The study of the production of milk-clotting enzyme by bacillus subtilis Natto

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ABSTRACT

Due to acute shortage of calf rennet in recent years, bacterial rennets have received wide acceptability as one of the calf rennet substitutes. This study investigated the environmental factors which affect milk-clotting activity (MCA) by Bacillus subtilis natto Takahashi in solid state fermentation and liquid state fermentation, followed by optimizing the production of milk-clotting enzyme by response surface methodology (RSM), partial purification and molecular determination was carried out by ultrafiltration concentrate system and SDS-PAGE, factors affected of enzyme activity was also investigated. The highest milk-clotting ratio was obtained when the Bacillus subtilis natto were cultivated in solid state fermentation containing rice bran and basal salts at 37°C, pH 6 and moisture 70% for 72 hr. In liquid state fermentation, the highest milk-clotting ratio was obtained when the Bacillus subtilis natto were cultivated in medium which containing starch, corn steep solids, soybean meal, dry milk and basal salts at 37°C, pH 6 and shaking at 175 rpm for 72 hr. The result of RSM showed that the highest milk-clotting activity was 1048.02 SU/mL when the concentration of starch, corn steep solids, soybean meal and dry milk was 55.41g/L, 1.5g/L, 2.69g/L and 22.29g/L respectively. The result of Ultrafiltration and SDS-PAGE showed that the molecular weight range of milk-clotting enzyme by Bacillus subtilis natto was between 20000 and 30000 g/mole. The optimal milk-clotting enzyme activity by Bacillus subtilis natto was at 60°C and pH 6 in milk substance. When heating at 60°C for 60 min or 70°C for 5min, the milk-clotting enzyme lost 80% of its enzyme activity. The result effect of pH on enzyme activity showed that the milk-clotting activity was quite stable between pH 5.0 and pH 6.0. The effect of various metals on enzyme activity of milk-clotting enzyme by Bacillus subtilis natto showed that the monovalent positive ions had no obvious effect on enzyme activity, but the divalent positive ions had positive effect on milk-clotting activity. The enzyme activity was lost entirely when mercury ion (Hg) was added; mercury ion was the inhibitor of milk-clotting enzyme by Bacillus subtilis natto.

Keywords : milk-clotting enzyme、Bacillus subtilis natto、response surface methodology、milk-clotting ratio、enzyme activity 、inhibitor

Table of Contents

REFERENCES

1. 王聖耀。1997。利用根黴菌株凝乳?☆槔s扣碗酪。台灣大學畜產學研究所碩士論文。臺北市。 2. 王聖耀、林慶文。1997。牛乳酵素性凝固及其凝乳品質之影響。科學農業。45: 153-159。 3. 沈名豪。2004。生物合成聚離胺酸之研究。大葉大學環境工程所碩士論文。彰化縣。 4. 余佩璟。1997。Amylomyces rouxii 凝乳? ’ b米基質中之產生，純化，特性與其應用之探討。台灣大學食品科技研究所博士論文。臺北市。 5. 吳尉偵。2006。Bacillus subtilis var. natto 不同液態培養條件對其代謝物生成之影響。大葉大學生物產業科技所碩士論文。彰化縣。 6. 東尾侃二、吉岡八洲男。1981。凝乳酵素產生系狀　分離，固定　　變異株　誘起　　酵素特性　改良　　。日本農藝化學會誌。55: 561-571。 7. 林美貞、林慶文。1990。酸凝酪之質地與風味。科學農業。38: 251-255. 8. 林慶文。1987。乳品加工學，pp. 43-57。華香園出版社，台北市。林慶文。1993。乳製品之特性與機能，pp.79, 189-193。華香園出版社。台北市。 9. 林慶文、吳祥雲。1973。利用微生物凝乳酵素製造半硬質（Camembert 式）乾酪之研究。第一報影響凝乳張力之因子。中國農業化學會誌 11: 10-14。 10. 林慶文、李素珍、劉?X睿。2002。乳品微生物學，pp. 4-6, 19-24。國立編譯館。台北市。 11. 范宜琮。2001。以苔蘚桿菌生產巨麩胺酸之研究。大葉大學環境工程所碩士論文。彰化縣。 12. 徐泰浩。2004。瞭解生物科技。學銘圖書。台北縣。 13. 陳小玲。2000。酒釀菌元篩選與其凝乳?＊S性。台灣大學畜產學研究所博士論文。臺北市。 14. 陳白珍。1981。食品酵素學。pp. 154-158。復文書局。台南市。 15. 陳立達。2009。