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FOOD ENZYME PROTEASE FROM RAW MILK SAMPLES AND ITS ISOLATION, IDENTIFICATION AND PURIFICATION

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ABSTRACT
Twenty five raw milk samples were collected in and around Thrissur 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* were 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* were identified as proteolytic organisms. Raw milk was contaminated by bacteria and other microorganism and the main prtrease producer was identified as *Pseudomonas aeruginosa.* The organism was cultured and the protease produced was purified by Ammonium sulphate precipitation, Dialysis, Chromatographic techniques like Sephadex 200G and Uultra gel column. A purification fold of 52.75 and a yield of 40.27% was obtained. The samples homogeneity was checked by SDS PAGE and the purified sample had a molecular weight of 45kDa was obtained.

KEYWORDS: *Pseudomonas aeruginosa***,** Pectinase, Dialysis, Sephadex 200G chromatography, SDS PAGE.

INTRODUCTION

Milk is one of the widely consumed production world and are highly susceptible to contamination by microorganisms and it is also a suitable medium for the rapid growth and multiplication of bacteria at favorable conditions. A great care has to be taken in the collection and handling of milk samples to prevent any extraneous contamination and to control the growth of organisms during transportation and during the storage of the milk. Milk is the first food served on the earth, the most satisfactory single food substance elaborated by nature. It is the one food for which there seems to be no adequate substitutes. 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 theprimary source of nutrition for infants since it is easily digestible. Milk contains number of proteins such as alpha-casein, beta-casein and kappa-casein, also alpha lactalbumin and beta lactoglobulin, which are synthesized in the mammary gland and minor proteins are transferred from blood to milk such as immunoglobulins and serum albumin. There are seven types of bacteria which changes the properties of milk. Psychrotrophic microorganisms are able to grow at temperature below 7^0C. They are often proteolytic and lipolytic in nature.Theyinclude species of *Micrococci, Bacilli, Staphylococci, Lactobacilli, Pseudomonas and Coliforms.* Spore forming bacteria can withstand greater extremes of acidic, alkaline pHs and temperatures. 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 micro organisms to milk or from blood to milk. Some proteases are secreted is an inactive form which becomes active by autolysis (or) by limited proteolysis by other protease. Milk is one of the widely consumed products, that is why it is called the 'Liquid Diamond'. It is an excellent culture medium for growth and reproduction of micro organisms.Milk becomes contaminated with several types of micro organisms, which originates from soil, water,skinand the hair of the animalsor from milk maiders. Temperature plays a vital role in the spoilage of milk. Micro organisms such as psychrotrophs even grow at refrigeration temperature 7^0C. They are distributed in diversified habitats, as water, soil, utensils and vegetation. 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 include 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.

Composition of Milk

Milk is a complex mixture of Lipids, Carbohydrates, Proteins and many other organic compounds and inorganic salts dissolved or dispersed in water

(Srilakshmi,1999).Chemical composition of milk varies due to numerous factors such as species, breed of animals, climate etc.

Water

The water in the milk is the same as any other water. Its function is to hold the solids of the milk partly in solution and partly in suspension.

Milk Fat

Milk is a true emulsion of oil in water. Each globule of fat is surrounded by a thin layer which is composed of a lipid protein complex and a small amount of carbohydrate. The lipid portion includes both phospholipids and triglycerides. Fat globules vary widely in size from 2-10 µm. Milk fat is a mixture of glycerides of fatty acids other lipid materials present in milk are phospholipids, sterols, free fatty acids, carotenoids, and fat soluble vitamins (Srilakshmi, 1999).

Milk Proteins

Milk contain casein and whey protein. Casein constitutes 80% of the total protein in milk. Casein occurs in milk as a colloidal protein – calcium phosphate complex. Whey proteins are made up ofα Lactalbumin and β Lactoglobulin, bovine serum albumin and immunoglobulins. Whey also contains small amounts of Lactoferrin and serum transferrin.

Milk Sugars

The disaccharide lactose is the predominant and distinctive carbohydrate of milk but there are in addition low concentration of monosaccharide including glucose and galactose.

Salts

Chlorides, phosphates, citrates, sulphates and bicarbonates of sodium, potassium, calcium and magnesium are present.

Trace elements

Milk contains trace elements like copper, zinc, aluminum, molybdenum, and Iodine.

ENZYMES

Alkaline Phosphates

This enzyme is inactivated by normal pasteurization procedures and its activity is tested to determine the effectiveness of pasteurization.

Lipase

More than one type of lipase occurs in milk. Milk Lipase is responsible for rancid flavors in milk. Bacterial lipase is responsible for serious quality defects.

Xanthin oxidase

This enzyme degrades FAD and gives FMN and riboflavin

Catalase

It decompose hydrogen peroxide to water and molecular oxygen

Lactoperoxidase

It catalyses the transfer of oxygen from peroxides

Casein Hydrolysis

Casein, the major milk protein, is a macromolecule composed of amino acid subunits linked together by peptide bonds (CO-NH). Before their assimilation into the cell, proteins must undergo step by step degradation into peptones, polypeptides, dipeptides, and ultimately into their building blocks amino acids. This process is called peptonization or proteolysis, and it is mediated by extracellular enzymes called proteases. The function of these proteases is to cleave the peptide bond CO-NH by introducing water into the molecule. The reaction then liberates the amino acids.

