CELLULASE PRODUCTION BY *Aspergillus japonicus* URM5242 USING RICE STRAW AS SUBSTRATE IN SEQUENTIAL FERMENTATION

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ABSTRACT

This study explored the production of fungal cellulases using Sequential Fermentation (SF) with rice straw as a lignocellulosic substrate. Utilizing the fungus *Aspergillus japonicus* URM5242**,** a 2^3 factorial design was employed to assess the effects of varying moisture levels, substrate amounts, and glucose concentrations. The optimal cellulolytic activity, 0.570 UI/ml, was observed with conditions of 70% moisture, 5 g substrate, and 30 g/L glucose. The results demonstrated an inverse relationship between the substrate amounted moisture for enzyme production and a synergistic effect between glucose and substrate. The rice straw is an economical substrate for enzyme production, promoting sustainable agricultural practices and mitigating environmental issues related to rice straw disposal.

Keywords: Lignocellulosic biomass. Endoglucanase. Fungi. Enzymatic production.

1 INTRODUCTION

The fungal potential to produce enzymes depends on the species, the lignocellulosic biomass used as a substrate, the cultivation method, and its conditions. Since cellulase is a high-value enzyme in the market, costs can be reduced by using naturally available biomass as a carbon source. The availability, low cost, and abundance of lignocellulosic biomass are the qualities that make it stand out as a versatile substrate for enzyme production [1]. Due to their higher production, most microorganisms explored for cellulase production are fungi [2]. Fungi commonly used in enzyme production include the genera *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, and *Phanerochaete* [3], with *Aspergillus*, a filamentous fungus, being highlighted as an excellent producer of exo and endoglucanases [4]. Sequential Fermentation (SF) methodology, when compared to conventional methods (solid-state fermentation and/or submerged fermentation), has proven superior for cellulase production, with SF presenting great potential for cellulolytic enzyme production and enabling better utilization of lignocellulosic substrates [5]. Rice is a significant crop worldwide. Approximately half of the world's population, especially in developing countries, depends on rice as a primary food source. The Food and Agriculture Organization (FAO) predicts that global demand for rice will reach approximately 760 million tons by 2025. However, the accumulation of rice straw residues can lead to serious environmental contamination if mishandled. The rice straw is an abundant agricultural residue that requires proper management, its improper disposal can cause environmental pollution [6]. Each kilogram of rice produced results in approximately 0.7 kg to 1.4 kg of rice straw, depending on the varieties, stubble height, and moisture content during harvesting [7]. Characterization of rice straw has identified its composition in terms of cellulose (36.8%), hemicellulose (28.6%), lignin (5.8%), and moisture (9.8%) [8]. Thus, the present study aimed to evaluate the production of fungal cellulase and determine the best conditions for enzyme activity using SF, with rice straw as the lignocellulosic substrate.

2 MATERIAL & METHODS

Microorganism

The fungus *Aspergillus japonicus* URM5242, originating from the culture collection of the Federal University of Pernambuco (Recife, Brazil), was selected. The culture medium used for maintaining the microorganism strain was Malt Extract Agar, with subculturing performed every thirty days. The culture medium used for sporulation was the Czapek medium [9]. The medium was carried out in an autoclave at 121°C, 1 atm, for 20 minutes. Fungal growth occurred in an incubator at 30°C for seven days.

Standardization and Characterization of Substrates

The substrate used for performance analysis was the rice straw. It was acquired at "Tupi" store, which operates as a distributor of various types of rice straw in Garanhuns, Pernambuco, Brazil. The substrate underwent a standardization procedure, initially involving moisture removal in an oven at a set temperature of 65°C, with measurements taken on an analytical balance every 24 hours until the weight stabilized. After drying, the substrate was ground in a knife mill and standardized using Mesh 10 and Mesh 32 sieves, with both granulometries stored. After standardization, the dried substrate was characterized by parameters of moisture content (%), a series of solids (total, fixed, and volatile) according to WHO (1978), and pH.

