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Simultaneous Amyloglucosidase and Exo-polygalacturonase Production by *Aspergillus niger* using Solid-state Fermentation

Jorge Alberto Vieira Costa[1]*, Eliane Colla[1], Glênio Magagnin[1], Lucielen Oliveria dos Santos[1], Mauricio Vendruscolo[1] and Telma Elita Bertolin[2]

[1]Laboratório de Engenharia Bioquímica; Departamento de Química; Fundação Universidade Federal do Rio Grande; C. P. 474; 96201-900; dqmjorge@furg.br; Rio Grande - RS - Brasil. [2]Centro de Pesquisa em Alimentação; Universidade de Passo Fundo; C. P. 611; 99070-010; Passo Fundo - RS - Brasil

ABSTRACT

Amyloglucosidase (AMG) and exo-polygalacturonase (exo-PG) were simultaneously produced by two different strains of Aspergillus niger *in solid-state fermentation (SSF) using defatted rice-bran as substrate. The effect of* Aspergillus niger *strain (t0005/007-2 and/or CCT 3312), inoculum type (spore suspension or fermented bran) and addition of inducers (pectin and/or starch) to the culture media was studied using a $3^2 \times 2^1$ factorial experimental design. The production of AMG and exo-PG was significantly affected by fungal strain and inoculum type but inducers had no effect. The maximum yields obtained were 1310 U/g_{dm} for AMG using a spore suspension of A. niger CCT 3312 and 50.2 U/g_{dm} for exo-PG production, using A. niger t0005/007-2 and fermented bran as inoculum. The yields obtained represented acceptable values in comparison with data available in the literature and indicated that defatted rice-bran was a good nutrient source.*

Key words: Amyloglucosidase, exo-polygalacturonase, rice-bran, solid-state fermentation

INTRODUCTION

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites (Gabiatti Jr. et al., 2006; Raghavarao et al., 2003). Agro-industrial residues are generally considered the best substrates for the process, including enzyme production, based on SSF (Ellaiah et al., 2002).

Compared with submerged fermentation, the use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity (Bertolin et al., 2001). The fact that the water content is quite low in SSF because the water present is absorbed, bound or complexed within the solid matrix, instead of being in the free liquid state, may also be more advantageous for growth due to more efficient oxygen transfer, because the microorganism remains in contact with gaseous oxygen, which is not the case in submerged fermentation (Smf) (Raghavarao et al., 2003). Castilho et al. (2000) state that the conditions in solid-state fermentation were closer to those found in the natural habitat of filamentous fungi, which were, thus, able to grow better and

* Author for correspondence

excrete larger quantities of enzymes. This can be of special interest in those processes where the crude fermented product may be used directly, as the enzyme source. According to Raghavarao et al. (2003) a further factor is that SSF is especially suited for growing mixed cultures of microorganisms because symbiosis stimulates better growth and productivity.

Starch-degrading enzymes and pectinases extracted from microorganisms have various industrial applications (Ramachandran et al., 2004). Extracellular amyloglucosidase (AMG) is of major importance to the starch-processing industry because it can convert starch to dextrins and glucose, but even though AMG is found in a wide variety of microorganisms, commercial AMG is mainly obtained from filamentous fungi (Ramadas et al., 1996; Pandey et al., 2000). Pectinases, a complex set of enzymes that includes polygalacturonase (PG), pectinesterase (PE) and pectin lyase (PL), can degrade pectinaceous substances and reduce viscosity, because of which they are extensively used in the industrial clarification of wine and fruit juices (Blandino et al., 2001; Sathyanarayana and Panda, 2003) and to improve oil extraction and de-gum fiber (Taragano and Pilosof, 1999).

Industrially, pectinases and amyloglucosidase have been produced using both submerged and solid-state fermentation, although solid-state fermentation has been found to be more advantageous because it is cheaper and produces larger amounts of enzymes (Singh and Soni, 2001). According to Soccol and Vandenberghe (2003), a further factor is that not only the application of agro-industrial residues in bioprocess provides alternative substrates, but also helps solving pollution problems. The production of amyloglucosidase and pectinases is highly influenced by the components of the growth medium, especially the carbon source (Nair and Panda, 1997; Blandino et al., 2001). Most extracellulary induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium (Nair and Panda, 1997). Several studies have shown maltose and starch to be typical inducers of glucoamylase production during both submerged and solid-state fermentation, with maximum enzyme yield occurring in media containing 1.25% soluble starch (Bertolin et al., 2001; Ramadas et al., 1996). Pectinolytic enzymes have been reported to be induced by several substrates including pure pectin as well as in complex media containing substances such as beet sugar, wheat-bran, groundnut meal and citrus fruit peel (Nair and Panda, 1997).

