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Production of amylases from *Coprinus comatus* under submerged culture using wheat-milling by-products: Optimization, kinetic parameters, partial purification and characterization

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ABSTRACT

Amylases are enzymes that hydrolyze starch, and are mostly produced from microbial sources. This study aimed to evaluate the potential of utilization of wheat milling by-products for the production of amylases by a macromycete strain, *Coprinus comatus*, recently discovered as a potential producer of amylases under submerged culture. The best results of enzymatic activity (6.07 U mL^{-1} for α-amylase and 0.64 U mL^{-1} for glucoamylases) were obtained in a medium containing 10 g L^{-1} of low-grade flour (LGF) as substrate. The composition of the culture medium was optimized by experimental designs. The enzymatic activities obtained in the optimized culture medium (105 g L^{-1} LGF, 3.76 g L^{-1} urea and 7.52 g L^{-1} K_2HPO_4) were 59.40 ± 0.56 U mL^{-1} and 12.65 ± 0.09 U mL^{-1} for α-amylases and glucoamylases, respectively, representing an increase of 9.78 and 19.77 fold in the initial enzymatic production. High productivity (0.59 U mL^{-1} h^{-1} for α-amylase and 0.11 U mL^{-1} h^{-1} for glucoamylase) and product formation yield on substrate consumed (814.28 U gds^{-1} for α-amylase and 231.24 U gds^{-1} for glucoamylase) were obtained in the kinetic study. These results show that the use of LGF medium represents a low-cost alternative for the production of amylases by the species studied. The characterization of the partially purified enzymes showed optimal activity at temperature of 50 °C and pH 5.0, in addition to the independence of the calcium ion, a desirable characteristic for amylases produced for industrial application.

1. Introduction

Amylases are enzymes that catalyze the hydrolysis of starch into dextrins, maltose and glucose (Simair et al., 2017). These enzymes are applied in several industrial processes, like processes in textile, pharmaceutical, pulp and paper, feed, detergent and food industries (Sindhu et al., 2017; Tiwari et al., 2015). In the food industry, amylases play an important role in the production of syrups and sweeteners, in baking, in cereals processing and beverage production, for example (Sundarram and Murthy, 2014; Tiwari et al, 2015; Karam et al., 2017). Due to their wide application range, the amylases represent 25% of the world enzyme market (Naili et al., 2016; Sindhu et al., 2017).

Commercially and industrially used amylases are mostly obtained from microbial sources. In recent years, the investigation of new amylase producing microorganisms has been of great interest. However, there are only a few reports of the use of macromycetes fungi, or mushrooms, for amylase production, although they are known as potential enzyme producers (Spier et al., 2015; Peralta et al., 2017).

The submerged culture (SmC) has been more used for the production of several microbial metabolites for being easy to handle and allowing greater control of process parameters when compared to the solid-state culture (SC) (Gangadharan et al., 2008; Naili et al., 2016). The efficiency of amylase production in SmC is influenced not only by the microbial source, but also by the correct manipulation of a set of parameters, such as the composition of the culture medium, pH, agitation, aeration, and temperature (Suárez-Arango and Nieto, 2013). The process for the production of enzymes from microbial sources under SmC can present high costs, which have been attributed mainly to the formulation of culture media and energy consumption of bioreactors (Mouna-imen and Mahmoud, 2015; Panesar et al., 2016). In this way, the optimization of bioprocess parameters and the use of agro-industrial residues in the production of amylases have been studied in recent years, as alternatives to reduce process costs.

Among the various agro-industrial segments, wheat processing is

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one of the most relevant. In the 2017/2018 harvest, 750 million tons of this grain were produced (USDA, United States Department of Agriculture, 2018), with an approximate yield of 75% for flour production. The remaining 25% (which would correspond to more than 180 million tons of the world production) are considered as agro-industrial waste and by-products. Wheat bran, germ, low-grade flours and mill cleaning waste are examples of processing residues (Neves et al., 2006; Clément et al., 2018), which are usually intended for animal feed production, even with the high potential for use in biotechnological processes. Wheat bran, for example, has already been used in studies for the production of macromycete amylases (El-Zalaki and Hamza, 1979; Kekos et al., 1987; Zilly et al., 2012). However, there are no records of the utilization of other wheat processing by-products for amylase production from macromycete species.

In previous studies (Paludo et al., 2018) *Coprinus comatus*, an edible macromycete strain, was screened as a potential producer of amylases by submerged culture using a synthetic culture medium. In this way, the aim of this work was to characterize the different residues and by-products obtained from the wheat milling process, to verify the potential of their use for the production of amylases from *Coprinus comatus* by submerged culture and then to optimize the composition of the culture medium using experimental designs. In addition, the parameters of the optimized bioprocess were calculated and compared with the preliminary studies using this species, and the enzymes produced were partially purified and characterized.

2. Material and methods

2.1. Wheat milling by-products

The wheat milling by-products used as substrates for the production of amylases in submerged culture, as well as the stage they were obtained and the destination of each of them, are presented in Table 1. The residues were characterized for starch content (Association of Official Analytical Chemists, 1995), moisture, protein and ash (Association of Official Analytical Chemists, 2005). Quantification of minerals (Calcium, Phosphorus, Magnesium, Potassium and Sodium) was performed according to the AOAC methodology 999.10 (2005).

2.2. Strain and inoculum

The strain *Coprinus comatus* was donated by the Mushroom Bioprocess Laboratory (LBC) of the *Midwestern* State University (UNICENTRO, PR) and maintained in Potato Dextrose Agar (PDA, Neogen®, USA) pH 6.0, previously autoclaved (121 °C/ 15 min) and inclined. The strain was stored at room temperature for up to 3 months. For reactivation, mycelial fragments of the sloped tubes were peeled on the surface of the same medium in Petri dishes and incubated at 28 ± 2 °C for 7 days.

The pre-inoculum was prepared initially on Czapek medium sterile containing (g L^{-1}): Sucrose (30.00), $NaNO_3$ (2.00), K_2HPO_4 (1.00), $MgSO_4{\cdot}7H_2O$ (0.50), KCl (0.50), $FeSO_4$ (0.01), with pH adjusted to 6.0 (± 0.2) and the flasks incubated at 28 ± 2 °C for 5 days (Spier et al., 2015). After the first experiments (results shown in Section 3.1), the greater adaptation of the strain and greater production of biomass in liquid medium containing the low-grade flour were verified. Therefore, the pre-inoculum for the other experiments (Section 3.2 onwards) was prepared in medium formulated only from the residue itself at a concentration of 5 g L^{-1}. Erlenmeyer flasks containing 100 mL of each medium were autoclaved (121 °C/ 15 min). They were then inoculated with 5 mycelial fragments (5 mm diameter) and incubated in a rotary shaker (TE-421, Tecnal®, Brazil, 28 °C - 120 rpm). For the experimental tests, the biomass of the pre-inoculum was drained with the aid of a sterile stainless steel sieve and used to inoculate the test vials in the proportion of 0.5% (m/v).

2.3. Substrate selection for amylases production

To evaluate if any of the by-products described in subitem 2.1 would be more favorable for the production of *C. comatus* amylases, media containing 10 g L^{-1} of each residue were formulated without any type of supplementation. One hundred mL of each medium were dispensed in 250 mL Erlenmeyer flasks and autoclaved. The pre-inoculum in Czapek medium was drained and inoculated as described above (item 2.2). Culture samples were taken every 24 h for 7 days to determine the enzymatic activities (α-amylase and glucoamylase, subitem 2.6.1).

