Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(2):167-174

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Semi-purification and kinetic study of microfungal rennet biosynthesized by local isolate of *Rhizomucor nainitalensis* using solid-state fermentation system: Concentration methods and determinant factors in clotting activity

Khademi Farshad[a*], Abachi Soheila[b] and Malekzadeh Fereydoon A.[b]

[a]*Department of Microbiology, Agriculture Research Center, Tabriz, Iran*
[b]*Department of Food Science, Agriculture & Natural Resources Research Center, Qom, Iran*

ABSTRACT

Our laboratory, previously, isolated and preserved at hermophilic Zygomycete, an indigenous Rhizomucor nainitalensis, demonstrating a high level of milk clotting activity for its external aspartic proteases. Following the determination of the optimum cultural conditions for the production of the microfungalrennet in solid state fermentation (SSF) system, enzymatic properties of crude rennet and the determinant factors in clotting activity were examined. The extracted rennet revealed the highest milk coagulating activity (1920 SU/ml) when was cultured on wheat bran at optimized fermentation condition of 40˚C, 50% humidity, and inoculated with 10^3 spore/g wheat bran for 110 hours. The pH of 4.6 was found to be the optimum pH for clotting activity. Milk clotting activity increased significantly as calcium concentration in the enzyme environment was raised from 0.001 M to 0.05 M and the reaction temperature from 25˚C to 65˚C. It was also found that, at 75˚C and the optimum pH, the enzyme becomes inactive in a period of 30 minutes. At pH of 4 and 5, the rennet maintains 100% of its activity level for 48 hours at 65˚C. In addition, like the commercial rennet, the produce drennet reaches its highest level of proteolytic activity at pH of 4. As a disadvantage, the proteolytic activity of crude rennet produced in our experiment was considerably stronger than the commercial rennet. Amongst physical and chemical methods of enzyme concentration, precipitation method with 40% and 90% saturated ammonium sulfate yielded the highest level of clotting activity.

Keywords: Kinetic, Microfungal Rennet, *Rhizomucor nainitalensis*, Cheese, Chymosin

---

INTRODUCTION

In general classification and nomenclature of Enzyme, EC 3.4.23.4 (rennin) is an endopeptidase enzyme classified under acid or acylaspartic-type proteinases as a subclass of hydrolases[1]. The enzyme is responsible for hydrolysis of polypeptide chain and, unlike many endopeptidases, does not contain a metal atom[2, 3]. Although hemoglobin hydrolysis by rennin is as extensive as hydrolysis by pepsin and trypsin, but in hydrolysis of casein, rennin specifically acts on the peptide bond connecting the phenylalanine residue in position 105 at the C-terminal of para-kappa-casein and the methionyl residue in position 106 at the N-terminal of kappa-casein macropeptides[4-6].

Insufficient supply of rennet has directed an extensive research into finding proper proteolytic enzymes as rennin-substitute, however only a few number of such enzymes, including swine and bovine pepsins and the proteases obtained from *Mucor miehi*, *Mucor pusillus*, and *Endothiaparasitica*, were introduced as potential substitute for rennin[7-9]. The proteinases biosynthesized from microbial order of Mucorales, such as microfungal rennets, has attracted more attentions amongst all substitute rennets since it has properties that make it comparable to calf rennet [10, 11].

Despite the unpleasant taste and undesirable texture of some types of cheese produced by thermostable *Mucor* rennets, which cannot be deactivated by thermal shock in cheese processing, they are widely used in industrial scale and even traditional cheese production worldwide[12-15]. Many commercial rennin substitutes are crude or a complex of several proteases with different levels of clotting and non-specific protease activities. They may contain enzymes such as esterase, lipase, cellulase, amylase, and β-galactosidase[16-18].Despite the aforementioned facts, non-stop efforts have been made to increase the ratio of milk clotting activity to the proteolytic activity (MCA/PA ratio) of such proteases[19, 20].

