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Studies on bacteriological quality of milk with special reference to *B.cereus*

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Abstract: *Milk is an inevitable thing due to its high nutrition and protien content. Along with the nutrients present, the market milk also contains several contaminants from different sources such as udder of cow, handlers of milk, soil, water, etc. Among them microbial contamination plays a major role due to its deteriorative effect on milk proteins and other contents. In addition the microbial contamination may lead to serious health issues. This study is attempted to flock together the microbial load present and its deteriorative effect on milk. The quality parameter was checked with raw milk using dye reduction and plating method. The milk was pasteurized until there is no microbial load. The milk inoculated with test organism was analysed for casein degradation, lipolytic activity, protein degradation. Finally the deterioration of milk was analysed using polyacrylamide gel electrophoresis and the results were recorded and were compared with normal pasteurized milk containing all nutrients.*

Keywords: *Bacteriological quality, casein degradation, dye reduction test, lipolytic activity, microbial deterioration, polyacrylamide gel electrophoresis.*

1. INTRODUCTION:

Only few bacteria are present in milk when it leaves the udder of healthy cow. However milk gets contaminated during milking process, from the exterior of udders and adjacent areas. Bacteria present in manure, soil and water may also contaminate the milk. The most prominent sources of contamination are dairy utensils and milk contact surfaces. Contamination in market milk may be due to adulteration with water. Undesirable bacteria in milk include lactic streptococci, coliform bacteria, psychotropic bacteria and those that survive pasteurization. Pathogenic bacteria include *M.tuberculosis*, *C.burnetti*, *Salmonella sp*. etc. [1] Though pasteurization and cold temperature eliminate many of the above pathogens, aerobic spore formers survive and multiply. Such bacteria have gained lot of importance in relation to milk deterioration. Apart from spore formers, psychrotrophic bacteria are also found in milk resulting in spoilage. They altogether lead to degradation of fats and proteins. Microbial enzymes causes protein degradation and these proteolytic enzymes are classified as serine proteinases, metalo proteinases, sulfhydryl proteinases and acid proteinases. Some of these proteinases have also been found to be useful in dairy technology. Bacteriological deterioration even occurs on storage. The current research work focussed on the study of the quality of milk samples collected from Coimbatore city by methylene blue reduction test and plate count. The organisms were isolated and identified by staining, etc. The role of spore formers in deterioration of milk was also analysed.

2. METHODOLOGY

2.1 Collection of milk samples:

About 75 samples were collected aseptically from different parts of Coimbatore city. The samples were bought from private distributors, Tamilnadu Dairy Corporation and private societies.

2.2 Quality testing:

2.2.1 Methylene blue reduction test: [2]

The samples were brought to the laboratory and tested immediately for their quality by methylene blue reduction test. The methylene blue was prepared as follows: 1gram dissolved in 25000ml of distilled water (or) 10milligram in 250ml distilled water. Using sterile pipettes, about 10ml of raw milk was taken in screw cap tube and 1ml of the dye was added and stoppered. The tubes were gently inverted and placed at 37°C in water bath. The time taken for discolouration of dye indicates the quality of milk. The quality is graded as follows

Grade 1: Reduction within 30 minutes – very poor quality
Grade 2: Reduction between 1 and half to 2 hours – poor quality
Grade 3: Reduction between 2 to 6 hours – fair quality
Grade 4: Reduction between 6 to 8 hours – good quality

2.2.2 Plating method:

About 3 different media such as Tryptone glucose extract agar, nutrient agar and eosin methylene blue agar was prepared and sterilised. The samples were serially diluted and plated on to media and incubated at 37°C for 24-48 hours and the colonies were counted.

2.3 Isolation and identification of the organism: (Cappuccino laboratory manual)

The colonies were isolated and identified for the organism present.

Gram staining: Smears of the bacterium on clean microscopic glass slides were heat fixed over a flame. The smears was flooded with crystal violet (for 1 minute) and then with gram's iodine (for 1 minute), after washing with distilled water. The smears were destained with absolute ethanol and counter stained with safranin. The cells were viewed under microscope. The cells appearing violet were considered as gram positive and red were considered as gram negative.

Endospore staining: Bacterial smears were heat fixed on glass slides. Malachite green solution (10% malachite green in distilled water) was flooded over the smear and heated for about 15minutes without letting it dry. After washing excess stain, the smear was counter stained with safranin. Endospores will appear green while vegetative cells appear red.

