Kirkuk University Journal for Agricultural Sciences
ISSN:2958-6585
https://kujas.uokirkuk.edu.iq

RESEARCH ARTICLE
https://doi.org.10.58928/ku24.15429

Study The Optimum Conditions, Purification, and Characterization of The Acid Protease Rennet Substitute Produced from *Rhizomucor Miehei.*

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Received: 09/10/2024 **Revised: 20/11/2024** **Accepted: 28/11/2024** **Published: 01/12/2024**

ABSTRACT

An isolate of *Rhizomucor Miehei* strain MMO was identified, and registered with accession number PP955195.1 in NCBI, which was distinguished by its production of acid protease rennet substitute, and then compared with four commercial preparations in soft cheese-making, all of which were done in a previous study. Continuation of that study, the highest enzyme proteolytic activity and productivity were achieved on rice bran using several agricultural and industrial wastes. The optimum conditions for the production of acid protease by solid-state fermentation included the hydration of rice bran with distilled water (1:2) V:W at an initial pH of 6.0, the inoculation of 10^6 spores per gram of dry substrate, and an incubation temperature of 40°C for 3 days. The enzyme was purified by ethanol (50–75%), DEAE-Cellulose ion exchange, and gel filtration on a Sephadex G-100 column, giving the final 13.59-fold purification and 32% yield. The purity of the enzyme to homogeneity was confirmed by polyacrylamide gel electrophoresis under non-denaturing conditions. When studying the characterization of the enzyme, it was found that the molecular weight of the purified enzyme, as determined by gel filtration, was 37.15 kDa, and the isoelectric point of the enzyme was 5.2. The optimum pH for proteolytic activities on casein was 5.5, while the milk-clotting activity was pH-dependent and increased with decreasing pH of reconstituted skim milk. The enzyme was most stable in a pH range of 4.0–6.0. The maximum proteolytic and milk-clotting activities were observed at 60°C. In the thermal stability study, when treated with heat at 35°C for 60 minutes, the enzyme maintained full proteolytic and milk-clotting activities.

***Keywords*:** proteolytic; milk-clotting; NCBI; yield; fold.

INTRODUCTION

The need for alternatives to substitute rennet or calf rennet emerged during World War II. This need escalated after the war ended, reaching its peak at the beginning of the 1960s due to the neglect that affected the herds of cows and the resulting desire to raise calves, which caused a real scarcity in the production of traditional rennet [1]. In return for this scarcity, milk production increased, increasing the demand for rennet to make cheese. Initially, the researchers proposed immediate solutions to confront this scarcity, including using the rennet of large animals in addition to the rennet of calves, improving the methods of extracting the rennet of calves to ensure high productivity while reducing costs, or adding calcium chloride to the milk to reduce the amount of traditional rennet used in the cheese process. Some researchers have found the possibility of using extracts from some plant sources as natural alternatives to traditional rennet [2;3]. The production of proteases from various sources constitutes more than 50% of the total enzymes produced on a commercial scale. Most of their production currently depends on microorganisms, estimated at 500 tons a year [4]. Several researchers believe that whoever wants to break through the barrier of obtaining a new isolate that can secrete the acid protease with specifications close to traditional rennet or microbial coagulants offered in local markets at present must prove that his product is distinguished from other coagulants in many aspects, the most important of which is the reduction in the cost of production through the use of agricultural or industrial waste and using it in the manufacture of multiple types of cheese [5]. Solid-state fermentation involves the use of insoluble materials (such as agricultural waste) to grow microorganisms and produce metabolic compounds. This method is one of the oldest methods used by humans to produce many different foods and materials [6;7]. Solid-state fermentation is sometimes known as surface cultures, solid or surface media fermentation, or the Koji process [8;9]. Among the filamentous fungi that have gained special importance in solid-state fermentation applications are the species and genera of the phycomycota, such as Mucor and Rhizopus [10]. The reason for the suitability of fungi and molds in particular for solid-state fermentation media is due, on the other hand, to their ability to form fungal threads that penetrate the particles of the medium, benefiting from its various components, especially since the nature of solid media makes the microorganisms restricted in movement, just as the medium's nutrients are also restricted [11;12]. Before starting to study the characteristics of the enzyme, it must be purified to the point of homogeneity, getting rid of the materials present with the enzyme to fear of falling into the wrong conclusions. Purification is only achieved through a set of gradual and studied steps, as many studies have used the purification of acid proteases produced from mold, and the common denominator in the methods used in purification

is ion exchange chromatography and gel filtration [13]. Therefore, this study aims to determine the optimum conditions for acid protease rennet substitute production by growing the isolated mold on several types of agricultural and industrial waste (rice, soybeans, wheat, barley, lentils, chickpeas, and wheat bran) solid-state fermentation characterized by its low cost and study other important factors affecting the growth and production of metabolic compounds, including the ratio of hydration, pH, temperature, mold inoculum size, and fermentation time, and then purification to perfect purity and characterization by estimating its molecular weight, isoelectric point (PI), and determining the optimal pH and temperature for proteolytic and milk-clotting activity and stability.

Materials and methods

This study used a *Rhizomucor Miehei* strain 33M isolate obtained in a previous study identified by molecular method and registered in the National Center for Biotechnology Information at accession number PP955195.1.

Note: All medium in this study was autoclaved at 121°C and 15 pounds/inch^2 for 15min.

