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INTENSIFICATION OF PRODUCTION AND APPLICATION OF LIPASE-RICH FERMENTED SOLIDS IN A MULTIPURPOSE FIXED-BED BIOREACTOR

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ABSTRACT

This study demonstrated the technical feasibility of producing solid fermented biocatalysts rich in lipases through the cultivation of the filamentous fungi *Rhizomucor miehei* and *Rhizopus oryzae* in a multipurpose fixed-bed bioreactor. The developed biocatalysts exhibited high catalytic efficiency, with *R. miehei* achieving about 80% conversion in 48 hours of fermentation and *R. oryzae* 73% in 16 hours of fermentation. The integration of biocatalyst production and application at esterification reaction catalysis was successfully achieved – eliminating the additional processing steps such as transportation, grinding, and purification. Scaling up to a larger volume bioreactor, maintained the same efficiency of the biocatalysts (conversion of approximately 79% in 24 hours), indicating the robustness of the process and uniformity in biocatalyst production across different scales. The findings highlight the efficiency of the MFB for the production and direct application of lipase-rich biocatalysts in integrated processes, underscoring its potential to simplify bioprocesses and enhance economic viability through process intensification.

Keywords: Fixed-bed bioreactor 1. One-pot system 2. Lipases 3. Esterification 4. Scale-up 5.

1 INTRODUCTION

Lipases, due to their specificity and catalytic efficiency, have become indispensable biocatalysts in various industrial sectors, including food, pharmaceuticals, and biofuels, with an expected significant market growth. However, the high costs associated with their production and purification processes pose challenges to the economic feasibility of many potential industrial applications. In this context, enzyme production through solid-state fermentation (SSF), using agro-industrial residues as the cultivation medium, and the use of fermented solids as biocatalysts, represents an economical and environmentally friendly alternative. This method not only eliminates the need for enzyme extraction, purification, and immobilization but also leverages low-cost substrates for enzyme production, thereby enhancing the economic viability of the bioprocess.[1] In this scenario, the multipurpose fixed-bed bioreactor represents a significant advancement in process intensification, integrating enzyme production via SSF and subsequent biocatalysis in a single equipment. This innovative approach minimizes equipment use, streamlines the process, and reduces the risk of contamination, aligning with green chemistry principles to promote economic and environmental sustainability.[2] Utilizing agro-industrial residues as substrates for SSF not only offers economic advantages due to their abundance and low cost but also contributes to waste valorisation, further highlighting the environmental benefits of this biotechnological approach. This study aimed to explore lipase production through SSF using the fungi *Rhizomucor miehei* and *Rhizopus oryzae* on different agro-industrial residues, employing a multipurpose fixed-bed bioreactor, evaluating its effectiveness in esterification reactions for the synthesis of industrially relevant esters, and assessing strategies to scale up the process.

2 MATERIAL & METHODS

Experimental Setup: Fixed-bed bioreactors of two sizes were utilized: large (h = 20 cm; Ø = 4 cm) and small (h = 10 cm; Ø = 2 cm). The temperature of the bioreactor and the temperature of forced aeration were controlled. Palm kernel cake and fiber were used as the cultivation medium (in different proportions) and supplemented with urea (1.5% w/w dry). Raw cottonseed cake was also employed as a fermentation substrate, without supplementation. The autoclaved substrate was inoculated with 10^7 spores/g of dry cake mass; the amount of inoculated substrate was 7 g for the small column and 30 g for the large column. The initial moisture content of the substrate was 65% for palm kernel fiber and cake, and 60% for cottonseed cake. For the large columns, an airflow rate of 0.3 L/min and a temperature of 35 °C were used, and for the small columns, 0.15 L/min and 30 °C. All fermentations were conducted in duplicate.

Evaluation of the Catalytic Capacity of Fermented Solids: The reactions were conducted in stirred reactors. Dry fermented solid (10% w/w of oil) was used as the biocatalyst in the esterification reaction of oleic acid and 95% ethanol, at a temperature of 40 °C, under agitation, for 24 hours. Samples were taken at different times during the reactions. The supernatant from the samples was weighed and dissolved in 40 mL of acetone/ethanol solution (1:1 v/v), and the residual free fatty acids were titrated with NaOH solution (0.04 mol/L) in an automatic titrator until pH 11. The reactions were conducted in duplicate and the samples were analysed in triplicate.