Kim and Goepfert agar for *B.cereus* cultivation:

Yolk of an egg was homogenised with sterile distilled water at 1:4 ratio. The homogenate was held at 45°C for 2 hours and left overnight at 4°C for setting the precipitate. The supernatant was filtered through Millipore filter unit. After sterilization, the KG media was cooled and 100ml of egg yolk emulsion was added. The organism was inoculated on to the media and incubated for about 24-48hours and observed for growth.

The biochemical tests were performed according to Cappuccino manual for the identification of organisms.

2.4 Sterilisation of milk: [3]

Milk samples were collected with utmost care to maintain sterility. The samples were directly collected in conical flask and stoppered immediately with cotton plug. They were plated on to nutrient agar to find the population of microbes. The samples were pasteurized at 70°C for 15minutes in water bath. Plating was done after cooling the sample. Pasteurization was done until there was no growth on plating. This sterile milk was refrigerated was future use.

2.5 Inoculation of pasteurized milk with test organism:

A known quantity of *B.cereus* cells estimated by hemocytometer was inoculated into known quantity of milk using sterile pipette.

2.6 Determination of cell number:

B.cereus isolated from poor quality milk was inoculated in nutrient broth and centrifuged after 24hours to separate the bacteria. The sedimented bacterium was washed repeatedly using phosphate buffer saline (pH 7.2) and the concentration was adjusted in the final suspension to give a cell number of 10^5 cells/ml of the buffer under sterile conditions. The cell number was calculated using a hemocytometer and were used for further experiments.

2.7 Growth of *B.cereus* in milk:

A known concentration of organism (10^5 cells/ml of milk) was used to study the growth in milk in several 100ml Erlenmeyer flasks. Growth was measured by viable plate count by plating known quantity of milk at different intervals from 0 to 6 hours.

2.8 Study of deterioration of milk: [4]

A known volume of the bacteria (10^5 cells/ml) was inoculated into sterile milk and the deterioration was studied every 2hours once for 6hours. Milk deterioration due to bacteria in room temperature 30°C / refrigerated temperature (4-8°C) were studied.

2.8.1 Methods of studying casein degradation due to *B.cereus*:

A known quantity of commercially obtained casein was dissolved in 0.01M phosphate buffer saline (pH 7.2). Insoluble casein was removed and soluble casein was estimated by Lowry method [5].

2.8.2 Methods of studying lipolytic activity due to *B.cereus*:

Lipolytic activity was studied using measurement of the saponification value of fat present in milk. Accurately weighed 1gram of milk in a beaker was dissolved in 3ml of the fat solvent (equal volumes of 95% ethanol and ether). To the contents 25ml/l alcoholic KOH was added and the flask was attached to a reflux condenser and boiled in boiling water bath for 30 minutes. After cooling to room temperature the contents were titrated with 0.5mol/l hydrochloric acid with phenolphthalein as indicator. A blank (consist all chemicals except the fat) and control (uninoculated sterile milk)

was also maintained along with the above test. Milk incubated with *B.cereus* was analysed after 0, 2, 4 and 6 hours. The difference in reading between the blank and test and test and control gave the number of ml of 0.5mol/l KOH required to saponify fat present in 1gram of milk.

2.8.3 Protein estimation: [5]

The insoluble casein was removed by centrifugation at 12000 rpm for 15minutes at 0°C and the concentration in the supernatant of casein suspension was estimated and the concentration of milk protein after removal of fats.

Protein concentration and casein was determined by lowry et al method (1951), which consisted of colour development. About 0.2ml of milk/casein and 5ml of reagent C were taken together in a test tube, mixed well and allowed to stand for 10 minutes. Then 0.5ml of diluted folin ciocalteau reagent (equal amount of reagent mixed with distilled water) was added and allowed to stand for 30 minutes in dark and absorbance was determined at 520nm. Control consisting of 0.2ml of uninoculated milk and reaction mixture was also determined. Standard graph was prepared with BSA. Unknown samples were read by extrapolating values with standard graph. Protein was estimated in milk/casein after 0, 2, 4 and 6 hours of *B.cereus* growth in milk both at room temperature and refrigerated temperature.

