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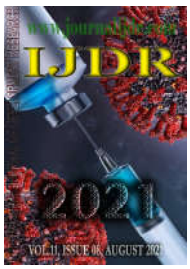

RESEARCH ARTICLE OPEN ACCESS

AMYLASE PRODUCTION BY *Aspergillus spp.* IN SUBMERGED FERMENTATION USING MALT BAGASSE RESIDUE

Diego G. De L. Lemos[1], Rosileide F. S. Andrade[1], Hilário J. B. Lima Filho[2] and Carlos A. A. da Silva[1]

[1]Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife, Brazil; [2]Department of Chemical Engineering – DEQ, Catholic University of Pernambuco, Recife, Brazil

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****Corresponding author:***
Diego G. De L. Lemos,

ABSTRACT

The objective of this work was use brewer's malt bagasse residue as an amylase production inducer by *Aspergillus* spp. (UCP1275, UCP6119, UCP1295, and UCP1261) in submerged fermentation. Initially, production was analyzed in standard culture medium (SCM) containing starch as the only carbon source. Then, the strains were tested for bioconversion potential in a new culture medium containing malt bagasse residue (MBM). A factorial design was applied with the selected strain to define the ideal conditions for cultivation and production, varying the initial pH (5 to 7), temperature (24 to 32ºC), and concentration of malt bagasse residue (5 to 15g/L). According to the results, all strains of *Aspergillus* spp. tested demonstrated amylolytic activity in a standard medium. In the alternative medium containing malt bagasse (MBM), strain 1 (*Aspergillus* spp. UCP 1275) was selected for its high potential in the production of amylase (2.264 U/mL). In planning condition 1 (pH 5, temperature 28ºC, and 5g/L of malt bagasse residue), this strain produced 7.59U/mL of amylase. From the data obtained, *Aspergillus* spp. UCP 1275 proved to be a promising microorganism for amylase production in a sustainable culture medium in submerged fermentation.

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INTRODUCTION

The residues produced by food manufacturers have a high content of organic matter and are rich in nutrients. When these industrial wastes are not adequately treated, they cause serious environmental pollution problems. In addition, when discarded into the environment, these residues represent a loss of valuable nutrients(KUMAR *et al*., 2020). The main waste generated by the brewing industry is malt bagasse. Brazil is the third-largest beer producer globally and, therefore, one of the largest generators of malt bagasse(MAIONE, 2019). Barley malt bagasse results from the initial brewing process, generated from the filtration of the wort before boiling. Malted barley grain husks constitute this bagasse. Its use has been studied in biotechnological processes(RODRIGUES, 2021).According to Mussatto (2006) e Silva (2019) malt bagasse is a rich lignocellulosic biomass containing about 20-30% proteins and 70-80% fiberswith hemicellulose, cellulose, and lignin as the primary fiber components. This residue is used as a substrate for fermentation and the production of enzymes by filamentous fungi(ALVES *et al*., 2019).Filamentous fungi are easily cultivated microorganisms. They have a high potential for enzymatic production and secrete many extracellular enzymes with potential for numerous industrial and biotechnological applications (NASCIMENTO *et al*., 2014).

The *Aspergillus* genus stands out among other fungal genera for having the ability to survive in different conditions and produce metabolites that can decompose agro-industrial residues into bioproducts with satisfactory efficiency in this conversion process (GUSMÃO *et al*., 2014).The objective of this work was to identify the potential of different strains of *Aspergillus* spp. in the production of amylase from malt bagasse residue bioconversion using submerged fermentation.

MATERIALS AND METHODS

Four samples of *Aspergillus* spp. (UCP 1275, UCP 6119, UCP 1295, and UCP 1261) were obtained of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco with registered in World Federation for Culture Collections - WFCC. The *Aspergillus* spp. samples were isolated from soil of the Caatinga of Pernambuco.The substrate used was brewer's malt bagasse residue acquired of the craft beer industry located in the municipality of Jaboatão dos Guararapes, Pernambuco, Brazil. For chemical analysis the substrate (malt bagasse residue) was dried in an convection oven, for 72 hours at 60° C, according to Lemos *et al*. (2020), obtaining the following parameters: Dry matter - DM %; Mineral matter - MM %; Total nitrogen - TN %; Total protein