Esterification Reaction in the Multipurpose Fixed-Bed Bioreactor: Columns with dry fermented solid (dried through forced aeration) were used, in the proportion of 20% (w/w) of fermented solids relative to distilled fatty acids from soybean oil. The reaction conditions were set at a temperature of 50 °C and a flow rate of 12 mL/min in a multipurpose fixed-bed system. The reaction medium was stored in an agitated reservoir, and a peristaltic pump was used to transfer the medium to the fixed-bed column. The system maintained the reaction medium in constant circulation throughout the reaction period. Samples were periodically collected from the bioreactor outlet at defined intervals over a period of 24 hours. The reactions were conducted in duplicate.

3 RESULTS & DISCUSSION

The cultivation of filamentous fungi in a multipurpose fixed-bed bioreactor was conducted following a cultivation kinetics of up to 96 hours, during which the lipase production profile with potential for esterification was monitored. In this kinetic study, SSF was conducted in the fixed-bed bioreactor, and the esterification capacity of the fermented solids was evaluated in a stirred system, using oleic acid and 95% ethanol (1:1) as substrates, at 40 °C, with 10% (w/w) of the biocatalyst. The kinetic data suggest the production of fermented solids rich in lipases, demonstrating excellent synthesis activity (Figure 1).

The fungus *R. miehei* cultivated in both the mixture of palm kernel cake/fiber (80:20) and in cottonseed cake showed maximum lipase production at 48 hours of fermentation: the fermented solid showed approximately 80% conversion in the 24 hours esterification reaction. This conversion remained stable up to 96 hours of fermentation, indicating stability in the catalytic capacity of the fermented solids throughout the fermentation process.

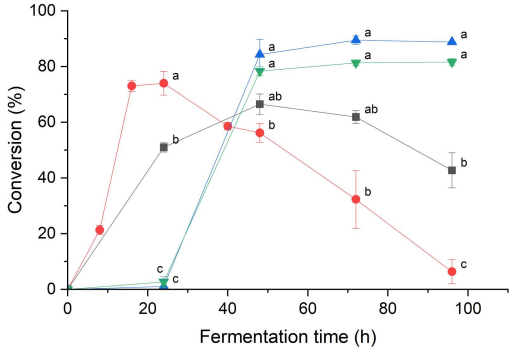

Figure 1. Catalytic capacity of biocatalysts produced by *Rhizopus oryzae* using palm kernel cake/fiber (80:20) (■) and cottonseed cake (●) and *Rhizomucor miehei* using palm kernel cake/fiber (60:40) (▼) and cottonseed cake (▲).

However, the biocatalyst developed by cultivating *R. oryzae* in cottonseed cake exhibited an early peak in conversion compared to *R. miehei*, reaching a maximum (about 70% conversion) in just 16 hours of fermentation, followed by a progressive decrease up to 96 hours of cultivation. This result highlights the effectiveness of *R. oryzae* in producing solid fermented biocatalysts rich in lipases in cottonseed cake, achieving high conversion rates in a short fermentation period. However, the subsequent decline in catalytic activity may indicate the generation of secondary metabolites, such as proteases, which compromise the integrity of the previously produced lipases.[3,4] Therefore, it is crucial to determine the optimal cultivation time for *R. oryzae* to prevent the loss of activity in the fermented solids. Additionally, based on the kinetic results, it becomes essential to examine the stability of the fermented solids during storage to ensure they retain their properties and are suitable for long-term storage.

To validate the multipurpose fixed-bed bioreactor, an esterification reaction involving distilled fatty acids from soybean oil and ethanol was conducted in the multipurpose fixed-bed bioreactor. The bioreactor was used to ferment *R. miehei* in a mixture of palm kernel cake/fiber (60:40) for a period of 72 hours, after which the fermented bed was dried through forced aeration until it reached approximately 5% moisture content. Esterification reactions with distilled fatty acids from soybean oil (DFASO) and 95% ethanol were evaluated at three different molar ratios of ethanol to DFASO (1:1, 2:1, and 4:1). It was observed that an excess of ethanol negatively affects the conversion in the fixed bed, achieving optimal conversion at a molar ratio of 1:1 (Figure 2).

