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ISOLATION AND IDENTIFICATION OF PROTEOLYTIC BACTERIA FROM RAW MILK SAMPLES

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ABSTRACT
Twenty five raw milk samples were collected in and around Kancheepuram and processed to identify the extent of contamination by methylene blue reduction test. The samples were subjected for identification of the number of bacterial contaminants by standard plate count method. The similar predominant colonies from each plate were isolated and identified using routine bacteriological technique. Bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Micrococcus luteus* and *Serratia marcescens* are identified. All those bacterial isolates were subjected for the identification of proteolytic activity. Bacteria such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Micrococcus luteus* and *Serratia marcescens* are identified as proteolytic organisms. Raw milk was contaminated by bacteria and other microorganism. Sources of the contaminations were utensil, handlers, udders and teats of cow and buffalos. Proper cleaning of the vessels and cleaning of the udder and teats of the cow and buffalo minimize the contamination. Water is the source of contamination. Pure water is used to clean the vessel and processing machines. Protein is the major component in the milk. Proteolytic bacteria affect the nutritive value of milk. Psychrotrophic proteolytic bacteria survive in the milk during low temperature storage. Spore forming proteolytic bacteria resist at high temperature. Proteolysis is generally affecting the nutritive value of milk. So care must be taken to avoid proteolysis in each and every step involving collection transportation, storage etc., of milk. Proper pasteurization of milk destroys microorganisms in milk. Avoidance of post pasteurization contamination with microorganism improves the quality of milk. Proteolytic bacteria affect nutritive value of milk and lead to cause health hazards.

KEYWORDS: methylene blue, bacteriological technique, proteolytic bacteria, health hazards.

INTRODUCTION

Milk is one of the widely consumed products. Milk is highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable temperatures. It is necessary to use very great care in the collection and handling of milk samples to prevent any extraneous contamination and to control the growth of organisms during transportation and storage of the milk. Milk is the first food served on the earth, the most satisfactory single food substances elaborated by nature. It is the one food for which there seems to be no adequate substitute. Milk is a complex mixture of carbohydrates, proteins, lipids and other organic compounds and inorganic salts dissolved (or) dispersed in water, (Srilakshmi, 1999). In addition milk is extremely easily digested as is shown by the fact that it forms the stable article of diet for infants. Milk contains number of proteins such as alpha-s-casein, beta-casein and kappa-casein, also alpha lactalbumin and beta lactoglobulin, which are synthesized in the modified sebaceous glands *i.e.* in the mammary gland major proteins are transferred from blood to milk such as immunoglobulins and serum albumin (Muean Aslam and Walter Hurley, 1996). There are seven types of bacteria which changes the properties of milk. Psychrotrophic microorganisms are able to grow at temperature below 7ºC. They are often proteolytic and lipolytic. They include species of *Micrococci, Bacilli, Staphylococci, Lactobacilli, Pseudomonas and Coliforms.* Spores forming bacteria can withstand greater extremes of acidity, temperature and desiccation. Enzymes are biological catalysts that accelerate the rate of biochemical reactions. Bacterial enzymes are most significant to milk spoilage and cheese ripening. Psychrotrophic bacteria produce heat stable enzymes. Proteases are a type of enzyme which act on proteins and cause their breakdown to produce smaller fragments. There are several different types of proteases present in the milk. Which are derived from somatic cells in the milk (Muean Aslam and Walter Hurley, 1996). Some proteases are secreted is an inactive from by autolysis (or) by limited proteolysis by another protease. Milk is one of the widely consumed products, that why it is called it is 'Liquid Diamond'. It is an excellent culture medium for growth and reproduction of microorganisms. Such balanced diet milk becomes contaminated with several types of microorganisms, which originate in the soil, water (or) skin and the hair of the animals (or) milk maiders. Temperature plays a vital role in the spoilage of milk. Microorganisms such as psychrotrophs may grow at refrigeration temperature 7°C. They are distributed in diversified habitats, as water, soil, utensils and vegetation. When the milk is stored under low temperature it gets contaminated and frequently

