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Production, Partial Characterization, and Immobilization in Alginate Beads of an Alkaline Protease from a New Thermophilic Fungus *Myceliophthora* sp.

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Thermophilic fungi produce thermostable enzymes which have a number of applications, mainly in biotechnological processes. In this work, we describe the characterization of a protease produced in solid-state (SSF) and submerged (SmF) fermentations by a newly isolated thermophilic fungus identified as a putative new species in the genus *Myceliophthora*. Enzyme-production rate was evaluated for both fermentation processes, and in SSF, using a medium composed of a mixture of wheat bran and casein, the proteolytic output was 4.5-fold larger than that obtained in SmF. Additionally, the peak of proteolytic activity was obtained after 3 days for SSF whereas for SmF it was after 4 days. The crude enzyme obtained by both SSF and SmF displayed similar optimum temperature at 50°C, but the optimum pH shifted from 7 (SmF) to 9 (SSF). The alkaline protease produced through solid-state fermentation (SSF), was immobilized on beads of calcium alginate, allowing comparative analyses of free and immobilized proteases to be carried out. It was observed that both optimum temperature and thermal stability of the immobilized enzyme were higher than for the free enzyme. Moreover, the immobilized enzyme showed considerable stability for up to 7 reuses.

***Keywords*:** alkaline protease, immobilized enzyme, *Myceliophthora* sp., solid state fermentation, thermophilic fungus

Enzymes have a wide range of biotechnological, biomedical, and pharmaceutical applications. Proteases constitute the most prominent group of commercially available enzymes (Joo *et al.*, 2002); they account for about 65% of the worldwide sale of industrial enzymes in the world market (Johnvesly and Naik, 2001). Microorganisms are a valuable source of proteases mainly due to their short generation time, the ease of bulk production and genetic manipulation (Patel *et al.*, 2005). Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Rao *et al.*, 1998), and among them alkaline proteases have ample biotechnological potential for industrial sectors like laundry detergents, leather processing, brewing, food and pharmaceutical industries (Kembhavi *et al.*, 1993).

Thermophilic fungi are known to produce thermostable enzymes. The use of these enzymes may exhibit several advantages due to the high processing temperatures that can be applied, which are related to increased reaction rates, improved solubility of reagents, and a reduction in mesophilic contamination. Besides thermal stability, these enzymes also exhibit higher stability towards other protein denaturating conditions when compared to similar mesophilic enzymes (Gusek and Kinsella, 1988).

The available literature shows that a large amount of work has been devoted to the immobilization of enzymes in polymeric carriers (Kennedy *et al*., 1990). The main advantage of using immobilized enzymes as biocatalysts is that it is possible to reuse them since they can be easily recovered, thereby making the process economically feasible (da Silva *et al*., 2009). Therefore, for industrial applications, the immobilization of enzymes can provide several advantages and may contribute to an increase in their stability, making feasible their widespread use in industry.

For immobilization purposes, alginates are one of the most used polymers due to their mild gelling properties and non-toxicity (Won *et al.*, 2005). It is a water-soluble anionic linear polysaccharide composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid in different proportions in sequential arrangements, which can be precipitated by the addition of Ca^{2+} ions (Smidsrod and Skajak-Braek, 1990; Ertesvag and Valla, 1998) allowing the formation of microspheres with considerable strength and flexibility (De Queiroz *et al.*, 2006).

In this work we describe the isolation of a thermophilic fungus and protease production through solid-state and submerged fermentations, and present a comparative study on the properties of the protease produced in both fermentation systems. Additionally, we show data on the immobilization of the enzyme from SSF on beads of calcium alginate and also compare the properties of the free and immobilized proteases.

Materials and Methods

Materials

Casein, glycine, agar, and $CaCl_2$ were obtained from Vetec (Brazil).

