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The role of the type of substrate, particle size, and coagulations analytical method on microbial rennet synthesis by *Mucor miehei* Cooney & R. Emers., 1964 (Fungi: Zygomycota) via solid-state fermentation

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Abstract. This study was performed to assess an alternative source of fungal rennet. This study aimed to investigate the influence of the substrate (wheat bran and broken gelatinized rice), bran particle size (fine and coarse), the addition of casein and the coagulation analysis method from *Mucor miehei* Cooney & R. Emers., 1964 (Fungi: Zygomycota) via solid-state fermentation. Cultivation of the micro-organism was performed in 10 g of substrate inoculated with a spore solution and incubated at 35 °C for 72 h. We opted to use Itambé skim milk for the coagulation analysis. Greater enzyme activity (2133.33 US) was found after 48 h using fine wheat bran with casein. The particle size of the substrate influenced the enzyme activity, wherein fine wheat bran was better compared to coarse wheat bran. In experiments employing broken gelatinized rice with casein, low enzymatic activity was observed, whereas no activity was detected using this substrate without casein. It is noteworthy that the enzyme activity found with fine wheat bran and casein was higher compared to other studies, indicating the need for further investigation.

Keywords: *Mucor miehei*, Protease, Microbial rennin, Cheese, Fermentation process.

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Introduction

Cheese is made from enzymatic coagulation (irreversible) or acid coagulation (reversible) of milk with later separation of the serum and coagulated portion (Spreer, 1975; Aquarone et al., 2001). The characteristics of cheese manufacturing are linked to the geographical region, climate, raw materials and other factors that may influence the process (Spreer, 1975; Aquarone et al., 2001).

Milk is composed of whey proteins such as lactalbumin and lactoglobulin and casein protein (Aquarone et al., 2001). Casein is organized into micelles that are arranged in the calcium-sensitive fraction, also called paracaseins: α_{s1}-casein, α_{s2}-casein, β-casein and colloidal calcium phosphate. Externally to the micelles is k-casein, which provides a protective effect to

the other fractions due to their non-precipitation in the presence of calcium ions. Enzymatic action breaks this protective effect, allowing for the process of cheese manufacturing through the coagulation of protein fractions (Spreer, 1975; Aquarone et al., 2001).

Enzymatic coagulation occurs at pH 6.6, allowing the proteolytic enzyme to act on k-casein, destabilizing it and exposing the other caseins to milk calcium. The proteolysis of the k-casein fraction and paracasein generate glycomacropeptide, which reacts with calcium ions, yielding calcium paracaseinate that aggregates and forms the coagulum for manufacturing cheese (Aquarone et al., 2001). Based on the rate of the reaction, it is possible to measure the enzymatic activity (Lima et al., 2001).

Proteases are proteolytic enzymes capable of catalyzing the cleavage of peptide bonds in other proteins (Sumantha et al., 2006). These biocatalysts have several applications in the textile, laundry, health services and food processing industries (Rao et al., 1998; Sumantha et al., 2006). Rennin may also be called acid proteases and may be isolated from different sources, such as animals, plants or micro-organisms (Scriban, 1985; Khademi et al., 2013). In the past, rennet was obtained from the fourth stomach (abomasum) of unweaned calves. Currently, due to the unavailability of animal rennin allied with increased consumption and production of cheese, research has been carried to obtain alternative sources of rennin (Preetha and Boopathy, 1994; Sumantha et al., 2006; Silveira, 2007).

Micro-organisms such as *Rhizomucor pusillus, R. miehei, Endothia parasitica*, *Aspergillus oryzae* and *Irpex lactis* (Neelakantan et al., 1999) are known to produce microbial rennin (Sumantha et al., 2006), and these enzymes possess proteolytic activity with commercial value (Neelakantan et al., 1999; Sumantha et al., 2006). The fungus *Mucor miehei* synthesizes various enzymes used industrially, mainly microbial rennet and lipases from submerged fermentation (FMS) or solid-state fermentation (SSF) systems (Silva et al., 1998; Silveira, 2007). Rennin-like enzymes synthesized by *R. miehei* produce cheeses with chemical, physical and organoleptic properties similar to cheeses made with bovine rennin (Reed, 1993).