undergoes spoilage due to proteinases and lipases released by the microbes present in the milk *i.e*. psychrotropic bacteria. The presence of these organisms in milk indicated not only unsanitary conditions, but also the yard stick to measure the quality of the products. The psychrotrophs are readily killed by HTST pasteurization their extra cellular enzymes are heat stable to varying degrees when sufficient activity may remain to degrade the fats and proteins of milk. The psychrotropic spore formers are to be one of the food poisoning agents in dairy products, which is isolated from pasteurized milk. It is believed that contamination takes place after pasteurization from equipment, cans, bottles and water. They are cold loving bacteria being capable of multiplying at 5°C and below regardless of their optimum temperature. They are therefore capable of growing at refrigeration temperature and are primarily responsible for limiting the keeping quality of milk and many milk products in which they may produce a wide variety spoilage defects.The defects may result in the production of many off-flavors which are characterized as fruity, stale, musty, bitter, rancid and even putrid. They are most commonly encountered as members of the genera *Achromobacter, Aerobacter, Alcaligenes, Escherichia, Flavobacterium, Pseudomonas,* and *Vibrio.* The general consensus that *Pseudomonas* is the most commonly encountered and this is true not only for milk products but also for meat, fish, poultry and eggs. An attempt has been made to throw light on the following aspects.

To check the quality of milk, to enumerate the bacteria in milk, to isolate and identify the bacteria in milk, and to identify the proteolytic activity of bacterial isolates.

MATERIALS & METHODS

Sample Collection (Bhattacharyya, 1986), Methylene blue reduction test is based on the method of Bhattacharyya, 1986, Standard plate count (Bhattacharyya, 1986), Isolation of Specific bacteria (Bhattacharyya, 1986), Proteolysis (Cappuccino and sherman, 1999), Grams staining (Sundararaj, 2002).

Motilyty test (Sundararaj, 2002).

Catalase test (Sundararaj, 2002).

Oxidase test (Sundararaj, 2002).

Methyl red voges - proskauer test (Sundararaj, 2002).

Citrate utilization test (Sundararaj, 2002).

Urease test (Sundararaj, 2002).

Indole test (Sundararaj, 2002).

Coagulase test (Sundararaj, 2002).

Carbohydrate fermentation test (Sundararaj, 2002).

RESULTS

Results on analysis of quality of milk samples by methylene blue reduction test are shown in Table 1. Among 25 raw milk samples analyzed 4 samples were identified as poor grade and remaining 21 samples were found to be fair quality. Results on analysis of raw milk samples by methylene blue reduction test are shown in Figure 1-6. Results on the enumeration of bacteria in 25 different milk samples are given in Table 2, among which 12 samples showed TNTC at 10^{-1} dilutions. Bacterial colonies of 69 to 98 were observed in remaining samples. Numbers of bacterial colonies were reduced in all milk samples at 10^{-2} dilutions, which varied from 9 to 205. The highest number of bacterial colony in 10^{-3} dilutions was 122. These bacterial colonies were counted in sample number 13. Lowest number of bacterial colony in 10^{-3} dilutions was 4 in first sample. Dilution range of 10^{-4} and 10^{-5} showed least number of colonies. Result on the enumeration of bacteria in milk sample is given in figure II. Results on the identification of different bacterial isolates in raw milk samples by biochemical reaction are given in Table 3. Morphology of *Escherichia coli* bacterial colony is shown in Figure 3. It showed metallic sheen in Eosin methylene blue agar. Red pigment produced by *Serratia marcescens* in Nutrient agar plate is shown in Figure 4. Totally 7 different bacterial species were isolated from different raw milk samples and they have showed different proteolytic activities as shown in Table 4. Proteolytic activity of *Pseudomonas* in skim milk agar is shown in Figure 5 and 7.