2.4. Optimization of culture medium composition

2.4.1. Fractional Factorial Design

Preliminary tests (data not shown) indicated that the addition of urea, K_2HPO_4, $MgSO_4$ and $CaCl_2$ in the culture medium containing 10 g L^{-1} LGF increased the production of amylases from *C. comatus*. A fractional factorial design 2^{5-1} (3 central points, totaling 19 runs) was then applied to evaluate the effect of the concentration of LGF, urea, K_2HPO_4, $MgSO_4$ and $CaCl_2$ as components of the culture medium in the production of amylases from *C. comatus*. Each variable was studied at three levels (-1, 0 and +1, Table 2). The levels chosen for urea, K_2HPO_4, $MgSO_4$ and $CaCl_2$ were based on concentrations previously used in synthetic medium and for the supplementation tests. The highest level (+1) for LGF was kept in 35 g L^{-1} due to the high viscosity acquired by the medium after autoclaving when higher concentrations were used.

2.4.2. Central Composite Rotatable Design (CCRD)

The significant variables ($p \leq 0.05$) from the Fractional Factorial Design were evaluated in a Central Composite Rotatable Design (CCRD) with three replicates at the central point and six axial points (2^3 plus star configuration, with 16 runs each). The variables studied and the coded and decoded levels are given in Table 3.

To achieve the optimization in CCRD, and based on preliminary experiments, the LGF, urea and K_2HPO_4 concentration range was increased. In view of the high concentrations studied (Table 3), the LGF was autoclaved separately from liquid media containing urea and K_2HPO_4 to avoid excessive viscosity of the culture medium after autoclaving. The study ranges for urea and K_2HPO_4 were also increased (level −1 near the maximum amounts studied in fractional factorial design and level −1.68 kept at 0, considering possible interaction with high substrate concentrations).

The production of amylases was carried out in submerged culture in 250 mL Erlenmeyer flasks with 100 mL of culture medium, at pH 6.0, in a rotary shaker (TE-421, Tecnal®, Brazil) at 28 °C and 120 rpm. After

Table 1
Wheat milling by-products: Wheat bran (WB), Low Grade Flour (LGF), Glue Flour (GF), Clean-out Flour (CF). Origin and destination.

By-product	Initials	Description
Wheat Bran	WB	Waste from the outer layers of wheat grain. It is usually intended for the production of animal feed.
Low-Grade Flour	LGF	Remaining flour at the end of the milling and reduction systems, which contains high levels of non-endosperm material.
Glue Flour	GF	Common wheat flour out of the standard of quality. It is forwarded to adhesive manufacturing industry.
“Clean-out” Flour	CF	Residual flour from silos, pipes and floor. It does not have a defined destination.

Table 2
Coded and decoded levels of the independent variables used in Fractional Factorial Design 2^{5-1} to optimize the production of amylases from *Coprinus comatus* in SmC. X1 = LGF; X2 = Urea; X3 = K_2HPO_4; X4 = $MgSO_4$ and X5 = $CaCl_2$.

		Coded and decoded levels		
Variables (g L^{-1})		-1	0	+ 1
LGF	X1	5.00	20.00	35.00
Urea	X2	0.30	0.80	1.30
K_2HPO_4	X3	0.30	1.05	1.80
$MgSO_4$	X4	0.30	0.75	1.20
$CaCl_2$	X5	0.30	0.75	1.20

Table 3
Proposed levels for the independent variables (LGF, Urea, and K_2HPO_4 concentration) studied in the Central Composite Rotatable Design.

	Coded and decoded levels				
Independent variables (g L^{-1})	-1.68	-1	0	+ 1	+ 1.68
LGF	10.00	48.50	105.00	161.50	200.00
Urea	0.00	1.00	2.50	4.00	5.00
K_2HPO_4	0.00	2.00	5.00	8.00	10.00

120 h of culture, samples were taken for the α-amylase and glucoamylase activity assay (subitem 2.6.1).

Experimental designs were developed and analyzed using Statistica Software® 7.0, for Windows (Statsoft®, United States). All experiments were performed randomly, with responses of α-amylase and glucoamylase activities. The fit of the equation of the second order model was expressed by the coefficient of determination R^2, and the statistical significance was determined by the F-test (ANOVA).

The mathematical model was externally validated by conducting experiments using the optimal values of the variables predicted by the response surface. The desirability index, also obtained by Statistica Software® 7.0, was applied in order to find the optimum process condition for both responses (α-amylase and glucoamylase activities).

2.5. Final kinetics of the optimized medium

After the definition of the ideal conditions for the production of the enzymes, a final kinetics were performed using the optimized medium with duration of 240 h (10 days). The tests were conducted in 250 mL Erlenmeyer flasks incubated at 28 °C and under 120 rpm of orbital shaking. Samples were collected every 12 h and analyzed for enzymatic activities of α-amylase and glucoamylase (subitem 2.6.1), amount of substrate (quantified in starch, subitem 2.6.2), biomass (subitem 2.6.3) and pH (subitem 2.6.4).

2.6. Analytical methods

2.6.1. Enzymatic activities

The reaction medium for the determination of the α-amylase and glucoamylase activities contained 250 µL 1% starch solution (m/v) in 0.1 M sodium acetate buffer (pH 6.0) and 250 µL crude enzyme extract (CEE), properly diluted in the same buffer. The reaction was conducted in a water bath at 50 °C for 15 min. The activity of α-amylase (saccharifying activity) was determined by the iodine-iodide method, modified from Xiao et al. (2006) and Fuwa (1954), based on the decrease in the blue color of the starch-iodine complex. One unit of α-amylase was defined as the amount of enzyme capable of convert 0.1 mg of substrate per min under the assay conditions. The activity of glucoamylase was determined by the dinitrosalicyclic acid method (DNS), according to Miller (1959). One unit (U) of glucoamylase activity was defined as the amount of enzyme that releases 1 µmol of glucose per min under the assay conditions.

2.6.2. Starch determination

The quantification of starch in the culture samples was performed by enzymatic hydrolysis, following a methodology adapted from the AOAC (996.11). Culture samples were taken every 12 h and kept in a boiling bath for 10 min for inactivation of the amylases. Two mL of each sample were transferred to 15 mL polypropylene tube. 8 mL 95% (v/v) ethanol was added in order to extract the glucose and dextrins present in the sample. The tubes were vortexed and held at room temperature for 30 min. After centrifugation (Centribio® centrifuge, Brazil) at 1000 g/ 15 min, the supernatant was discarded and the pellet retained was diluted in 1 mL water. The samples were then hydrolyzed with α-amylase (Spring Alfa, Granotec®, Brazil, at a 100 U mL^{-1} concentration) and glucoamylase (Spring AG, Granotec®, Brazil, at a 330 U mL^{-1} concentration) diluted, and the glucose produced by the hydrolysis of the remaining starch was measured colorimetrically (λ = 505 nm) using glucose oxidase/peroxidase reagent (Bioliq Glucose, Laborclin®, Brazil). The starch content of the samples was calculated by multiplying the final glucose result by 0.9 (to convert free glucose to starch), and the results were presented in g L^{-1} of culture medium.

2.6.3. Biomass determination

The biomass was determined using the thermogravimetric method. The liquid culture was filtered through a porcelain funnel with filter paper (Whatman n°1), kitassate and vacuum pump (Suctron Eletronic, Schuster®, BR). The filter paper containing the biomass retained after filtration was dried at 105 °C until constant weight.

2.6.4. pH determination

The determination of pH throughout the process was carried out by direct measurement of the fermented broth in pHmeter (Luca®, Brazil).

