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Covalent Immobilization of α-Galactosidase from *Penicillium griseoroseum* and its Application in Oligosaccharides Hydrolysis

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Abstract Partially purified α-Galactosidase from *Penicillium griseoroseum* was immobilized onto modified silica using glutaraldehyde linkages. The effective activity of immobilized enzyme was 33%. Free and immobilized α-galactosidase showed optimal activity at 45 °C and pH values of 5 and 4, respectively. Immobilized α-galactosidase was more stable at higher temperatures and pH values. Immobilized α-galactosidase from *P. griseoroseum* maintained 100% activity after 24 h of incubation at 40 °C, while free enzyme showed only 32% activity under the same incubation conditions. Defatted soybean flour was treated with free and immobilized α-galactosidase in batch reactors. After 8 h of incubation, stachyose was completely hydrolyzed in both treatments. After 8 h of incubation, 39% and 70% of raffinose was hydrolyzed with free and immobilized α-galactosidase respectively. Immobilized α-galactosidase was reutilized eight times without any decrease in its activity.

Keywords *Penicillium griseoroseum* · α-Galactosidase · Enzyme immobilization · Modified silica · Raffinose oligosaccharides · Soybean products

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Introduction

Immobilized enzymes are currently an object of considerable interest. The main advantages of immobilized enzyme systems are stability, reusability, and separation of the enzymes from the products. Easy separation of enzymes from the product simplifies enzymes applications and supports a reliable and efficient reaction technology. Also, the reuse of enzymes reduces production costs [1]. Furthermore, immobilized enzymes have longer half-lives and predictable decay rates [2].

Chemical immobilization generally involves enzyme attachment to a matrix via cross-linking or covalent bonding. A variety of matrixes have been used as support materials for enzyme immobilization [3]. The use of silica-based gels for enzyme immobilization has received increased attention for industrial manufacturing of enzyme-processed products [4]. Silica gels possess relatively higher thermal and chemical stabilities under conditions encountered in industrial processing, and their inert nature also renders them resistant to microbial contamination or degradation [5].

Soy is an inexpensive source of high-quality protein [6], and recent studies have suggested that soy may have other beneficial features, including prevention against breast cancer [7], cardiovascular risks [8], antioxidant activity [9], and bone loss in postmenopausal women [10]. However, considerable amounts of flatulence-causing raffinose oligosaccharides 1 in soybean-based foods limit their biological value and acceptability. Raffinose and stachyose (α-galactosides) are the major oligosaccharides associated with flatulence production [11]. Many investigators have studied the application of microbial α-galactosidases for the degradation of raffinose oligosaccharides present in legume food [12–16]. In addition, the utilization of immobilized α-galactosidases for the reduction of raffinose oligosaccharide content in soybean-base products is reported in the literature [12, 17, 18]. Removal of oligosaccharides from soy food is therefore a major factor in improving its nutritive value and acceptability [13].

This study focused on the application of free and covalently immobilized *Penicillium griseoroseum* α-galactosidase in silica for the treatment of oligosaccharides in defatted soybean flour. In addition, the properties of immobilized and free enzymes were compared, and the advantages of the use of immobilized α-galactosidase were discussed.

Materials and Methods

Materials

Silica gel with pore size 150 Å used as immobilization supports was obtained from the Aldrich Chemical Company. The substrate *p*-nitrophenyl-α-D-galactopyranoside (pNPGal), glutaraldehyde, and 3-aminopropyltriethoxysilane (APTS) locust bean gum was purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Microorganism and Growth Conditions

P. griseoroseum was obtained from the Microbiology Department's Fungal Collection at the Federal University of Viçosa, MG, Brazil. For enzyme production, spores (10^7 mL^{-1}) were transferred to 1.0 L of liquid medium containing (*w*/*v*): 0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.001% $ZnSO_4$, and 1% locust bean gum as a carbon source. The culture was incubated under constant agitation at 120 rpm for 168 h at

28 °C. Culture filtrates were collected using filter paper, concentrated by lyophilization, and kept at 5 °C until use.

α-Galactosidase Purification

The lyophilized enzymatic sample was loaded in a Sephacryl S-200 column (Amersham Biosciences, Uppsala, Sweden; 87.5 cm×2.5 cm) and equilibrated with a 25 mM sodium acetate buffer with a pH of 5.0. The proteins were eluted with the same buffer at flow rate of 30 mL/h at 4 °C. Fractions containing α-galactosidase activity were pooled and used in the immobilization process.

Protein Determination

The protein concentration in the enzymatic extract was determined according to the Coomassie Blue binding method with bovine serum albumin as standard [19].

