Vol.48, n. 6 : pp. 931-937, November 2005
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY

A N I N T E R N A T I O N A L J O U R N A L

# Microbial Rennet Produced by *Mucor miehei* in Solid-State and Submerged Fermentation

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## ABSTRACT

*The aim of this work was to study the effect of carbon and nitrogen sources in submerged fermentation, and the casein effect on solid-state fermentation on rennin production by Mucor miehei. Biomass peaks reached 6.7; 8.1 and 8 g/L and enzymatic production peaks of 1,066; 857 and 480 Soxhlet Units (S.U.) for glucose concentration of 18; 25 and 35 g/L respectively. Flasks with baffles showed biomass peaks of 6.7; 8.3 and 10 g/L and enzyme activity peaks of 648; 279 and 300 S.U. for the same glucose concentration. The values of 923 and 667 S.U. were obtained when corn steep liquor and Proflo (Cottonseed Nutrients from Traders ®) were used. In SSF system the enzymatic activities were 414, 264 and 167 S.U., when using HCl 0.2 N, 0.3 N and 0.4 N respectively. SSF experiments using 1 and 2 g of casein/gram wheat bran (10% moisture) showed an increase in the enzymatic production (966 and 1,117 S.U.). The results suggested that increase in glucose concentration affected the enzyme synthesis, and casein was the prime factor in the enzyme synthesis induction. SSF showed to be a good system for rennin production.*

**Key words:** *Mucor miehei*, rennin, clotting activity, casein, solid-state fermentation, submerged fermentation

## INTRODUCTION

Cheese production, as well as the use of exogenous enzymes in their manufacture date back to 6000 B.C (Neelakantan et al., 1999). The rennin acts on the milk protein in two stages, through enzymatic and non-enzymatic action, resulting in the coagulation of the milk. In the enzymatic phase, the milk becomes a gel, due to the calcium ions influence and the temperature used in the process. Calf rennet obtained from the fourth stomach of suckling calves (Nagodawithana and Reed, 1993; Scriban, 1985) is used all over the world to manufacture most of the cheese varieties. The recent growth in the cheese industry and the scarcity on calf rennet have stimulated the research for milk clotting enzyme from alternative sources (Escobar and Barnett, 1993; Fox, 1991). Many microorganisms are known as producers of rennet such as proteinases, which can substitute the calf rennet. Microorganisms like *Rhizomucor pusillus*, *Rhizomucor miehei*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lactis* are extensively used for rennet production in cheese manufacture [Bailey and Siika-Aho, 1988;, Escobar and Barnett, 1993; Kan et al., 1979; Kolaczkowska et al., 1988; Lasure, 1980; Neelakantan et al., 1999, Thakur et al.1990). The aspartyl protease from *Mucor miehei* is commonly

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used as a chymosin substitute in cheese making. This enzyme has a high ratio of MCA/PA (milk clotting activity/proteolytic activity) (Escobar and Barnett, 1995; Thakur et al., 1990), an important requirement to substitute calf rennet. Researchers have investigated the enzyme production by *Mucor miehei* through submerged fermentation (Bailey and Siika-Aho, 1982; Beyenal et al., 1999 Escobar and Barnett, 1995;Lasure, 1980; Seker et al., 1999; Streets and Ingle, 1972) studying the effects of the medium composition, pH, concentration of each constituent, and operational parameters, such as agitation and dilution rate, temperature on the enzyme production in batch and continuous system. These systems showed that high agitation rate increased the enzyme production (Escobar and Banett, 1993). On the other hand, high glucose concentration inhibited the enzyme production (Beyenal et al., 1999; Seker et al., 1999) and high concentration of amino acids and sulfate repressed the enzyme activity (Lasure, 1980). Enzyme production has also been studied through solid state fermentation (Fernandez-Lahore et al., 1999, Fernandez-Lahore et al., 1998; Pandey et al., 1999; Pandey et al., *2000;* Preetha and Boopathy,1994; Thakur et al.,1990; Thakur et al.,1993) verifying the effects of operational parameters such as temperature, influence of the moisture content in solid substrate medium, effect of nutritional parameters. Studies of the medium composition effects on the rennin production have been elucidated mostly in batch submerged fermentation or solid-state systems (Kan et al., 1979; Kolaczkowska, 1988; Lasure, 1980; Thakur et al., 1990). For *Mucor miehei*, the use of casein in the medium induced high enzyme yields, and the lack of glucose resulted in a significant decrease in the enzyme production. On the other hand, the enzymatic preparation obtained by solid-state fermentation showed a lower proteolytic and lipolytic activity (Arima and Iwasaki, 1970). In this study the fungus *Mucor miehei* was grown in a submerged fermentation. In these experiments, glucose concentration was modified, and casein concentration was maintained constant. The enzyme production in submerged fermentation was compared with the milk clotting activity obtained in solid-state fermentation where the casein concentration was modified. Solid-state fermentation was also compared when wheat bran was supplemented with casein and pre-treatment with HCl solution.