There are reports of applied mutation and genetic engineering techniques in the enzyme industry for modification and improvement of rennin-like proteases[21-23].However, optimization of fermentation conditions and advancements made by novel techniques in post-production (fermentation down-stream) processes, (including enzyme-based product purification and chemical modifications in the enzyme structure) have helped researchers to the introduction of high-quality proteases for the production of different types of cheese in the global market[24-26]. Due to the developments in animal and dairy industries in developing and third world countries , the constantly growing demand for rennet is met through importing microbial rennet from several industrial countries without any consideration for the quality compliance of the rennet with the type of cheese produced in the consuming countries[27].

The present study assesses the determinant factors in rennet biosynthesis using freshly isolated *Rh. Nainitalensis* species in solid state fermentation (SSF)system and briefly reviews the downstream processes and the parameters that influence the rennet's kinetic and enzyme activity [28, 29].Clearly, production of microbial rennet at industrial scale requires extensive and accurate research in determining effective parameters in SSF and submerged fermentation (SF), differences between the bio-products of each fermentation process in terms of quality and quantity, parameter settings to achieve the highest MCA/PA ratio, and studying the available semi-purification methods of rennet.

MATERIALS AND METHODS

1.1 Enzyme Source

In this project, crude enzyme biosynthesized by *Rh. nainitalensis* (previously isolated in this laboratory and cultured using SSF system)was examined in the laboratory to determine its properties. At the optimum cultivation time, the fermentation was stopped and the enzyme extract was obtained by addition of cold phosphate buffer solution (pH 6, 0.1 M). The extract was blended with Sodium Azide (0.02%) after filtration; in two stages by Whatman filter papers No. 40 and then No. 1, in order to prevent the growth of contaminant microorganisms. The mixture was centrifuged at 6000 rpm for 20 minutes to remove particles and colloids, and was stored at 2-4˚C prior to examination of the enzyme activity[30-32].

1.2 Enzyme activity

1.2.1 Clotting activity

Clotting activity of the rennet was evaluated and represented in Soxhlet unit (SU)using the method of Arima[33]. One Soxhlet unit is defined as the amount of enzyme that clots 1 ml of substrate (0.1g skim milk powder and 1.47 mg $CaCl_2$) in 40 min at 35˚C.

1.2.2 Specific milk clotting activity (SMCA)

First, protein concentration in each ml of the enzyme extract was determined and then clotting activity was calculated for each milligram of protein dissolved in the enzyme extract.

1.2.3 Protease activity

For each liquid sample of*Rh. Nainitalensis* rennet, liquid concentrations of NOVO fungal rennet with coagulation capacity equal to that sample were prepared to graph protease activity of the produced rennet versus pH and to compare it with commercial rennet. Since casein substrate cannot be used to measure protease activity at the isoelectric point of casein (pH4.6), the method of Anson[34]with hemoglobin substrate was employed in addition to the method of Kunitz[35]. By definition, one unit is the amount of enzyme that increases light absorption by 0.001 units at 30˚C, at the wavelength of 280 nm, and a pH of 3.6 for hemoglobin substrate and pH of 6.2 for casein. In a similar work, milk clot hydrolyzing activity (CHA) method developed by Bailey[36]was employed to recombined milk in an effort to simplify the process of protease activity measurement. The enzyme-substrate mixture was incubated for 24 hours at 35˚C. The following formula was used to compare the dry weight of precipitation after centrifuging with the dry weight at time zero:

$$CH(\%) = \frac{(mA - mB)}{mA} \times 100\%$$

Protein concentration per each ml of enzyme extract was determined by the method developed by Lowry[37], with Folin–Ciocalteu reagent as indicator.

1.3 Determinant factors in enzyme activity

1.3.1 Temperature and enzyme activity

Similar to the Arima method of MCA assay, substrate was prepared by dissolving skimmed milk powder (10%) in 0.01 M $CaCl_2$ solution. 5 ml of the solution was put into a test tube that, together with another tube containing 0.5 ml of enzyme solution, was water bathed for 10 minutes at 25 to 65˚C. Time and enzyme activity were calculated after mixing the substrate and the enzyme.

1.3.2 pH activity

To examine the effect of hydrogen ion concentration, a substrate of fat-free milk with a pH range of 4to4.5was prepared using the method developed by Arima[38]. Coagulation capacity of the rennet was determined while the other conditions were kept unchanged.