2.7. Bioprocess parameters

The bioprocess parameters duplication time (t_d), maximum specific growth rate ($\mu_{máx}$) and maximum productivity in product ($Y_{pmáx}$) were calculated from the experimental data (Eqs. 1–3) during the logarithmic phase of the growth and production of the enzymes (from 36 to 120 h). The total biomass productivity and total product productivity (amylases) were calculated in total culture time, using Eqs. 4 and 5.

$$\mu_{máx} = \frac{\ln X_f - \ln X_i}{t_f - t_i} \quad (1)$$

$$t_d = \frac{\ln 2}{\mu_{max}} \quad (2)$$

$$\gamma_{pmax} = \frac{P_{flog} - P_{ilog}}{t_{flog}} \quad (3)$$

$$\gamma_{xtotal} = \frac{X_f - X_i}{t_t} \quad (4)$$

$$\gamma_{Ptotal} = \frac{P_f - P_i}{t_t} \quad (5)$$

Where: t_d = duplication time (h); $\mu_{máx}$ = maximum specific growth rate (h^{-1}); t_i and t_f = initial and final time (h); X_i and X_f = initial and final biomass concentration (g L^{-1}); Y_{xtotal} = total biomass productivity (g L^{-1} h^{-1}); t_t = total culture time (h); Y_{pmax} = maximum productivity (α-amylase and glucoamylase) (U mL^{-1} h^{-1}); P_{Ilog} and P_{Flog} = product concentration in initial and final times of log phase; Y_{ptotal} = total product productivity (α-amylase and glucoamylase) (U mL^{-1} h^{-1}); P_i and P_f = initial and final product concentration (g L^{-1});

Table 4
Enzymatic activity, maximum activity and productivity of α-amylase and glucoamylase by the strain *C. comatus* at 72, 96 and 120 h of culture in medium containing 10 g L^{-1} of the different residues tested in culture at 28 °C and 120 rpm agitation.

Cultivation medium /Time (h)		α-amylase			Glucoamylase		
		Activity (U mL^{-1})	Maximum activity (U mL^{-1})	Productivity (U mL h^{-1})	Activity (U mL^{-1})	Maximum activity (U mL^{-1})	Productivity (U mL h^{-1})
WB	72	3.69 ± 0.07	5.06 ± 0.14[b]	0.05	0.29 ± 0.21	0.56 ± 0.08[a]	0.004
	96	4.41 ± 0.16		0.05	0.51 ± 0.10		0.005
	120	5.06 ± 0.14		0.04	0.56 ± 0.08		0.005
LGF	72	4.48 ± 0.92	**6.07 ± 0.03[a]**	0.06	0.26 ± 0.02	**0.64 ± 0.03[a]**	0.004
	96	5.90 ± 0.06		0.05	0.46 ± 0.02		0.005
	120	6.07 ± 0.03		0.05	0.64 ± 0.03		**0.005**
GF	72	4.52 ± 0.24	4.97 ± 0.07[b]	0.06	0.20 ± 0.01	0.46 ± 0.01[ab]	0.003
	96	4.72 ± 026		0.05	0.37 ± 0.01		0.004
	120	4.97 ± 0.07		0.04	0.46 ± 0.01		0.004
CF	72	4.39 ± 0.23	4.88 ± 0.06[b]	0.06	0.24 ± 0.02	0.40 ± 0.13[ab]	0.003
	96	4.84 ± 0.88		0.05	0.37 ± 0.08		0.004
	120	4.88 ± 0.06		0.04	0.40 ± 0.13		0.003

Mean of the experiments ± standard deviation. Different letters in the same column represent mean values with significant difference between them ($p \leq 0.05$) and equal letters represent mean values without significant difference ($p > 0.05$).

2.8. Yield factors

The biomass yield on substrate ($Y_{X/S}$), product formation yield on substrate consumed ($Y_{P/S}$) and product formation yield on biomass formation ($Y_{P/X}$) were calculated using Eqs. 6 – 8.

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{P_f - P_i}{S_i - S_f} \quad (6)$$

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X_f - X_i}{S_i - S_f} \quad (7)$$

$$Y_{P/X} = \frac{\Delta P}{\Delta X} = \frac{P_f - P_i}{X_f - X_i} \quad (8)$$

Where: X_i and X_f = initial and final biomass concentration (g L^{-1}); P_i and P_f = initial and final product concentration (U mL^{-1}); S_i and S_f = initial and final substrate concentration (g L^{-1});

2.9. Partial purification of the enzyme extract

The produced amylases were partially purified (low-resolution purification) from the crude enzyme extract obtained from the optimized medium culture, following the methodology described by Spier et al. (2015). This process uses the precipitation of the protein by the salting-out method with 80% saturation ammonium sulfate followed by desalination and concentration in centrifugal ultrafiltration units (cut-off - 10 kDa, Vivaspin 6, GE®, UK). The concentrated enzymatic extract (retentate fraction) was used to test the effect of temperature, pH and metallic ions.

2.10. Effect of pH and temperature on α-amylase and glucoamylase activity

To evaluate the pH effect on α-amylase and glucoamylase activities, sodium acetate buffers 0.1 M (pH 4.5, 5.0, 5.5 and 6.0) and sodium phosphate buffers 0.1 M (pH, 6.5 and 7.0) were used. The time and temperature conditions of the enzymatic assays were the same as those used in the standard assay described above (subitem 2.6.1). The temperature effect on the amylolytic activity was then determined using the standard pH and reaction time of the enzymatic assay at 40 and 60 °C, in addition to the standard 50 °C. The relative activity (%) was expressed as the ratio between the enzymatic activity obtained in the respective pH and temperature and the activity obtained in the standard conditions.

2.11. Effect of metal ions on the amylase activity

The effect of metal ions (Ca^{2+}, Mg^{2+} and Mn^{2+}) on the α-amylase and glucoamylase activity was evaluated using 0.1 M acetate buffer, pH 5.0. The ions in the final concentration of 5 mM were added to the buffer used in the enzymatic reaction in the form of calcium chlorides (Prolab®, Brazil) and magnesium and manganese sulfates (Synth®, Brazil). The activity without addition of the metal ions was considered as 100%.

2.12. Statistical analysis

Replicates were performed for each experiment and the results were expressed as mean ± standard deviation. Data were analyzed according to analysis of variance (ANOVA). The homogeneity of the groups was identified and the difference between means was performed using the Tukey test at a 95% confidence level ($p \leq 0.05$). Analyzes were performed using Statistica® 7.0 software for Windows (Statsoft®, USA).

3. Results and discussion

3.1. Substrate characterization and selection for amylase production

Amylase production using alternative media containing 10 g L^{-1} of each residue under study was evaluated throughout 7 days of culture. The results of enzymatic activity obtained at 72, 96 and 120 h culture are shown on Table 4. After 120 h of culture, the enzymatic activity decreased considerably, so the results were not shown.

For all the media formulated with the wheat by-products, the maximum enzymatic activity for the α-amylase and glucoamylase was reached at 120 h culture. Statistically significant difference ($p \leq 0.05$) was verified between the α-amylase activity obtained in the media formulated with the residue. The highest enzymatic activity for this enzyme was obtained in the medium containing low-grade flour (LGF), corresponding to 6.07 ± 0.03 U mL^{-1} (α-amylase) and 0.64 ± 0.03 U mL^{-1} (glucoamylase).

The by-products used were characterized as to their moisture, ashes, proteins and starch content and the profile of some minerals. The results obtained are shown on Table 5. The low-grade flour (LGF) presents the highest protein content (18,02%) and balanced ashes and starch content (approximately 2,7% and 63%, respectively).

LGF is a flour with low purification degree, obtained at the end of the milling and reduction systems (Neves et al., 2006). Flours of this kind might present varied composition, depending on the process used.

Table 5
Composition of wheat by-products used for the production of amylases from *Coprinus comatus*. Ash, protein and starch contents expressed as % of dry matter.

Composition (%)					Profile of minerals (mg/100 g)				
Residue	Moisture	Ashes	Proteins	Starch	Ca^{2+}	P	Mg^{2+}	K^{+}	Na^{+}
WB	12.98	5.02	15.80	43.95	122.002	379.499	153.603	727.387	31.885
LGF	13.24	2.68	18.02	63.91	64.593	760.136	272.259	816.837	34.708
GF	13.24	0.85	15.18	78.98	35.137	149.135	43.331	182.241	27.416
CF	13.48	0.92	14.57	81.07	50.751	156.794	46.825	219.279	252.841

However, they are mainly constituted of the endosperm extremities and the layers known as sub-aleurone and aleurone, rich in proteins and ashes contents (Clément et al., 2018). This justifies the protein content in the flour and the largest quantities of important minerals to the microbial growth and enzyme production, such as phosphorus (760 mg/ 100 g), magnesium (272.259 mg/ 100 g) and potassium (816 mg/ 100 g).