The low molecular weight, soluble amino acids can now be transported through the cell membrane into the intracellular amino acid pool for use in the synthesis of structural and functional cellular proteins.

MATERIALS & METHODS

Sample Collection (Bhattacharyya, 1986)

Twenty five raw milk samples were collected during June – July 2014 from various local milk venders in and around Thrissur. Thorough mixing of milk was done by 25 completed up and down motion of about 1 ft in 7 seconds that reach entire depth of the liquid. Milk samples are collected in a sterilized test tube under aseptic conditions.

Methylene blue reduction test (Bhattacharyya, 1986).

Three clean and sterilized test tubes were taken. To each of the test tubes 10 ml of milk to be tested was added. Second and third tubes were placed in boiling waterbath for 3 minutes to destroy the natural reducing system of the milk. These two tubes will serve as controls. Then 1 ml of certified methylene blue of 1: 25,000 dilutions to the first tube and 1 ml of tap water to the third tube was added. Mix the content thoroughly in each tube. The milk in the first two tubes will look blue and in the third tube, the milk will remain white. Incubated all three tubes in waterbath at 37^{o} C and note the time. Mixed the contents of each tube by stirring and examined the colour change every half an hour.

The second and the third tubes which serve as controls will not show any colour change. Milk in the second tube will remain blue and milk in the third tube will remain white, while milk in the first tube will gradually become colourless except at the top where the milk is in contact with air. The second tube as control will indicate when colour change starts in the first tube and the third tube will indicate when the colour change is complete.

MBRT is the interval between the placing of the tubes in the water bath at 37^{o} C and the disappearance of the blue colour of the milk. The shorter the time interval greater is the number of active bacteria in the milk and the lower is the quality of the milk. A reduction time between 6 to 8 hours indicates the quality of milk as good and less than 2 hours indicates poor with large number of bacteria. The following table may be used as an interpretation of the result.

Sl.No.	Reduction time	Quality
1.	More than 8 hours	Excellent
2.	6 – 8 hours	Good
3.	2 – 6 hours	Fair
4.	Less than 2 hours	Poor

Serial dilution of the sample (Bhattacharyya, 1986).

Six graduated sterile pipettes and six sterile petridishes were taken. Do not unwrap the before the actual start of the experiment. Also six sterile test tubes containing 9

ml of sterile saline were taken. Arrange them and mark them 1 – 6.

Sterile plate count agar was melted and cooled to 45° C. With a sterile pipette remove 1 ml of milk and transfer to No. 1 tube shake the tube vigorously. Discard the pipette, the tube now contains 1 ml of the original milk diluted 10 times mark dilution factor 10^{-1}. Now with another sterile pipette remove and transfer 1 ml of the 10^{-1} sample and transfer 1 ml to sterile petridish perform all operation under aseptic conditions. One ml of diluted milk sample at 10^{-1} was removed and transferred tube No:2 and vigorously mixed. Discard the pipette. Using another sterile pipette 1 ml of diluted sample from tube number 2 and transfer 1 ml to petridish. Similarly, continue the dilution upto 10^{-5} and pipette the diluted samples to corresponding petridish. The sixth tube containing sterile water blank will serve as a control.

Standard plate count (Bhattacharyya, 1986).

Milk sample was shakedatleast making 25 completed up and down motion of about 1 ft in 7 seconds. The sample is then diluted to 1:100, 1:1000 and 1:10,000 using sterile saline. Diluted samples were shaked again, using sterile pipette each time 1 ml was transferred to sterile petridish. As soon as the milk samples have been transferred into the petridishes, 10 to 12 ml of melted (43 to 45°C) plate count agar was poured onto each petridish. Sample with the medium was thoroughly mixed and allowed the medium to solidify. Plates were incubated in inverted position at 37°C for 48 hours. After incubation plates were selected having 30-300 colonies and their numbers were counted.

Isolation of Specific bacteria (Bhattacharyya, 1986)

Isolated bacteria were inoculated into appropriate selective media such as EMB agar, egg-yolk agar, cetrimide agar, mannitol salt agar, nutrient agar.

Proteolysis (Cappuccino and sherman, 1999)

Skim milk agar plates were prepared for inoculation. Bottom of the petridish was divided into two sections. Using sterile technique, single line streak inoculation of each test organism on the agar surface of its appropriately labeled section on agar plate was made.

Plates were incubated in an inverted position for 24 – 48 hours at 37°C. Reincubated all negative cultures for an additional 5 days. Milk agar plate culture for the presence or absence of a clear area or zone of proteolysis, surrounding the growth of test organism was examined. Based on the observation, organisms capable of hydrolysing the milk protein casein were determined.

