PRODUCTION AND PURIFICATION OF PROTEASE FROM *Bacillus subtilis* AND *Bacillus coagulans* AND USING THE ENZYME FOR IMPROVEMENT AND ACCELERATING DOMIATI CHEESE RIPENING

BY

Neweigy, N.A.*; Abou Aly, H.E.*; Azza A.Ismail,.; Tewfike,T.A.* and Nayl, A.A.***

* Agric. Botany Dept., Fac. Agric. Moshtohor, Zagazig Univ., Egypt

** Food Technology & Dairying Dept., NRC, Cairo, Egypt

ABSTRACT

Two highly protease-producing isolates, identified as *Bacillus subtilis*M14 and *Bacillus coagulans*M53, were selected from sixty isolates of bacteria from different environments for extracellular protease production and used in this investigation. Both strains were studied for their protease activity on seven culture media. The two strains gave maximum protease production on Malik and Mathur medium at 30˚C and pH 7.0 for 5 days. The optimum incubation period for protease production was established after 3-4 days by both two strains. Maximum protease activity and specific activity of protease were obtained through acetone precipitation (60% saturation). Optimum pH for protease activity of *B. subtilis*M14 and *B. coagulans*M53 was found to be 8 and 7, respectively, while optimum temperature for activity of the two proteases were 35 and 30˚C, respectively. Protease from *B.subtilis*M14 retained 65% of its activity after 30 min at 65˚C, while the enzyme from *B. coagulans*M53 retained 45% of its activity after 30 min at the same temperature.

Proteases of the two strains were added to Domiati cheese milk at a rate of 500, 1000 and 1500 U/kg. Obtained results showed that, acidity, Shilovich index, tyrosine, tryptophane and soluble nitrogen as ripening indices were increased in cheese treated with both two enzymes than control. The quality of cheese greatly improved by increasing protease concentration reaching the highest scores (1500 U/kg milk) at the end of the ripening period. From the repining indices results it can be concluded that, protease from both strains can be used for improving the quality of Domiati cheese.

INTRODUCTION

Proteases are enzymes that under appropriate conditions specifically hydrolyze the peptide bonds of protein. Wide varieties of microorganisms are well known to produce extracellular protease (Priest, 1977). The genus of *Bacillus* contains a number of industrially important species and an approximately half of the present commercial production of bulk protease enzymes derives from the strains of *Bacillus* (Beg and Gupta, 2003). These enzymes play an important role in cheese ripening, leather industry, insulin production, meat tenderization and food processing (Poldermans and Gist-Brocades, 1984). Microbial proteinases are used in the dairy industry mainly to accelerate cheese ripening and for the production of enzyme-modified cheese or casein hydrolysates (Godfrey, 1996 and Kilcawley et al, 1998).

Cheese ripening is a complex series of biochemical phenomenon related to enzyme concentration that enhanced the flavour development of cheese by hydrolysis of casein (El-Soda and Saada, 1986). Proteolysis is one of the most important phenomena that takes place during cheese ripening (Fox, 1989) and contributes directly to the texture and flavour of the matured cheese. The cheese conditions through the hydrolysis of the proteins increases the pH and increase in the retention of water by the amino and carboxyl groups that are formed during the protein degradation. The contribution to the flavour takes place by increasing the free amino acids and peptides. In its turn, the amino acids can catabolize forming amines, acids, thiols, thioesters, etc.

The aim of this work is to study the production, purification and some characteristics of protease from some proteolytic bacterial strains, using the produced enzyme to accelerate Domiati cheese ripening.

MATERIALS AND METHODS

Microorganisms:

Two bacterial isolates were selected from sixty isolates (from Egyptian environments) for their protease production by selective screening on skim milk agar plates. They were identified according to Bergy's Manual of Systematic Bacteriology (1984). The methods used for identification were performed according to methods of

Lelliott and Stead (1987) and Collins *et al*. (1995). The stock cultures of the two strains were maintained on nutrient agar at 5 C and transferred monthly.