## MATERIALS AND METHODS

### Microorganism and culture

*Mucor miehei* NRRL 3420 was obtained from Fundação André Tosello and maintained on Sabouraud Agar slants at $15^0$C. To recover the spores, Roux flasks containing 100 mL of Sabouraud Agar were inoculated and incubated at $35^0$C. After 72 h, the inoculum was obtained by scraping the agar surface in the presence of 200 mL sterile water. The spore suspension ($10^6$ spores/mL, determinated by Neubauer Chamber) was transferred to 500 (SLF) and 250 (SSF) mL erlenmeyer flasks containing sterilized (121°C/20 min.) growth medium.

### Culture media

### Submerged liquid fermentation medium

The composition of growth medium was (g/L): glucose (18, 25 and 35); peptone (8); casein (8); $KH_2PO_4$ (2); corn steep liquor [CSL] (1.1; 2.2; 4.4; 8.8 and 17.6) and Proflo [Cottonseed Nutrients from Traders ®] (4 and 8). The initial pH was 6.0.

### Solid-state fermentation medium

The composition of growth medium was (g/g wheat bran): wheat bran (10); casein (0.5, 1.0, 1.5, 2.0, 3.0, 5.0 and 10.0) plus ten milliliters of water or ten milliliters of HCl 0.2 N; 0.3N or 0.4 N per 10 grams wheat bran. The initial moisture of wheat bran was 10%.

### Culture Conditions

### Submerged liquid fermentation (SLF)

Erlenmeyer flasks (500mL) and baffled flasks (Erlenmeyers with four baffles) containing 90 mL of medium (sterilized at 121°C for15min.) were inoculated with a spore suspension (10% v/v) prepared from *Mucor miehei* Roux culture ($10^6$ spores/mL). The flasks were incubated in orbital shaker (News Brunswick) at 35 °C and 150 rpm. The initial pH was 6.0. Submerged Fermentation was also carried out in reactor of 5 liters with working volume of 2 liters and temperature (35 °C, agitation (380 rev.min$^{-1}$), aeration (2vvm) and pH (6.0) controlled. The inoculum ($10^6$ spores/mL) of reactor medium was 10% (v/v).

**Solid-state fermentation (SSF)**
Erlenmeyer flasks (250 mL) containing 10 g of wheat bran plus a range of casein concentration (sterilized at 121°C by 15 min.) were inoculated with 10.0 mL of spore solution ($10^6$ spores/mL) and incubated at 35 °C under static condition for 72 h. 250 mL Erlenmeyer flasks containing 10 g of wheat bran were moistened with 10 mL of 0.2, 0.3 and 0.4 N HCl solutions and sterilized at 121°C for 15 minutes. The medium was inoculated with $10^6$ spore / g wheat bran.

**Enzyme extraction in submerged liquid fermentation and solid-state fermentation**
The samples were taken each 24 h of cultivation and separated from the mycelium through a Filtrak GmbH paper filter (∅ 90 mm). After centrifugation (5,000 g) the filtrate was used for enzyme assay. The enzyme recovery in solid-state fermentation was obtained by adding 100 mL of cold sterile water (Fernandez-Lahore, 1998). The mycelium and solid medium were separated by cotton filtration, and the filtrate was centrifuged and used for enzyme assay.