Grams staining (Sundararaj, 2002)

Smear was prepared for gram strain. A drop of crystal violet was added to the smear it is allowed for one minute. It is rinsed with tap water. A drop of grams iodine is added to smear. It is allowed for 30 seconds to 1 minute. It is rinsed with tap water. It is decolorised with 95% of ethanol. Alcohol was added drop by drop with the help of a dropping bottle on the top of the slide so that the alcohol runs over the smear and decolorises it. Alcohol addiction was stopped and immediately washed it with under a running tap water. This decolorisation may take 30 seconds to 1 minute depending upon the density of the smear. Smear was covered with safranine and allowed to act for 1 minute. It is rinsed with tap water. Blot dried and examined under oil immersion objective.

Motility test (Sundararaj, 2002).

A drop of broth culture was placed in a cover slip. It is keep it in a concave slide. It was observed under low power microscope. Corner of the cover slip was located. Hanging drop method was followed.

Catalase test (Sundararaj, 2002).

1 ml of hydrogen peroxide was taken in 12 X 100 mm test tube. Small amount of bacterial culture was inoculated into the fluid with the help of a glass rod or plastic loop and the releases of air bubbles were observed and it is compared with the controls.

Oxidase test (Sundararaj, 2002)

1% of p-phenylenediaminedihydro chloride was prepared in distilled water T, C+, C – marks were made on the filter paper strips. Filter paper was soaked with few drops of the reagent and it's kept on a slide or petridish. With the help of a clean glass rod or plastic loop or platinum wire colony was picked from 24 hrs growth of the test organisms and controls and it is rubber over the filter paper. Different loops were used for each organism. Color change was observed to blue or purple within 10 seconds.

Methyl Red Voges - Proskauer test (Sundararaj, 2002)

Organisms were inoculated into MR/VP broth, it is incubated at 37°C for 48 hours broth was divided into two equal half and to one 0.5 ml of MR reagent was added to the other of 0.2 ml of VP reagent A and 0.2 ml of VP reagent B was added it is mixed and allowed it to stand for 15 minutes. Formation of red color indicates positive results. And yellow color indicates negative result of MR test formation of pink color indicates positive for VP test and yellow or colorless indicates negative result for VP test.

Citrate utilization test (Sundararaj, 2002)

Citrate agar was melted and distributed in 1 to 2 ml quantities in 12 X 100 mm test tubes. It is autoclaved at 121°C for 15 minutes and it is allowed to solidify in a slanting position. A drop of 4-6 hour old culture was inoculated in to the medium and it is incubated for 18-24 hours and result was read. Formation of blue color indicated positive results. No change indicated negative result.

Urease test (Sundararaj, 2002)

A drop of 4-6 hour growth of bacterium was inoculated into the urea agar slant and incubated at 37°C for 18-24 hours. Formation of pink color indicated positive result.

Indole test (Sundararaj, 2002).

Organisms were inoculated into the tubes of tryptone broth and incubated for 24-48 hours at 37°C. 0.2 ml of Kovac's reagent was added and it is allowed to stand for few min and result was read. Formation of red ring indicates positive results. No change in color indicates negative result.

Coagulase test (Sundararaj, 2002)

Organism was inoculated into blood agar on nutrient agar for 18-24 hours. 0.5 ml of sterile rabbit EDTA plasma was taken into three 10 X 100 mm test tubes and labeled P, N and T. Single colony of *S.aureus* into plasma in tube P and *S.epidermidis* into tube N and the

test organism into tube T was inoculated. Incubated at 37^0C for 1-4 hours formation of clots indicates positive results. No clot formation indicates negative results.

Carbohydrate fermentation test (Sundararaj, 2002)

Single colony of the test organism was inoculated into nutrient broth incubated at 37^0C for 4-6 hours. Using sterile pasteur pipettes one drop of the culture was inoculated into individual carbohydrate tubes. Tubes were incubated at 37^0C for 18-24 hours. Result was read. Formation of yellow color indicates acid protection no color change indicates negative result.

REAGENTS

Gram's stain

I. Methyl Violet

(Hucker's ammonium oxalate crystal violet)

Crystal Violet - 2 grams
Ethyl alcohol -120 ml
Dissolve the dye completely – Solution A
Ammonium Oxalate -0.8 grams
Dissolve the salt Solution B
Mix solution A and B

II. Gram's Iodine

Potassium Iodide -2.0 grams
Distilled water -10 ml
Dissolve
Add Iodine -1.0 gram
Dissolve completely
Distilled water -290 ml
Make up the solution to 300 ml
III. Ethanol -95%

IV. Safranine -1%

Safranine -1 gram
Distilled water -100 ml

Catalase test

3% Hydrogen peroxide

Oxidase test

p-phenylenediaminedihydrochloride -1 gram
Distilled water - 100 ml

Methyl red voges - Proskauer test

MR REAGENT

Methyl red -0.1 gram
Ethyl alcohol - 300 ml
Distilled Water - 200 ml

VP REAGENT

VP reagent A
Alpha naphthol -5.0 gram
Ethlyl alcohol -100 ml

Dissolve alpha naphthol in small amount of alcohol first and then add the remaining alcohol to 100 ml. Stored in brown bottle at 4^0C.

VP reagent B
Potassium hydroxide -40.0 gram
Distilled Water -100 ml

Cool the volumetric flask/cylinder in a cold water bath with 80 ml of water, add KOH crystals, dissolve and make up to 100 ml store in polythene bottles at 4^0C.

