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Protease Production by Different Thermophilic Fungi

Mariana M. Macchione · Carolina W. Merheb · Eleni Gomes · Roberto da Silva

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Abstract A comparative study was carried out to evaluate protease production in solid-state fermentation (SSF) and submerged fermentation (SmF) by nine different thermophilic fungi – *Thermoascus aurantiacus* Miehe, *Thermomyces lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus flavus* 1.2, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, *Rhizomucor pusillus* 13.36 and *Rhizomucor* sp. 13.37 – using substrates containing proteins to induce enzyme secretion. Soybean extract (soybean milk), soybean flour, milk powder, rice, and wheat bran were tested. The most satisfactory results were obtained when using wheat bran in SSF. The fungi that stood out in SSF were *T. lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, and *Rhizomucor* sp. 13.37, and those in SmF were *T. aurantiacus*, *T. lanuginosus* TO.03, and 13.37. In both fermentation systems, *A. flavus* 1.2 and *R. pusillus* 13.36 presented the lowest levels of proteolytic activity.

Keywords Protease · Thermophilic fungi · Solid state fermentation · Submerged fermentation · Wheat bran

Introduction

Proteases are enzymes that break down protein molecules through peptide bond hydrolysis [1]. They are commercially employed in many industrial processes. In foods, proteases have two main applications: in the processing of traditional food products and in the processing of new protein-based ingredients called functional foods [2]. Proteases are also used in other industrial segments such as leather industry, pharmaceutical, waste management, and the detergent industry. Currently, microbial proteases make up approximately 40% of total enzyme sales [3, 4].

M. M. Macchione · C. W. Merheb · E. Gomes · R. da Silva (✉)
Laboratório de Bioquímica e Microbiologia Aplicada,
IBILCE - Instituto de Biociências Letras e Ciências Exatas, UNESP - Universidade Estadual Paulista,
Rua Cristóvão Colombo, 2265 São José do Rio Preto, São Paulo CEP 15054-000, Brazil
e-mail: dasilva@ibilce.unesp.br

Proteinaceous material such as horn, feather, nail, hair, and cheese whey occur in nature as waste and can be converted, by proteases, into liquid concentrates or dry solids with high protein content and of nutritional value for food and feed. Thus, proteases provide potential application for the management of residues from various food processing industries such as poultry and cattle slaughterhouses and fishing and dairy industries [5, 6].

Microorganisms are capable of producing extracellular proteases that degrade proteins into amino acids to make their assimilation possible [7]. Many of them exhibit a very wide production due to their fast growing rate and thus have become potential sources of industrial enzymes [1].

Thermophilic fungi are known to produce thermostable enzymes. The use of these enzymes may present many advantages, especially in the food industry, due to the high processing temperatures that could be applied, which are related to an increase in reaction rates, improved solubility of reagents, and a decrease in mesophilic contamination. Besides thermal stability, these enzymes also exhibit higher stability towards other protein denaturating conditions such as extreme pH values and compounds such as ionic detergents and organic solvents, when compared to similar mesophilic enzymes [8].

In this study, nine thermophilic fungi were screened for the production of protease in solid and submerged cultivation mediums. Tested fungi were: *Thermoascus aurantiacus* Miehe, *Thermomyces lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus flavus* 1.2, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, *Rhizomucor pusillus* 13.36 and *Rhizomucor* sp. 13.37. Five different substrates were used: soybean extract (soybean milk), soybean flour, milk powder, rice, and wheat bran.

Materials and Methods

Microorganisms and Inoculum

The fungi *T. aurantiacus*, *T. lanuginosus*, *T. lanuginosus* TO.03, *A. flavus* 1.2, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, *R. pusillus* 13.36, and *Rhizomucor* sp. 13.37, belong to the culture bank of the Laboratory of Applied Biochemistry and Microbiology, *Instituto de Biociências Letras e Ciências Exatas*, *Universidade Estadual Paulista*. They were inoculated in test tubes with Sabouraud and were incubated at 45°C for 4 days until complete growth. Afterwards, they were kept at room temperature until further use.

The mycelium was suspended in 7 mL of sterilized nutrient solution made up of 0.1% (w/v) $(NH_4)_2SO_4$, $MgSO_4.7H_2O$ and NH_4NO_3, and 1 mL of this mycelial suspension was used to inoculate the culture medium.