2.8.4 Polyacrylamide gel electrophoresis: [6, 7]

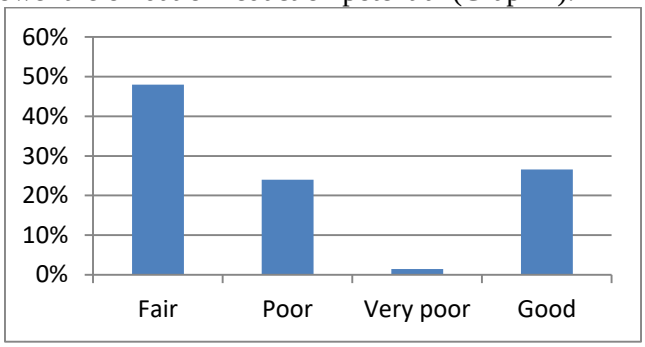
Fat was removed by centrifugation at 12000 rpm for 15 minutes at 0°C and was used for electrophoresis. Stock and working solutions were prepared. The gel was polymerised and the wells were cut. The samples were loaded in the wells and power supply was connected and adjusted till the tracking dye migrates to two-third of the running gel.

The gels were removed and stained for protein and viewed. It was fixed, left overnight and destained later until the bands were clear and stored.

3. RESULTS AND DISCUSSION

3.1 Microbial quality of milk samples:

The main aim of analysing the microbial quality of milk sample is to gather information on bacteriological quality of milk in order to fix acceptable standards for milk, standardise the processing conditions and improving the keeping quality. The microbial quality was assessed by methylene blue reduction test. The test revolves around the principle of decreased concentration of dissolved oxygen in milk by large number of metabolically active microorganisms which greatly lower the oxidation reduction potential (Graph 1).



Graph 1 showing that most of the milk samples were of fair quality. Only minimum samples were of very poor quality.

3.2 Bacteriological quality:

As methylene blue reduction test is less sensitive, total bacterial plate counts, aerobic spore count and gram negative bacterial count were taken specifically for poor and very poor samples. A known volume of milk samples were plated on Tryptone glucose extract agar for total plant count and aerobic spore count and on Eosin methylene blue agar for gram negative bacteria (coliforms). Aerobic spore formers were counted after confirming by gram and endospore staining (Table 1)

Sample number	Total plate count	Spore count	Colonies on Eosin methylene blue agar
1	6.4×10^5	13×10^4	8
2	2.9×10^6	12×10^4	150
3	4.8×10^5	16×10^4	100
4	2.5×10^6	38×10^4	298
5	2.2×10^5	31×10^4	300
6	6.6×10^5	1.6×10^5	19
7	5×10^6	19×10^4	300

Table 1 representing number of colonies from fair and poor quality milk samples.

3.3 Isolation and identification of aerobic spore forming bacteria:

Spore forming bacteria were observed to be responsible for spoilage of milk and milk products. Cold tolerant aerobic spore formers were also found to be important in spoilage of milk. These organisms normally find their way into the milk through soil and dung and reduce the shelf life even when pasteurised or stored below 3°C. Hence, study of aerobic spore formers appears important in milk microbiology, due to their ability to withstand heat and also cold. Our studies (experiment 1 and 2) also indicated the prevalence of aerobic spore formers in milk samples. Typical bacteria found in large numbers were plated on to nutrient agar and isolated. This purified bacterium developed dull white colonies that are rhizoidal with lobate margin on nutrient agar. The elevation of the colony was convex papillate, smooth and produced mucous. Microscopic observations indicated them to occur in chains and were of bacilli type. They measured 4µm in length and 1µ in breadth. The colony turned wrinkle after 48-96 hours. Wrinkling appeared from the centre and proceeded towards the margins. The colony characters demonstrated by the organism indicate it to be a *Bacillus*. Wrinkling observed could be due to production of endospore.

On blood agar, clear zones were observed around the bacterial colonies indicating haemolytic activity. On K.G egg yolk agar, turbid areas around bacterial colonies were observed indicating lecithinase activity (**Table 2**).

Biochemical test	**Result**
Indole production	Negative
Methyl red	Positive
Voges proskauer	Negative
Citrate utilisation	Positive
Gelatin hydrolysis	Positive
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Oxidase	Negative
Catalase	Positive
Hydrogen sulphide production	Negative
Nitrate reduction	Positive
Carbohydrate fermentation	
Glucose	Negative
Lactose	Negative
Maltose	Positive
Sucrose	Positive
Mannitol	Positive