以固定化技術生產果糖聚合物之研究。大葉大學環境工程所碩士論文。彰化縣。 16. 陳妍?。2004。生薑蛋白? ′ 〝吨尬v響因子及其應用於牛乳凝膠製品之試製。台灣大學畜產學研究所碩士論文。臺北市。 17. 陳順宇。1999。STATISTICA 手冊(Ⅱ):工業統計。pp. 6.1-6.58。華泰書局。台北市。 18. 陳淑華。1987。微生物酵素的生產分離與純化。食品工業。19: 36-44。 19. 陳詩侶。1996。酒釀萃之特性及其影響凝乳堅實度之因子。台灣大學食品科技研究所碩士論文。臺北市。 20. 游芸悌。2003。以納豆菌生產生物性高分子之研究。大葉大學環境工程所碩士論文。彰化縣。 21. 廖國森。2007。納豆菌生產果糖聚合物之研究。大葉大學生物產業科技所碩士論文。彰化縣。 22. 潘氏蘭瑛。2007。納豆菌發酵產物之乳化及凝乳活性之探討。大葉大學環境工程所碩士論文。彰化縣。 23. 盧盈瑜。2008。Bacillus subtilis var. natto 和 Rhizopus oligosporus 發酵大豆凝乳?〃 滲瞻　M凝乳活性之研究。大葉大學生物產業科技所碩士論文。彰化縣。 24. 謝佳慧。1991。凍存逆滲透濃縮乳製作酸凝乳與甜凝乳之物性與凝析。台灣大學畜產學研究所碩士論文。臺北市。 25. 蘇遠志、陳文彬。1970。利用微生物生產凝乳酵素之研究。生產菌株之篩選試驗與酵素之製備。中國農業化學會誌 8: 73-83。 26. Amal M. Hashem. 1999. Optimization of milk-clotting enzyme productivity by Penicillium oxalicum. Bioresource Technology. 70:203-207. 75:219-222. 27. A.O.A.C. 1980. Offical methods of analysis. Association of official anylytical chemists. pp. 344-345. Washington, D. C. 28. Arima K. 1970. Milk-clotting Enzyme from Mucor pusillus var. Lindt. Methods in enzymology 19:446-459. 29. Arnon R. 1970. Papain. Methods Enzymol. Methods in enzymology 19: 226-244. 30. Asakura T., H. Watanabe, K. Abe and S. Arai. 1997. Oryzasin as an aspartic proteinase occurring in rice seeds: purification, characterization, and application to milk clotting. J. Agric.Food Chem. 46: 1070-1075. 31. A. D ’ Ambrosio, R. Rossano, N. Ungaro, P. Riccio. 2003. Proteolytic and milk clotting activities in extracts odtained from the crustaceans Munida. Journal of Molecular Catalysis B. Enzymatic 22:145-150. 32. A.M.S. Ismail, S.A. Elassar, A.F. Abdel-Fattah. 1984. Production of milk-clotting and proteolytic enzymes by fungi, Agric. Wastes 10:95-102. 33. Bashir H. Yousif, Donald J. McMahon & Khalid M. Shammet. 1996. Milk-clotting Enzyme from Solanum dobium Plant. Elaevier Science Limited. Int. Dairy Journal 6:637-644. 34. Birch, G. G., N. Blakebrough and K. J. Parker. 1981. Enzymes and food processing. pp. 33-38. Appied Science Publishers LTD. London. 35. Broome, M. C. and G. K. Y. Limsowtin. 1998. Milk coagulants. Aust. J. Dairy Technol.53:188 – 190. 36. Brunner, J. R. 1977. Milk-proteins. In “ Food Protein ”, pp.175-208. ed. By J. R. Whitaker and S. R. Tannenbaum. AVI Publishing Co. New York. U.S.A. 37. Cheryan, M., P. J. van Wyk, N. F. Olson and T. Richardson. 1975. Secondary phase and mechanism of enzymatic milk coagulation. J. Dairy Sci. 58: 477-481. 38. Dalgleish, D. G. 1979. Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin. J. Dairy Res. 46: 653-661. 39. Dalgleish, D. G. 1987. The enzymatic coagulation of milk. In: P. F. Fox.(Ed.). Cheese: chemistry, physics and microbiology. Vol. I, general aspects pp. 63-96. Elsevier Applied Science, London. 40. Dalgleish, D. G. and A. J. R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. J. Dairy Res. 55: 529-538. 41. Dill, C. W. and W. M. Roberts. 1959. Relationships of heat treatment, solids-not-fat, and calcium chloride to the curd tension of skim milk. J. Dairy Sci. 42: 1792-1799. 42. Famelart, M. H. 1994. Rennet coagulation of milk in the presence of sucrose. J. Dairy Res. 61: 473-483. 43. F Ayhan, Serdar S Celebi and A Tanyolac. 2001. The effect of fermentation parameters on the production of Mucor miehei acid protease in a chemically defined medium. Fournal of Chemical Technology and Biotechnology. 76:153-160. 