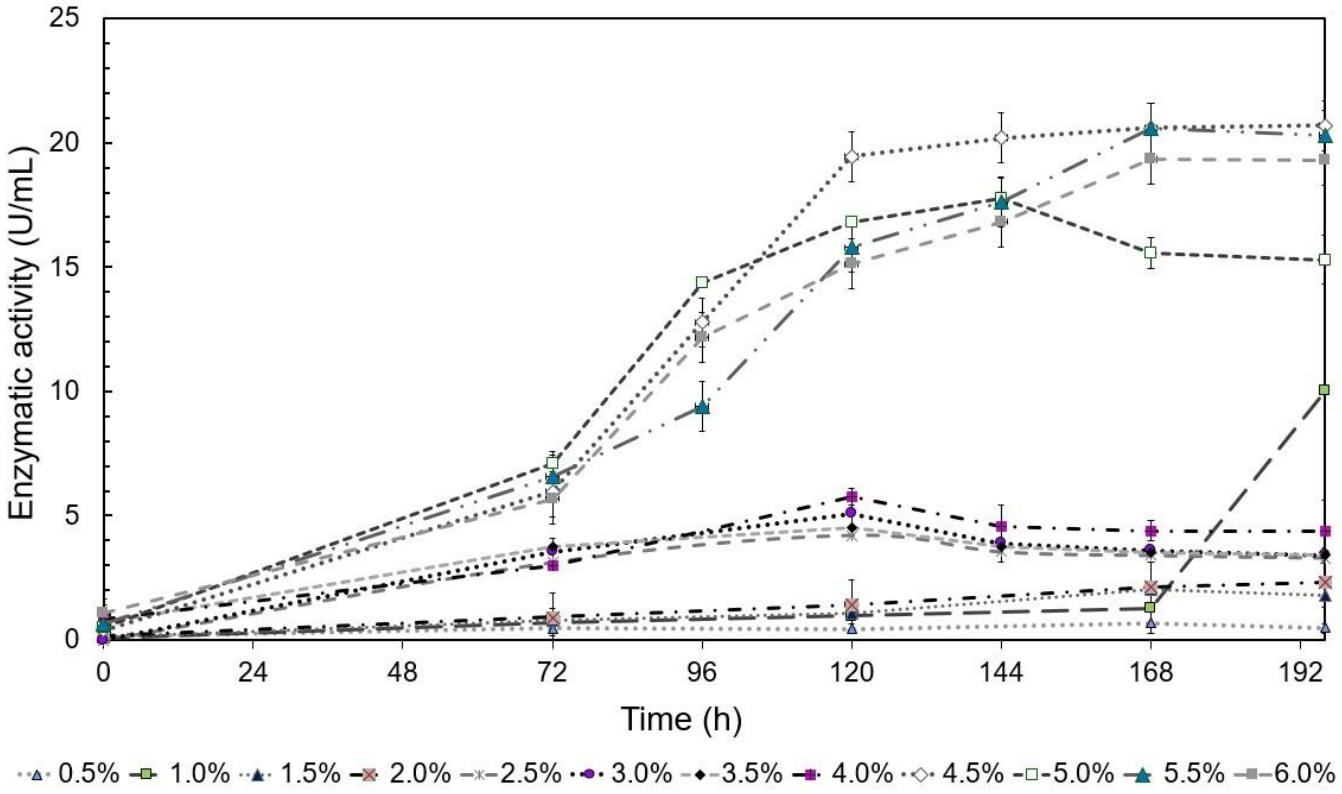



**Figure 2.** Enzymatic activity (U/mL) under submerged cultivation (SmC) using liquid media containing soluble starch as the sole carbon source in different concentrations. The best strain previously selected *Ganoderma applanatum* MR-56 cultivation was carried out at 120 rpm, 30 ˚C for 8 days.

The enzyme productivity tended to decrease when 6% (w/v) starch was used, an observation that may also affect the feasibility of the process. Higher starch concentrations may repress or inhibit enzymes synthesis, or also affect mass and heat transfer, also may affected the oxygen dissolved during aeration process, and contributed to an increase in fermentation cost.

Concentrations higher than 6% (w/v) were not tested, since no statistically significant increase in enzyme activity was detected after 168h as compared to that of the 4.5% (w/v) starch concentration ($p < 0.05$). On the other hand, enzyme activity before 168 h for kinetics with 4.5% (w/v) starch was higher than those with 5.0, 5.5, and 6.0% (w/v) starch.