**Enzyme activity**
Milk-clotting activity (MCA) was determined according to the method of Arima and Iwasaki, (1970) and expressed in terms of Soxhlet Unit.

**Biomass measure**
For biomass determination, samples of the culture fluid (30 mL) were filtered through dried paper filters [Filtrak GmbH paper filter (∅ 90 mm)], and mycelial dry-mass was determined after drying at $80^0$C for 24 h.

## Sugar determination
Total reducing sugars were determinated as a glucose according methods of Miller (1959).

## RESULTS AND DISCUSSION

Table 1 showed optimum glucose concentration (over 18 g/L) on the enzyme synthesis, showing that an inhibition by substrate occurred.

**Table 1** - Microbial rennin production through SLF in flasks with/without baffles after 120 hours fermentation, temperature 35°C, 150 rpm.

| Flasks type | Glucose concentration (g/L) | Highest clotting activity (S.U.) |
|---|---|---|
| Baffled flasks | 0 | 0 |
| | 9 | 58 |
| | 18 | 648 |
| | 25 | 279 |
| | 35 | 300 |
| Flasks without baffles | 0 | 0 |
| | 9 | 81 |
| | 18 | 1066 |
| | 25 | 857.4 |
| | 35 | 357 |

Beyenal et al. (1999); Seker et al.(1999), Silveira and Contiero (2001) also observed this fact. The use of baffled flasks permited more aeration with an increased in the growth and a decrease in the enzyme production. Experiments with sucrose as main carbon source, carried out in reactor with pH control, showed poor enzyme production (<168.0 S.U.). It must be evident the effort of microorganism to hydrolyze this carbon source to obtain the glucose to induce the enzyme synthesis. Similar fact occurred when using molasses as main carbon source, where the maximum activity was 250 S.U. (Total Reducing Sugars concentration 18 g/L). Table 2 shows the influence of casein concentration and Proflo on the rennin production. High levels of free amino

acids (1%) appeared to repress enzyme activity, as observed by Lasure (1980). The high activity, 666.7 S.U., was reached when the initial concentration of both, casein and Proflo were 4 g/L, where the low nitrogen concentration and low pH have a positive contribution for the production and the enzymatic stability. The use of 8 g/L of Proflo in the medium resulted lower activity (270.3 S.U.), and high biomass production, showing a probable metabolic deviation for biomass synthesis in the metabolism. Seker et al. (1999) observed this using high carbon concentration. The usage of low Proflo concentration and casein showed to be useful for the enzyme production. Experiments were also carried out to study the effect of corn steep liquor in the medium as a nitrogen source for rennin production (Table 3).

**Table 2 -** Rennin production at 96 hour by SLF using *Mucor miehei* at several Proflo and casein concentrations.

| Nitrogen Source | Glucose concentration (g/L) | Highest Clotting activity (S.U.) | Biomass (g/L) | pH |
|---|---|---|---|---|
| Proflo 4 g/L | 18 | 585.4 | 9.6 | 7.5 |
| Proflo 8 g/L | 18 | 270.3 | 10.6 | 7.4 |
| Proflo 4 g/L + Casein 4 g/L | 18 | 666.7 | 7.5 | 5.2 |

Table 3 showed the highest enzymatic activity (923 S.U.) was obtained when 2.2 g corn steep liquor/L was used in the medium. There was a decrease in the enzyme production when the concentration increased. The increase in corn steep liquor concentration was followed by an increase in biomass concentration. A decrease in the enzymatic activity also observed at concentrations around and below 1.1 g corn steep liquor/L.

