Revista Internacional de Investigación e Innovación Tecnológica

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Simultaneous production of *Aspergillus niger* biomass and amylolytic enzymes under submerged fermentation using agro-industrial by-products

Producción simultánea de biomasa de *Aspergillus niger* y enzimas amilolíticas por fermentación sumergida utilizando subproductos agroindustriales

Nazari, M.T.[a], Rigueto, C.V.T.[b], Kreling, N.E.[a], Reinehr, C.O.[b], Colla, L.M.[a,b]

[a] Graduate Program in Civil and Environmental Engineering (PPGEng), Faculty of Engineering and Architecture, University of Passo Fundo (UPF), Passo Fundo RS, Brazil.
[b] Graduate Program in Food Science and Technology (PPGCTA), Faculty of Agronomy and Veterinary Medicine, University of Passo Fundo (UPF), Passo Fundo RS, Brazil.
nazari.eas@gmail.com; cesartoniciolli@gmail.com; naiarakreling@hotmail.com; reinehr@upf.br; lmcolla@upf.br

Industrial Application area: Sustainability.

Received: november 30th, 2020
Accepted: november 30th, 2021

Resumen

Varios bioproductos pueden ser obtenidos a partir de los cultivos de hongos filamentosos, lo que hace viable la utilización de hongos para aplicaciones alimentarias, farmacéuticas y medioambientales. Además, los hongos filamentosos son capaces de cultivarse utilizando como sustrato residuos y subproductos agroindustriales, mejorando la economía circular y la valorización de estos residuos. En este contexto, nuestro objetivo fue producir simultáneamente biomasa fúngica y enzimas amilolíticas en fermentación sumergida (SmF) utilizando como sustrato subproductos agroindustriales (salvado de trigo y cáscara de papa). *Aspergillus niger* DAOM fue la cepa utilizada en este estudio. Se realizaron diseños factoriales para lograr una mayor producción de biomasa fúngica (evaluada por concentración de biomasa) y amilasas (evaluada por actividad amilolítica) bajo SmF realizado en dos pasos. En el Paso 1 y en el Paso 2 se aplicó un diseño factorial fraccionado (DFF 2^{5-1} con puntos centrales) y dos diseños factoriales completos (Diseño 2 y Diseño 3), respectivamente. Los niveles de significancia y los efectos estimados se analizaron a un nivel de confianza del 90% y el 95% para los Pasos 1 y 2, respectivamente. Se pudo verificar las mejores condiciones para producir biomasa fúngica y enzimas amilolíticas bajo SmF utilizando dos subproductos agroindustriales. El salvado de trigo mostró mejores resultados tanto para la concentración de biomasa como para la actividad amilolítica en comparación con la cáscara de papa (Paso 1). En el Paso 2, la mayor producción de biomasa fúngica ocurrió a los dos días para ambos sustratos. La concentración de biomasa

fue mayor en el medio compuesto por salvado de trigo que con cáscara de papa + salvado de trigo (Paso 2). Finalmente, se pudo determinar que las mejores condiciones para producir biomasa fúngica son 1% de $NaNO_3$ y pH 7 para salvado de trigo, mientras que para cáscara de papa + salvado de trigo son 1% de $NaNO_3$ y pH 5.

Palabras clave: Amilasas, *Aspergillus niger*, Cáscara de papa, Salvado de trigo.