Using the *Ganoderma applanatum* strain, the current work applied a surface fit to the data acquired and obtained the following equation:

$$P=C+A_1S+A_2t+A_3S^2+A_4St+A_5t^2+A_6S^2t+A_7St^2+A_8t^3+A_9S^2t^2+A_{10}St^3+A_{11}t^4+A_{12}S^2t^3+A_{13}\,St^4+A_{14}t^5$$

Where the Residual Sum of Squares is 0.015, the Reduced ChiSq is 0.008, Pearson's r is 0.998, $R^2$ is 99.55%, and Adjusted $R^2$ is 0.964. The constants of the equation obtained from the surface fit are: A1=21.73; A2=-813.68; A3=-0.55; A4=7888.85; A5=18.77; A6=0.004; A7=-172.10; A8=-0.11; A9=$-0.18E^{-4}$; A10=0.88; A11=0.0003; A12=$0.45E^{-7}$; A13=-0.001; A14=$-0.26E^{-6}$; and A15=$-0.49E^{-10}$. The Figure 3 shows the adjusted surface for α-amylase production during SmC at 30 °C using a liquid medium containing soluble starch as the sole carbon source, using the MR-56 strain under constant agitation at 120 rpm for 8 days (192h).

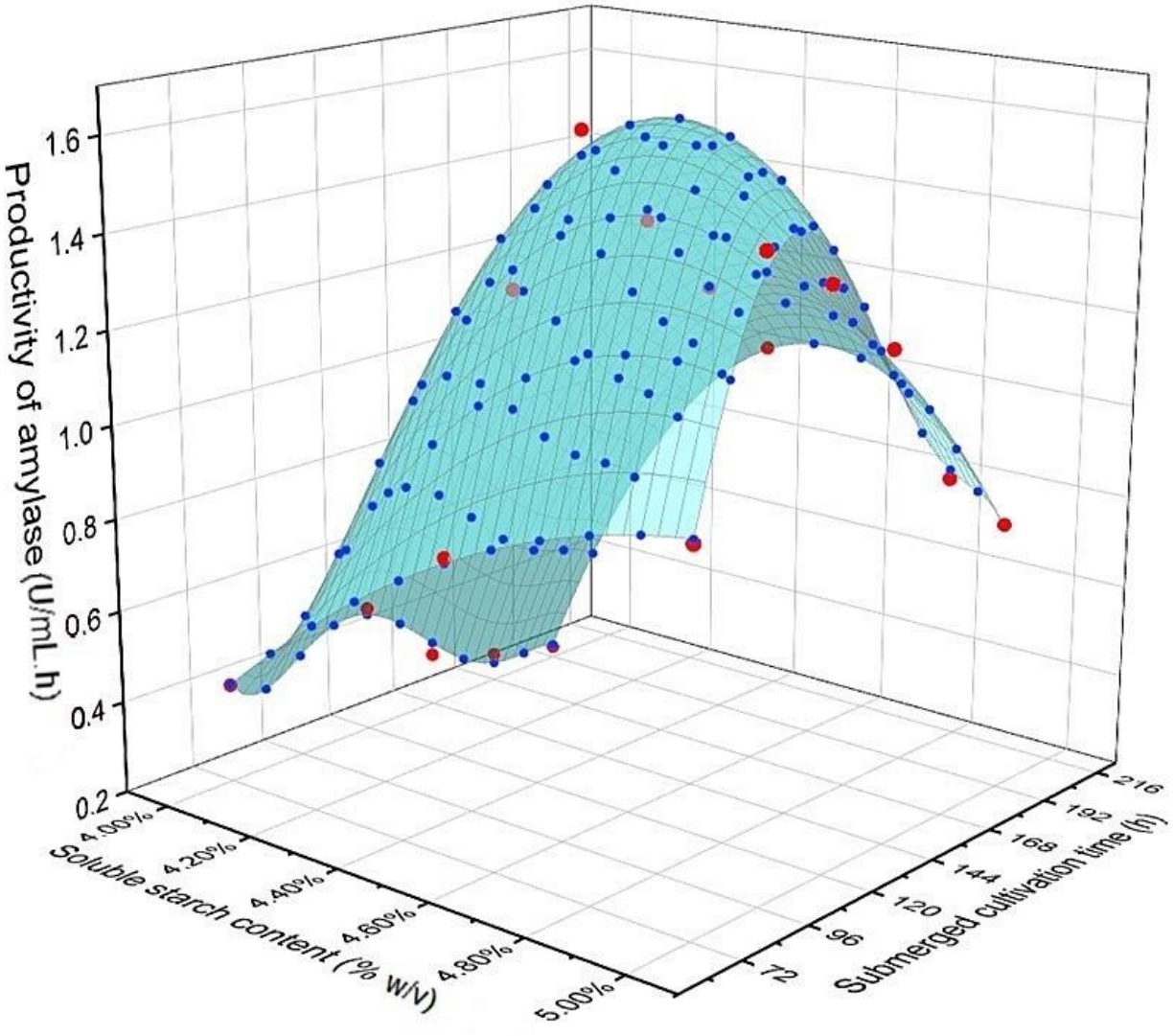



**Figure 3.** Surface fit for α-amylase production during submerged culture (SmC) using liquid medium containing soluble starch as the sole carbon source, using strain MR-56, under constant stirring conditions at 120 rpm, 30 °C for 8 days (216h).

A multivariable optimization was performed based on a Direct Search Method in the Engineering Equation Solver® software. The optimum soluble starch fraction was obtained (4.6% w/v) for the *Ganoderma applanatum* strain, with an optimal SmC time of 109.4 h.

**Analysis of the use of various wheat wheat milling by-products for α-amylase production**

*G. applanatum* (MR-56) was evaluated for α-amylase production in four different culture media, three of which were composed of different wheat milling by-products (GF, CF4, and LGF), and a control medium (4.5% (w/v) of soluble starch P.A.) as described in Table 1. The α-amylase activity (U/mL), Table 4, was used to determine which by-products are best for enzymatic synthesis. The increase in enzyme activity was observed on the 120 h of cultivation using GF with a 32% increase in α-amylase activity (Figure 4).