44. Flink, J. M. 1975. Application of freeze drying for preparation of dehydrated powders from liquid food extracts. In: Goldblith, S. A., L. Rey and W. W. Rothmayr (Ed.). Freeze Drying and Advanced Food Technology. pp. 309.329. Academic press,London New York San Francisco. 45. Grag, S. K. and B. N. Johri. 1994. Rennet: current trends and future research. Food reviews int. 10: 313-355. 46. Glazer, A. N. and E. L. Smith. 1971. Papain and other plant sulfhydryl proteolytic enzymes. The Enzymes 3, 501-547. 47. Gordin, S. and I. Rosenthal. 1978. Efficacy of chicken pepsin as a milk clotting enzyme. J. Food Prot. 41: 684-688. 48. Green, M. L. 1987. Effect of manipulation of milk composition and curd-forming conditions on the formation, structure and properities of milk curd. J. Dairy Res. 54: 303-313. 49. Green, M. L. and G. Crutchfield. 1971. Density-gradient electrophoresis of native and of rennet-treated casein micelles. J. Dairy Res. 38: 151-164. 50. Green, M. L. and R. J. Marshall. 1977. The acceleration by cationic materials of the coagulation of casein micelles by rennet. J. Dairy Res. 44: 521-531. 51. Green, M. L., D. G. Hobbs, S. V. Morant and V. A. Hill. 1978. Intermicellar relationships in rennet-treated separated ilk II. Process of gel assembly. J. Dairy Res. 45: 413-422. 52. Grundelius, A. U., K. Lodaite, K. ?零tergren, M. Paulsson and P. Dejmek. 2000. Syneresis of submerged single curd grains and curd rheology. Int. Dairy J. 10: 489-496. 53. Guilherme Garcia da Silvera, Gustavo Monteiro de Olivera, Eloizio Julio Riberiro Rubens Monti and Jonas Contiero. 2005. Microbial Rennet Produced by Mucor miehei in

Solid-State and Submerged Fermentation. Brazilian Archives of Biology and Technology. Vol.48, n. 6:pp. 931-937. 54. G.A. Somkuti, F.J. Babel. 1967. Conditions influencing the synthesis of acid protease by Mucor pusillus Lindt. Appl. Microbiol. 15:1309-1312. 55. G.D. Venera, C. Machalinski, H. Zum-arrage, M.J. Biscoglio de Jimenez Bonino. 1997. Further characterization and kinetic parameter determination of a milk-clotting protease from Mucor bacilliformis. Appl. Biochem. Biotechnol. 68:207-216. 56. Habibi-Najafi, M. B. and B. H. Lee. 1996. Bitterness in cheese: a review. Crit. Rev. Food Sci. Nutri. 36: 397-411. 57. Harwalkar, V. R. and M. Kalab. 1980. Milk gel structure. XI. Electron microscopy of glucono- δ -lactone-induced skim milk gels. J. Texture Studies 11: 35-49. 58. Ing-Lung Shin, Yi-Tsong Van. 2001. The production of poly-(γ -glutanmic acid) from micro-organisms and its various applications. Bioresource Technology 79:207-225. 59. J. Escobar, S. Barnett. 1993. Effect of agitation speed on the synthesis of Mucor miehei acid Protease. Enzyme Microb. Technol. 15:1009-1013. 60. J.M. ageitos, J.A. Vallejo, A.B.F. Sestelo, M. Poza and T.G. Villa. 2007. Purification and characterization of milk-clotting protease from Bacillus Licheniformis strain USC13. Journal of Applied Microbiology. 103:2205-2213. 61. Kei Arima, Juhyun Yu, Shinjiro Iwasaki and Gakuzo Tamura. 1968. Milk-clotting Enzyme from Microorganisms.Applied Microbiology. pp. 1727-1733. 62. Kimmel, J. R. and E. L. Smith. 1954. Crystalline papain. I. Preparation, specificity, and activation. J. Biol. Chem. 207: 515-531. 63. Kowalchyk, A. W. and N. F. Olson. 1977. Effect of pH and temperature on the secondary phase of milk clotting by rennet. J. Dairy Sci. 60: 1256-1259. 