This study was designed to determine the effect of the use of different *Aspergillus niger* strains, type of inoculum and inducers on the simultaneous production of amyloglucosidase (AMG) and exo-polygalacturonase (exo-PG) during solid-state fermentation using defatted rice-bran as substrate.

MATERIALS AND METHODS

Fungal strains

Two *Aspergillus niger* strains were used, *A. niger* t0005/007-2 (strain one isolated from soil) maintained on potato dextrose agar (PDA), and *A. niger* CCT 3312 (Coleção de Culturas Tropicais), provided by Fundação André Tosello, Brazil (strain two), which was maintained on nutrient agar (NA), containing (g/L): 20 glucose; 20 malt extract; 20 agar; 1 peptone. In both cases, agar slants were made, inoculated with the appropriate strain and incubated at 30°C for 5 days.

Culture media for the enzyme production experiments

The basal rice-bran medium (RBM) consisted of defatted rice-bran as the main carbon source with 15% (w/w) of rice-straw being added to decrease the density of the medium, resulting in a better oxygen transfer. The RBM plus 1.25% (w/w) corn starch (RBSM) was used to test for AMG induction (Bertolin et al., 2001) and RBM plus 10% (w/w) pectin (RBPM), for exo-PG induction (Linde, 2000). A combined medium containing both inducers (RBSPM) at the same concentrations as they were in the individual media was also prepared in order to assess the effect of induction. In all cases, 45 mL of mineral salts solution (containing (g/L): 2 KH_2PO_4; 1.8 Urea; 1 $MgSO_4$) was added to each 100 g of dry medium and the moisture content adjusted to 50% (w/w) with distilled water. All media were autoclaved for 15 min at 121°C before use.

Inoculum

Two types of inoculum, spore suspension and fermented bran, were tested for each fungal strain. An initial spore suspension was prepared by washing spores from agar slants (PDA for strain one and NA for strain two) using 5 mL of 0.2% (v/v) aqueous Tween 80.

To prepare spore suspensions for the enzyme production experiments, 0.5 mL of the initial spore suspension was spread on the surface of 150 mL of PDA medium contained in 500 mL flasks, which were incubated at 30°C for 96 h. After incubation, 50 mL of 0.2% (v/v) aqueous Tween 80 was added and the spores suspended by gentle shaking before counting the number of spores by direct microscopy using a Neubauer chamber.

The fermented bran inoculum was prepared by inoculating RBM with sufficient spore suspension (described in the previous paragraph) to produce $4x10_6$ spores/g of RBM, and the mixture incubated under the same conditions of temperature and time as those described below for the enzyme production experiments.

Culture conditions for the enzyme production experiments

Cultures were grown in 500 mL Erlenmeyer flasks containing 100 g of RBSM, RBPM or RBSPM medium adjusted to different pH values by the addition of HCl 1.5 M, according to the *A. niger* strain used, i.e. pH 3.5 for strain one, pH 5.5 for strain two and pH 4.5 for mixed cultures. The solution of HCl 1.5 M was added directly in the medium because rice bran and rice straw possess strong buffering capacity. The pH values were different for each strain due to the better conditions for grown and sporulation (defined previously). For the single strain experiments using spore suspension as the inoculum, flasks were inoculated with spore suspension to give $4x10_6$ spores/g of medium (Hasan et al., 1998; Linde, 2000), while for the mixed strain experiments, flasks were inoculated with $2x10^6$ spores of each strain per gram of medium.

For the single strain experiments using fermented bran as inoculum, flasks were inoculated with sufficient fermented bran to give 1 ´ 107 spores/g of medium (Hasan et al., 1998), while for the mixed strain experiments, flasks were inoculated with sufficient fermented bran inoculum to give 5 ´ 106 spores of each strain per gram of medium. In all cases, incubation was carried out at 30°C for 96 h. The total number of different experimental runs assayed was 18.