Immobilization of α-Galactosidase

The α-galactosidase was immobilized by the covalent linkage method in chemically modified silica gel with 3-aminopropyltriethoxysilane. Silica gel (10 g) was mixed with 100 mL of toluene and 2 mL of APTS. The suspension was incubated at 80 °C for 12 h with constant mixing, washed thoroughly with same the solvent, and dried at 120 °C for 2 h. One gram of silanized silica beads was mixed with 50 mL of glutaraldehyde solution 5% (*w*/*v*) and was agitated for 10 min. Silica gel was separated by decantation and washed with distilled water five times. Enzyme solution (10 mL) containing 8.9 U mL^{-1} was added to the silica gel, and the reaction was carried out for 10 min while stirring to allow the covalent linkage of enzyme with the aldehyde groups. α-Galactosidase immobilized onto silica gel was recovered by decantation, washed with distilled water five times, and stored in 0.1 M sodium acetate buffer, pH 5.0, at 4 °C. The enzyme loading was determined from the difference between the initial and residual α-galactosidase activity within the adsorption medium using standard enzyme assay. The efficiency of immobilized α-galactosidase was calculated by dividing the activity of immobilized enzyme by theoretical value of activity contained in an appropriate aliquot of support.

Enzyme Assay

The assay for free α-galactosidase consisted of 650 μL of 0.1 M sodium acetate buffer, pH 5.0, 100 μL of enzyme solution (50 μg protein), and 250 μL of 2 mM pNPGal. The reaction was carried out for 15 min at 40 °C and concluded with the addition of 1 mL of 0.5 M sodium carbonate.

The immobilized α-galactosidase assay was performed in a batch reactor. The reaction system contained 2 mL of 2 mM pNPGal solution prepared in a 0.1 M sodium acetate buffer (pH 5.0) and 10 mg of silica gel containing the immobilized enzyme (≈100 μg protein). The reactions were run in a water bath shaker for 10 min at 40 °C. After being removed from the shaker, 0.5 mL of the reaction mixture (without silica) was transferred to a test tube and mixed with 0.5 mL of sodium acetate buffer and 1 mL of 0.5 M sodium carbonate. The amount of *p*-nitrophenol (PNP) released in both assays was determined to be 410 nm. These procedures were defined as the standard assay for free and immobilized α-galactosidase, respectively.

One unit of enzyme activity (U) was defined as the amount of protein required to produce 1 μmol of *p*-nitrophenol/min under the conditions stated above.

Effect of pH and Temperature

The effect of pH on free and immobilized enzyme activities was determined within the pH range of 3.0–7.0 using McIlvaine buffer (citric acid/sodium phosphate) [20] at 40 °C under otherwise standard enzyme assay conditions. The optimum temperature was determined to be within the range of 35–60 °C, at pH 5.0.

For the investigation of thermal stability, samples of free and immobilized α-galactosidases were pre-incubated for varying time periods at 40 °C and 45 °C. Enzyme thermal stability was evaluated by measuring the residual activity using the standard assays. Results of the analyses are presented as mean±SD for three measurements.

K_M Values of Free and Immobilized Enzymes

Kinetic experiments were performed at 40 °C and pH 5.0. The Michaelis–Menten constant (K_M) and V_{max} for pNPGal were calculated by using the Lineweaver–Burk plot. The substrate concentrations ranged from 1 to 15 mM, and the amount of free and immobilized enzymes added to each assay was kept constant.

Enzymatic Treatment of Defatted Soybean Flour

Enzymatic treatment of defatted soybean flour (Bunge Alimentos, RS, Brazil) was performed in a batch reactor. Soybean flour was mixed with deionized water (1:10 *w*/*v*), and samples were incubated with free and immobilized α-galactosidase. A 5-mL volume of free α-galactosidase (10 U) was added to 25 mL of soybean flour mixture. In the case of immobilized α-galactosidase, 0.2 g of silica (10 U) was added to 25 mL of the soybean flour mixture. The hydrolysis reactions were carried out at 40 °C in an incubator shaker (100 rpm) for 0, 2, 4, 6, and 8 h. After incubation, an aliquot of the reaction mixture was taken out and immediately frozen at −80 °C. Control experiments were performed in the same manner with distillate water replacing the free enzyme. Samples were lyophilized, and the soluble sugars were extracted from 20–30 mg of dried powder with 80% aqueous-ethanol (*v*/*v*) according to Guimarães et al. [21]. The solvent was evaporated at 50 °C, and the sugars were resuspended in 1 mL of 80% ethanol and analyzed by HPLC.