64. Lagoueyte, N., J. Lablee and B. Tarodo De La Fuente. 1994. Temperature affects microstructure of renneted milk gel. J. Food Sci. 59: 956-959. 65. L.B. Areces, M.J. Biscoglio de Jimenez Bonino, M.A.A. Parry, E.R. Fraile, H.M. Fernandez, O. Cascone. 1992. Purification and characterization of a milk-clotting protease from Mucor baciliformis, Appl. Biochem. Biotechnol. 37:283-294. 66. Lin, C. W., C. H. Hsieh and H. P. Su. 1994. Breaking stress and syneresis of rennin curds from reconstituted skim milk frozen concentrate. J. Food Sci. 59: 952-955. 67. Lodaite, K., K. ?零tergren, M. Paulsson and P. Dejmek. 2001. One-dimensional syneresis of rennet-induced gels. Int. Dairy. J. 10: 829-834. 68. Lucky, J. A. and P. F. Fox. 1993. Importance of calcium and phosphate in cheese manufacture: a review. J. Dairy Sci. 76: 1714-1724. 69. Macedo, I. Q., C. J. Faro and E. M. Pires. 1993. Specificity and kinetics of the milk-clotting enzyme from cardoon toward bovine κ -casein. J. Agric. Food Chem. 41: 1537-1540. 70. Macedo, I. Q., C. J. Faro and E. M. Pires. 1996. Caseinolytic specificity of cardosin, an aspartic protease from the cardoon Cynara cardunculus L.: Action on bovine αs- and β -casein and comparison with chymosin. J. Agric. Food Chem. 44: 42-47. 71. Magda a. EL-Bendary, Maysa E. Moharam and Thanaa H. Ali. 2007. Purification and Characterization of Milk Clotting Enzyme Produced by Bacillus sphaericus. Jurnal of Applied Sciences Research. 3(8):695-699. 72. Marshall, R. J. 1982. An improved method for measurement of the syneresis of curd formed by rennet action on milk. J. Dairy Res. 49: 329-336. 73. Marshall, R. J. and M. L. Green. 1980. The effect of the chemical structure of additives on the coagulation of casein micelle suspensions by rennet. J. Dairy Res. 47: 359-369. 74. McMahon, D. J. and B. H. Yousif. 1993. Effect of whey protein denaturation on structure of casein micelles and their rennetability after ultra-high temperature processing of milk with or without ultrafiltration. Int. Dairy J. 3: 239-256. 75. McMahon, D. J. and R. J. Brown. 1983. Milk coagulation time: linear relationship with inverse of rennet activity. J. Dairy Sci. 66: 341-344. 76. McMahon, D. J., G. H. Richardson and R. J. Brown. 1984. Enzymic milk coagulation: role of equations involving coagulation time and curd firmness in describing coagulation. J. Dairy Sci. 67: 1185-1193. 77. McMahon, J. D. and R. J. Brown. 1984. Enzymatic coagulation of casein micelles: a review. J. Dairy Sci. 67: 919-929. 78. McMahon, D. J., P. A. Savello, R. J. Brown and M. Kalab. 1991. Effect of phosphate and citrate on the gelation properties of casein micelles in renneted ultra-high temperature（UHT）sterilized concentrated milk. Food Struct. 10: 27-36. 79. McMahon, D. J. and W. R. McManus. 1998. Rethinking casein micelle structure using electron microscopy. J. Dairy Sci. 81: 2985-2993. 80. Mickelsen, R. and N. L. Fish. 1970. Comparing proteolytic action of milk clotting enzymes on caseins and cheese. J. Dairy Sci. 53: 704-710. 81. Mitchel, R. E. J., I. M. Chaiken and E. L. Smith. 1970. The complete amino acid sequence of papain. J. Biol. Chem. 245: 3485-3492. 82. M. Ghareib, H.S. Hamdy, A.A. Khalil. 2001. Production of intracellular milk-clotting enzyme in submerged cultures of Fusarium subglutinans. Acta Microbiol. 50:139-147. 