Fermentation Medium and Culture Conditions

Protease Production Profile by Solid-State Fermentation Mediums containing 10 g of wheat bran hydrated with 15 mL of nutrient solution, to approximately 60% moisture, were sterilized (120°C/40 min) in 250-mL Erlenmeyer flasks. The mediums were inoculated with mycelial suspension from each fungus and cultivated at 45°C for 8 days. Samples were taken every 24 h. The crude enzyme solution was obtained by adding 25 mL of water to the fermented material. Solids were removed by filtering through Whatman no. 1 filter paper and centrifuging at 10,000 rpm/20 min, and finally, the clear solution was assayed.

Protease Production Profile in Different Substrates by Solid-State Fermentation Mediums containing 10 g of soybean extract (soybean milk), soybean flour, milk powder, rice, and

wheat bran hydrated with 15 mL of nutrient solution, to approximately 60% moisture, were sterilized (120°C/40 min) in 250-mL Erlenmeyer flasks. The mediums were inoculated with mycelial suspension from each fungus and cultivated at 45°C during maximum production period as determined earlier. Experiments were carried out in duplicate; the results shown are average values. The crude enzyme solution was obtained by adding 25 mL of water to the fermented material. Solids were removed by filtering through Whatman no. 1 filter paper. For soybean extract, soybean flour, milk powder, and rice, centrifugation (10,000 rpm/20 min) was carried out prior to filtering.

Protease Production Profile by Submerged Fermentation Mediums containing 2.5 g of wheat bran hydrated with 22.5 mL of nutrient solution, to approximately 90% moisture, were sterilized (120°C/40 min) in 125-mL Erlenmeyer flasks.

The mediums were inoculated with mycelial suspension from each fungus and cultivated on rotary shaker (150 rpm) at 45°C for 8 days. Samples were taken every 24 h. The crude enzyme solution was obtained by centrifuging (12,000 rpm/10 min) and filtering through Whatman no. 1 filter paper.

Protease Production Profile in Different Substrates by Submerged Fermentation Mediums containing 2.5 g of soybean extract (soybean milk), soybean flour, milk powder, rice, and wheat bran hydrated with 22.5 mL of nutrient solution, to approximately 90% moisture, were sterilized (120°C/40 min) in 125-mL Erlenmeyer flasks. The mediums were inoculated with mycelial suspension from each fungus and cultivated on rotary shaker (150 rpm) at 45°C during maximum production period, as determined earlier. Experiments were carried out in two sets; the results shown are average values. The crude enzyme solution was obtained by centrifuging (12,000 rpm/10 min) and filtering through Whatman no. 1 filter paper.

Proteolytic Activity

Proteolytic activity was assayed as described by Kembhavi et al. [9], with modification. The reaction mixture was made up of 0.4 mL of casein (Sigma) 0.5% (w/v) in distilled water and 0.4 mL 0.2 M acetate buffer, pH 5.0, to which 0.2 mL of the crude enzyme solution was added. The reaction was carried out at 60°C and stopped after 30 min with 1 mL of 10% trichloroacetic acid (TCA). Test tubes were centrifuged at 5,000 rpm/5 min, and the absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared in which the TCA was added before the enzymatic solution. One unit of enzyme activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.01 in absorbance at 280 nm under the assay conditions.

Results and Discussion

Protease Production Profile by Solid-State Fermentation

Figure 1 shows the protease production profile in solid medium through 8 days, and it can be seen that six fungi, *T. lanuginosus*, *T. lanuginosus* TO.O3, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, and *Rhizomucor* sp. 13.37, exhibited maximum production on the third day. *Thermoascus aurantiacus* revealed maximum production from the third day up to the fourth. *Rhizomucor pusillus* 13.36 showed a plateau of production from the third day up to the fifth. *Aspergillus flavus* 1.2 showed a peak at the fifth day. The highest

production peak in Fig. 1 was reached by *T. lanuginosus*, with 945.2 U/mL followed by *Aspergillus* sp. 13.33, *Rhizomucor* sp. 13.37, *T. lanuginosus* TO.03, *Aspergillus* sp. 13.35, *Aspergillus* sp. 13.34, and *T. aurantiacus* with 844.6, 770.9, 730.0, 640.5, 469.6, and 258.3 U/mL, respectively, all on the third day. The lowest peak was obtained by *A. flavus* 1.2 with 117.6 U/mL on the fifth day. From these results, an incubation period of 3 or 4 days was chosen for the experiment of protease production in different substrates (Fig. 2). The variability of the results obtained from the sets was below 5% for each experiment.