Media:

The following media were used:

Modified Luria broth (Zamost *et al*., 1990) [yeast extract 0.5%, tryptone 1%, $CaCl_2$ 0.037% and dextrin 1%]; **Malik and Mathur (1984) medium** [yeast extract 0.25%, tryptone 1%, KH_2PO_4 0.1% and K_2HPO_4 0.1%]; **Tryptone soy broth medium** (Oxoid) [tryptone 1.7%, soytone 0.3% NaCl 0.5%, K_2HPO_4 0.25% and dextrose 0.25%]; **Sidler and Zuber (1977) medium** [beet extract 0.3%, tryptone 0.2%, maltose 0.3%, NaCl 0.1%, NH_4Cl 0.1%, $CaCl_2$ 0.018% and $FeCl_3$ 0.0003%]; **Routine medium**, El-Shafei and Rezkallah (1998) [bactpeptone 0.5%, yeast extract 0.2%, Difco casein 0.2%, glucose 0.2% and K_2HPO_4 0.07%]; **Sweet whey medium** El-Shafei and Rezkallah (1998) [lactose 0.41-0.52%, fat 0.03-0.05%, protein 0.018-0.11% and total solids 0.8-1.0%] and **Salted whey medium**, Shady and Abdel-Razik, 1996 [skim milk 0.2% NaCl 0.5% and whey from Domiati cheese manufactured]. Each medium was adjusted to pH 7.0 and sterilized.

Protease production:

One hundred milliliter of the medium in 500-ml Erlenmeyer flasks was inoculated with 3 ml of a 48h old culture and incubated at 30˚C up to 7 days intervals. The culture was centrifuged to remove cells and the supernatant was assayed for protease activity.

Protease assay:

Protease activity was determined by the method of Keay and Wildi (1970). The reaction mixture consisting of 1 ml diluted enzyme and 1 ml of casein solution for 10 min, then the reaction was stopped by the addition of 2.0 ml 24% trichloroacetic acid. After 30 min at room temperature, the undigested casein was removed by filtration. The amount of trichloroacetic acid-soluble casein breakdown fragments were determined using the method of Hindazlothink *et al*. (1983). One unit of protease activity was defined as the amount of enzyme required to release trichloroacetic acid-soluble casein fragments giving blue colour equivalent to 1.0 µg

of tyrosine per min under conditions of the assay. Protein assay was determined according the method of Lowry *et al.* (1951).

Purification of the protease:

Acetone was added to the supernatant to give final concentration ranging from 30-80%. The mixture was left overnight at 4˚C for precipitation then, centrifuged, resuspended in 0.05M tris-HCl buffer, (pH 7.5) and dialyzed against the same buffer. The supernatant was concentrated 10 fold. The concentrated enzyme was applied to a sephadex G-100 column (2.5 X 70 cm) which had been equilibrated with 0.05M tris-HCl buffer, pH 7.5. The column was eluted with the same buffer and 5 ml fractions were collected (Jensen *et al*., 1980). Protease activity and protein content were determined and the purified enzyme was used for further studies.

Effect of pH:

The optimum pH of protease activity was determined over a pH range 5-10 with casein as substrate.

Effect of temperature:

The enzyme solution at pH 7.5 was incubated at different temperatures (30-70˚C) for 20 min to obtain the optimum temperature of protease activity. Thermal stability of protease was determined by exposition enzyme solution to 65˚C in a water bath at different times (15-120 min). The residual activity was measured.

Cheese manufacture:

The milk was pasteurized at 65˚C for 30 min. Pasteurized milk was then salted with 8% NaCl. The rennet was added at the rate of 1 ml/ kg milk. The milk was divided into 4 portions. The first one was served as a control, the other three potions were treated with the protease enzyme for cheese making at the rate of 500, 1000 and 1500 units/kg milk. The resultant curd was left to drain then the cheese was cut and stored.

Cheese analysis:

Cheese samples were analyzed when fresh and after 7, 14, 21, 28, 35 and 42 days intervals for moisture, titratable acidity, total and soluble nitrogen as described

by Ling (1963). Shilovich numbers were determined by the method of Abd El-Tawab and Hofi (1966). Method of Vakaleries and Price, 1959 was used to determine soluble tyrosine and tryptophane.