TABLE 1: Methylene blue reduction test for milk sample

Sample No.	Reduction time (hours)	Grade
1	4	Fair
2	30 Minutes	Poor
3	20 Minutes	Poor
4	3½	Fair
5	3½	Fair
6	2½	Fair
7	2	Fair
8	2	Fair
9	2	Fair
10	3½	Fair
11	3	Fair
12	2	Fair
13	22 Minutes	Poor
14	4	Fair
15	2	Fair
16	3½	Fair
17	3	Fair
18	3	Fair

19	20 Minutes	Poor
20	3	Fair
21	3	Fair
22	2	Fair
23	4	Fair
24	3½	Fair
25	2	Fair

TABLE II: Standard plate count of milk samples

Sample No.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1	80	9	4	3	1
2	TNTC	196	82	40	25
3	TNTC	172	65	32	24
4	94	13	8	5	3
5	98	17	7	5	2
6	TNTC	186	76	45	22
7	TNTC	161	48	35	15
8	TNTC	176	68	54	26
9	TNTC	181	64	56	19
10	69	25	11	5	3
11	76	23	16	3	1
12	TNTC	151	45	26	14
13	TNTC	205	122	64	47
14	64	47	29	7	3
15	TNTC	196	89	47	32
16	90	47	23	7	2
17	85	45	20	6	4
18	92	48	23	7	2
19	TNTC	174	78	56	29
20	76	44	32	6	2
21	83	39	26	5	3
22	TNTC	196	79	36	22
23	89	46	27	6	1
24	83	44	22	5	1
25	TNTC	186	87	29	16

TNTC – Too Numerous To Count

10^{-1}, 10^{-2}, 10^{-3} ,10^{-4} ,10^{-5} - Dilution factors

More than 8 hours	-	Excellent
6 – 8 hours	-	Good
2 – 6 hours	-	Fair
Less than 2 hours	-	Poor

TABLE 3: Results on biochemical characteristics of bacterial isolates

	Indole	MR	VP	Citrate	Cogulase	Catalase	Sugar Fermentation					Urease	Oxidase	Gram Reaction	Motility
Bacteria							M	G	L	MAN	S				
Staphylococcus aureus	-	+	+	-	+	+				+		+	+	Gram positive cocci	Non motile
Pseudomonas aeruginosa	+			+		+		+					+	Gram Negative rod	Motile
Bacillus cereus	-		+			+		+		-				Gram Positive rod	
Proteus mirabilis	-	+	-	+	-	+	-	+	-	-	-	+	-		Motile
Serratia marcescens			+	+											
Micrococcus luteus				-		+		-	-						

MR – Methyl Red
VP – Voges Proskauer
M – Maltose
G – Glucose
L – Lactose
MAN – Mannitol
S – Sucrose
+ – Positive
- – Negative

TABLE 4: Proteolytic activities of isolates in skim milk agar

S.No.	Microorganism	Proteolytic activity
1	*Escherichia coli*	Negative
2	*Staphylococcus aureus*	Negative
3	*Bacillus cereus*	Positive
4	*Pseudomonas aeruginosa*	Positive
5	*Proteus mirabilis*	Positive
6	*Serratia marcescens*	Positive
7	*Micrococcus luteus*	Positive (Slow proteolytic)

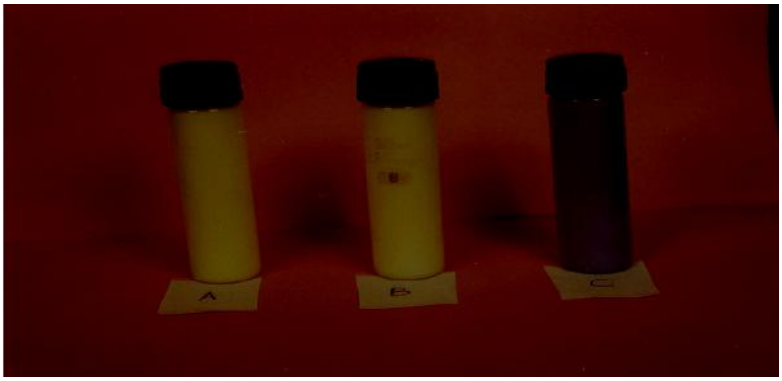

FIGURE 1: Methylene blue reduction test for raw milk sample
A – Sample - Showing disappearance of methylene blue dye
B – Positive Control - Showing disappearance of methylene blue dye
C –Negative Control – Showing no color change