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Yeast extract was obtained from Oxoid (England). NaCl, Na_2CO_3, $MnCl_2{\cdot}4H_2O$, $FeSO_4{\cdot}7H_2O$, and sodium phosphate were obtained from Synth (Brazil). K_2SO_4 and $MgCl_2$ were obtained from Nuclear (Sao Paulo, Brazil). Glycerol, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, EDTA, H_3BO_3, $(NH4)_2HPO_4$, $CoCl_2{\cdot}5H_2O$, and $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ were obtained from Merck (Germany). $(NH_4)_2SO_4$, trichloroacetic acid, Hepes, Taps, Caps, and BSA were obtained from Sigma (USA). $MgSO_4{\cdot}7H_2O$ and NH_4NO_3 was obtained from Dinamica (Brazil). Sodium citrate dehydrate and sodium acetate were obtained from Mallinckrodt Baker (USA). Sodium alginate was obtained from Fluka (Germany).

Isolation and identification

Several thermophilic fungal strains were isolated from box fat, poultry, poultry litter, and a composting plant. Approximately, 1 g or 2 ml of collected material was transferred directly to flask containing the following nutrient medium adapted from Jensen *et al.* (2002) and Sandhya *et al.* (2005): 0.5% (v/v) glycerol, 2% casein, 0.3% yeast extract, 0.5% NaCl, 0.15% Na_2CO_3, 0.1% K_2SO_4, 0.02% $MgCl_2$, 0.3% $(NH_4)_2HPO_4$ in the proportion (w/v) and a 0.5% (v/v) solution of trace elements [2.2% $ZnSO_4{\cdot}7H_2O$, 1.1% H_3BO_3, 0.5% $MnCl_2{\cdot}4H_2O$, 0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $CoCl_2{\cdot}5H_2O$, 0.16% $CuSO_4{\cdot}5H_2O$, 0.11% $(NH_4)Mo_7O_{24}{\cdot}4H_2O$, 5% EDTA in the proportion (w/v)]. After 24, 48 or 72 h of incubation at 45°C, the samples were transferred by means of grooves, to Petri dishes. After the emergence of fungi colonies, they were separated based on the appearance of the mycelium, color of spores and other characteristics of the obverse and reverse of the colonies. These colonies were reinoculated by streaking to obtain the pure cultures.

The fungal isolate that showed the highest protease activity was identified by conventional and molecular approaches. Cultures grown in 2% (w/v) malt agar for seven days at 45°C (Van Oorschot, 1980) were observed under a stereomicroscope (Zeiss, Stemi SV6) in order to study the colony morphology. In addition, details on the micromorphology were observed under light microscope (Zeiss, Axioskop 40) using squashed lumps stained with cotton blue. Molecular identification was carried out by sequencing the ITS1-5.8S-ITS2 rDNA region coupled with phylogenetic analyses as described in Sette *et al.* (2006).

Enzyme production in solid-state (SSF) and submerged (SmF) fermentations

Erlenmeyer flasks (250 ml) containing media composed of 4.75 g of wheat bran and 0.25 g of casein and hydrated with 7 ml of DW and 3 ml of nutrient solution 0.1% (w/v) $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and NH_4NO_3 were inoculated with 2 ml of a spore suspension and cultivated at 45°C for 168 h, taking samples every 24 h. The fermented material was mixed with 30 ml of distilled water per 5 g of fermented material, stirred for 30 min, filtered, and centrifuged at 10,000×g, at 6°C. The supernatant was used as a crude enzyme solution for proteolytic assays.

The submerged fermentation (SmF) was carried out in 125 ml Erlenmeyer flasks containing 25 ml of medium composed by 2% (w/v) casein, 0.1% (w/v) $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and NH_4NO_3. This medium was inoculated with 2 ml of a spore suspension and incubated at 45°C under agitation of 100 rpm, for 168 h. After each 24 h period of fermentation, the material in a flask was vacuum filtered using Whatman paper Nº1, centrifuged at 10,000×g for 40 min at 4°C and the supernatant was used as crude enzyme solution for testing proteolytic activity. The fermentation experiments were performed in triplicate and the results are reported as mean averages.