Repeat Batch

Soybean flour mixture (25 mL) and 20 U of immobilized α-galactosidase (0.4 g silica) were mixed in Erlenmeyer flasks and placed in an incubator shaker (100 rpm) at 40 °C. After an incubation period of 6 h, aliquots of the soybean flour mixture were taken, and the oligosaccharide concentration was determined by HPLC. Silica containing immobilized enzyme was recovered by decantation, washed with distilled water, and reapplied in a new treatment.

Determination of Oligosaccharides

The sugars were analyzed using a HPLC Shimadzu series 10A chromatograph. An analytical column [aminopropil (NH_2)] was used for this purpose, eluted with an

acetonitrile–water isocratic mixture (80:20 *v*/*v*) at 35 °C with a flow rate of 1 mL min^{-1} according to Guimarães et al. [21]. The amounts of sucrose, raffinose, and stachyose were quantified by comparison with the retention times and standard sugar concentrations.

Results and Discussion

Purification and Immobilization of α-Galactosidase

Locust bean gum grown *P. griseoroseum* secreted high levels of α-galactosidases, similar to other species such as *Penicillium simplicissimum* [22], *Penicillium* sp. [23, 24], and other fungi [25–27]. The concentrated culture supernatant was subjected to gel filtration chromatography, resulting in the separation of one protein fraction with α-galactosidase activity (Fig. 1). This step resulted in considerable specific activity enhancement, generating a purification factor of 9.5% and a 75% recovery level of the original α-galactosidase activity.

The semipurified α-galactosidase was immobilized on surface of silica modified by silanization with APTS. The use of silica gel for enzyme immobilization has received increased attention for industrial manufacturing of enzyme-processed products [28]. Silica gels offer a number of advantages over "soft gels" for use in industrial processes, including high mechanical strength [29], high thermal and chemical stabilities, resistance to microbial contamination or degradation [5], and large surface area with elevated porosity, which in turn can be used to enhance enzyme loading and accessibility [28]. In this work, we employed APTS to modify silica gel surface and supply a spacer arm to enzyme immobilization. The use of space arms could prevent undesirable side interactions between large enzyme molecules and support. In this way, more areas for the immobilized enzyme could become accessible to substrate [30, 31].

The chemical immobilization of 89 U semipurified α-galactosidase in modified silica resulted in an enzyme loading of 49 U/g of silica, corresponding to 55% of immobilization

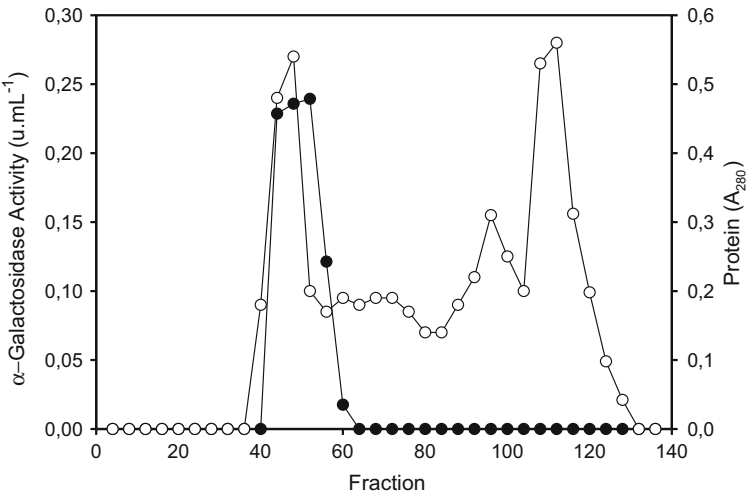

Fig. 1 Elution profile of the α-galactosidase from *P. griseoroseum* in a Sephacryl S-200 column. α-Galactosidase activity (*filled circles*); A_{280} (*open circles*)

yield. The effective activity of immobilized α-galactosidase was 16.13 U/g of silica resulting in an activity yield of 33%. New attempts need to be tested to optimize these parameters. Ettalibi and Baratti [32] showed that *Aspergillus ficuum* inulinase covalently immobilized on glass beads exhibited an activity yield of 70–77%, depending on pore diameters. The lost of activity detected in immobilized α-galactosidase may have been caused by unfavorable enzyme orientation support linkages and by changes in the protein structure.