RESULTS AND DISCUSSION

Isolation and identification of microorganisms:

From sixty bacterial isolates, two isolates designated as M14 and M53 showed higher hydrolysis of casein on skim milk agar plates. The two isolates were selected for further study. From the morphological appearance, staining properties, growth under anaerobic conditions and physiological characteristics as described by the methods of Lelliott and Stead (1987) and Collins *et al*. (1995), which present in table(1) cleared identified that the two bacterial isolates were belong to *Bacillus subtilis* and *Bacillus coagulans*.

Table (1): Taxonomic properties of the two protease producing isolates.

The test	Isolate (M14)	Isolate (M53)	The test	Isolate (M14)	Isolate (M53)
Form	Rod shape	Rod shape	**Gas formation**		
Gram stain	+	+	Glucose	+	+
Spore formation	+	+	Sucrose	+	+
Anaerobic growth	-	+ facultative	Maltose	+	+
Growth at NaCl			Ethanol	-	+
5%	+	-	**Hydrolysis of**		
7%	+	-	Casein	+	+
10%	-	-	Gelatin	+	-
Acid formation			Starch	+	+
D-glucose	+	+	**Growth at**		
L-arabinose	+	-	5˚C	-	-
D-mannitol	+	-	10˚C	-	-
D-xylose	+	-	30˚C	+	+
Starch	+	+	40˚C	+	+
Growth at pH			50˚C	-	+
6.8	+	+	55˚C	-	+
5.7	+	+	65˚C	-	-

Effect of media:

The effect of different media on the enzyme production by *Bacillus subtilis*M14 and *Bacillus coagulans*M53 are presented in Table (2). The highest protease specific activity was found when both bacterial strains were grown on medium of Malik and Mathur (1984) at 30˚C and pH 7.0 for 5 days. This medium was followed by routine medium for *B. subtilis*M14 and Trypton Soy broth medium for *B.*

coagulans M53. These results are in accordance with Abd El-Hafez (1994) and El-Shafei and Rezkallah (1998) who used the previous media for protease production.

Table (2): Effect of different media on protease production by the two *Bacillus* strains.

Culture media	*B. subtilis* M14			*B. coagulans* M53		
	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
M1	66.551	0.3608	184.45	68.09	0.2577	264.22
M2	65.507	0.0859	762.59	55.940	0.0775	721.80
M3	68.945	0.3256	211.75	109.13	0.3672	297.19
M4	24.143	0.3736	64.62	37.823	0.6648	56.893
M5	50.135	0.0721	695.35	49.793	0.2667	186.68
M6	28.589	0.5048	56.634	27.221	0.4152	65.56
M7	34.061	0.6224	54.725	18.329	0.3051	60.07

M1: modified Luria broth medium. M2: Malik and Mathur medium.
M3: Tryptone soy broth medium. M4: Silder and Zuber medium.
M5: Routine medium. M6: Sweet whey medium.
M7: Salted whey medium.

Effect of incubation period:

Protease production by *B. subtilis*M14 and *B. coagulans*M53 grown in Malik and Mathur (1984) medium at different incubation periods was studied. (Table 3). The maximum protease production for the two strains of Bacillus was obtained after 3 days of incubation, followed by gradual slow decrease after 4 and 5 days, while it was sharply decreased after 6 days of incubation. These results are similar to those obtained by El-Bendary *et al.* (2002) who found that maximum enzyme levels were obtained from *Bacillus sphaericus* after 3 days of incubation.

Table (3): Effect of time-course on protease production by the two *Bacillus* strains grown on Malik and Mathur medium.

Cultivation time (days)	*Bacillus subtilis*M14			*Bacillus coagulans*M53		
	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg).	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg).
2	53.70	0.103	521.35	18.9	0.067	282.08
3	66.53	0.087	764.71	60.4	0.082	736.58
4	59.97	0.084	713.92	60.2	0.082	734.14
5	56.45	0.091	620.33	57.6	0.081	711.10
6	33.20	0.134	247.76	25.7	0.081	317.30
7	10.66	0.120	88.83	6.77	0.066	102.57

Protease precipitation:

The protease enzyme was precipitated from *B. subtilis*M14 and *B. coagulans*M53 cultures supernatant using acetone fractionation. The data presented in

Table (4) indicates that the highest protease activity, specific activity and recovery were at the 60% saturation of acetone for both supernatants of culture strains. Abd El-Hafez (1994) found that 55% (v/v) acetone was the best level for enzyme recovery.