Figure 1 shows that maximum enzyme production occurred on the third day for most fungi. The subsequent decrease in enzyme activity as fermentation time increased was probably due to degradation of the extracellular enzymes. When dealing with proteases, autolysis may occur, and in this case, it was probably higher than the production of new enzymes by the fungi, reaching an equilibrium as seen by the low and fairly constant activity remaining after the fifth up to the eighth day. Also, depletion of nutrients available to the microorganisms [10] or even cessation of production, because enzymes are primary metabolites [11], might have contributed to the decrease in enzyme activity.

Protease Production Profile in Different Substrates by Solid-State Fermentation

Substrates rich in proteins may act as protease inducers [12, 13]. Thus, a group of these substrates was chosen to verify their potential as protease producers.

Fermentation medium that yielded highest protease production by all fungi tested was wheat bran, as shown in Fig. 2. In addition to being a good inducer for proteolytic enzyme production, wheat bran is an agro-industrial residue from wheat flour production, which is of low cost and always available. Other factors that make wheat bran an excellent substrate for protease production in solid-state fermentation (SSF) are, besides its composition, its texture, which gives it an adequate surface area with good porosity, acting as physical support and allowing the fungi to access the nutrients [14].

The worst substrate tested was milk powder. This probably occurred due to its thin granularity, which ends up allowing a gathering of the particles when moist, causing stickiness. Moreover, the heat treatment during sterilization may have promoted Maillard reactions, which maybe affected the availability of amino acids in the medium, and also caramelization reactions, both leading to a darkening and modification of the medium's texture.

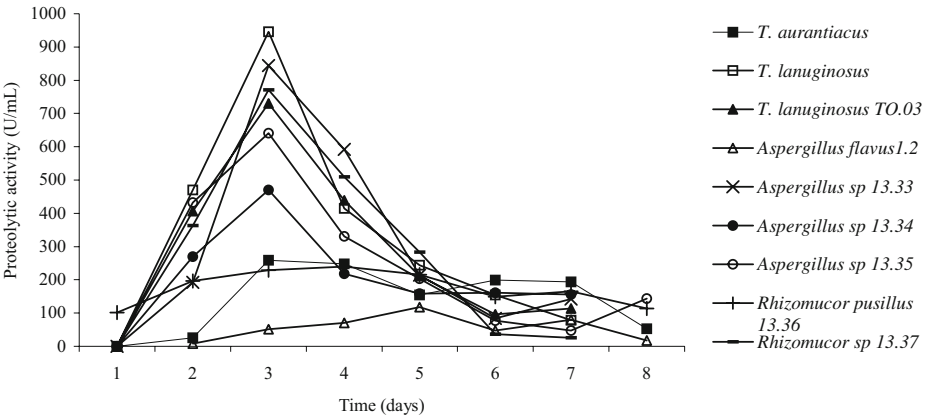

Fig. 1 Protease production profile by different thermophilic fungi in SSF using wheat bran as substrate

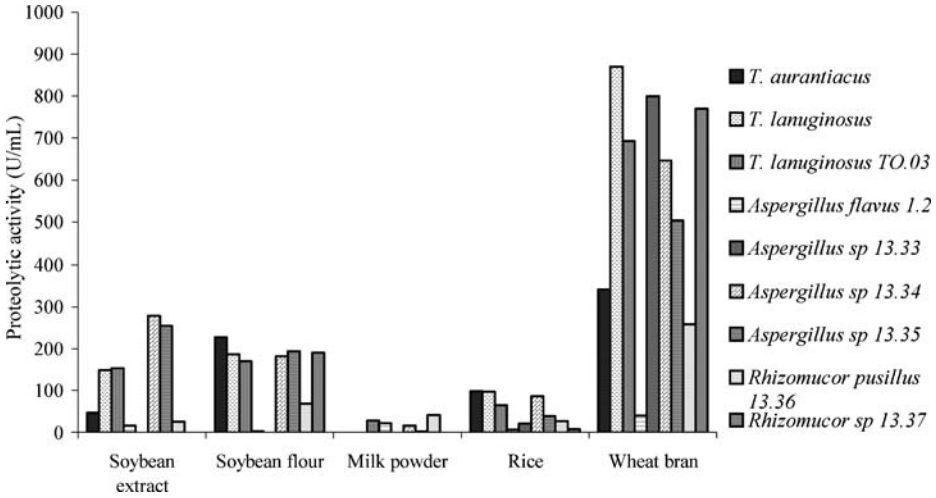

Fig. 2 Protease production by different thermophilic fungi in SSF using different substrates. *Thermoascus aurantiacus*, *T. lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus* sp. 13.33, *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, and *Rhizomucor* sp. 13.37: 3-day fermentation period. *Aspergillus flavus* 1.2 and *R. pusillus* 13.36: 4-day fermentation period

Soybean flour and soybean extract also exhibited, as main problems, thin granularity. The problem with rice was the difficulty in spreading the inoculum equally throughout the medium.

