ELECTRON MICROSCOPE STUDIES ON YOGHURT FROM COW AND BUFFALO MILKS WITH PARTICULAR REFERENCE TO CASEIN MICROSTRUCTURE AND MICROBIAL DISTRIBUTION

PH.D. THESIS

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ELECTRON MICROSCOPIC STUDIES ON YOGHURT PREPARED FROM COW AND BUFFALO MILKS WITH PARTICULAR REFERENCE TO CASEIN MICROSTRUCTURE AND MICROBIAL DISTRIBUTION

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This is to certify that work reported in the Thesis entitled ELECTRON MICROSCOPIC STUDIES ON YOGHURT PREPARED FROM COW AND BUFFALO MILKS WITH PARTICULAR REFERENCE TO CASEIN MICROSTRUCTURE AND MICROBIAL DISTRIBUTION was carried out by Mr. SUDHIR KUMAR TOMAR, for the requirement of the Degree of DOCTOR OF PHILOSOPHY in the Faculty of Dairying, Animal Husbandry and Agriculture, under my guidance.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Lactic acid bacteria ferment the milk into a variety of milk products for human consumption with increased shelf-life viz. Sour Milk, Dahi, Acidophilus Milk, Kumiss and Kefir etc. Yoghurt is one such product made from milk with high level of solid content. The role of starter culture is shared by Lactobacillus bulgaricus and Streptococcus thermophilus in variable proportions. Modification of the conventional product with the use of Lactobacillus acidophilus and Bifidobacterium bifidum besides Yoghurt flora (Schioppa et al., 1981 and Zoikowski, 1981) is not uncommon now-a-days. The use of Yoghurt is not restricted to Balkan states where it finds its origin but has proliferated round the globe. Advertisement to Kissle, an uncultured dairy product as a challenger to Yoghurt's supermacy in United States speaks itself for the high consumption of yoghurt in the Western market of dairy products.

In India, Dahi is the comparable fermented product, being consumed since ancient times. Yoghurt excels better in certain aspects in comparison with Dahi i.e. short duration product, firm body, characteristic flavour, high nutritive and therapeutic values (Deeth and Tamime, 1981). The consumers are fascinated by its suitability to lactose and milk protein intolerant persons and adult women etc. Yoghurt therefore is a relatively new introduction to Indian dietary system and gaining wider acceptance gradually.

In Yoghurt, the milk protein exists as casein in the form of micelles and possesses the ability of interaction among these micelles and whey protein. Subsequently, these micelles undergo hydrolysis and aggregation under the individual or combined effect of pH, high heat and the presence of proteolytic enzymes. Thus, interaction among casein, fat additives, whey and lactic acid bacteria results in the development of typical microstructure of Yoghurt. With the advancement of knowledge, a significant correlation between the physical, rheological properties and the microstructure of Yoghurt and other dairy products is being established.

The microstructure of Yoghurt, infact, is influenced by different manufacturing processes and storage conditions. Any alteration in the microstructure, is translated into variation in physical properties. Hence, a study of microstructure and ultrastructure of yoghurt coagulum and its components will help in recognition of food resources utilization, optimization of manufacturing processes and evaluation of quality.

The microstructure of yoghurt can be studied by electron microscopy envisaging the use of both scanning electron microscope (SEM) and transmission electron microscope (TEM). The transmission electron microscopy facilitates the identification of multi-molecular structure of proteins and protein complex, whereas, scanning electron microscopy

comperatively with less resolution but higher depth of focus is able to discern sub-microscopic moieties particularly the surface or 3-dimensional morphology. This is indeed a very fascinating area of modern research which allows fine structural analysis and molecular close ups. However, only limited information is available on this aspect. In view of this, a systematic study on yoghurt milk gel has been undertaken to investigate the comperative microstructure of yoghurt from fresh, unfortified cow and buffalo whole milks pre-heated at different time-temperature combinations employed by manufacturers and subsequently stored for different period till about a week at low temperature. The study is being mainly confined to structure and distribution of casein and lactic acid bacteria. Most of the structural studies are restricted to SEM but transmission informations have also been incorporated wherever considered necessary.

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CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Yoghurt is a typical biological system comprising of lactic acid bacteria and protein, hence is liable to be influenced by various environmental and compositional factors. These factors include composition of milk, heating of milk, starter cultures, incubation time-temperature, stabilizers and storage of the product etc. The physical and textural properties are the product of all these variables. The electron microscopy particularly scanning was not much commercialised for the study of dairy products before the year, 1965. This technique can be successfully used to visualise the relation between microstructure and properties of the finished yoghurt.

2.1 YOGHURT MANUFACTURE

Yoghurt can be prepared from all kinds of bovine milk available now-a-days. There is common practice to heat the milk prior to inoculation with culture for yoghurt manufacture. The heating of milk results in reduction of water content accompanied by increase in total solid content of milk and improvement in the texture of finished product.

Storgards (1964) rated sterilization at 134C inferior to pasteurization at 80C for 30 minutes for yoghurt preparation.

Pasteurization of cow milk at 85C for 20-30 minutes was observed to yield the best hydrophilic properties of the protein in Yoghurt while pasteurization at higher temperature (90-95C) for the same period appeared to bear deteriorative impact on this characteristic (Grigorov, 1966; Hrabova and Hylmar, 1974; Hong and Goh, 1979). Hydrophilic properties for which the yoghurt gel is best known are the outcome of modification in protein structure. This modification comes into existence owing to interaction among protein components (Humphreys and Plunkett, 1969; Rasic and Kurmann, 1978). Huhn et al. (1981) opted heating the buffalo milk at 90C for 30 minutes for Yoghurt manufacture. A pre-heating temperature of 80-90C for 10 minutes was recommended by Dolezalek and Vokacova (1981).

Heating of milk not only effects the pH of milk initially but influences the pH of milk at gelation point during Yoghurt manufacture too. Kalab et al. (1976) observed that unheated skim milk gelled significantly slower than heated milk. First sign of gelation appeared at pH 5.14 and gelation was in full swing at pH 4.92 in unheated milk. For heated skim milk, pH values corresponding to these changes were 5.36 and 5.17 respectively.

Viscosity and consistency of the product are the index of quality and have been found to be influenced by heating temperature-time combination of milk in case of Yoghurt. Stadhouders and Hassing (1973) found that

substitution of batch heat treatment of yoghurt milk with HTST process rendered the product of lower viscosity. The apparent viscosity of milk and cultured yoghurt thermally treated by UHT and Vat systems was measured by Labropoulos et al. (1981_a). The values for Yoghurt prepared from UHT milk, milk heated at 63C and 82C was 0.8, 1.8 and 3.9 C.P. after 14 minutes of shearing respectively. A specific change in whey proteins brought by Vat process only and missing in UHT milk has been postulated as the cause of formation of firm structure (Labropoulos et al, 1981b). The significant role of SH-group in disulphide bond formation and whey protein denaturation has already been deciphered and discussed (Lyster, 1970; Parry et al., 1974).

The use of Lactobacillus bulgaricus and Streptococcus thermophilus strains as yoghurt starter culture has been well documented. A symbiotic relationship between these lactic acid bacteria in milk enabled them to play their significant role in Yoghurt production. Bautista et al. (1966) attributed this biological relationship to the accumulation of growth factors comprising glycine and histidine in L. bulgaricus culture. Streptococcus thermophilus, in turn was reported stimulating L. bulgaricus by producing a factor similar to formic acid (Galesloot et al., 1968). According to a recent report, L. bulgaricus requires not less than 31 mg Co_2 per kg of milk for its optimal growth (Driessen et al., 1981). In milk, S. thermophilus furnishes Co_2 in excess for the growth of its counterpart. Valerie et al. (1982)

succeeded in manufacturing Yoghurt from single starter, either L. bulgericus or S. thermophilus using milk to which casein hydrolysate had been added.

Incubation temperature-time combination of the cultured milk also plays no less important role in Yoghurt preperation. Hrabova and Hylmar (1974) recommended the low temperature incubation (30C for 17 h) to avoid any damage to yoghurt structure. Bottazzi (1977) in an attempt to evaluate new microbiological and technological aspects of yoghurt production, opted for 42C as temperature of incubation till the pH of milk reached 4.0. Magdesi (1979) preferred incubation temperature of 36C rather than conventional temperature of 42C. Out of three different coagulation temperatures tried 30, 37 and 45C, Dolezalek and Vokacova (1981) found 45C the most suitable incubation temperature. Huhn et al. (1981) also favoured this temperature of incubation for Yoghurt from buffalo milk. Harris (1982) incubated the milk at 44C for 6.5 h and 30C for 18 h for Yoghurt preperation. He came to the conclusion that samples incubated at 44C had a firmer body with more free whey, whereas, those incubated at 30C had improved acid, flavour and aroma.

Post-incubation operations are the factors finally responsible for the quality of Yoghurt. Sternberger (1973) studied the structural damages Yoghurt suffered during

its passage through filling plant. He found that various factors viz. stirring of the product in the incubation vessel, passage through pipes and constrictions contributed to the damage cumulatively. Lumps were observed in Yoghurt by Galesloot and Hassing (1973_b) and it was concluded that viscosity of gel before and after stirring and bacterial mucus played important role in lumps formation. These lumps are found to consist of a protein matrix only slightly denser than the surrounding medium (Kalab, 1979). Different compositional and manufacturing factors are found responsible for shaping the problem of nodulation in stirred yoghurt (Robinson, 1981).

Yoghurt is well known at the same time for its typical aroma and flavour. These characteristics bank upon the production of carbonyl compounds during the fermentation of milk by lactic acid bacteria. In Yoghurt L(+) lactic acid is the major isomer formed by these starters. Forty percent of the total acid has been found of D(−) configuration in this product (Alm, 1982). Other important compound contributing to the flavour of Yoghurt is acetaldehyde (Law, 1981). Acetaldehyde values between 23 and 41 ppm give the optimum flavour and its rate of production depends on the level of acidity (Green and Manning, 1982_a). But Gyosheva (1982) was of the view that aroma is determined by aroma complex formed from volatile aromatic compounds and volatile free fatty acids.

Towler *et al.* (1980) dehydrated the Yoghurt into yoghurt powder and observed 0.1 percent and 0.001 percent survival of *S. thermophilus* and *L. bulgaricus* respectively in the powder. The protective effect of cryoprotective agents, total solids and stabilizers on starter bacteria during freeze-drying in Yoghurt has been visualised by Cabrini *et al.* (1982).

Yoon (1981) analysed the liquid yoghurt for carbohydrate and minerals content. Sucrose and lactose, are the main carbohydrates, whereas , Calcium, Sodium, Potassium and Phosphorous are the chief minerals found in product.

2.2 USE OF ELECTRON MICROSCOPY IN MILK PRODUCTS

The electron microscopy envisaging both transmission and scanning finds its wider application in the study of structure of milk and milk products at molecular and three-dimensional levels. The selection of a technique to be employed for a particular product depends upon the composition of product and type of informations required.

Knoop (1972) carried out EM studies to understand the process of crystallization of fat globule and formation and modification of casein micelles.

Using Scanning electron microscopy, a variety of food gels has been studied by Kalab and Harwalkar (1973). Casein micelles linked by short thin fibres in heat induced

Chabot (1979) summarised various methods used for the preparation of food sample for both SEM and TEM and the various problems encountered. The SEM techniques used for dried milk, dried whey protein, yoghurt, cottage cheese were later reviewed by Kalab (1979). Kalab (1981) discussed the different techniques employed in both SEM and TEM in milk products summarised as under:

Technique	Nature of specimen	Example of milk product for which suitable
	SCANNING ELECTRON MICROSCOPY	
Conventional	Dry	Powdered milk, whey, buttermilk etc.
	Dried	Low-fat milk products (Yoghurt, Cottage cheese, some cheeses)
		Cheese (fat extracted)
		Cheese (trypsin-etched)
	Freeze-fractured and replicated with gold	Viscous and high-fat milk products (cream, butter, cheese etc.)
Cold stage	Freeze-fractured and coated with carbon and/or gold	Viscous, whipped, high-fat products (ice cream, whipped cream, butter etc.)
	TRANSMISSION ELECTRON MICROSCOPY	
Negative staining	Suspension	Fluid milk, cream
Metal shadowing	Suspension	Fluid milk, cream
	Suspension	Liquid products (microcapsulation)
Thin-sectioning	Solid	Products solid by nature(cheese) or solidified by mixing with agar (cream)
Freeze-fracturing (Freeze-etching)	All products	All products
Replication of dried specimen	Solid	Milk products based on protein

Replication of freeze-fractured surface with heavy metals like platinum is another useful technique employed recently (Katoh, 1979; Kalab, 1980a). Freeze-fracturing has been found equally suitable for structural and compositional analysis of dried milk products (Buchheim, 1981).

Double fixation of yoghurt gels in gluteraldehyde and osmic acid were found suitable for TEM study (Kalab et al., 1976; Davies et al., 1978).

2.3 CASEIN MICROSTRUCTURE IN FRESH AND STORED MILK PRODUCTS

2.3.1 Milk gels:

Electron microscopy has been widely used to study the microstructure of milk gels and effect of storage on high heat treated milk.

Under the influence of heating, the casein micelles swell and are linked together by short bridges into long chains. The spatial arrangement of these long chains finally result into a three-dimensional structure (Kalab et al., 1973). The total solid content of milk including protein plays an important role in skim milk gel formation. The skim milk with high total solid content (60 percent) has been found comprising of fused micelles and yielded a gel of considerably high strength (Kalab and Harwalkar, 1974).

The pH of milk is another important factor which influences the aggregation of milk protein in heated skim milk. Creamer et al. (1978) observed a number of thread-like structures in the milk heated at high pH (6.80). However, there was chemically no difference in composition of protein aggregates of heated milk from the unheated milk. High pH of milk is associated with high negative electrostatic charge on protein and high repulsive forces between protein aggregates. Thus, high pH prevents the attachment of heat induced complexes with micelles. Acid gels prepared from heated and unheated skim milk give the similar results. Higher the temperature of gelation of milk adjusted to a definite pH, firmer is the gel formed. Harwalkar and Kalab (1981) elaborated that gels made at 90C were three times more firmer than gel made at 70C at pH 4.6. At higher pH, the gap between firmness of gels heated at different temperatures becomes wider.

Gel formation is a common defect encountered during storage of concentrated milk. Schmidt (1968) compared ultra-high temperature short time (UHTST) sterilized milk with that of conventionally sterilized concentrated milk. In UHTST milk, casein micelles confluenced into net which remained independent in sterilized milk even after longer storage.