Effect of pH on α-Galactosidase Activity

Free and immobilized enzymes had an optimum pH of 5.0 and 4.0, respectively (Fig. 2). The pH value for maximal free α-galactosidase activity is in agreement with the values reported for other fungal α-galactosidase [16, 25, 33]. The immobilization process affected the pH-activity profiles of *P. griseoroseum* α-galactosidase. The optimum pH of immobilized enzymes was found to be one point more acidic. Consequently, the change in the optimum pH and p*K* values may have been caused by the microenvironmental effect on the functionalized silica surface, which contains ionic groups such as the amino groups, creating an uneven distribution of hydrogen ions between the surface and bulk solution [34]. The immobilized enzyme maintained 70% of its maximal activity in the pH range of 4–7, while free enzyme maintained 70% activity levels in the pH range of 5–6. The pH profile change in immobilized enzymes is very common and was confirmed by other authors [12, 35, 36]. It was found that the immobilized α-galactosidase was much more stable than free enzymes, indicating that free enzymes are more sensitive to the pH changes, proving that the enzyme stability in different pH concentrations was significantly improved by immobilization.

Effect of Temperature on α-Galactosidase Activity

The effects of temperature on the activity of free and immobilized α-galactosidases was investigated from 35 °C to 60 °C (Fig. 3). Both the free and immobilized enzymes showed maximum activity at 45 °C. However, the free enzyme completely lost its activity at 55 °C, probably due to thermal denaturation of the enzyme, while the immobilized α-galactosidase maintained about 75% of its original activity at the same temperature. Other publications confirmed that microbial immobilized α-galactosidases showed increased resistance to high

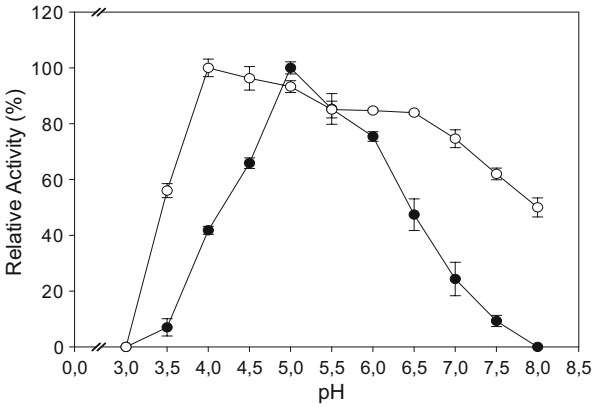

Fig. 2 Effect of pH on free (*filled circles*) and immobilized (*open circles*) α-galactosidase from *P. griseoroseum*

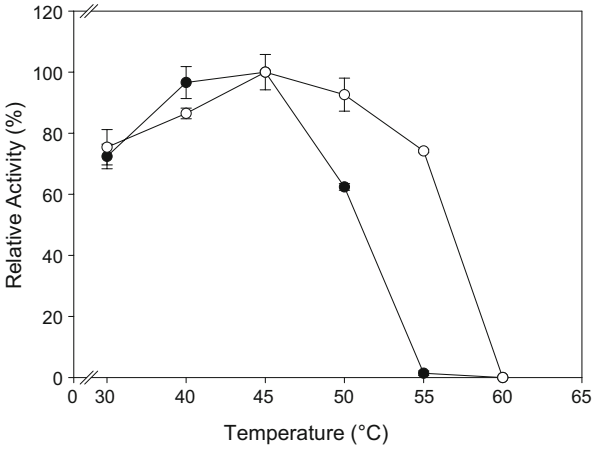

Fig. 3 Effect of temperature on free (*filled circles*) and immobilized (*open circles*) α-galactosidase from *P. griseoroseum*

temperatures [37]. This increase in stability is convenient, since high temperatures diminish microbial contamination during the treatment of soybean products [38].

Thermal Stability

Thermal stability of the free and immobilized α-galactosidases was examined at 40 °C and 45 °C (Fig. 4). After 24 h of incubation at 40 °C, free and immobilized enzymes retained 32% and 100% of their activity, respectively. At 45 °C, free enzyme was totally inactive after 4 h of incubation, while the immobilized enzyme maintained 51% of its original activity after 24 h of incubation. When of the both enzyme forms, free and immobilized, were pre-incubated at 50 °C, about 30% of initial activities were maintained. Thermal stability of *P. griseoroseum* α-galactosidase was increased when immobilized, in agreement with previous results [18, 39–41]. Enhanced thermal stability and a wide pH range were observed after α-galactosidase immobilization on silica. This could be explained by the strong covalent linkages formed between the enzyme molecules and the support. These linkages aid in the stabilization of intramolecular forces, which are responsible for maintaining the three-dimensional conformation of the enzyme molecule [35]. The increase

