

Optimization of *Mucor miehei* Rennin Production and Recovery

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In this paper, fungal rennin production has been carried out by solid state fermentation of *Mucor miehei* NRRL 3420. The optimum dilution ratio, temperature and fermentation time of the enzymatic solution were determined to be 1:30, 40°C and 4 days after the beginning of the fermentation process, respectively. Also, the enzyme recovery by precipitation was studied using (NH₄)₂SO₄, acetone and ethanol, respectively. The effects of pH, salt and solvent concentrations on rennin recovery were studied and the optimum pH value as well as the optimum salt concentration were determined. The maximum specific activity of the enzyme was obtained by precipitation at pH of 6.5 and 50% saturated solution of (NH₄)₂SO₄. When organic solvents were used for rennin recovery, the maximum specific activity of the enzyme was observed for acetone solution at pH of 6.5 and 65% saturation which is 17% less than the optimum specific activity obtained by 50% saturated solution of (NH₄)₂SO₄. In comparison to ethanol, the use of acetone decreases the denaturation effect and the amount of the solvent needed for rennin recovery. The lower cost of the salt, higher stability of the recovered enzyme, more simple operation as well as more specific activity of the enzyme precipitated by (NH₄)₂SO₄ in comparison to ethanol or acetone suggest salt precipitation as a reliable method for rennin purification.

INTRODUCTION

The curdling or coagulation of milk is accomplished with rennets at pH values other than the isoelectric of casein. The milk-curdling rennet enzymes may be derived from vegetable and the fourth stomach of ruminants. The active milk-coagulating enzyme present in calf rennet extract is an acidic protease which is denoted as calf chymotrypsin (rennin; aspartyl proteinase, EC 3.4.23.4). Other crude proteases which can coagulate milk and form relatively stable curds are also called rennets or, if pure, rennins [1-4]. Due to

a sharp decrease in the general availability of vells, restrictions on the export of rennets and an increase in worldwide cheese consumption, calf rennet substitutes are needed. Such substitutes must satisfy the following requirements: effective coagulation of milk without undue hydrolysis of curd, proper flavor, body and texture development in curd, considerable shelf life, proper color and odor and absence of toxins and pathogens in cheeses. Three major successful rennet calf substitutes are microbial protease *Endothia parasitica*, *Mucor pusillus* and *Mucor miehei* [5-10]. The microbial rennet has a higher nonspecific proteolysis than animal rennet which causes a bitter flavor in aged cheeses. The protease of *Mucor miehei* mold (EC 3.4.23.10) is the preferred substitute for true calf rennet because of its specificity in splitting similar peptide bonds in kappa-casein, high ratio of milk-coagulating activity, identical calcium requirements and good cheese quality [8-10]. *Mucor miehei* is well suited for protease production by submerged cultivation. The protease of *Mucor miehei* is an acid-aspartate protease having a molecular weight of about 38,000

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[11,12]. The molecule consists of a single peptide chain and contains about 6% carbohydrate [13,14]. The enzyme is stable at 38°C and pH values between 3 to 6 with an optimum pH of 4.5 for both stability and activity against denatured hemoglobin [2,9]. The recent reviews include the study of temperature effects on the microstructure of renneted milk gel [4], the effect of agitation speed on the synthesis of *Mucor miehei* acid protease [2] and the production of fungal rennet by *Mucor miehei* [1]. In this paper, the optimum dilution ratio of the enzymatic solution of rennin from *Mucor miehei*, the optimum temperature for activity measurement and the optimum fermentation time were determined. Also, the enzyme recovery by precipitation was studied using $(\text{NH}_4)_2\text{SO}_4$, acetone and ethanol, respectively. The effects of pH, the salt $((\text{NH}_4)_2\text{SO}_4)$ and solvent (acetone and ethanol) concentrations on rennin recovery were studied and the optimum pH value as well as the optimum salt concentration were determined.