Abstract

Several bioproducts can be obtained from the cultivations of filamentous fungi, which makes viable the utilization of fungi for food, pharmaceutical and environmental applications. Besides, filamentous fungi are capable of grown using agro-industrial wastes and by-products as substrate, improving the circular economy and valorization of these residues. In this context, we aimed to produce simultaneously fungal biomass and amylolytic enzymes under submerged fermentation (SmF) using agro-industrial by-products as substrate (wheat bran and potato peel). *Aspergillus niger* DAOM was the strain used in this study. Factorial designs were performed to achieve higher production of fungal biomass (evaluated by biomass concentration) and amylases (evaluated by amylolytic activity) under SmF performed in two steps. A fractional factorial design (FFD 2^{5-1} with central points) and two full factorial designs (Design 2 and Design 3) were applied in Step 1 and Step 2, respectively. The significance levels and estimated effects were analyzed at a confidence level of 90% and 95% for Step 1 and 2, respectively. It was possible to verify the best conditions to produce fungal biomass and amylolytic enzymes under SmF using wheat bran and potato peel. Wheat bran showed better results for both biomass concentration and amylolytic activity when compared to potato peel (Step 1). In the Step 2, the highest fungal biomass production occurred in two days for both substrates. Biomass concentration was higher in a medium composed by wheat bran (Step 2). Finally, it was possible to determinate that the best conditions to produce fungal biomass are 1% of $NaNO_3$ and pH 7 for wheat bran, while for potato peel + wheat bran are 1% of $NaNO_3$ and pH 5 (Step 2).

Keywords: Amylases, *Aspergillus niger*, Potato peel, Wheat bran.

1. Introduction

Brazil is known for its high agricultural activity. This sector stands out due to its wide variety of products grown and sold in the country, and in parallel, high volumes of waste are generated in the processing of these raw materials [1-3].

The wastes and by-products from industrial and agro-industrial activities can affect the environment, since they are organic raw materials generated in high quantities, and during decomposition the fermentation process occurs, generating a bad smell and proliferation of vectors. Therefore, for the final disposal of this waste in landfills, drying becomes necessary, which requires additional costs [2, 4-6].

In this context, researchers around the world have reported alternative uses for the use of wastes from agro-industrial activities, showing that these have the potential to generate a variety of value-added products, such as biofuels [7-9], biofertilizers [10-11], extraction of bioactive compounds [3] and substrate for microbial growth [12-14].

Regarding the use for microbial growth, the filamentous fungi of the genus *Aspergillus* and, more specifically, the species *Aspergillus niger*, has the potential to produce numerous enzymes, such as amylases, cellulases, pectinases, peroxidases, lipases, xylanases, proteases, besides products of biotechnological interest, such as biopeptides, biosurfactants, fungal chitosan and bioflocculant for microalgae harvesting [15-16]. Besides, the optimization of fungal biomass production is essential since we aimed to apply this biomass for microalgae harvesting in a more eco-friendly biorefinery approach called bioflocculation [16-17]. In this sense, the aim of this work was to optimize the simultaneously production of fungal biomass and amylolytic enzymes under submerged fermentation using agro-industrial by-products as substrate.

2. Material and Methods

2.1. Cultivation of *Aspergillus niger* for production of fungal biomass and amylases

Aspergillus niger DAOM [18], stored in potato dextrose agar (PDA) in test tubes at 4 °C, was reactivated by adding 5 mL of a 0.1% Tween solution to obtain a spore suspension. Then, 0.5 mL of the spore suspension was transferred to Petri dishes with PDA and incubated in an oven at 30 °C for 6 days.

The inoculums obtained were used to inoculate the culture media and, thus, to carry out the cultivation of the *A. niger* via submerged fermentation (SmF). The SmF was studied in two stages, being the fungal biomass concentration and amylolytic activity the response variables for both steps. The first step (Step 1) was evaluated using a fractional factorial design (2^{5-1}), with four repetitions at the central point, totalizing 20 experiments. The parameters studied in the Step 1 of SmF were: a) substrate (wheat bran and potato peel); b) nitrogen source (sodium nitrate and urea); c) nitrogen source concentration (0.5% and 2%); d) pH (4 and 7); e) micronutrients addition or not. The micronutrient composition was adapted from Djekrif-Dakhmouche et al. [19], containing: 1 g L^{-1} de $CaCl_2$; 0.2 g L^{-1} $MgCl_2 6H_2O$; 0.1 g L^{-1} $FeSO_4$, $7H_2O$; 0.1 g L^{-1} $MnSO_4$.

Regarding the preparation of the culture medium, a substrate suspension in distilled water was prepared with the amount of substrate necessary to start each SmF with 15 g L^{-1} of starch, considering the starch contents of wheat bran [20] and potato peel [21], which were obtained from a local mill and restaurant, respectively. The mediums were cooked at 100 °C for 30 min, with subsequent filtration to remove insoluble solids. Then, the other adjustments were made according to the experimental designs and, finally, the mediums were sterilized at 121 ºC for 20 min.