Analytical Determinations

Samples were collected from each flask at 24, 48, 72 and 96 h. The moisture content, AMG and exo-PG activity were determined. Moisture content of the SSF material was determined gravimetrically by drying the samples at 105ºC until constant weight, according to AOAC (1997). Amyloglucosidase (AMG) was extracted by placing 1 g of culture material in a flask, adding 15 mL of distilled water, agitating at 230 rpm for 3 h at 30ºC and removing debris by filtering through Whatman #41 filter-paper. The AMG activity in the filtrate was assayed using a reaction mixture containing an appropriate dilution of the filtrate along with 4% (w/v) starch solution dissolved in 0.2 M acetate buffer (pH 4.6), which was incubated at 60ºC for 60 min. Reducing groups released were determined by the DNS method (Miller, 1959) using glucose as a standard. One AMG unit (U) was defined as the amount of enzyme required to produce 1 mmol of glucose per minute in the presence of 4% (w/v) soluble starch solution at 60°C and pH 4.6 (Bertolin et al., 2003).

Exo-polygalacturonase (exo-PG) was extracted by placing 1 g of culture material in a flask, adding 9 ml of distilled water, agitating at 100 rpm for 30 minutes at 30ºC and removing debris by filtering through Whatman #41 filter-paper. The exo-PG activity in the filtrate was assayed using a reaction mixture containing an appropriate dilution of the filtrate along with 0.5% (w/v) poligalacturonic acid dissolved in 0.2 M phosphate-citric acid buffer (pH 5.0), which was incubated at 37ºC for 60 min. Reducing groups released were determined by the DNS method (Miller, 1959) using galacturonic acid as a standard. One exo-PG unit (U) was defined as the amount of enzyme required to produce 1 mmol of D-galacturonic acid per minute in the presence of 0.5% (w/v) polygalacturonic acid, at 37°C and pH 5.0.

For comparison, both AMG and exo-PG activities were converted to units per gram of dry medium (U/gdm).

Experimental design and statistical analysis

A 3^2x2^1 factorial experimental design with 18 experimental runs (Table I) was used to study the influence of inoculum type (spore suspension or fermented bran), *A. niger* strain (strain one, strain two or both) and inducer (pectin, starch or both) on the production of AMG and exo-PG in solid-state fermentation. The AMG and exo-PG activities were evaluated statistically at all cultivation times (24, 48, 72, and 96 h) to determine the effect of each variable on enzyme activity.

RESULTS AND DISCUSSION

Amyloglucosidase production

The amyloglucosidase (AMG) activities in units per gram of dry medium (U/g_{dm}) at 24, 48, 72 and 96 h for the different experimental runs are shown in Table 1. The maximum AMG activity observed was 1310 U/g_{dm}, which occurred at 72 h in run 7 using a spore suspension inoculum of *A. niger* CCT 3312 (strain two) with pectin as the inducer. Similar result was obtained at 96 h in run 5 using a mixed spore suspension inoculum (equal numbers of spores of strain one and strain two) with both pectin and starch being present as inducers. In addition to that, the results observed in runs 15 (mixed spore suspension and starch as inducer) and 17 (spore suspension of strain two and pectin and starch as inducer) were higher than that obtained by Ramadas et al. (1996), who found an AMG activity of 450 U/gdm at about 96 h cultivation in SSF using oat-bran as substrate and 225 U/gdm using a wheat-bran substrate. Fig. 1 shows the kinetics for runs 5, 7, 15 and 17.

Table 1- Experimental variables and amyloglucosidase (AMG) activities (U/g_{dm}) for all runs.