2.1 Preparation and Activation of *Rhizomucor Miehei*

The mold inoculum was prepared by growing it on Modified Czapek Dox Agar (CZA). This medium was prepared by melting the ingredients (30 g glucose, 15 g casein, 0.5 g K2HPO4, 0.5 g NaCl, 0.5 g MgSO4, 0.01 g Fe2SO4, and 20 g agar) in 1000 ml of distilled water. Adjust the pH to 6.0, and the mixture was autoclaved. After that, at 50°C, penicillin was added at a final concentration of 20,000 U/L, and streptomycin 0.5 g/L was also added [14]. in a 250-ml flask at 45°C for 5-7 days. After that, 50 ml of sterile 0.01% Tween 80 was added and mixed well until the fungal hyphae were removed from their spores from the surface of the medium. The mixture was filtered through a cotton swab, and the filtrate was received in a glass funnel previously sterilized by autoclaving. The hemocytometer was used to determine the number of spores, and the suspension was stored at 7°C until use.

2.2 Determine The Optimum Condition for Acid Protease in Production

The studied optimum conditions are the important factors affecting the growth and production of metabolic compounds, including factors such as medium, ratio of hydration, pH, temperature, inoculum size, and fermentation time. At the end of the incubation period for each condition, the enzyme was extracted. The clear supernatant was prepared by adding 50 ml of phosphate buffer 2.0M, pH 6.0; the fermentation media was filtered by Whatman No. 1 and then centrifuged at 2500 g at 4°C for 20 min to obtain the crude enzyme and then estimated the proteolytic and milk-coagulant activity and productivity.

2.2.1 The Effect of Medium Types

The several types of agricultural and industrial wastes used as media for enzyme production by solid-state fermentation included wheat bran, rice bran, sunflower meal, soybean meal, wheat straw, and yellow corn meal, then ground and passed through a 1.5 mm filter to homogenize their parts, then dried at 100°C for 24 hours and stored at 30°C until use. The medium production contained 10 g of waste with 30 ml of distilled water, was divided into 250 ml Erlenmeyer flasks, the pH was calibrated at 6.4 and autoclaved, then 1 ml/L containing 10^6 spores/gm of the dry weight of the medium (before hydration), and was incubated at 40°C for 3 days [14].

2.2.2 The Effect of Ratio of Hydration

Distilled water was added to the rice bran, the optimum medium production in proportions ranging from 1:0.5 to 1:3.5 (ml: gm of the medium) with an increase of 1:3.5 each time, and autoclaved. The inoculum was added at 10^6 spores/gm of medium and incubated at 40°C for 3 days.

2.2.3 The Effect of The pH of the Medium

Rice bran media with pH values ranging from 3.0-7.0 were prepared by adding distilled water at a ratio of 1:2 after adjusting the pH to the desired number using a 1.0 M sulfuric acid solution and then autoclaved. The inoculum was added at 10^6 spores per gram of medium and incubated at 40°C for 3 days.

2.2.4 The Effect of The Inoculum Size

Rice bran media were prepared, moistened in a ratio of 1:2 (V:W) using distilled water with a pH of 6.0, inoculated with spore sizes ranging from 10^5 to 10^7 spores per gram of medium, and then incubated at 40°C for 3 days.

2.2.5 The Effect of The Temperature of Incubation

Rice bran media were prepared, moistened at a ratio of 1:2, using distilled water with a pH of 6.0, and inoculated with 10^6 spores/gm of medium, then incubated at 30-50°C for 3 days.

2.2.6 The Effect of The Fermentation Time

Inoculate 1:2 (V:W) moistened rice bran medium with 10^6 spores/gm of medium and incubated at 40°C for 1-8 days.

2.3 The Milk-Coagulant Activity

The milk-clotting activity was carried out using [15]. The 5 ml skim milk medium contains 10 g of skim milk in 100 ml of CaCl2 at pH 6.4. The solution was incubated in a water bath at 35°C for 10 minutes and mixed with 0.5 ml of crude enzyme. The coagulation time was estimated in seconds from the moment the enzyme was added until the clot appeared as a thin layer. The milk-clotting activity was determined using the formula: (U/ml)=(2400)/(The time of coagulant in seconds)×Enzyme dilution agent. One unit of enzyme milk-coagulant activity is the amount of enzyme that coagulates 10 ml of 10% skim milk solution recovered in 0.01 M CaCl2 within 40 min at 35°C in the experimental conditions. The milk-clotting specific activity (U/mg) = milk-clotting activity (U/ml)/protein co. (mg/ml).

2.4 The Proteolytic Activity Assay

They used the method followed by [16]. The standard curve of tyrosine was prepared between 0-100 µml by diluting the stock tyrosine (L-tyrosine 100 µml/0.2 M of HCL) at an absorbance of 275 nm. The reaction of the acid protease activity was estimated by mixing 0.2 ml of a crude enzyme with 1.8 ml of 1% casein substrate (1 g of casein in 100

phosphate buffer 0.2 M at pH 6.5). In the 10 min, the mix reaction was incubated at 35°C and then stopped by adding 3 ml of 5% TCA (trichloroacetic acid) and centrifugation at 2500 g for 20 min, with the separation of the sediment absorbance at 275 nm. The blank was prepared using the same steps, except adding the TCA to the reaction solution before adding the crude enzyme. One unit of proteolytic activity was defined as producing 1 µml of tyrosine per minute in the experimental conditions.

2.5 Protein Assays

Protein concentrations were measured using the Bradford method [17]. The specific activity (U/mg)=enzyme activity (U/ml)/protein co. (mg/ml).

2.6 Productivity Assays

The productivity was measured using the equation: Productivity (coagulation Unit/gm of the medium)=Coagulant activity (U/ml)×Volume of a crude enzyme (ml)/Amount of medium used in production (gm).