The prepared culture media (100 mL) were added in erlenmeyers of 250 mL. The inoculation was carried out by transferring two mycelial disks of fungus (1 cm in diameter) from the inoculum preparation. SmF (Step 1) was performed for four days on an orbital shaker (TE-421 model, Tecnal, Brazil) at 30 ºC, at 0,97*g* (120 rpm).

Based on the results obtained in Step 1, two full factorial designs (2^2), Designs 2 and 3 were realized in the Step 2. In view of the fact that potato peels were excluded in the Step 2, since wheat bran was the best substrate in the Step 1, it was decided to use potato peels in one of the designs in a combination with wheat bran. Design 2 was made using only wheat bran as substrate, while Design 3 was carried out with a proportional mixture of wheat bran and potato peel. For both designs of the Step 2, the sodium nitrate was used as nitrogen source and the solution of micronutrients was note added, following the results of the estimated effects of the Design 1. The stirring and temperature conditions were the same as in Step 1, however, three

fermentation times were studied in Step 2 (0 (before starting the fermentation), 2, and 4 d), totalizing 42 experiments.

2.2. Determination of biomass concentration and amylolytic activity

At each sampling point, the fermented media were centrifuged (5810 model, Eppendorf, Germany) at 2,588*g* for 15 min. The solid fraction was dryed at 70 °C until constant weight in air circulation oven (TE-394-1 model, Tecnal, Brazil) to determinate fungal biomass concentration. The liquid fraction was used to amylolytic activity determination, according to the methodology proposed by Rodrigues et al. [22] and Rempel et al. [23].

It was defined that one unit of amylolytic activity (U) is the amount of enzyme capable of releasing 1 μmol glucose per minute under the conditions of the proposed method [24]. Amylolytic activities were calculated from Equations 1 and 2.

$$[RS] = \big((0.2135 * Abs) + 0.0089\big) * FD \quad \textbf{(Eq. 1)}$$

$$AA = \frac{[RS]}{t_R} * \frac{1000}{180} \quad \textbf{(Eq. 2)}$$

Where,
[RS]: Concentration of reducing sugars;
Abs: Absorbance at 546 nm;
FD: factor of dilution (175);
AA: Amylolytic activity (U);
t_R: Time of reaction (30 minutes)

2.3. Data treatment and statistical analysis

Experiment designs were evaluated by the main and interaction effects of the studied variables. The significance levels and estimated effects were analyzed at a confidence level of 90% and 95% for Step 1 and 2, respectively.

3. Results and Discussion

Table 1 shows the results of biomass concentration (g L^{-1}) and amylolytic activity (U) obtained from the fractional factorial design (2^{5-1}) of Step 1. In Table 1 we present the results of the Fractional Factorial Design 2^{5-1}. When using the statistical designs approaches, specially fractionary designs, confounding effects can occur in the matrix, so the ideal is not to carry out a discussion based on the comparison of the experiments individually. Therefore, when using a statistical methodology of experimental design in order to verify the effect of variables, the ideal is the evaluation of the effects of the variables, which is presented in Table 2, that shows the significance levels (p) and the estimated effects of the variables studied in Step 1 on the biomass concentration and amylolytic activity.

Table 1. Biomass concentration and amylolytic activity on the 4th day of fermentation obtained in the fractional factorial design (2^{5-1}).