	Variable			Enzyme activity (U/g_{dm}) [d]			
Run	**Inoculum [a]**	**Inducer [b]**	**Strain [c]**	**AMG_{24}**	**AMG_{48}**	**AMG_{72}**	**AMG_{96}**
1	-1	-1	-1	32.9 ± 2.5	72.4 ± 5.7	129 ± 4.3	372 ± 3.6
2	+1	0	-1	21.0 ± 1.4	47.2 ± 3.6	72.9 ± 5.1	192 ± 2.6
3	-1	+1	-1	43.7 ± 0.4	148 ± 2.1	327 ± 3.7	527 ± 6.2
4	+1	-1	0	176 ± 1.4	227 ± 2.1	330 ± 4.5	576 ± 7.3
5	-1	0	0	55.8 ± 2.1	542 ± 5.3	875 ± 3.8	1310 ± 9.1
6	+1	+1	0	135 ± 3.1	262 ± 5.8	306 ± 6.7	332 ± 1.2
7	-1	-1	+1	249 ± 1.4	831 ± 11.5	1309 ± 15.3	1001 ±16.1
8	+1	0	+1	467 ± 6.4	677 ± 5.8	819 ± 5.0	921 ± 5.5
9	-1	+1	+1	150 ± 3.8	276 ± 2.1	227 ± 5.6	230 ± 6.9
10	+1	-1	-1	41.1 ± 2.3	50.9 ± 2.6	163 ± 6.7	207 ± 5.8
11	-1	0	-1	15.2 ± 0.1	56.8 ± 4.0	143 ± 2.9	285 ± 3.1
12	+1	+1	-1	42.4 ± 0.6	53.4 ± 5.1	111 ± 1.1	239 ± 4.8
13	-1	-1	0	98.6 ± 5.2	507 ± 6.1	923 ± 3.7	1014 ± 12.2
14	+1	0	0	134 ± 4.2	209 ± 5.8	362 ± 5.7	630 ± 7.5
15	-1	+1	0	309 ± 3.4	1040 ± 4.5	1104 ± 6.5	1294 ± 8.3
16	+1	-1	+1	745 ± 4.7	1197 ± 7.3	936 ± 10.2	1093 ± 12.1
17	-1	0	+1	313 ± 10.2	846 ± 17.1	993 ± 16.2	1232 ± 20.1
18	+1	+1	+1	228 ± 3.7	352 ± 7.2	497 ± 8.1	573 ± 8.7

Note: For AMG the subscript shows the cultivation time (hours).
[a] (-1) Spore suspension; (+1) Fermented bran
[b] (-1) Pectin; (+1) Starch; (0) Pectin and Starch
[c] (-1) *A. niger* strain t0005/007-2; (+1) *A.niger* strain CCT 3312; (0) strain t0005/007-2 and strain CCT3312
[d] Average of triplicate runs

The AMG activities obtained in this work were also higher than the 59 U/g_{dm} detected by Bertolin et al. (2001) using a fixed-bed bioreactor with wheat-bran and a mineral salts solution as medium, and starch as inducer. The results of the statistical analysis with the effects of each variable studied in the experimental design on AMG activity, at different cultivation times, are shown in Table 2. Evidently, the type of inducer was not a significant variable at the 95% probability level, and the addition of inducers did not necessarily result in higher AMG activity. Bertolin et al. (2001) reported the induction of glucoamylase when soluble starch or maltose were used as secondary carbon source by *A. awamori* in a solid-state fermentation, although Mackenzie et al. (1994) stated that maltose did not show induction in solid-state fermentation, possibly due to the limited availability of water in solid media, which led to a much higher build-up of glucose than in the case of submerged culture.

The variable strain had a positive effect during cultivation and strongly affected AMG activity. In this way, utilization of *A. niger* CCT 3312 produced higher activities of this enzyme. Type

of inoculum was significant at 48, 72 and 96 h, and the spore suspension inoculum produced higher AMG activity than the fermented bran inoculum.

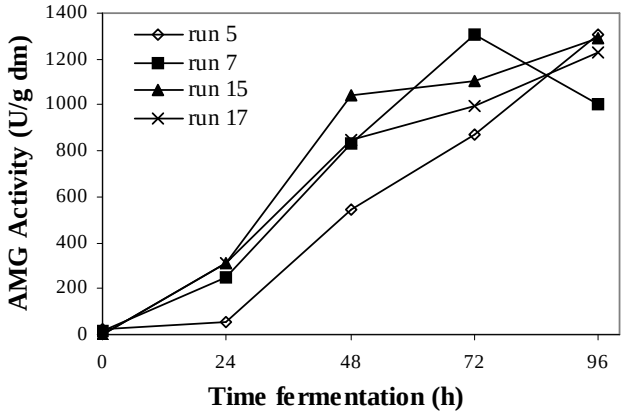

Figure 1: Time course of amyloglucosidase (AMG) production for runs 5, 7, 15 and 17.

Table 2 - Effect of the variables studied on the amyloglucosidase (AMG) activities (U/g_{dm}) at different cultivation times for the $3^2 \times 2^1$ factorial experimental design.