2.7 The Purification Steps of Acid Protease

After determining the optimal conditions for producing the acid protease, the crude enzyme was subjected to purification to study its characterization. All steps of enzyme purification were performed at a temperature of 7°C. After each purification step, the milk-clotting activity, protein concentration, and volume of solution were estimated. The purification steps included three steps: concentration by ethanol, then ion exchange with DEAE-cellulose, and gel filtration with Sephadex G-100.

2.7.1 Concentration by Ethanol

The cooled ethyl alcohol at -15°C was used gradually to concentrate the crude enzyme with stirring to obtain a graduated percentage ranging from (50-75)%, and then centrifuged at 6000 g for 10 min at 7°C. The supernatant was separated, and the precipitate formed at each step of adding the converter was dissolved in a small amount of sodium acetate buffer (0.01 M) at pH 6.0 and then dialyzed by dialysis bags (12–14 kDa).

2.7.2 Ion Exchange DEAE-Cellulose

The DEAE-cellulose was prepared according to the method described by [18]. by suspending 20 grams (Whatman Co., England) in 500 ml of distilled water and allowing it to settle. The liquid upper was then poured off, and the exchanger was washed several times with distilled water until the overlying liquid became clear. It was filtered through a Buchner funnel under vacuum and suspended in a 0.25 M sodium hydroxide 0.25 M sodium chloride solution. The exchanger was washed several times with distilled water after filtration. Then washed with a 0.25M hydrochloric acid and distilled water solution, then equilibrated with a 0.1M sodium acetate buffer solution of pH 6.0 containing 0.3M sodium chloride and 0.02% sodium azide and filled to give a column of dimensions 1.5×30cm with a flow rate of 39ml/h; they were collected in 5 ml tubes. Then it was eluted by a NaCl gradient from 0.05 to 0.5 M solution at pH 6.0. The absorbance of the recovered fractions was read at 280 nm. The fractions that showed enzyme activity were subjected to the next step.

2.7.3 Sephadex G-100

They Prepared according to the instructions of the company (Pharmacia Co., Sweden) by suspending 40 grams in a liter of distilled water and heating the suspension in a water bath at a temperature of 85–90°C for 3 hours with continuous stirring, then left at a temperature of 7°C until the next day. After removing the air, it was filled in a column to give dimensions of 2.6×78 cm with a flow rate of 30 ml/h; they were collected in 5 ml tubes. Then it was equilibrated with a 0.1 M sodium acetate buffer solution of pH 6.0 containing 0.3 M sodium chloride and 0.02% sodium azide. The absorbance of the recovered fractions was read at 280 nm. The active fractions were collected and dialyzed for concentration by sodium acetate solution buffer (0.01 M) at pH 6.0.

2.8 Enzyme Purity Determination

The purity of the enzyme to homogeneity was confirmed by polyacrylamide gel after the purification steps performed under non-denatured conditions, according to [19]. The Polyacrylamide gel was stained with 0.025% (coomassie blue R-250) and run at 30 mA/gel for 6–8 h at 4 °C. Then the gel was immersed in the fixing solution for 30 minutes, then in the staining solution for 18 hours, and finally in the destaining solution until the dye is removed and read band by a UV device.

2.9 The Characterization of Acid Protease - Rennet Substitute

2.9.1 Molecular weight

Gel filtration Sephadex G-100 estimated the molecular weight (MW) with the same conditions mentioned for the purification enzyme. The blue dextran concentrically (3 mg/ml) was used to determine the void value (Vo) on (600 OD) and then the elution value (Ve) on (280 OD) using standard proteins, which included lysozyme (14 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa), with a concentration of 5 mg/ml. The molecular weight value was calculated using the straight-line equation of the relationship between the logarithm of the MW of the standard proteins and the (Ve/Vo).

2.9.2 Isoelectric point (PI)

According to [20], the isoelectric point was determined by mixing 0.3 ml of ampholyte and 3 ml of acrylamide-bis-acrylamide solution (7.5% concentration) with 8 ml of distilled water. The air was removed from the solution. Then 0.7 ml of ammonium persulfate was placed in the tube system of isoelectric focusing and allowed to solidify, and 2 ml of the pure enzyme was used with three repetitions: the first was used to determine the pH, the second replicate was used to estimate the coagulation activity, and the third replicate was used to locate the enzyme. The device operated at 200 volts and a current of 30 mA for 4 hours. Then immerse in the staining solution (Coomassie blue R-250) and the

destaining solution.

2.9.3 Determination of The Optimum pH for Proteolytic Activity

To determine the optimal pH for the enzyme's activity, 1 ml of casein 1% dissolved in acetate-phosphate buffer with pH values ranging from 4.0–8.0 at 0.05 M was added to 1 ml of the purified enzyme, and then the proteolytic activity was determined.

2.9.4 The Effect of The Optimum pH for Enzyme Milk-Clotting Activity

To determine the effect of the optimal pH on the milk-clotting activity of the enzyme, the pH of the recovered skimmed milk solution (10gm of skimmed milk in 100 ml of 0.1M calcium chloride solution) was adjusted with pH values ranging from 5.8-7.0 pH and with a difference of 0.2 pH from one solution to another, either using hydrochloric acid or sodium hydroxide solution at 1M. Then the milk-clotting activity was estimated.

2.9.5 Determine The Optimum pH for Enzyme Stability

To determine the optimum pH for enzyme stability, a known volume of purified enzyme solution was mixed with an equal volume of solution buffer in pH values ranging from 2.0-8.0 and incubated at 35°C for 30min and then transferred to an ice bath and used to estimate relative of the proteolytic and milk-clotting activities.

2.9.6 Determine The Optimum Temperature for Proteolytic Activity

The enzyme had proteolytic and milk-clotting activities were estimated over a temperature range of (30-75)°C.