Experiment	Substrate	N source	N concentration (%)	pH	Micronutrients (g L^{-1})	Biomass concentration (g L^{-1})[a]	Amylolytic activity (U)
1	WB (–1)	$NaNO_3$ (–1)	0.5 (–1)	4 (–1)	1.4 (+1)	4.54±0.25	0.22±<0.01
2	PP (+1)	NaNO3 (–1)	0.5 (–1)	4 (–1)	0 (–1)	0.68±0.04	0.20±0.01
3	WB (–1)	Urea (+1)	0.5 (–1)	4 (–1)	0 (–1)	3.17±0.89	0.21±<0.01
4	PP (+1)	Urea (+1)	0.5 (–1)	4 (–1)	1.4 (+1)	0.46±0.09	0.20±<0.01
5	WB (–1)	$NaNO_3$ (–1)	2 (+1)	4 (–1)	0 (–1)	3.89±0.33	0.22±<0.01
6	PP (+1)	NaNO3 (–1)	2 (+1)	4 (–1)	1.4 (+1)	0.98±0.02	0.22±0.01
7	WB (–1)	Urea (+1)	2 (+1)	4 (–1)	1.4 (+1)	2.31±0.25	0.31±0.01
8	PP (+1)	Urea (+1)	2 (+1)	4 (–1)	0 (–1)	0.27±0.03	0.22±0.01
9	WB (–1)	$NaNO_3$ (–1)	0.5 (–1)	7 (+1)	0 (–1)	3.48±1.76	0.47±0.02
10	PP (+1)	NaNO3 (–1)	0.5 (–1)	7 (+1)	1.4 (+1)	0.88±0.20	0.23±0.01
11	WB (–1)	Urea (+1)	0.5 (–1)	7 (+1)	1.4 (+1)	3.43±0.45	0.24±0.01
12	PP (+1)	Urea (+1)	0.5 (–1)	7 (+1)	0 (–1)	0.33±0.04	0.22±0.01
13	WB (–1)	$NaNO_3$ (–1)	2 (+1)	7 (+1)	1.4 (+1)	4.35±1.49	0.23±0.02
14	PP (+1)	NaNO3 (–1)	2 (+1)	7 (+1)	0 (–1)	1.04±0.04	0.22±<0.01
15	WB (–1)	Urea (+1)	2 (+1)	7 (+1)	0 (–1)	1.88±0.78	0.30±0.01
16	PP (+1)	Urea (+1)	2 (+1)	7 (+1)	1.4 (+1)	0.58±0.20	0.22±0.01
17	PP/WB (0)	$NaNO_3$/U (0)	1.25 (0)	5.5 (0)	0.7 (0)	1.37±0.78	0.25±0.01
18	PP/WB (0)	$NaNO_3$/U (0)	1.25 (0)	5.5 (0)	0.7 (0)	1,49±0.60	0.24±<0.01
19	PP/WB (0)	$NaNO_3$/U (0)	1.25 (0)	5.5 (0)	0.7 (0)	2.07±0.37	0.26±0.02
20	PP/WB (0)	$NaNO_3$/U (0)	1.25 (0)	5.5 (0)	0.7 (0)	5.08±0.94	0.24±0.01

Legend: WB: wheat bran; PP: potato peel; PP/WB: potato peel and wheat bran; N: Nitrogen; $NaNO_3$: sodium nitrate; $NaNO_3$/U: sodium nitrate and urea. [a]: Dry basis.

It is possible to verify a wide variation in the production of fungal biomass between experiments, with some experimental conditions generating 4 and 5 g L^{-1} and others less than 1 g L^{-1}. In general, low amylolytic activity was observed for all experiments, with 0.47 ± 0.02 U being the highest activity obtained. However, fractional designs are commonly used to select variables of interest, as they are able to identify which factors should be explored in greater detail in subsequent complete factorial designs, making the experiment more economical and efficient [25].

Table 2. Significance levels (p<0.1) and estimated effects of the variables studied in the first SmF design.

Factor	Biomass concentration (g L^{-1})		Amylolytic activity (U)	
	p	Effect	p	Effect
Substrate	0.000046	-2.72986	0.000128	-0.056581
N source	0.068352	-0.92931	0.141207	-0.007097
N concentration (%)	0.663696	-0.20903	0.185052	-0.006201

pH	0.929087	-0.04264	0.000418	0.041848
Micronutrients (g L^{-1})	0.468459	0.35069	0.003134	-0.024661

It is observed that the substrate showed a significant ($p<0.1$) and negative effect among the factors tested for both response variables (Table 2). This indicates that the wheat bran substrate obtained better results, both for biomass concentration and for amylolytic activity, when compared to potato peel. The production of amylolytic enzymes by *A. niger* in solid and submerged fermentations using wheat bran has been used as controls in our research group, so this result was already expected. Previous works have used wheat bran as the main culture media of producing amylases, as Rodrigues et al. [22].