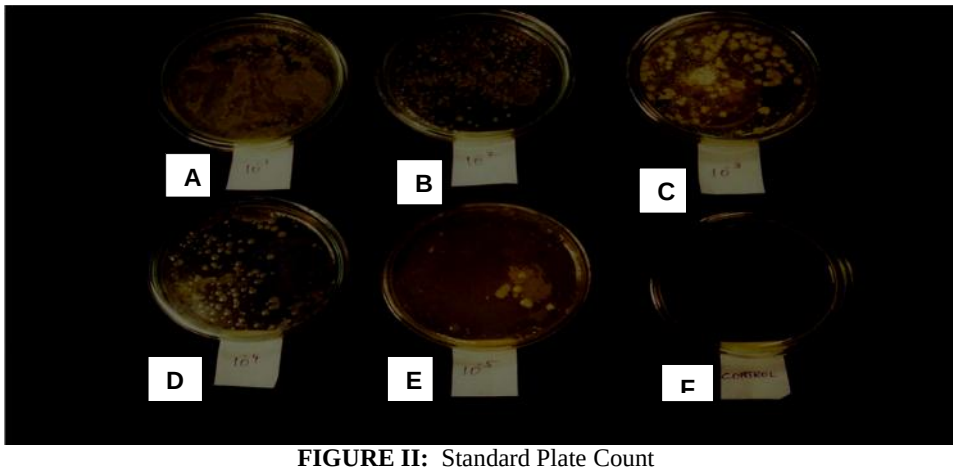

FIGURE II: Standard Plate Count
A - 10^{-1} dilution factor, B - 10^{-2} dilution factor, C - 10^{-3} dilution factor, D - 10^{-4} dilution factor, E - 10^{-5} dilution factor
F – Control Plate

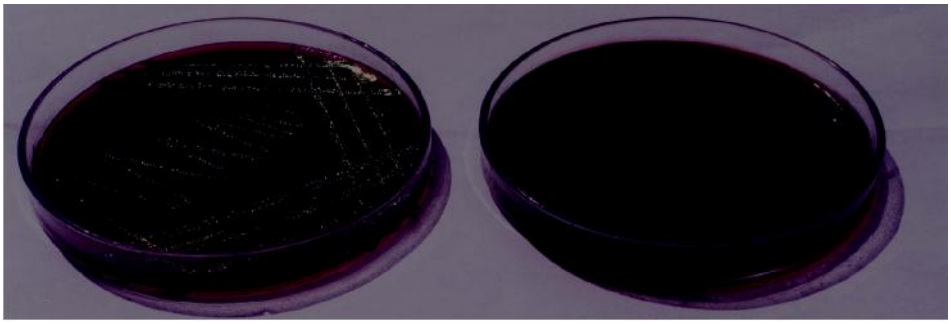

FIGURE 3. *Escherichia coli* in Eosin methylene blue agar plate
A – Showing metallic sheen colonies produced by *Escherichia coli*
B – Control plate

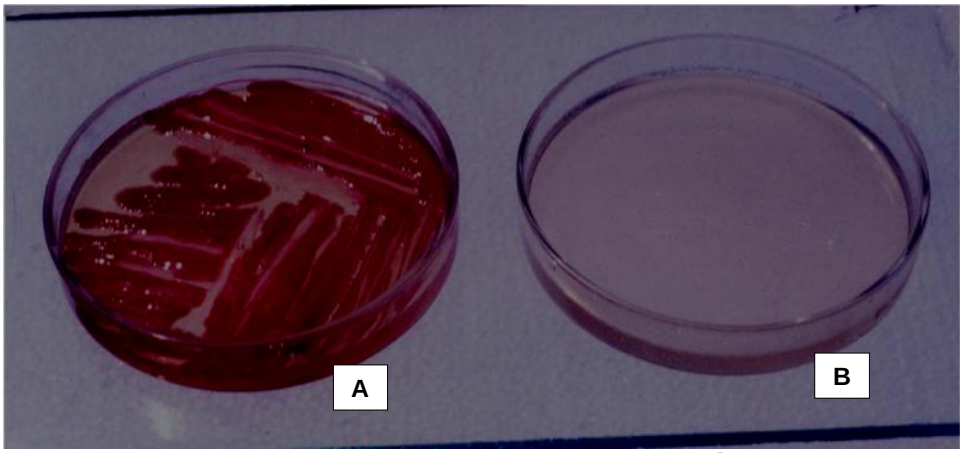

FIGURE 4: *Serratia marcescens* in nutrient agar plate
A – Showing red pigments produced by *Serratia marcescens*, B – Control plate