	Effects (U/g_{dm}) at different cultivation times			
Variable	**AMG_{24}**	**AMG_{48}**	**AMG_{72}**	**AMG_{96}**
Inoculum	-7.2	-496*	-617*	-777*
Inducer	85.1	284	78.4	18.3
Aspergillus strain	372*	710*	798*	838*

Note: For AMG the subscript gives the cultivation time in hours.
* Significant variable ($p<0.05$).

Fig. 1 showed that runs 5, 7, 15 and 17 presented similar behaviour to AMG production. This could be explained for the fact that these runs were performed at the same level of the inoculum (spore suspension) and with the strain *A. niger* CCT 3312.

Exo-polygalacturonase production

The exo-polygalacturonase (exo-PG) activities for the experimental runs are shown in Table 3. The maximum exo-PG activity was 50.2 U/g_{dm} at 72 h in run 10 using fermented bran inoculum of strain one with pectin as inducer. Solis-Pereyra et al. (1996) studied the influence of different inicial glucose concentration on the production of pectinase by *A. niger* strain CH4 and found a maximum pectinase activity of 35 U/g_{dm} at 72 h in a medium containing 100 g/L of glucose. Taragano and Pilosof (1999) observed a polygalacturonase activity of 12.2 U/g_{dm} in solid-state culture with wheat-bran as substrate and pectin as inducer and 53.3 U/g_{dm} in submerged culture using 10% (w/v) of the same substrate.

The results of the statistical analysis with the effects of each variable studied in the experimental design on exo-PG activity, at different cultivation times, are shown in Table 4. For fermentation times of 24, 48 and 96 h the variable *Aspergillus* strain showed significant negative effects, which indicated that an increase in exo-PG activity was obtained by the use of strain one. Addition of inducers to the media did not influence significantly the exo-PG production, which could have occurred because exo-PG was constitutive in *A. niger* and would not have been significantly affected by the presence of inducers.

According to Blandino et al. (2001), exo-PG production depends on the fungal strain and the culture medium, but the presence of pectin as inducer appears to be less important, supporting the view that this enzyme is inded constitutive.

Table 3 - Experimental variables and exo-polygalacturonase (Exo-PG) activities (U/g_{dm}) for all runs.

	Variable			Enzyme activity (U/g_{dm}) [d]			
Run	**Inoculum [a]**	**Inducer [b]**	**Strain [c]**	**Exo-PG$_{24}$**	**Exo-PG$_{48}$**	**Exo-PG$_{72}$**	**Exo-PG$_{96}$**
1	-1	-1	-1	17.3 ± 0.8	29.7 ± 0.5	26.1 ± 0.4	26.7 ± 0.8
2	+1	0	-1	27.4 ± 0.8	33.6 ± 1.3	33.8 ± 1.4	40.5 ± 0.8
3	-1	+1	-1	11.0 ± 0.3	34.3 ± 0.6	36.3 ± 0.5	30.8 ± 0.5
4	+1	-1	0	18.6 ± 0.4	20.3 ± 0.2	19.8 ± 0.3	24.2 ± 0.6
5	-1	0	0	14.2 ± 0.5	16.2 ± 0.6	24.1 ± 0.5	23.2 ± 0.6
6	+1	+1	0	16.8 ± 0.4	23.4 ± 1.6	22.7 ± 0.8	17.4 ± 0.5
7	-1	-1	+1	13.7 ± 0.5	15.3 ± 0.6	15.3 ± 1.3	17.0 ± 0.6
8	+1	0	+1	15.9 ± 0.6	20.1 ± 0.7	21.3 ± 0.2	17.3 ± 1.2
9	-1	+1	+1	9.4 ± 0.4	10.4 ± 0.1	11.0 ± 0.2	11.4 ± 0.7
10	+1	-1	-1	32.7 ± 0.2	41.7 ± 0.9	50.2 ± 1.3	44.9 ± 1.1
11	-1	0	-1	15.4 ± 1.0	32.1 ± 0.6	31.7 ± 0.1	29.1 ± 0.8
12	+1	+1	-1	24.2 ± 0.3	29.0 ± 0.2	29.9 ± 1.1	31.3 ± 1.6
13	-1	-1	0	12.6 ± 0.3	13.6 ± 0.2	14.8 ± 1.1	16.9 ± 0.4
14	+1	0	0	20.9 ± 1.1	27.1 ± 1.8	26.7 ± 1.8	23.4 ± 1.9
15	-1	+1	0	12.7 ± 0.4	13.3 ± 0.4	13.8 ± 0.4	13.8 ± 0.2
16	+1	-1	+1	13.6 ± 0.2	13.8 ± 0.2	12.5 ± 0.5	13.9 ± 0.6
17	-1	0	+1	11.9 ± 0.1	11.9 ± 0.5	12.5 ± 0.5	12.2 ± 0.2
18	+1	+1	+1	10.5 ± 0.4	9.7 ± 0.4	9.6 ± 0.5	9.5 ± 0.3