Table (4): Effect of different concentrations of acetone on precipitation process of proteolytic enzyme from two *Bacillus* strains.

Acetone %	*B. subtilis* M14				*B. coagulans* M53			
	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Recovery %	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Recovery %
30	70.297	0.5078	138.423	18.09	66.449	0.3926	169.853	23.52
40	62.516	0.3772	156.737	20.48	73.717	0.2902	254.021	35.18
50	67.817	0.3414	198.620	25.96	71.152	0.277	256.866	35.57
60	73.289	0.1980	370.146	48.38	76.282	0.2032	375.403	51.99
70	66.449	0.3952	168.140	21.97	67.646	0.244	276.557	38.85
80	70.297	0.3952	177.877	23.25	72.007	0.321	224.32	31.06

Effect of pH on protease activity:

The activity of protease produced by *B. subtilis*M14 and *B. coagulans*M53 at different pH values was presented in Table (5). The activity of both proteases of *B. subtilis*M14 and *B. coagulans*M53 strains was increased as the pH increased reaching the maximum at pH 8.0 and 7.0, respectively. Further increase in pH caused a sharp decline in the enzyme activity of both strains. These results are similar to those reported by Ghorbel *et al*. (2003) who found that the optimum pH for protease activity from *Bacillus cereus* was 8.0. On the other hand, Ali, (1994) mentioned that the maximum protease activity was at pH 7.5 for *Bacillus subtilis* protease.

Table (5): Effect of different pH values on protease activity by the two strains of *Bacillus*.

pH	*B. subtilis* M14			*B. coagulans* M53		
	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
5	28.93	0.1281	225.83	18.31	0.2022	90.55
6	32.47	0.1046	310.42	26.22	0.1453	180.45
7	65.50	0.0858	763.51	56.67	0.0784	722.83
8	66.94	0.0874	765.90	56.58	0.0785	720.76
9	34.52	0.0929	371.58	28.31	0.1284	220.48
10	25.31	0.1942	130.32	20.52	0.1275	160.94

Effect of temperature:

Table (6) shows the optimum temperature for proteases produced by *B. subtilis*M14 and *B. coagulans*M53. It could be noticed that, the proteases of both

strains gave similar activity at most of the incubation temperatures selected in this study. The optimum temperature for both protease activities was 30-35˚C. At temperature above 40˚C, the proteolytic activity for the two proteases markedly decreased. At higher temperature, the activity for both proteases decreased. These results are in agreement with those reported by El-Shafei and Rezkallah, 1998 and Zin El-Din and Shady, 1998.

Table (6): Effect of different temperatures on protease activity by the two strains of *Bacillus.*

Temperature ˚C	***B. subtilis* M14**			***B. coagulans* M53**		
	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
20	29.37	0.1047	280.51	33.57	0.1121	299.416
30	62.73	0.0823	762.21	56.46	0.0783	721.07
35	64.32	0.0839	766.62	50.39	0.0718	701.81
40	52.81	0.0776	680.54	42.42	0.0771	550.19
45	46.34	0.1152	402.25	31.97	0.1229	260.13
50	27.43	0.1052	260.74	27.14	0.1284	211.37
55	27.42	0.1053	260.39	20.98	0.1895	110.71
60	25.98	0.1441	180.29	28.39	0.279	101.75
65	3.965	0.4023	9.855	9.71	0.629	15.437
70	0.358	0.4092	0.874	1.150	0.598	1.923

Heat stability:

The heat stability of protease of the two strains is presented in Fig. (1). After heating at 65˚C for different periods (15, 30, 60, 90 and 120 min), the activities of the enzymes slightly decreased, reached to 70% for *B. subtilis*M14 and 50% for *B. coagulans*M53, when they were heated for 15 min at 65˚C, while treatment at the same temperature for 30 min markedly decreased the activity. In addition, the sharp decrease in the activity of both proteases was noticed after 60 min *B. subtilis*M14 protease maintained with activity higher than that noticed for the protease of *B. coagulans*M53. The obtained results are in agreement with those reported by Ali (1994) who found that *B. subtilis* protease retained 75% of their maximal activity after 30 min at 62.5˚C.