Protease Production Profile by Submerged Fermentation

Figure 3 shows protease production profile in liquid medium by submerged fermentation (SmF) through 8 days. It can be seen that most fungi exhibited maximum protease secretion

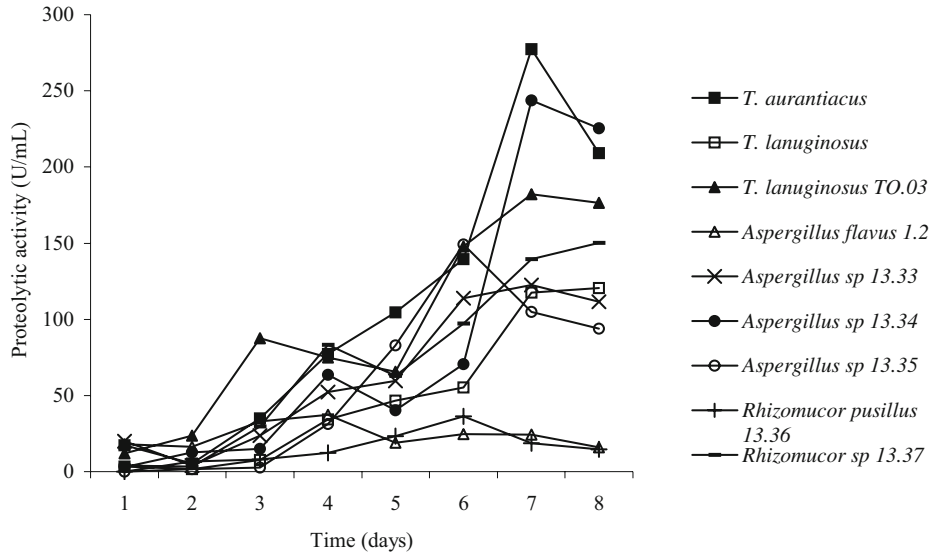

Fig. 3 Protease production profile by different thermophilic fungi in SmF using wheat bran as substrate

between the sixth and eighth days. The fungi *Aspergillus* sp. 13.35 and *R. pusillus* 13.36 showed maximum activity on the sixth day; fungus *T. aurantiacus* with the highest production (277.35 U/mL) and *T. lanuginosus*, *T. lanuginosus* TO.03, *Aspergillus* sp. 13.33, and *Aspergillus* sp. 13.34, also with high production (243.60 U/mL), showed maximum activity on the seventh day; and fungus *Rhizomucor* sp. 13.37 showed maximum activity on the eighth day. Hence, a 7-day incubation period for all fungi was adopted to continue with the SmF experiments using different substrates. It can also be seen in Fig. 3 that the lowest enzyme production was exhibited by *A. flavus* 1.2, with 37.22 U/mL on the fourth day.

Protease Production Profile in Different Substrates by SmF

Soybean extract and soybean flour were good inducers for protease production in SmF, as shown in Fig. 4. When compared to SSF, these results were similar. However, in SmF, there was a significant decrease in production when the substrate was wheat bran. Other researches have revealed that the SSF technique gives greater enzyme yield than SmF [10, 15]; however, there is not any established scale or method to compare product yield in SSF and SmF, and the exact reason for higher production in SSF is not well known yet [14]. It is known that the amount of enzyme produced varies with the cultivation medium used [16], meaning that the microorganism will behave differently according to the different types of substrates and conditions used for fermentation; thus, no defined medium has been established for the optimum production of protease from different microbial sources [17]. In SSF, the conditions may favor the growth of filamentous fungi, which generally grow in nature on solid material such as wood, leaves, and roots and other organic matter [18], explaining the higher enzyme yields. The major activity peaks occurred for *T. aurantiacus* on soybean extract (262.95 U/mL) and wheat bran (244.00 U/mL) and for *T. lanuginosus* TO.03 on soybean extract (185.02 U/mL) and soybean flour (193.55 U/mL).