It appears that coalescing of casein micelles marks the begining of gel formation during storage of evaporated milk. This fusion is confined to the outer layers of the particles, at the nuclei of these particles later on and thus a three-dimensional network comes into existence (Schmidt and Buchheim, 1968).

The casein micelles increase in size during the process of concentration. Fresh, untreated skim milk was found to comprise of casein micelles of size range of 500-2500Å, while micelles doubled in size (1500-4000Å) in concentrated skim milk (Carroll et al., 1971). According to this group of research workers, heating of milk may influence the size of casein micelles in the following ways:-

a) interaction of heat denatured whey proteins with casein micelles;
b) deposition of insoluble serum protein on casein micelles;
c) increase in Calcium content leading to calcium bridging; among micelles;
d) increase in the number of casein micelles due to splitting of the micellar surface.

Davies et al. (1978) also emphasised that heat treatment of milk resulting in denaturation of whey protein and interaction of these with micelle surface determines the micelle fusion and gel strength.

Temperature of storage plays a pivotal role in gelation of evaporated milk during storage. Storage of evaporated milk at elevated temperature before sterilization for canning improves the shelf-life of the canned product (Heintzberger et al., 1972). Andrews et al. (1977) studied the properties of aseptically packed ultra-heat treated milk during storage and observed gel formation after 34 months of storage. There is an increase in size of casein micelles with increase in temperature of storage.

Microstructure of spray-dried, roller-dried and freeze-dried skim milk powders and gels prepared from these powders were studied by Kaleb and Emmons (1974). Gel prepared from spray dried powder showed individually distributed casein micelles linked by bridging materials, while roller-dried skim milk powder gel contained both fused as well as sparsely distributed casein micelles. The freeze-dried powder exhibited the firmest gels formed by a network of casein micelles aggregated together.

2.3.2 Cheese:

A good number of cheese variety is manufactured and consumed round the globe. Electron microscopy has been employed to study the development and microstructure of a few of them.

Cheeses consist of spongy microstructure, the proteins attain the form of network of casein micelles, cavities in

between filled by fat and whey (Mudder et al., 1966). The structure development is initiated by fusion of individual micelles to form long chains. These chains grow into multiple strands either by shrinkage of the chains or by aggregation of adjacent chains (Kimber et al., 1974).

Kneep and Peters (1971) studied sub-microscopic structural variations in Camembert cheese and reported four different kinds of casein breakdown in the structure development. Rennet and bacteria were found playing about insignificant role in the process of ripening. Smooth curd formation probably due to the deposition of -lactoglobulin on the casein particles was observed by Prokopek et al. (1976) in Camembert cheese made from variously treated cream-enriched skim milk.

The texture and microstructure of Cheddar cheese has been studied by a number of workers. Hall and Creamer (1972) placed Cheddar cheese in the intermediate positim with respect to Gouda and Cheshire cheeses in terms of size of globular units forming the protein matrix. Kalab and Emmons (1975_a) observed fibrillar structure in the cheese giving chicken breast muscle texture to the product. After cheddaring is complete, this structure gets transformed into marble-like structure due to formation of casein mass between the fat globules by fusion of casein fibres at sub-microscopic level (Kalab and Emmons, 1978). Emmons et al. (1980) asserted that cheese with reduced fat

containing more protein matrix is liable to be firmer than full fat cheese.

The considerable difference in the microstructures of Gruyere and Emmental cheeses was observed by Ruegg and Blanc (1972). Gruyere cheese maintains the regular fine structure with no sub-microscopical cavities (Ruegg et al., 1980).

Fresh cheese, Gouda cheese and Processed cheese were compared by Kimura and Taneya (1975). Casein micelles measured 200-600 nm in diameter in curd and Gouda cheese, whereas 20 mm in processed cheese. Heertage et al. (1981) elaborated further the structure of processed cheese. They reported material of approximately 300 nm length with 10 nm in diameter formed by dissociation and association of casein micelles. These string-like structures are found missing in soft type processed cheese (Taneya et al., 1980).

Comparing the structures of Harzer, Tilsit and Camembert cheeses, Knoop and Buchheim (1980), emphasised that there would be basic structural differences between acid curd cheeses (Harzer) and rennet cheeses (Tilsit and Camembert). The explanation for this phenomenon lies in the fact that proteolysis is done by bacterial enzymes in acid curd and rennin in rennet cheeses.

Glaser et al. (1979) studied the skin formation around the curd in cottage cheese during curing procedure as

reported earlier in the literature. They ruled out this phenomenon in cottage cheese and even if existed at all was due to high heating treatment and not due to conventional curing procedure according to their view.

The ripening process in cheeses comprises two phases- the dissociation of casein micelles and setting of whey into the disintegrated mass of protein. The rate of disintegration process increases with the decrease in calcium content. The osmotic pressure produced by casein micelles may be causing the penetration of whey into the casein matrix (Brooker, 1979). In general, the structure controls the behaviour composition, body, texture and yield of cheeses. Proteolysis brought out by coagulant, bacterial and milk enzymes has significant impact on microstructure during ripening (Green and Manning, 1982).

Most of the hard cheeses were found to contain microscopic crystalline inclusions. These inclusions are normally confined along curd junctions though distributed throughout the body of the cheese. Ruegg and Blanc (1972) reported these crystalline inclusion bodies in Emmental cheese and amino acids were suggested the basic building units. It was also suggested that the decayed lactic acid bacteria appears to provide crystallization nuclei leading to the formation of crystalline inclusions in cheese (Kalab, 1980_b). Bottazzi *et al.* (1982) reported compact and needle shaped structures in Grana cheese. Compact

bodies measured 10-20 nm in diameter of calcium phosphate. The needle like crystals were 20-25 nm in length and were composed of calcium lactate as revealed by X-ray microanalysis.

Brooker et al. (1975) reported curd granules composed of calcium phosphate in cheese. Similar granules were also observed in cheddared curd, Mozzarella and Provolone cheeses by Kalab (1977). SEM micrographs revealed that the junctions consist of compacted protein zones less fat than the interior of the curd granules. Separation of these types of curd granules at junctions is rather feasible. Improper homogenisation can be one possible explanation for curd granules formation. That is the reason why fat globules are lost at the granules surfaces leaving junctions protein rich (Emmons et al., 1980).

2.3.3 Yoghurt:

Yoghurt is a protein based product. The liquid phase is immobilised to form coagulum. The coagulum consists of protein matrix which in turn is composed of casein micelles. These micelles are fused together and thus form chains and clusters.(Kalab & Emmons, 1975_b and Kalab, 1979).

Kalab et al. (1976) studied electron microscopically the relationship between microstructure of yoghurt and heating of milk used for its preparation. Skim milk heated to 90C produced Yoghurts much firmer than those of unheated skim milk. Corresponding values of curd tension of yoghurt samples were 64-68 g per probe and 26-33 g per probe respectively. The size

of casein micelles were bigger in the Yoghurt from unheated skim milk (mean diameter, 0.46 μm) than from skim milk heated to 90C (mean diameter, 0.23 μm). In heated skim milk, the changes were not so prominent but quite conspicuous in untreated skim milk. About half an hour before the gelation starts, the micelles started expanding and enlarging in their size. Short projections appeared on the smooth surface of casein micelles about 16 minutes prior to gelation. Non-micellar protein from the solution probably got accumulated on the outside of micelle, hence forming a temporary ragged surface. After 2-3 minutes, micelles started aggregating into larger units. When a three-dimensional net work was interwoven at the termination of this stage, projections were lost. The outlines of casein micelles forming gel reappeared smooth and distinct. Further examinations of yoghurts prepared from skim milk heated to different temperatures showed that there is a critical temperatures which affect both casein and whey proteins. With these findings, the authors postulated that fusion of caseins in bigger micelles would result in fewer chains and points of junction, thus offering a much more open structure. The bigger micelle formation obviously results in soft coagulum of Yoghurt susceptible to syneresis.

Morr (1973) proposed the temperature-dependent alterations in micellar and soluble casein resembling Ca-dependent dissociation- association of soluble casein with micelles in milk. The associated materials do not

seem to indulge in complex formations within the micellar interstices but remains outside the casein micelles. Findings of various groups of workers support the view that heating of milk results into denaturation of whey proteins which associate subsequently with K-casein through disulphide bonds (Parry, 1974; Morr, 1975 and Fox and Morrissey, 1977).

Ruegg and Blanc (1978) studied the influence of pasteurisation and UHT processing upon the size distribution of casein micelles in milk and asserted that there is an increase of number of free sub-micelles upon heating. The severity of the heating methods can be ranked in the order HTST > UHT direct > UHT indirect.

A heat induced change in the ultrastructure of milk and its effect on gel formation in Yoghurt was visualised by Davies et al. (1978). They observed that micelles from raw milk possessed smooth contours while micelles from heated milk bore appendages composed of denatured β-lactoglobulin. These appendages appear to inhibit coalescence of micelles and thus facilitating the formation of a firmer curd with lower tendency to syneresis. Mehriz and Ganguli (1980) also asserted that forewarming of buffalo milk improves heat stability.

Redema and Van (1973) assessed the effect of different thickening agents in yoghurt manufacture. Gelatin, sodium caseinate and amylopectin were found giving satisfactory

results. Kalab and Emmons (1975_b) reported that out of the various thickening agents used, Carrageenan and starch altered the Yoghurt microstructure which could easily be detected by TEM and SEM. In the presence of starch, flat sheets and smaller casein micelles were the typical structures observed.

Kalab et al. (1976) calculated the size of casein micelles in Yoghurt of different origin after the storage at 6C for one week and results were as follows:-

		Pre-heating temperature	
		44C	90C
A.	One day Yoghurt:		
	Fresh	0.467 µm	0.237 µm
	Spray-dried	0.566 µm	0.270 µm
	Roller-dried	0.342 µm	0.319 µm
	Freeze-dried	1.099 µm	0.280 µm
B.	One week Yoghurt:		
	Fresh	0.440 µm	0.226 µm
	Spray-dried	0.476 µm	0.289 µm
	Rollder-dried	0.306 µm	0.298 µm
	Freeze-dried	0.757 µm	0.258 µm

In general, the micelles shrank during storage to some extent, the effect being more prominent in case of freeze-dried skim milk.

2.4 FATE OF LACTIC ACID BACTERIA DURING MANUFACTURE AND STORAGE OF MILK PRODUCTS

Lactic acid bacteria when added to milk lower the pH of milk to curdling point through the production of lactic acid. After the process of curd formation is complete,

the association of these bacteria with coagulum is the subject of great curiosity. These starter bacteria whether simply form part of the microstructure of are actively involved in the processing of dairy products is the another point needs to be elucidated.

Dawson and Feagon (1957) observed uniform distribution of S. lactis and S. diacetilactis, whereas S. cremoris were found in the form of colonies in curd of Cheddar cheese. Uneven distribution of starter bacteria in cheese has been reported (Dean et al., 1959 and Rammell, 1960). Lactobacilli were found multiplying fast in the initial stage of curing (Johns and Cole, 1959).

Cheese samples prepared using single and mixed starters showed a decline in total bacterial count after three months of ripening (Ranganathan and Laxminarayana, 1972). Kalab (1979) observed the formation of pockets by colonisation of bacterial cells in the protein matrix of Yoghurt. Dhingra (1981) also supported the microcolony formation in Yoghurt coagulum on the basis of light microscopic observation.

The electron microscopic studies have revealed the mucus production by ropy strains of starter bacteria in milk products. A correlation has been established between viscosity of Yoghurt and mucus production by bacteria (Galesloot & Hassing, 1973a,b,c and Kroger, 1976). Kimber et al. (1974) spotted the starter bacteria entrapped in

casein near the fat-casein interface in Cheddar cheese. TEM observations revealed the tenuous filament connections between bacteria and casein matrix. Brooker (1976) asserted that an extracellular material secreted by bacterial cell wall is extended into these long filaments connecting casein micelles and milk fat globules. This extra-cellular material was found to be acidic carbohydrate in nature. These filaments are proposed to be connected matrix in the microstructure and prevents their removal from the curd with the whey during syneresis. Such filaments have been reported in Gouda, Edam and Mozzarella cheeses (Kalab, 1977) and in Yoghurt (Bottazzi, 1977; Kalab, 1979). Ruegg et al. (1980) observed these filaments among bacteria covering the rind of Gruyere cheese. This group of workers also described the dimensions of lactic acid bacteria. Streptococci measured to 0.94 μm in diameter, while lactobacilli measured 0.78 and 2.6 μm in diameter and length respectively. All these findings support the observation of Kalab and Emmons (1975_b) that lactic acid bacteria are not merely the incorporation in the micro-structure but are also associated with milk gel structure significantly.

Bottazzi (1980) examined the microflora of Kefir granules. He observed most richly colonized part near the exterior of the granules which contained mainly bacteria

and a few yeasts. In the centre of the granule, the ratio between yeasts and bacteria was found reversed.

Borraquio *et al.* (1981) studied the keeping quality of Yoghurt and found yeast and mold as contaminants during storage.

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3. MATERIALS AND METHODS

3.1 YOGHURT MANUFACTURE

3.1.1 Collection of milk samples

With the objective of controlling the history of milk samples, fresh cow and buffalo milk samples were collected directly from Cattle Yard, N.D.R.I., Karnal in clean and sterilized sample bottles.

3.1.2 Pre-heating of milk samples

Milk samples were filtered and heated without any fortification at 70C and 90C for 30 minutes in a water bath equipped with thermostat.

3.1.3 Inoculation and incubation of milk samples with starter cultures

Yoghurt cultures, Lactobacillus bulgaricus (W) and Streptococcus thermophilus (H) were obtained from National Collection Centre of Dairy Organisms, N.D.R.I., Karnal. Cultures were maintained in sterilized litmus milk tubes. Prior to inoculation, the cultures were propagated in sterilized skim milk in larger volume of 100 ml.

The milk samples of cow and buffalo (4 samples in duplicate) were removed from water bath after heating and cooled to 42C. At this temperature, these samples were inoculated with starter cultures in the ratio of 1:1 at 3 percent level and mixed thoroughly with the help of a clean electrically operated glass stirrer. Subsequently, the contents were subjected to incubation at 42C ± 0.5C for 4 h. For electron microscopy, the inoculated milk samples were distributed in small dishes of 10 cm depth and 50 cm diameter and microbeakers of 15 cm depth and 20 cm diameter. For pH and viscosity measurements, the yoghurts were prepared in 100 ml beakers.

3.1.4 Storage of Yoghurts

After incubation period, the set yoghurt samples were removed from the incubator and cooled to about 7C and transferred to refrigerator at 4-6C for further storage till 120 h. Various steps involved in Yoghurt manufacture are summarised in flow Diagram I.