LGF is then a good source of carbon, minerals and a better nitrogen source when compared to other by-products. This justifies the better results of α-amylase and glucoamylase activity obtained with the use of this residue, since the carbon, nitrogen and mineral sources are important for the regulation of growth and production of microbial metabolites.,

According to Neves et al. (2006), bran, low-grade flours and germ are the most representative wheat processing by-products and correspond to around 25% of the volume processed. Wheat bran (WB) was used by El-Zalaki and Hamza (1979), Kekos et al. (1987) and Zilly et al. (2012) in studies aiming at producing amylases with macromycetes. But there are few studies related to the production of amylases using other wheat by-products, mainly using fungi from the macromycete group. Among the few reports found, Kammoun et al. (2008) and Naili et al. (2016) used a wheat milling by-product with composition similar to the LGF employed in this study (60% starch, 5% other carbohydrate and 13.65% protein) to produce amylases under submerged culture of fungi of the genus *Aspergillus*. Kammoun et al. (2008), employing a concentration of 2.5% substrate supplemented by peptone, yeast extract, NaCl, KH_2PO_4, $MgSO_4$ and $CaCl_2$, obtained 21.81 U mL^{-1} α-amylase activity, at 25 °C and 150 rpm. The results are higher than the ones found in this study, but it is relevant to mention that they were obtained with higher concentrations of substrate and in the presence of other components in the culture medium.

The results obtained revealed that all the wheat by-products evaluated are able to induce the production of amylases from *C. comatus*. However, taking into consideration the results of the enzymatic activity, the easiness to manipulate the residue and the formulation of culture media, in addition to the inexistence of other studies employing this kind of material in the production of amylases using fungi from the macromycete group, the low-grade flour (LGF) was selected for the following phases of optimization of the amylase production.

3.2. Fractional factorial design

The matrix of tests carried out with the codified values of the variables under study and the responses obtained for the α-amylases and glucoamylases are presented in Table 6.

For α-amylase, the highest response obtained in the design was 37.85 U mL^{-1} (test 8). In contrast, the lowest response was 0.76 U mL^{-1} (test 11), in which LGF and K_2HPO_4 were found at level −1. The glucoamylase activities varied between 0.08 U mL^{-1} (test 5) and 5.76 U mL^{-1} (test 8). From the results obtained, the effects of each of the variables were calculated on the α-amylase and glucoamylase, which are presented in Table 7.

The results presented in the Table 7 show that the concentration of substrate, urea and K_2HPO_4 presents significant and positive effects ($p \leq 0.05$) in both responses evaluated (α-amylase and glucoamylase activities), that is, the higher the concentration of these components is, within the concentration range studied,the higher the responses obtained are. The effect of $MgSO_4$ and $CaCl_2$ concentrations was not significant for any of the responses. For α-amylase these effects were positive, which indicates that the higher the $MgSO_4$ and $CaCl_2$ concentrations are, the higher this enzyme activity is. This occurs inversely for glucoamylases, in which the effects of $MgSO_4$ and $CaCl_2$ concentrations were negative.

Table 6
Fractional Factorial Design 2^{5-1} used to evaluate the effects of the components of the culture medium: X1 = LGF; X2 = Urea; X3 = K_2HPO_4; X4 = $MgSO_4$ and X5 = $CaCl_2$ in the production of amylases and glucoamylases from *Coprinus comatus* in SmC, pH 6.0, at 28 °C and 120 rpm.

	Variables					Enzymatic activity (U mL^{-1})	
Runs	X1	X2	X3	X4	X5	α-amylase[a]	Glucoamylase[a]
1	− 1	− 1	− 1	− 1	1	10.43 ± 0.450	1.68 ± 0.010
2	1	− 1	− 1	− 1	− 1	14.05 ± 0.710	3.78 ± 0.020
3	− 1	1	− 1	− 1	− 1	1.05 ± 0.060	1.00 ± 0.010
4	1	1	− 1	− 1	1	20.07 ± 0.510	4.64 ± 0.010
5	− 1	− 1	1	− 1	− 1	6.45 ± 0.570	0.08 ± 0.010
6	1	− 1	1	− 1	1	15.79 ± 0.130	4.72 ± 0.030
7	− 1	1	1	− 1	1	26.60 ± 1.100	2.14 ± 0.010
8	1	1	1	− 1	− 1	37.85 ± 0.630	5.76 ± 0.040
9	− 1	− 1	− 1	1	− 1	11.66 ± 0.180	1.97 ± 0.030
10	1	− 1	− 1	1	1	15.73 ± 0.240	2.91 ± 0.040
11	− 1	1	− 1	1	1	0.76 ± 0.200	0.88 ± 0.010
12	1	1	− 1	1	− 1	20.72 ± 0.790	3.98 ± 0.050
13	− 1	− 1	1	1	1	16.04 ± 1.780	1.98 ± 0.003
14	1	− 1	1	1	− 1	20.46 ± 0.430	3.61 ± 0.060
15	− 1	1	1	1	− 1	25.84 ± 0.350	2.84 ± 0.003
16	1	1	1	1	1	33.65 ± 1.130	3.72 ± 0.050
17	0	0	0	0	0	11.86 ± 0.410	3.14 ± 0.020
18	0	0	0	0	0	13.44 ± 0.120	3.35 ± 0.010
19	0	0	0	0	0	12.63 ± 0.110	2.95 ± 0.200

[a] The results are expressed as mean ± standard deviation. Average of three determinations.

Among the several limiting factors of growth and microbial metabolic regulation, the sources of carbon and nitrogen are considered the most important. Phosphate also plays an important regulating role, it is present in specific transportation chains for several micro-organisms, in the composition of nucleic acids and cell membranes (Harder and Dykhuizen, 1983; Gupta et al., 2003; Ramesh and Murty, 2014). This fact justifies the significant and positive effects obtained for these variables in the fractional design.

The presence of magnesium is important in the growth and microbial metabolite production and the presence of calcium has been reported as significant for the production of amylases (Kammoun et al., 2008; Ramesh and Murty, 2014; Sundarram and Murthy, 2014; Dutta et al., 2016). However, the quantity of these minerals naturally present in the LGF substrate (Table 5), might have supplied the needs of the culture when its concentrations varied, which justifies the non-significant effects of the concentrations under study.

The results obtained in the fractional factorial design showed which variables were really relevant and allowed the exclusion of unnecessary compounds for the next optimization phase. From the effects evaluated,

Table 7
Effect of the factors studied in Fractional Factorial Design 2^{5-1} on α-amylase and glucoamylase activity (U mL^{-1}). X1 = LGF; X2 = Urea; X3 = K_2HPO_4; X4 = $MgSO_4$ and X5 = $CaCl_2$.

	α-amylase				Glucoamylase			
Factors	**Effect**[a]	**Standard error**	**t (3)**	**p – value**	**Effect**[a]	**Standard error**	**t (3)**	**p – value**
Mean	16.58468	0.99698	16.63485	0.00047*	2.90009	0.07124	40.70814	0.00003*
X1	9.93837	2.17288	4.57383	0.01961*	2.56844	0.15527	16.54221	0.00048*
X2	6.98984	2.17288	3.21686	0.04870*	0.53058	0.15527	3.41725	0.04193*
X3	11.02678	2.17288	5.07474	0.01478*	0.50158	0.15527	3.23044	0.04820*
X4	1.57226	2.17288	0.72358	0.52164	− 0.23924	0.15527	− 1.54082	0.22101
X5	0.12337	2.17288	0.05678	0.95829	− 0.04428	0.15527	− 0.28522	0.79404

[a] Effects expressed in U mL^{-1}.
* $p \leq 0.05$.

the LGF, urea and K_2HPO_4 concentrations were optimized using a central composite rotatable design (CCRD).

3.3. Central Composite Rotatable Design

The matrix of tests carried out in the CCRD, containing the levels studied and the values observed for the α-amylase and glucoamylase activities is presented in Table 8.