1.3.3 The impact of calcium on rennet activity

In this part of study, similar $CaCl_2$ substrates with different molarities (0.001-0.05 M) were prepared and milk-clotting activities were recorded for each calcium concentration, and so clotting activity was determined.

1.3.4 Thermostability

Similar to other parts of the study, the enzyme used was in liquid state at milk clotting optimum pH of 4.6. The enzyme-containing tubes were placed in water bath at the specified temperatures, then samples were taken at certain intervals, and activity levels were determined[39].

1.3.5 pH stability

A relatively thick suspension was prepared using the enzyme powder and after complete dialysis, an enzyme solution was prepared using 0.1 M buffer solutions of acetate, phosphate, and citrate with different pH levels so that a clotting activity of 1,000 SU/ml could be obtained at pH of6. After treatment at 65˚C, activity of enzyme residues were calculated at specified intervals.

1.3.6 Electrophoresis of Rh. Rennet and its comparison to commercial rennet

Samples of commercial rennet (Novo) and the rennet produced by the isolated *Rh. Nainitalensis* were electrophoresed through 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) at pH7.1 in a vertical cell as previously described[40].

1.4 Enzyme concentration and partial-purification of enzyme extract

To increase the concentration of enzyme proteins per unit, the following physical and chemical methods were applied:

1.4.1 Salting out

At this step, consecutive rounds of 40-90% ammonium sulfate saturation were performed. Residual enzyme activity and specific clotting activity of the ammonium sulfate-precipitated extracts were compared to those of the protein obtained through 100% saturation. To produce enzyme powder, the precipitated extract was puton aluminum foil and then dried for 48 hours in a desiccator containing P_2O_5.

1.4.2 Ethanol precipitation

Protein was precipitated by adding ethanol to enzyme extract in the ratio (3:1) at 20˚C. The protein was then separated by refrigerated centrifugation at 10,000 rpm for 10 min.

1.4.3 Acetone precipitation

Two units of acetone with a temperature of 4˚Cwere added to 100 ml enzyme extract cooled down to 2˚C. The precipitated protein was then separated by refrigerated centrifugation at 10,000 rpm for 10 min.

1.4.4 Freezing and thawing

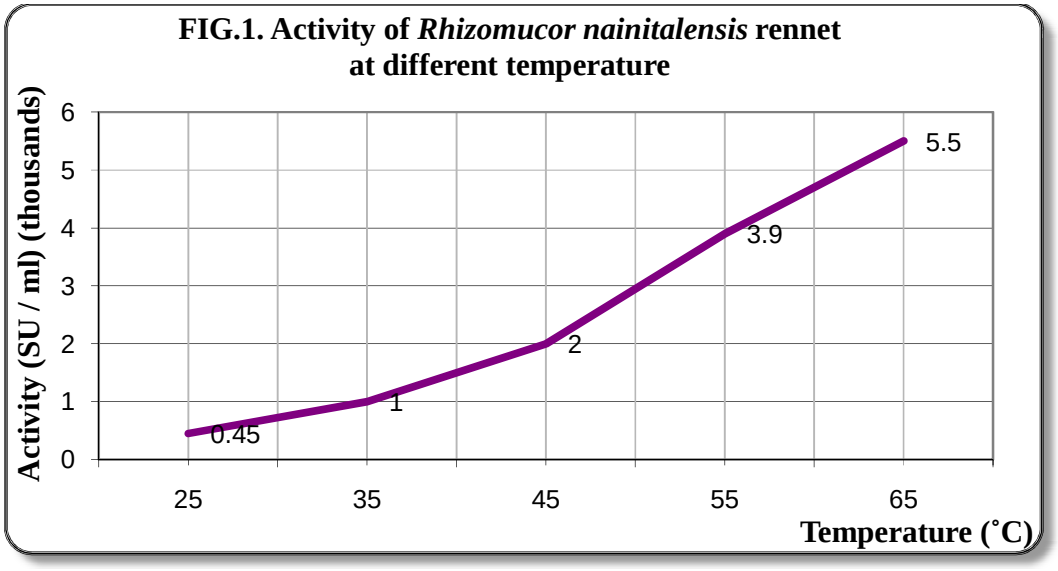
By slow freezing and thawing of the enzyme suspension, twice, the protein contained in the extract was thrust toward the end of the suspension tube and then separated by refrigerated centrifugation at 10,000 rpm for 10 min.