- TP %; Acid detergent fiber - ADF %; Neutral Detergent Fiber - NDF %; Non-nitrogen extract - NNE %; Total fiber - TF %; Ethereal extract - EE %; Starch – AM %; Sodium – Na^{+} %; Potassium – K^{+} %; Calcium – Ca^{2+} %; Magnesium – Mg^{2+} %, according to Official Methods of Analyzes (AOAC, 2007). The dried material was manually crushed and sieved to obtain 0.5mm uniform particles.Strains of *Aspergillus* spp. were acclimated in Sabouraud medium supplemented with malt bagasse (0.2%). The inoculum was made frommycelia of fungal strains grown in Sabouraud medium supplemented with malt bagasse (0.2%). After 72 hours of growth at 28ºC, 20 discs of 8mmwere obtained and used as inoculum in the production medium(PERA *et al*., 1998).Amylase production was investigated by *Aspergillus*spp. strains grown on standard medium, according with Adms (1990). In the alternative medium to the standard medium was replacement of starch by malt bagasse in same concentration. According to the methodology the DNS method was employed to perform the amylolytic activity(ANVISA, 2012; FRANÇA, 2021). This method is based on quantifying reduced sugars released by the starch hydrolysis reaction catalyzed by amylases. For this purpose, the tubes were prepared with a mixture of 1.5 mL of soluble starch (1% w/v), 0.05 M citrate-phosphate pH 6 buffer solution, and 1.5 mL of crude enzymatic solution. The reaction was carried out at 50° C for 15 minutes. Subsequently, the reaction was stopped by adding 1.5 mL of 3,5-dinitrosalicylic acid reagent. The mixture was boiled for 5 min and the solution was cooled to room temperature. Additionally, 15 mL of distilled water was also added. Absorbance was read at 550 nm in a UV-VIS spectrophotometer. A unit of enzyme activity was defined as the amount of enzyme required to catalyze the release of reduced sugar equivalent to 1µmol of D-glucose per minute under the test conditions.

bagasse residue through factorial design. Thereforein the planning the minimum (-1), maximum (1) and intermediate (0) levels and 4 repetitions in central point were defined as described in Table 1. The independent variables were: initial pH, temperature (ºC) and the concentration of malt bagasse residue (g/L). The response variables were: Amylase Enzyme Activity ($UA.mL^{-1}.min^{-1}$), final pHand biomass yield (g). A Statistica12 software was used to support the execution of this step.

RESULTS AND DISCUSSION

Soluble starch was used in standard culture medium (SMC) using only carbon sourcetoinvestigate*Aspergillus* spp. strains regarding the potential of amylase production in submerged fermentation (SF). The results of the analyzes are described in Table 2. All strains of *Aspergillus* spp. strains tested demonstrated amylolytic activity. The maximum amylolytic activity in the medium with soluble starch (standard medium - SMC) after 96h of fermentation was 0.760 U/mL with strain 2 (*Aspergillus* spp. UCP6119).

The coefficient of variation analysis with the results obtained with strain 2 was statistically significant with a confidence level of 95% and R^2 of 0.9829. The results of replicas of strain 2 (*Aspergillus* spp. UCP6119) were considered statistically valid due the absence of statistical distortions through the outlier values obtained.Strains of *Aspergillus* spp. able of assimilate the soluble starch present in the standard medium (SMC) were tested in a new culture medium containing malt bagasse residue (MBM) as carbon source replacing soluble starch.

Table 1. Factorial design for evaluation of pH, temperatureand malt bagasse residue concentration in amylolytic activity by selected strain

Conditions	Independent variables		
	pH_0	Temperature (°C)	Conc. Residue (g/L)
1	5 (-1)	24 (-1)	5 (-1)
2	7 (1)	24 (-1)	5 (-1)
3	5 (-1)	32 (1)	5 (-1)
4	7 (1)	32 (1)	5 (-1)
5	5 (-1)	24 (-1)	15 (1)
6	7 (1)	24 (-1)	15 (1)
7	5 (-1)	32 (1)	15 (1)
8	7 (1)	32 (1)	15 (1)
9	6 (0)	28 (0)	10 (0)
10	6 (0)	28 (0)	10 (0)
11	6 (0)	28 (0)	10 (0)
12	6 (0)	28 (0)	10 (0)