In SSF, the growth and metabolism of micro-organisms occur in free water with the absence or near absence on a solid substrate. The solid substrate not only provides essential nutrients for the culture, but is also plays a role as support for microbial cells (Del Bianchi et al., 2001; Damaso et al., 2008; Couto and Sanromán, 2006). Many factors can influence growth and microbial activity during the process; among these, the main factors are the type of substrate, particle size, water level/humidity and temperature (Couto and Sanromán, 2006).

The increase in agricultural activities has set off the need for research into the use of agro-industrial residues that can often be considered candidates as substrates for SSF (Bortolazzo, 2011). Among these, for the production of proteases, we highlight sunflower meal, coffee hulls, soybean meal, rice bran and husks, corn bran, yam residue and wheat bran (Pandey et al., 1999), considered to be the most suitable and commonly used substrates in bioprocess (Pandey et al., 2000).

In the milk clotting process, to verify the enzymatic activity, milk powder is used (Preetha and Boopathy, 1994; Apocada, 1994; Silveira et al., 2005; Silveira, 2007). Such milk powders have different values and nutritional contents (Itambé, 2013; Nestle, 2013). Likely, over time, the composition of powdered milk can be changed in market dairy products, so it is necessary to standardize milk calcium concentration used in experiments to avoid distorted results between different studies. Therefore, the aim of this study was to investigate the influence of the substrate (wheat bran and broken gelatinized rice), bran particle size (fine and coarse), the addition of casein, and the method of coagulation analysis using SSF for microbial rennin production.

Materials and methods

Micro-organism and culture

Mucor miehei NRRL 3420 was obtained from the Industrial Microbiology Laboratory of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP Rio Claro-SP, Brazil) and maintained on Sabouraud agar slants. The inoculum consisted of a spore solution obtained by adding 200 mL of distilled water followed by scraping the agar surface of the mycelium grown for 72 h (35 °C) in a Roux flask using the same culture medium for maintenance. After filtration of the solution through a funnel with glass wool, the solution was standardized to 10^6 spores/mL by counting in a Neubauer Chamber.

Solid-state fermentation

Fermentation was carried out in duplicate using Erlenmeyer flasks (250.0 mL) containing 10.0 g of substrate (coarse wheat bran, fine wheat bran or broken gelatinized rice) with and without casein (2.0 g), in a bacteriological incubator at 35 °C for up to 72 h of cultivation. The inoculum consisted of 5 or 10 mL of the spore solution (10^6 spores/mL) added to the substrate of broken rice or wheat bran, respectively. All the material used was previously sterilized (121 °C for 20 min). It is noteworthy that, in experiments with rice, 5 mL of water was added to the substrate before autoclaving to promote gelatinization.

Extraction of the enzyme from the culture solution

After time intervals of 24, 48 and 72 h of cultivation, the enzyme was extracted from the culture solution by adding 100 mL of distilled water to each Erlenmeyer flask with a fermentation substrate followed by homogenization and extraction of the enzyme by filtration through a funnel.

Determination of enzyme activity

The enzyme activity was determined using a technique modified from Arima et al. (1970), obtaining results in Soxhlet Units (US). In this procedure, 1 mL of the filtered fermentation broth solution, adjusted to pH 6.5, was added to 10 mL of Itambé skim milk powder solution containing 0.01 M $CaCl_2$, preheated to 35 °C, by noting the exact time to the onset of clotting in the test tube.

Two brands of milk powder were tested to verify the influence on clotting time: Molico Total Calcium Nestlé® skim milk powder and Itambé® skim milk powder.

Statistical analysis

Two-way ANOVA was used for statistical analysis with the Bonferroni post-test for comparing the groups. Differences were considered significant when $p < 0.05$. All analyses were performed using GraphPad Prism 5.0 software.

Results and discussion

Analytical method to assess the enzymatic coagulation of milk

From the results obtained in comparative tests corresponding to different brands of milk powder for the determination of enzymatic activity, it was found that the Itambé® skim milk powder demonstrated higher enzymatic activity when compared with the Molico Total Calcium Nestlé® skim milk powder. For all types of milk powder tested, enzymatic coagulation after 48 h of cultivation was higher than at 24 and 72 h. The effect of the calcium concentration in the milk powder on coagulation was compared. It was observed that the Molico milk (500 mg of calcium) provided lower activity than the Itambé milk (300 mg of calcium) (Figure 1).