3.2 MEASUREMENT OF P^H OF MILK GELS DURING YOGHURTS PREPARATION

The pH of inoculated milk samples were measured at half an hour interval regularly till setting of coagulum using EC-Digital pH meter.

3.3 MEASUREMENT OF VISCOSITY AND JUDGEMENT OF YOGHURT COAGULUM QUALITY

To have an idea of consistency of fresh yoghurts, the viscosity of fresh samples was measured using constant stress rotation viscometer (Associated Instrument Manufacturers (India) Private Ltd.). In addition to it, Yoghurts were judged visually in terms of body of coagulum and degree of syneresis in each sample.

3.4 MEASUREMENT OF YOGHURTS ACIDITY DURING STORAGE

The titrable acidity of fresh and stored Yoghurts was determined according to the procedure given in I.S.I. (1960). Ten g of the sample mixed with equal volume of distilled water was titrated against N/9 NaOH using 1.0 ml of phenolphthalein (0.5%) as indicator. The acidity was expressed as % lactic acid (by wt.).

3.5 ELECTRON MICROSCOPY

3.5.1 Preparation of Yoghurt samples for electron microscopy

3.5.1.1 Preparation of reagents:

3.5.1.1.1 Preparation of cacodylate buffer:- To prepare 0.1 M cacodylate buffer, 1.6 g of sodium cacodylate (Fluka Batch No.13530I-120) was dissolved in 100 ml glass distilled water. The pH was adjusted to 7.2 using a EC-Digital pH meter.

3.5.1.1.2 Preparation of buffered Osmic acid solution:-
The osmium tetroxide (Sigma, Batch No.127C-0502) is commonly available as 1 g sealed glass vial. The surface of vial was thoroughly cleaned with soap and distilled water to remove any organic matter present on glass surface. The glass vial was cracked in the middle into two halves using iron file under a hood. The content was transferred with no loss of time to a clean amber coloured glass. stoppered bottle containing 25 ml of deionised water. The stopper of bottle was well tightened and stored at low temperature in dark place. All the precautions were taken not to come in direct contact with solution or fumes. The final concentration of 2 percent osmium tetroxide was prepared by mixing equal volumes of stock solution and 0.1 M cacodylate buffer adjusted to pH 7.2.

3.5.1.1.3 Preparation of buffered glutaraldehyde solution:-
(Sabitini et al., 1963)

Following composition was adopted to prepare 2.5 percent buffered glutaraldehyde:-

25% Glutaraldehyde EM grade (Polysciences, Batch No. 99C-5029)	2.5 ml
0.1 M Cacodylate buffer	12.5 ml
Distilled water	10.0 ml
Total volume	25.0 ml

3.5.1.1.4 Preparation of graded series of ethanol-water mixtures:-

For the purpose of dehydration of Yoghurt, samples like any other biological specimens, a graded series of ethanol-water mixture of 30, 50, 70, 90, 100 percent was prepared. Absolute acetone and propylene oxide were used for further dehydration.

3.5.1.2 Procedure:

3.5.1.2.1 Sampling of Yoghurts:- In addition to fresh Yoghurts were sampled at 24, 72 and 120 h intervals of storage. For sampling, undisturbed coagulum in small dishes and microbeakers was cut into small cubes. The cubes were further trimmed into 2mm x 2mm x 2 mm sizes.

3.5.1.2.2 Fixation:- Fragmented curd cubes were transferred to labelled microbeakers conteining 2.5 percent buffered glutaraldehyde fixative. The fixation continued in cold for 3 h. The cubes were further trimmed to 1 x 1 x 1 mm size to facilitate the diffusion of fixative into coagulum. The fixed samples were buffered with 0.05 M cacodylate at 4-6 C for 6 h. The buffered samples were doubly fixed in 2 percent osmic acid at 4-6C for 2 h. Each solution was replaced gradually from one to another in the same container during the whole process.

3.5.1.2.3 Dehydration:- After fixation of Yoghurt specimens, these were subjected to dehydration as per the schedule given below:-

Concentration of mixture	Duration
30 percent ethanol	5-10 minutes in cold
50 percent ethanol	5-10 minutes in cold
70 percent ethanol	5-10 minutes in cold
90 percent ethanol	5-10 minutes in cold
100(Absolute) ethanol	30-45 minutes in cold
100 (Absolute) ethanol	1 h at room temperature

3.5.2 Scanning Electron Microscopy

3.5.2.1 Drying of the Yoghurt samples:

3.5.2.1.1 Air drying: - For air drying, the specimens were further processed through absolute acetone for 20 minutes twice and subsequently dehydrated in propylene oxide. The dried samples were transferred to desiccator for overnight.

3.5.2.1.2 Freeze-drying(Kalab,1978):- For freeze-drying, the samples were diverted from the routine course after fixation in Osmic acid. Samples were lyophilised in Toshniwal Lyophiliser. This process took about one to one and half an hour for completion.

3.5.2.2 Coating of specimens:

Freeze-dried and air-dried samples were mounted on

stubs with adhesive and sputter coated with gold at approx. 100-200 Å thickness on Hitachi IB-3 ion-coater. The ion current was kept at 6mA at fine vacuum of 0.05-0.07 torr for 2-4 minutes.

To visualise the internal microstructure of coagulum, the air-dried specimens were fractured with the help of a sharp blade and coated the fractured surface. The portion with blade mark was totally avoided during examination.

3.5.2.3 SEM Observations:

The samples on aluminium stubs were placed in specimen holder and inserted into chamber under vacuum. Hitachi S-405A Scanning Electron Microscope was operated at 15 kv using secondary electron mode.

3.5.3 Transmission Electron Microscopy

3.5.3.1 Embedding of Yoghurts:

3.5.3.1.1 Preparation of embedding mixture (Mascorro et al., 1976):-

Spurr's low viscosity embedding medium was used for the purposes of embedding Yoghurt specimens in the present study. The composition and preparation of medium were as follows:

Vinylcyclohexenedioxide (VCD-Epoxy resin)	5.0 g
n-Hexenyl succinic anhydride (HXSA, Hardner)	10.0 g
1,4-butanedioldiglycidyl ether (Araldite RD-2, Modifier)	0.75g
Diethylaminoethanol (DMAE, Catalyst)	0.15g

All the above components were poured into a dry beaker with the help of pipete, pipet filler attached at other end as per the above mentioned schedule. Proper mixing of the embedding mixture was accomplished by the use of electrically operated glass stirrer. Air incorporetion during agitation was considered highly undesirable.

3.5.3.1.2 Procedure:- For embedding, the dehydrated specimens were processed through the following schedule:-

1:1 ratio of ethanol-embedding mixture	5 minutes at room temperature
Pure embedding mixture	10 minutes at room temperature

3.5.3.2 Ultramicrotomy:

LKB Ultramicrotome-V was used and glass knives were employed for the purpose of ultrathin sectioning. The ribbons containing grey and silver sections werr stretched with the help of toluene.

New copper grids (200 mesh) were cleaned in 1N Hcl for 3-4 minutes to ensure complete removal of greasy material. Grids were thoroughly washed with distilled water to remove residual acid and subsequently rinsed in absolute alcohol several times and dried in desiccator.

Ultrathin sections were picked up on clean and dry copper grids.

3.5.3.3 Staining of ultrathin sections:

3.5.3.3.1 Preparation of stains:

3.5.3.3.1.1 Preparation of lead citrate(Reynolds, 1963):-

Lead nitrate	1.33 g
Sodium citrate	1.76 g

The ingredients listed above were added to a 50 ml volumetric flask with 30 ml distilled water. The suspension so prepared was agitated vigorously for one minute and allowed to stand with intermittent shaking to ensure the complete conversion of lead nitrate to lead citrate. After an interval of 30 minutes, 8.0 ml of 1N NaOH was added to the content of flask. The suspension was diluted further to 50 ml with addition of diluted water and mixed by inversion. Final pH of staining solution was found to be of pH 12.00 $\pm$ 0.1.

3.5.3.3.1.2 Preparation of Uranyl acetate:-

Uranyl acetate	0.5 g
Deonized water	100.0 ml

The ingredients were put in 250 ml conical flask. The mixture was subjected to vigorous shaking and was kept overnight for complete dissolution. Subsequently, the solution was filtered and stored in a glass stoppered bottle at low temperature.

3.5.3.3.2 Staining procedure:- Hydrophobic plastic sheet was used for the purpose of staining the ultrathin sections. A few drops of uranyl acetate were poured on the sheet and drops maintained etheir convex meniscus. The copper grids with ribbon were floated over the drops for 10-15 minutes, and switched to lead citrate staining for 4 minutes in the similar fashion. Grids after passing through the operation of staining were washed with distilled water while holding these in fine tip twizzers carefully. The grids were made free of excess water and dried. The grids were stored in a grid box and labelled.

3.5.3.4 TEM Observations:

Hitachi S-405A* Electron microscope was operated at 25 kV transmission mode for observing the yoghurt ultrathin sections mounted on copper grids. Some work was taken up at the Institute of Pathology, Delhi using Jeol -100CX Transmission Electron microscope.

3.6 ELECTRON MICROGRAPHY

3.6.1 Processing of photographic film

The observations were recorded on ORWO 35 mm perforated film (40 ASA) with the help of attached camera assembly. The film was developed in film developer (Kodak D-19) for 8-10 minutes at 20C and fixed in acid hardening fixer

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* Specifications and scale of Hitachi S-405A Electron microscope are given in Annexure I and II.

(Agfa-301) for 10-15 minutes. The film was thoroughly washed in running water and dried in air.

3.6.2 Printing of Electron micrographs

The prints were made on Agfa-brovira normal/hard photographic paper. Standerd (Agfa-100) and high contrast (Agfa-108) developers were used for developing the prints. Developing was followed by fixation in acid fixing bath (Agfa-300) and washing thoroughly under running water. The prints were finally dried on glazing machine.

3.6 ANALYSIS OF ELECTRON MICROGRAPHS

Electronmicrographs were visually observed with all precision and accuracy. The measurement of individual component of microstructure was made from a large number of micrographs using precision scale. Average values of all such observations were recorded and further interpreted.

A summary of the methods used for electron microscopy has been given in flow diagram-II.

I. FLOW DIAGRAM FOR YOGHURT MANUFACTURE

Collection of cow or buffalo milk

↓

Clarification

↓

Heating of milk samples at 70C or 90C for 30 minutes

↓

Inoculation of milk with starter culture at 42C

↓

Incubation at 42 ± 0.5C for 4 h

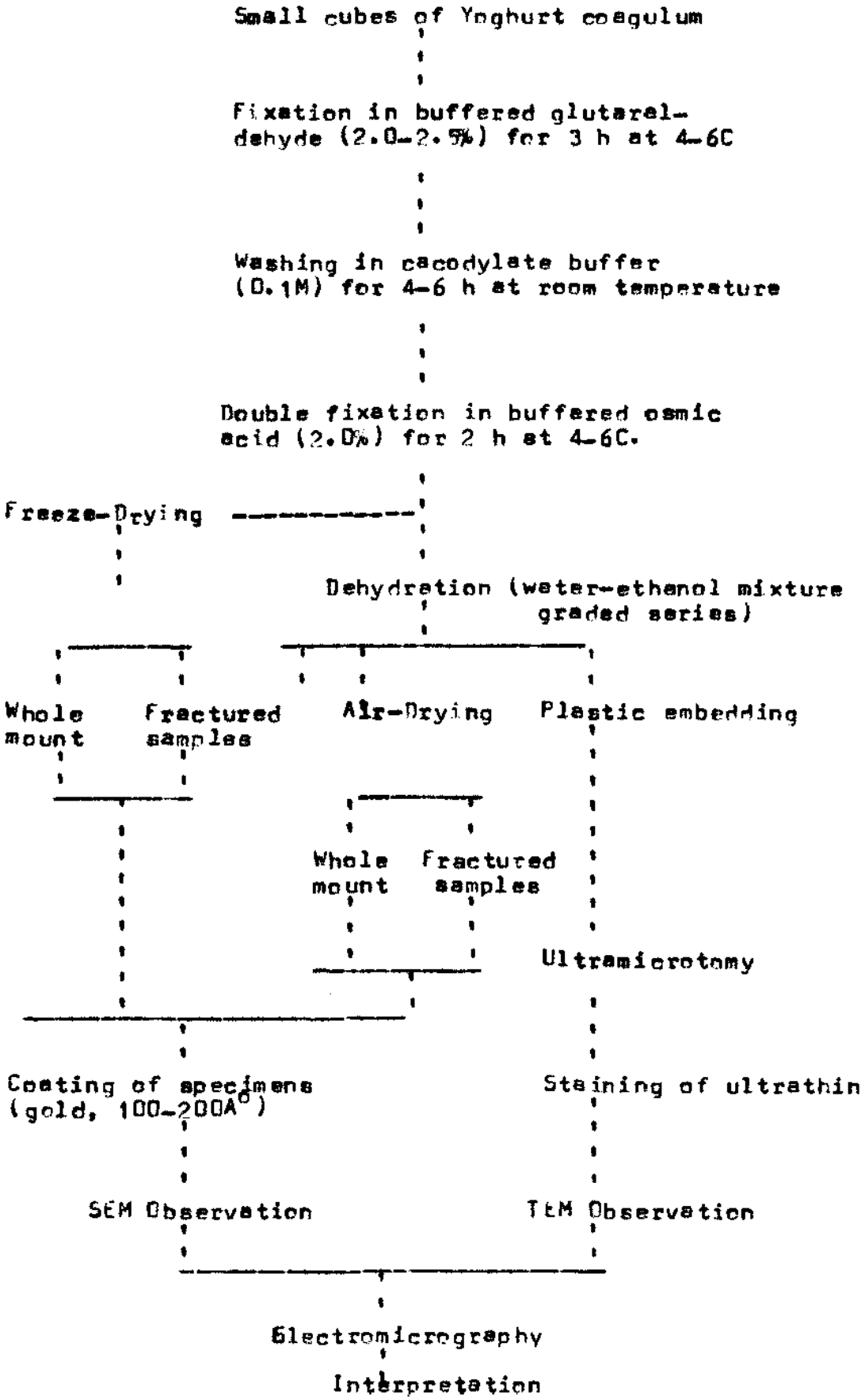
↓

Cooled to 7-10 C

↓

Stored in refrigerator at 4-6C

II. SUMMARY OF THE METHODS USED FOR ELECTRON MICROSCOPY



CHAPTER 4

RESULTS

4. RESULTS

Yoghurts of acceptable quality were prepared from fresh, unfortified cow and buffalo milks pre-heated at 70C and 90C for the holding period of 30 minutes and subsequently subjected to storage till 120 h. Samples were taken fresh and at 24 h, 72h and 120 h intervals of storage and processed further for scanning and transmission electron microscopic observations. Besides, pH, acidity and viscosity of yoghurt samples were measured to have an idea of development of yoghurt coagulum under different manufacturing and storage conditions.