Table 2 showing biochemical characteristics exhibited by *Bacillus cereus*

3.4 Deterioration of milk:

Milk is usually stored at about 4-10°C which is selective for psychrotrophic bacteria. Even aerobic spore formers are able to grow in pasteurised milk as well. Hence certain spore formers such as *B.cereus* have been recognised both as psychrotrophic and thermophilic. Deterioration of milk was usually studied in terms of protein hydrolysis and fat degradation in milk through lipase. It was indicated that *B.cereus* is an important organism in milk deterioration. Hence studies were carried out on deterioration of milk especially proteins due to *B.cereus.*

3.5 Growth of *B.cereus* in milk:

It has been already indicated that *B.cereus* causes milk deterioration. For an organism to cause deterioration, it should have the capacity to grow and multiply in the food-milk. Its ability to grow in milk was evaluated in the present experiment. As milk is either distributed fresh or kept in refrigerated condition for later distribution it is essential to study bacteria's growth both in room and refrigerated temperature. Milk was sterilised and a known number of *B.cereus* was inoculated. The load of inoculum used corresponded to the number of *B.cereus* present in fair quality milk (**Table 3** & **Graph 2**)

Number of cells	Fridge Temperature	Room Temperature
10^5	1.8	2
10^6	4	6.2
10^7	5	7.2

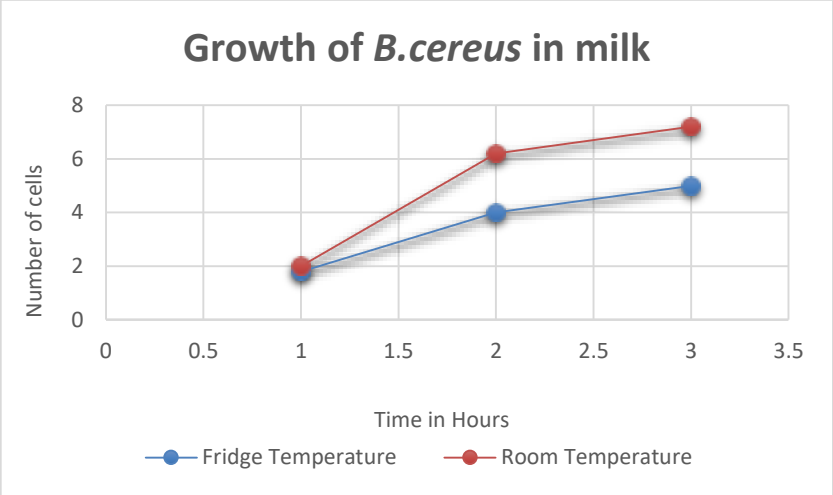


Table 3 & **graph 2** indicating the growth at room and refrigerated temperature. The growth patterns recorded were identical but the rate of growth was more in room temperature compared to refrigerated temperature.

3.6 Lipolytic activity of *B.cereus*:

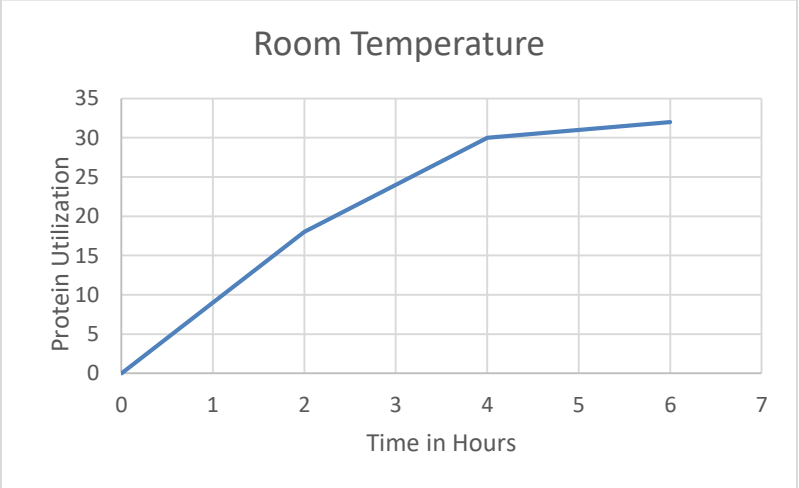
Lipolysis is the enzymatic hydrolysis of milk lipids to free fatty acid which may give rise to off flavours. Estimation of saponification value of milk lipids in terms of milligrams of potassium hydroxide required to neutralise the fatty acids released will indicate (saponification value) the amount of free fatty acids released. Saponification value of milk lipids were estimated after every 2 hours of growth of the organism, both at room and refrigerated temperature. Very less lipolytic activity was observed indicating that *B.cereus* doesn't produce lipolytic enzyme.