Table 2. Potential of *Aspergillus* spp. in production of amylase in medium containing soluble starch

Strains of *Aspergillus*spp.	Culture medium	Enzyme Index (U/mL)	Coeff. Variation (CV)	Outlier			Confidence Interval (95%)
				Rep1	Rep2	Rep3	
Strain1 (UCP1275)	SMC	0.410	0.26	1.122	0.798	0.324	0.144 +/- 0.0945
Strain2 (UCP6119)	SMC	0.760	0.30	1.137	0.394	0.743	0.213 +/- 0.3132
Strain3 (UCP1295)	SMC	0.004	0.30	0.429	0.714	1.143	0.063 +/- 0.0522
Strain4 (UCP1261)	SMC	0.500	0.30	0.551	0.604	1.154	0.161 +/- 0.1413

*Standard medium (SMC)

*CV ≤ 0.1 → low dispersion; 0.1< CV ≤ 0.2 → medium dispersion; 0.2< CV ≤ 0.3 → high dispersion; CV >0.3 → data discard or repeat. And Out ≤ 2value without discrepancy (LEMOS et al., 2020)

The results of amylolytic activity were expressed in$UA.mL^{-1}.min^{-1}$ according to Equation 1. The experiments were performed in triplicate with values statistically validated by: R^2, coefficient of variation (CV), outlier (OUT) and $T_{student}$ at 95%.

$$\text{Amylolytic Activity } [AU.mL^{-1}.min.^{-1}] = \frac{C * Vm}{Tr} \quad \text{(Equation 1)}$$

Where:C = Concentration of reduced sugars in the sample (µmol ml^{-1}); Vm = volume of reaction mixture; Tr = Reaction time

The maximum amylase production was investigated with the selected strain varying the pH, temperature, and concentration of the malt

According to the data obtained, strain 1 (*Aspergillus* spp. UCP1275) was effective in the bioconversion of nutrients present in malt bagasse for amylase enzyme production resulting in 2.264 $U/mL^{-1}.min^{-1}$ amylolytic activity (Table 3).

Additionally, this strain showed an increase of more than 500% in the value of the amylolytic activity. The obtained data coefficient of variation analysis in all experiments in medium with malt bagasse residue (MBM) was statistically significant with a confidence level of 95% and R^2 of 0.9467.The results of replicas of strain 1 (*Aspergillus* spp. UCP1275) were considered statistically valid due to the absence of statistical distortions through the obtained outlier values.

Table 3. Selection of the *Aspergillus* spp. strain with greater amylolytic potential in malt bagasse medium

Strains of *Aspergillus* spp.	Culture medium	Enzyme Index (U/mL)	Coeff. Variation (CV)	Outlier			Confidence Interval (95%)
				Rep1	Rep2	Rep3	
Strain1 (UCP1275)	MBM	2.264	0.17	1.135	0.385	0.750	0.512 +/- 0.2174
Strain2 (UCP6119)	MBM	2.253	0.27	0.334	0.791	1.124	0.510 +/- 0.3425
Strain3 (UCP1295)	MBM	1.252	0.17	1.130	0.772	0.358	0.311 +/- 0.1320
Strain4 (UCP1261)	MBM	1.979	0.06	1.069	0.913	0.156	0.456 +/- 0.0690

* Malt Bagasse Medium (MBM);
*CV ≤ 0.1 → low dispersion; 0.1< CV ≤ 0.2 → medium dispersion; 0.2< CV ≤ 0.3 → high dispersion; CV >0.3 → data discard or repeat. And Out ≤ 2, value without discrepancy

Table 4. Results of the bromatological composition of malt bagasse residue

Components	Amount (%)	Method
Dry Matter – DM	78.30	
Mineral Matter – MM	34.22	
Total Nitrogen – TN	3.90	
Total Protein – TP	24.37	
Acid Detergent Fiber – ADF	28.83	AOAC 2007
Neutral Detergent Fiber - NDF	50.30	
Non-nitrogen Extract – NNE	43.16	
Total Fiber – TF	24.13	
Ethereal Extract – EE	04.12	
Starch – AM	07.46	
Sodium - Na^+	00.03	