MATERIALS AND METHODS

Microorganism and Materials

The strain of *Mucor miehei* used in the present study was provided by NRRL (Northern Regional Research Laboratories at Peoria, Illinois, USA) and is called *Mucor miehei* NRRL 3420. The stock culture was maintained on potato dextrose agar slants. All reagents were analytical grade and purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Fungal Rennin Production

Mycelia from the slants of the stock culture of *Mucor miehei* NRRL 3420 were inoculated on potato dextrose agar containing potato extract 4 g/l, dextrose 20 g/l and agar 15 g/l [1]. The mold was added to the preculture, which was incubated at 25-30°C for 4 days [1]. The preculture consisted of a standardized wheat bran medium with 40% moisture content (17 ml water was used to moisten 25 g of raw wheat bran) in a 500 ml flask sterilized in an autoclave at 121°C for 20 min [1]. The main culture medium composition was the same as the preculture. The fermentation was carried out at 37°C for 3, 4, 5, 6 and 7 days, respectively. All the experiments were carried out in duplicate and the average of the two values was stated. The crude enzyme solution was obtained by adding 125 ml distilled water to the solid fermentation medium and centrifuging the diluted solution at 6000 rpm and 4°C for 20 min. The obtained crude enzymatic solution was concentrated using a forced circulation evaporator [1].

Determination of the Protein Content and Enzyme Activity

Protein content was determined by the method of Lowry using Folin phenol reagent, as described in [15]. Milk clotting activity is usually determined according to the method of Arima and is expressed in terms of Soxhlet Units (SU) [16,17]. One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00147 g calcium chloride in 40 min at 35°C [16]. The procedure used for milk clotting assay was a modification of Arima technique and one unit of the enzyme activity, U, corresponds to the amount of enzyme which could coagulate 1 ml of the substrate per min. The substrate was 12% skim milk (Difco) dissolved in 0.01 M CaCl_2 solution. This solution was kept at 4°C for 20 h and then the contents were placed at 35°C. Curd formation was observed by manually rotating the test tube from time to time, so as to form a thin film on its inner surface. The end-point was considered as the instant when discrete particles were discernible. All results are expressed in U unless specified.

Optimization of Dilution Ratio and Temperature of the Enzymatic Solution

The effect of enzymatic solution concentration on activity measurement was studied using enzymatic solutions with dilution ratios (v/v) of 1:5, 1:10, 1:20, 1:30, 1:40, 1:50 and 1:60, respectively. The corresponding clotting time for each dilution ratio was measured and used for optimization study of the dilution ratio of the enzymatic solution. The effect of temperature on clotting time was studied using 1:30 diluted enzymatic solution at the temperature range of 30°C to 70°C (5°C intervals). The corresponding clotting time for each temperature value was measured and used for optimization study of temperature of the enzymatic solution.

Determination of Optimum Fermentation Time

In order to determine the optimum fermentation period, the fermentations were carried out for 3, 4, 5, 6 and 7 days, respectively, at 37°C. The total protein content and enzyme activity were measured as described earlier.

Determination of Optimum pH and $(\text{NH}_4)_2\text{SO}_4$ Concentration for Fungal Rennin Recovery

In order to determine the optimum pH and salt concentration used for recovery of the enzyme by the precipitation method, the protein of the mycelial

extract was precipitated using $(\text{NH}_4)_2\text{SO}_4$ at 20, 40, 50, 60, 70 and 80% saturation, respectively. Also, the pH of each solution was adjusted at 5.0, 5.5 and 6.0, respectively, using 0.1 M phosphate buffer. The precipitate was centrifuged at 10000 rpm for 10 min and dialyzed against 0.1 M phosphate buffer for 48 h at 4°C. The protein thus concentrated. The protein content and specific activity of the enzyme were measured.

Fungal Rennin Recovery by Organic Solvents

In addition to precipitation by $(\text{NH}_4)_2\text{SO}_4$, enzyme recovery by organic solvents was carried out using acetone and ethanol, respectively. The procedure is the same as that of the precipitation by salt except that there is no need to dialyze the enzymatic solution when acetone or ethanol is used for recovery. All the experiments were carried out at -10°C when organic solvents were used. The precipitated enzyme was separated from the additional solvent using a vacuum separator. The protein content and activity of the concentrated enzymatic solutions were measured.