**Table 3 -** Maximum clotting activity, at 96 hour by SLF in several corn steep liquor concentrations.

| CSL concentration (g/L) | Highest Clotting activity (S.U.) | Biomass (g/L) |
|---|---|---|
| 1.1 | 685.7 | 6.3 |
| 2.2 | 923 | 6.4 |
| 4.4 | 705.9 | 5.3 |
| 8.7 | 500 | 7.1 |
| 17.5 | 171.4 | 8.0 |

**Fermentations using sucrose and molasses as carbon source**

Flasks experiments using molasses and sucrose for enzyme production showed a pH increase during the fermentation. High pH affects the enzyme stability. Experiments with molasses and sucrose in medium composition carried out in reactors (5 liters), pH (6.0), aeration (2 vvm) and agitation (380 rpm) did not reach high rennin production. Both, sucrose and molasses were not considered as good carbon sources. The results of the enzymatic activity were 138 S.U. for sucrose at 25 g/L and 233 S.U. for molasses at 35 g/L total reducing sugars.

**Enzyme production by solid-state fermentation**

Table 4 shows the behavior of the microorganism related to the medium composition. Highest enzymatic activity occurred at 48 h of cultivation. Figure 1 showed the milk clotting activity until 72 h, where the enzyme activity was better when the medium was supplemented with 2 grams of casein and with 0.3 N HCl the enzyme activity increased after 48 h of fermentation.

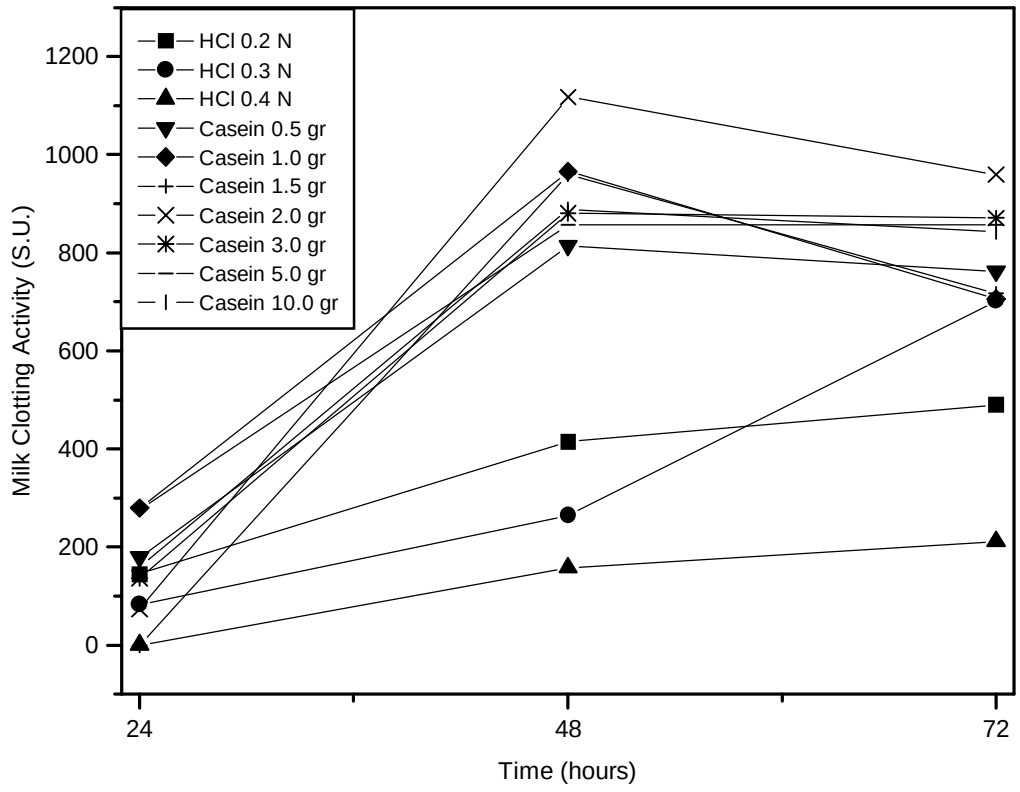


**Figure 1 -** Milk clotting activity for *Mucor miehei* on SSF fermentation during 72 h and 35°C.

**Table 4 -** Maximum rennin production by *Mucor miehei* during 48 hours of cultivation on wheat bran added with HCl and casein concentrations at 35 °C.