A considerable number of studies do not describe the brand of milk used for the analysis of enzymatic coagulation (Apocada et al., 1994; Preetha and Boopathy, 1994; Escobar and Barnett, 1995; Silveira et al., 2005). Data obtained from this study confirm the need for standardization of the milk used, since the nutrient components of different brands of milk are present in different concentrations, which may influence the coagulation

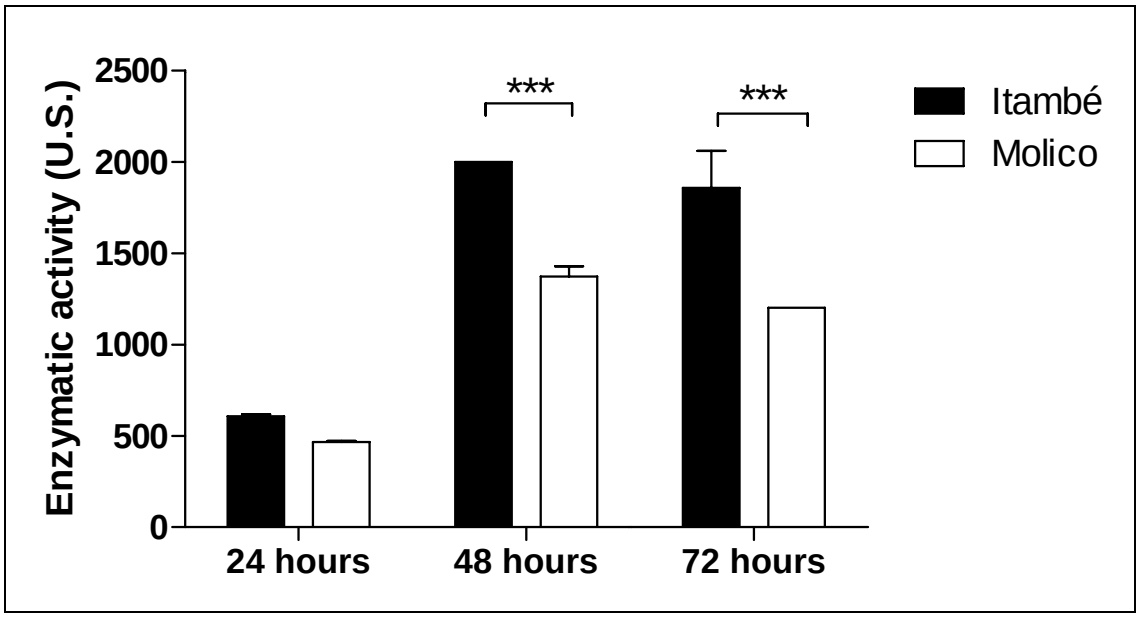

Figure 1. Effect of calcium concentration on the milk clotting enzyme analysis. *p < 0.05.

process as well as differences in the results between studies.

The milk composition exhibits a direct effect on curd formation since milks from different regions and breeds of animals do not have exactly the same nutritional composition. In other words, some milk is deficient or less coagulant than others and, in addition, the covariance in milk components levels characterizes the effects of the natural variation of these constituents (Law and Tamime, 2010). Therefore, there are difficulties in establishing convincing inferences about the effects of such components individually in rennet coagulation (Law and Tamime, 2010).

Substrates and supplementation

The absence of casein in experiments with SSF using previously gelatinized broken rice showed no enzymatic coagulation within 5 min. However, with the addition of 2 g of casein to the substrate, significantly higher enzymatic activity was observed after 48 h (384 US) and 72 h of culture (872.72 US) ($p < 0.05$) (Figure 2).