The α-amylase activities varied between 10.57 and 55.48 U mL^{-1} in tests 9 and 12, respectively. For the glucoamylase, the lowest activity was observed also in test 9 (3.81 U mL^{-1}), while the highest activity was seen in test 7 (12.80 U mL^{-1}).

The enzymatic activity highest results (in bold in the table) were obtained in tests in which at least one of the variables was at level 0, + 1 and + 1.68. From the analysis of effects (data not presented), the α-amylase production was observed to be mostly influenced by the LGF and urea concentrations, whose linear terms exercised significant and positive effects (p = 0.007 and p = 0.0005, respectively). On the other hand, the quadratic term (X_1^2) of the variable LGF (p = 0.010) had a negative significant effect.

The glucoamylase production was significantly influenced by the three variables studied. The linear terms of the variables urea and K_2HPO_4 presented significant and positive effects (p = 0.029 and p = 0.047). In contrast, the LGF quadratic term presented negative effect (p = 0.013). The LGF linear term presented the value p = 0.058.

Due to the influence of the variables in the production of amylases and that the exclusion of any of the terms could hamper the ability of the model to describe the process behavior, the variance analysis (ANOVA) was carried out considering all the terms (linear and quadratic). The ANOVA results are presented in Table 9.

Table 8
Central Composite Rotatable Design (CCRD) for production of α-amylase and glucoamylase from *Coprinus comatus* in SmC at pH 6.0, 28 °C and 120 rpm.

	Variables			Enzymatic Activity (U mL^{-1})	
Runs	LGF	Urea	K_2HPO_4	α-amylase[a]	Glucoamylase[a]
1	− 1.00	− 1.00	− 1.00	22.09 ± 0.10	7.06 ± 0.21
2	− 1.00	− 1.00	1.00	21.65 ± 0.00	7.68 ± 0.15
3	− 1.00	1.00	− 1.00	49.01 ± 0.05	8.64 ± 0.49
4	− 1.00	1.00	1.00	**54.78 ± 0.79**	**11.36 ± 0.17**
5	1.00	− 1.00	− 1.00	35.88 ± 0.20	7.15 ± 0.44
6	1.00	− 1.00	1.00	45.26 ± 0.40	9.80 ± 0.34
7	1.00	1.00	− 1.00	**50.55 ± 0.15**	**12.80 ± 0.65**
8	1.00	1.00	1.00	**54.26 ± 0.05**	**11.42 ± 0.36**
9	− 1.68	0.00	0.00	10.57 ± 0.35	3.81 ± 0.07
10	1.68	0.00	0.00	43.58 ± 0.79	8.72 ± 0.67
11	0.00	− 1.68	0.00	16.60 ± 0.84	7.87 ± 0.20
12	0.00	1.68	0.00	**55.48 ± 0.39**	10.95 ± 0.21
13	0.00	0.00	− 1.68	34.73 ± 0.05	5.89 ± 0.35
14	0.00	0.00	1.68	**54.54 ± 0.15**	**12.47 ± 0.45**
15[a]	0.00	0.00	0.00	52.23 ± 0.29	**12.71 ± 0.67**
16[a]	0.00	0.00	0.00	54.57 ± 0.20	**12.35 ± 0.41**
17[a]	0.00	0.00	0.00	46.52 ± 0.54	11.18 ± 0.43

[a] The results are expressed as mean ± standard deviation. Average of three determinations.

The R^2 values obtained by the ANOVA indicate that 90.97% and 81.04% of the variation in the α-amylase and glucoamylase, respectively, can be explained by the models obtained. According to Kaur and Satyanarayana (2005), R^2 vary between 0 and 1, and obtaining values above 0.75 indicates the aptitude of the model.

In addition, the F calculated for both models was significant ($F_{cal} > F_{tab}$) and the F value obtained by the lack of fit (3.386 for α-amylase and 6.337 for glucoamylase) was not significant ($F_{tab5;2} = 19.30$ confirming that the experimental data fitted satisfactorily to the models. Therefore, an equation was obtained for each model using the multivariate polynomial regression technique (Eqs. 9 and 10)

$$Y_1 = 50.6766 + 6.878X_1 - 7.01041X_1^2 + 10.917\ X_2\ - 3.841X_2^2 + 3.788\ X_3 - 0.804X_3^2 - 4.548X_1X_2 + 0.969X_1X_3 + 0.068X_2X_3 \quad (9)$$

$$Y_2 = 11.987 + 1.075X_1 - 1.738X_1^2 + 1.295\ X_2\ - 0.625X_2^2 + 1.146X_3 - 0.708X_3^2 + 0.250X_1X_2 - 0.258X_1X_3 - 0.241X_2X_3 \quad (10)$$

The model equations obtained were employed to the construction of response surface graphs (Fig. 1) and their respective contour curves. These graphic expressions are used to interpret the interactions between two variables and find the optimal point of each of them (Jhample et al., 2015; Gajdhane et al., 2016). For α-amylase, the response surface was obtained through the interaction between the LGF and urea concentration, which presented significant effects, keeping the K_2HPO_4 concentrations (not significant) in its central point. Regarding the glucoamylase, response surfaces were generated through the interaction between LGF and urea / LGF and K_2HPO_4, keeping the third variable in its central point.

Based on the response surfaces obtained, the optimal values to maximize α-amylase production verified, considering only the significant variables, were: 119.8 g L^{-1} LGF and 4.4 g L^{-1} urea. For glucoamylase, the optimal conditions found were: 124.7 g L^{-1} LGF, 4.0 g L^{-1} urea and 6.8 g L^{-1} K_2HPO_4. Tests were carried out in these conditions to validate the models obtained. For α-amylase, the result obtained was 62.95 ± 0.23 U mL^{-1} and for glucoamylase, the result was 12.87 ± 0.08 U mL^{-1}. These results did not present statistically significant difference from the results predicted by the model (63.46 U mL^{-1} and 13.13 U mL^{-1}, respectively) in the Tukey test at 95% significance.

The optimal conditions to maximize the α-amylase and glucoamylase production from *C. comatus* jointly were obtained by the desirability profile (Fig. 2).

The values corresponding to the optimal points presented in Fig. 2 are: 105 g L^{-1} LGF, 3.76 urea and 7.52 K_2HPO_4. In such conditions, the values predicted for α-amylase and glucoamylase were 59.81 U mL^{-1} and 12.92 U mL^{-1}, respectively. The values observed after the

Table 9
Analysis of variance (ANOVA) of the quadratic model obtained by CCRD for the production of α-amylase and glucoamylase.

	α-amylase				Glucoamylase			
Source of variation	SQ[a]	GL[b]	MQ[c]	F ratio[d]	SQ[a]	GL[b]	MQ[c]	F ratio[d]
Regression	3271.877	9	363.542	8.964	92.741	9	10.304	3.799
Residual	324.445	8	40.556		21.700	8	2.712	
Lack of Fit	290.167	5	58.033	3.386	20.412	5	4.082	6.337
Pure Error	34.278	2	17.139		1.288	2	0.644	
Total	3596.322	16			114.441	16		
	R^2 = 0.9097; $F_{0.05;\ 9,8}$ = 3.39; $F_{tab5;2}$ = 19.30				R^2 = 0.8104; $F_{0.05;\ 9,8}$ = 3.39; $F_{tab5;2}$ = 19.30			

[a] Sum of squares.
[b] Degrees of freedom.
[c] Mean square.
[d] *F ratio* = model significance (regression/residual).

experiments had been carried out in these conditions were 59.4 ± 0.56 U mL^{-1} and 12.652 ± 0.09 U mL^{-1}. These values did not differ statistically from the values predicted at 95% significance in the Tukey test, which indicates that the model obtained is efficient to predict the α-amylase and glucoamylase activities.