The nitrogen source had a significant ($p<0.1$) and negative effect on the biomass concentration, not being significant for amylolytic activity. Based on the estimated effects, sodium nitrate was the selected nitrogen source to be used in subsequent tests. The pH and the addition of micronutrients were also significant on the amylolytic activity, the effects being positive for pH and negative for the addition of micronutrients. Thus, pH 7 and the non-addition of micronutrients proved to be the best conditions for amylolytic activity.

Although the nitrogen concentration did not show significance on the two response variables, it was decided to study it in the second and third experimental designs under the conditions of non-insertion of sodium nitrate (0%) and the addition of 1% of this source. Nitrogen is an essential nutrient for fungal growth, playing an important role in the development of fungi and in the secondary production of metabolites [26-27]. El-Enshasy [28] considers that, as well as the type of nitrogen source, the concentration of this source applied to cultivation also influences fungal growth.

Therefore, two new experimental designs were proposed, selecting the concentration of the nitrogen source and the pH as study variables, since the pH was significant on the amylolytic activity of the extracts. In Design 2, only wheat bran was used as substrate and, in Design 3, a mixture of wheat bran and potato peel was used. Although the potato peel did not show good results, it was decided to continue studying this residue according to the amount of starch available in this source.

Figures 1a and 1c show, respectively, the values obtained for biomass concentration (g L^{-1}) and amylolytic activity (U) in Step 2, with fermentations carried out with wheat bran (Design 2), while Figures 1b and 1d refer to crops with potato peel and wheat bran (Design 3).