2.9.7 Determine The Thermal Stability of The Enzyme

The pure enzyme was dissolved in acetate-phosphate buffer solution 0.05M with a pH of 5.5 and treated at a temperature ranging from (30-70)°C for 15min, then transferred to an ice bath, and the relative proteolytic and milk-clotting activities were estimated after the temperature treatments.

Results

3.1 Determine The Optimum Condition for Acid Protease Production

3.1.1 Determine The Optimal Medium for Production

The results of determining the optimum medium used several locally available agricultural and industrial wastes at the lowest cost to produce acid protease. It is noted from (Figure 1) that rice bran gave the highest milk-clotting specific efficiency, reaching 19.3 U/mg, with a productivity of 253 (coagulation U/gm of the rice bran medium).

The reason for variation in enzyme production in different media is due to several reasons, the most important of which is the variation in the components of the organic and inorganic materials and the degree of complexity or the formation of complexes with the inorganic components of the medium of salts and minerals, in addition to the physical properties of the materials used in solid-state fermentations, which have varying effects on the production of enzymes and other metabolic compounds, such as the size of particles, surface area, and porosity of the medium [21].

3.1.2 Determine The Optimal Ratio of Hydration (Water Content)

It is noted from Figure (2) that productivity increases with increasing hydration ratio up to 1:2 if the milk-clotting specific activity reaches 27.3 U/mg and productivity reaches 321 (coagulation U/gm of the medium) when it is moistened at the mentioned rate.

The effects of the hydration ratio or water content of the medium on enzyme production by solid-state fermentation cannot be discussed in isolation from the type of organism used, the size and type of inoculum, or the environmental conditions applied in the production system, such as temperature. The transfer of nutrients from the medium to the cells depends on what is available in a water-soluble form, which is likely to decrease with the aging of the incubation period at relatively high temperatures (40 °C, for example) due to evaporation, which reduces metabolic activities and stimulates mold to form spores early, especially at low levels of hydration ratios. Also, increasing the humidity level beyond its ideal limits has negative effects that lead to the dilution of nutrients and the limitation of oxygen transfer, which affects the lack of space available for the penetration of mold threads into the medium [22].

3.1.3 Determine The Optimal Initial pH for Production

It was noted from Figure 3 that the ability of the mold to secrete the enzyme increased with the increase in pH, and the increase reached its maximum at pH 6.0. The specific coagulation activity was 26.5 U/mg with a productivity of 583 coagulation U/gm of the rice bran medium. Therefore, this value was adopted as the best pH for enzyme production in the later stages of determining the optimal conditions. There were no significant changes or differences in the proteolytic and milk-clotting specificity activities or productivity in the initial pH ranges studied, which ranged between 3.0 and 7.0. Although the nature of the effect of pH on the growth and metabolic activity of molds is a matter of great complexity, some aspects of this effect on the production of exoenzymes are clear in two aspects: one is the effect of pH on the solubility of nutrients, and the other is the stability of the enzyme produced for pH [23].

3.1.4 Determine The Optimal Temperature for Production

Figure (4) shows the effect of temperature on enzyme production. It is noted that the specific coagulation activity and productivity increased with the increase in temperature to 40°C and then decreased significantly at higher temperatures. The milk-clotting-specific activity and productivity reached 27.3 U/mg and 590 (coagulation U/gm), respectively.

These results are consistent with what was reached by [24], who found that the optimum temperature for the production of the coagulant enzyme from Mucor miehei on wheat bran medium was 42°C and that a decrease or increase in temperature beyond this limit leads to a clear deterioration in the coagulation activity of the enzyme and productivity. The temperature of enzyme production is intertwined with other environmental factors, as the optimum temperature and its ranges are correct only under specific conditions of incubation time and nutrient medium. Also, the nutritional needs

of some microorganisms change during growth at temperatures above the optimum [25].

3.1.5 Determine The Optimal Inoculum for Production

The results showed in Figure 5 that adding the inoculum to the production medium at levels ranging from 105 to 107 spores/gm of the medium showed that the ideal inoculum volume for enzyme production ranged from 5×10^6 to 10^6 spores/gm if the milk-clotting specific activity of the enzyme reached at these two levels (26.1 and 26.8 U/mg, respectively), with a productivity of 590 (coagulation U/gm of the medium). These results are consistent with most of the studies that included, in part, studying the effect of inoculum size on the production of metabolic compounds using solid-state fermentation using molds [26].

3.1.6 Determine The Optimal Fermentation Time for Production

Figure 6 shows the results that productivity increases with time and reaches its maximum after three days of incubation if the milk-clotting specific activity reaches 30.1 U/mg and productivity 590 (coagulation U/gm of the medium), an increase in its value more than five times from the first day 6.1 (U/mg and 95 coagulation U/gm of the medium). This result is almost consistent with several studies that have addressed enzyme production by solid-state fermentation in determining the optimum period for production between 2 and 7 days [28 ؛27;16].

Table 1 shows the development in acid protease productivity over the six stages of determining the optimum conditions for production using the solid-state fermentation method.