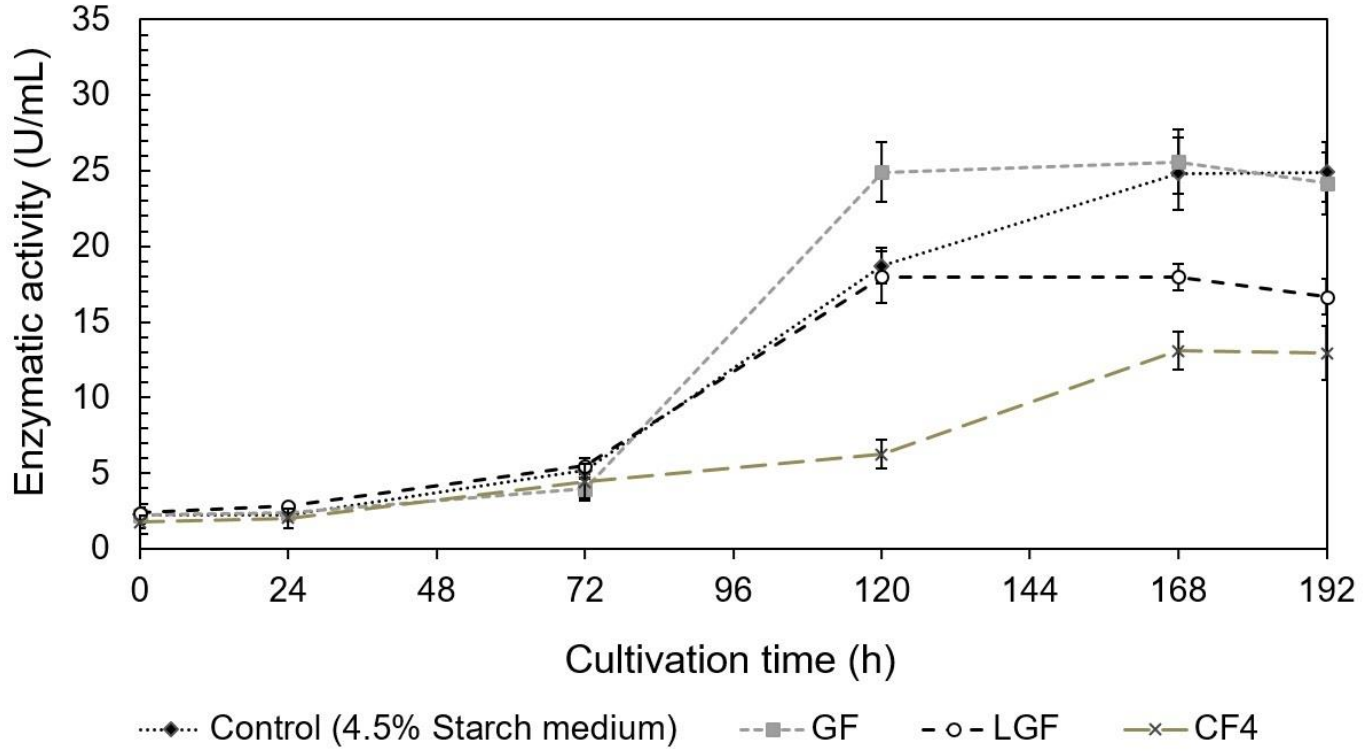



**Figure 4**. Evolution of SmC for α-amylases production by *G. applanatum* MR-56 strain over 8 days (192 h) at 30 °C and 120 rpm using industrial by-products GF, CF4, and LGF and Control (4.5% starch medium).

All three wheat milling by-products (evaluated individually) were capable of inducing α-amylase production. Enzyme production increases significantly (approximately 6-fold) from the 3rd (72 h) to the 5th (120 h) day of cultivation (Table 4).

**Table 4.** α-amylase activity and productivity achieved by *Ganoderma applanatum* MR-56 in SmC during 192 h of cultivation at 120 rpm and 30ºC, comparing synthetic medium (4.5% w/v soluble corn starch P.A.) with different by-products (GF, CF4, and LGF).

| Cultivation | Starch 4.5% | | GF | | CF4 | | LGF | |
|---|---|---|---|---|---|---|---|---|
| time (h) | A | P | A | P | A | P | A | P |
| 0 | $2.27\pm0.32^{b}$ | 0.00 | $2.22\pm0.02^{b}$ | 0.00 | $1.79\pm0.23^{c}$ | 0.00 | $2.38\pm0.15^{a}$ | 0.00 |
| 24 | $2.25\pm0.18^{b}$ | 0.09 | $2.39\pm0.22^{b}$ | 0.10 | $2.03\pm0.17^{c}$ | 0.08 | $2.85\pm0.02^{a}$ | 0.12 |
| 72 | $5.18\pm0.23_{b}$ | 0.07 | $3.99\pm0.35^{d}$ | 0.05 | $4.43\pm0.33^{c}$ | 0.06 | $5.49\pm0.11^{a}$ | 0.07 |
| 120 | $18.72\pm0.14^{b}$ | 0.16 | $24.89\pm0.07^{a}$ | 0.21 | $6.28\pm0.01^{d}$ | 0.05 | $17.98\pm0.38^{c}$ | 0.15 |