Sequential Fermentation, 2^3 factorial design and statistical analysis

Sequential Fermentation (SF) was initially carried out through the growth of the fungus *A. japonicus* URM5242 in Solid-state Fermentation (SSF) for 24 hours. Subsequently, the process continued as Submerged Fermentation (SmF) by adding liquid

culture medium to the substrate (rice straw) cultivation for 48 hours. During the SSF cultivation stage, the moisture content was adjusted to 70% by adding nutrient medium (2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.30 g/L $CaCl_2$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 5.0 g/L yeast extract, 2 g/L yeast extract, 0.30 g/L urea, 0.10% Tween 80, and 0.10% saline solution (5 mg/L $FeSO_4 \cdot 7H_2O$, 1.4 mg/L $ZnSO_4 \cdot 7H_2O$, and 2.0 mg/L $CoCl_2$)) enriched with 10 g/L glucose, in a 250 mL Erlenmeyer flask containing 3 g substrate. A volume of spore suspension was added, 10^7 spores per gram of dry substrate, with cultivation maintained under SSF static conditions for 24 hours at 30°C. For the SmF, a volume of nutrient solution with the same composition as described previously was added, following 40 mL of nutrient medium per gram of solid ferment, with cultivation continued in an orbital incubator for 48 hours at 30°C, with continuous agitation at 120 rpm.

To determinate better conditions to be applied in the Sequential Fermentation (SF) to the production of fungal cellulases, a 2^3 factorial design was carried out, in which the conditions of moisture content (%), substrate (g), and glucose (g/L) present in the nutrient solution were varied. With the use of the Statistica software, it became possible to conduct statistical analysis of the results obtained from the 2^3 factorial design, including the development of the Pareto Chart and of the cubic graph.

Determination of Enzymatic Activity

The cellulolytic activity was determined through total cellulase activity on endoglucanase (CMCase) [11]. The endoglucanase (CMCase) activity was carried out using 1% sodium carboxymethyl cellulose in 0.05 M citrate buffer (pH 4.8) was used as the substrate. The substrate (0.5 mL) was placed in test tubes, followed by the addition of 0.5 mL of enzyme extract. The enzymatic reaction occurred at 50°C for 30 minutes. Subsequently, the amount of glucose released was measured using the 3,5-dinitrosalicylic acid (DNS) reaction. Control for the colorimetric reaction (enzyme blank) and substrate (reaction blank) were used in both activities. Absorbances were converted to glucose using a previously established standard curve. One international unit (IU) was defined as equivalent to 1 µmol of glucose released per minute. The activity was performed by reading absorbance at 540 nm on a spectrophotometer. The glucose concentration released was identified through the reaction with DNS [12].

3 RESULTS & DISCUSSION

Substrate Characterization

The substrate applied for SF was characterized, considering that its physicochemical characteristics may influence the efficiency of SF and the enzymatic production results. The pH of rice straw was identified as acidic. Regarding Moisture, rice straw showed low moisture content (8.04%±0,16). The results obtained regarding Volatile Solids (%) were of 89,6±0,00. Similar results were shown about rice straw characterization, with Volatile Solids (%) results of 84.1±0.39 and Moisture of 6.49%±0.20 [13].