1.5 Dialysis

To remove ammonium sulfate and other unwanted ions, cellophane dialysis tubing (cut off of 12,000D) was used with acetate buffer at 0˚C and a volume of 1,000 ml. Dialysis was repeated for several times until no barium sulfate was produced by adding barium chloride to dialysis buffer.

RESULTS AND DISCUSSION

Following the determination of optimum conditions for synthesizing rennet by *Rh. Nainitalensis* using SSF system, rennet properties with considerable industrial importance were also identified. To determine optimum conditions for milk clotting activity of enzymes and to compare their activity in different situations, it was required to examine factors such as pH, calcium and substrate concentration, enzyme thermostability and pH-stability, which may affect the enzyme activity.

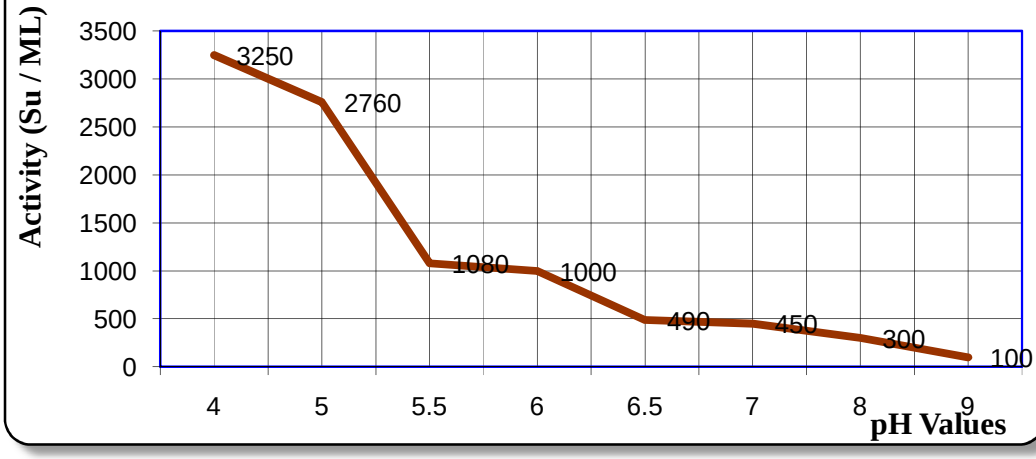


Given the fact that almost all industrial enzymes are marketed without purification, and that the microbial rennets are used in the crude form as commercial enzymes in dairy industry, rennet properties and the effective factors in enzyme activity were examined using the crude rennet obtained from culture extract. Analysis of temperature indicated that, within the range of 25-55˚C, an increase in temperature by 10˚Calmost doubled the enzyme activity

with a relatively consistent growth rate. Furthermore, the analysis was performed for temperatures equal to, or lower than 65˚C since variations in equilibrium of salts and changes in milk proteins' structure at the temperatures over 70˚C could lead to deceitful results (Fig.1).