Protease assay

The proteolytic activity was assayed as described by Sarath *et al.* (1996) with modifications. The reaction mixture was made up of 0.2 ml (or 0.5 g immobilized) crude enzyme and 0.8 ml of 1% (w/v) casein dissolved in glycine buffer (50 mM, pH 9.0). The reaction was carried out at 50°C and stopped after 30 min by adding 0.5 ml of 15% trichloroacetic acid (TCA). Test tubes were centrifuged at 15,000×g for 30 min and the absorbance of the supernatant was measured at 280 nm using a Cary 100 (Varian) spectrophotometer. A control was prepared by adding TCA before the enzyme solution. One unit of enzyme activity (U/ml) was arbitrarily defined as the amount of enzyme required to cause an absorbance increase of 0.01 at 280 nm under the assay conditions (Merheb *et al*., 2007). The experiments were performed in triplicate.

Entrapment of the protease in calcium alginate beads

The procedure for enzyme immobilization in alginate was adapted from Betigeri and Neau (2002). Predetermined quantities of sodium alginate (2, 2.5, 3, 3.5%, w/v) were prepared in 4 ml of 50 mM glycine buffer pH 9.0. To this solution 1 ml of crude protease was added and thoroughly mixed. The mixture was added drop wise to 5 ml $CaCl_2$ solutions (0.04, 0.06, 0.08, 0.1 M). The cure time was fixed in 20 min, and thereafter, the formed microcapsules were washed with the buffer twice, to remove non-encapsulated enzyme. A similar method was followed for preparation of control alginate beads in the absence of the enzyme. The ideal concentrations of alginate and calcium were established guided by the highest activity of the immobilized enzyme. The amount of immobilized enzyme was estimated by subtracting the value of specific activity determined in the solution after immobilization from the value of specific activity (8 U/mg) used for immobilization (Worsfold, 1995). The total protein concentration was determined using the method of Bradford (1976) and bovine serum albumin as a standard.

Effect of pH and temperature on free and immobilized enzyme activity

The effect of temperature and pH on the activity of free and immobilized protease was investigated. The enzyme was incubated at 50°C in 0.05 M buffer solutions containing 1% (w/v) casein: sodium citrate dehydrate (pH 5), sodium acetate (pH 5.5 and pH 6), sodium phosphate (pH 6.5 and pH 7), Hepes (pH 7.5), TAPS (pH 8 and pH 8.5), Glycine (pH 9 and pH 9.5), CAPS (pH 10, pH 10.5, and pH 11). Optimum temperature was determined by incubating the reaction mixture at temperatures ranging from 0 to 75°C at the optimum pH.

Effect of temperature on free and immobilized enzyme stability

The thermal stability was investigated by measuring the enzyme activity after keeping the enzyme solution for 24 h in the absence of substrate, at temperatures between 30 and 70°C. Remaining protease activity was determined at optimum pH and temperature.

Reusability of protease immobilized in alginate beads

The initial activity of the immobilized enzyme was measured and the conjugate was then subjected to cycles of repeated use. The results of pH, temperature and reusability stability of free and immobilized protease are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