INDOLE TEST

A. Kovac's reagent
Paradimethylaminobenzaldehyde -5.0 gram
Amyl alchol -75.0 gram
Concentrated HCl -25.0 gram

Dissolve the aldehyde in alcohol and add acid slowly and store in brown bottles.

COAGULASE TEST

Rabbit EDTA plasma

Carbohydrate fermentation test

A. Base

Peptone - 1.0 gram
Beef extract -1.0 gram
NaCl -0.5 gram
Distilled Water -100 ml

B. Carbohydrate solution

Carbohydrate -10.0 grams
Distilled Water -100 ml

C. Indicator

Bromocresol Purple -1.6 grams
Ethanol -100 ml
A = 900 ml
B = 100 ml
C = 1 ml

Mix dispenses in 1 ml amounts in 12 X100 mm test tubes and autoclave for 10 min at 121^0C.

Media employed

Eosin Methylene Blue Agar:

Peptone -10.0 gm
Lactose -5.0 gm
Sucrose -5.0 gm
K2HPO4 -2.0 gm
Eosin Y -0.4 gm
Methylene blue -0.06 gm
Agar -15.0 gm
Distilled Water to make upto 1 liter.

Skim-Milk Agar

Tryptone -5.0 gm
Yeast extract -2.5 gm
Glucose -1.0 gm
Raw skim milk -20.0 gm
Agar -15.0 gm
Water to make1 liter.
Autoclave for 20 minutes at 15 psi. Cool rapidly

Egg-yolk Agar

Egg-yolk emulsion -10.0 ml
NaCl -1.0 gm
Yeastrel agar or
Nutrient agar -100.0 ml

To the basal medium add the salt and sterilize by autoclaving at 121^0C for 15 minutes. Cool to 45^0C and add aseptically egg yolk emulsion. Mix thoroughly and pour on to plate. For egg-yolk broth omit yeastrel agar and add nutrient broth.

Mannitol salt agar

Beef extract -1.0 gm
Peptone -10.0 gm
Sodium chloride -75.0 gm
d-Mannitol -10.0 gm
Agar -15.0 gm
Phenol red -0.025 gm

MR/VP broth

Peptone -7.0 gm
Glucose -5.0 gm
DipotassiumPhosphate -5.0 gm
D.water -1000 ml

Final pH	-6.9

Dissolve the ingredients; distribute in 5 ml quantities in 15 X 125 mm tubes and autoclave at 121^0C for 15 min.

Citrate agar

Sodium chloride	-5.0 gm
Magnesium sulphate	-0.2 gm
Ammonium dihydrogen phosphate -	1.0 gm
Dipotassium phosphate	-1.0 gm
Sodium citrate	-2.0 gm
Bromothymol blue	-0.08 gm
Distilled water	-1000 ml
pH	-6.9

Christensen's Urea Agar

Peptone	-0.1 gm
Glucose	-0.1 gm
Sodium chloride	-0.5 gm
Mono potassium phosphate	-0.2 gm
Phenol red (1.2%)	-1.0 ml
Agar	- 2.0 gm
p^H	-6.8

Prepare the base, sterilize by autoclaving at 121^0C for 15 min. Cool to 50^0C in water bath and then add 5 ml of filter sterilized 40% urea solution. Mix, distribute in 2-4 ml amounts in 12 X 100 mm test tubes. Allow the medium to solidify in a slanting position in such a way to get half inch butt and one inch slant.

Preparation of Tryptone broth

Tryptone	-1.0 gm
Sodium chloride	-0.5 gm
Distilled Water	- 100 ml
pH	-7.4

Dissolve the ingredients, distribute in 2 ml quantities in 12 X 100 mm test tubes and autoclave at 121^0C for 15 min.

Nutrient Agar

Peptone	-	1.0 gram
Beef extract	-	1.0 gram
Sodium chloride	-	0.5 gram
Agar	-	1.5 gram
Distilled water	-	100 ml

Preparation of Crude extract

The culture showing proteolytic activity was culture in suitable medium supplemented with milk. The microorganisms along with the culture medium were triturated in a mortar with acid-washed sand. This was centrifuged and the supernatant was collected. The residue was triturated again with distilled water, centrifuged and the supernatant was mixed with the previous extract. The total liquid collected was made up to a known volume and analyzed for proteolytic activity.

Extraction of Proteinase

The microorganisms were grown in liquid medium in 250 Erlenmeyer flasks. The mycelial mat were filtered through cheese cloth and dried in an incubator. The filtrate was centrifuged and taken for further studies. Extraction was carried out at 20^0 C for 1hr. The supernatants are separated by centrifugation (20min at 10,000rpm) and reached to the 2.5ml volume with buffer A (TrisHCl20mM).

Estimation of Protein

Total protein was determined by the method of Lowry et al, (1951) using BSA as the standard. The procedure was explained in general materials and method

Determination of Proteinase enzyme

Proteinase activity was estimated according to the method of Kurnitz(1947) using case in as the substrate. In this method, a protein substrate is subjected to enzymatic hydrolysis. The tyrosine liberated by the hydrolysis is quantitatively estimated by measuring the absorbance at 275 nm. The amount of tyrosine liberated is directly proportional to the enzyme activity.