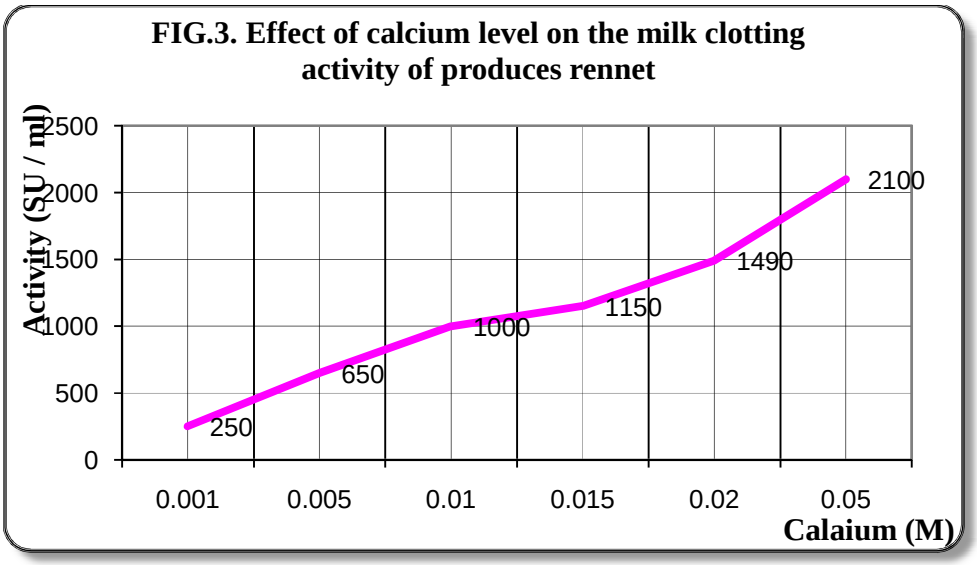
For milk clotting proteases, optimum pH for enzyme activity is of great importance and among different types of rennet; the ones with high levels of clotting activities at a pH close to the pH of bovine milk (6.5-6.8) are considered better substitutes. It was also observed that *Rh. nainitalensis* rennet, like other acid proteases, had greater activity at acidic pH. The activity level increases as pH of the environment decreases (Fig. 2). In this study, clotting activity of rennet was examined for pH values as low as 4.6.Since this pH is the isoelectric point of casein in bovine milk then it starts to precipitate at this point.

FIG.2. Variation of milk coagulation capacity with pH valus for *Rhizomucor nainitalensis* rennet



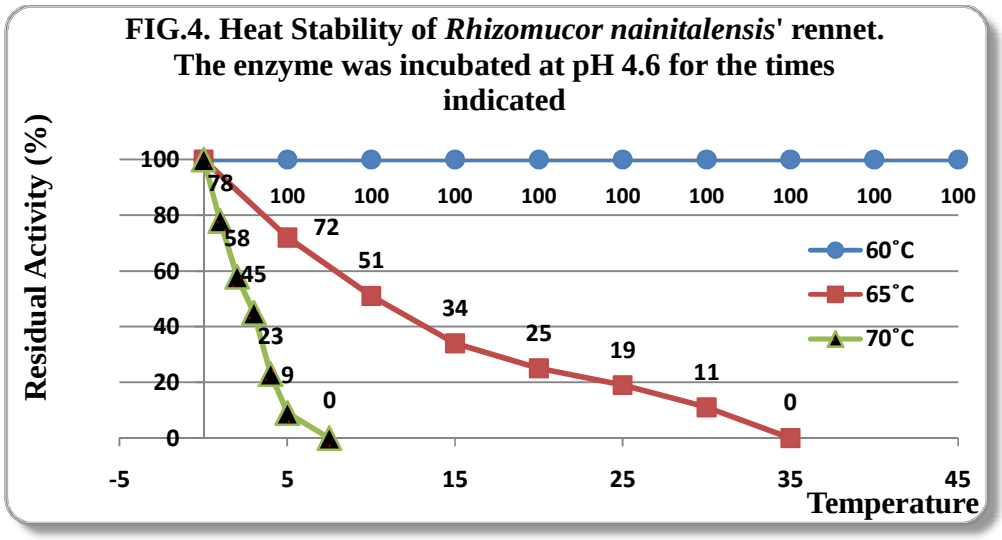
Clotting activity of rennin and majority of substituting enzymes varies as calcium concentration changes. As shown in Fig. 3, clotting activity of the rennet, like activity of rennin and many microbial rennets, increases as calcium level escalates.