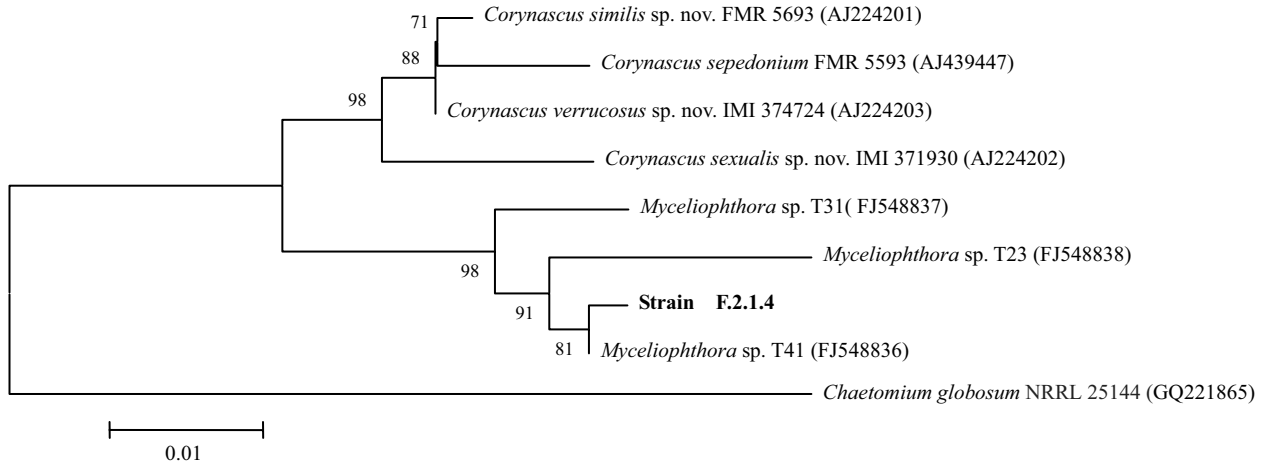

Fig. 1. Phylogenetic tree based on ITS-rDNA analyses showing closest relatives of thermophilic fungus strain F.2.1.4 (Kimura two-parameter model; Neighbor-Joining algorithm). Bootstrap values (1,000 replicate runs) greater than 70% are listed.

Results and Discussion

Isolation and identification

We isolated thirty thermophilic fungi with the ability to grow at 45°C on medium containing wheat bran and casein as carbon sources and produced proteases in both SSF and SmF (data not shown). The fungus strain F.2.1.4 presented the highest protease activity and was subjected to taxonomic characterization.

Analyses of macro- and micro-morphological characteristics accommodated the isolate F.2.1.4 in the genus *Myceliophthora* due to the presence of spreading colonies with dense aerial mycelium and blastic conidia often borne in ampulliform swellings (Van Oorschot, 1980). Particularly, this isolate produced pulverulent pale mycelium, obovoid conida, measuring 5.65-7.91×3.39-4.52 µm as well as finely ornamented conidia walls. Based on such characteristics the isolate F.2.1.4 is similar to *Myceliophthora thermophila*, however, some slight morphological differences were observed, for instance, *M. thermophila* has pale brown, woolly colonies, and a variable range of conidia sizes 4.5-11×3.0-4.5 µm (Van Oorschot, 1977).

Data derived from BLAST (ITS-rDNA region) showed that the isolate F.2.1.4 had high sequence similarities (99-96%) with three unidentified *Myceliophthora* spp. isolated from geothermal soils in China; and 95-94% of sequence similarities with different strains of the Ascomycota genus *Corynascus* (one of the *Myceliophthora* teleomorphic states), including three new species: *C. verrucosus* sp. nov., *C. similis* sp. nov., *C. sepedonium*, *C. sexualis* sp. nov. (Stchigel *et al.*, 2000). The phylogenetic tree showed that the isolate F.2.1.4 clustered with the three *Myceliophthora* strains from China (Fig. 1).

Molecular and phylogenetic results corroborate the data from morphological analyses and considering the combined methods used for fungus identification, the thermophilic isolate F.2.1.4 was identified as *Myceliophthora* sp. Species in this genus are commonly found in soil, molding open-stack alfalfa ensilage, fir and spruce pulpwood chips and wheat straw compost (Van Oorschot, 1980). Moreover, results derived from conventional, molecular and phylogenetic analyses suggest that isolate F.2.1.4 likely represent a new species in the genus *Myceliophthora*. Additional taxonomic analyses will be performed in order to confirm the new species hypothesis.