83. M.R. Khan, J.A. Blain, J.D.E. Patterson. 1979. Extracellular protease of Mucro pusillus. Appl.Environ. Microbiol. 37:719-724. 84. M.S. Thakur, N.G. Karanth and Krishna Nand. 1990. Studies on the production of microbial rennet by solid state fermentation. Trans. Mycol. Soc. R.O.C.. 5:13-28. 85. M.S. Thakur, N.G. Karanth and Krishna Nand. 1990. Production of fungal rennet by Mucor miehei using solid state fermentation. Applied Microbiology Biotechnology. 32:409-413. 86. M.T.H. Cavalcanti, C.R. Martinez, V.C. Furtado, B.B. Neto, M.F. Teixeira, J.L. Lima Filho and A.L.F. Porto. 2005. Milk-clotting protease production by Nocardiopsis sp. In an inexpensive medium. World Journal of Microbiology & Biotechnology. 21:151-154. 87. Nakajima, H. 1985. Characteristics of fermented milk produced by slime-forming Lactococcus lactis subsp. Cremoris. Snow Brand R&D reports. 104: 97-169. 88. Nelson, J. H. 1975. Symposium: application of enzyme technology to dairy manufacturing. J. Dairy Sci. 58: 1739-1750. 89. Ohashi, T., S. Haga, K. Yamauchi and N. F. Olson. 1982. Effects of pH, calcium and unheated casein micelle on physical properties of high-temperature heated milk rennet curd. Nippon Shokuhin Kogyo Gakkaishi, 29: 70-77. Cited by Hsieh, 1991. 90. Okigbo, L. M., G. H. Richardson, R. J. Brown and C. A. Ernstrom. 1985. Interactions of calcium, pH, temperature, and chymosin during milk coagulation. J. Dairy Sci. 68: 3135-3142. 91. Olson, N. F. 1977. Rheology of milk gels formed by milk clotting enzymes. J. Food Sci. 42: 669-673. 92. Patel, M. C., D. B. Lund and N. F. Olson. 1972. Factors affecting syneresis of renneted milk gels. J. Dairy Sci. 55: 913-918. 93. Payens, A. J. 1979. Casein micelles: the colloid-chemical approach. J. Dairy Res. 46: 291-306. 94. Pearse, M. J. and A. G. Mackinlay. 1989. Biochemical aspects of syneresis: a review. J. Dairy Sci. 72: 1401-1407. 95. Pearse, M. J., P. M. Linklater, R. J. Hall and A. G. Mackinlay. 1985. Effect of heat induced interaction between β -lactoglobulin and κ -casein on syneresis. J. Dairy Res. 52: 159-165. 96. Pearse, M. J., P. M. Linklater, R. J. Hall and A. G. Mackinlay. 1986. Effect of casein micelle composition and casein dephosphorylation on coagulation and syneresis. J. Dairy Res. 53: 381-390. 97. Pei-Jing Yu and Cheng-Chun Chou. 2005. Factors Affecting the Growth and Production of Milk-clotting Enzyme by

Amylomyces rouxii in Rice Liquid Medium. Food technol. Biotechnol. 43(3):283-288 98. Peri, C., E. Pagliarini, S. Iametti and F. Bonomi. 1990. A study of surface hydrophobicity of milk proteins during enzymic coagulation and curd hardening. J. Dairy Res. 57: 101-108. 99. P.A. O ' Leary, P.F. Fox. 1974. A method for the quantitative analysis of the enzyme complement of commerical rennets. 41:381-387. 100. Reed, G. 1975. Enzymes in food processing, pp. 154-156. 2nd ed.Universal Foods Corporation Milwaukee, Wisconsin. 101. Renault, C., E. Gastaldi, A. Lagaude, J.L. Cuq and B. Tarodo de la Funete. 1997. Mechanisms of syneresis in rennet curd without mechanical treatment. J. Food Sci. 62: 907-910. 102. Ruegg, M. and U. Moor. 1984. Effect of calcium on the hydration of casein. I. Water vapour sorption and fine structure of calcium caseinates compared with sodium caseinated in the pH range 4.6-8.0. J. Dairy Res. 51: 103-111. 