3.6.1 Alterations in total milk protein:

Since proteins constitute a bulk percentage in milk, its hydrolysis was estimated both at room and refrigerated temperature.

(**Table 4, 5** & **Graph 3,4**)

	Time in Hours	Room Temperature
	0	0
1	2	18
2	4	30
3	6	32



Time in Hours	Refrigerated Temperature
0	0
2	10
4	18
6	19

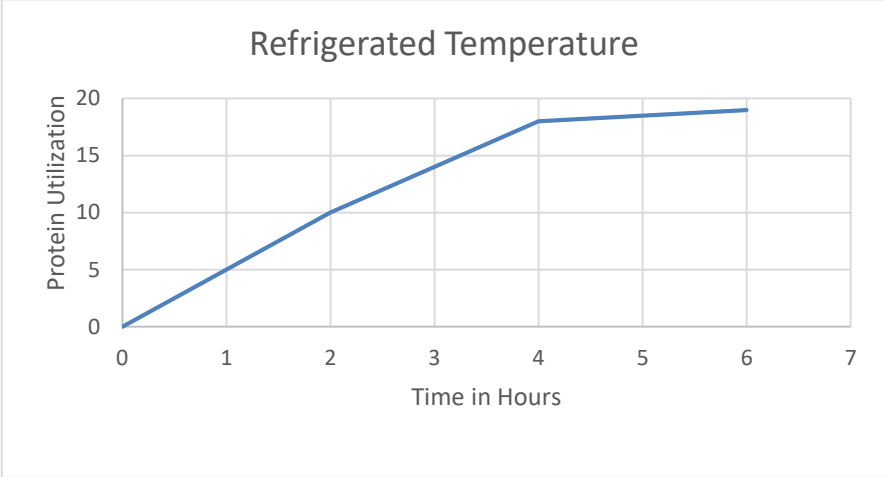
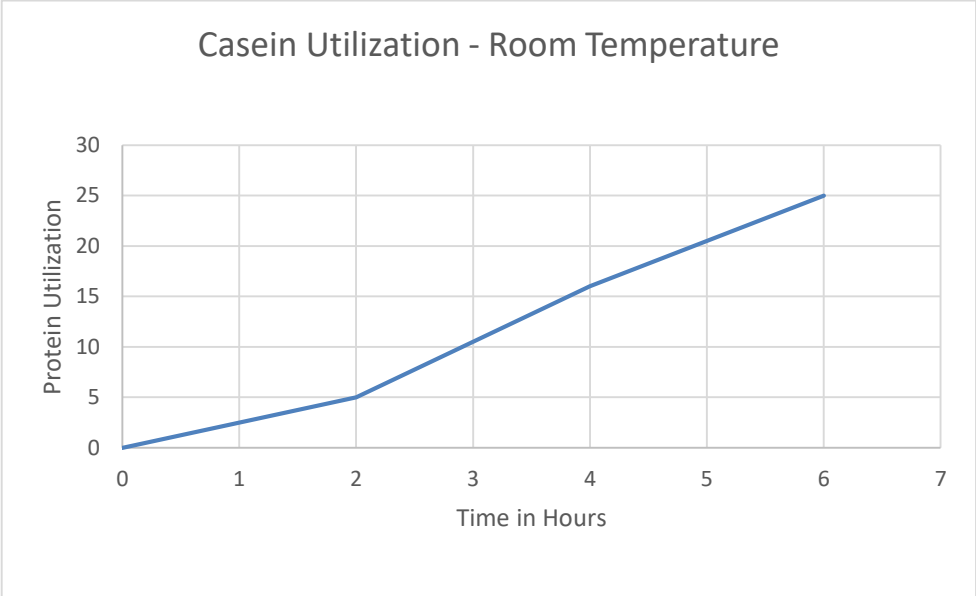


Table 4,5 & **graph 3,4** showing alteration in total milk protein in room and refrigerated temperature respectively