| * | Initial pH | Final pH | Clotting activity (S.U.) |
|---|---|---|---|
| HCl 0.4 N | 4.0 | 4.02 | 157.0 |
| HCl 0.3 N | 4.5 | 5.12 | 264.0 |
| HCl 0.2 N | 5.3 | 6.27 | 414.0 |
| 0.5 gram of Casein | 6.00 | 6.84 | 813.6 |
| 1.0 gram of Casein | 5.57 | 7.1 | 966.0 |
| 1.5 grams of Casein | 5.35 | 6.88 | 960.0 |
| 2.0 grams of Casein | 5.25 | 7.02 | 1,117.0 |
| 3.0 grams of Casein | 5.06 | 7.64 | 880.8 |
| 5.0 grams of Casein | 4.82 | 7.65 | 857.1 |
| 10 grams of Casein | 4.51 | 7.77 | 888.9 |

*Addition of HCl and casein

These results when compared to Preetha and Boopaathy (1994) and Thakur et al. (1990) showed a time decreased on enzyme production. When HCl at 0.4 N was used, the activity was 157 S.U. while at 0.2 N and 0.3 N it was 414 and 264 S.U. respectively, showing that a possible catabolic repression occurred because of the free carbohydrate from wheat bran hydrolysis (Thakur et al., 1990). In these experiments the highest activity occurred after 72 h of fermentation. Table 4 showed that casein was an important factor in the enzyme production. This could be observed from rise on casein concentration, which resulted increase in coagulant activity. Between 1 and 2 gr of casein, the high enzymatic production was reached (1,117 S.U.) during 48 h of fermentation; this value is close to the submerged fermentation; however, over 2 g of casein a decreased in enzyme production was observed, probably because of catabolic repression. Preetha and Boopathy (1994) studied the influence of the powder skim milk inclusion in solid medium and observed that concentrations over 2% of this resulted an increase of 3.5 times in the coagulant activity related to the control. Thakur et al. (1990) observed that the higher coagulant activity was obtained using 5.0 % of powder skim milk. Casein is the constituent in most expressive quantities in powder skim milk, thus proving the role in the induction of the enzyme synthesis.

## CONCLUSION

In this study can observed that the solid-state fermentation was a good method for rennin enzyme production. The results were closed to submerged fermentation when supplemented with casein, which showed that casein had an important role in rennin production. The use of corn steep liquor instead of peptone showed to be a better nitrogen source for enzyme production in submerged fermentation. Sucrose and molasses did not show to be good carbon sources for enzyme production.

## ACKNOWLEDGEMENTS

This work was financially supported by FAPESP and CNPq. We thank São Paulo State University (UNESP) for their technical support.

## RESUMO

A produção de renina microbiana por *Mucor miehei* foi estudada através de Fermentação Submersa e em Estado Sólido. O objetivo deste trabalho foi verificar o efeito de diferentes fontes de carbono e nitrogênio, utilizando Fermentação Submersa e da adição de caseína utilizando Fermentação em Estado Sólido na produção da renina microbiana. Os picos de biomassa foram de 6,7; 8,1 e 8 g/L e atividade enzimática de 1.066; 857 e 480 Unidades Soxhlet (U.S.) para as concentrações de glicose: 18, 25 e 35 g/L respectivamente. Em frascos aletados, os picos de biomassa foram de 6,7; 8,3 e 10 g/L e atividade enzimática de 648, 279 e 300 U.S., para a mesma concentração de glicose. Quando se utilizou Proflo (Farinha de semente de algodão, Traders ®) e Água de Maceração de Milho os picos de atividade enzimática foram de 667 e 923 U.S., respectivamente. Nos experimentos utilizando Fermentação em Estado Sólido com a adição de HCl 0,2 N a máxima atividade enzimática foi de 414 U.S. e, quando utilizou-se caseína (1 e 2 gramas), verificou-se valores mais altos de atividade: 966 e 1117 U.S respectivamente. Os resultados sugerem que o aumento na concentração de glicose afeta a síntese da enzima e que a caseína é um importante fator na indução neste processo. Fermentação em Estado Sólido pode ser considerada uma boa opção para a produção de renina.

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Received: May 26, 2004;
Revised: August 11, 2004;
Accepted: August 04, 2005.