| 168 | $24.83\pm0.10^{b}$ | 0.15 | **$25.61\pm0.43^{a}$** | 0.15 | $13.09\pm0.03^{d}$ | 0.08 | $17.98\pm0.43^{c}$ | 0.11 |
| 192 | $24.93\pm0.08^{a}$ | 0.13 | $24.17\pm0.28^{b}$ | 0.12 | $12.96\pm0.21^{d}$ | 0.07 | $16.68\pm0.36^{c}$ | 0.08 |

A= Amylase activity (U/mL); P= productivity (U/mL/h); Different letters on the same line means significant difference ($p<0.05$).

GF medium exhibited the highest activity values (25.61 U/mL) at the end of the 7th day (168 h), with a productivity of 0.15 U/mL/h. However, the highest yield value (0.21 U/mL/h) was obtained on the 5th day (120 h) of fermentation (24.89 U/mL). The Tukey test showed a statistical difference ($p<0.05$) for the two highest activities.

The analysis of the by-products enriched with nutrients ($KH_2PO_4$, $(NH_4)_2SO_4$, $CaCl_2$, $MgSO_4$, and Urea) was also evaluated (Table 5) at 120 h of cultivation (the time was maintained the same as previous studies). This time was determined after taking into consideration the previous analyses.

Both GF and LGF flours demonstrated potential for α-amylase production. The increase in α-amylase activity for GF (disregarding activity at 0 h), was 31.79 U/mL, and activity for LGF was 30.98 U/mL. These values exhibited no significant differences ($p<0.05$). Both results were obtained with wheat milling by-products diluted to 4.5% (w/v) without supplementation with other nutrients (nitrogen and salt sources).

