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Lipase Production by *Penicillium restrictum* Using Solid Waste of Industrial Babassu Oil Production as Substrate

Marcia B. Palma,[1] Annette L. Pinto,[2] Andreas K. Gombert,[3] Karina H. Seitz,[4] Silvia C. Kivatinitz,[5] Leda R. Castilho,*[,2] and Denise M. G. Freire[6]

[1]Department of Chemical Engineering, University of Blumenau, Brazil; [2]Program of Chemical Engineering/COPPE, Federal University of Rio de Janeiro, Brazil, E-mail: LEDA@GBF.DE; [3]Department of Chemical Engineering, University of São Paulo, Brazil; [4]Department of Chemical Engineering, National University of Mar del Plata, Argentina; [5]Department of Biochemistry, National University of Córdoba, Argentina; and [6]Faculty of Pharmacy, Federal University of Rio de Janeiro, Brazil

Abstract

Lipase, protease, and amylase production by *Penicillium restrictum* in solid-state fermentation was investigated. The basal medium was an industrial waste of babassu oil (*Orbignya oleifera*) production. It was enriched with peptone, olive oil, and Tween-80. The supplementation positively influenced both enzyme production and fungal growth. Media enriched with Tween-80 provided the highest protease activity (8.6 U/g), whereas those enriched with peptone and olive oil led to the highest lipase (27.8 U/g) and amylase (31.8 U/g) activities, respectively.

Index Entries: Lipase; protease; amylase; solid-state fermentation; *Penicillium restrictum.*

Introduction

Solid-state fermentation presents many advantages over submerged fermentation *(1)*. In solid-state fermentation, microorganisms grow under conditions closer to their natural habitat, therefore being able to synthesize certain enzymes and metabolites that usually would not be or would be only poorly produced in submerged fermentation *(2,3)*.

*Author to whom all correspondence and reprint requests should be addressed.
Current address: Gesellschaft für Biotechnologische Forschung (GBF), Biochemical Engineering Division (BVT), Mascheroder Weg 1, 38124 Braunschweig, Germany.

Solid-state fermentation has been gaining increasing attention not only as a way to produce enzymes, mushrooms, fermented food, animal feed, and fine chemicals *(4–11)*, but also as a way of upgrading the value of agroindustrial byproducts and residues *(12,13)*. Oily residues and other solid substrates have been recently used for the production of lipases by solid-state fermentation *(11,14,15)*.

Currently, lipases account for about 5% of the world enzyme market, and a significant growing trend is expected, because a wide range of applications has been or is being developed. Lipases are used in the food industry for flavor modification, in the oils and fats industry for modification of fats and ester synthesis, and in the detergent industry for the removal of oily and fatty stains. Possible medical applications are under consideration, such as the use of lipases as digestive enzymes or clinical reagents. Other applications, such as the production of chiral compounds for the agrochemical and pharmaceutical industries and the production of optically pure polymers, will probably result from the stereospecific properties of some lipases. Among these applications, it is important to observe that the detergent industry accounts for about 35% of the world market for industrial enzymes. Apart from lipases, proteases and amylases are also employed in this industry. Proteases are the most widely used enzymes in detergents, enabling the removal of protein stains. Amylases are employed in laundry as well as in dishwashing detergents to remove residues from starchy foods. Preparations with these three enzyme activities can therefore be of great interest in the detergent industry. Additionally, microbial sources of protease and lipase occurring simultaneously are of great importance for water treatment and for the dairy food industry *(16,17)*.

Considering the usually claimed economic advantages of solid-state fermentations *(2,5)*, the aim of the present work was to develop a cheap process for the production of lipases, proteases, and amylases. Therefore, an agroindustrial babassu waste was employed as the main raw material, and a fungal strain recently isolated from this waste and identified as lipase producer *(18,19)* was used. The babassu waste was enriched with different substances—Tween-80, peptone, and olive oil—resulting in media with varying C:N ratios. The influence of the different supplementations and specially the role of Tween-80 (polyoxyethylene sorbitan monooleate) in accelerating enzyme production are discussed.

