INVESTIGATION OF PROTEASE PRODUCTION BY *YARROWIA LIPOLYTICA* FROM BREWING INDUSTRY WASTES

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ABSTRACT

Proteases are enzymes that participate in the hydrolysis of peptide bonds and exhibit specificity and selectivity in protein modification. The yeast *Yarrowia lipolytica* can be used in the production of this enzyme, and because it is non-pathogenic, its products can be considered safe for use in the food industry. Therefore, the aim of this research was to study the feasibility of producing protease by *Y. lipolytica*, using the malt bagasse, a brewing industry waste, and test media supplementation with different nitrogen sources. Submerged fermentation was carried out in Erlenmeyer flasks with medium containing 25 g/L of malt bagasse, with and without different organic and inorganic nitrogen sources at a concentration of 2 g/L. All tested media exhibited proteolytic activity, but the highest protease productivity (0.87 U/L.h) was detected in medium composed of urea (2 g/L) and malt bagasse (25 g/L). Therefore, culture media composition is essential for *Y. lipolytica* (IMUFRJ 50682) protease production, and production parameters can be adjusted for large-scale protease production.

Keywords: protease production. agro-industrial residues. nitrogen sources

1 INTRODUCTION

In recent years, studies on enzyme production from microbial origin have been explored to optimize processes and increase productivity because the use of enzymes, especially proteases, in the industrial sector still faces limitations due to high production costs.[1] One of the factors that significantly influences protease production costs is the raw material used in the fermentative process. Thus, the search for cheaper substrates, mainly from agro-industrial residues, is being explored. [2]

Proteases are enzymes that participate in the hydrolysis of peptide bonds and exhibit specificity and selectivity in protein modification. They are important for both metabolic processes and their use in various industries, such as the food industry. The yeast *Yarrowia lipolytica* can be used in the production of this enzyme, and because it is non-pathogenic, its products can be considered safe for use in food and consumption. [1]

Brazil is one of the countries with the highest agricultural production, and many residues from this production, such as peels, seeds, cakes, and pomaces from fruits, vegetables, and cereals, can be used as raw materials and carbon sources in fermentative processes, contributing to more sustainable production and development. One of these byproducts is malt bagasse, derived from beer production, both in large-scale and small-scale industries. Since this residue is rich in carbon source compounds (hemicelluloses, celluloses, and carbohydrates in general), [3] its application as a substrate in fermentative processes would be a way to utilize this byproduct and reduce the storage difficulties and quality control challenges already identified by the brewing industry.

A supplementation with malt bagasse in media to produce lipase and other enzymes such as glucanases provided higher enzyme activities in previous studies and became an agro-industrial residue with good viability as a substrate for large-scale fermentative processes.[4,5] However, this waste material has never been tested for protease production with *Y. lipolytica*. Therefore, the aim of this study was to assess the feasibility of protease production by *Y. lipolytica* in submerged fermentation with malt bagasse and different nitrogen sources.

2 MATERIAL & METHODS

The malt bagasse used was provided by a brewery located in the city of Rio de Janeiro. The collected material, already dried, was stored frozen until its use. For the fermentation process, the residue was milled in a Wiley-type knife mill (TE-680), sieved using a mesh sieve with a 0.50-mm mesh size and stored at -20 °C in polyethylene bags until the time of use.

A wild strain of *Y. lipolytica* (IMUFRJ 50682), isolated from the Guanabara Bay, Rio de Janeiro (Brazil), was used. The cells were precultured in YPD medium (w/v: yeast extract 1%; peptone, 2%; glucose, 2%) at 28ºC, 160 rpm, for 72 hours. The precultured cells were used in sufficient quantities to initiate protease production with 1 g of cells (dry weight)/L.

Protease production was performed in 500 mL Erlenmeyer flasks (submerged fermentation) with 200 mL of medium containing 25 g/L of malt bagasse and 2 g/L of different organic and inorganic nitrogen sources (urea, ammonium chloride, ammonium sulfate, corn steep liquor, yeast extract, tryptone, soy tryptone, peptone, peptone/yeast extract, and urea/yeast extract), based on

preliminary studies. [7] The flasks were incubated at 28 °C, 250 rpm for 96 h. Samples were collected every 24 h, centrifuged (4000 rpm, 20°C, 10 min), and the resulting supernatants (enzyme extract) were stored at -20 °C.

For protease activity, 1.0 mL of the supernatant was mixed with 1 mL of 0.5% azocasein solution in glycine-NaOH buffer (0.05M, pH 9.0). The mixture was incubated at 40°C for 30 min, and then 1 mL of trichloroacetic acid (10%) was immediately added to stop the reaction. The reaction mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was mixed with 2 mL of 0.5 N NaOH solution, and the absorbance at 428 nm was determined. The blank was obtained by mixing TCA with the substrate before adding the enzyme extract. One unit of enzymatic activity (1U) is defined as the increase of 0.01 in absorbance compared to the blank per minute under the reaction conditions.