**Table 5**. α-amylase activity using wheat by-products GF, CF4, and LGF at 4.5% (w/v), with and without nutrient supplementation $KH_2PO_4$, $(NH_4)_2SO_4$, $CaCl_2$, $MgSO_4.7H_2O$, and Urea. In the control experiment, 4.5% (w/v) soluble starch medium containing the salts $KH_2PO_4$, $(NH_4)_2SO_4$, $CaCl_2$, $MgSO_4.7H_2O$, and Urea. The cultivation was carried out at 30 ºC and 120 rpm of agitation in a rotatory shaker. Samples were collected at 0 h and 120 h.

| Conditions | α-amylase Activity (U/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (Starch 4.5%) | | GF | | CF4 | | LGF | |
| | 0 h | 120 h | 0 h | 120 h | 0 h | 120 h | 0 h | 120 h |
| Without supplementation | $0.89^{F}$ | $9.58^{f}$ | $1.11^{E}$ | **$32.90\pm0.31^{b}$** | $2.79^{A}$ | $8.46\pm0.05^{g}$ | $2.32^{B}$ | **$33.3\pm0.13^{a}$** |
| With supplementation | | | $1.38^{D}$ | $27.66\pm0.21^{c}$ | $1.94^{C}$ | $22.83\pm0.18^{d}$ | $1.90^{C}$ | $17.97\pm0.45^{e}$ |

A Tukey test with $p<0.05$ was carried out, comparing the cultivation times of each treatment. The uppercase letters refer to the time 0h and the lowercase letters to the time 120 h.

The culture medium containing CF4 showed higher enzyme activity (20.89 U/mL) with supplemented than the control experiment (8.69 U/mL). The use of by-product alone did not induce enzyme production compared to the by-product supplemented with salts.

GF may be considered one of the best by-product alternatives for amylase synthesis since it is currently used only to manufacture glues and adhesives. It is therefore a low-cost raw material and consequently contributes to a decrease in industrial production costs. The second-best enzyme activity result was observed for LGF, whose activity was only 2.5% less than that of GF. In contrast, CF4 exhibited 34.28% less enzyme activity than GF.

## DISCUSSION

The qualitative selection was an essential step for the evaluation of the studied strains. Souza and coauthors [25] reported α-amylase production by *Ganoderma sp*. obtained by means of the qualitative test. The degradation halos exhibited a diameter of 7.0 mm after 120 h of incubation. These results were less pronounced than those obtained in the present study for *Ganoderma applanatum* (MR-56), 6.5 cm in 96 h (Table 1). Goud and coauthors [26] qualitatively studied 50 strains of basidiomycetes. The authors also analyzed MR-56 and MR-01 strains but did not observe α-amylase production, perhaps due to the different composition of the culture medium used. The composition of the medium used by Goud and coauthors [26] did not contain $CaCl_2$, a source of calcium and Urea. $Ca^{2+}$ ions are essential, because this enzyme may interact with charged amino acids [27]. This ion is also reported in the literature as being an important α-amylase stabilizer [28]. According to the study by Krupodorova and coauthors [29], *Ganoderma applanatum* exhibited amylase activity, when characterized qualitatively, as did *Schizophyllum commune*. However, the authors of the study did not include the values of the halos formed by the strains, making the comparison with the present work difficult. Although all strains have the potential for α-amylase production, MR-56 exhibited the best halo formation.

The fact that few studies have been published involving α-amylase production by basidiomycetes in SmC highlights the importance of the current work. The quantitative test demonstrated that the MR-56 strain exhibits a high potential for α-amylase production, a fact confirmed by the high levels of enzyme activity (5.10 U/mL in 192 h), which are higher than those of other studies available in the literature. Paludo et al. [4] observed a maximum activity of 2.314 U/mL for *Coprinus comatus* after 48 h. Tatsumi et al. [30], in their analysis of *Lentinus edodes*, observed an activity of 1.79, and Jonathan and Adeyo [31] observed an activity of 0.6 U/mL for *Agaricus blazei*.

Moreover, Frantz and coauthors [23], who studied *C. comatus,* obtained an activity of 62.95 U/mL. These works demonstrate the potential for α-amylase production and the need for new studies whose aim is to increase the enzyme production. The differences in reported activity may be explained by differences in the nutritional and physical requirements of the strains, although they belong to the same fungal species. Among the strains evaluated in the present study, MR-56 exhibited the greatest α-amylase production and therefore was chosen for further experiments.