3.2 The Purification of Acid Protease - Rennet Substitute

Table 2 shows that the number of purification folds and the enzyme recovery obtained after the enzyme precipitation step with ethyl alcohol between the 50–75% addition ratios were 3.18 and 59%, respectively. Then the number of purifications folds to 5.73, and recovery decreases to 45% after the ion exchange chromatography step. It was noted that the parts not bound to the exchanger (due to washing) were completely free of activity, which confirms the association of the enzyme with the negative ion exchanger and that the net charge carried on the enzyme under the conditions used is negative (Figure 7). The acid protease recovery from the ion exchanger was not achieved until the concentration of sodium chloride in the acetate buffer solution reached 0.2 to 0.3 M. As for the last step of enzyme purification by gel filtration (Figure 8), it was possible to obtain purification folds of 13.59, where the enzyme recovery reached 32%. Many previous studies have purified the acid protease enzyme from various microbial sources, showing diverse properties and potential applications. Purification methods typically involve chromatography techniques, resulting in varying yields and purification folds. For instance, Aspergillus oryzae BCRC 30118 produced a cysteine protease with a purification fold of 6.6 and a 15.1% yield [29]. *Pediococcus acidilactici* yielded an acidic protease with a 2.26-fold purification increase using gel filtration chromatography [30]. *Aspergillus carbonarius* produced a cysteine protease with a 10-fold purification and a specific activity of 485.47 U/mg protein [31].

3.3 Enzyme Purity Determination

After the purification steps, the acid protease was analyzed by polyacrylamide gel performed under non-denatured conditions; the result showed a single band, as shown in Figure 9, which indicates that the enzyme was pure to the point of homogeneity. Another evidence of enzyme purity is that the activity peak matches the absorption peak at 280 nm, which is considered to be the concentration of the enzyme in the recovered solution. The activity peak matches the absorption peak when proving the presence of a covalent bond between the carbohydrate and protein parts of the enzyme [32].

3.4 The Characterization of Acid Protease

3.4.1 Molecular weight

The molecular weight was determined by the gel filtration method by calculating the value of the elution value/void value, which amounted to 1.50, and then applying the straight-line equation of the standard curve. The results show that the logarithm of the MW of the acid protease was 1.57. Thus, the molecular weight is 37.15 kDa (Figure 10). Acid proteases from various fungal sources have been characterized, revealing diverse molecular weights and properties. The molecular weights of these enzymes range from 34 kDa in Monascus kaoliang [33] to 70 kDa in *Rhizopus stolonifer* RN-11 [34], with intermediate values of 41 kDa for Aspergillus oryzae BCRC 30118 [29] and 50 kDa for Aspergillus niger I1 [35].

3.4.2 Isoelectric point (PI)

The study of the isoelectric point of the enzyme (Figure 11) showed that it is equal to 5.2, which is higher compared with the isoelectric points of several studies that were reviewed. Acid proteases from various organisms exhibit diverse characteristics and isoelectric points. Two acidic proteases from Sporotrichum pulverulentum had isoelectric points of 4.7 and 4.2 [36]. It is worth noting that the variation in the isoelectric points of identical enzymes from different sources is due to the difference in their content of amino acids, especially the acidic ones (glutamic and aspartic acid), and the basic ones (arginine, histidine, and lysine), and the difference in the proportion of these two groups with each other [37].