RESULTS AND DISCUSSION

Characterization of the Enzyme Activity Measurement (Optimization of Dilution Ratio and Temperature of the Enzymatic Solution)

The results obtained for clotting time values at each dilution ratio of the enzymatic solution are shown in Figure 1. The optimum dilution ratio of the solution was found to be 1:30 (Figure 1) where the measured values for activity, protein content, specific activity and purification factor were 29.65 U.ml⁻¹, 0.3 mg.ml⁻¹, 98.83 U.mg⁻¹ and 3.71, respectively. The range of

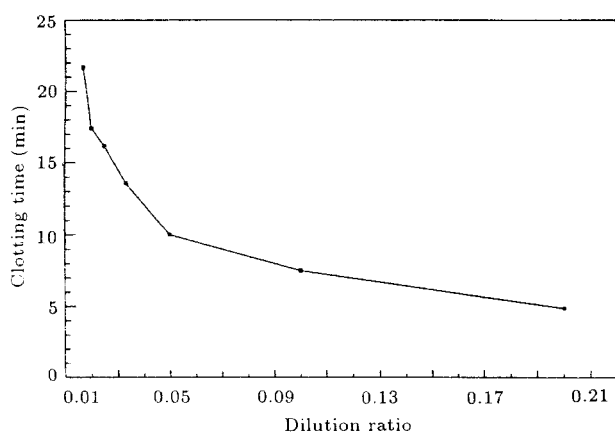


Figure 1. The clotting time versus dilution ratio of the enzymatic solution of *Mucor miehei* rennin (the values of rennin activity, total protein content, specific activity and purification factor at 1:30 dilution ratio are 29.65 U.ml⁻¹, 0.3 mg.ml⁻¹, 98.83 U.mg⁻¹ and 3.71, respectively).

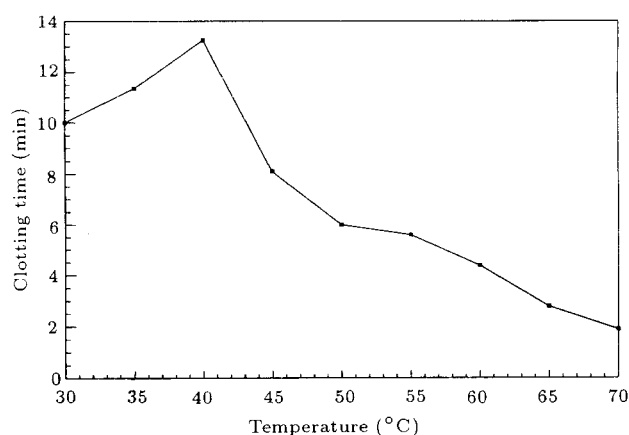


Figure 2. The clotting time versus temperature of the enzymatic solution of *Mucor miehei* rennin (dilution ratio 1:30).

dilution ratio over which a linear response in terms of dilution occurs is from 1:50 to 1:20 (Figure 1) and the best clotting time is 13 min after the addition of the enzyme to the milk solution as it is mentioned by Sardinas [7]. The corresponding dilution for 13 min clotting time (i.e., the optimum dilution ratio) was obtained at 1:30 dilution ratio of the enzymatic solution.

The results show that the optimum temperature for clotting is 40°C (Figure 2) and for higher temperatures both clotting time and stability of the enzyme decrease; therefore, the enzyme is deactivated at higher temperatures [1].

Optimization of Fermentation Time

The total protein content and enzyme activity for different fermentation time intervals, between 3 to 7 days, were measured and the results are shown in Figure 3. The maximum activity and the specific activity of the enzyme are observed 4 days after the beginning of the fermentation process (Figure 3). That is when the fungi growth passed the lag phase and the enzyme production started [1,2].

Optimization of pH and Salt Concentration for Fungal Rennin Recovery

The results obtained for protein content and specific activity of the enzyme precipitated by $(\text{NH}_4)_2\text{SO}_4$ solutions are shown in Figure 4. It is known that the enzyme of *Mucor miehei* is unstable at pH values greater than 6.5 and it was also demonstrated that the total loss of enzyme activity occurs at pH of 8.0 after 72 h at 38°C [2,9]. While at pH values less than 5.0, no detectable activity was observed as was mentioned by Escobar et al. [2].