An analysis of the concentrations of starch used as a substrate revealed that excess starch might negatively influence enzyme production. Paludo et al. [4] also observed negative effects of high starch concentrations, values greater than 2.7%, although the strain they studied requires lower starch concentrations than those used in the present study (approximately 13 mg/mL). High concentrations resulted in the suppression of α-amylase production. Glucose, which is released when the macromycete degrades the starch, is an inhibitor of the various α-amylases produced by fungi strains, resulting in a decrease in

enzymatic activity [23]. This may explain why higher starch concentrations (Figure 2) result in decreased α-amylase activity. In contrast, the study by Frantz and coauthors [23] reported that the ideal concentration observed was approximately 6.7% starch using wheat milling by-products.

The *Ganoderma applanatum* strain is one of those reported as having the highest enzymatic activity (31.79 U/mL), as compared to *Coprinus comatus* (5.84 U/mL) in a synthetic medium [4]. This characteristic makes *Ganoderma applanatum* a promising source for industrial-scale production. *G. applanatum* is still little explored in the literature regarding α-amylase production, and could be grown in wheat milling by-products by SmC.

The use of wheat milling by-products was proven effective in increasing α-amylase activity. It should also be noted that these substrates may be purchased at lower prices than synthetic ones. In the current study, *Ganoderma applanatum* exhibited a higher enzymatic activity (31.79 U/mL) with the by-product used without supplementation. The activity was less than that reported by Frantz and coauthors [23] (approximately 62 U/mL), who used the same type of culture medium with *C. comatus.* Further studies need to be conducted to determine whether supplementation with nitrogen sources could be a viable alternative for reducing this difference observed between the strains.

In contrast, the strain studied exhibited higher activity (0.16 U/mL/h) after 120h when grown in a synthetic medium, 18.72 U/mL, as compared to *C. comatus* grown in a synthetic medium, which exhibited a peak activity of 5.84 U/mL after 48 h (0.12 U/mL/h). Few reports exist in the literature regarding α-amylase production using this strain. Moreover, the possibility of strain cultivating in wheat milling by-products and by SmC demonstrates its potential for industrial exploitation.

The kinetics of the by-products GF and LGF exhibiting higher enzymatic activities did not require supplementation. The characteristic was also observed by Frantz and coauthors [23], who observed that a nitrogen source was sufficient to increase activity and that calcium and magnesium sources were unnecessary. The results of this study demonstrated that wheat milling by-products could be used as an excellent alternative to replace synthetic media.

This, in turn, is related to its centesimal composition and the average cost of the synthetic medium. Wheat milling by-products have a protein concentration of approximately 14%, composed of amino acids that are essential for the maintenance and regulation of the strain metabolism. Although the percentage of vitamins and other mineral salts in the by-products has not been quantified, it has been reported in the literature that such components are present in these by-products. Peron-Schlosser and coauthors [32] state that the lipid concentration in GF is approximately 1.63% ± 0.43. This compound, even in small amounts, is vital for fungal metabolism and enzymatic synthesis, since it participates in processes such as fatty acid biosynthesis.

Phosphate and magnesium components, present in greater amounts in the by-products than in the synthetic medium, are essential for growth and biomolecule production since these compounds participate in the cellular transport chain [33]. The growth of the organism is likewise influenced by ions such as $Ca^{2+}$, $Na^{2+}$, $Cl^{-}$, $SO_4^{2-}$ and $Mg^{2+}$ [34], and enzymatic synthesis is influenced by macronutrients such as $K^{1+}$, $S^{2-}$, $P^{1+}$, and Mg, as well as by the micronutrients $Mn^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Mo^{3+}$ and $Cu^{1+}$ [35]. All these factors, in addition to the fact that the average cost of producing 1 L of the substrate using by-products is currently R$0.10, whereas the cost of producing the same amount using synthetic substrate is R$ 4.20, contribute to make this substrate a promising material for this application. Furthermore, its use resulted in a 1.5-fold increase in enzymatic activity as compared to that observed for the synthetic medium. An increase that was also observed by Frantz and coauthors [23], who observed increases of up to 11-fold in the production of enzymes with the use of a by-product as a medium, as compared to the use of a synthetic medium.

When compared to Table 4, the increase in α-amylase production shown in Table 5, despite the same medium and strain being used, appears to be related to adaptations of the strain. It is common for microbial strains to undergo genetic changes after several replication procedures. This may occur as a result of its adaptation to the culture medium in which it is inoculated. Differences in biomolecule production, whether increased production or even inhibition of production, may have been a result of the above factors.