Enzyme production in solid-state (SSF) and in submerged (SmF) fermentations

Figure 2 shows the profile of protease production by *Myceliophthora* sp. in SSF and SmF. The maximum enzyme production was obtained in the third day of fermentation in SSF (1.78 U/ml) and in the fourth day in SmF (0.38 U/ml). The higher protease production achieved in SSF corroborate data reported by several investigators and reinforce the hypothesis that production of extracellular enzymes is higher in SSF since the conditions of this fermentation system are similar to natural fungal growth media (Ramesh and Lonsane, 1991; Morita and Fujio, 1999; Aguilar *et al.*, 2004; Silva *et al.*, 2007). The data also showed that wheat bran that contains approximately 18% protein, 5% fat, and 62% carbohydrate was an effective medium for protease production because it supplies adequate nutrients for microorganisms (Beg *et al.*, 2000; Ellaiah *et al.*, 2004). Protease production in state solid and submerged fermentation by thermophilic fungi have been reported by Germano *et al.* (2003), Sandhya *et al.* (2005), Macchione *et al.* (2008), O'Donoghue *et al.* (2008), and Merheb *et al.* (2007).

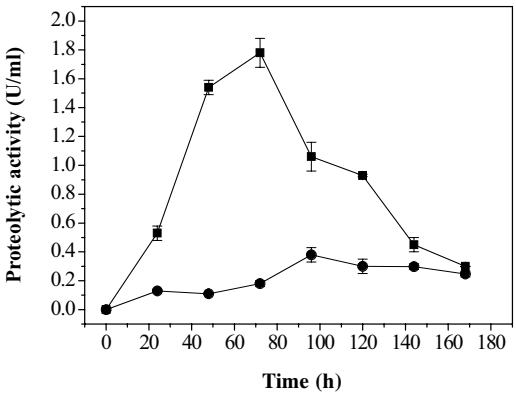

Fig. 2. Production of protease by *Myceliophthora* sp. in SSF (■) and SmF (●) fermentations. Each symbol represents the Mean±SD.

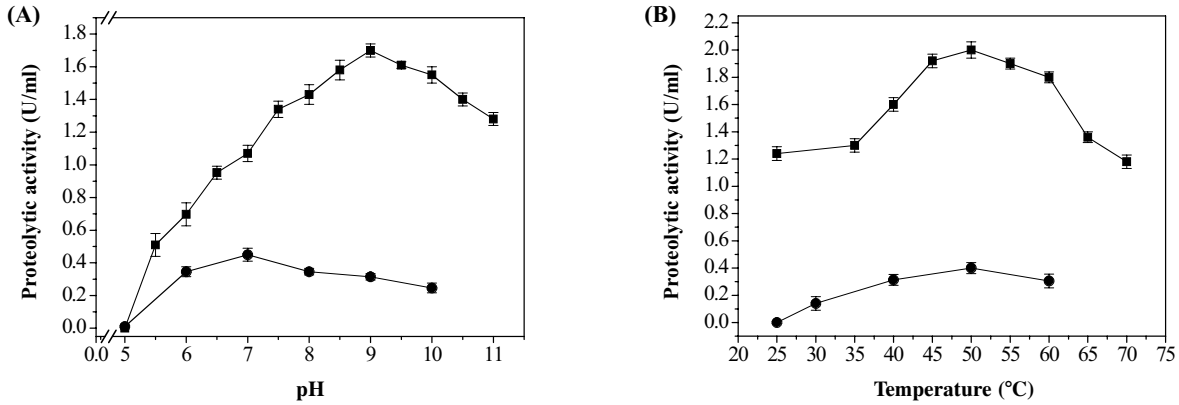

Fig. 3. Effect of pH (A) and temperature (B) on free crude proteolytic activity produced by *Myceliophthora* sp. in SSF (■) and SmF (●). For the pH effect the samples were incubated at 50ºC using different buffers, and for the temperature analysis the samples were incubated at the optimum pH. Each symbol represents the Mean±SD.

Also Badhan *et al.* (2007) reports the production of protease by *Myceliophthora* sp. IMI 387099.