4.1 MEASUREMENT OF P^H OF MILK GELS DURING YOGHURT PREPARATION

Using EC digital pH meter, pH of different gels from cow and buffalo milks pre-heated at different temperatures was measured at every half an hour interval till setting of coagulum. Initiation of curdling of milk was taken as curdling point for that particular milk. The pH values corresponding to the curdling points of buffalo milk pre-heated at 90C (B_{90}) buffalo milk pre-heated at 70C (B_{70}), cow milk pre-heated at 90C (C_{90}) and cow milk pre-heated at 70C (C_{70}) were 5.22, 5.28, 5.00 and 5.05 respectively (Table 1). In all the samples, curdling took place between

Table 1. pH measurement of milk gels during Yoghurts preparation

Sample		Sampling intervals in h							
Type of milk	Pre-heating temperature (holding period, 30 minutes)	0.0	0.5	0.10	01.5	2.0	2.5	3.0	3.5
	70C	6.88	6.58	6.00	5.76	5.58	5.30	5.05*	4.88**
Cow milk									
	90C	6.88	6.50	5.94	5.57	5.35	5.00*	4.73**	-
	70C	6.90	6.75	6.30	5.85	5.57	5.28*	5.08**	-
Buffalo milk									
	90C	6.90	6.72	6.22	5.73	5.22*	5.05**	-	-

* - Curdling point of milk

** - Setting point of coagulum.

2-3 h of incubation.

Setting of coagulum was observed in all the samples. The term " setting of coagulum" here implied the completion of coagulation process. It completed first in sample B_{90} (2.5 h), followed by B_{70} (3.0 h), C_{90} (3.0 h) and C_{70} (3.5 h). Corresponding pH values of setting points for different samples were 5.05, 5.08, 4.73 and 4.88, respectively.

4.2 MEASUREMENT OF VISCOSITY AND JUDGEMENT OF YOGHURTS COAGULUM QUALITY

In order to have an idea of quality and consistency of yoghurt coagulum, fresh Yoghurts were judged visually. In addition ot it, the viscosity of yoghurts was measured by constent stress rotation viscometer and expressed in terms of centipoise. On the basis of body of coagulum as well as degree of syneresis, yoghurts were graded as follows: B_{90} B_{70} C_{90} C_{70}. Sample C_{70} was found giving typical off flavour.

The values of viscosity for different yoghurt samples showed that B_{90} was firm as much as two times as compered to B_{70} . Similarly, cow milk yoghurt C_{90} was four times firmer than C_{70} (Table 2).

Table 2. Measurement of viscosity and judgement of coagulum quality of fresh yoghurts

Sample		Viscosity in centipoise	Coagulum quality
Type of milk	Pre-heating temperature (holding period, 30 minutes)		
Cow milk	70C	13 ± 2.0	+
	90C	49 ± 5.0	++
Buffalo milk	70C	238 ± 10.0	+++
	90C	550 ± 15.0	++++

4.3 MEASUREMENT OF YOGHURTS ACIDITY DURING STORAGE

The acidity of yoghurts was estimated at different intervals of storage. Samples were titrated against N/9 NaOH using phenolphthalein as indicator and acidity was expressed in terms of percent of lactic acid (by wt.). The results were presented in Table 3.

The acidity increased to different levels in various samples as storage period proceeded. There was little variation in acidity of yoghurts prepared from buffalo and cow milks pre-heated at 90C, whereas, the increase in acidity was substantial in yoghurts from buffalo and cow milks pre-heated at 70C during storage. The percent increase in acidity of different samples at 120 h interval of storage over fresh B_{70}, C_{70}, B_{90} and C_{90} samples was 33.3, 30.0, 9.4 and 6.0, respectively.

4.4 STUDY OF MICROSTRUCTURE OF FRESH AND STORED YOGHURTS AS REVEALED BY ELECTRON MICROSCOPY

The microstructure of fresh and stored samples of yoghurts prepared from cow and buffalo milks subjected to different heat treatments was studied by electron microscopy. Three dimensional informations were obtained by SEM whereas ultrastructural details through TEM. The coagulum of various yoghurts was observed comprehensively and only the representative micrographs have been included in this chapter.

Table 3. Acidity measurement of yoghurts during storage (as percent lactic acid)

Sample		Storage period			
Type of milk	Pre-heat temperature (holding period, 30 minutes)	Fresh	24 h	72 h	120 h
Cow milk	70C	0.70	0.75	0.80	0.91
	90C	0.83	0.84	0.87	0.88
Buffalo milk	70C	0.75	0.90	0.98	1.00
	90C	0.85	0.90	0.92	0.93

4.4.1 Fresh Yoghurts

After completion of fermentation, different samples of Yoghurt were removed from incubator and cooled to room temperature. At this stage, these samples were processed for scanning and transmission microscopy to study the microstructure and ultrastructure of fresh Yoghurts.

4.4.1.1 Yoghurt from cow milk preheated at 70C:

To study the three-dimensional surface structure of cow milk yoghurt, specimen was scanned under Scanning Electron Microscope. At lower magnification, the coagulum of Yoghurt was compact in structure. However, at higher magnification the same microstructure appeared as a three-dimensional network of casein micelles fused together in form of clusters and aggregates separated by interspaces throughout the coagulum. The interspaces were invariably wide and measured 3 u to 6 u in dimension. This typical organisation of microstructure is very well depicted in figures 1 and 2.

Casein micelles were observed in form of spherical and cylindrical shapes measuring 360-900 nm in diameter, with average dimension of 400 nm. The surface of unfractured coagulum seemed to be covered by aggregates of small globular structures presumably of coagulated whey protein.

FIGURES 1-8. ELECTRON MICROSCOPY OF FRESH YOGHURT FROM COW MILK PRE-HEATED AT 70C FOR 30 MINUTES (C_{70}).

* Fig.1. Scanning electron micrograph of fresh yoghurt at low magnification.

Fig.2. Porous microstructure of yoghurt coagulum showing pockets (white arrow) and interspaces (black arrow).

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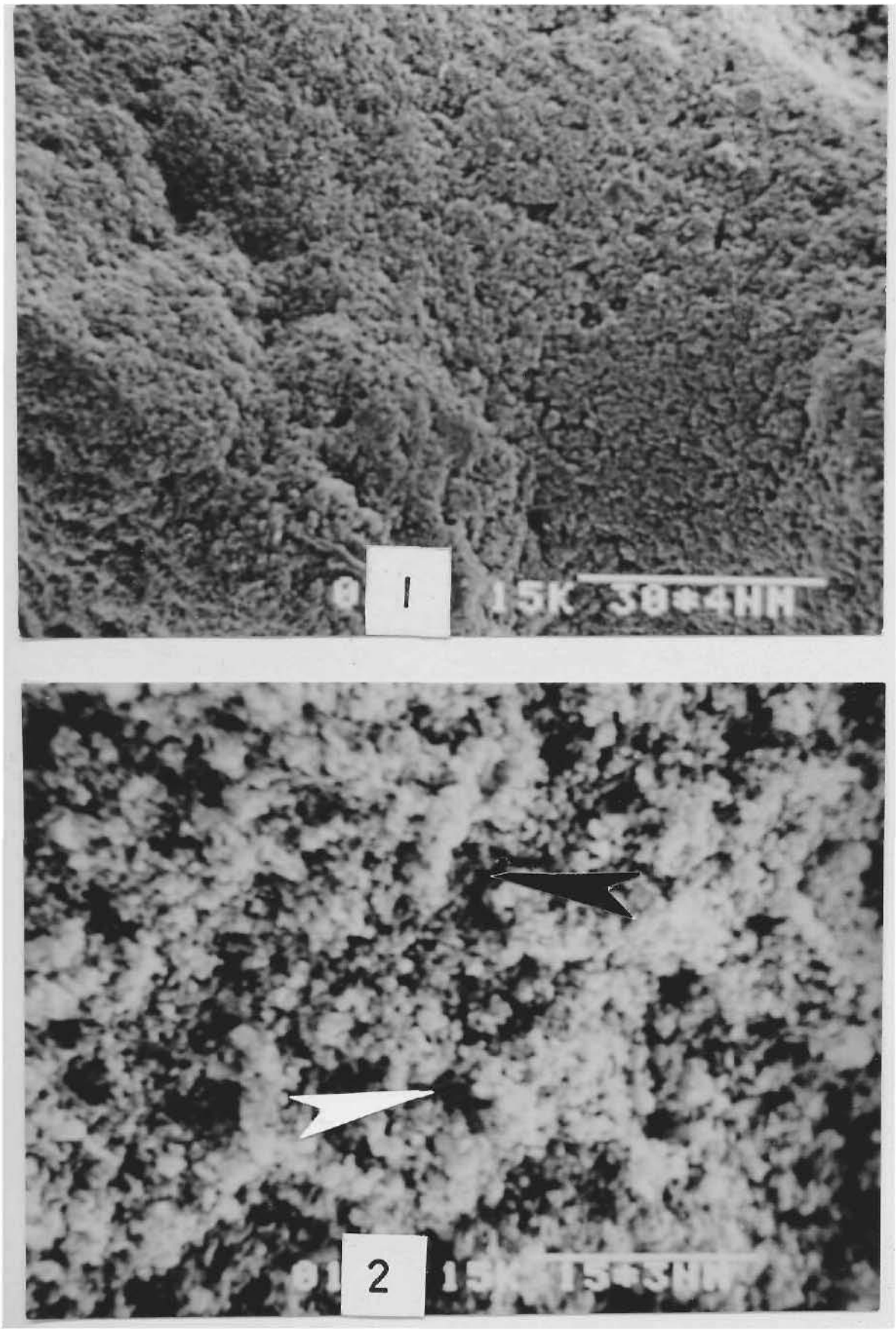
* Key to Micrograph Scales
15 K - 15 kV Operating voltage
30 * 4 NM - 30 x 10^4 nm corresponding to the length of bar above it.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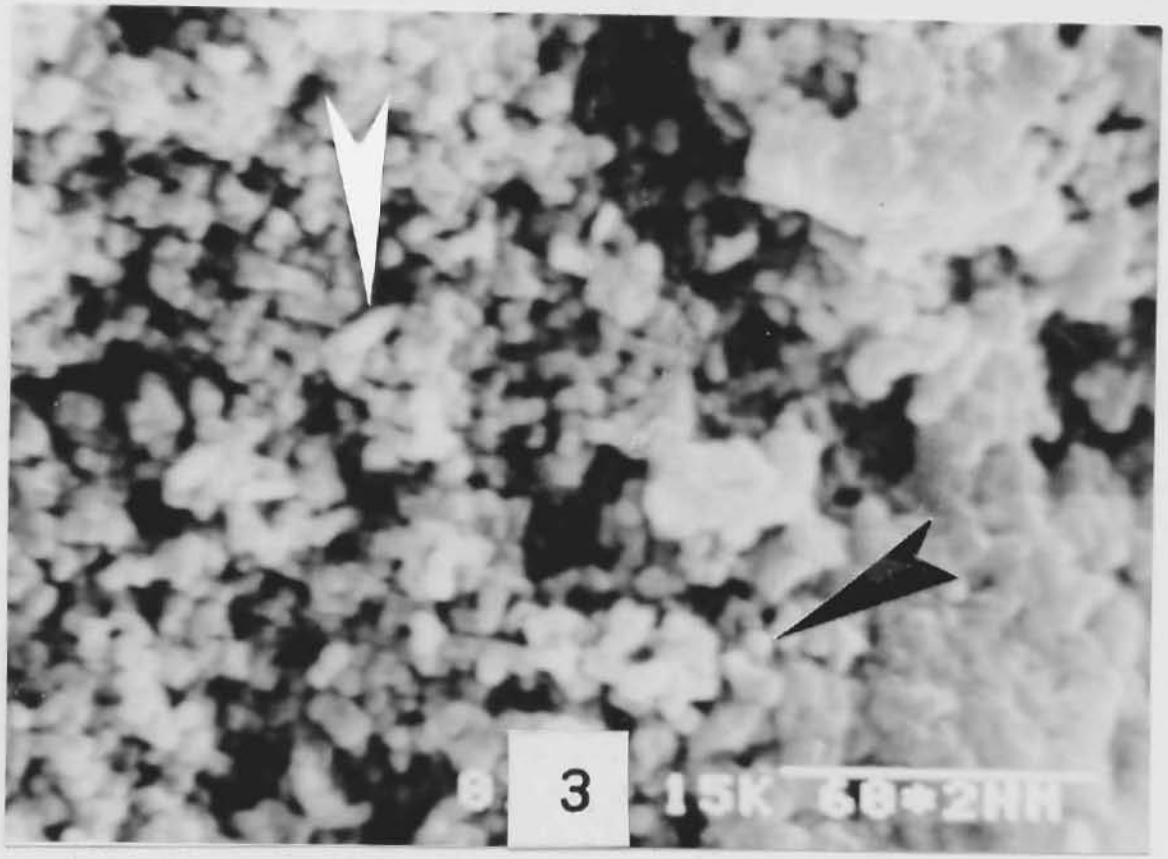
However, these structures were not observed in fractured coagulum (Figs. 3,4). The lactic acid bacteria - Streptococcus thermophilus and Lactobacillus bulgaricus were frequently spotted on surface and in pockets of coagulum without any connection between their surface and protein matrix. On scanning of different microfields, it was found that streptococci outnumbered the lactobacilli overall. Streptococcal chains of unusual length of 12-14 cells, not common in in vitro conditions were seen colonised in the form of micro-colonies at certain places. On close examination, surfaces of these bacteria were found covered with structure less deposits, whereas surface of lactobacillus cells were observed comparatively clean. Further, the sample was found free of contaminants as no microbes other than yoghurt starter were seen throughout the protein matrix.

Ultrathin sections of fresh cow milk yoghurt were studied under Transmission Electron Microscope to observe the ultrastructural details of the sample. Transmission electron micrographs endorsed the presence of casein agglomerations of various sizes and shapes as shown in Figs. 5,6,7. Aggregates of micelles appeared as discrete entities interconnected by long fibrils like structure. Besides casein clusters, large fat globules made their appearance with the ruptured electron dense

Fig.3. Micrograph showing both spherical (black arrow) and cylindrical (white arrow) casein micelles.