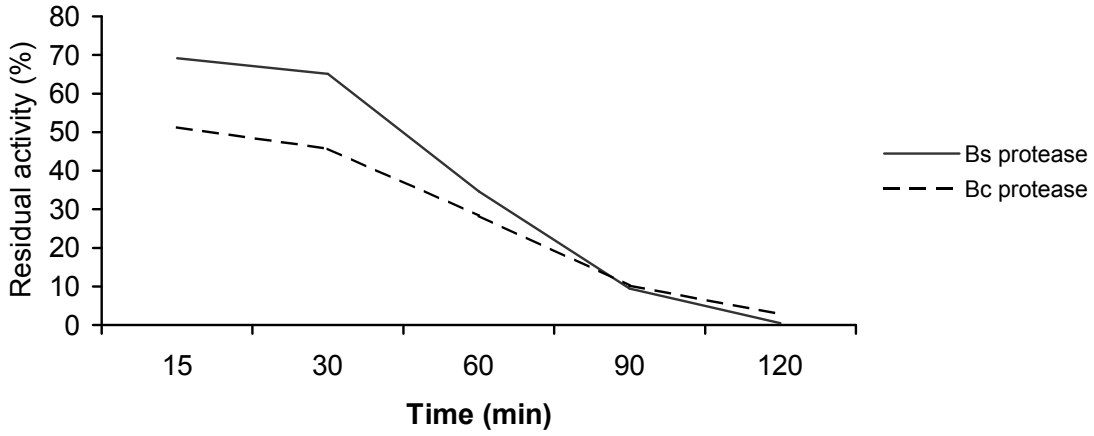

Fig.(1): Thermal stability of protease from two *Bacillus* strains.
Bs: *B. subtilis* M14. Bc: *B. coagulans*M53.

Accelerating of Domiati cheese ripening with protease:

It is shown in Table (7) that the moisture content of Domiati cheese treated with the two proteases or control one decreased gradually. It could be remarked from the obtained data that, addition of protease during manufacture of Domiati cheese had no effect on moisture content. In addition, data in Table (7) shows that, titratable acidity and Shilovich index (as protein breakdown indicator) increased gradually throughout the storage period. Addition of *B. subtilis*M14 protease increased titrable acidity and Shilovich index more than that noticed by protease of *B. coagulans*M53 treatment. Acidity and Shilovich index increased by increasing protease concentration up to the highest concentration (1500 U/kg milk).

Changes in tyrosine, tryptophane, soluble nitrogen /total nitrogen and soluble nitrogen/dry weight (as a measure of proteolysis during ripening) of Domiati cheese treated with protease of the two strains are presented in Tables (8 and 9). The obtained results indicate that, Domiati cheese treated with the two proteases had higher proteolysis than the control. In general protease of *B. subtilis*M14 increased proteolysis of Domiati cheese more than that obtained by protease of *B. coagulans*M53, while proteolysis by both protease treatments were much faster than in the control. Generally, these results are in agreement with those obtained by Ali (1994) and El-Sawah and Zin El-Din (2000). In conclusion, protease from *B. subtilis* and *B. coagulans* can be used to accelerate cheese ripening and to improve the quality of Domiati cheese.

REFERENCES

Abd El-Hafez, A.A. (1994): Production of some microbial enzymes. M.Sc. Thesis Fac. Agric. Ain Shams Univ., Cairo, Egypt.

Abd El-Tawab, G.A. and Hofi, A.A. (1966): Testing cheese ripening by rapid chemical technique. Indian J. Dairy Sci., 19:39.