The results for precipitation of the enzyme by

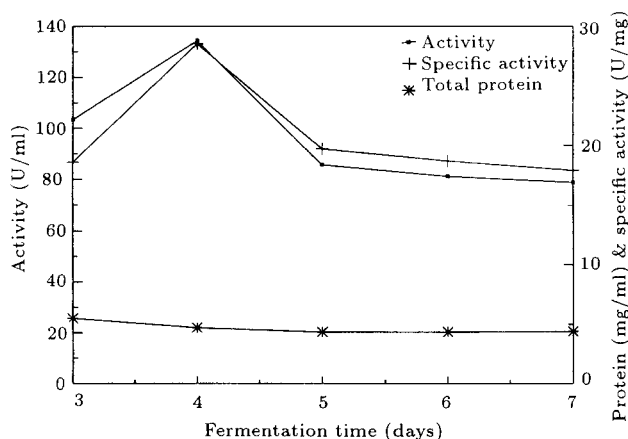


Figure 3. The activity, specific activity and protein content of *Mucor miehei* rennin solution versus fermentation time (dilution ratio 1:30 and 40°C).

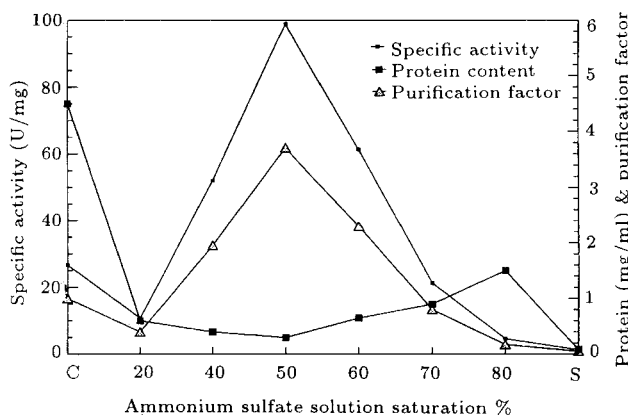


Figure 4. The specific activity, protein content and purification factor of *Mucor miehei* rennin solution at different saturation concentrations of $(\text{NH}_4)_2\text{SO}_4$ solution and pH=6.5 ("C" for the crude rennin solution and "S" for the supernatant of 80% saturated solution of acetone).

$(\text{NH}_4)_2\text{SO}_4$ show that for all the reported pH values, the purification factor (the ratio of the specific activity of the sample to that of the crude) of the supernatant of 80 % saturated solution of $(\text{NH}_4)_2\text{SO}_4$ is negligible i.e., most of the enzyme precipitated and there is no considerable amount of rennin in the supernatant phase (Figure 4). In addition, the maximum specific activity ($98.83 \text{ U}\cdot\text{mg}^{-1}$) of the enzyme was obtained at pH of 6.5 and 50% saturated solution of $(\text{NH}_4)_2\text{SO}_4$, where the minimum amount of the total protein was observed (Figure 4). At pH values of 5.5 and 5.0, the concentration of $(\text{NH}_4)_2\text{SO}_4$ solution must be increased to 70% and 80% saturation to yield the 89.0 and $71.38 \text{ U}\cdot\text{mg}^{-1}$ values for the specific activity of the enzyme, respectively. That is, at optimum pH and saturation concentration of $(\text{NH}_4)_2\text{SO}_4$ (pH 6.5 and 50% saturation), higher specific activity is obtained using lower amounts of the salt. However, for other pH

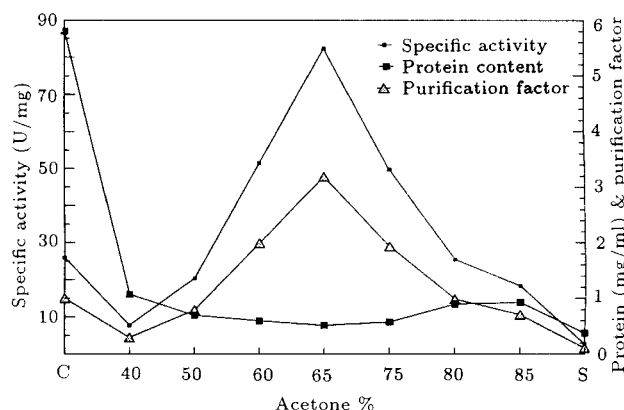


Figure 5. The specific activity, protein content and purification factor of *Mucor miehei* rennin solution at different concentrations of acetone solution and pH=6.5 ("C" for the crude rennin solution and "S" for the supernatant of 85% saturated solution of acetone).

values, lower specific activity was obtained at higher saturation concentrations.