Considering the enzymatic activity obtained after the simultaneous optimization of both responses, an increase of 9.78 times in the α-amylase enzymatic activity and 19.77 times in the glucoamylase enzymatic activity was observed, which confirms the efficiency of the several phases in the optimization of the production of these enzymes using the species *C. comatus*. The optimal medium obtained by the desirability profile was used in the following phases of the bioprocess kinetic study and partial characterization of the enzymes.

3.4. Final kinetics

The kinetics of growth and amylases production of *Coprinus comatus* in the optimized medium were carried out throughout 240 h (10 days), and the results obtained are presented in Fig. 3.

The lag, log and stationary phases of the growth of the species *C. comatus* and the amylases production could be identified. The lag phase (adaptation period) occurred in the first 36 h of culture. Between 36 and 120 h of culture, logarithmic growth was observed. The biomass in this phase ranged between 0.67 and 3.04 g L^{-1}, and after 120 h it was kept constant, reaching 3.63 g L^{-1} in 240 h.

Similar behavior was observed in the enzymatic production. The highest increase in the α-amylase enzymatic activity occurred between

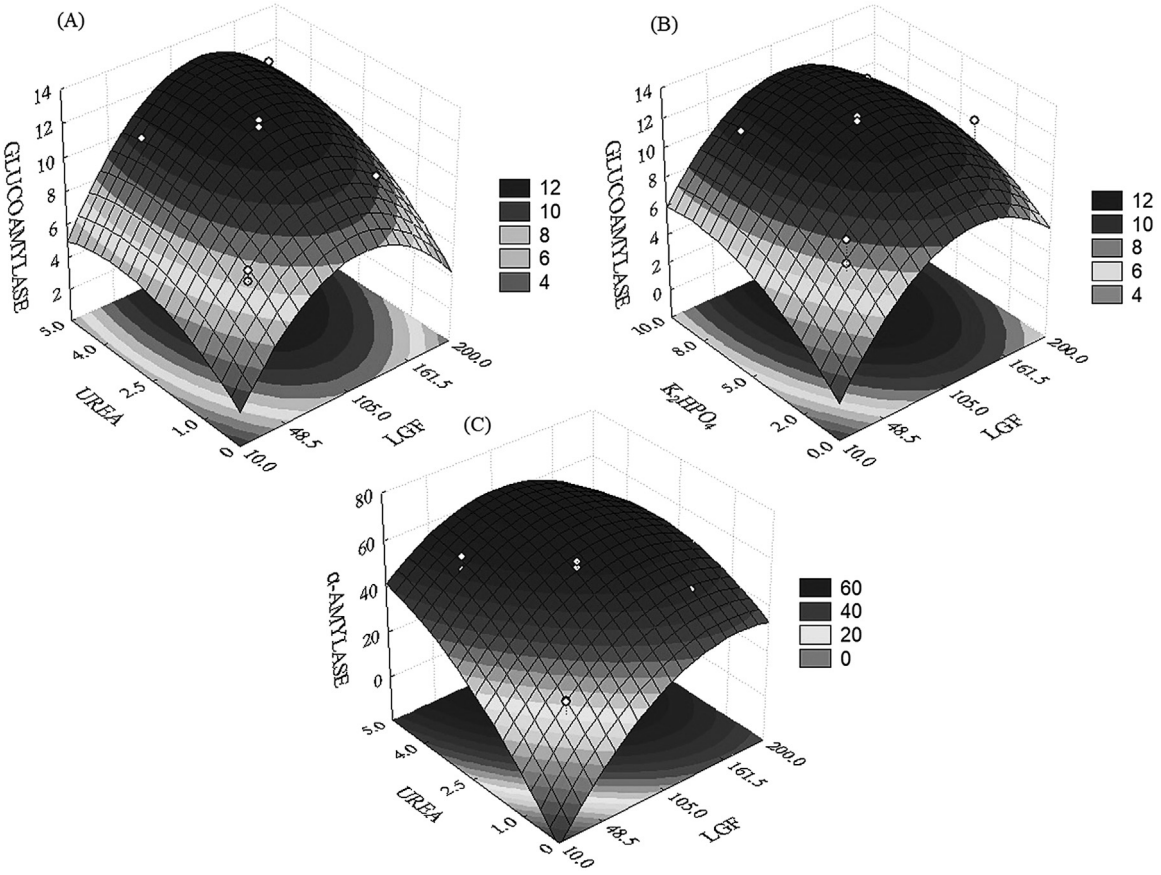

Fig. 1. Response surface graphs showing (A) Interaction between LGF and Urea concentration and (B) LGF and K_2HPO_4 concentration in glucoamylase production (C) Interaction between LGF and Urea concentration in α-amylase production from *Coprinus comatus* in SmC. Culture conditions: at pH 6.0, 28 °C and 120 rpm of orbital agitation.

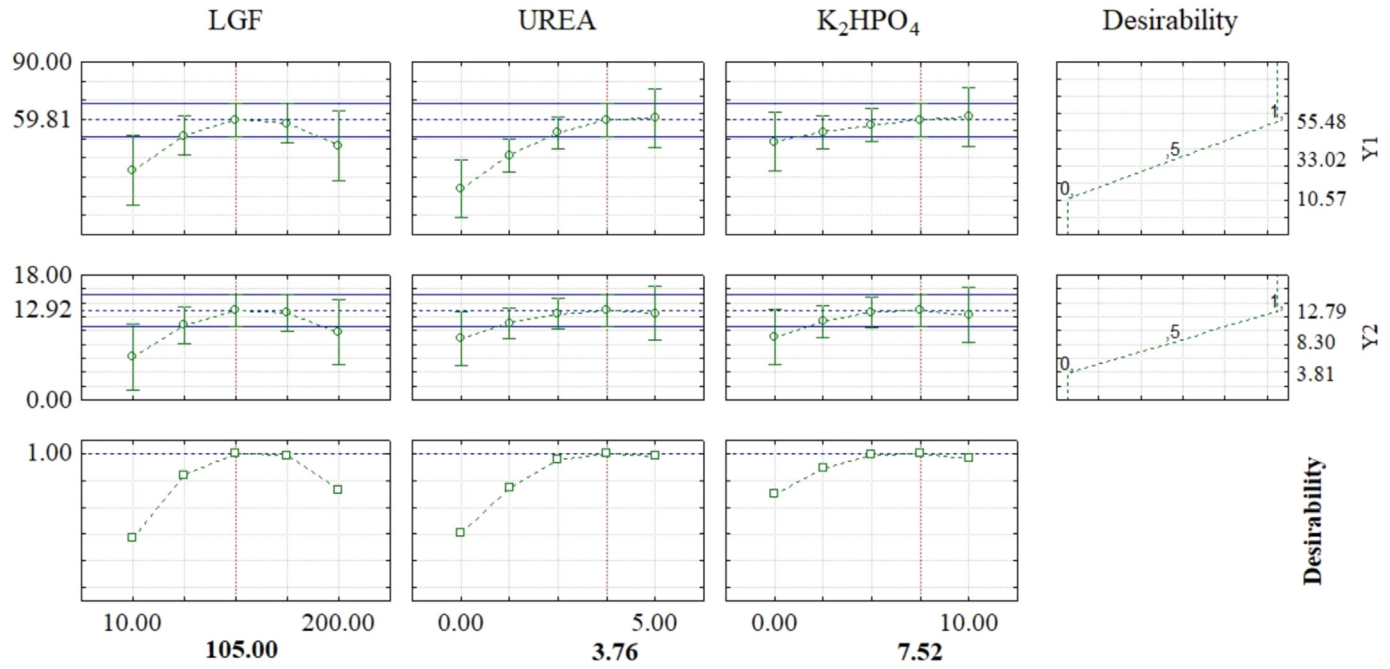

Fig. 2. Desirability profiles for α-amylase (Y1) and glucoamylase production (Y2) along with the desirability levels for LGF, Urea and K_2HPO_4 concentration for optimum production of both enzymes from *Coprinus comatus*.