Materials and Methods

Microorganism

The *Penicillium restrictum* strain employed in this work was previously isolated from wastes of the Brazilian babassu oil industry (babassu cake) and identified as lipase producer in submerged fermentation *(18,19)*. After cultivation for 7 d at 30°C on agar slants (2% soluble starch, 2% olive oil, 0.1% yeast extract, 0.5% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCO_3$, 1% agar), the microorganism was maintained at 4°C.

Solid-State Fermentation

The basal fermentation medium was babassu (*O. oleifera*) cake, which was dried, ground, and sieved to provide particle sizes between 0.21 and 0.42 mm. The composition of the babassu cake was 6.6% (w/w) water, 22.8% (w/w) proteins, 61.8% (w/w) overall carbohydrates, 4.5% (w/w) lipids, and 4.3% (w/w) ashes. This medium was supplemented with different carbon and nitrogen sources (different C:N ratios) at a 1% (w/w) concentration, as shown in Table 1. Experiments enabled an analysis of the effects of increasing (olive oil, Tween-80) and reducing (peptone) the C:N ratio of the basal medium. Furthermore, the experiments allowed a comparison of the effects of two different substances (olive oil and Tween-80) enriching the basal medium at an equal C:N ratio.

The experiments were conducted aseptically in polypropylene beakers covered with microbiological filter tissue (Lamino Padding Roll, ACCO®, Tidi Products). Each beaker containing 10 g of solid substrate (dry basis) was sterilized at 111°C for 20 min and then inoculated with 10^8 spores from spore suspensions *(20)*. The moisture content of the media was adjusted to 70%. Incubation was carried out for 15–64 h at 30°C in an incubation chamber, in which humidified air was continuously injected.

Sampling and Enzyme Extraction

At selected time intervals, whole beakers were taken as samples. The contents of each beaker were comminuted in order to have uniform samples. In each beaker, three 0.5-g portions were then withdrawn for assessing moisture content, glucosamine content, and pH. Smits et al. *(21)* have shown that accurate determination of moisture content and glucosamine can be achieved with samples as small as 0.4 and 0.1 g, respectively.

Enzyme extraction was carried out by adding to each beaker 45 mL of 50 m*M* phosphate buffer (pH 7.0), and then shaking the mixture in a rotary shaker (200/min) for 30 min at 37°C. The raw extract was obtained using a manual press and was subsequently centrifugated (1600*g*, 2 min). The supernatant was used to determine enzymatic activities.

Glucosamine Assay

As an indirect measurement of cell growth *(22,23)*, glucosamine was determined according to the method proposed by Aidoo et al. *(24)*.

Moisture Content

Moisture was determined by drying 0.5 g of fermented material at 75°C until constant weight was obtained.

pH Measurement

A volume of 5 mL of distilled water was added to 0.5 g of fermented material and the mixture agitated vigorously. After a 10-min interval for settling, the pH of the supernatant was determined with a pH meter.

Table 1
Carbon and Nitrogen Amount and C:N Ratio in Fermentation Media

Medium	C (% w/w)	N (% w/w)	C:N ratio
Babassu cake (BC)	44.8	3.6	12.4
Cake + 1% peptone (CP)	46.8	4.0	11.7
Cake + 1% Tween-80 (CT)	47.8	3.6	13.3
Cake + 1% olive oil (CO)	47.8	3.6	13.3

Enzyme Activity Assays

Lipase

Determination of lipase activity in the raw enzyme extract followed the procedure described in previous works *(18,19)*. One unit of lipase activity was defined as the amount of enzyme that produces the equivalent of 1 μmol of fatty acids/min under the assay conditions.

Protease

Proteolytic activity was determined in the raw enzyme extract following the method proposed by Charney and Tomarelli *(25)*. One unit of proteolytic activity was defined as the quantity of enzyme that produces a 1-U difference in absorbance between the reaction blank and the sample per minute under the assay conditions.

Glucoamylase

Glucoamylase activity was determined as described in ref. *26* by reacting the enzyme extract with a 4% soluble starch solution at 45°C and pH 4.2. One unit of enzyme was defined as the amount of enzyme that produces 1 μmol of reducing sugars/min under the assay conditions *(27)*.