FIG.3. Effect of calcium level on the milk clotting activity of produces rennet

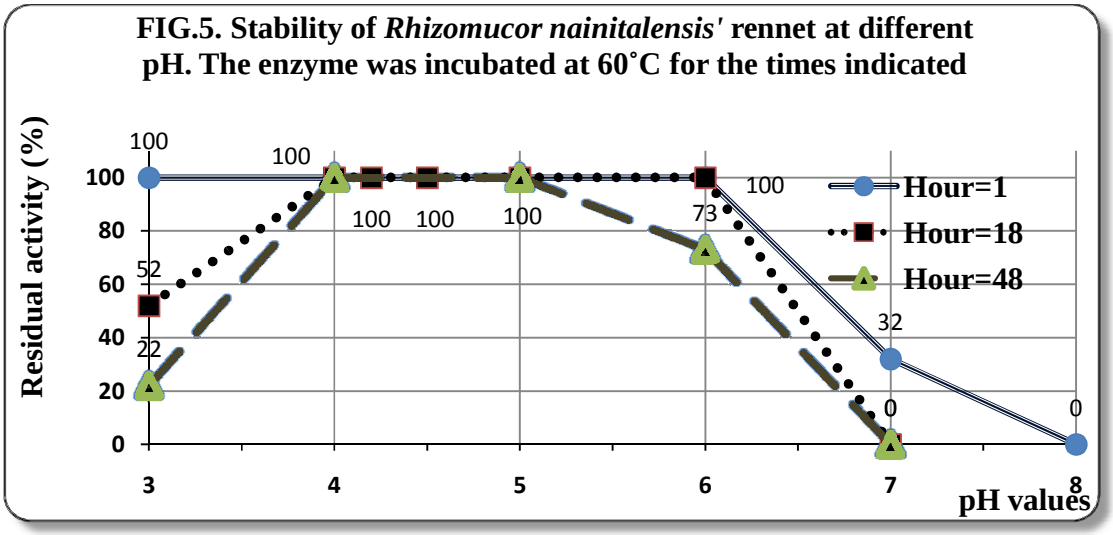


For kinetic and thermostability analysis, an enzyme solution at pH of4.6 (optimum pH for milk clotting activity) was employed. As shown in Fig.4, no change was observed in clotting activity of the enzyme solution at 60˚C and lower

temperatures, at optimum pH, and for a period of 48 hours. However, at heat treatment within higher temperatures significant reduction in milk clotting activity was observed. At 75˚C, the rennet lose 56% of its clotting activity in 10 minutes, and then its activity decreases to 18% in 20 minutes and less than 7% in 30 minutes compared to the control sample.



Like thermostability, pH-stability of enzyme is an important specification of rennin used in cheese industry. One reason for this importance relates to the shelf-life of the enzyme within ascertain temperature and pH and also it relates to the rennet residual activity in the milk clot based on the type of cheese and pH value. In addition, pH stability of substitute rennin is of importance because of the possibility of rennet deactivation through thermal shock of the produced cheese in a specific pH. As shown in Fig. 5, the rennet became completely deactivate at pH values of 8 and 9 within one hour. Reduction of pH led to significant increase in residual activity; however no decrease in activity was recorded at pH of 4-5 compared to zero time. Unpredictably, as the environment became more acidic (pH of 3), rennet activity dropped to 50% and 23% of the activity level compared to the control sample at the hours 18 and 48, respectively.

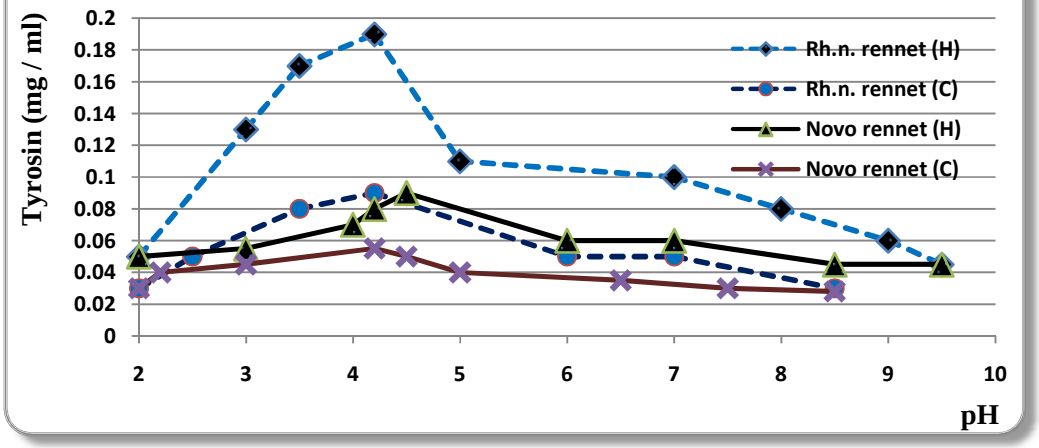


In cheese industry, substitute rennet's proteolytic activity is very important characteristic since the higher non-specific proteases activity in crude rennet results in lower yield and less organoleptic quality of the produced cheese.