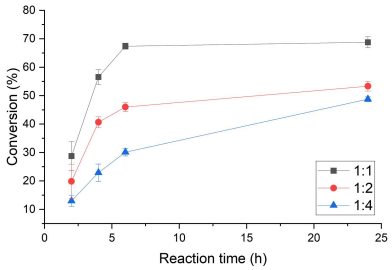

Figura 2. Esterification reaction in the fixed-bed bioreactor with DFASO and 95% ethanol conducted at 50°C in three different molar ratios of ethanol (1:1, 1:2, and 1:4).

The presented results confirm the robustness of the multipurpose fixed-bed bioreactor, which facilitates the integration of the biocatalyst production process by fermentation with downstream steps and the application of the fermented solids. These steps aim to dry the biocatalyst and apply it directly in reactions, eliminating the need for handling the fermented solids. To expand the production capacity, a fixed-bed bioreactor with a volume eight times larger than the bioreactor used in the initial studies was developed. To evaluate the reproducibility of fermentation for catalyst production in this new bioreactor, comparative fermentations were conducted between the two bioreactor models—the larger and the smaller fixed-bed bioreactors. In the scale-up fermentation process, the microorganism *Rhizomucor miehei* was used under the fermentation conditions of 72 hours, and the mixture of palm

kernel cake and fiber in the ratio of 60:40, as obtained in previous results. The aeration rate was adjusted proportionally to the height of the fixed bed. Thus, for the fermentation in the smaller fixed-bed bioreactor, a flow rate of 0.15 L/min was adopted, and for the larger bioreactor, 3.00 L/min. The performance of the biocatalysts after 24 hours of reaction was evaluated, produced in fixed-bed bioreactors with volumes of 31.42 cm^3 and 251.33 cm^3, respectively.

The biocatalysts obtained in the small and large bioreactors achieved a maximum conversion of approximately 79% in 24 hours of reaction (Table 1). Comparative analysis between the biocatalysts produced in fixed-bed bioreactors of different scales revealed no significant differences in efficiency between them, showing the same conversion profile for both biocatalysts. Additionally, the fractionated analysis of the fixed bed shows the uniformity in biocatalyst production in the large-volume multipurpose fixed-bed bioreactor, indicating that the fermentation parameters were adequately adjusted for the new scale. This result suggests that the scaling-up process was successful, maintaining the desirable characteristics of the biocatalysts. The absence of significant variation reinforces the robustness of the method employed, indicating that the increase in volume did not compromise the quality and catalytic capacity of the biocatalysts.

Tabela 1. Conversion profile of biocatalysts produced in the scale-up process of the multipurpose fixed-bed bioreactor using the fungus *R. miehei* over palm kernel cake and fiber (60:40), applied in a standard esterification reaction for 24 hours.

Biorreactor	Conversion (%)			
	Base	Middle	Top	Average ± (SD)
Small	79.24	-	78.81	79.0 ± 0.3
Larger	79.04	79.20	78.72	79.0 ± 0.2

4 CONCLUSION

The use of a multipurpose fixed-bed bioreactor for the cultivation of filamentous fungi, specifically *Rhizomucor miehei* and *Rhizopus oryzae*, resulted in the efficient production of solid fermented biocatalysts rich in lipases. These biocatalysts demonstrated high catalytic activity, with *R. miehei* achieving conversion rates of approximately 80% and *R. oryzae* 70%. The integration of the reaction step in the multipurpose fixed-bed bioreactor was successfully carried out, using the reaction between DFASO and ethanol at a molar ratio of 1:1. This ratio was found to be optimal for esterification efficiency, while an excess of ethanol negatively impacted the conversion.

The scalability of the process was demonstrated by increasing the bioreactor volume by eight times, without significant loss in the efficiency of the biocatalysts, which maintained a maximum catalytic capacity of about 79% in 24-hour reactions. The uniformity in biocatalyst production in the large-volume bioreactor indicates that the scaling-up process was successful, maintaining the desirable characteristics of the biocatalysts and reinforcing the robustness of the scaling-up method. This suggests that the multipurpose fixed-bed bioreactor system is a promising platform for integrating biocatalyst production with direct industrial applications, without the need for additional processing.

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