Rennin Recovery by Organic Solvents

The results for protein content and activity of the concentrated enzymatic solutions are shown in Figures 5 and 6 for acetone and ethanol, respectively. When organic solvents were used for rennin recovery, the maximum specific activity of the enzyme ($82.42 \text{ U}\cdot\text{mg}^{-1}$) was observed for acetone solution at pH of 6.5 and 65% saturated solution (Figure 5), which is 17% less than the optimum specific activity obtained by 50% saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The lower value of the specific activity of the enzyme recovered by acetone in comparison to that of the $(\text{NH}_4)_2\text{SO}_4$ solution is mostly due to the denaturation effect of acetone. Furthermore, in comparison to ethanol, the use of acetone will cause a decrease in the amount of the solvent needed for rennin recovery (Figures 5 and 6). Due to lower value of dielectric constant of acetone, a small amount of acetone causes more precipitation of proteins compared to ethanol. Therefore, the denaturation effect is decreased when acetone is used instead of ethanol for rennin recovery. The lower cost of the salt, higher stability of the recovered enzyme, more simple operation as well as more specific activity of the enzyme precipitated by $(\text{NH}_4)_2\text{SO}_4$ in comparison to ethanol or acetone lead to applying the salt precipitation as a reliable method for rennin purification.

CONCLUSIONS

In this paper, the optimum dilution ratio of the enzymatic solution of rennin from *Mucor miehei*, the optimum temperature for activity measurement and the optimum fermentation time were determined. Also,

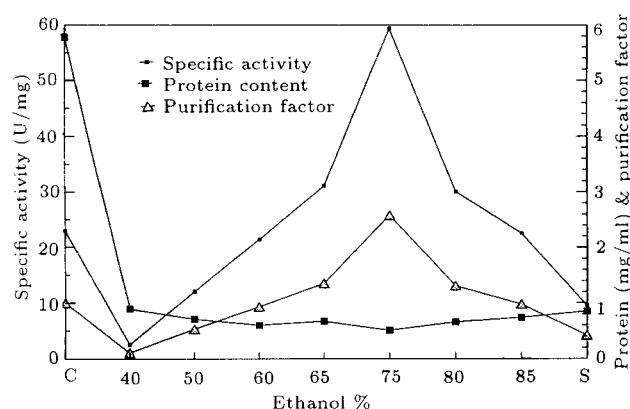


Figure 6. The specific activity, protein content and purification factor of *Mucor miehei* rennin solution at different concentrations of ethanol solution and pH of 6.5 ("C" for the crude rennin solution and "S" for the supernatant of 85% saturated solution of ethanol).

enzyme recovery by precipitation was studied using $(\text{NH}_4)_2\text{SO}_4$, acetone and ethanol. The effects of pH as well as the salt and solvent concentrations on rennin recovery were studied and the optimum pH value and the optimum salt concentration were determined. The obtained results show that the optimum dilution ratio, temperature and fermentation time of the enzymatic solution is 1:30, 40°C and 4 days after the beginning of the fermentation process, respectively. The results for precipitation of the enzyme by $(\text{NH}_4)_2\text{SO}_4$ reveal that the maximum specific activity ($98.83 \text{ U}\cdot\text{mg}^{-1}$) of the enzyme was obtained at pH of 6.5 and 50% saturated solution of $(\text{NH}_4)_2\text{SO}_4$. While using organic solvents, the maximum specific activity of the enzyme ($82.42 \text{ U}\cdot\text{mg}^{-1}$) was observed for acetone solution at pH of 6.5 and 65% saturated solution which is 17% less than the optimum specific activity obtained by 50% saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The lower value of the specific activity of the enzyme recovered by acetone, in comparison to that of the $(\text{NH}_4)_2\text{SO}_4$ solution, is mostly due to the denaturation effect of acetone. In comparison to ethanol, the use of acetone will cause a decrease in the amount of the solvent needed for rennin recovery. Due to lower value of dielectric constant of acetone, the denaturation effect is decreased when acetone is used instead of ethanol for rennin recovery. The lower cost of the salt, higher stability of the recovered enzyme, more simple operation as well as more specific activity of the enzyme precipitated by $(\text{NH}_4)_2\text{SO}_4$ in comparison to ethanol or acetone suggest salt precipitation as a reliable method for rennin purification.

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