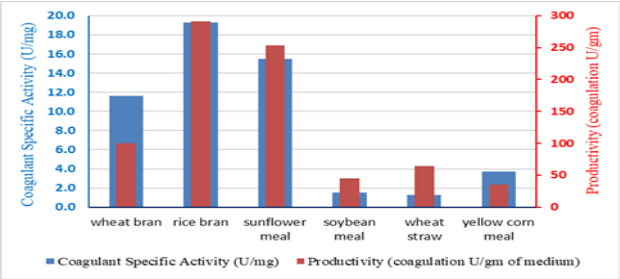

Figure 1: The Optimal of Medium for The Production

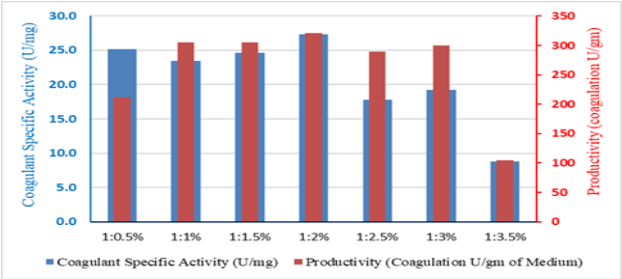

Figure 2: The Optimal Ratio of Hydration

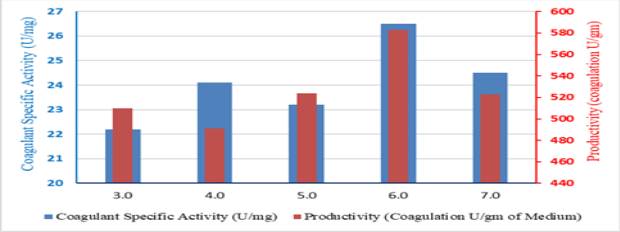

Figure 3: The optimal Initial pH for Production

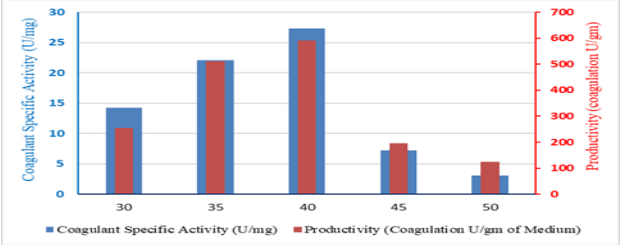

Figure 4: The Optimal Temperature for Production

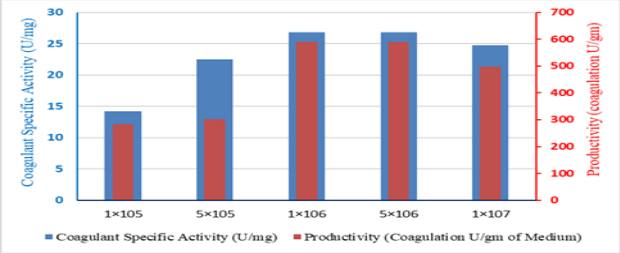

Figure 5: The Optimal Inoculum for Production

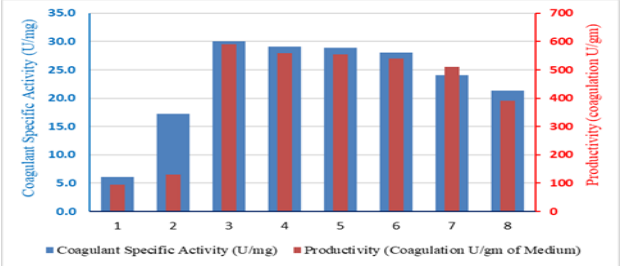

Figure 6: The Optimal Fermentation Time

Table 1: Development of Acid Protease - Rennet Substitute - Production by *Rhizomucor miehei* Strain 33M After the Six Stages of Determining the Optimum Conditions

The Stage	Productivity (coagulation U/gm)	Source of Information
Medium Production (rice bran)	291	Figure 1
The ratio of Hydration (1.2%)	321	Figure 2
pH (6.0)	583	Figure 3
Temperature (40°C)	590	Figure 4
Inoculum (10^6 spores/gm)	590	Figure 5
Fermentation Time (3 days)	590	Figure 6

Table 2: Acid Protease Purification Steps - Rennet Substitute - Produced by Solid-State Fermentation

Purification Steps	Volume (ml)	Coagulant Activity (U/ml)	Protein concentration1 (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Recovery (%)
Crude enzyme	80	122	4.28	29	9760	1.00	100
Precipitation 50-75%*	24	240	2.65	91	5760	3.18	59
DEAE-cellulose*	55	80	0.49	163	4400	5.73	45

Sephadex G-100*	50	62	0.16	388	3100	13.59	32

* The results represent after dialysis in 0.01M acetate buffer at pH 6.0 for 18 h.

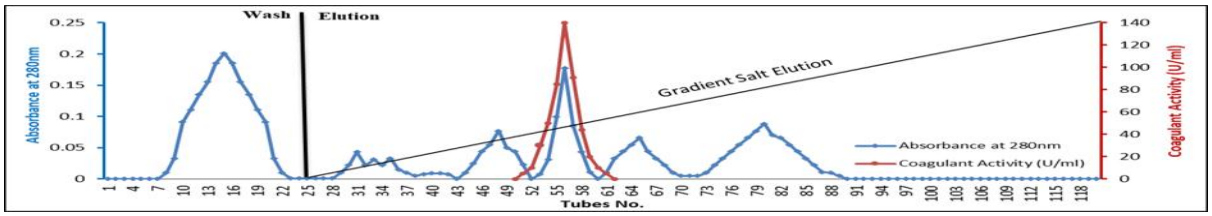

Figure 7: Acid protease - Rennet Substitute - Purification by DEAE-cellulose Step

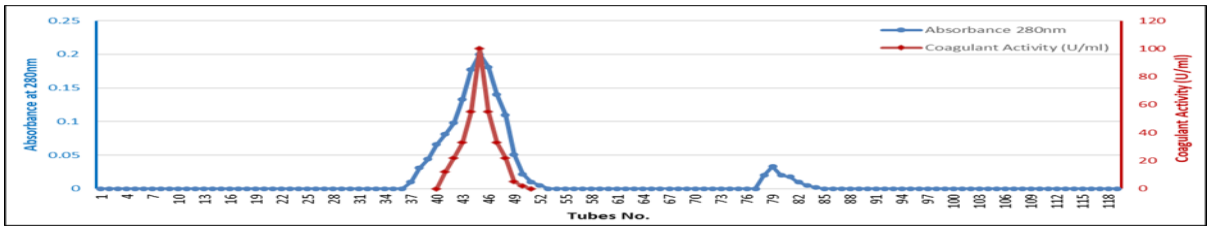

Figure 8: Acid protease - Rennet Substitute - Purification by Sephadex G-100 Step

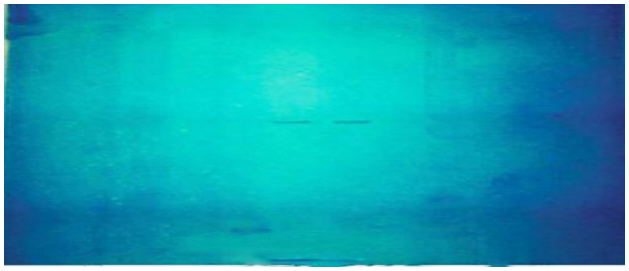

Figure 9: The Electrophoresis of The Sample of Fractions Recovered from Sephadex Column G-100 in a Polyacrylamide Gel in Non-Denaturing Conditions

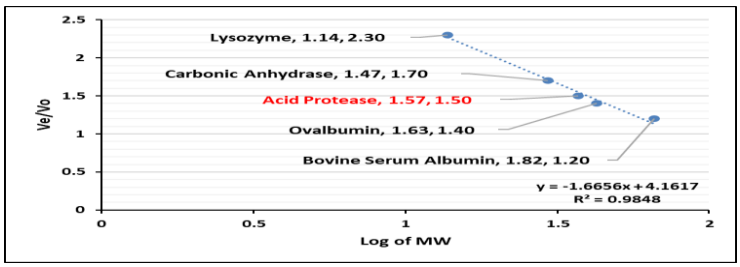

Figure 10: Molecular Weight Determination of Acid Protease - Rennet Substitute - by Gel Filtration Method Sephadex G-100