Results and Discussion

Table 2 shows maximum enzyme activities and glucosamine content achieved in the different culture media. The microorganism was able to grow and produce the three enzymes in the basal medium without any supplementation, indicating that the nutrients present in the babassu cake could be enough to allow the growth of the fungus.

The peak of lipolytic activity appeared after 24 h of cultivation in all media, except in the Tween-80-enriched medium (CT medium) in which the peak of lipase activity was observed after 20 h of fermentation (Fig. 1).

Supplementation of the basal medium with peptone, olive oil, and Tween-80 resulted in lipase activities 4.3, 2.6, and 1.7 times higher, respectively (Table 2). Therefore, an increase in the availability of nutrients seems to have a positive effect on lipase production. Although Tween-80 has been commonly employed as a nutrient source in lipase production *(28–30)*, the

Table 2
Maximum Lipase, Protease, and Glucoamylase Activities
and Glucosamine Content Achieved in the Different Culture Media

	Maximum activity (U/g of IDW[a])			Glucosamine content (mg/g of IDW[a])	
Medium	Lipase	Protease	Glucoamylase	Initial	Final
Babassu vake (BC)	6.5	4.8	18.8	2.3	5.3
Cake + 1% peptone (CP)	27.8	5.4	28	2.3	6.7
Cake + 1% olive oil (CO)	17.2	4.8	31.8	2.3	6.7
Cake + 1% Tween-80 (CT)	11.2	8.6	17.1	[b]	[b]

[a]IDW, initial dry weight.
[b]Tween-80 apparently interfered with production and/or measurement of *N*-acetyl-glucosamine in the fungal wall, hindering the assay.

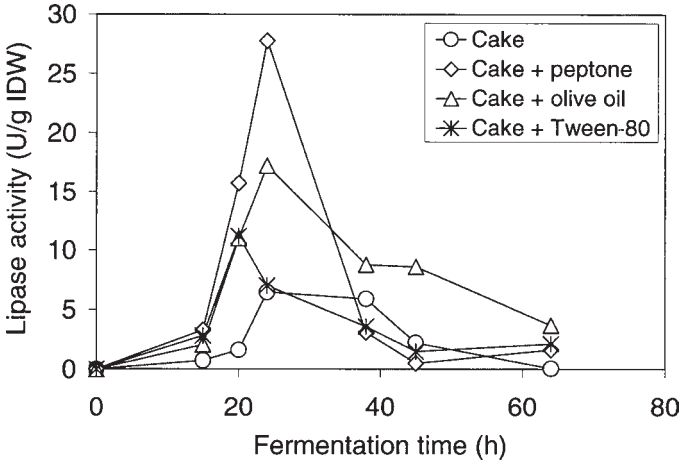

Fig. 1. Effect of different medium composition on lipase production by *P. restrictum* in solid-state fermentation.

present results show that it is the least effective of the three kinds of supplementation tested. The highest lipase activity (27.8 U/g of IDW) was achieved with peptone enrichment (CP medium), because this substance contains aminoacids and cofactors needed for lipase biosynthesis *(18)*. Salleh et al. *(31)* compared the use of eight different nitrogen sources for the production of an extracellular lipase and also observed that peptone was the most adequate.

The highest protease (8.6 U/g of IDW) and amylase (31.8 U/g IDW) activities were obtained in media enriched with Tween-80 (CT medium) and olive oil (CO medium), respectively (Figs. 2 and 3). In CO and CP media, amylase activity was approx 1.7 and 1.5 times higher than in the basal medium, respectively, but it remained almost constant in the CT

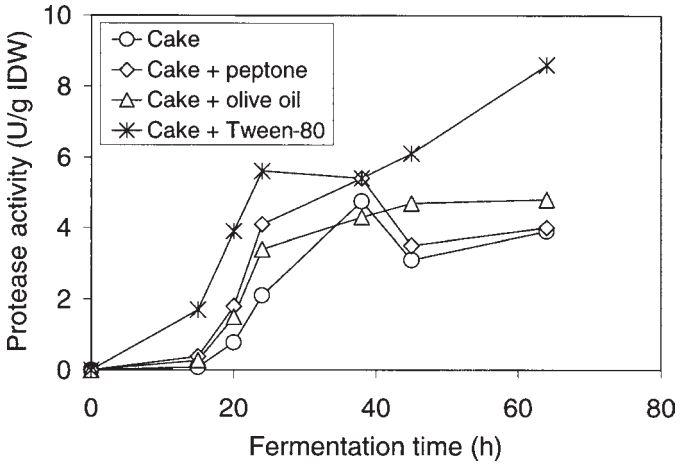

Fig. 2. Effect of different medium composition on protease production by *P. restrictum* in solid-state fermentation.