Ali, A.A. (1994): Accelerating cheese ripening with bacterial proteinases. Ph. D. Thesis Fac. Agric. Ain Shams Univ., Cairo, Egypt.

Beg, Q.K. and Gupta, R. (2003): Purification and characterization of an oxidation-stable, thiol- dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme and Microbial Technology, 32: 294-304.

Bergey's Manual of Systematic Bacteriology (1984): Volume II Williams and Wilkins Co., London.

Collins, C.H.; Lyne P.M. and Grange J.M. (1995): "Microbiological Methods". Butterworth-Heinemann Ltd. Linacre House, Jordan Hill, Oxford OX2 8DP.

El-Bendary, M.A.; Moharam, M.E. and Foda, M.S. (2002): Studies on production of alkaline serine protease from *Bacillus sphaericus*. J. Agric. Sci., Mansoura Univ., 27(2): 1231-1246.

El-Sawah, M.M.A. and Zin El-Din, M. (2000): Production and properties of thermostable alkaline protease from *Bacillus licheniformis* and its importance in Domiati cheese ripening. Annals Agric. Sci., Ain Shams Univ., Cairo, 45(1):113-127.

El-Shafei, H.A. and Rezkallah, L.A. (1998): Production and properties of alkaline protease by *B. thuringiensis* var *alesti*. Egypt. J. Microbiol., 33(4):541-556.

El-Soda, M. and Saada, M.(1986): Accelerate ripening of Egyptian cheese varieties (A review). Egypt. J. Dairy Sci., 14:115.

Fox, P.F. (1989): Proteolysis during cheese manufacture and ripening. J. Dairy Sci., 72:1379-1400.

Ghorbel, B.; Kamoun, A.S. and Nasri, M. (2003): Stability studies of protease from *Bacillus cereus* BGI. Enzyme and Microbial Technology 32:513-518.

Godfrey, T. (1996): Protein Modification. In Industrial Enzymology, 2nd Ed., Godfrey, T., West, S., Eds. Macmillan Press, London. 301-325.

Hindazlothink, Y.; Hiroshi, I.; Shigek, M. and Shinichi, I. (1983): Purification and flurometric assay of proteinases A from yeast. Annal. Biochem., 134(1):210.

Jensen, S.E.; Fecyez, I.T. and Cample, J.N. (1980): Nutritional factors controlling extracellular protease production by *Pseudomonas aeroginosa.* J. Bacteriology 144:844-847.

Keay, L. and Wildi, B.S. (1970): Protease of genus *Bacillus* L.Neutral protease.Biotech. Bioeng., 12:179.

Kilcawley, K.N.; Wilkinson, M.G. and Fox, P.F. (1998): Enzyme modified cheese. Int. Dairy J., 8:1-10.

Lelliott, R.A. and Stead, D.E. (1987): Methods for the Diagnosis of Bacterial Diseases of Plants in: Methods in plant pathology. Ed. Preece, T.F., Blackwell Scientific Publications, Oxford, London.

Ling, E.R. (1963): A Text Book of Dairy Chemistry. Vol.2, Practical (3rd Ed.), Chapman&Hall, LTD., London, England, pp. 58-65.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951): Protein measurement with folin phenol reagent. J. Biol. Chem., 193:265-270.

Malik, R.K. and Mathur, D.K.(1984): Purification and characterization of a heat-stable protease from *Pseudomonas*. J. Dairy Sci., 67:522-530.

Poldermans, B. and Gist-Brocades, N.V. (1984): Proteolytic enzyme. P.108-118. (Gerhartz, W. ed.). Enzyme in industry, production and application. VCH. Publishers.

Priest, F.G. (1977): Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriological Review. 41:711-754.

Shady, T.S.M. and Abdel-Razik, M.B. (1996): Utilization of whey for protease biosynthesis by some bacterial strains. J. Agric. Sci., Mansoura Univ., 21(11):4045-4053.

Sidler, W. and Zuber, H. (1977): The production of extracellular thermostable neutral proteinases and alpha amylase by *B. stearothermophilus* grown at 40ºC and at 50ºC. FEBS Lett. 25:292-294.

