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# Effect of fermentation time and acid casein concentration as nitrogen source on microbial rennet production

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## Research Article

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**Abstract**

We evaluated the effects of fermentation time and acid casein content on the microbial rennet obtained by solid-state fermentation using wheat bran as the carbon source. The experiments used two fermentation times (72 and 96 h), while acid casein content was 1.5, 2.0, 2.5, and 3.0 g. Rennet strength from eight enzymatic extracts was measured using pasteurized whole milk. Rennet strength of samples from 72 h of fermentation showed an increase when acid casein content increased. The rennet strength increased at 96 h of fermentation with increasing amount of casein (up to 2.5 g), and then decreased with the largest addition (3.0 g) of casein. Coagulation time for the sample with highest rennet strength was 420 s.

## Introduction

Cheese producers use some enzyme coagulants, obtained mainly from animal sources such as calves, in order to curd milk. Enzymes that coagulate milk are known as rennin or rennet, which is mixture of two enzymes: chymosin and pepsin in a 90/10 ratio. Since the 1960s some cheeses have been produced using coagulants obtained from non-animal sources such as microbial fermentation (fungi, and bacteria) and vegetable sources (like fruits). More than one hundred different microbial coagulants have been reported (Garg and Johri, 1994; Mandy *et al*., 2011).

Microbial rennet is an enzymatic proteolytic consortium including enzymes very similar to those extracted from calves' rumen, which can unfold the 105–106 phenyl-methionine link from κ-casein. There are diverse sources of these coagulant enzymes such as vegetables, animals, and microorganisms. Enzyme coagulant is an acid enzyme kind (EC: 3.4.23.10) produced by a diverse variety of microorganisms such as *Mucor miehei*, *Bacillus subtilis*, *Pseudomonas* sp., and *Rhizomucor miehei*. The last one is a fungus that has shown an excellent production capacity of coagulant enzymes (Crueger and Crueger, 1990). Fungi are isolated from several environments since they are present everywhere (Tubesha and Al-Delaimy, 2003). *R. miehei* can produce an aspartic protease that has a chemical structure similar to chymosin, an enzyme extracted from calf abomasum. More than 33% of the dairy industry around the world use enzymes from fungi fermentation due to its ability to coagulate milk (Khademi *et al*., 2013). There are several ways to measure the enzymatic activity of coagulant extracts, for example by measuring the rennet strength, which is defined as the quantity of milk in milliliters that coagulate after 40 min at 36 ± 1 °C after the addition of 1 ml of curd. This work aimed to evaluate the effects of fermentation time and acid casein content on the microbial rennet obtained from *R. miehei* by solid-state fermentation using wheat bran as the carbon source.

## Materials and methods

### *Inoculum preparation*

*R. miehei* was inoculated on Petri dishes with potato dextrose agar (PDA) and incubated four days at 30 °C. A spore suspension with a concentration of $1 \times 10^6$ spores/ml was used as the inoculum for the solid state cultures.

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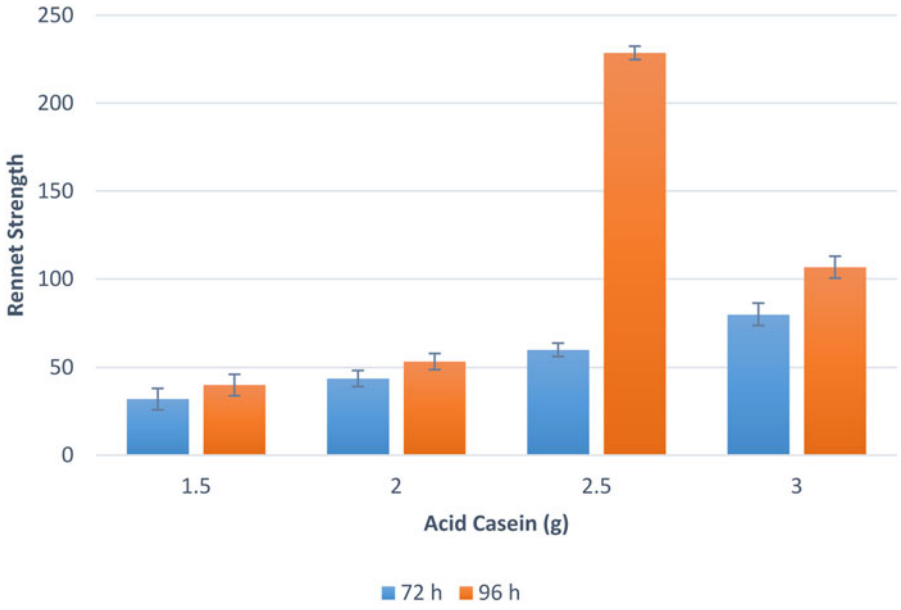



**Fig. 1.** Rennet strength of enzymatic extracts at 72 and 96 h of fermentation with 1.5, 2.0, 2.5 and 3.0 g of acid casein. The error bars show the standard deviation.