In the next step of examining factors affecting the rate of *Rhizomucor* rennet activity, comparison of proteolytic activity of *Rh. nainitalensis'* rennet to commercial rennet (Novo) at various pH indicated a similar trends for both rennets(Fig. 6).

Proteolytic activity reaches its maximum at pH of 4 and by shifting towards alkaline environment, activity level dropped dramatically. Additionally, proteolytic activity of *Rhizomucor* rennet is much higher than the activity of commercial rennet due to the presence of non-specific proteases in the crude rennet. The proteolytic activity can be significantly reduced through low-cost physical and chemical treatments while maintaining the high clotting activity of the enzyme.

FIG.6. Proteolytic Activity of *Rhizomucor nainitalensis'* rennet compared with Novo rennet



The next step involved enzyme concentration through a number of different methods and then these methods were scaled based on the specific clotting activity and residual activity of the enzyme.

Concentration means increasing the amount of enzyme in a fixed volume by removing the water and the contaminants, non-specific enzymes, from the enzyme solution. In this project, different economical precipitation methods including salting out (40-90% saturation along with 100%consecutive saturations), ethanol precipitation, acetone precipitation, and freezing-thawing method were studied. Table I indicates that amongst these methods ammonium sulfate-salting out method is considered as the best rennet concentration method due to its highest residual activity (98%) and its largest specific clotting activity (595 SU/mg protein).

Finally, SDS-PAGE electrophoresis technique was utilized for the determination of Novo rennin purity, and its comparison to the biosynthesized rennet by *Rh. nainitalensis*. Commercial sample developed numerous bands comparable to those of *Rh. Nainitalensis* rennet, in terms of bandwidths.

It was concluded that the commercial rennet also was not purified and sold in partial-purified form like *Rh. Nainitalensis* rennet. This was an indication that the substrates and production process employed for production of *Rh. Nainitalensis* rennet were similar to those of commercial rennet.

Table I: A comparison of enzyme concentration methods

Concentration method Enzyme activity	Moldy Bran extract	Ammonium sulfate (100% saturation)	Ammonium sulfate (40-90% saturation)	Ethanol	Acetone	Freezing &Thawing
Residual activity (ml)	1,920	1,873	1,352	1,286	998	635
residual activity (%)	100	98.1	70	67	52	33.1
Specific clotting activity (SU/mg protein)	480	474	595	346	254	160

Acknowledgment
We greatly thank the Agricultural Research Center of the province of East Azerbaijan for their financial support and collaboration to perform this research project.