103. Sardinas, J. L. 1972. Microbial rennets. Adv. Appl. Microl. 15: 39-73. 104. Schmidt, R. H. and H. A. Morris. 1984. Gelation properties of milk proteins, soy proteins, and blended protein system. Food Technol. 38: 85-88. 105. Shammet, K. M., R. J. Brown and D. J. McMahon. 1992. Proteolytic activity of proteinases on macropeptide isolated from κ-casein. J. Dairy Sci. 75: 1380-1388. 106. Shammet, K. M., R. J. Brown and D. J. McMahon. 1992. Proteolytic activity of some milk-clotting enzyme on κ-casein. J. Dairy Sci. 75: 1373-1379. 107. Shalabi, S. I. and P. F. Fox. 1982. Influence of pH on the rennet coagulation of milk. J. Dairy Res. 49: 153-157. 108. Singh, H. and P. F. Fox. 1987. Heat stability of milk: influence of colloidal and soluble salts and protein modification on pH-dependent dissociation of micellar κ-casein. J. Dairy Res. 54: 523-534. 109. Smith, M. and D. J. McMahon. 1996. Aseptic rennet coagulation of ultra-high temperature processed milk concentrates. J. Dairy Sci. 79: 1513-1520. 110. Sousa, M. J., Y. Ard? and P. L. H. McSweeney. 2001. Advances in the study of proteolysis during cheese ripening. Int. Dairy J. 11:327-345. 111. Storry, J. E., A. S. Grandison, D. Millard, A. J. Owen and G. D. Ford. 1983. Chemical composition and coagulating properties of renneted milks from different breeds and species of ruminant. J. Dairy Res. 50: 215-229. 112. Storry, J. E. and G. D. Ford. 1982. Some factors affecting the post clotting development of coagulum strength in renneted milk. J. Dairy Ses. 49: 469-477. 113. Supannee Chitpinityol, Derek Goode & M. James C. Crabbe. 1998. Site-specific mutations of calf chymosin B which influence milk-clotting activity. Elsevier Science. Fppd Chemistry, Vol. 62, No. 2, pp. 133-139. 114. Tamine, A. Y. and H. C. Deeth, 1980. Yogurt: technology and biochemistry. J. Food Prot. 43: 939-977. 115. T. Hosoi, K. Kiuchi, Production and probiotic effects of natto, in: E. Ricca, A.O. Henriques, S. Cutting (Eds.). 2004. Bacterial Spore Formers:Probiotics and Emerging Applications, Horizon Bioscience, Norwich, UK. Chapter 12. 116. T.M. D ' Souza, L. Pereira. 1982. Production and immobilization of a bacterial milk-clotting enzyme. 65:2074-2081. 117. Uchikoba, T. and M. Kaneda. 1995. Milk-clotting activity of cucumisin, a plant serine protease from melon fruit. Appl. Biochem. Biotechnol. 56: 325-330. 118. Walstra, P., H. J. M. van Dijk and T. J. Geurts. 1987. The syneresis of curd. In: P. F. Fox (Ed.). Cheese: Chemistry, Physics and Microbiology. Vol. I, general aspects pp. 135-177. Elsevier Applied Science, London. 119. Yaw-Nan Chang. 2001. Use of response surface methodology to optimize culture medium for production of lovastatin by Monascus ruber. Enzyme and Microbial Technology 30 (2002) 889-894. 120. Y.C. Hung, C.C. Chou. 1997. Growth and milk-clotting enzyme production in submerged culture of Amylomyces rouxii. 35:422-432. 121. Y.C. Su, W.P. Chen. 1970. Studies on milk-clotting enzymes from microorganisms. Part 1. Screening tests and the production of the enzymes. 8:73-83. 122. Z. A. Tubesha and K. S. Al-delaimy. 2003. Rennin-like milk coagulant enzyme produced by a local isolate of Mucor. Society of Dairy Technology. 56:237-241. 123. Zhang, Z. P. and T. Aoki. 1995. Effect of alkaline earth metals on the crosslinking of casein by micellar calcium phosphate. J. Dairy Sci. 78: 1665-1672.