Fig.4. Lactic acid bacteria colonised on the surface and in the pockets (Streptococci- black arrow; Lactobacillus - white arrow).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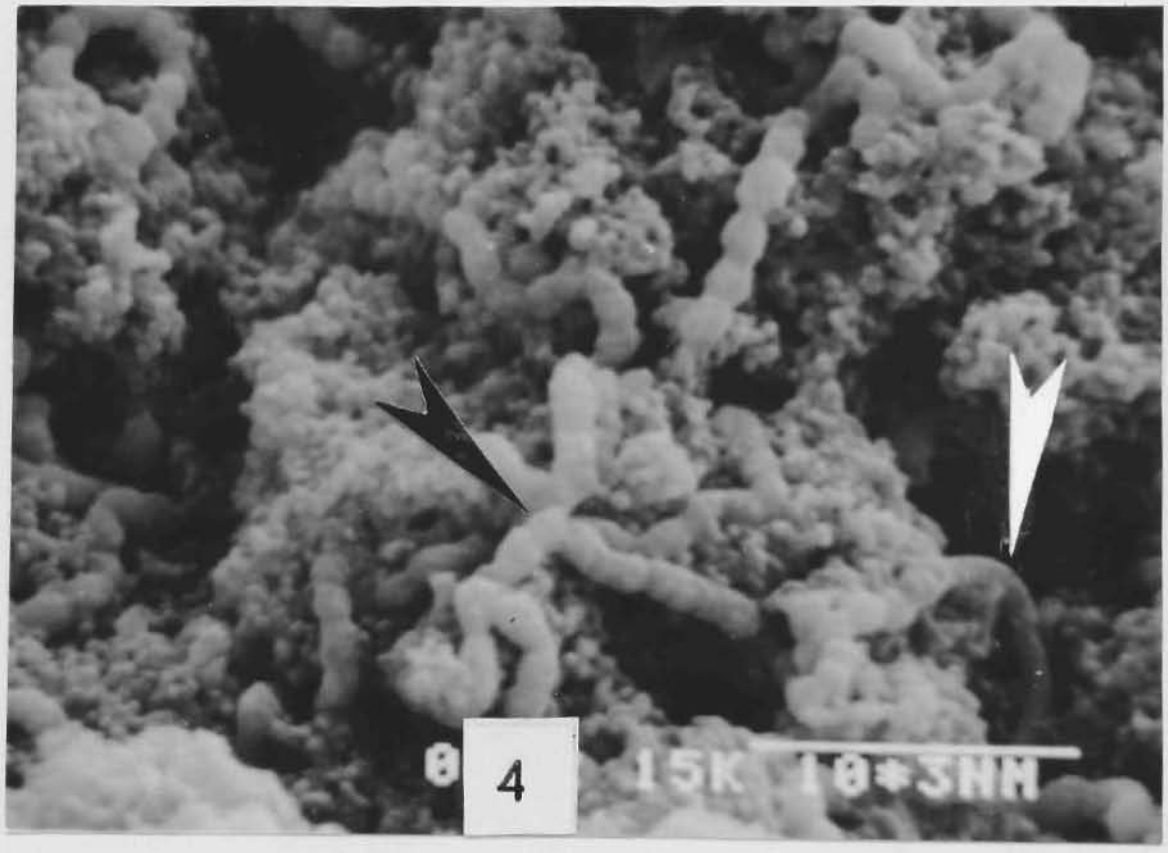
15K 10*2NM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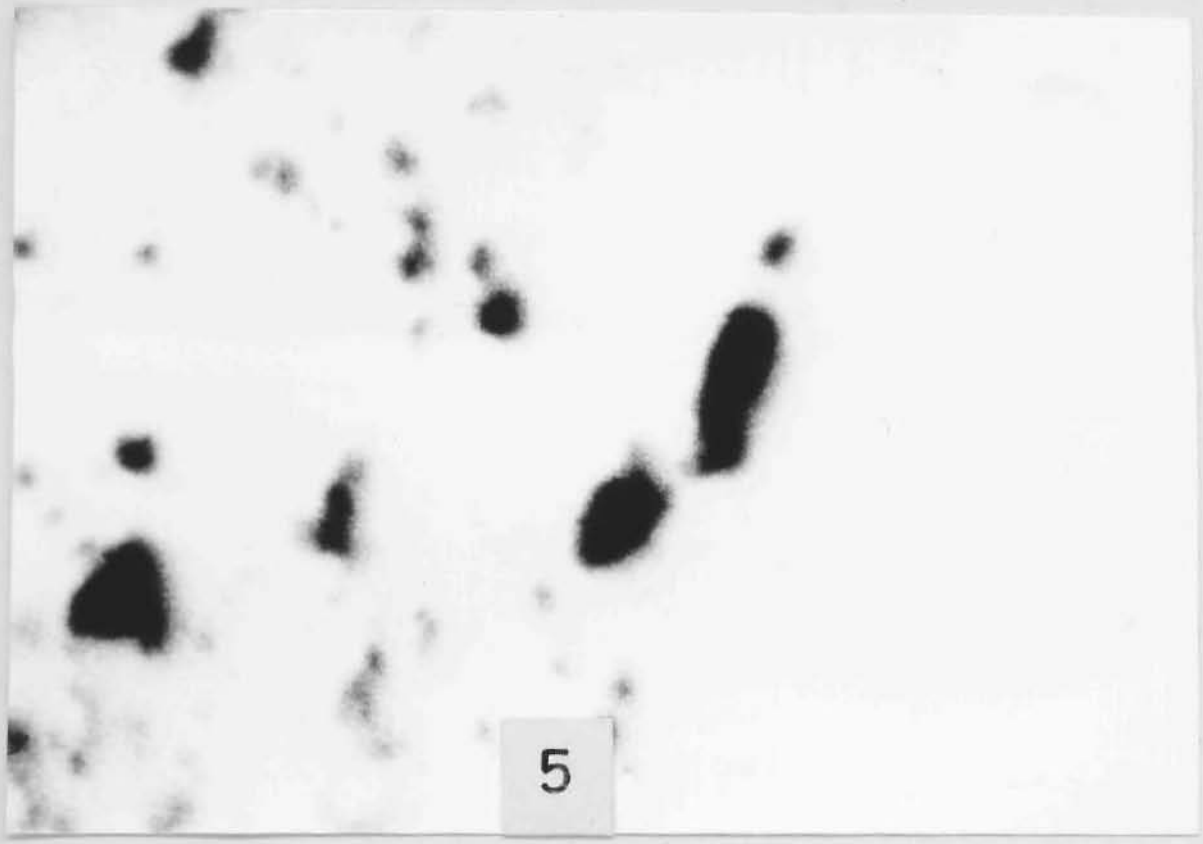
15K 10*3NM

Fig.5. Transmission electron micrograph of cow milk yoghurt showing microstructure overall (X 1,000).

Fig.6. Micrograph showing joining of casein aggregates through fibrillar structure (black arrow) present on the surface (X 10,000).



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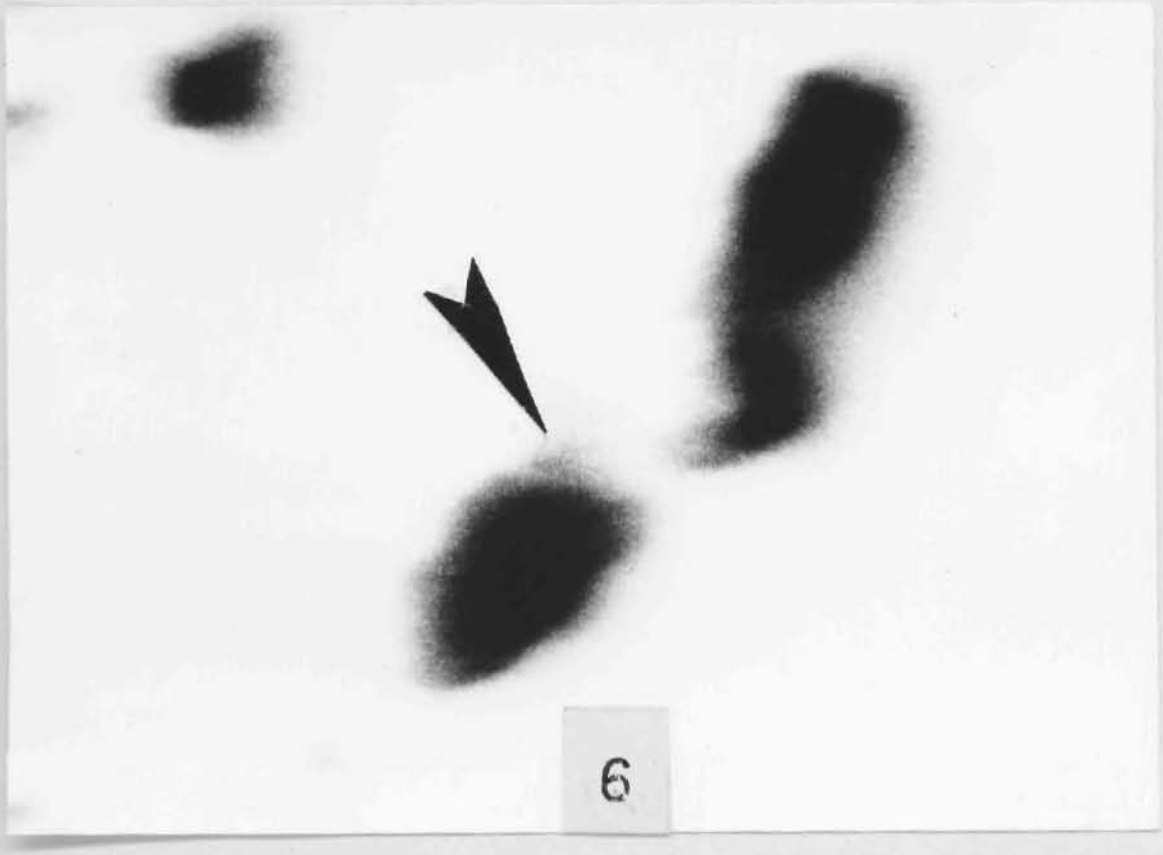

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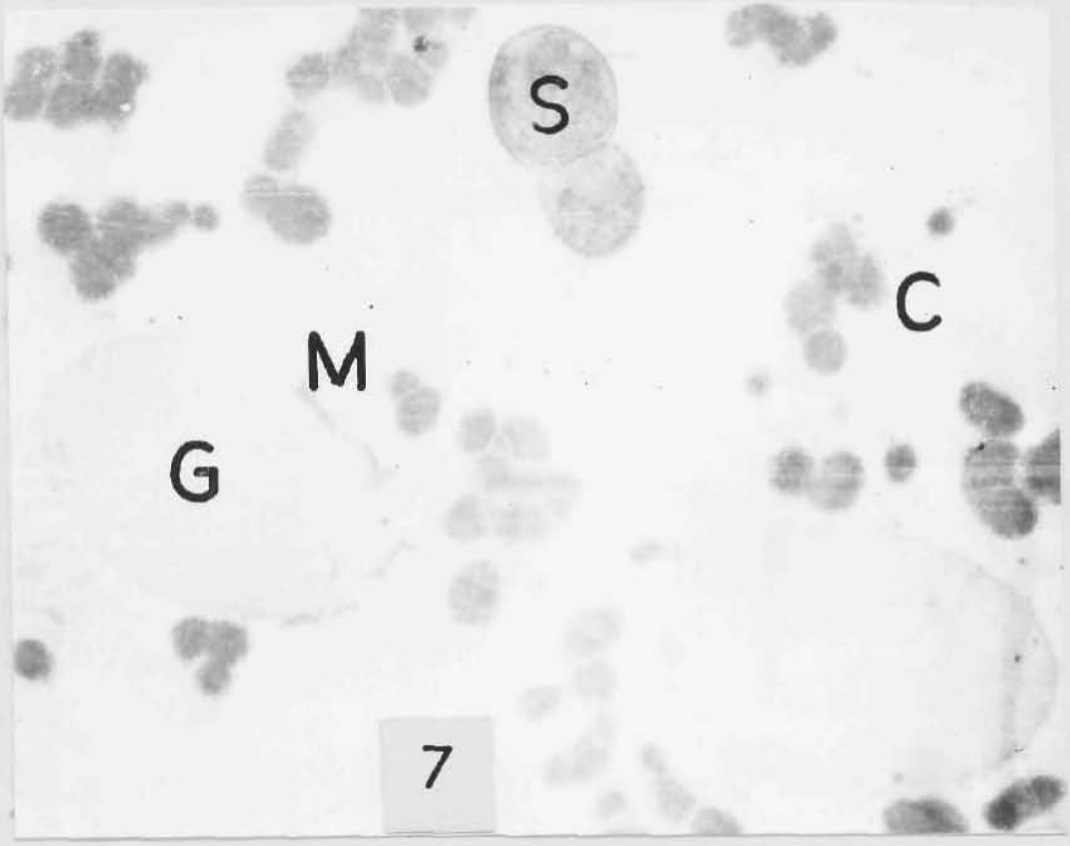
Fig.7. TEM micrograph showing casein aggregates (C); milk fat globule (G); milk fat globule membrane (M) ruptured at different places and Streptococcal cell (S) (X 7,200).

Fig.8. Ultrastructure of streptococcal cell showing surface surrounded by minute structures (X 25,000).