## CONCLUSION

The current study evaluated the potential of basidiomycete strains for α-amylase production, presenting to the scientific and industrial community the alternative of using agro-industrial by-products as an important strategy for increasing enzyme production. It should be noted that GF is a potential source for use in microbial α-amylase production, mainly by mushrooms of the genus *Ganoderma spp.* The work also demonstrated that CF4 and LGF wheat milling by-products might also be used as potential substrates for α-amylase production under SmC conditions. These by-products cost, on average, US$ 96.96 per ton, a lower cost compared to

other starch sources currently used in industrial enzyme production. The current work opens possibilities for process optimization and scale-up. Also, the use of different microbial strains and reuse of starch from wheat milling by-products which have commercial applications.

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## REFERENCES

1. Kaur K, Verma RK. Fungal resources: Current utilization, future prospects, and challenges. In: Singh J, Gehlot P, editors. New and Future Developments in Microbial Biotechnology and Bioengineering. 1th ed. Elsevier; 2021. p. 15–38.
2. Pinya S, Ferriol P, Tejada S, Sureda A. Mushrooms. reishi (*Ganoderma lucidum*), shiitake (*Lentinela edodes*), maitake (*Grifola frondosa*). In: Pinya S, Pere Ferriol ST, Sureda A, editors. Nonvitamin and Nonmineral Nutritional Supplements. 1th ed. Academic Press; 2019. p. 517–26.
3. Spier MR, Behsnilian D, Zielinski A, Konietzny U, Greiner R. Studies Towards the Stabilisation of a Mushroom Phytase Produced by Submerged Cultivation. Protein J. 2015 Oct; 34(5):367-79.
4. Paludo LC, Frantz SC, Ançay R, Stutz H, Dantas TLP, Spier MR. Optimization, kinetic and bioprocess parameters of amylases production from *Coprinus comatus* under submerged culture using starch-based simple medium: Partial enzyme characterization. Biocatal Agric Biotech. 2018 Oct;16(2018):529-37.
5. United States Department of Agriculture (USDA) [Internet]. c2020 [cited 2023]. Available from: https://apps.fas.usda.gov/psdonline/circulars/production.pdf.
6. Abitrigo [Internet]. Wheat is the energy for our body. c2020 [cited 2023]. Available from: http://www.abitrigo.com.br/conhecimento. 2020. Portuguese.
7. Abitrigo [Internet]. Wheat grinding presents stability in Brazil in 2021. c2022 [cited 2023]. Available from: https://www.abitrigo.com.br/moagem-de-trigo-apresenta-estabilidade-no-brasil-em-2021/. Portuguese
8. Silva JR, Angelo JA. Perspectiva: safra de trigo 2022. Análises e Indicadores do Agronegócio [Internet]. 2021 Jun [cited 2023]; 17(6):1-7. Available from: http://www.iea.agricultura.sp.gov.br/out/TerTexto.php?codTexto=16046#:~:text=A%20produ%C3%A7%C3%A3o%20mundial%20de%20trigo,o%20estimado%20para%202021%2F22. 2022. Portuguese.
9. Research and Markets [Internet]. Food Enzymes – Global Market Outlook (2017-2026). 2017 [cited 2023]. Available from: https://www.researchandmarkets.com/reports/4613533/food-enzymes-global-market-outlook-2017-2026#pos-38.
10. Peralta RM, Silva BP, Côrrea RCG, Kato CG, Seixas FAV, Bracht A. Enzymes from Basidiomycetes — Peculiar and Efficient Tools for Biotechnology. In: Brahmachari G, editor. Biotechnology of Microbial Enzymes - Production, Biocatalysis and Industrial Applications. 1th ed. Academic Press; 2017; p. 119-49.
11. Tiwari S, Srivastava R, Singh CS, Shukla K, Singh RK, Singh P, Singh R, Singh NI, Sharma R. Amylases: an Overview With Special Reference To Alpha Amylase. J Global Biosc. 2015;4(1):1886–901.
12. Grujić M, Dojnov B, Potočnik I, Duduk B, Vujčić Z. Spent mushroom compost as substrate for the production of industrially important hydrolytic enzymes by fungi *Trichoderma* spp. and *Aspergillus niger* in solid state fermentation. Int Biodeterior Biodegrad. 2015;104:290–8.
13. Research and Markets [Internet]. Alpha-amylase baking enzyme market analysis by source [Fungi, Bacteria (Maltogenic, G4), Plant-Based], by application (Breads, Cookies & Biscuits, Desserts) and segment forecasts to 2024. 2019 [cited 2023]. Available from: https://www.researchandmarkets.com/reports/3765920/alpha-amylase-baking-enzyme-market-analysis-by#pos-2.
14. Gois IM, Santos AM, Silva CF. Amylase from *Bacillus* sp. produced by solid state fermentation using cassava bagasse as starch source. Braz Arch Biol Technol. 2020;63:1–9.
15. Abdelkrim T, El-Mokhtar DA, Radia A, Bouziane A. Screening and Characterization of *Bacillus* Strains Producing Highly Thermostable Amylase from Various Hot Springs of Algeria. Braz Arch Biol Technol. 2021; 64:1–9.
16. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial α-amylases: A biotechnological perspective. Process Biochem. 2003 Jun 30;38(11):1599–616.
17. Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enz Microb Technol. 2010 Jun 7;46(7):541–9. Epub 2010 Jun 7.
18. Sundarram A, Murthy TP. α-Amylase production and applications: A review. J Appl Environ Microb. 2014 Jun 15;2(4):166–75.
19. Anto H, Trivedi UB, Patel KC. Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. Bioresour Technol. 2006 Jul;97(10):1161–6.