3 RESULTS & DISCUSSION

Protease production by *Y. lipolytica* (IMUFRJ 50682) with malt bagasse was detected in the absence or the presence of all nitrogen sources tested (Figure 1). The highest protease activity was identified for the medium supplemented with yeast extract and urea after 72 h (27.13 U/L ± 1.25). However, higher productivity was found for the medium supplemented with urea only (0.87 U/L.h) after 24 h, similar to that of medium supplemented with yeast extract and urea after 24 h (0.76 U/L.h).

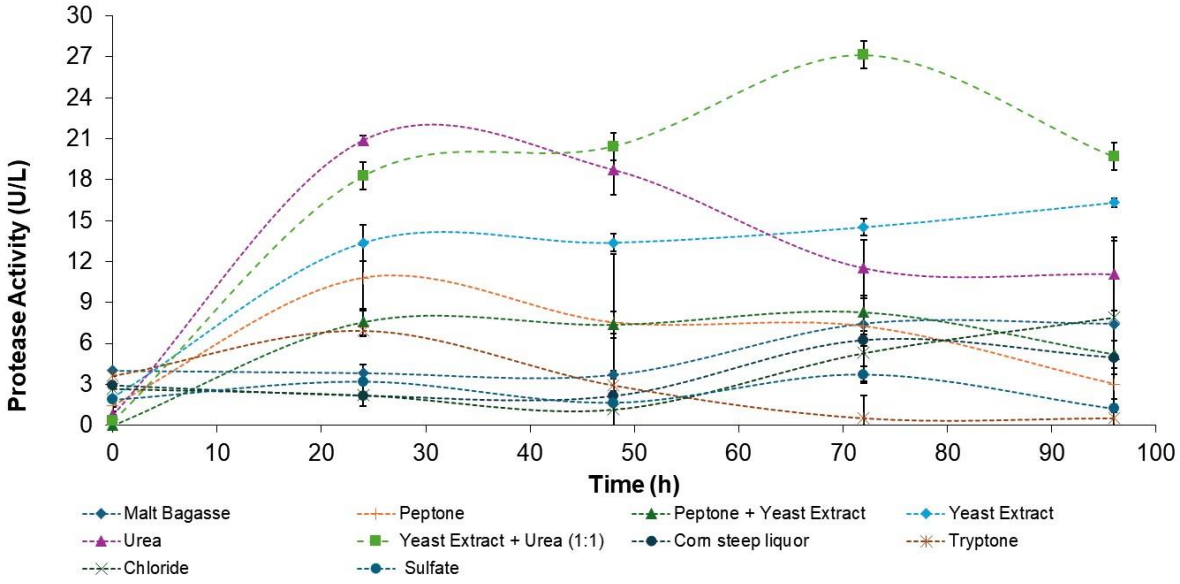

Figure 1: Extracellular protease activity during submerged cultivation of *Y. lipolytica* (IMUFRJ 50682) in Erlenmeyer flasks with malt bagasse with and without supplementation of nitrogen sources.

The culture medium composed solely of distilled water and malt bagasse did not result in high protease production. This is possibly due to deficiencies in some essential elements for yeast growth and enzyme production, primarily vitamins and nitrogen. On the other hand, media containing tryptone, peptone, peptone + yeast extract, as well as media enriched with chloride, sulfate, and corn steep liquor, were not effective in protease production.

Protease production with mushrooms of the *Lentinus crinitus* lineage (L.) was also observed in all the media studied. However, the highest activity (190 U/mL ± 1.33) was observed in the medium composed of 2% glucose (w/v) + 0.5% gelatin (w/v) + 0.5% yeast extract (w/v), while the medium containing ammonium sulfate (0.5%) and starch (2%) showed lower activity (77.33 U/mL ± 2.31). [11] The composition of the medium substantially affected protease production by *Bacillus* sp. EBTA6, especially yeast extract, which was identified as the most significant factor (190 U/mL ± 1.33). [12] Crude extracellular alkaline protease production from *Yarrowia lipolytica* (YlTun15) was influenced by the addition of specific metal ions, such as Ca^{2+}, Mn^{2+}, and Mg^{2+}, which also increased and stabilized protease activity. This could be a strategy in this research to achieve more significant protease activity results in media containing malt bagasse.

4 CONCLUSION

A culture medium composed solely of malt bagasse was not sufficient for protease production by *Yarrowia lipolytica* (IMUFRJ 50682). It is concluded that the composition of the culture medium with carbon and nitrogen sources is essential for *Y. lipolytica* (IMUFRJ 50682) protease production. Media containing yeast extract, and especially urea and malt bagasse as substrates,

showed the best proteolytic activities, indicating that malt bagasse, as a byproduct of beer production, can be reused in the production of an enzyme that can still return as an ingredient in the industry for beer production. Additionally, production protocols and parameters can be adjusted for large-scale protease production.

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