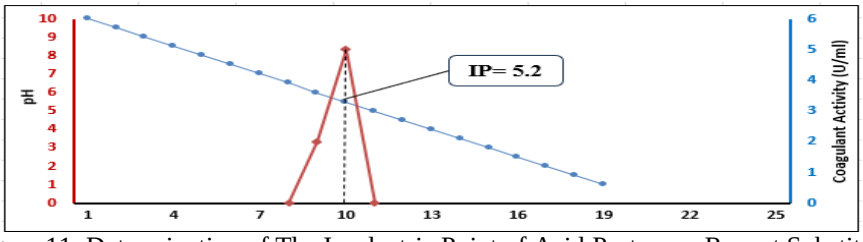

Figure 11: Determination of The Isoelectric Point of Acid Protease - Rennet Substitute

3.4.3 Determine The Optimum pH for Proteolytic Activity

Figure (12) shows the estimation of the optimal pH for proteolytic activity and the use of casein as a substrate. It was found that it is impossible to accurately determine this value due to the precipitation of casein at pH below 5.0 because the isoelectric point is 4.6. Therefore, substrates not affected by low pH must be used, such as hemoglobin and bovine serum albumin [38].

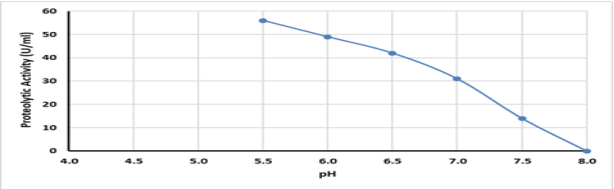

Figure 12: Optimal pH Curve for Proteolytic Activity of Acid Protease - Rennet Substitute

3.4.4 The Effect of The Optimum pH for Enzyme Milk-Clotting Activity

By studying the effect of pH on the coagulation activity of the enzyme (Figure 13), it was found that the coagulation activity increases sharply with a decrease in pH from 7.0 to 5.8 and that the coagulation activity increases about 4.5 times at pH 5.8 from its coagulation activity at the natural pH of the skimmed milk used, which is 6.0. From reviewing previous studies, it was found that the coagulating enzymes from molds decompose casein in a wide range of pH numbers ranging from 5.5 to 7.5 and that the effectiveness of these enzymes and the coagulating enzymes from animal sources increases with the decrease in the pH of the milk below its natural pH, although their sensitivity to the pH deviates towards basicity [39].

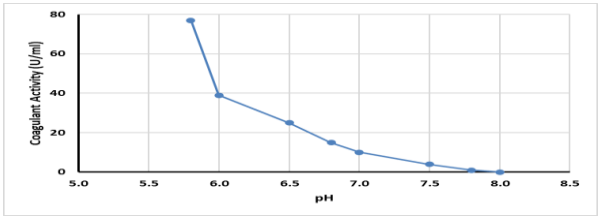

Figure 13: Optimal pH Curve for Milk-Clotting Activity of Acid Protease - Rennet Substitute

3.4.5 Determine The Optimum pH for Enzyme Stability

It is clear from Figure 14, which represents the stability of the enzyme towards the pH numbers, that the pH number for the stability of the enzyme ranges between 4.0 and 6.0, as the enzyme retained 90% of its proteolytic and milk-clotting activity when treated with the mentioned pH numbers for 30min at 35°C. This range falls within the ranges determined by other studies that dealt with the stability of coagulant enzymes from different molds. Aspergillus oryzae MTCC 5341 produced high acid protease activity under optimized solid-state fermentation conditions at pH 5.0 and 30°C [40]. Similarly, Aspergillus sp. showed optimal enzyme production at pH 5.0 and 30°C, with the purified enzyme exhibiting maximum activity at pH 5.0 and 50°C [41]. *Rhizopus stolonifer* RN11 produced an acid protease with optimal activity at pH 2.5 and 50°C, remaining stable between pH 2.0-4.0 [34], The marine Antarctic yeast *Rhodotorula mucilaginosa* L7 secreted an extracellular protease with optimal activity at pH 5.0 and 50°C, demonstrating stability in high salt concentrations [42]. These studies highlight the diverse sources and characteristics of acid proteases, with most showing optimal stability and activity in acidic conditions and moderate temperatures.

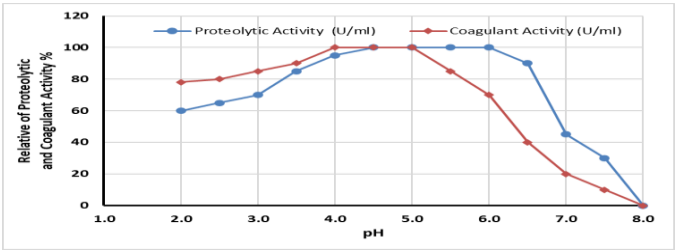

Figure 14: Optimum pH Stability Curve for Relative Proteolytic and Milk-Clotting Activity for Acid Protease - Rennet Substitute

3.4.6 Determine The Optimum Temperature for Enzyme Activity

Figure (15) shows the optimum temperature curve for the proteolytic and milk-clotting activity in the range between 30-75°C, where it is noted that the activity of the enzyme increases rapidly from 30-45°C and increases slowly between 45-60°C, where it reached its maximum at this last temperature. The coagulant activity of the enzyme reached its maximum at 60°C and then decreased and the enzyme lost its full coagulant activity at 70°C. It is worth noting that the formation of a complete clot was not observed in the samples selected at high temperatures (55-65°C) despite the formation of a thin layer of clot particles on the inner surfaces of the test tubes, perhaps due to the effects of high temperatures on the components of the milk and thus on the aggregation of casein particles.