### *Solid state fermentation*

Ten grams of wheat bran purchased at a local store were put in a 250 ml flask. Acid casein was used as the nitrogen source at four concentrations (0.15, 0.2, 0.25, and 0.30 g per g of wheat bran). The spore suspension of *R. miehei* was added as the inoculum to initiate the solid substrate fermentation. Culture pH was adjusted at 5.5, using chloridric acid 0.2 N. Flasks were incubated for 72 and 96 h at 37 °C. The enzyme was extracted after fermentation using 10 ml of phosphate buffer pH 5. Whatman filter paper number 1 was used to recover the enzymatic extract. A high speed centrifuge with temperature control was used to centrifuge the enzymatic extract at 15 000 rpm, 4 °C for 10 min. Coagulation tests with supernatant were performed.

### *Coagulation test*

Pasteurized whole milk was used to perform coagulation tests. Twenty ml of milk and 0.5 ml of enzymatic extract were mixed in a flask. The temperature was set at 37 °C and coagulation time was measured. All tests were carried out in triplicate. Clotting time is the time when milk phases begin to separate and agglomerate formation is evident. The rennet strength was calculated from the clotting time using Equation 1, described by Osorio *et al.* (2008).

$$\mathrm{RS} = \frac{V \times 2400}{C \times t}$$

where RS: Rennet strength, $V$: Volume of milk, ml, 2400: Coagulation time for milk at 37 °C using animal rennet, s, $C$: Volume of enzymatic extract, ml, and $t$: Coagulation time of sample, s.

## Results

This work demonstrates the feasibility of rennet production from the fungus *R. miehei* in solid state fermentation. Rennet strength results of the enzyme extract samples obtained are shown in Figure 1. Rennet strength was affected by both casein content and incubation time with a maximum value at 2.5 g of casein per g of wheat bran and 96 h. This combination represented an increment of 614% compared to the lowest value, which was observed at 1.5 g of casein per g of wheat bran and 96 h. Samples obtained at fermentation time of 96 h showed an increase in the rennet strength with the increase of the casein content, until reaching a maximum at 0.25 g/g; after that a decrease was observed.

Increase of fermentation time from 76 to 96 h caused an increment of rennet strength over all samples. This is probably due to the fact that the microorganism is still in its exponential growing phase, producing and excreting the diastase (an enzyme capable of clotting milk) to broth culture. The lag phase of fungi is around 24–48 h, at this time coagulation enzyme is not produced (or produced at a low rate).

## Discussion

The increase in the amount of casein added to the fermentation from 1.5 to 2.5 g caused an increase in rennet strength. After a further increase in the amount of acid casein, a decrease in the strength of the rennet was observed in comparison to the maximum values. De Castro *et al.* (2014) reported results similar to these obtained here. They evaluated the production and biotechnological properties of secreted proteases by *Aspergillus niger* in solid state fermentation with different substrates and found a higher protease production when bran wheat was used as carbon source at 96 h of fermentation time. On the other hand, enzyme activity reported by Morillo et al. (2015) used a strain of *Rhizomucor* sp., which is recognized as a strain with a higher excretion of milk coagulant proteins. These researchers reported a rennet strength of 148.15, using glucose and casein as carbon and nitrogen source, respectively. This result is lower than the maximum rennet strength reported here, which could indicate that wheat bran supply nutrients necessary for protease production.

Other authors (Harboe *et al.*, 2000; Kurutahalli *et al.*, 2010) have reported values of rennet strength higher than those reported in this work. The differences may be due to the use of calcium carbonate ($CaCO_3$) to enhance coagulation because the presence of calcium ions favors the increase of milk coagulant activity,

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even at concentrations as low as 50 mmol/l (Ding *et al*., 2011). Morrillo *et al*. (2014) reported an increase in the rennet strength of enzymatic solutions by increasing the concentration of $CaCl_2$, as they obtained even higher values than commercial coagulant enzymes (Maxiren® and BIOVEN®). Maigua (2017) reported rennet strength values from 932.24 using industrial enzymes extracted from young cattle. Cordova and Paitan (2013), performed enzymatic extractions obtaining values of 7.69 Soxhlet units using 3% sodium chloride at a pH of 4.8.

All samples with a fermentation time of 72 h showed an increase in the rennet strength when addition of acid casein was increased. The same was true of 96 h up to 2.5, but thereafter rennet strength decreased. The increases are due to the fact that acid casein participates as an inducer in the synthesis of enzymes by the microorganism, producing a variable proteolytic activity with an increase in the production of milk coagulant proteases (Dutt, *et al*., 2008). More than 2.5 g of acid casein causes a decrease on rennet strength probably due to the carbon/nitrogen ratio not being the optimum for this particular fungus.

In conclusion, the feasibility of rennet production from the fungus *R. miehei* in solid state fermentation was demonstrated, and we found that 2.5 g of casein per g of wheat bran and 96 h maximized the rennet production.

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