1ml of casein solution (1% w/v in 0.1 m Tris-HCl buffer pH 9.0) was mixed with 0.5 ml of suitably diluted enzyme solution and incubated at 45° C for 10 min. 50% TCA was then added to arrest the enzyme action. The mixture was warmed and filtered through whatman no.1 filterpaper. The filtrate was read at 275 nm. A control was run in an identical manner except that the enzyme was added after the addition of TCA.

The molar absorbance coefficient of tyrosine was determined as 1400 m-1 cm-1 one unit of proteinase activity was the amount of proteinase activity was the amount of enzyme which liberated 1 micro mole of tyrosine in 10 minute under the assay conditions

ENZYME PURIFICATION

Ammonium Sulphate Precipitation

The organism was grown for 48 hours as described previously. The cells were separated by centrifugation (10 000 rpm, 15 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1M Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer.

Dialysis

The protein suspension was transferred to visking dialysis tubing and dialyzed against two changes of buffer against 200ml of buffer A and allowing 8-10 h for equilibration after adjustment the pH (pH.8 as the buffer A). Dialysis was repeated against the distilled water 200ml for 2h. The dialyzed sample was centrifuged at 5000g for 15min. The dialyzed ammonium sulphate precipitates (40 to 60 %) were loaded on to a DEAE - Sephadex G - 200 column. Proteins where eluted using a 10mM tris buffer (pH 8.0) and 02-1.0 M NaCl at pH of 8 and eluted proteins where collected fractions were cooled, absorbents read, and tested for specific protease activity

Sephadex G-200 Gel Filtration Chromatography

The protein pellet obtained after saturation with ammonium sulphate between 50% and 70% was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5 ×24cm) (Sigma-Aldrich, St Louis, MO) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 mL/h with a

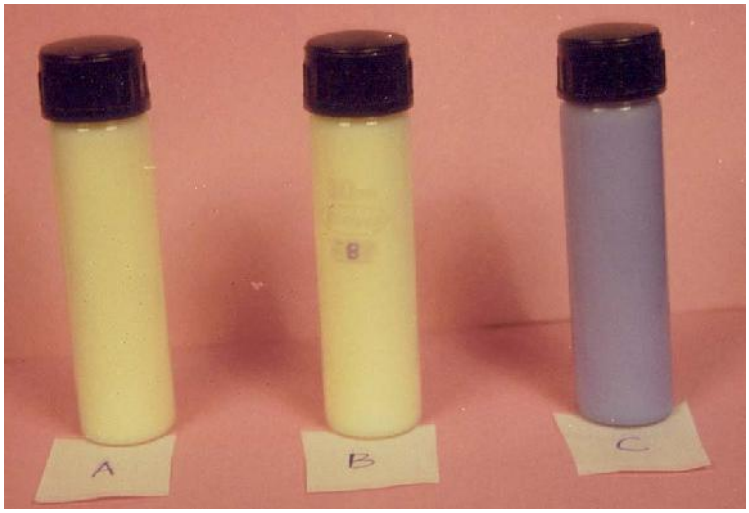
1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at a NaCl concentration of 0.6M. Fractions (19-23) with high protease activities were pooled, dialyzed, and concentrated by lyophilization (lyophilizer) and used for further studies.

Sodium DodoecylSulphate-Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis has become a routine laboratory technique for determining the homogeneity and molecular weight of the protein. Because of the simplicity of the technique, the small amount of protein required and the high resolution obtained, it is regarded as a powerful tool for the molecular characterization of the proteins. The procedure was explained in general materials and method.

RESULTS

Results on analysis of quality of milk samples by methylene blue reduction test is shown in Table – I. Among 25 raw milk samples analysed, 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 and other biochemical tests are shown in figures1.



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 1: Methylene blue reduction test for raw milk sample

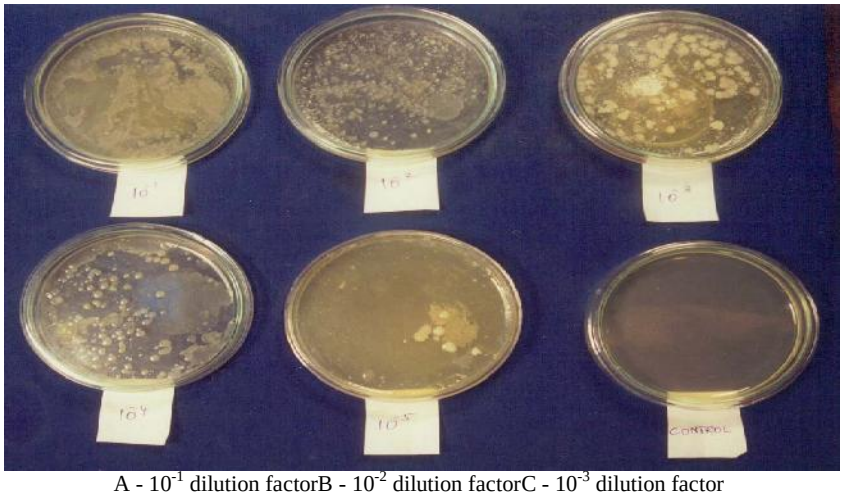
Results on the enumeration of bacteria in 25 different milk samples are given in Table 2, among which 12 samples showed TNTC at 10^{-1} dilutions. Bacterial colonies of 69 to 98 were observed in remaining samples. Numbers of bacterial colonies were reduced in all milk samples at 10^{-2} dilutions, which 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.