REFERENCES

[1] M.N. Raymond, J. Garnier, E. Bricas, *Biochimie*, **1972**, 54(2):145-154.
[2] J. Garnier, *Biochim Biophys Acta*, **1963**, 66:366-377.
[3] S. Radha, R.H. Babu, A. Sridevi, N. Prasad, G. Narasimha, *Eur J Exp Biol*, **2012**, 2(5):1517-1528.
[4] S. Visser, P.J. Van Rooijen, C. Schattenkerk, K.E. Kerling, *Biochim Biophys Acta*, **1976**, 438(1):265-272.
[5] H.B. Drohse, B. Foltmann, *Biochim Biophys Acta*, **1989**, 995(3):221-224.
[6] E.S. Dennis, R.G. Wake, *Biochim Biophys Acta*, **1965**, 97:159-162.
[7] Z. Xiu-ling, *Resource Development & Market*, **2009**, 3:007.
[8] R.A. Srinivasan, M.K. Iyengar, I.J. Babbar, S.C. Chakravorty, A.T. Dudani, K.K. Iya, *Appl Microbiol*, **1964**, 12:475-478.
[9] I. Braun, H. Kunath, *Nahrung*, **1988**, 32(4):375-381.
[10] M.H. Alves, G.M. de Campos-Takaki, K. Okada, I.H. Ferreira-Pessoa, A.I. Milanez, *Rev Iberoam Micol*, **2005**, 22(2):114-117.
[11] J. Escobar, S. Barnett, *Process Biochem*, **1995**, 30(8):695-700.
[12] C. Blecker, J.M. Habib-Jiwan, R. Karoui, *Food Chem*, **2012**, 135(3):1809-1817.
[13] S. Yegin, M. Fernandez-Lahore, A. Jose Gama Salgado, U. Guvenc, Y. Goksungur, C. Tari, *Appl Microbiol Biotechnol*, **2011**, 89(4):949-960.
[14] S. Neelakantan, A. Mohanty, J.K. Kaushik, *Curr Sci*, **1999**, 77(1):143-148.
[15] T. Guinee, M. Wilkinson, *Int J Dairy Technol*, **1992**, 45(4):94-104.
[16] G.A. Somkuti, F.J. Babel, *J Bacteriol*, **1968**, 95(4):1407-1414.
[17] M.S. Thakur, N.G. Karanth, K. Nand, *Biotechnol Adv*, **1993**, 11(3):399-407.
[18] A.C. Oliveira, J.M. Cabral, *J Chem Technol Biotechnol*, **1993**, 56(3):247-252.
[19] J. Adler-Nissen, *Trends Biotechnol*, **1987**, 5(6):170-174.
[20] D. Jaros, M. Jacob, H. Rohm, *European Dairy Magazine*, **2009**(7).
[21] S. Yegin, M. Fernandez-Lahore, *Mol Biotechnol*, **2012**:1-12.
[22] Y. JIANG, J. WANG, Y. LI, D. LI, Z. YANG, *China Dairy Industry*, **2010**, 2:003.
[23] N.H. Loc, N.S. Cong, N.T.Q. Do Van Giap, H.T. Hoa, N.D.H. Quang, *Eur J Exp Biol*, **2012**, 2(4):913-918.
[24] S. Branner-Joergensen, P. Eightved, P. Schneider, **1982**:287-288.
[25] M. Joly, W. Kauzmann, A. Klibanov, T. Barta, V. Mozhaev, N. Kaplan, M. Kamen, K. Martinek, V. Goldmarcher, *Biochim Biophys Acta*, 485:13-28.
[26] B. Sasirekha, T. Bedashree, K. Champa, *Eur J Exp Biol*, **2012**, 2(1):95-104.
[27] J. Winwood, *Int J Dairy Technol*, **1989**, 42(1):1-2.
[28] F. Khademi, S. Abachi, A. Mortazavi, M. Ehsani, M. Tabatabaie, F. Malekzadeh, *IOSR J Agri Vet Sci*, **2013**, 2(2):30-37.
[29] F. Khademi, S. Abachi, A. Mortazavi, M. Ehsani, M. Tabatabaei, F. Malekzadeh, *IOSR J Pharm Bio Sci*, **2013**, 5(3).
[30] C. Unakal, R.I. Kallur, B.B. Kaliwal, *Eur J Exp Biol*, **2012**, 2(4):1044-1052.
[31] S. Abachi, F. Khademi, H. Fatemi, F. Malekzadeh, *IOSR J Dent Med Sci*, **2013**, 4(3):17-26.
[32] S. Abachi, F. Khademi, H. Fatemi, F. Malekzadeh, *IOSR J Dent Med Sci*, **2013**, 4(1):59-63.
[33] K. Arima, J. Yu, S. Iwasaki, *Methods Enzymol*, **1970**, 19:446-459.
[34] M.L. Anson, *J Gen Physiol*, **1938**, 22(1):79-89.
[35] M. Kunitz, *J Gen Physiol*, **1947**, 30(4):291-310.
[36] M.J. Bailey, M. Siika-aho, *Biotechnol Lett*, **1988**, 10(3):161-166.
[37] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J Biol Chem*, **1951**, 193(1):265-275.
[38] K. Arima, J. Yu, S. Iwasaki, G. Tamura, *Appl Microbiol*, **1968**, 16(11):1727-1733.
[39] P. Tiwari, M.K. Upadhyay, *Eur J Exp Biol*, **2012**, 2(3):464-467.
[40] N. Asato, A. Rand Jr, *Anal Biochem*, **1971**, 44(1):32-41.