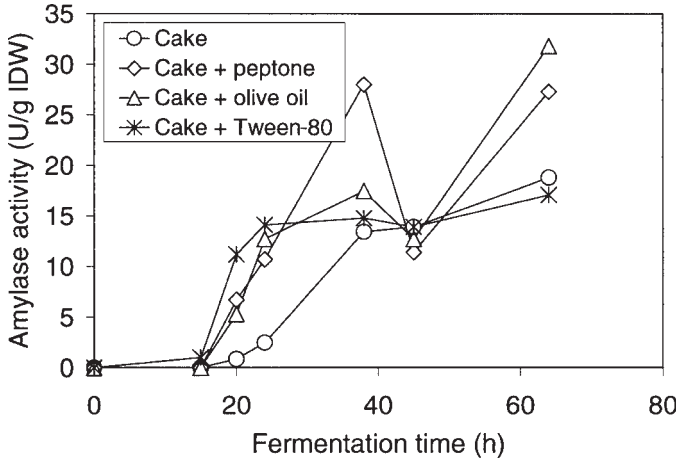

Fig. 3. Effect of different medium composition on glucoamylase production by *P. restrictum* in solid-state fermentation.

medium. On the contrary, no significant increase in protease activity was observed in CO and CP media, but it practically doubled in the CT medium.

Babu and Satyanarayana *(32)*, studying the production of amylase by *B. coagulans*, also observed that the use of Tween-80 did not result in any significant improvement in amylase production. On the other hand, Banerjee and Bhattacharyya *(33)*, investigating the effect of inducers on protease biosynthesis by *Rhizopus oryzae*, noted a 4.2-fold increase in protease production when Tween-80 was added to the medium. In the present case, the addition of Tween-80 enabled a twofold increase in protease level.

The greater proteolytic levels achieved in the CT medium probably increased the inactivation of lipases and amylases, resulting in maximum

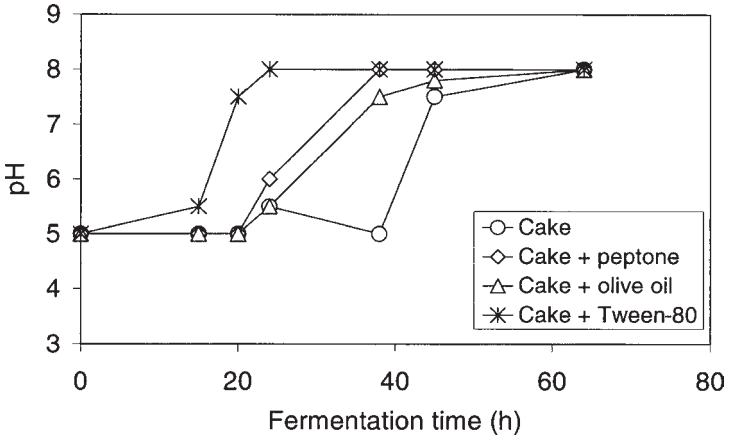

Fig. 4. Effect of different medium composition on pH during solid-state fermentation with *P. restrictum*.

lipolytic and amylolytic activities that are significantly lower than in CP and CO media (Table 2).

In all media, pH increased after 20 h of cultivation, except for the CT medium in which an increase was noted earlier (Fig. 4). The alkalinization of culture media is observed together with an increase in protease level (Fig. 2). This behavior is probably related to proteolysis, which generates amino acids that are deaminated, liberating ammonia to the culture medium *(18)*. This fact can be easily noted in the CT medium, in which both protease activity and pH rose significantly after 15 h (Figs. 2 and 4).