Note: For Exo-PG the subscript shows the cultivation time (hours).
[a] (-1) Spore suspension; (+1) Fermented bran
[b] (-1) Pectin; (+1) Starch; (0) Pectin and Starch
[c] (-1) *A. niger* strain t0005/007-2; (+1) *A.niger* strain CCT 3312; (0) strain t0005/007-2 and strain CCT3312

Table 4 - Effect of the variables studied on the exo-polygalacturonase (exo-PG) activities (U/g_{dm}) at different cultivation times for the $3^2 \times 2^1$ factorial experimental design.

	Effects (U/g_{dm}) at different cultivation times			
Variable	**Exo-PG$_{24}$**	**Exo-PG$_{48}$**	**Exo-PG$_{72}$**	**Exo-PG$_{96}$**
Inoculum	6.2*	11.4*	5.5	4.7
Inducer	-0.8	1.4	0.9	-5.0
Aspergillus strain	-7.5*	-16.8*	-15.9	-20.1*

Note: For exo-PG the subscript gives the cultivation time in hours.
* Significant variable ($p<0.05$).

The variable inoculum presented positive and significant influence only at 24 and 48 h of fermentation. This indicated that fermented bran (level +1) produced higher results for exo-PG activity than spore suspension (level -1). The reason for variable inoculum to have been significant only at 24 and 48 h could be explained by the possible pre-adaptation of the fungi to the culture medium when the fermented bran was used as inoculum, leading to a decrease in the lag phase as compared to the use of spore suspension as inoculum. Also, when fermented bran was used as inoculum there might already have been some exo-PG activity.

CONCLUSIONS

The feasibility of producing AMG and exo-PG, using *Aspergillus* species and defatted rice-bran as substrate in solid-state fermentation was demonstrated by the results presented in this work. Defatted rice-bran is a cheap and readily available raw material. It gave high yields of

both AMG and exo-PG in comparison with the results obtained by other researchers. Of variables studied in this work, fungal strain and inoculum type showed significant effects on exo-PG and AMG production, but the synthesis of these enzymes using both *Aspergillus* strains studied was not significantly affected by incorporation of pectin and starch to the defatted rice bran, demonstrating the constitutive nature of these enzymes. The maximum amyloglucosidase (AMG) activity was 1310 U/g_{dm} using *A. niger* strain CCT 3312 as strain producer and a spore suspension as inoculum, while the maximum exo-polygalacturonase (exo-PG) production was 50.2 U/g_{dm} using *A. niger* strain t0005/007-2 as strain producer and fermented bran as inoculum.

RESUMO

As enzimas amiloglicosidase (AMG) e exo-poligalacturonase (exo-PG) foram produzidas simultaneamente por duas cepas de *Aspergillus niger*, através de fermentação em estado sólido usando farelo de arroz desengordurado como substrato. Foram avaliados os efeitos da cepa de *Aspergillus niger*, tipo de inóculo e adição de indutores no meio de cultura, utilizando-se um planejamento experimental fracionário $3^2 \times 2^1$. O máximo rendimento obtido foi 1310 U/g_{ms} para a produção de AMG e 50,2 U/g_{ms} para a exo-PG. Comparando-se estes resultados com dados da literatura pode-se dizer que os rendimentos obtidos foram aceitáveis e indicam que o farelo de arroz desengordurado é uma boa fonte de nutrientes. A produção de AMG e exo-PG foi significativamente afetada pelas variáveis cepa de *A. niger* e tipo de inóculo, enquanto a variável indutor não apresentou influência significativa na produção destas enzimas.

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