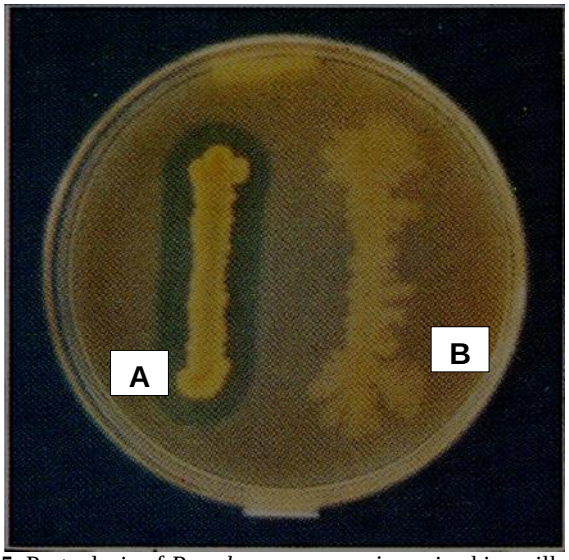

FIGURE 5. Proteolysis of *Pseudomonas aeruginosa* in skim milk agar plate
A – Proteolysis – Zone formation
B – Control – No Zone formation

DISCUSSION

Milk is a nutritious food, and it act as a medium for microorganisms. In this present study there are twenty five raw milk samples were analysed by methylene blue reduction test for checking its quality. Among these twenty five samples, four samples showed poor quality remaining twenty one samples showed fair quality. Methylene blue reduction times (h) in thirteen milk samples were 3 – 4 hours. Methylene blue reduction time (h) in eight samples was 2 hours. Only four samples showed colour change within 30 minutes. Methylene blue reduction time observed in this study are lower than these reported by Ramesh *et al*. (2001) which were reported to be more than 4 hours. In this present study there are twenty five raw milk samples were subjected to standard plate count. The bacterial counts of the inoculated samples showed high in 10^{-1} dilution and the remaining dilutions showed decreased number of bacterial colonies. Among the twenty five samples twelve samples revealed TNTC at 10^{-1} dilution. Bacterial colonies of 64 – 98 were observed in remaining samples. Numbers of bacterial colonies were reduced in all milk samples at 10^{-2} dilutions, which varied from 9 to 205. The highest number of bacterial colony in 10^{-3} dilution was 122. These bacterial colonies were counted in sample number 13, and lowest number of bacterial colony 10^{-3} dilution was 4 in first sample. Dilution range of 10^{-4} and 10^{-5} showed least number of colonies. More number of colonies in 10^{-1} dilution is due to contamination of milk with bacteria. But in case of remaining dilutions numbers of colonies were decreased because load of bacteria was diluted. Standard plate counts observed in this study are lower than those reported by Favale *et al*. (1994). Seven different bacterial species were isolated from different raw milk samples. *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia Coli, Serratia marcescens, Micrococcus luteus, Proteus mirabilis* were isolated from raw milk sample. Among the seven isolates five isolates showed proteolytic activity in skim milk agar. Two isolates does not show proteolytic activity in skim milk agar. *Escherichia coli*

and *Staphylococcus aureus* showed negative result to proteolysis because these two isolates were not hydrolysed milk protein casein so no zone formed around the culture. *Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis, Serratia marcescens*, showed zone formation in skim milk agar, due to proteolytic activity. But *Micrococcus luteus* hydrolyse the milk protein slowly. *Pseudomonas aeruginosa* showed proteolytic activity was observed in this study. But *Pseudomonas fluorescens* sowed proteolytic activity was reported by Wieldmann *et al*. (2000). Lira *et al*. (2000) isolated Protease enzyme responsible for milk protein hydrolysis from *Pseudomonas fluorescens.* In this present study *Bacillus cereus* showed proteolytic activity at 37ºC was reported. *Bacillus cereus* showed proteolytic activity at different environmental conditions such as temperature at 2 to 37ºC and P^H 4 to 7.3 was reported by Braun and Fehlhaber (2002). *Proteus mirabilis* and *Micrococcus luteus* showed proteolytic activity at 37ºC was observed in this study. But these species showed proteolytic activity at different environmental conditions such as temperature at 2 to 37ºC and P^H 4 to 7.3 were observed by Braun and Fehlhaber (2002).