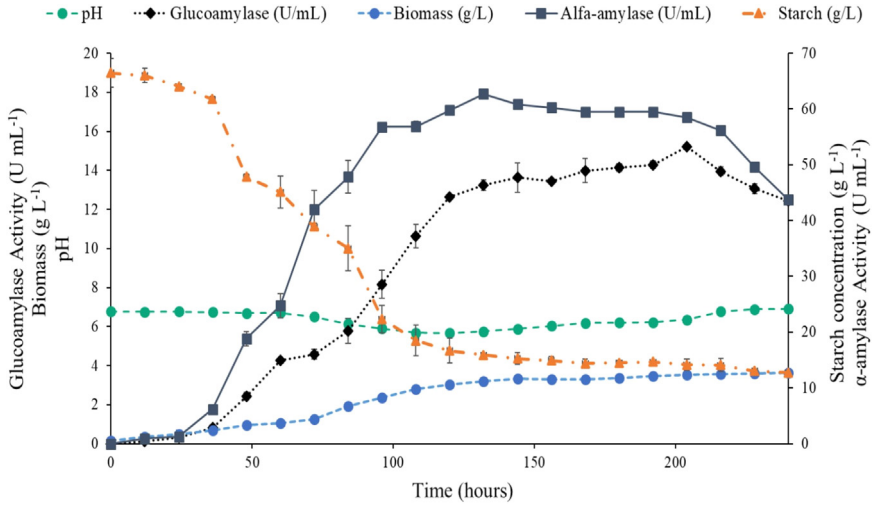

Fig. 3. Kinetics of α-amylase and glucoamylase (U mL^{-1}) production, biomass (g L^{-1}), substrate consumption (g L^{-1}) and pH throughout 240 h of *C. comatus* culture in CSm, using optimized LGF medium. Culture conditions: temperature 28 °C; agitation: 120 rpm.

36 and 96 h of culture (6.18 – 56.81 U mL^{-1}). Next, there was gradual increase, reaching 59.78 U mL^{-1} in 120 h of culture. Similarly, the production of glucoamylases presented greater increase in this period, varying from 0.81 to 12.65 U mL^{-1} between 36 and 120 h of culture.

The enzymatic activity was seen to stabilize between 120 and 216 h of culture, which might be due to the high concentration of the substrate used in the optimized culture medium (quantified as starch in kinetics). The remaining starch was noticed to reduce to about 16 g L^{-1} in 120 h, and from that point on to decrease slightly, reaching the final concentration of 12.76 g L^{-1}.

The glucoamylase activity increased after 120 h of culture, and presented a peak (15.21 U mL^{-1}) in 204 h. This suggests a mechanism associated to the action of both enzymes throughout the culture. Studies report that the production of glucoamylase could be stimulated by the products of the starch hydrolysis released by the α-amylase, mainly maltose and dextrins (Barton et al., 1972; Carlsen and Nielsen, 2001; Rajoka and Yasmeen, 2005). The results obtained in this study suggest that during the culture, a hydrolysis of the starch chains occurred through the α-amylase initial action. Next, the glucoamylase acted hydrolysing the maltose and dextrins into glucose, which justifies the late peak of enzymatic activity obtained for this enzyme.

The initial pH of the culture medium (6.77) decreased slightly, presenting the lowest value in 120 h (pH = 5.67) and increased at the end of the culture (pH = 6.9). The low pH alteration might be ascribed to the presence of K_2HPO_4, acting as a buffer when added to the culture medium (Kammoun et al., 2008; Ramesh and Murty, 2014).

Based on the results presented, the parameters of the amylase production bioprocess were calculated and are shown in Table 10. The results obtained were compared to results of previous studies (Paludo et al., 2018), which optimized a synthetic culture medium for the production of α-amylases and glucoamylases separately.

The results presented in the Table 10 show that the biomass production by the species *C. comatus* in synthetic culture medium (starch, urea, $FeSO_4$ and K_2HPO_4 based) was lower than the production in

Table 10
Kinetic parameters of the *C. comatus* amylase production in synthetic medium (previous studies) and LGF medium optimized in this study.

Parameters	Optimized synthetic media Paludo et al. (2018)		Optimized LGF medium (this study)	
	α-amylase	Glucoamylase	α-amylase	Glucoamylase
Biomass (g L^{-1})	0.77	0.65	3.78	
μ_{max} (h^{-1})	0.03	0.03	0.018	
Td (h)	22.13	25.24	38.61	
Maximum activity (U mL^{-1})	5.84 (48 h)	8.87 (44 h)	62.7 (132 h)	15.20 (204 h)
Y_{pmax} (U $mL^{-1}h^{-1}$)	0.12 (48 h)	0.20 (44 h)	0.59	0.11
γ_{xtotal} (g L^{-1} h^{-1})	0.01	0.008	0.01	
γ_{Ptotal} (U mL^{-1} h^{-1})	0.05	0.03	0.18	0.05
$Y_{P/S}$ (U gds^{-1})[a]	323.01	192.61	814.28	231.24
$Y_{X/S}$ (gx gds^{-1})	0.06	0.05	0.06	
$Y_{P/X}$ (U gx^{-1})[b]	5260.02	3755.92	12542.34	3561.78

[a] gds = g substrate.
[b] gx = g biomass.

optimized medium containing LGF, urea and K_2HPO_4 (0.77, 0.65 and 3.78 g L^{-1}). Studies report that this species growth is lower in media that do not contain strong nutrient complexes (such as the yeast extract, for example) (Eddy, 1958; Adebayo-Tayo and Ugwu, 2011). In this case, the LGF acts as the main carbon source and also a source of nitrogen and other minerals, as previously described, which justifies the greater biomass production. Also, the biomass production by *C. comatus* in the LGF medium is lower when compared to other macromycete species in CSm culture, such as *Ganoderma lucidum* (around 30 g L^{-1}) (Salmon et al., 2016). In the LGF medium, the strain presents lower growth specific rate (μ_{max} = 0.018 h^{-1}) and longer duplication time (38.61 h) than that in the synthetic media.

Since the enzyme production profile seems to be associated to the species growth profile, lower biomass production by *C. comatus* in the synthetic medium might have influenced the production of amylases in this medium. The maximum enzymatic activities were lower than those found in the LGF medium (5.84 and 8.87 U mL^{-1} in synthetic medium and 60.82 and 15.20 U mL^{-1} in LGF medium). The α-amylase productivity was γ_{pmax} = 0.12 U mL^{-1} h^{-1} in synthetic medium and 0.59 U mL^{-1} h^{-1} in the LGF medium. However, due to the short period of time needed to reach the glucoamylase maximum activity, the maximum hourly productivity was higher in the synthetic medium (0.20 U mL^{-1} h^{-1} compared to 0.11 U mL^{-1} h^{-1}).

Regarding productivity, the synthetic medium should be more appropriate for the production of glucoamylase. However, the analysis of other parameters indicated the contrary. The conversion factors of substrate into product ($Y_{P/S}$) were higher for both enzymes in the medium (LGF) (814.28 U gds^{-1} for amylase and 231.24 U gds^{-1} for glucoamylase, which means that for each gram of consumed substrate, 814 U α-amylase and 231 U glucoamylase were produced). This indicates that there is higher efficiency in the substrate conversion (starch) when LGF is used. In a bioprocess this factor might mean cost reduction and, jointly with productivity parameters, it is important in the choice of microorganisms used, culture media and operation parameters. Considering that obtaining LGF represents lower costs, this medium was observed to be the most appropriate for the production of α-amylases and glucoamylases with *C. comatus* when compared to the synthetic medium used in previous studies.

3.5. Partial purification and characterization of amylases

After the *salting-out*, desalination and concentration in ultra-filtration membranes, a recovery of about 70% of the enzymes was seen in both enzymatic activity quantification methods. The quantification of proteins using the Bradford method (1976) and calculation of specific activity (expressed in U per protein mg) confirms the partial purification of the enzymes. Considering the α-amylase dosages, the specific activity before and after the concentration procedure were 378.90 U mg^{-1} and 557.97 U mg^{-1} respectively. Regarding glucoamylases, the specific activities were 82.47 and 111.73 U mg^{-1} prior and after partial purification, respectively.