Previous results with submerged fermentation have shown that *P. restrictum* lipase is not stable at alkaline pH values and that the addition of a protease inhibitor (phenylmethylsulfonyl fluoride) reduces the decrease in lipase activity *(18,19)*. Proteolysis, pH inactivation, or both phenomena usually explain the decrease in lipolytic activity observed during cultivation. In the medium enriched with Tween-80, the accelerated release of proteases and the early increase in pH can therefore explain the early decrease in lipase activity.

A common feature for the three enzyme activities in the CT medium was the earlier detection of enzymes in the fermented medium. Although its mechanism of action has not yet been clearly elucidated *(34)*, Tween-80 probably stimulates enzymatic release because of its detergent properties and its interaction with the cell membrane *(29,35)*. According to Henriette et al. *(17)*, the inclusion of Tween-80, a surface active agent, in the medium lowers the interfacial tension around the microorganisms, thereby reducing transport resistances. Other investigators *(36,37)* affirm that Tween-80 causes an increase in the degree of unsaturation of membrane fatty acids and therefore enhances membrane fluidity and permeability.

Considering the different culture media and C:N ratios tested, one can note that the different C:N ratios of CP and CO medium had practically no

influence on maximum amylase and protease levels achieved (Tables 1 and 2). Additionally, media of equal C:N ratios (13.3) but different kinds of supplementation (CO and CT media) led to quite different maximum amylase and protease levels. This shows that the production of amylases and proteases, in the present case, is influenced by the type of enriching substance rather than by the C:N ratio.

On the other hand, the results obtained for lipase, in which the greatest relative concentration of nitrogen (CP medium) resulted in the highest maximum lipase level, could lead to the conclusion that C:N ratio has a strong influence on the production of this enzyme. However, regarding the basal medium, in which the relative amount of nitrogen is higher than in CO and CT media but the maximum lipase level is lower (Tables 1 and 2), one can conclude that the adequate kind of nitrogen-containing substances present in peptone *(18)* are responsible for the enhanced lipase activity in the CP medium.

Conclusion

The present results show that the fungus *P. restrictum* is able to grow under solid-state fermentation, using as substrate a waste of the industrial production of babassu oil. The kind of supplementation of the fermentation medium proved to have more influence on the production of lipases, proteases, and amylases than the C:N ratio, within the interval tested.

For lipase production, peptone was the most effective enriching substance, confirming that certain peptides and amino acids present in peptone match the physiological requirements of *P. restrictum* for lipase biosynthesis. Supplementation with Tween-80 caused an earlier detection of the enzyme peaks, an increase in the maximum protease activity achieved, and an early rise in the pH level of the medium. This can be explained by its surfactant properties and its interaction with the cell membrane, increasing membrane fluidity and permeability.

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References

1. Pandey, A. (1992), *Process Biochem.* **27,** 109–117.
2. Nigam, P. and Singh, D. (1994), *J. Basic Microbiol.* **34,** 405–423.
3. Lonsane, B. K., Ghildyal, N. P., Budiatman, S., and Ramakrishma, S. V. (1985), *Enzyme Microb. Technol.* **7,** 258–264.
4. Sircar, A., Sridhar, P., and Das, P. K. (1998), *Process Biochem.* **33,** 283–289.
5. Tao, S., Beihui, L., Peng, L., Deming, L., and Zuohu, L. (1998), *Process Biochem.* **33,** 419–422.
6. Zakaria, Z., Hall, G. M., and Shama, G. (1998), *Process Biochem.* **33,** 1–6.
7. George, S., Raju, V., Krishnan, M. R. V., Subramanian, T. V., and Jayaraman, K. (1995), *Process Biochem.* **30,** 457–462.