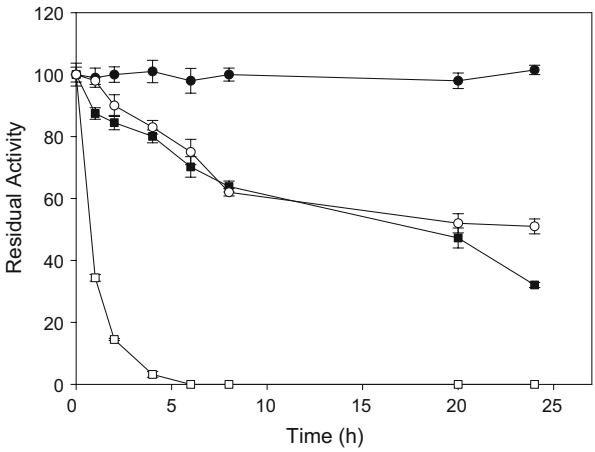

Fig. 4 Influence of temperature on stability of free and immobilized α-galactosidase from *P. griseoroseum*. Free α-galactosidase at 40 °C (*filled squares*), free α-galactosidase at 45 °C (*open squares*), immobilized α-galactosidase at 40 °C (*filled circles*) and immobilized α-galactosidase at 45 °C (*open circles*)

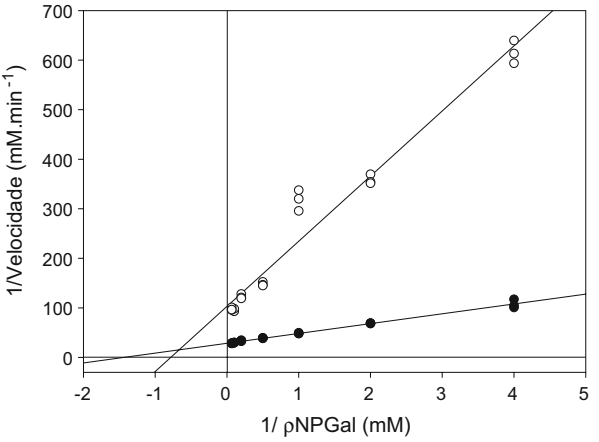

Fig. 5 Lineweaver–Burk plots for the free (*filled circles*) and immobilized (*open circles*) α-galactosidase

in thermostability of α-galactosidase could be a great advantage for industrial application, especially when removing RO from soymilk and other soy-based products [38]. Despite of improvement in immobilized enzyme compared with free enzyme, our results with immobilized enzyme were not expressive, compared with results reported in the literature [42, 43].

Determination of Kinetic Constants

The initial reaction rates for free and immobilized α-galactosidase were determined at different substrate concentrations ranging from 1 to 15 mM. The K_M values for immobilized and free enzymes were 1.27 and 0.65 mM, respectively (Fig. 5). The V_{max} values of both immobilized and free enzymes were 0.009 and 0.034 mM min^{-1}, respectively. Increase in K_M values was reported by Prashanth [18] after *Aspergillus oryzae* α-galactosidase immobilization on calcium alginate. Similarly, Sanjay et al. [35] reported an increase in K_M and decrease in V_{max} values upon immobilization of invertase on montmorillonite activated with APTS. The change in enzyme affinity for its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by the lower accessibility of the substrate to the active site of the immobilized enzyme [44].

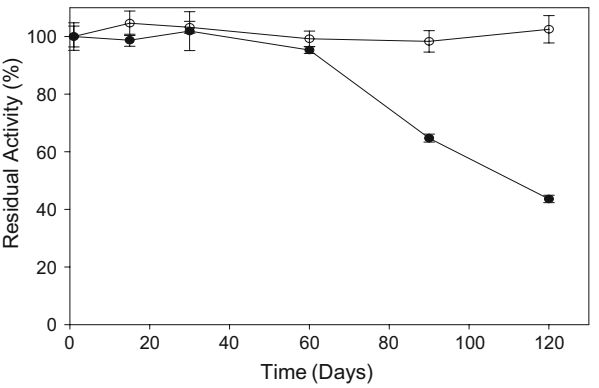

Fig. 6 Storage stability at 5 °C of free (*filled circles*) and immobilized (*open circles*) α-galactosidase from *P. griseoroseum*

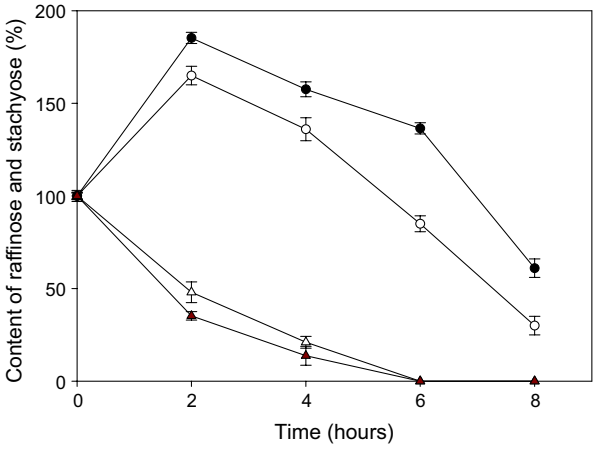

Fig. 7 Raffinose and stachyose content (percent) during defatted soybean flour treatment with free and immobilized α-galactosidase from *P. griseoroseum*. Amount of raffinose (*filled circles*) and stachyose (*filled triangles*) during treatment with free enzyme. Amount of raffinose (*open circles*) and stachyose (*open squares*) during treatment with immobilized enzyme