TABLE 1: 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

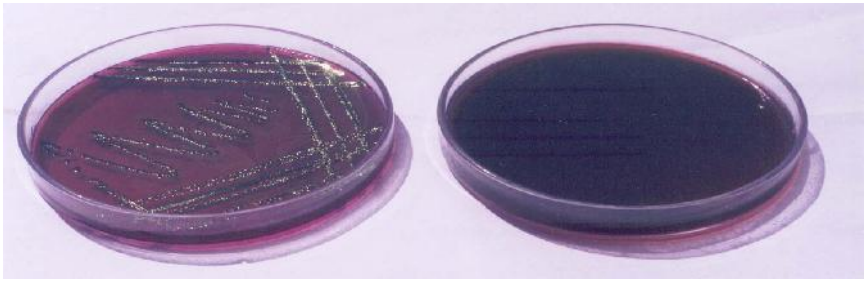
19	TNTC	174	78	56	29
20	76	44	32	6	2
21	83	39	26	5	3
22	TNTC	196	79	36	22
23	89	46	27	6	1
24	83	44	22	5	1
25	TNTC	186	87	29	16

TNTC – Too Numerous To Count
10^{-1},10^{-2},10^{-3} ,10^{-4} ,10^{-5}- Dilution factors

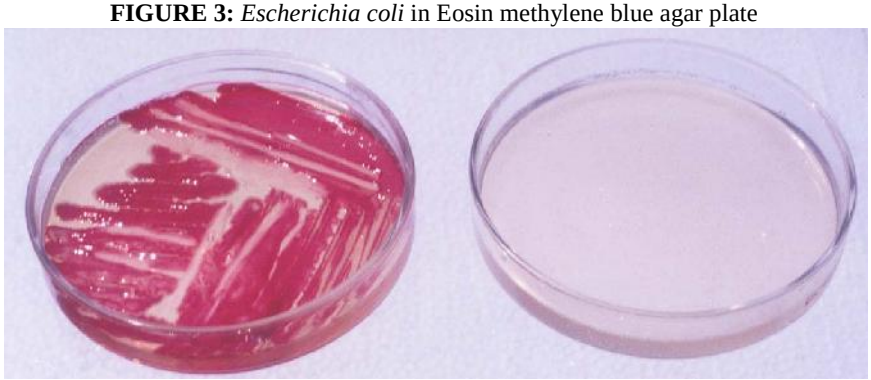


A - 10^{-1} dilution factorB - 10^{-2} dilution factorC - 10^{-3} dilution factor
D - 10^{-4} dilution factorE - 10^{-5} dilution factor, F – Control Plate
FIGURE 2: Standard Plate Count

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 *Serratiamarcescens* in Nutrient agar plate is shown in figure 4.



A – Metallic sheen colonies B – Control plate
FIGURE 3: *Escherichia coli* in Eosin methylene blue agar plate



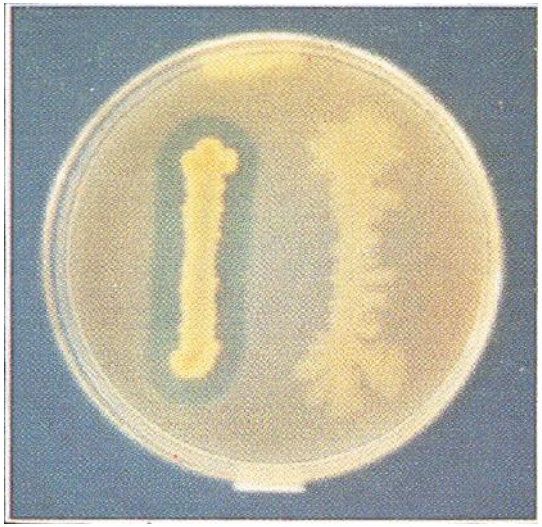
A – Showing red pigments produced by *Serratiamarcescens*, B – Control plate
FIGURE 4: *Serratiamarcescens* in nutrient agar plate

TABLE 2: Biochemical Reactions

Bacteria	Indole	MR	VP	Citrate	Cogulase	Catalase	Sugar Fermentation					Urease	Oxidase	Gram Reaction	Motility
							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
Serratiamarcescens			+	+											
Micrococcus luteus				-		+		-	-						

TABLE 3: 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	*Serratiamarcescens*	Positive
7	*Micrococcus luteus*	Positive (Slow proteolytic)



A – Proteolysis – Zone formation, B – Control – No Zone formation

FIGURE 5: Proteolysis of *Pseudomonas aeruginosa* inskim milk agar plate

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 *Pseudomonasaeruginosa* in skim milk agar is shown in Figure 5.