Vakaleries, D.G. and Price, W.V. (1959): A rapid spectrophotometric method for measuring cheese ripening. J.Dairy Sci., 42(b):264-268.

Zamost, B.L.; Brantly, Q.I.; Elm, D.D. and Bek, C.M. (1990): Production and characterization of thermostable protease produced by an asporogenous mutant of *B. stearothermophilus*. J. Indust., Microbiol., 5:303-312.

Zin El-Din, M. and Shady, T.S.M. (1998): Purification and some properties of *Pseudomonas fluorescens* protease produced on Domiati cheese whey medium. J. Agric. Sci. Mansoura Univ.

إنتاج وتنقية إنزيم البروتييز من *Bacillus subtilis* و *Bacillus coagulans* واستخدامه فى تحسين وإسراع تسوية الجبن الدمياطى

نسيم عبد العزيز نويجى*–حامد السيد أبوعلى*–عزة عبد العزيز اسماعيل–طه عبده توفيق*–أحمد عبد الخالق نايل***

* قسم النبات الزراعى– كلية الزراعة بمشتهر– جامعة الزقازيق/فرع بنها– مصر

** قسم الصناعات الغذائية والألبان– المركز القومى للبحوث– القاهرة – مصر

تم اختيار عزلتين متفوقتين فى إنتاج إنزيم البروتييز من بين ٦٠ عزلة بكتيرية معزولة من بيئات مختلفة وتم تعريفهما على أنهما *Bacillus subtilis* M14 و *Bacillus coagulans* M53 وتم استخدامهما لإنتاج إنزيم البروتييز ودراسة بعض خواصه واستخدامه فى تحسين وإسراع تسوية الجبن الدمياطى, وكانت النتائج كالتالى:

كلا السلالتين تم تنميتهما على سبعة بيئات مختلفة لاختيار أفضلها فى إنتاج إنزيم البروتييز, ولقد كانت بيئة Malik and Mathur أفضل بيئة فى إنتاج الإنزيم لكلا السلالتين. كما وجد أن أنسب وقت لإنتاج البروتييز كان بعد ٣ و ٤ أيام من نمو الميكروب على البيئة السابقة. ولقد أمكن الحصول على أقصى تنقية للإنزيم عند ٦٠% تشبع بالأسيتون لكل من الإنزيمين.

وقد وجد أن أقصى نشاط للإنزيم تم الحصول علية على درجة تركيز أيون الهيدروجين ٨ و ٧ لكلا الإنزيمين من السلالتين *B. subtilis*M14 و *B. coagulans*M53 على التوالى. بينما وجد أن درجات الحرارة ٣٥° و ٣٠° م هى الأنسب للحصول على أقصى نشاط لكلا الإنزيمين على التوالى. كما وجد أن الأنزيم المنتج من *B. subtilis*M14 احتفظ ب ٦٥% من نشا طه عند تعرضه لدرجة حرارة ٦٥° م لمدة ٣٠ ق بينما وجد أن البروتييز من *B. coagulans*M53 أحتفظ ب ٤٥% من نشاطه عند تعرضه لنفس درجة الحرارة لنفس المدة.

ولقد تم معاملة اللبن المستخدم فى تصنيع الجبن الدمياطى بكلا الإنزيمين بمعدلات ٥٠٠ و ١٠٠٠ و ١٥٠٠ وحدة/ كجم لبن. وقد أدت المعاملة بهذين الإنزيمين إلى زيادة فى الحموضة ومعامل التسوية فى الجبن الناتج. كما أدت إضافة الإنزيم إلى زيادة فى الحمضيين الأمينيين التيروسين والتربتوفان وكذلك فى النيتروجين الذائب بالنسبة للنيتروجين الكلى أو للوزن الجاف فى الجبن الناتج عن الكنترول.

أيضا تحسنت جودة الجبن الدمياطى بزيادة تركيز الإنزيم لكلا الإنزيمين حتى وصلت أعلاها مع تركيز ١٥٠٠ وحدة / كجم لبن. ومن هذا توصى الدراسة باستخدام هذين الإنزيمين لتحسين جودة الجبن الدمياطى والإسراع فى تسويته.