The effects of pH and enzymatic reaction temperature in the partially purified extracts can be seen in Fig. 4.

Optimal temperature and pH of enzyme action depend on its origin. The α-amylase obtained from *Bacillus laterosposrus*, for example, demonstrated optimal action at 60 °C and pH 7.0 (Kumar et al., 2013). Amylases obtained from macromycetes, such as *Ganoderma lucidum, Ganoderma tsuage,* and *Hericium erinaceum* presented 5.5, 4.5 and 6.0 pH of action and 50, 35 and 40 °C optimal temperatures, respectively (Do and Kim, 1985; Irshad, 2012; Du et al., 2013).

Amylases produced from *C. comatus* presented optimal action at 50 °C and pH 5.0. This profile was similarly reported by Do and Kim (1985) for glucoamylases produced from *Ganoderma lucidum*, and it is common for amylases originated from several fungal species such as

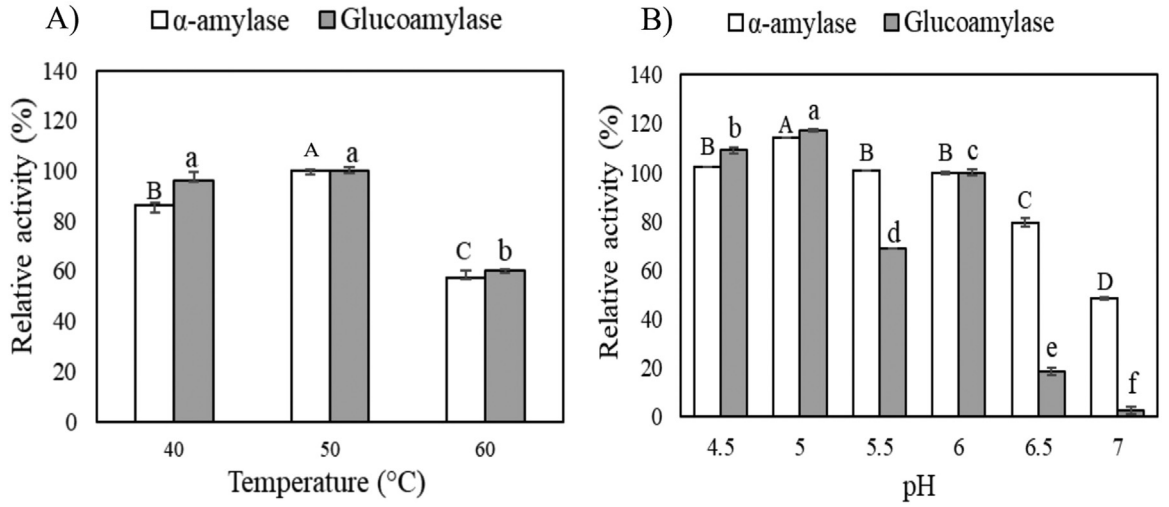

Fig. 4. Influence of reaction temperature (A) and pH (B) on the enzymatic activities of α-amylase and glucoamylase produced by *C. comatus* ($\bar{X}$ ± standard deviation). * Equal letters in the columns represent values of relative enzymatic activity with no significant difference ($p > 0.05$) and different letters represent values with significant difference ($p \leq 0.05$). ** Capital letters indicate Tukey's range test for relative activity of α-amylase and lowercase letters for glucoamylase.

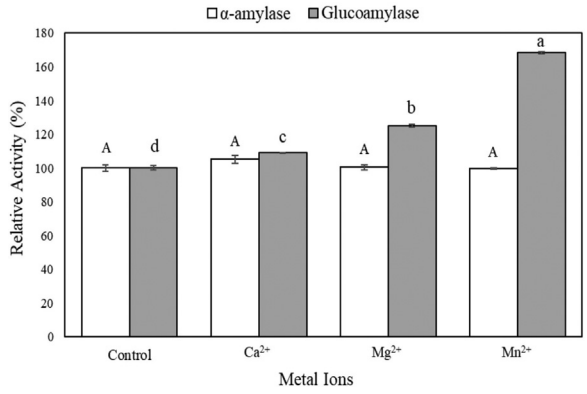

Fig. 5. Influence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions on the activity of α-amylases and glucoamylases produced by *C. comatus*. * Equal letters in the columns represent values of relative enzymatic activity with no significant difference ($p > 0.05$) and different letters represent values with significant difference ($p \leq 0.05$). * * Capital letters indicate Tukey's range test for relative activity of α-amylase and lowercase letters for glucoamylase.

Aspergillus oryzae (Dey and Banerjee, 2015) and *Aspergillus awamori* (Karam et al., 2017). The characterization of the crude extract obtained from the culture in synthetic medium of *C. comatus* presented the same optimal temperature of action for both enzymes. The optimal pH observed in the characterization of the crude extract, however, was 6.0 and 6.5 for α-amylase and glucoamylase, respectively. This difference might be related to the interference of some component in the culture medium or even the buffer used (buffer acetate, 0.5 M).

The influence of the presence of Ca^{2+}, Mg^{2+}, Mn^{2+} ions in the relative activity of α-amylase and glucoamylase can be seen in Fig. 5.

It is well established that the presence of several metal ions might promote a better interaction between the enzyme catalytic site and its respective substrate (Pasin et al., 2017). Several studies report that α-amylase is a metal enzyme that contains Ca^{2+} as the prosthetic group, and that the presence of this ion is important for its activity and stability (Gupta et al., 2003; Souza and Magalhães, 2010). In this study, the addition of ions was not shown significant in the α-amylase activity, which indicates that this is a calcium independent enzyme. This type of α-amylase is important in the production of fructose syrup, since the glucose isomerase used in the process might be inhibited by the presence of this ion (Tonkova, 2006; Singh et al., 2012; Mouna-imen and Mahmoud, 2015).

The Ca^{2+}, Mg^{2+} and Mn^{2+} ions had a positive effect on the enzymatic activity of glucoamylase, stimulating by 9%, 25% and 68% of the activity of this enzyme produced from *C. comatus*. Manganese, the main activator of this enzyme was also reported as an important activator of other glucoamylases produced by fungal species such as *Aspergillus phoenicis* (Benassi et al., 2014), *Aspergillus japonicus* (Pasin et al., 2017) *Aspergillus brasiliensis* and *Rhizopus oryzae* (Almeida et al., 2017).

The characteristics described for the amylases from *C. comatus*, such as temperature profile and pH and its independence from calcium, may be an indication of their potential for application in several relevant processes in the food industry, such as bread production and the already mentioned production of fructose syrups. Therefore, the bioprocess developed in this study represents some advancement to obtain amylases from a new microbial source. Further details of the downstream and enzyme characterization phases deserve attention in future studies.

4. Conclusions

Based on the results obtained, the wheat milling by-products were seen to represent good alternative substrates for the production of *C. comatus* amylases, in particular, the low-grade flour (LGF). The use of two stages of statistical design allowed the optimization of the culture medium, called LGF medium, containing 105 g L^{-1} LGF, 3.76 g L^{-1} urea and 7.52 g L^{-1} K_2HPO_4. The use of the LGF medium enabled the achievement of enzymatic activity in 120 h of culture of 59.40 ± 0.56 U mL^{-1} and 12.65 ± 0.09 U mL^{-1} for α-amylase and glucoamylase, values that are even higher in longer culture time, such as demonstrated in the final kinetics. The comparison of the parameters of the bioprocess developed in this study with the ones obtained in synthetic medium in previous studies demonstrates that this medium allows higher productivity and conversion factors and represents an alternative to reduce costs for the production of amylases. Finally, the partial characterization of the enzymes revealed a profile similar to several fungal amylases used in industrial processes. Further studies on the purification and characterization of these amylases are necessary to show its potential for industrial application.

Acknowledgments

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