Storage Stability

Enzymes are generally not stable during storage in solution, and their activity decreases gradually over time. Immobilized enzymes are more stable than free enzymes [45]. The storage stability of free and immobilized α-galactosidase of *P. griseoroseum* was further studied by keeping the enzymes in a sodium acetate buffer, 100 mM, pH 5.0, at 4 °C for 3 months (Fig. 6). The immobilized enzyme maintained full activity during this period, while the free enzyme maintained only 44% of its original activity.

Enzymatic Treatment of Defatted Soybean Flour

The enzymatic hydrolysis of raffinose family sugars present in defatted soybean flour by free and immobilized α-galactosidase from *P. griseoroseum* is shown in Fig 7. During an incubation period of 6 h, stachyose was completely hydrolyzed by free and immobilized α-galactosidase. However, after 8 h of incubation, only 39% and 70% of raffinose were hydrolyzed utilizing free and immobilized α-galactosidase, respectively. Interestingly, during the first 6 h of treatment, an increase in raffinose content was observed in defatted

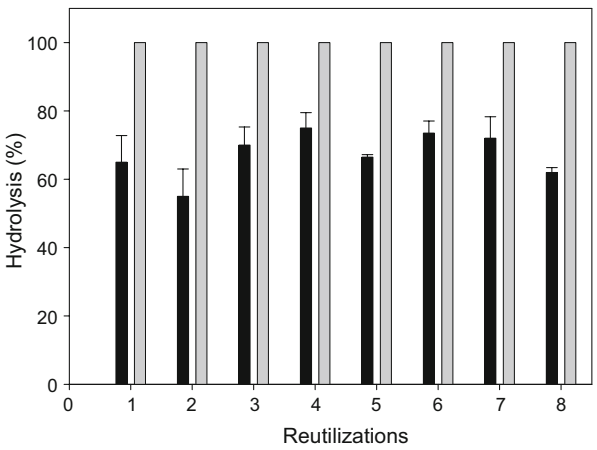

Fig. 8 Hydrolysis of raffinose (*black bars*) and stachyose (*gray bars*) in defatted soybean flour by immobilized α-galactosidase from *P. griseoroseum* obtained from eight enzyme reuses

soybean flour. This may have been caused by raffinose accumulation, which is formed after stachyose hydrolysis. A similar situation was described by Viana et al. [38] utilizing α-galactosidase from *Debaryomces hansenii* immobilized onto modified silica to reduce RO in soymilk. In this work, the time of treatment necessary for total RO hydrolysis present in fat-free soybean flour could be reduced by use of higher amount of α-galactosidase. This approach could prevent possible contaminations during the processing of soybean flour.

Generally, immobilized α-galactosidases shows a poorer substrate hydrolysis performance in comparison with free α-galactosidases due to diffusional limitations (resistance of substrate and products to diffuse into the immobilization matrix and resistance of the products to diffuse out) [18, 38, 46]. Oddly, in this work, it was observed that immobilized α-galactosidase was most efficient than free enzymes. This fact could be explained by the higher thermostability of immobilized α-galactosidase compared to free enzyme. The increase in thermostability of immobilized α-galactosidase from *P. griseoroseum* might compensate diffusional limitations, improving the catalytic efficiency of the immobilized enzymes.

Recently, several microbial α-galactosidases, free or immobilized, have been tested for RO reduction in soybean products. Immobilization of *Gibberella fujikuroi* α-galactosidase in polyacrylamide gel promoted a RO reduction of 71% in soymilk after 12 h of treatment [12]. In the same way, *A. oryzae* α-galactosidase entrapped in gelatin blends with alginate hydrolyzed 93% of the RO in defatted soybean flour after 12 h of treatment [47].

Operational Stability

The major advantage of enzyme immobilization is the easy reuse. Operational stability is the key parameter which determines the possible application of immobilized enzymes in large scale processes. The operational stability of immobilized α-galactosidase from *P. griseoroseum* was evaluated in a repeated batch process. The immobilized enzyme was submitted to eight consecutive cycles, each lasting 6 h. Even after continuous use for 48 h, the immobilized enzyme maintained its initial activity (Fig. 8). *A. oryzae* α-galactosidase immobilized on calcium alginate [18] and on kapa-karrageenan [46] maintained 83% and 61% of its initial activity after 24 and 16 h of treatment, respectively. The immobilization of *P. griseoroseum* α-galactosidase on modified silica was particularly advantageous for industrial applications due to its enhanced thermal stability, broadened pH range, and extended period of catalytic activity.