REFERENCES

Abdou, S.M., Dawood, A.H., Montasser, E.A. and Ismail, E.E.A. (2001) Evaluation of UHT milk during storage. *In: 8th Egyptian conference for Dairy science and Technology held at the International Agriculture centre, Cairo, Egypt, Research papers* (I)*:* 149 – 16

Aslam, M. and Hurley, W.L. (1996) Proteases in Milk. *Illinois Dairy Report Home Page.* 1 – 3.

Bhattacharyya, R. N. (1986) Experiment with Microorganisms. *Emkay publications, Delhi*. pp # 366.

Braun, P. and Fehlhaber, K. (2002) Combined effect of temperature, a_w and P^H on enzymatic activity of spoilage causing bacteria. *Milchwissenschaft*. 57(3): 134 – 136.

Carvalho, F. and Luchese, R.H. (2000) Profile of raw milk spoilage microbiota important for UHT milk processing. *Anais do XVII congresso Nacional de Laticinios. Perspectivas Advances em Laticinios.* 54(318): 188 – 195.

Celestino, E.L,Iyer, M, and Roginski,H.,1996,The effects of refrigerated storage on the quality of raw milk. *Australian J. Dairy Tech.,* 51(2): 59 – 63.

Dawood, A.H., Abdou, S.M., Montasser, E. A., and Ismail, E.A. (2001) Microbiological evaluation of Egyptian ultra-high temperature (UHT) milk. *In: 8th Egyptian conference for Dairy science and Technology held at the International Agriculture centre, Cairo, Egypt, Research paper* (I): 133 – 147.

Deeth, H. C., Datta, N. (2011) Heat treatment of Milk-Ultra High Temperature (UHT): Heating System Encyclopedia of Dairy Sciences (Second Edition), Pages 699-707.

Dekker, P.J.T., Daamen, C.B.G. (2011) Enzymes exogenous to milk in diary technology β-D-Galactosidase, Encyclopedia of Dairy Sciences (Second Edition), 2011, Pages 276-283.

Geetha, R. and Prasad, V. (2001) Studies on the performance of cultures of lactic acid bacteria in lactose hydrolysed buffalow skim milk. *Cheiron.* 30(3-4): 81 – 84.

Griswold, K. E. and Mackie, R.I. (1997) Degradation of protein utilization of the Hydrolytic products by a predominant Ruminol bacterium, *Prevotella ruminicola B14. J. Dairy Sci.,* 80:167 – 175.

Gurung, M., Nam, H. M., Tamang, M. D., Chae, M. H., Jang, G. C., Jung, S. C., Lim. S. K. (2013) Prevalence and antimicrobial susceptibility of Acinetobacter from raw bulk tank milk in Korea Original Research Article Journal of Dairy Science, Volume 96, Issue 4, April , Pages 1997-2002.

Hassan, A. N., Frank, J. F. (2011) Microorganisms associated with milk, Encyclopedia of dairy sciences (second edition) pages447- 457.

Herlekar, D. A., Shashikant, C. S., Gurjar, A. A., Jayarao, B. M. (2013) Presence of viral and bacterial organisms in milk and their association with somatic cell counts , Journal of Dairy Science Volume 96, Issue 10, October 2013, Pages 6336–6346.

Huang chien Jung and Huang, C.J. (1996) Effect of proteolytic products produced by other lactic acid bacteria in milk on the growth of *Bifidobacterium longum. Journal of the chinese society of Animal science*. 25(2): 221 – 230.

Ibrahim, S.A. (1998) Agglutination Behavior of Mesophilic starter cultures as a function of proteolysis. *J. Food prot.,* 61(7): 855 – 858.

Jung, W. (2002) Bacterial Flora in raw milk views of dairy personnel. *DMZ. Lebensittel – Industrie – und – Milchwirtschaft.* 123(10): 24 – 30.

Kavitha, N. S., Hilda, A., Gopinath, S. and Latha, K. (1997) Ecoethical Technology using extracellular enzymes of *chrysosporium* species. *Bioethics in India: Proceedings of the International Bioethics workshop in Madras.*

Kolakowski, P., Reps. A. and Fetlinski. A. (2000) Microbial quality and some physicochemical properties of high pressure – processed cow milk. *Polish journal of food and Nutrition Sciences.* 9(4): 19 – 26.