Table 5. Evaluation of pH, temperature and malt bagasse residueconcentration in amylolytic activity of the selected strain

Conditions	INDEPENDENT VARIABLES			RESPONSE VARIABLES		
	pH_0	Temperature (°C)	Conc. Residue (g/L)	Enzyme Activity (U/mL)	Final pH	Biomass (g)
1	5 (-1)	24 (-1)	5 (-1)	7.59	7.86	0.2272
2	7 (1)	24 (-1)	5 (-1)	6.85	8.33	0.3371
3	5 (-1)	32 (1)	5 (-1)	2.09	8.28	0.2483
4	7 (1)	32 (1)	5 (-1)	4.34	8.76	0.086
5	5 (-1)	24 (-1)	15 (1)	2.74	7.71	0.999
6	7 (1)	24 (-1)	15 (1)	3.37	8.33	0.803
7	5 (-1)	32 (1)	15 (1)	4.21	8.19	0.838
8	7 (1)	32 (1)	15 (1)	3.90	8.63	0.6063
9	6 (0)	28 (0)	10 (0)	6.36	8.18	0.6166
10	6 (0)	28 (0)	10 (0)	6.50	8.06	0.4333
11	6 (0)	28 (0)	10 (0)	6.34	8.18	0.4920
12	6 (0)	28 (0)	10 (0)	6.48	8.18	0.6798

According to Table 4malt bagasse residue proved to be an excellent carbon source for *Aspergillus* spp. UCP1275 (strain 1) due the its rich nutritional constitution in carbon source (starch), nitrogen source (total protein) and minerals present in the malt bagasse residue. Beer waste used by Eichler (2018) andMendoza (2021) demonstrates protein percentage values lower than those obtained in this study.The 2^3factorial design (Table 5) was applied with the selected strain (*Aspergillus* spp. UCP1275) to maximize the amylolytic activity. According with amylolytic activity response variable, planning condition 1 (malt bagasse residue with pH 5, temperature 28°C and 5g/L) resulted in maximum amylolytic activity (7.59UA.mL^{-1}.min^{-1}). In this planning condition, the final pH showed slightly alkaline valuesand the microbial growth was not significant proving no relationship between growth and amylase production.Comparing the high levels of enzymatic activity in this study with other works where the same production methodologies are applied (submerged fermentation with *Aspergillus*sp.), it is possible to confirm that the values of enzymatic activity for amylaseobtained in this study (Table 5) are higher than those mentioned in literature(VASCONCELOS *et al*., 2021), 0.91 U/mL (FRANÇA *et al*., 2021), $7.062x10^{-2}$ U/mL (YAHYA *et al*., 2021).According to the Pareto Diagram (Figure 1), it is possible to identify that all independent variables were significant with values above p. favoring amylase production. Figure 1 statistically demonstrates the influence of pH, temperature and concentration of malt bagasse residue on the amylolytic activity. The relationship between malt bagasse residue concentration and temperature variation was the one that most contributed to the increase in enzymatic activity.

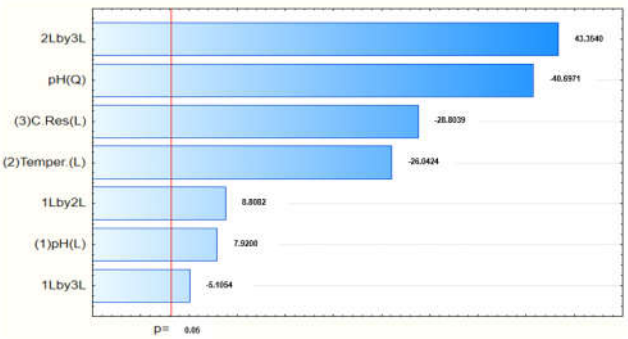

Figure. 1 Pareto diagram for statistical analysis of pH, temperature and residue concentration in amylolytic activity of*Aspergillus* spp. UCP 1275 (strain 1)

The effects of independent variables and interactions for enzyme activity response variable are shown in two-dimensional (Figure 2A) and three-dimensional (Figure 2B) graphs. They show statistically that malt bagasse residue concentration values of 4.0 g/L, pH 6 and temperature 23º C induce the maximum amylase production by strain 1 (*Aspergillus* spp. UCP1275). The influence of the pH, temperature and residue concentration on biomass production by *Aspergillus* spp. UCP1275 (strain 1) was also analyzed statistically (Figure 3). According to the results obtained in the Pareto Diagram (Figure 3), the independent variable that most influenced growth was malt bagasse concentration.