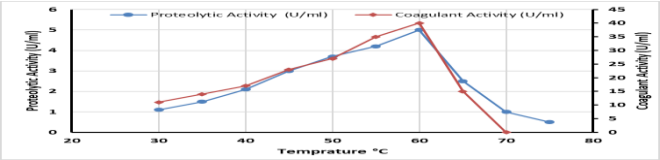

Figure 15: Optimum Temperature Curve for Proteolytic and Milk-Clotting Activity of Acid Protease - Rennet Substitute

3.4.7 Determine The Thermal Stability of The Enzyme

The enzyme, after determining its thermal stability, retained its full coagulation and lytic activity when treated with heat at 35°C for 60 minutes, but the activity began to decrease slightly after 45 minutes of heat treatment. The enzyme lost only 5% of its activity after treatment at 35°C for 60 min (Figure 16). The enzyme coagulation activity slowly increased after 45 minutes of heat treatment after 60 min. On the other hand, when the enzyme was treated for 15 minutes at different temperatures ranging from 30-70°C, it retained its full coagulant and activity at 40°C. The activity began to decrease with the increase in the temperature treatment to 45°C, so the enzyme lost 10% of its activity at this temperature. It was also observed that the coagulation and activity continued to deteriorate rapidly and sharply until they reached zero at 65°C.

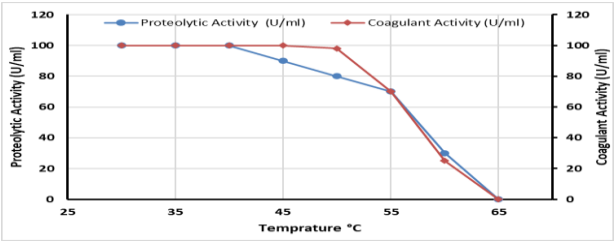

Figure 16: Effect of Heat Treatment on Proteolytic and Milk-Clotting Activity of Acid Protease - Rennet Substitute

Conclusion

The most important conclusions drawn from studying the optimal conditions for producing acid protease enzyme as an alternative to rennet are that using agricultural and industrial waste in the development of the production from the isolated microorganism has yielded excellent results and that rice bran is the best medium for production in terms of the specific coagulation activity (U/mg) and productivity (U/mg) using the solid-state fermentation method. Possibility of enzyme purification in short, simple, scalable steps. The enzyme produced in this study was found to be similar in its characterization to the properties of commercial coagulants available in the local dairy factories.

Acknowledgments

This study was conducted in the laboratories of the College of Agriculture, University of Kirkuk, Iraq. Great thanks to the staff in these labs for providing the equipment, requirements, and facilities.

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دراسة الظروف المثلى لإنتاج إنزيم البروتييز الحامضي بديل المنفحة المنتج من العفن *Rhizomucor Miehei* وتنقيته وتوصيفه.

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الخلاصة

أمكن الحصول على عزله من الفطر *Rhizomucor Miehei strain MMO* وتسجيلها برقم الوصول PP955195.1 في NCBI، التي تميزت بإنتاجها لإنزيم البروتييز الحامضي بديل المنفحة، ومن ثم تمت مقارنته بأربعة مستحضرات تجارية من إنزيم الرنين المستخدمة في صناعة الجبن الطري في معامل الألبان، كل هذا تم في دراسة سابقة. استكمالاً لتلك الدراسة، ولإنتاج الانزيم بأعلى فعالية بأسلوب تخمرات الحالة الصلبة وجد أن أفضل وسط لإنتاج الأنزيم هو سحاله الأرز، وتضمنت الظروف المثلى لإنتاج الأنزيم باستخدام نسبة ترطيب 1:2 (مليلتر ماء مقطر: غرام من الوسط) ورقم هيدروجيني ابتدائي 6.0 عند درجة حرارة 40مْ بعد تلقيح الوسط بلقاح مقداره 10^6 بوغ/غرام من الوسط مع الحضانة لمدة ثلاثة أيام. نقي الأنزيم بخطوات تضمنت الترسيب بالإيثانول بنسبة (50–75) %، وتبادل الأيوني باستخدامDEAE–Cellulose، والترشيح الهلامي على عمود Sephadex G–100. إذ بلغ عدد مرات التنقية 13.59 مرة وبحصيلة مقدارها 32%. جرى التأكد من نقاوة الأنزيم بطريقة الترحيل الكهربائي في ظروف غير ماسخة للبروتين، إذ ظهرت حزمة بروتينية واحدة في هلام متعدد اكريلامايد. أما عند دراسة توصيف الإنزيم، وجد أن الوزن الجزيئي للإنزيم بطريقة الترشيح الهلامي 37.15 كيلو دالتون، أما نقطة التعادل الكهربائي للأنزيم قد بلغت 5.2. في حين ان الرقم الهيدروجيني الأمثل للفعالية التحللية للأنزيم المنقى تجاه الكازين هو 5.5، بينما كانت الفعالية التخثرية معتمدة على الرقم الهيدروجيني، ويزداد مع انخفاض الرقم الهيدروجيني للحليب الخالي من الدسم المعاد تكوينه. كان الإنزيم أكثر استقرارًا في نطاق الرقم الهيدروجيني 4.0–6.0. لوحظ أقصى نشاط للفعالية تحللية وتخثرية للحليب عند 60 مْ. وفي دراسة الاستقرار الحراري، وجد عند المعاملة بالحرارة عند 35مْ لمدة 60 دقيقة حافظ الإنزيم على كامل فعاليته التحللية والتخثرية.

الكلمات المفتاحية: الفعالية التحللية، الفعالية التخثرية، الحصيلة الانزيمية، الرقم الهيدروجيني، اكريلامايد.