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References

1. Tischer, W., & Wedekind, F. (1999). *Biocatalysis—From discovery to application* (1st ed.). US: Springer.
2. Isik, S., Alkan, S., Toppare, L., Cianga, I., & Yagci, Y. (2003). *European Polymer Journal*, *39*, 2375–2381. doi:10.1016/S0014-3057(03)00184-8.
3. Girelli, A. M., & Mattei, E. (2005). *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *819*, 3–16. doi:10.1016/j.jchromb.2005.01.031.
4. Pierre, A. C. (2004). *Biocatalysis and Biotransformation*, *22*, 145–170. doi:10.1080/10242420412331283314.
5. Pang, J. B., Qiu, K. Y., & Wei, Y. (2002). *Journal of Inorganic Materials*, *17*, 665–671.

6. Suarez, F. L., Springfield, J., Furne, J. K., Lohrmann, T. T., Kerr, P. S., & Levitt, M. D. (1999). *The American Journal of Clinical Nutrition*, *69*, 135–139.
7. Wu, A. H., Wan, P., Hankin, J., Tseng, C. C., Yu, M. C., & Pike, M. C. (2002). *Carcinogenesis*, *23*, 1491–1496. doi:10.1093/carcin/23.9.1491.
8. Song, X. G., Pan, W. J., Noguchi, T., Yasui, N., Negishi, H., Takebe, M., et al. (2007). *Clinical and Experimental Pharmacology & Physiology*, *34*, S53–S54. doi:10.1111/j.1440-1681.2007.04778.x.
9. Rufer, C. E., & Kulling, S. E. (2006). *Journal of Agricultural and Food Chemistry*, *54*, 2926–2931. doi:10.1021/jf053112o.
10. Setchell, K. D. R., & Lydeking-Olsen, E. (2003). *The American Journal of Clinical Nutrition*, *78*, 593S–609S.
11. De Lumen, O. B. (1992). *Food Structure*, *11*, 33–46.
12. Thippeswamy, S., & Mulimani, V. H. (2002). *Process Biochemistry*, *38*, 635–640. doi:10.1016/S0032-9592(02)00010-9.
13. Gote, M., Umalkar, H., Khan, I., & Khire, J. (2004). *Process Biochemistry*, *39*, 1723–1729. doi:10.1016/j.procbio.2003.07.008.
14. Puchart, V., Vrsanska, M., Svoboda, P., Pohl, J., Ogel, Z. B., & Biely, P. (2004). *Biochimica et Biophysica Acta General Subjects*, *1674*, 239–250.
15. Garro, M. S., de Valdez, G. F., & de Giori, G. S. (2004). *Food Microbiology*, *21*, 511–518. doi:10.1016/j.fm.2004.01.001.
16. Viana, P. A., de Rezende, S. T., Marques, V. M., Trevizano, L. M., Passos, F. M. L., Oliveira, M., et al. (2006). *Journal of Agricultural and Food Chemistry*, *54*, 2385–2391. doi:10.1021/jf0526442.
17. Thananunkul, D., Tanaka, M., Chichester, C. O., & Lee, T. C. (1976). *Journal of Food Science*, *41*, 173–175. doi:10.1111/j.1365-2621.1976.tb01128.x.
18. Prashanth, S., & Mulimani, V. H. (2005). *Process Biochemistry*, *40*, 1199–1205. doi:10.1016/j.procbio.2004.04.011.
19. Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3.
20. McIlvaine, T. C. (1921). *Journal of Biology and Biochemistry*, *49*, 183–186.
21. Guimaraes, V. M., de Rezende, S. T., Moreira, M. A., de Barros, E. G., & Felix, C. R. (2001). *Phytochemistry*, *58*, 67–73. doi:10.1016/S0031-9422(01)00165-0.
22. Luonteri, E., Alatalo, E., Siika-aho, M., Penttila, M., & Tenkanen, M. (1998). *Biotechnology and Applied Biochemistry*, *28*, 179–188.
23. Varbanets, L. D., Malanchuk, V. M., Buglova, T. T., & Kuhlmann, R. A. (2001). *Carbohydrate Polymers*, *44*, 357–363. doi:10.1016/S0144-8617(00)00252-6.
24. Wang, C. L., Li, D. F., Lu, W. Q., Wang, Y. H., & Lai, C. H. (2004). *Letters in Applied Microbiology*, *39*, 369–375. doi:10.1111/j.1472-765X.2004.01594.x.
25. De Rezende, S. T., Guimaraes, V. M., Rodrigues, M. D., & Felix, C. R. (2005). *Brazilian Archives of Biology and Technology*, *48*, 195–202. doi:10.1590/S1516-89132005000200005.
26. Gote, M. M., Khan, M. I., Gokhale, D. V., Bastawde, K. B., & Khire, J. M. (2006). *Process Biochemistry*, *41*, 1311–1317. doi:10.1016/j.procbio.2006.01.003.
27. Falkoski, D. L., Guimaraes, V. M., Callegari, C. M., Reis, A. P., de Barros, E. G., & de Rezende, S. T. (2006). *Journal of Agricultural and Food Chemistry*, *54*, 10184–10190. doi:10.1021/jf0617162.
28. David, A. E., Wang, N. S., Yang, V. C., & Yang, A. J. (2006). *Journal of Biotechnology*, *125*, 395–407. doi:10.1016/j.jbiotec.2006.03.019.
29. Cabrera, K. (2004). *Journal of Separation Science*, *27*, 843–852. doi:10.1002/jssc.200401827.
30. Bulmus, V., Ayhan, H., & Piskin, E. (1997). *Chemical Engineering Journal*, *65*, 71–76. doi:10.1016/S1385-8947(96)03156-7.
31. Bayramoglu, G., Kaya, B., & Arıca, M. Y. (2004). *Food Chemistry*, *92*, 261–268. doi:10.1016/j.foodchem.2004.07.022.
32. Ettalibi, M., & Baratti, J. C. (2001). *Enzyme and Microbial Technology*, *28*, 596–601. doi:10.1016/S0141-0229(00)00342-2.
33. Ademark, P., Larsson, M., Tjerneld, F., & Stalbrand, H. (2001). *Enzyme and Microbial Technology*, *29*, 441–448. doi:10.1016/S0141-0229(01)00415-X.
34. Chaplin, M. F., & Bucke, C. (1990). *Enzyme technology*, (1st ed.). Cambridge, UK: Cambridge, University Press.
35. Sanjay, G., & Sugunan, S. (2005). *Clay Minerals*, *40*, 499–510. doi:10.1180/0009855054040187.
36. Bora, U., Kannan, K., & Nahar, P. (2005). *Journal of Membrane Science*, *250*, 215–222. doi:10.1016/j.memsci.2004.10.028.
37. Masoud, F., Nasrin, M., Saeed, M., Reza, M., Manouchehr, V., & Reza, B. M. (2002). *World Journal of Microbiology & Biotechnology*, *18*, 649–653. doi:10.1023/A:1016860032468.
38. Viana, P. A., de Rezende, S. T., Falkoski, D. L., Leite, T. D., Jose, I. C., Moreira, M. A., et al. (2007). *Food Chemistry*, *103*, 331–337. doi:10.1016/j.foodchem.2006.07.055.