Characterization of the free crude enzyme

Figure 3A shows that the optimum pH value for the free protease produced by SSF was 9. However, the protease produced in submerged fermentation exhibited the maximum activity at pH 7 with accentuate decreasing of the activity in alkaline pH, suggesting a different protease expression in both fermentation systems. Extracellular alkaline proteases from thermophilic and thermotolerant fungi have been described from *Thermomyces lanuginosus* (Li *et al.*, 1997), *Aspergillus*

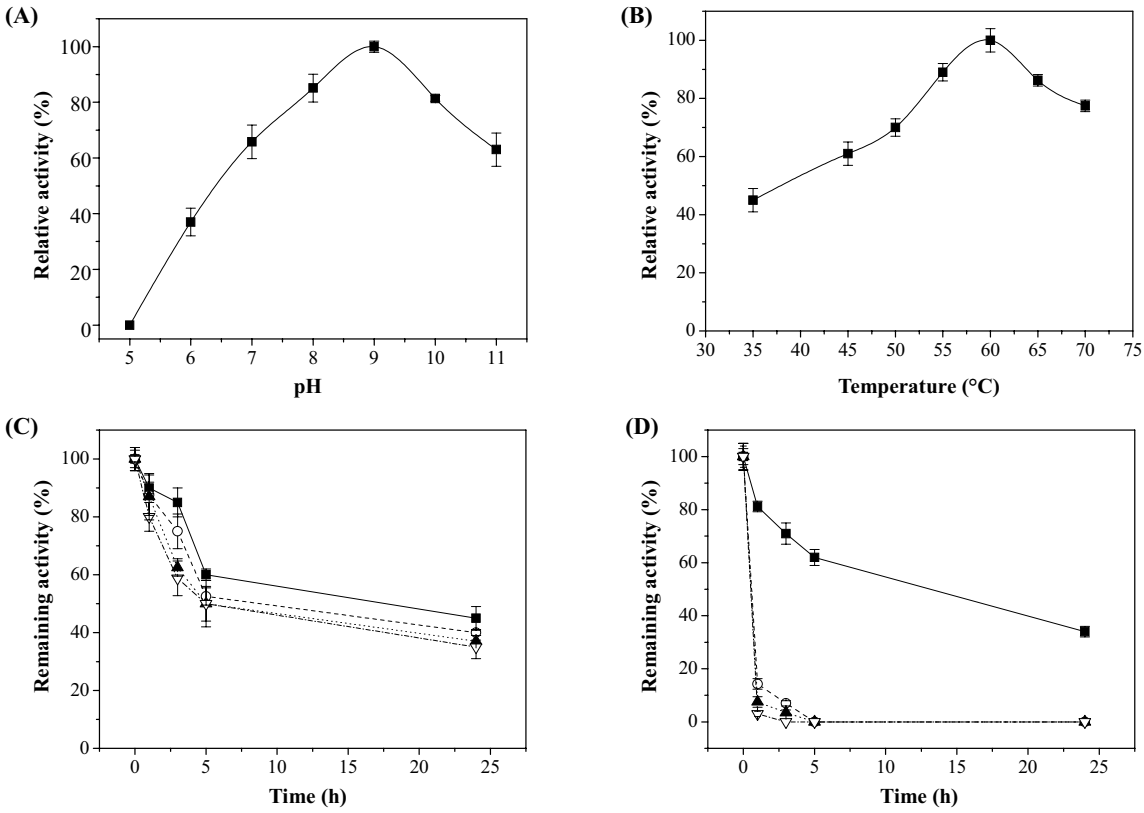

Fig. 4. Properties of the immobilized protease produced by SSF. (A) Effect of pH on immobilized enzyme activity, (B) Effect of temperature on immobilized enzyme activity, (C) effect of temperature on stability of immobilized enzyme when in the absence of substrate, (D) effect of temperature on stability of free enzyme when in the absence of substrate. (■) 50°C, (○) 55°C, (▲) 60°C, (△) 65°C. The results were expressed as perceptual relative activity (the ratio of activity of the sample to that of the maximum ×100). Each symbol represents the Mean±SD.

clavatus (Tremacoldi and Carmona, 2005), and *Fusarium culmorum* (Pekkarinen *et al*., 2002).

Alkaline proteases have many applications in the industry such as laundry detergents, leather processing, brewing, food and pharmaceutical industries therefore the alkalophilic properties exhibited by proteases from *Myceliophthora* sp. in SSF are an important feature to be explored.