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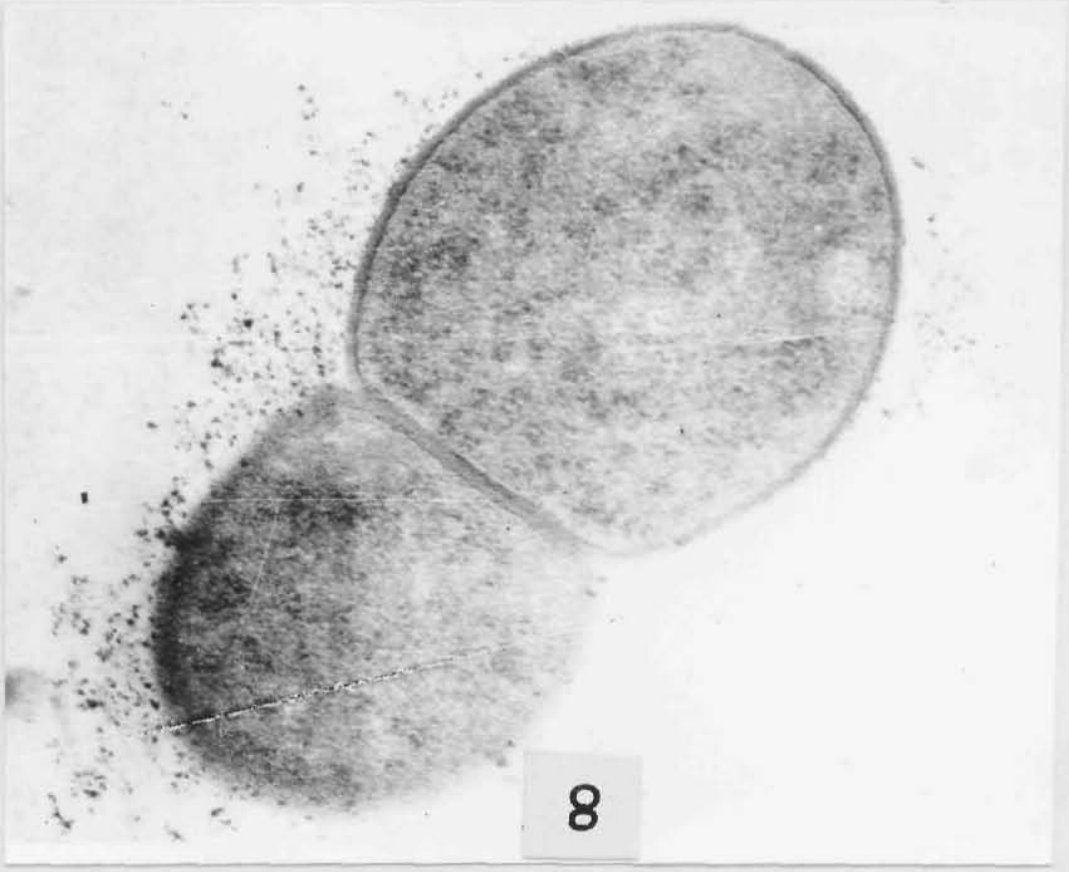

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milk fat globule membrane in the yoghurt sample prepared from whole milk. The section through streptococci revealed diplococcal cell with the cell wall covered by minute casein like structures (Fig. 8).

4.4.1.2 Yoghurt from cow milk preheated at 90C:

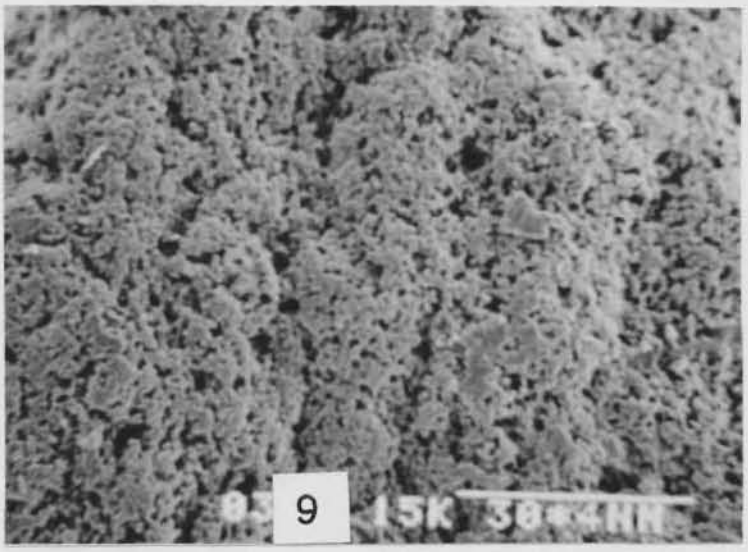
To visualise the impact of high heating of milk on cow milk yoghurt, sample was observed stereoscopically and ultrastructurally. A porous structure of coagulum marked by abundance of interspaces was visible at low magnification (Fig.9). The continuity of interspaces was frequently interrupted by casein micelles and thus resulting in formation of large and deep pockets as shown at higher magnifications. Casein micelles of dimensions of 300-700 nm with average diameter, 350 nm, were observed in the coagulum. Two tendencies of casein orientation- tendency of aggregation and chain formation were seen prevalent among micelles in the sample. Besides, big lumps and flat sheet like structures were also observed at some places in the coagulum (Fig.10). In respect of microflora, streptococci with surface deposits (diameter 1200 nm) and slender clean surfaced lactobacilli (diameter 500 nm) were observed in interspaces and on the surface as well. Diplococci cells of larger dimensions of approximately 1500 nm could be spotted in some microfields during the scanning of sample (Fig.11).

FIGURES 9-13 ELECTRON MICROSCOPY OF FRESH YOGHURT FROM COW MILK PRE-HEATED AT 90C FOR 30 MINUTES (C_{90}).

Fig.9. General view of microstructure of coagulum at low magnification.

Fig.10. Micrograph showing porosity and 3-dimensional network of protein matrix.

Fig.11. Big casein aggregates (black arrow) as seen at higher magnification; diplococci (white arrow) can also be spotted.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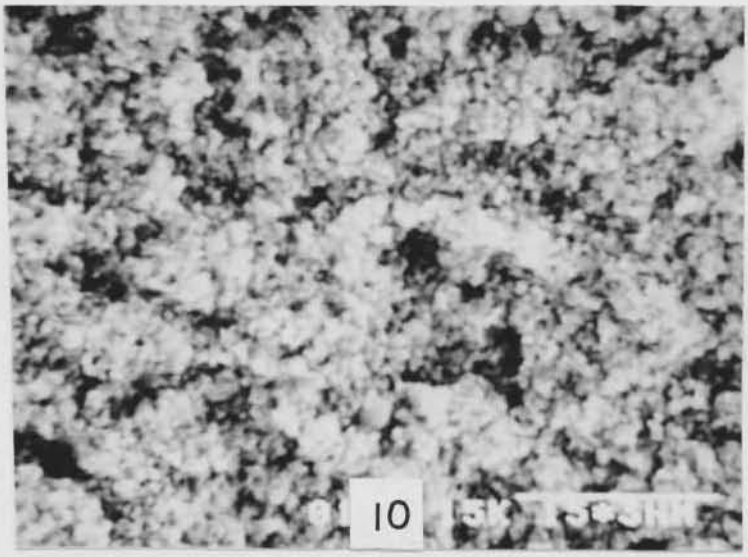
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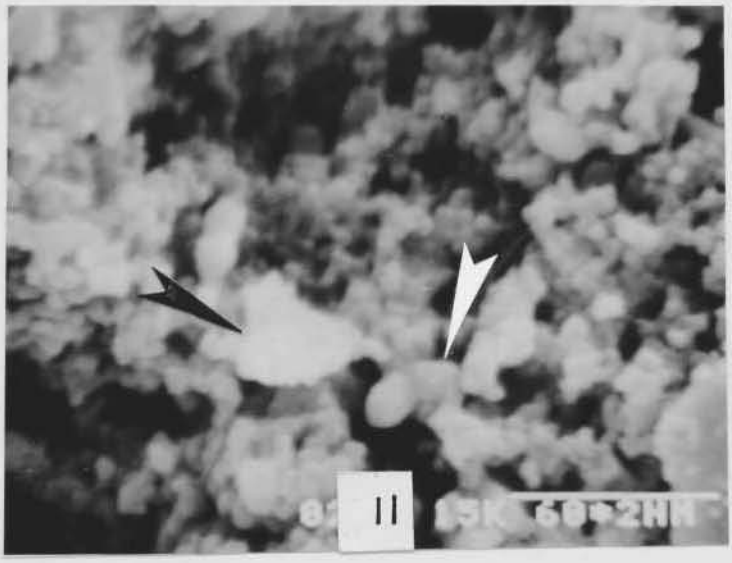
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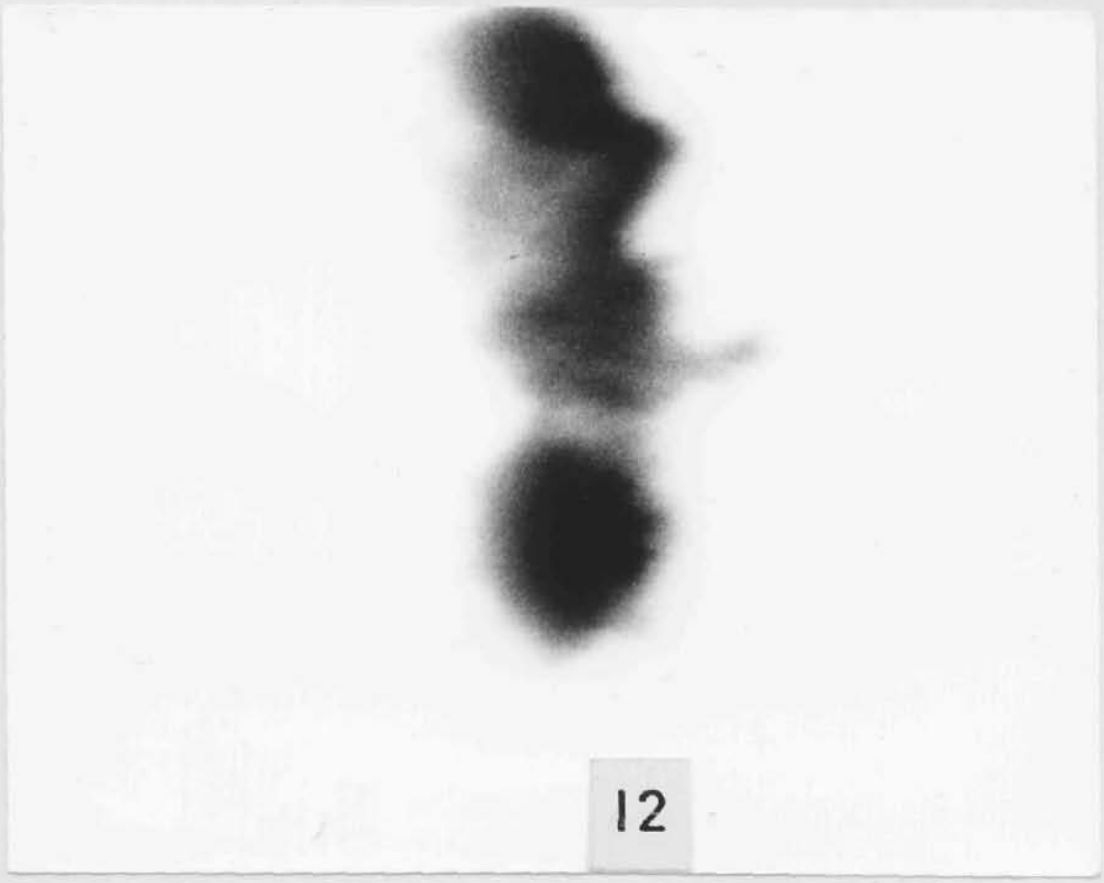
Compact clusters of casein surrounded by fibrillar material were frequently observed in TEM pictures. Fibrils are seen extended to adjacent casein aggregates in some fields. Big casein aggregates of about 1-2 μ dimension with appendages like structures attached on the surface are well documented in Fig. 12. Ultrathin sections through lactobacillus cells revealed the structures of different dimensions. At higher magnification, outlines of these structures looked fuzzy in appearance (Fig. 13).

4.4.1.3 Yoghurt from buffalo milk pre-heated at 70C:

The coagulum of buffalo milk yoghurt was found moderately porous; pockets being deep and small, ranged between 1-3 μ in size. Casein micelles were nearly spherical in shape and found isolated, interlinked and as larger aggregates under high magnification (Fig.14). The measurement of size of micelles showed considerable variation among free micelles ranging from 250-1350 nm in term of dimension with average diameter of about 300 nm. Occasionally, big inclusion bodies presumably of casein with needle like structures of calcium complex on the surface were observed in unfractur coagulum (Fig.15). A covering of coagulated globular aggregates was noticed in unfractured specimens, while fractured specimens were found free of any such struct Similar situation has been earlier reported in samp In various microfields, lactic acid bacteria made appearance invariably with surface covered.

Fig.12. TEM micrograph showing compact casein aggregates surrounded by fibriller structures (X 10,000).

Fig.13. A longitudinal and oblique section through lactobacilli showing cells of variable dimension (X 10,000).



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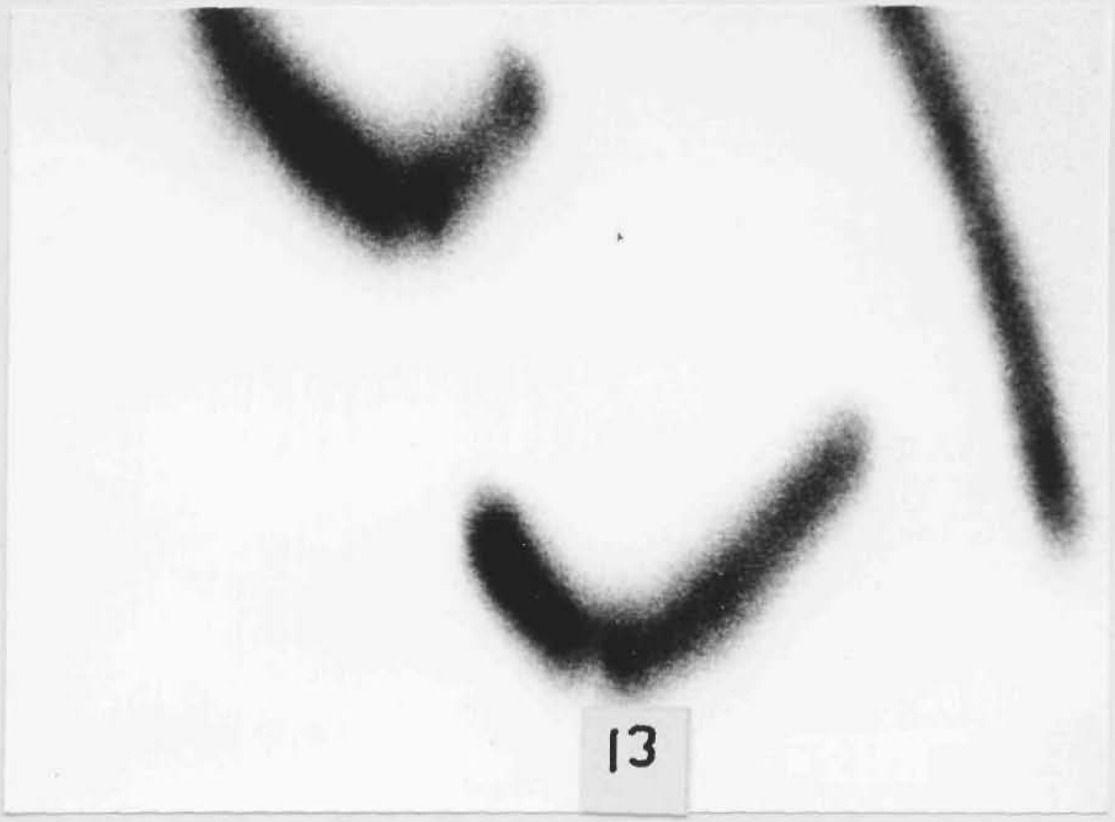