39. Park, S. W., Choi, S. Y., Chung, K. H., Hong, S. I., & Kim, S. W. (2002). *Biochemical Engineering Journal*, *11*, 87–93. doi:10.1016/S1369-703X(02)00031-1.
40. Hernaiz, M. J., & Crout, D. H. G. (2000). *Enzyme and Microbial Technology*, *27*, 26–32. doi:10.1016/S0141-0229(00)00150-2.
41. Akgol, S., Kacar, Y., Denizli, A., & Arica, M. Y. (2001). *Food Chemistry*, *74*, 281–288. doi:10.1016/S0308-8146(01)00150-9.
42. Arica, M. Y., Yavuz, H., & Denizli, A. (2001). *Journal of Applied Polymer Science*, *81*, 2702–2710. doi:10.1002/app.1716.
43. Arica, M. Y., Yavuz, H., Patir, S., & Denizli, A. (2000). *Journal of Molecular Catalysis. B, Enzymatic*, *11*, 127–138. doi:10.1016/S1381-1177(00)00223-X.
44. Arica, M. Y., & Hasirci, V. (1993). *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire)*, *58*, 287–292.
45. Amaya-Delgado, L., Hidalgo-Lara, M. E., & Montes-Horcasitas, M. C. (2006). *Food Chemistry*, *99*, 299–304. doi:10.1016/j.foodchem.2005.07.048.
46. Girigowda, K., & Mulimani, V. H. (2006). *World Journal of Microbiology & Biotechnology*, *22*, 437–442. doi:10.1007/s11274-005-9053-9.
47. Naganagouda, K., & Mulimani, V. H. (2006). *Process Biochemistry*, *41*, 1903–1907. doi:10.1016/j.procbio.2006.03.040.