Purification Procedures

Crude enzyme preparation

In the present study, the natural medium supplemented with milk was used for culturing the organism. To obtain maximum quantity of enzyme, the medium was taken in 5litre Haffkin's Flask. This was autoclaved in barslead (Boston) autoclave at 15 lb pressure for 20 minutes. After sterilization the flasks were cooled to 32 plus or minus 1º C. Then a pure culture slant of *Pseudomonas aeruginosa* was inoculated into medium in a sterile chamber. After 16 days of incubation at 32 plus or minus 1degree C the contents were taken out by adding 500 ml of 0.25M NaCl. This was blended in warning blender for 5 second at 4ºC. the blended juice was filtered through 2 layers of cheesecloth. The filtrate was centrifuged at 20,000 rpm for minutes at 4º C. The clear supernatant was dialysed against 2 liter. 0.01 M phosphate buffer at pH 7.0 for 48 hours with two changes at 4 ±1ºC.

Ammonium SulphatePrecipitaion

To 1 liter of dialysed supernatant solution ammonium sulphate (AR) was added slowly with a constant stirring in a magnetic stirrer for 4 to 6 hours at 4° C to give 0- 25 %, 25- 50%, 50- 75% and 75-100% saturation. After the desired percentage of saturation the contents of each step was centrifuged at 20,000 rpm for 20 minutes at 4°C. The supernatant solution was taken for the next ($NH_4)_2$ SO_4precipitation. The precipitate obtained from each step after centrifugation was dissolved in 0.01 M phosphate buffer at pH 7.0 containing 0.1 M NaCl. The solution thus obtained was dialysed at 4ºC for 24 hours against the same buffer with at least three changes of 3 liter buffer. The activity of protease and protein content was estimated and it is shown in table 4.

TABLE 4: Ammonium sulphate precipitation of crude enzyme preparation of protease from milk sample

Sl.No	Percentage of $(NH_4)_2SO_{4\,(\%)\,v}$	Volume (ml)	Protease activity units of tyrosine release /10min	Protein content µg/ml	Specific activity in units/µg
1	Culture filtrate	800	92.8	67.5	1.3
2	0-25%	40	159.1	39.3	4.04
3	25-50%	30	632.5	55.9	11.31
4	50-75%	15	243.2	48.3	5.03
5	75-100%	10	144.3	50.5	2.85

Dialysis

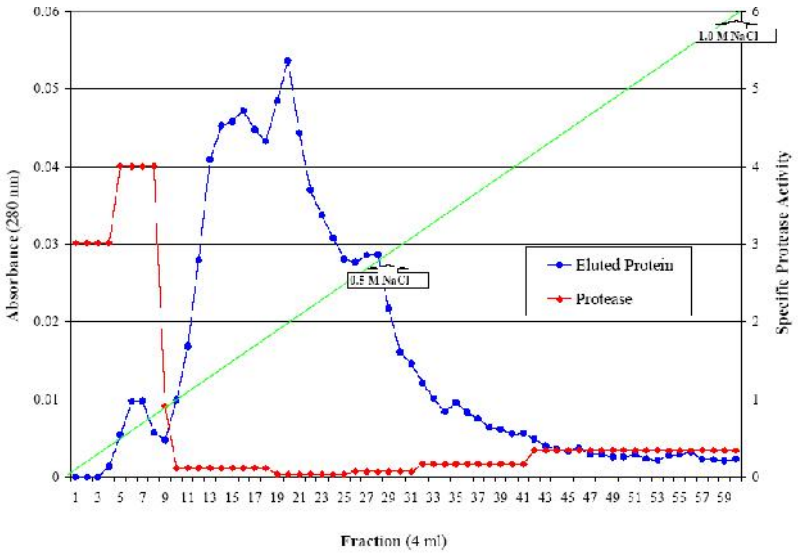
The protein suspension was transferred to visking dialysis tubing and dialyzed against two changes of buffer against 200ml of buffer A and allowing 8-10 h for equilibration after adjustment the pH (pH.8 as the buffer A). Dialysis was repeated against the distilled water 200ml for 2h. The dialyzed sample was centrifuged at 5000g for 15min.

Chromatographic procedures

The sample containing maximum protease activity was selected for further purification by chromatography.

Sephadex G -200 column chromatography

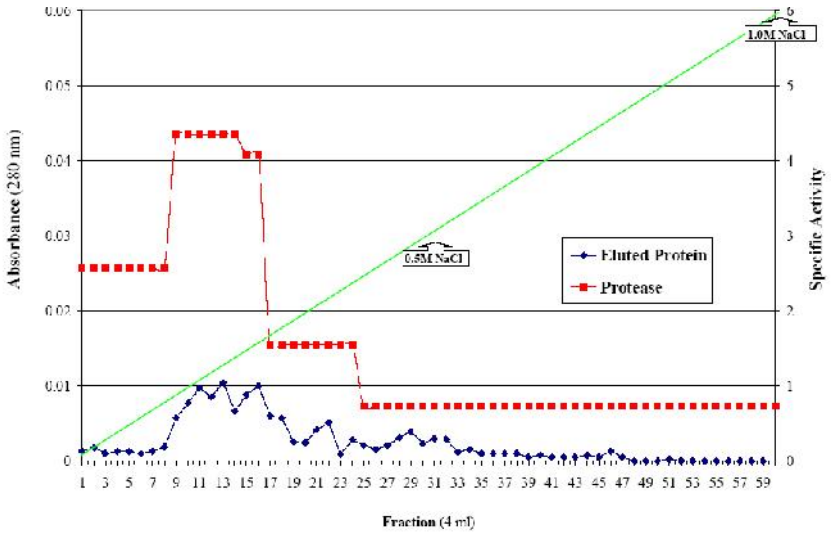
After Sephadex G-200 column chromatography, the fractions (19-23) showing the highest specific activity were dialyzed, lyophilized, and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).