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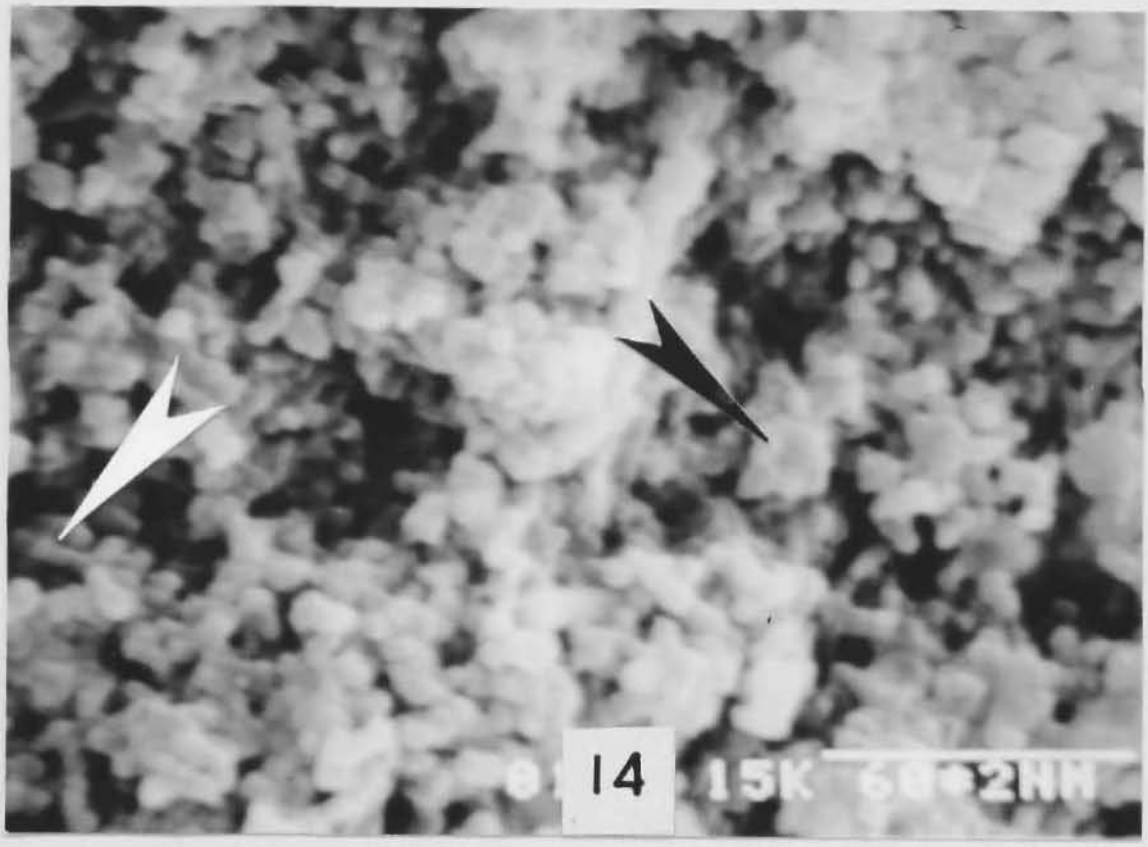
FIGURES 14–17. ELECTRON MICROSCOPY OF FRESH YOGHURT PREPARED FROM BUFFALO MILK PRE-HEATED AT 70C FOR 30 MINUTES (B_{70}).

Fig.14. SEM micrograph showing microstructure of fractured coagulum (Casein aggregates – black arrow; and micellea in chain – white arrow). Shallow pockets can also be seen.

Fig.15. Microstructure of unfractured coagulum showing deposition of small globular structures (black arrow); sheet like structures and needle shaped crystals. Lactobacillus can be observed protruding out of the pockets (white arrow).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15K

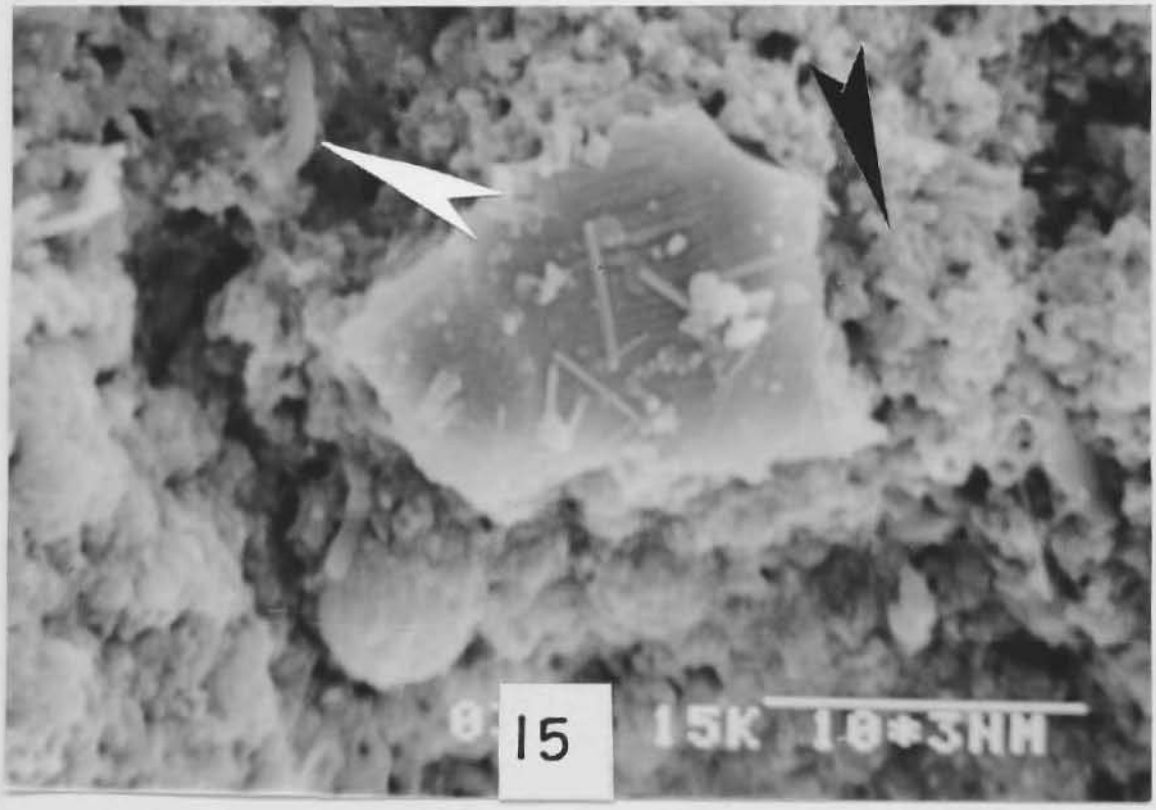
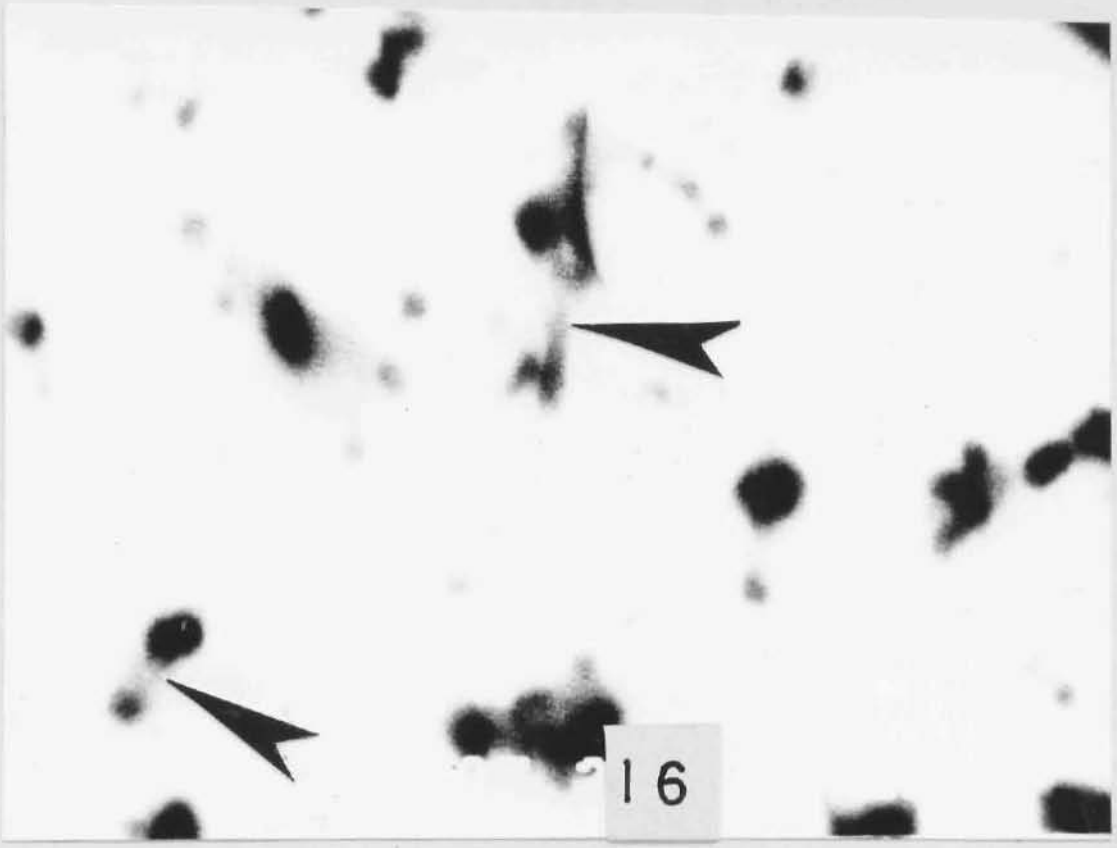

15
15K 10*3HH

Fig. 16. TEM picture showing the section through casein aggregates. Thread like structure of denatured whey protein (black arrow) between casein aggregates are frequently visible (X 5,000).

Fig. 17. In addition to casein aggregates linear chain of streptococci (white arrow) are shown in the micrograph. (X 4,000).

Fig. 16. TEM picture showing the section through casein aggregates. Thread like structure of denatured whey protein (black arrow) between casein aggregates are frequently visible (X 5,000).

Fig. 17. In addition to casein aggregates linear chain of streptococci (white arrow) are shown in the micrograph. (X 4,000).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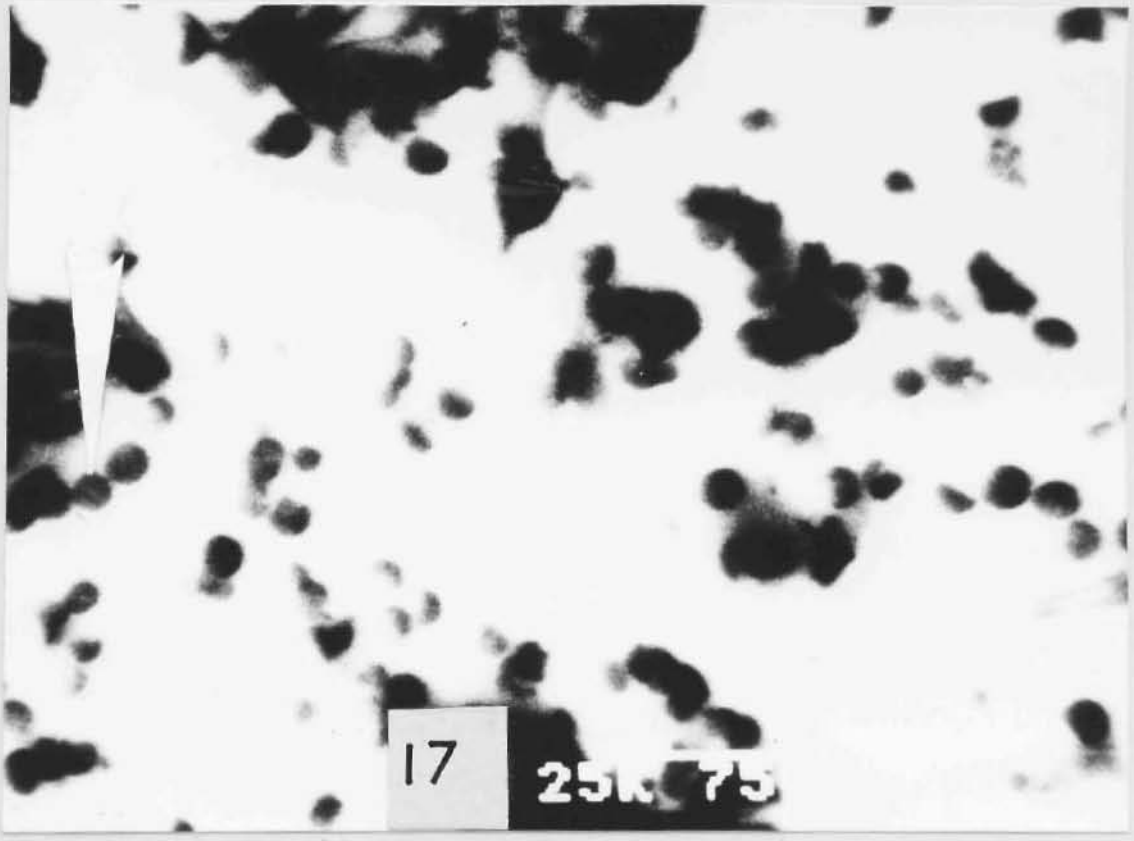
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Lactic acid bacteria with both clean and unclean surfaces were found in the coagulum as documented by different SEM micrographs (Figs. 18,19). Occasionally, lactobacillus cells with typical club-shaped terminal and were also observed. The cell diameter measured 700 nm in the middle region and increased to 900 nm towards the end of the cell. The size of streptococci was found as high as 1400 nm in some fields. Besides casein and yoghurt microflora, smooth, solid structure and cotton-like aggregated masses were seen scattered randomly in the microstructure (2Fig.20).