8. Trejo-Hernandez, M. R., Lonsane, B. K., Raimbault, M., and Roussos, S. (1993), *Process Biochem.* **28,** 23–27.
9. Rivera-Muñoz, G., Tinoco-Valencia, J. R., Sánchez, S., and Farrés, A. (1991), *Biotechnol. Lett.* **13,** 277–280.
10. Castilho, L. R., Medronho, R. A., and Alves, T. L. (2000), *Bioresource Technol.* **71,** 45–50.
11. Gombert, A. K., Pinto, A. L., Castilho, L. R., and Freire, D. M. G. (1999), *Process Biochem.* **35,** 85–90.
12. Paredes-Lopez, O. and Alpuche-Solis, A. (1992), in *Bioconversion of Waste Materials to Industrial Products*, Martin, A. M., ed., Elsevier Scientific, London, pp. 117–145.
13. Considine, P. J., Mehra, R. K., Hackett, T. J., O'Rorke, A., Comerford, F. R., and Coughlan, M. P. (1986), *Ann. NY Acad. Sci.* **469,** 304–311.
14. Kamini, N. R., Mala, J. G. S., and Puvanakrishnan, R. (1998), *Process Biochem.* **33,** 505–511.
15. Christen, P., Angeles, N., Corzo, G., Farres, A., and Revah, S. (1995), *Biotechnology Techniques* **9,** 597–600.
16. Banerjee, R. and Bhattacharyya, B. C. (1992), *Bioprocess Eng.* **7,** 225–228.
17. Henriette, C., Zinebi, S., Aumaitre, M. F., Petitdemange, E., and Petitdemange, H. (1993), *J. Ind. Microbiol.* **12,** 129–135.
18. Freire, D. M. G., Teles, E. M. F., Bon, E. P. S., and Sant'Anna, G. L., Jr. (1997), *Appl. Biochem. Biotech.* **63,** 409–421.
19. Freire, D. M. G., Gomes, P. M., Bon, E. P. S., and Sant'Anna, G. L., Jr. (1997), *Rev. Microbiol.* 28, 6-12.
20. Castilho, L. R., Alves, T. L. M., and Medronho, R. A. (1999), *Process Biochem.* **34,** 181–186; Erratum *Process Biochem.* **34,** 417.
21. Smits, J. P., Rinzema, A., Tramper, J., Schlösser, E. E., and Knol, W. (1996), *Process Biochem.* **31,** 669–678.
22. Pandey, A., Selvakumar, P., and Ashakumary, L. (1996), *Process Biochem.* **31,** 43–46.
23. Sakurai, Y., Lee, T. H., and Shiota, H. (1977), *Agric. Biol. Chem.* **41,** 619–624.
24. Aidoo, K. E., Hendry, R., and Wood, B. J. B. (1981), *Appl. Microbiol. Biotechnology* **12,** 6–9.
25. Charney, J. and Tomarelli, R. M. (1947), *J. Biol. Chem.* **171,** 501–505.
26. Freire, D. M. G. and Sant'Anna, G. L., Jr. (1990), *Appl. Biochem. Biotechnol.* **26,** 23–33.
27. Pandey, A., Selvakumar, P., and Ashakumary, L. (1996), *Process Biochem.* **31,** 43–46.
28. Maliszewska, I. and Mastalerz, P. (1992), *Enzyme Microb. Technol.* **14,** 190–193.
29. Espinosa, E., Sánchez, S., and Farrès, A. (1990), *Biotechnol. Lett.* **12,** 209–214.
30. Okeke, C. N. and Okolo, B. N. (1990), *Biotechnol. Lett.* **12,** 747–750.
31. Salleh, A. B., Musani, R., Basri, M., Ampon, K., Yunus, W. M. Z., and Razak, C. N. A. (1993), *Can. J. Microbiol.* **39,** 978–981.
32. Babu, K. R. and Satyanarayana, T. (1993), *Folia Microbiol.* **38,** 77–80.
33. Banerjee, R. and Bhattacharyya, B. C. (1993), *Biotechnol. Bioeng.* **41,** 67–71.
34. Thies, E., Jenkins, T., and Stutzenberger, F. (1994), *World J. Microb. Biotechnology* **10,** 657–663.
35. Macrae, A. R. and Hammond, R. C. (1985), *Biotechnol. Genet. Eng. Rev.* **3,** 193–217.
36. Asther, M. and Corrieu, G. (1987), *Enzyme Microb. Technol.* **9,** 245–249.
37. Jacques, N. A., Jacques, V. L., Wolf, A. C., and Wittenberger, C. L. (1985), *J. Gen. Microbiol.* **131,** 67–72.