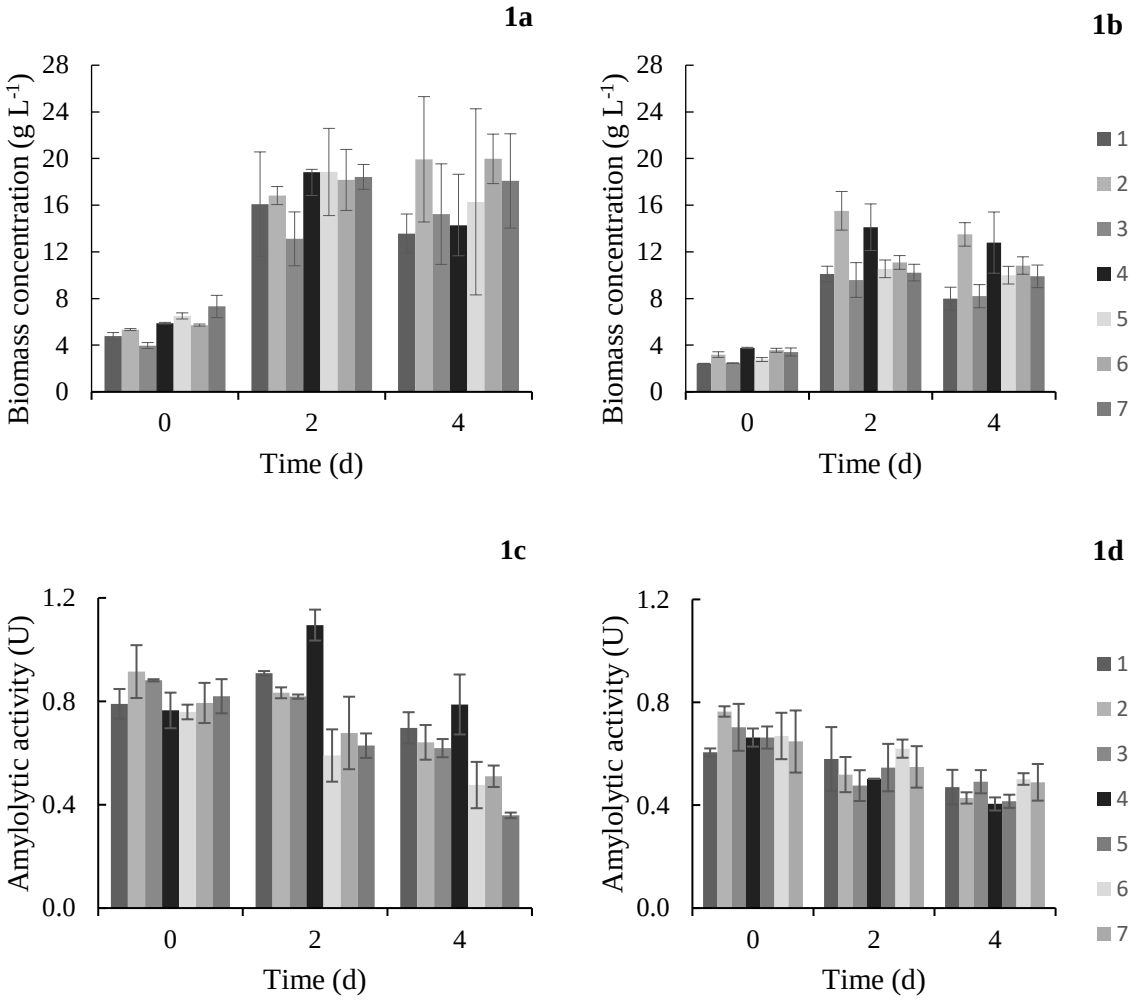

Figure 1. Biomass concentration (a and b) (g L^{-1}) and amylolytic activity (U) obtained in Design 2 (wheat bran)* and Design 3 (potato peel + wheat bran)*.

Figure 1a and 1c: Fermentation carried out with wheat bran; Figure 1b and 1d: Fermentation carried out with potato peel + wheat bran. *Experimental conditions: Exp. 1: 0 g L-1 NaNO3 and pH 5; Exp 2: 1.0 g L-1 NaNO3 and pH 5; Exp. 3: 0 g L-1 NaNO3 and pH 7; Exp. 4: 1.0 g L-1 NaNO3 and pH 7; Exp. 5, 6 and 7: 0.5 g L-1 NaNO3 and pH 6.

It is possible to observe in Figure 1 that the development of the fungus *A. niger* cultivated with wheat bran as substrate was better than the medium composed by potato peel and wheat bran, since higher concentrations of biomass and amylolytic activity were achieved, corroborating the results of the first design experimental. This can be explained by the high levels of carbohydrates and proteins present in these residues and, consequently, of carbon and nitrogen to be used by the fungus for its metabolic activities [29]. It is also possible to verify that the greatest production of fungal biomass occurred in two days of fermentation, for both substrates used.

Rodrigues et al. [22] also produced amylases through cultivation of *A. niger* in bioprocess and submerged using wheat bran as substrate, obtaining an amylolytic activity of 0.79 U in 24 hours of submerged fermentation. Although amylolytic activities superior to the study mentioned in 48 h have been achieved, it would be necessary to employ some method of purification to enable the direct use of fungal amylases in saccharification of microalgal biomass, as performed by Rodrigues et al. [22]. In order to optimize the production of fungal biomass and amylolytic activity of *A. niger* cultivated in

SmF, it was considered to choose the results obtained on the second day of fermentation to evaluate them according to the design methodology of experiments. The levels of significance ($p<0.05$) and estimated effects of Design 2 and Design 3 are shown in Table 3.

Table 3. Significance levels ($p<0.05$) and estimated effects of the variables studied in Design 2 and Design 3.

Factor	Biomass concentration (g L^{-1})			
	Wheat bran (Design 2)		Potato peel + wheat bran (Design 3)	
	p	Effect	p	Effect
Curvature	0.013189	4.53167	0.036273	-3.42633
(1) N concentration (%)	0.011242	3.21800	0.007724	4.96900
(2) pH	0.289754	-0.49100	0.160433	-0.96000
(1) x (2)	0.018627	2.48600	0.418504	-0.44400
Factor	**Amylolytic activity (U)**			
	Wheat bran (Design 2)		Potato peel + wheat bran (Design 3)	
	p	Effect	p	Effect
Curvature	0.014568	-0.542572	0.240660	0.105360
(1) N concentration (%)	0.212025	0.078452	0.730334	-0.016552
(2) pH	0.280168	0.063569	0.292026	-0.059251
(1) x (2)	0.070908	0.153994	0.410206	0.043168