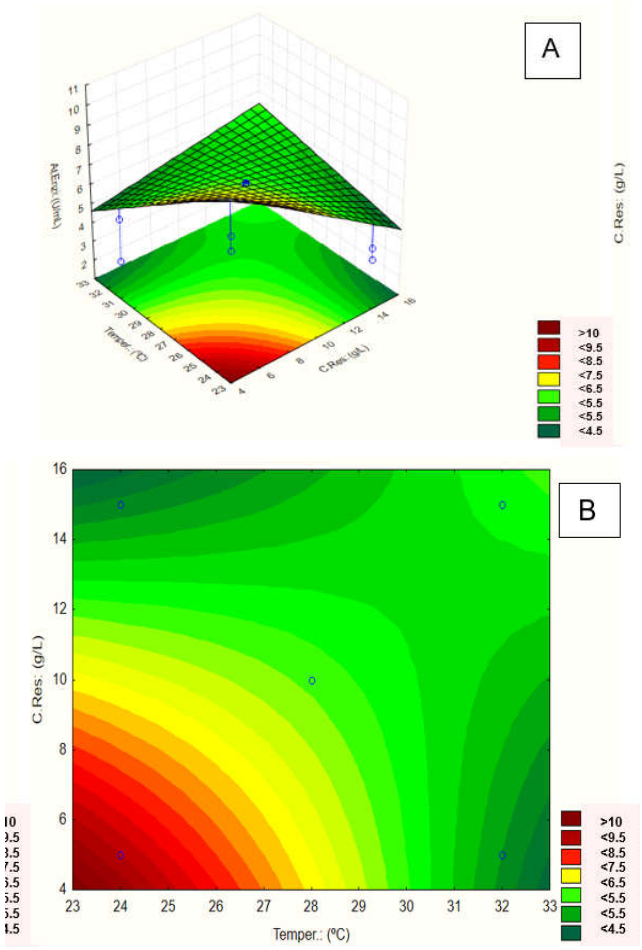

Figure 2. Enzyme activity as a function of temperature and residue concentration evaluated by surface response (A) and contour curve (B) graphs

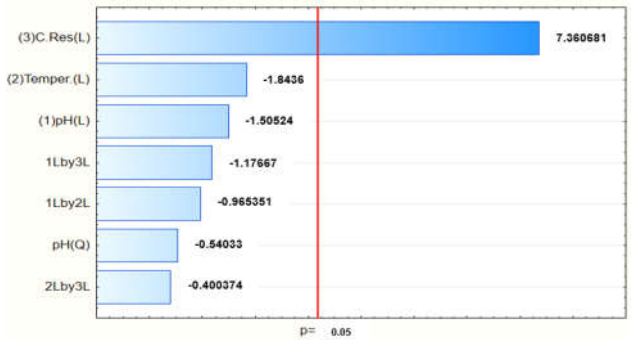

Figure. 3Pareto diagram for statistical analysisof biomass production after cultivation of *Aspergillus*spp. UCP1275 (strain 1) in medium containing malt bagasse (MBM)

Associating this information with the data in Table 5, it is possible to identify that the maximum level of malt bagasse residue (15g/l) favored the maximum growth of the fungus and consequent maximum biomass yield.

CONCLUSION

All strains of *Aspergillus* spp. studied demonstrated biotechnological potential for amylase production in standard medium (SMC) containing soluble starch. On the other hand, in the alternative medium containing malt bagasse residue (MBM) *Aspergillus* spp. UCP1275 demonstrated maximum potential for metabolizing malt bagasse residue for amylase production in submerged cultivation. The data obtained were statistically validated with an R^2 valuedemonstrating the robustness of the mathematical model.

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