3.7 Casein hydrolysis:

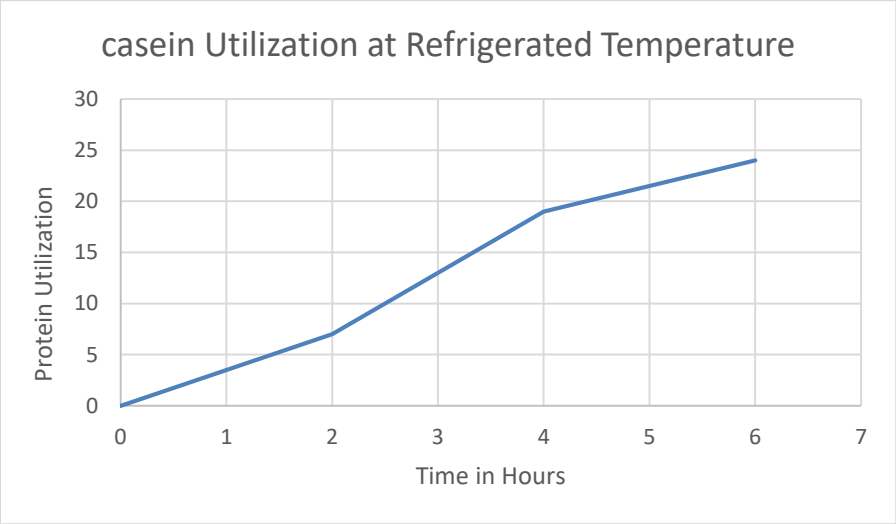
Casein is the main protein found in milk and is present at a concentration of about 35gram per litre. The concentration of casein in this study was the same amount of casein normally found in milk. Proteolysis was determined by estimating the total casein concentration after allowing *B.cereus* to grow for definite lengths of time at room temperature and refrigerated temperature (**Table 6,7** & **Graph 5,6**).

Time in Hours	Room Temperature
0	0
2	5
4	16
6	25



Graph 5 indicating that only about 20% of casein was degraded even after 6hours of growth of *B.cereus*. The degradation of casein appeared logarithmic with time and corresponded with growth of the bacterium in the initial stage till 4 hours.

Time in Hours	Refrigerated temperature
0	0
2	7
4	19
6	24



Graph 6 confirming the ability of *B.cereus* to hydrolyse casein proteins of milk at low temperature.

3.8 Electrophoresis:

To study the quantitative and qualitative utilisation of milk and casein proteins Polyacrylamide gel electrophoresis (PAGE) was performed with total milk and casein proteins. In this experiment we describe the protein patterns of sterile milk and casein. Milk was sterilised by repeated pasteurisation and a quantity of soluble casein in phosphate buffer was estimated and sterilised through millipore filter. Electrophoresis was performed in a refrigerator at 4°C using the above protein source. Equal amounts of the proteins were loaded on gels and electrophoretic pattern was visualised after staining with amido black. Six proteins could be identified in milk. Three of these proteins was visualised as casein proteins. For easier observation, the protein bands in milk are serially numbered and those appearing corresponding to those proteins in casein are given the same numbers based on the Ef values. One of the proteins – protein 7 appearing in casein was not detected in total milk.

Thus the protein patterns indicate '6' proteins in milk and three of these appeared to be of casein. The appearance of protein band '7' in casein alone indicates variation from the usual expected pattern.

3.9 Milk protein degradation by *B.cereus*:

Milk proteins separated by PAGE were incubated in suspensions of *B.cereus* in 0.01M phosphate buffer saline before fixing in TCA. The number of *B.cereus* cells was adjusted to 10^5 cells/ml of the buffer for proteolytic activity. In experiments conducted for studying degradation of milk proteins at refrigerated temperature, cooled buffer inoculated with *B.cereus* was used. The gel was incubated at room and refrigerated temperature for 6hours. Four of the proteins observed in the control (**Fig. 1**) did not appear in gels treated with *B.cereus* culture at room temperature and cold temperature. This indicates the initial degradation of the above proteins by *B.cereus* extracellular proteolytic enzymes. Two of the proteins remained unaltered, probably not degraded (**Fig. 2**). It appears that the organism demonstrates a specific proteolytic activity.

Fig. 1 Control

Fig. 2 showing disappearance of proteins bands due to proteolytic activity

4. CONCLUSION:

In conclusion, the milk samples collected were analysed for their quality and were distinguished among themselves. The sample was inoculated on to the media and observed for growth. Several aerobic spore formers and gram negative organism were observed. Later on, the test organism was inoculated into sterilised milk and were observed for the enzymatic activity. Deterioration of milk was seen and potent proteolytic activity was observed using polyacrylamide gel electrophoresis. Thus it was inferred that, though *B.cereus* occur in large numbers in milk, their ability to cause spoilage is confined to the proteolytic activity. Also, its ability to degrade milk proteins appears less important in conditions where milk is consumed in 6 hours of distribution. But, the pyschrophilic and spore forming capacity have a tremendous impact in relation to milk storage.

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