Table 3 shows that the curvature was significant ($p<0.05$) for both biomass concentration and amylolytic activity (with the exception of the cultivation carried out with potato peel + wheat bran for amylolytic activity response). Thus, it is possible to have maximum and minimum points between the factors studied. The concentration of the nitrogen source ($NaNO_3$) influenced the production of fungal biomass and, based on the estimated effects, nitrogen supplementation (1%) was beneficial for the two tested substrates, proving that the addition of a supplementary nitrogen source favors the fungal growth, as reported by Schmidt and Furlong [30].

From the level of significance ($p<0.05$) verified for the curvature of the response variables (biomass concentration and amylolytic activity), and with the objective of identifying the best experimental conditions of submerged fermentation for the production of biomass and of amylolytic activity simultaneously, the following response surfaces of Figure 2 were generated.

It can be seen that SmF made with wheat bran as substrate showed better results at the higher levels of the tested variables, that is, with the addition of 1% nitrogen and pH 7. On the other hand, the fungus grown with potato peel and wheat bran developed better at pH 5, but also with 1% $NaNO_3$ supplementation. Although the conditions mentioned above have been chosen, it must be understood that the pH was not significant separately in Design 2 and Design 3 (Table 3). However, Table 3 still shows a significant interaction between pH and concentration of the nitrogen source. From Figure 3, it can be seen that the interaction between the variables results in

an increase in the concentration of biomass when both are at the upper levels.

Thus, the conditions of cultivation in submerged fermentation showed viability for the production of fungal biomass using agro-industrial by-products. This study demonstrates the feasibility of reusing solid wastes generated from agricultural activities, making them feedstocks for bioprocesses to obtain several bioproducts. This reuse is considered sustainable and viable from a sustainability point of view, reducing waste disposal and contributing to the reduction of the environmental impact on agricultural activities.