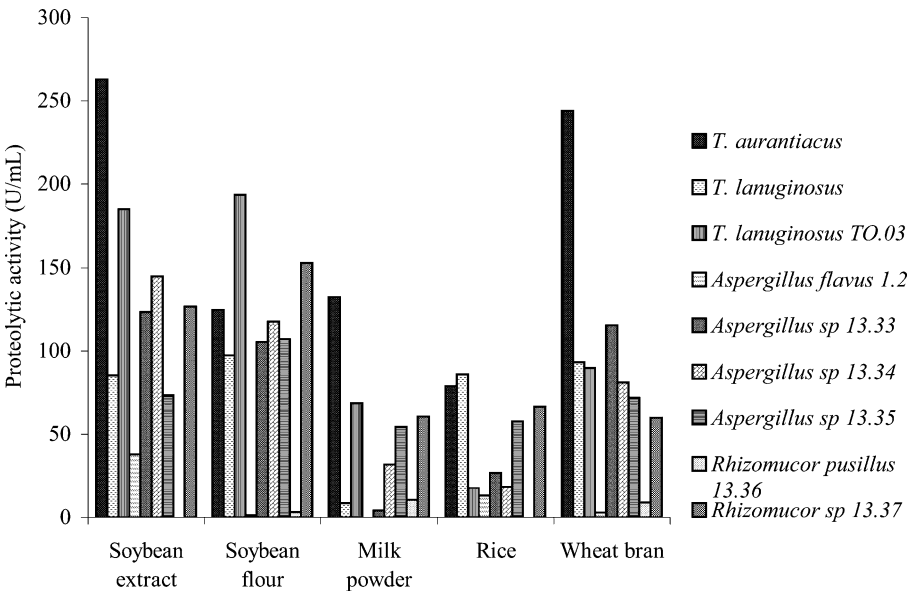

Fig. 4 Protease production by different thermophilic fungi in 7-day SmF using different substrates

The solubility of soybean extract, soybean flour, and milk powder had a positive influence on the results from SmF. For milk powder, the higher amount of water decreased the damaging effects of sterilization, especially regarding the texture, increasing enzyme production.

There was decrease in production in SmF when using rice, due to gelatinization, because it absorbed a lot of water during sterilization and incubation, decreasing the free water available for fermentation. The process was then similar to a SSF but with less substrate accessible for microbial growth. Similar results occurred when using wheat bran, where the biggest problem was probably the difficulty faced by the hyphae when penetrating the medium, besides the lower aeration due to the presence of water.

Cost and efficiency are major characteristics of enzymes for industrial application. Hence, it is desirable for the material used in the fermentation medium to be of low cost, available in large amounts, and continually renewable, which is exactly the case of agro-industrial residues, in addition to making microbial growth possible [19]. Thus, it should present in its composition carbon, nitrogen, and mineral sources, and, in the case of enzyme production, the presence of inducers is essential [18]. For the production of protease, substrates that contained proteins in its composition were chosen, and a nutrient solution was incorporated with the aim of making it suitable for the microorganism to grow in the desired fermentation [20].

Mediums used in submerged and solid fermentations present different characteristics. In SmF, the microorganism has more water available for its growth; there is the advantage of process control (pH, temperature, etc.) and it is easier to collect representative samples; and the heat and gases resulting from active growth can be easily dispersed, not causing a temperature increase. Some advantages of SSF are the possibility of using agro-industrial by-products offering the opportunity to process these residues, high product concentration, lower costs for enzyme recovery, less amount of liquid residues produced, and lower energy requirements [10, 14, 21].

Conclusions

The best condition for high protease production was using wheat bran in SSF. Soybean extract and soybean flour exhibited moderate production results for both types of fermentation; rice and milk powder did not present good results in either. Another type of sterilization can be suggested, such as filtration, when using medium made up of milk powder or even the addition of sterile water after the sterilization of dry material to improve the results. In spite of the fact that wheat bran was the best substrate for protease production, it should be noted that the inclusion of inducers such as the ones used in this work (powder milk, soybean flour, and soybean extract) might be beneficial as additives to the suitable solid substrate and should be further studied. SmF presented lower protease yields and longer fermentation time for maximum enzyme production.

Among the fungi tested, both strains of *T. lanuginosus* were revealed to be good protease producers in SSF. Fungi *Aspergillus* sp. 13.34, *Aspergillus* sp. 13.35, *Rhizomucor* sp. 13.37, *Aspergillus* sp. 13.33, and finally *T. aurantiacus* also stood out as good producers in this type of fermentation. Fungi *A. flavus* 1.2 and *R. pusillus* 13.36 exhibited the worst results due to the inconsistent curves and the low production peaks in all substrates tested. Thus, the most potent protease producer was *T. lanuginosus* in SSF with wheat bran.

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