Kirin, S. (2001) The effect of cooling procedure on the characteristics and quality of raw milk. *Mijekarstvo.* 51(2): 151 – 159.

Ma, Y., Barbano, D. M., Santos, M. (2003) Effect of

CO_2 Addition to Raw Milk on Proteolysis and Lipolysis at 4°C, Journal of Dairy Science,Volume 86, Issue 5, May , Pages 1616–1631.

Matta, H. and Punj, V. (1996) Isolation and identification of protelytic psychrotrophic sporeforming bacteria from milk. *Indian J. Dairy Sci.,* 49(10): 695 – 699.

Mehanna, N.M., Moussa, M.A.M. and Ahwall, R. (2001) Improving quality of milk during its cold storage. *Egyptian J. Dairy sci.,*29(1): 9 – 18.

Mohran, M.A., Fahmy, M.A., Elhoda Hanary, N. and Nanis, H.G. (2000) Influence of lactic acid bacteria starters on the proteolytic activity of thermoduric bacteria isolated from milk. *Assiut Journal of Agricultural sciences.* 31(1): 261 – 269.

Mohran, M. A., Fahmay, M.A., Elhoda Hanaf, N. and Gomah, N.H. (2000) Proteolytic activity of thermoduric bacteria isolated from milk. *Assiut Journal of Argicultural Sciences.* 31(3): 177 – 187.

Meisel, H. and Backelmann, W. (1999) Bioactive peptides encrypted in milk proteins, proteolytic activation and thropho-functional properties. *Antonievan Leeuwenhoek.* 76(1-4): 207 – 15.

Petrova, N. and Stancheva, N. (1995) Technological conditions for cleaning milking machines for ewes using a combined alkaline detergent, and their influence on the microbiological quality and biochemical properties of milk. *Zhivotnovdhi – Nauki.* 32(5 – 8): 325 – 328.

Prabha, R., Vijaya, G.V., Narayana reddy, K.V. and Bhat, G.S. (2002) Enhancement of functionality of milk proteins by extra cellular proteinases isolated from psychotropic organisms in milk. *Indian J. Dairy Sci.*, 55(2): 70 – 73.

Rankin, S. A., Lopez-Hernandez, A., Rankin, A. R. (2011) Liquid Milk Products Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk) Encyclopedia of Dairy Sciences (Second Edition), Pages 281-287.

Sawyer, L. and Holt, C. (1993) The secondary structure of milk proteins and their Biological function. *J. Dairy Sci.,* 76: 3062 – 3078.

Santos, E.de.S., Carvalho, Ep.de. and Abrev, L.R. de. (1999) Psychrotrophic bacteria consequences of their presence in milk and cheeses. *Boletim – da – Sociedade – Braisileria – de – ciencia – e – Techno – logia – de – Alimentos*. 33(2): 129 – 138.

Sundararaj, T. (2002) Microbiology Laboratory Manual. pp # 218.

Tatzel, R., Ludwig, W., Schleifer, K.H. and Wallnofer, R.P. (1994) Identification of *Bacillus* strains isolated from milk and cream with classical and nucleic acid hybridization methods. *J. Dairy Research.* 61: 529 – 535.

Vyletelova, M., Urbanova, E. and Benda, P. (2000) The incidence of psychrotrophic microorganisms in raw cow milk and the effect of proteolytic and lipolytic enzymes on technological traits of milk. *Vyzkum-V-Chovu – Skotu.* 42(3): 1 – 10.

Vyletelova, M. Hanus, O. Urbanova, E. and Kopunecz, P. (2000) The occurrence and identification of psychrotrophic bacteria with proteolytic and lipolytic activity in bulk milk samples on storage in primary production conditions. *czech Journal of Animal Science*. 45(8): 373 – 383.

Wiedmann, M., Weilmeier, D., Dineen, S., Ralyea, R. and Boor, K.J. (2000) Molecular and phenotypic characterization of *Pseudomonas* Isolated from milk. *Applied and Environmental Microbiology*. pp # 2085 – 2095.