2^3 Factorial Design

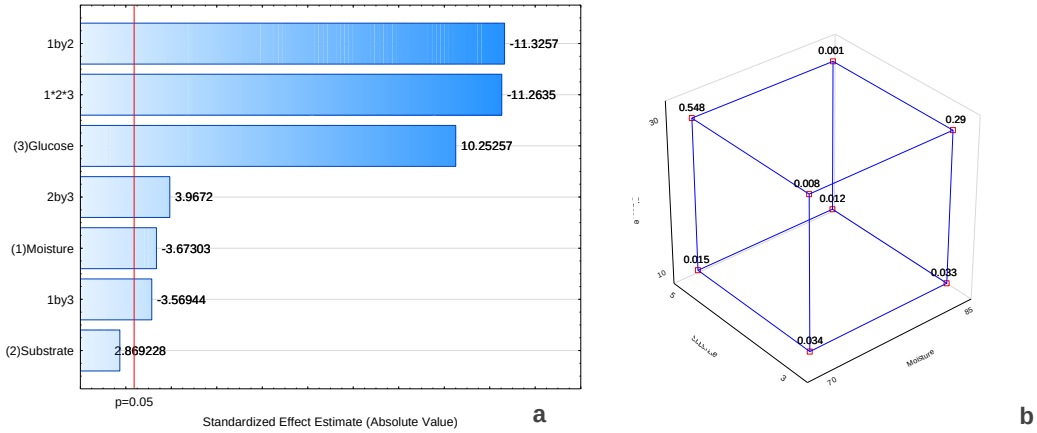
The 2^3 factorial design allowed the cellulase production under different conditions of moisture (%), substrate (g), and glucose (g/L) (Table 1).

Table 1. Cellulolytic Activity of the 2^3 factorial design by *Aspergillus japonicus* URM5242 in Sequential Fermentation

Assay	Moisture (%)	Substrate (g)	Glucose (g/L)	Endoglucanase (UI/mL)
01	70	3	10	0.056
02	85	3	10	0.056
03	70	5	10	0.037
04	85	5	10	0.034
05	70	3	30	0,030
06	85	3	30	**0.312**
07	70	5	30	**0.570**
08	85	5	30	0.023
09	78	4	20	0.055
10	78	4	20	0.065
11	78	4	20	0.111
12	78	4	20	0.060

The assay 7 achieved the best result, with CMCase activity of 0.570 UI/mL under conditions of 70% moisture, 5 g of substrate, and 30 g/L of glucose. The second highest cellulase production was obtained in Assay 6, with CMCase activity of 0.312 UI/mL under conditions of 85% moisture, 3 g of substrate, and 30 g/L of glucose. Similarly, in Assay 6, the moisture was higher (85%) and the substrate amount lower (3 g), highlighting the inversely proportional relationship of the variables and how they influence the cellulolytic activity results. Assay 8 obtained the lowest enzymatic activity, with a result of 0.023 IU/mL under conditions of 85% moisture, 5 g of substrate, and 30 g/L of glucose. The Pareto Chart analyzed the significance of the three factors in the design, as well as the interactions between them (Figure 1 a).

Figure 1. Pareto Chart (a) and cubic plot (b) describing the relationship between factor variation and enzymatic activity



Higher glucose (3) concentrations showed positive results regarding the enzymatic activity. In respect to the moisture (1) levels, the lower the moisture concentration, the higher the cellulose production. The interaction between Moisture and Substrate (1x2) (Figure 1 a) proved relevant to the behavior of cellulolytic activity, showing that with the simultaneous increase of glucose and decrease of moisture, a higher enzymatic production was found. The second most significant interaction involved the three factors varying together (Figure 1 b), with an inverse proportional relationship affecting the interaction of the three factors, meaning that the increase of moisture, glucose and substrate concentration (1x2x3) at the same time causes a lower endoglucanase production. The interaction between Glucose and Substrate (2x3) revealed a proportional relation, exhibiting that the simultaneous increase of these factors is favorable for cellulase production. Regarding the interaction between Moisture and Glucose (1x3), which also showed relevant significance levels (above $p \leq 0.05$), the inverse relation was found once again. The higher the amount of glucose, the more beneficial it is for enzymatic production if the moisture level is lower, as well as the other way around.

4 CONCLUSION

This study demonstrated the feasibility of producing fungal cellulases using rice straw as a lignocellulosic substrate through Sequential Fermentation (SF). The most valuable result of the Factorial Desing on terms of cellulase production were obtained under conditions of 70% moisture, 5 g of substrate, and 30 g/L of glucose. This research underscores the potential of using rice straw, an abundant agricultural residue, for cost-effective and efficient cellulase production. The findings provide valuable insights into optimizing fermentation conditions, paving the way for further advancements in industrial enzyme production and sustainable biomass utilization.

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