The maximum activity for both crude enzymes was at 50°C, but the crude enzyme from SSF maintained 95% of the maximum activity at 60°C while for the protease from SmF the activity was 79% (Fig. 3B). This property is consistent with the data reported for extracellular enzymes from thermophiilic fungi. Similar results were reported for proteases from *Thermoascus aurantiacus* var. *levisporus* (55°C) (Marcy *et al.*, 1984). Proteases from thermophilic *Scytalidium* (Hasbay and Ögel, 2002) and thermotolerant *Aspergillus fumigatus* (Santos *et al.*, 1996) showed optimal activities at 45°C.

Since proteolytic activity was higher in the crude enzyme solution from SSF and additionally, it showed alkaline optimum pH and activity at higher temperature range (50-65°C), it was used for the immobilization procedure.

Entrapment of the protease in calcium alginate beads

We tested several concentrations of sodium alginate (2, 2.5, 3, 3.5%, w/v) and $CaCl_2$ (0.04, 0.06, 0.08, 0.1 M). The best condition for immobilization of the protease was 2.5% alginate and 0.08 mM $CaCl_2$ (data not shown), obtaining 2 mm (average diameter) beads.

The immobilized enzyme was reevaluated concerning its physical-chemical properties (Fig. 4). The profile of activity of the immobilized protease was very similar to the free enzyme in terms of its optimum pH and temperature (Figs. 4A and B), but the thermal stability was strongly increased by the entrapment procedure. The immobilized enzyme maintained around 50% of the initial activity at 50 to 65°C for 5 h (Fig. 4C), but the free enzyme was stable at 50°C for 5 h but it was inactivated at temperatures above 55°C (Fig. 4D). This result suggested that the conformational flexibility of the enzyme was affected by immobilization causing an increase in enzyme rigidity which was reflected by increase in stability towards thermal denaturation, as proposed by Abdel-Naby (1993) and Worsfold (1995) for other proteases.

The immobilization of proteases in alginate beads was reported by Ko *et al.* (2008) and Ahmed *et al.* (2008), and the last author reported the increased thermal stability of an immobilized enzyme; at 65°C the free enzyme was inactivated, while the immobilized enzyme maintained around 80% of the initial activity.

Concerning pH stability, the free protease isolated from *Myceliophthora* sp. exhibited 95% of activity after 24 h at pH values from 8 to 11 at room temperature (data not shown).

Reusability of protease immobilized in the alginate beads

When comparing the performance of immobilized biocatalysts intended for industrial use, knowledge of their operational stability is very important. The stability of an immobilized enzyme without appreciable loss of enzyme activity is important mainly for the economic viability. Figure 5 shows the effect of repeated use on the activity of the immobilized protease; it decreased gradually during 7 cycles of reuse (its activity at the 7th cycle being greater than 50% of the original activity) and more sharply after that.

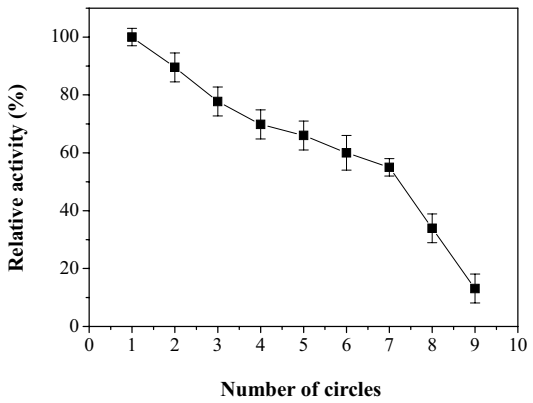

Fig. 5. Reusability of the immobilized protease at 50°C. The results were expressed as percentual relative activity (the ratio of activity of the sample to that of the maximum ×100). Each symbol represents the Mean±SD.

In conclusion, the thermophilic fungus *Myceliophthora* sp. was able to produce alkaline and thermostable proteases in SSF using wheat bran as culture medium. The protease immobilization in alginate not only maintained some properties, but significantly improved its thermal stability and permitted its reuse for seven cycles.

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