GRAPH 1: Shows the elution profile of protease by Sephadex G -200 column Chromotography

Ultra Sephadex G -200 column Chromatography

The absorption peaks at 280 nm were observed (Graph 2). The peaks containing the greatest protease activity was found in fractions between 9-16.



GRAPH 2: The elution profile of protease byUltraSephadex G -200 columnChromotography

The purification of protease in which the total protein activity, specific avtivity, purification fold and yield of the sample were summarized in table 6.

TABLE 5: Purification of Protease

Steps	Volume (ml)	Total Protein(µg)	Total protease activity (µg of thyrosine released)	Specific activity Units/µg protein	Purification fold	Yeild (%)
Culture filtrate	800	325	2130	6.5	1	100
25-50% $(NH_4)_2SO_4$ precipitation	600	61.5	1720	27.96	4.3	80.75
SephadexG-200 column fraction	400	15.75	1292	82.03	12.61	60.65
Ultragel column fraction	350	2.5	858	343.2	52.75	40.27

SDS -PAGE

Samples obtained from the protease purification steps eluted from ultra sephadex G 200 column chromatography where run under denaturing conditions on a 12% SDS – polyacrylamide gel and stained (Figure 6). The band obtained with the molecular weight of 45,000 Da as the protease activity band.

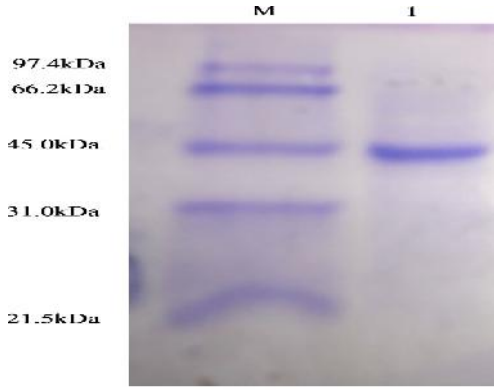


FIGURE 6: Shows the SDS-Poly acrylamide gel electrophoresis of the purified sample

M-Marker, 1-Sample purified

The theoretical molecular weight and isoelectric point of the enzyme proteinase was confirmed as 43387.78 and 7.82 respectively. This result was similar with the results of Bjellquist *et al.* (1993 and 1994).

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 time (h) in thirteen milk samples was 3 – 4 hours. Methylene blue reduction times (h) in eight samples were 2 hours. Only four samples showed colour change within 30 minutes.

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. Number of bacterial colonies was 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, number of colonies was decreased because load of bacteria was diluted. Standard plate count observed in this study is 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, Serratiamarcescens, 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* showed 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 study the enzyme was cultured in submerged fermentation conditions with medium supplemented with milk. In case of alkaline protease several nutritional and environmental parameter are affected for the production of enzyme in submerged and solid state fermentation (Kanupriya *et al*., 2017).The culture filtrate after incubation was purified by ammonium sulphateprecipitation, dialysis, Sephadex column chromatography and ultra gel column chromatography. These type of purification techniques were done on different researches (Solimar, 2016, Fran, 2017, Enling 2017). In this study the assay of protease activity was done using Kurnitzmethod. The purification methods like ammonium sulphate precipitation, dialysis and DEAE column chromatography, SDS-PAGE were for the purification of protease in different researches (Aysha 2015, Ramamoorthy, 2014). When crude enzyme extract from *Pseudomonas aeruginosa* was sequentially purified purification fold of 52.75 was obtained. The result of SDS-PAGE (Fig. 6) showed that the purified sample has a molecular weight of 45kDa. This molecular weight is similar to the purified protease enzyme isolated and purified in different researches (Solimar *et al*., 2016, Ramamoorthy *et al*., 2014). The broad application of proteases in different industries makes this study relevant.The large application of proteases are seen in food industries regarding low allergenic infant formulas, milk clotting and flavor. It has wide application in detergent industries in the removal of stains in fabrics (Kirk et al, 2002)

SUMMARY

Twenty five raw milk samples were collected in and around Thrissur 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 plates were isolated and identified using routine bacteriological technique like gram staining, catalase test, citrate test, urease test, oxidase test, MR-VP, indole test, coagulase test *etc*. The microbial strain *Pseudomonas aeruginosa* was isolated and cultured in nutrient medium containing milk for the production of protease. The crude culture was then filtered and precipitated with ammonium sulphate using the standard chart. Then after dialysis this was subjected to G-200 sephadex column chromatography and purified to the extent of 343.2 enzyme activity, 52.75 purification fold and 40.27% yield. The homogeneity was confirmed by the usual tests.

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