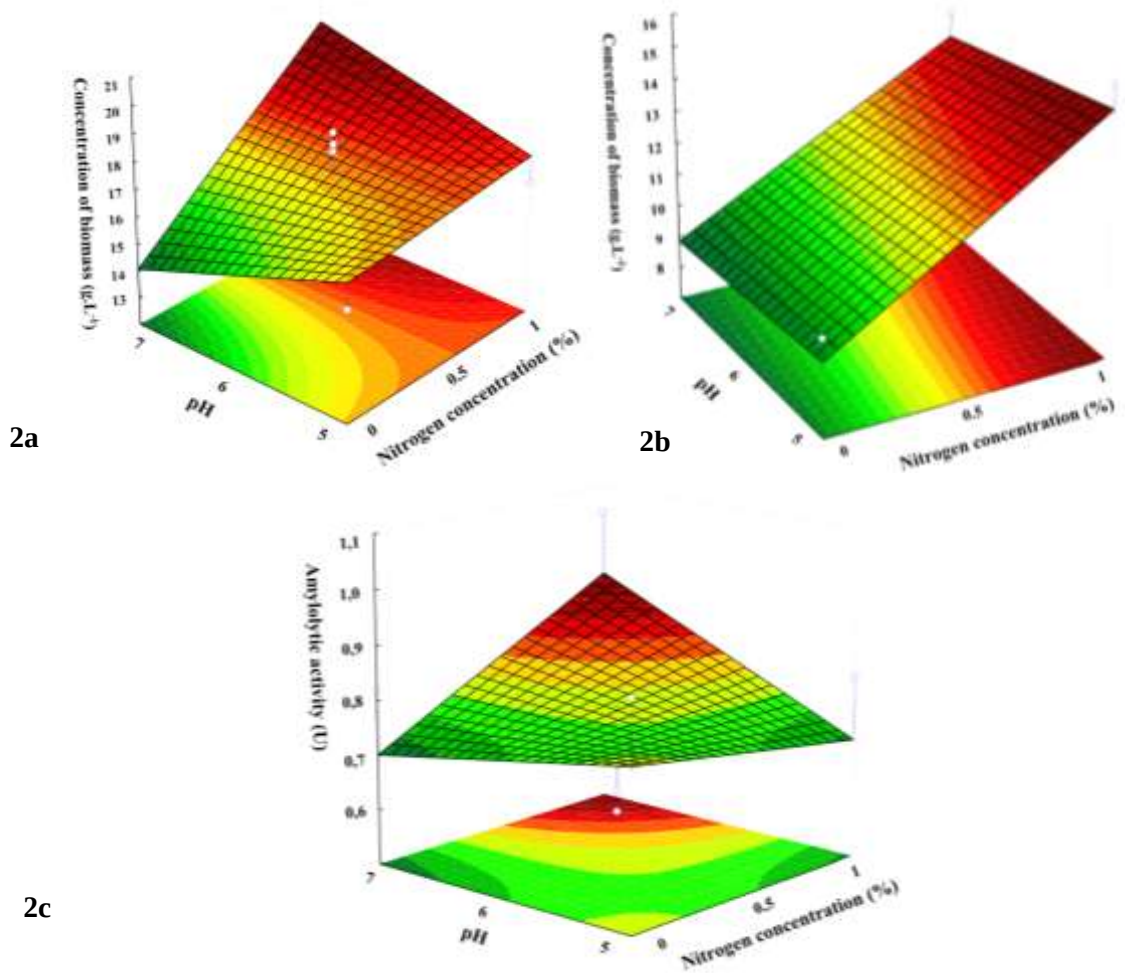

Figure 2. Response surfaces obtained from the Step 2 experiments ($p<0.05$)*.

*Experimental conditions:
2a: Biomass concentration of the fungus with wheat bran;
2b: Biomass concentration of the fungus with wheat bran and potato peel;
2c: Amylolytic activity in wheat bran used as substrate.

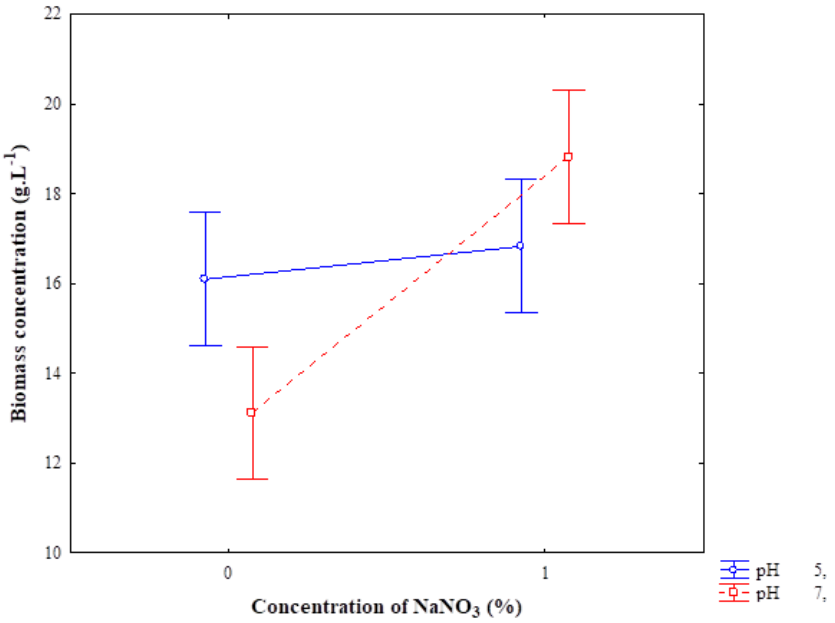

Figure 3. Interaction of the means of the variables in the second design.

4. Conclusions

It was possible to verify the best conditions to produce fungal biomass and amylolytic enzymes under SmF using two agro-industrial by-products. The highest fungal biomass production occurred in two days for both substrates. Biomass concentration was higher with a medium composed by wheat bran than potato peel + wheat bran. The best conditions to produce fungal biomass were 1% of $NaNO_3$ and pH 7 for wheat bran, while for potato peel + wheat bran were 1% of $NaNO_3$ and pH 5.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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