20. Aguiar MM, Ferreira LFR, Monteiro RTR. Use of vinasse and sugarcane bagasse for the production of enzymes by lignocellulolytic fungi. Braz Arch Biol Technol. 2010 Oct;53(5):1245-54.
21. Fuwa H. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J Biochem. 1954;41(5):583–603.
22. Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem. 1959; 31(3):426–8.
23. Frantz SC, Paludo LC, Stutz H, Spier MR. Production of amylases from *Coprinus comatus* under submerged culture using wheat-milling by-products: Optimization, kinetic parameters, partial purification and characterization. Biocatal Agric Biotech. 2019 Jan;17(2019):82-92.
24. Palit S, Banerjee R. Optimization of extraction parameters for recovery of α-amylase from the fermented bran of *Bacillus circulans* GRS313. Braz Arch Biol Technol. 2001 Mar;44(1):107–11.
25. Souza HQ, Oliveira LA, Andrade JS. Seleção de Basidiomycetes da Amazônia para produção de enzimas de interesse biotecnológico. Ciênc Tecnol Aliment. 2008;28:116–24. Portuguese.
26. Goud MJP, Suryam A, Lakshmipathi V, Charya MAS. Extracellular hydrolytic enzyme profiles of certain South Indian basidiomycetes. African J Biotech. 2009;8(3):354–60.
27. Linden A, Mayans O, Meyer-Klaucke W, Antranikian G, Wilmanns M. Differential Regulation of a Hyperthermophilic α-Amylase with a Novel (Ca,Zn) Two-metal Center by Zinc. J Biol Chem. 2003 DEc 12;278(11):9875–84.
28. Singh R, Kapoor V, Kumar V. Production of thermostable, $Ca^{+2}$-independent, maltose producing α-amylase by *Streptomyces* sp. MSC702 (MTCC 10772) in submerged fermentation using agro-residues as sole carbon source. Ann Microbiol. 2012;62(3):1003–12.
29. Krupodorova T, Ivanova T, Barshteyn V. Screening of Extracellular Enzymatic Activity of Macrofungi. J Microb Biotech Food Sci. 2014 Feb 1;3(4):315–8.
30. Tatsumi E, Konishi Y, Tsujiyama S. Application of residual polysaccharide-degrading enzymes in dried shiitake mushrooms as an enzyme preparation in food processing. Biotech Lett. 2016 Jul 20;38(11):1923–8.
31. Jonathan SG, Adeoyo OR. Evaluation of ten wild Nigerian mushrooms for amylase and cellulase activities. Mycobiol. 2011 Jun;39(2):103–8.
32. Peron-Schlosser B, Carpiné D, Jorge RMM, Spier MR. Optimization of wheat flour by product films: Atechnological and sustainable approach for bio-based packaging material. J Food Sci. 2021 Sep 25; 86(10):4522-38.
33. Harder W, Dykhuizen L. Physiological responses to nutrient limitations. Ann Rev Microbiol. 1983; 37:1-23.
34. Saranraj P, Stella D. Fungal amylase – A review. Inter J Microb Res. 2013; 4(2):203-11.
35. Flegg PB, Spencer DM, Wood DA. The biology and technology of the cultivated mushroom. 1th ed. Chichester: John Wiley, 1985.