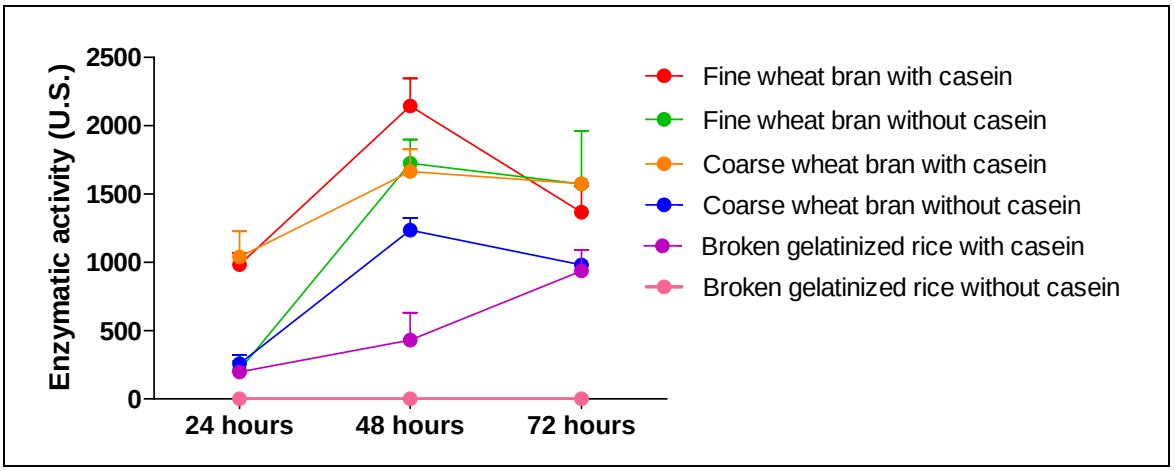

Figure 2. *Mucor miehei* enzyme activity by 24, 48 and 72 h, using wheat bran (coarse and fine) and broken gelatinized rice as the substrate with or without casein.

Schmidell et al. (2001) also reported proteolytic enzyme production using cooked rice as the substrate by studying other micro-organisms such as *Bacillus amyloliquefaciens*, *Aspergillus awamori*, and *A. oryzae.*

As shown in earlier studies, casein is a relevant factor in microbial rennin synthesis during fermentation processes (Thakur et al., 1990; Fernadez-Lahore et al., 1998; Silveira et al., 2005; Silveira, 2007). The most suitable concentration for coagulant activity is 2 g, since higher concentrations tend to restrict the activity (Silveira et al., 2005).

Commercial wheat bran with two different particles sizes, coarse and fine, were tested using the same inoculum with and without casein. In the absence of casein, it was observed that the wheat bran particle size influenced enzymatic synthesis; the outcome was significantly better for fine wheat bran without casein after 48 and 72 h of cultivation, as compared to the coarse wheat bran without casein in the same time period ($p < 0.05$) (Figure 2). As shown in previous studies, it is known that the particle size of the substrate can interfere with the fermentation process because the surface areas of the substrate particles is a limiting factor to fungal attack (Couto and Sanromán, 2006). Substrates with smaller particles favor have surface area for the colonization of micro-organisms; however, extremely tiny particles can result in the agglomeration of the substrate, limiting the surface area of the substrate granule and affecting oxygenation and cultivation, thus leading to weak microbial growth. Otherwise, larger particles provide better aeration (oxygen diffusion) (Couto and Sanromán, 2006). Besides the influence of particle size, moisture in the fermentation process should also be stipulated carefully, because high levels of humidity cause a decrease in porosity, resulting in mass transfer problems, especially in relation to system oxygenation. On the other hand, limited moisture levels reduce solid substrate solubility (Pinheiro, 2006).

Supplementation of 2 g of casein to experiments with coarse and fine wheat bran favored enzymatic synthesis and provided better results than testing without casein, confirming that this molecule has the ability to enhance the synthesis of microbial rennin.

The results of the casein addition experiments reiterate that fine wheat bran showed significantly higher enzymatic activity (2133.33 US) when compared to coarse wheat bran (1655.17 US); however this difference was observed only after 48 hours ($p < 0.05$) (Figure 2).

Special attention should be placed on the substrate employed in fermentation processes, as it should have characteristics that optimize the yield of the process, such as the degree of accessibility to the culture medium, the porosity, the size and shape of the particle, which should offer proper conditions for the micro-organism (Schmidell et al., 2001). The diameter of the fine wheat bran, used in this study, led to considerable differences in relation to enzymatic activity.

The results obtained for wheat bran with the addition of casein after 48 hours of culture provided the highest enzymatic activity (US 1655.17 and US 2,133) compared to the results of other researchers using the same experimental parameters (Silveira, 2007).

Conclusion

Among the experimental parameters that favor the synthesis of microbial rennin by *Mucor miehei* via solid-state fermentation, special attention should be given to the type of substrate, the particle size, supplementation with casein and the brand of powdered milk used for milk clotting because these factors directly influence the process. In this study, fine wheat bran with and without the addition of casein resulted in the highest enzyme activities, and was considered the best substrate analyzed. The peak of enzyme activity was 2133 US using fine wheat bran with casein, a higher result than what has

been observed in other studies. The broken gelatinized rice substrate did not provide significant enzymatic activity.

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Conflict of interest statement

Authors declare that they have no conflict of interests.

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