Small compact casein micelles with well defined outlines, sparsely distributed all over were observed under TEM (Fig. 21). Short hairy projections were present on the outer periphery as seen at higher magnification. Interconnecting fibrils or appendages on casein micelles were not so prominent in this sample. Bean shaped casein aggregates or micelles of 3000 x 2000 nm were spotted in different fields (Fig. 22).

4.4.2 Yoghurt after 24 h of storage

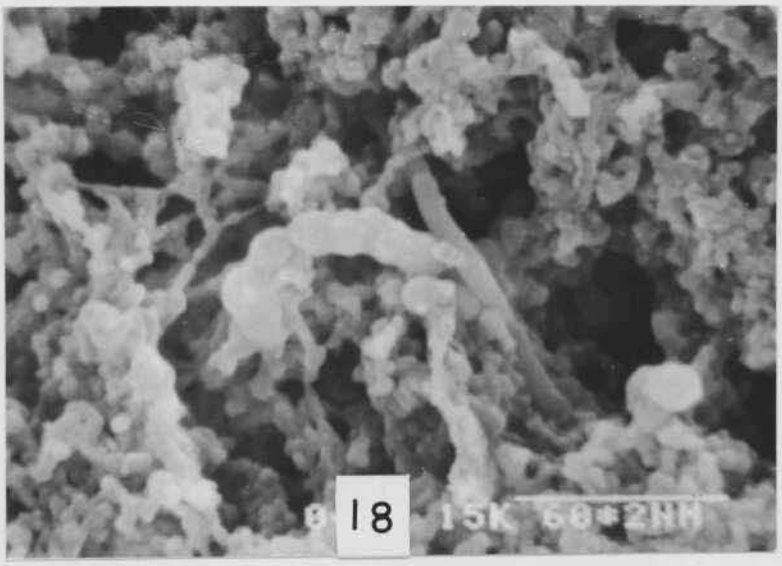
Electron microscopic observations of cow and buffalo milk yoghurts stored for 24 h at 4-6C were carried out to study the effect of storage on the microstructure of casein and yoghurt flora in yoghurt coagulum.

FIGURES 18-22. ELECTRON MICROSCOPY OF FRESH YOGHURT FROM BUFFALO MILK PRE-HEATED AT 90C FOR 30 MINUTES (B_{90}).

Fig. 18. SEM micrograph showing highly porous network of microstructure. Lactic acid bacteria in chains can be spotted in the centre.

Fig. 19. Microstructure of a fractured coagulum showing details of general matrix. Club shaped lactobacillus (black arrow) in association of Streptococci is seen.

Fig. 20. Lactose crystal (white arrow) and cotton like free aggregates presumably of denatured whey protein are seen entrapped in the 3-dimensional highly cross-linked network of protein matrix.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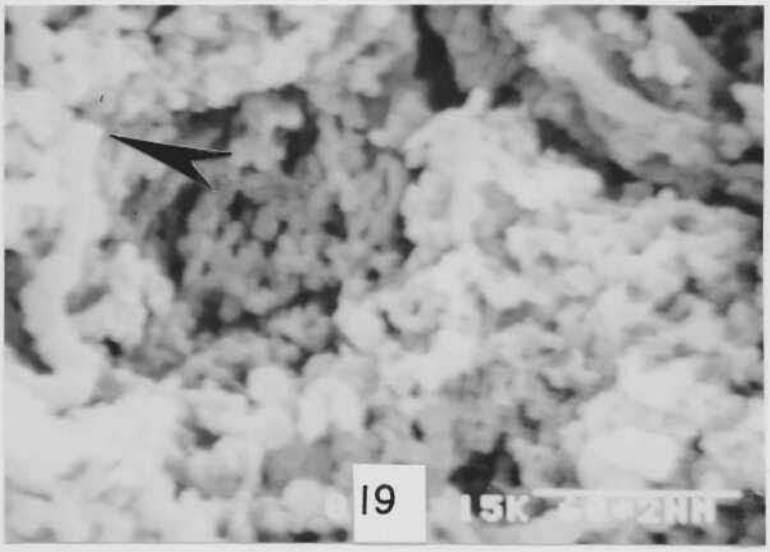
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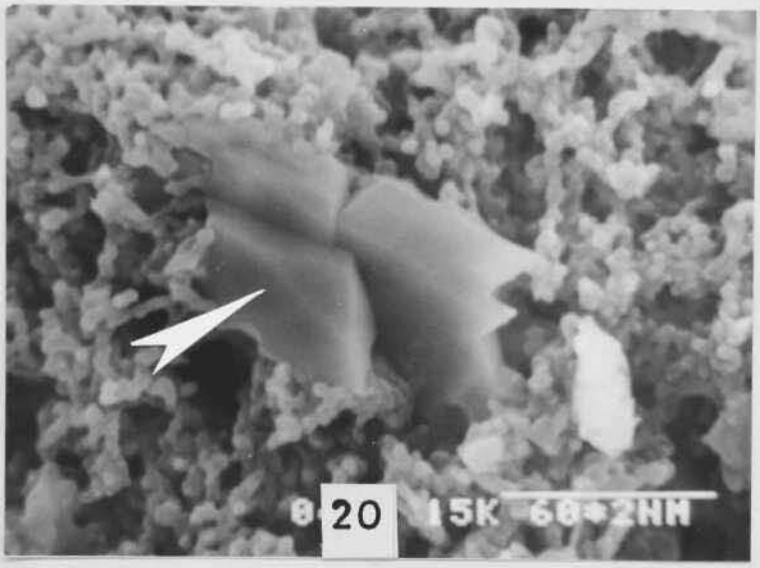
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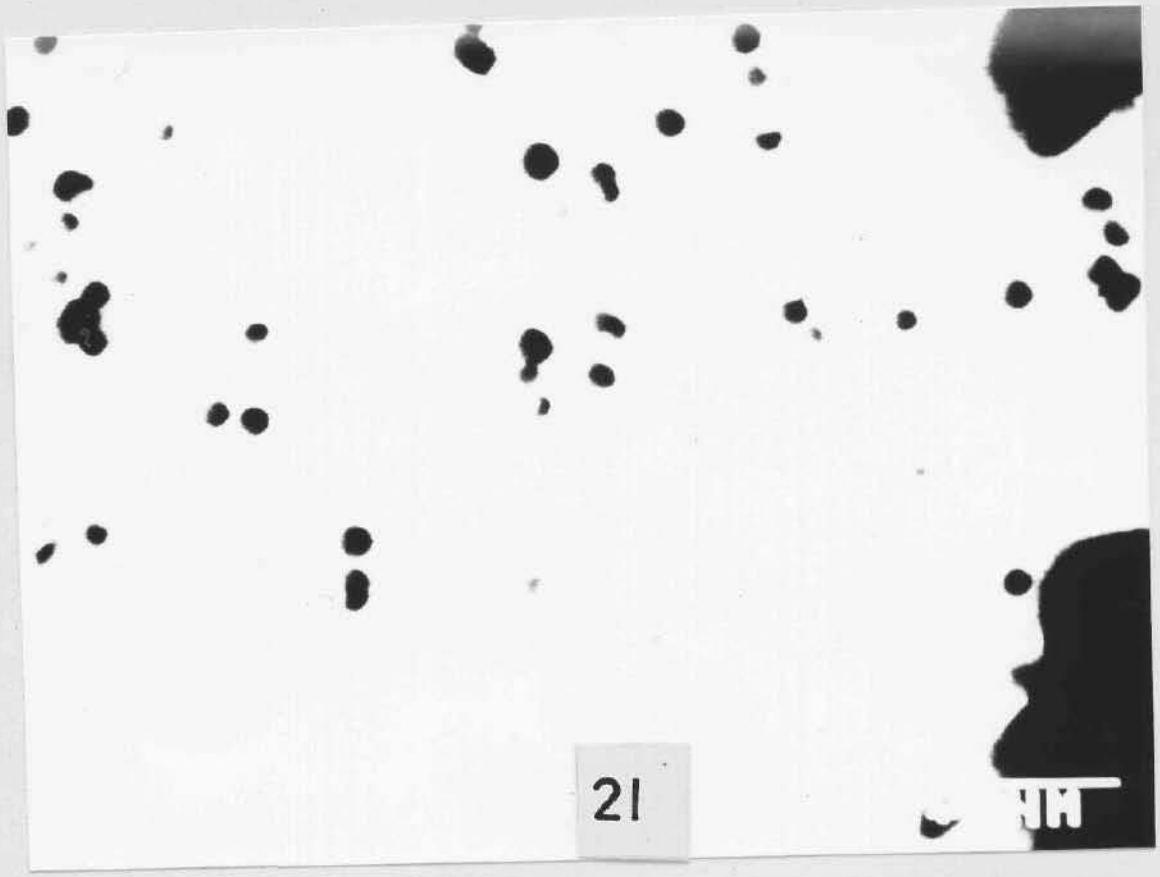
Fig. 21. TEM micrograph showing small compact and smooth casein micelles (X 5,000).

Fig. 22. Section through coagulum showing highly electron dense big micelles aggregates arranged in a linear fashion (X 5,000).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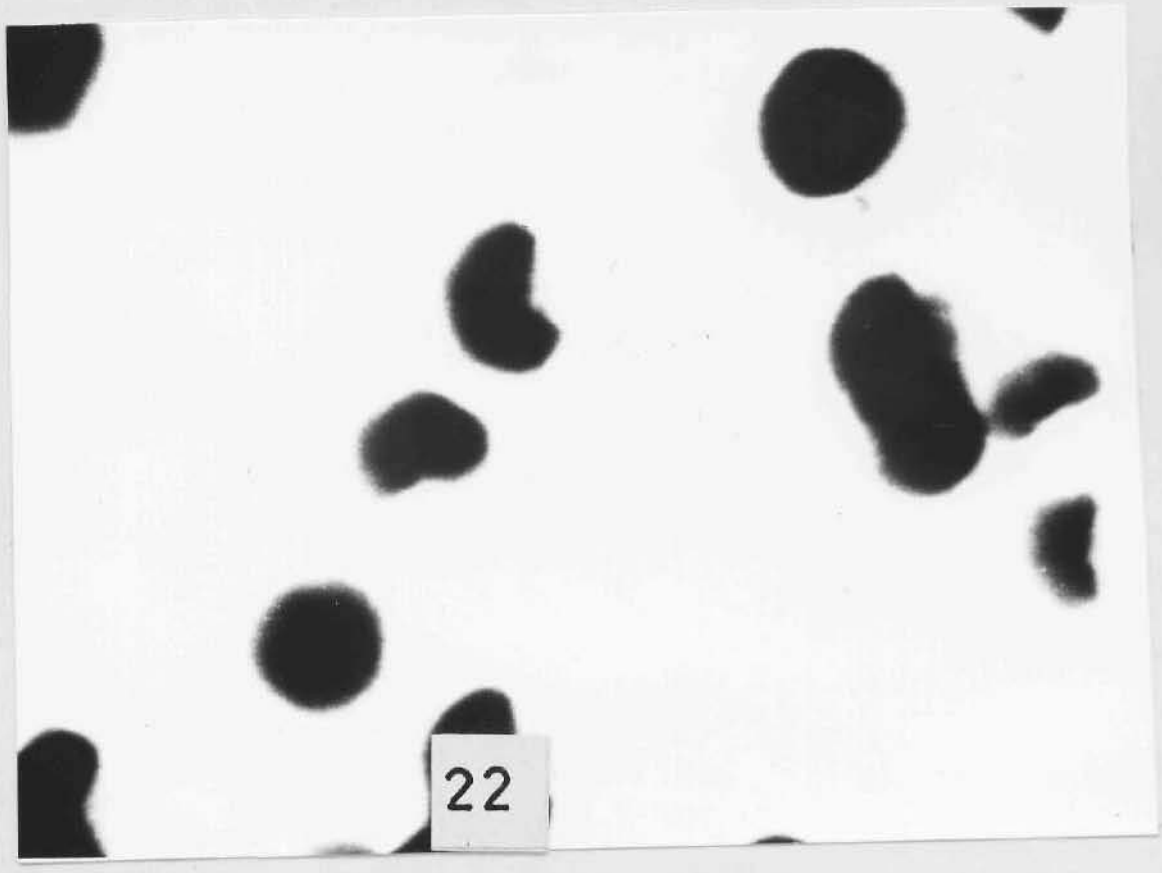
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4.4.2.1 Yoghurt from cow milk preheated at 70C:

At lower magnification, the microstructure looked compact similar to fresh cow milk yoghurt. The interspaces were found wider in the coagulum. The casein micelles were seen comparatively more fused giving rise to the formation of increased number of big clusters than fresh sample as observed as higher magnification (Fig. 23). Free micelles which were not frequent, measured 440-770 nm in diameter. In addition, globular clusters were also observed on the surface of unfractured coagulum. There was no visual difference in the morphology of lactic acid bacteria. Surface of yoghurt microflora showed deposits as reported earlier (Fig. 24).

TEM study showed the presence of fusion of micelles as observed in SEM. Big micelles of irregular shapes covered with appendages were observed. Typical heart shaped casein aggregates were spotted at few places (Fig. 25). Free aggregates of less electron dense protein like material also appeared frequently in the background of casein structures.

4.4.2.2 Yoghurt from cow milk preheated at 90C:

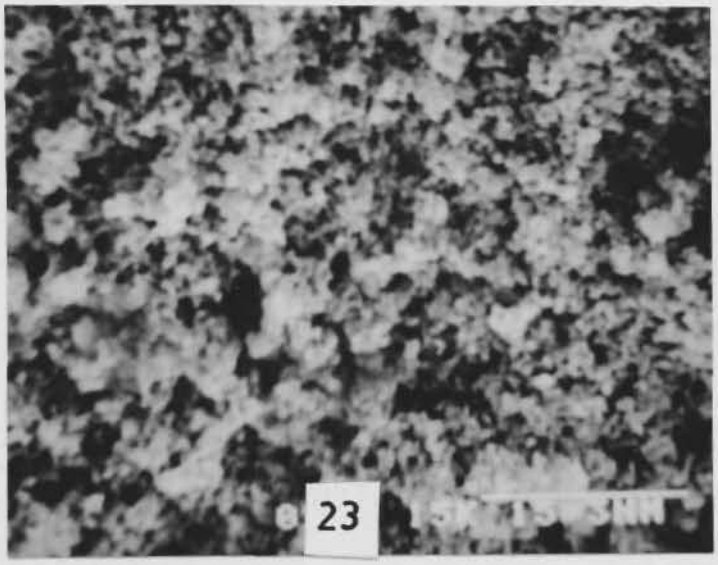
The yoghurt prepared from cow milk pre-heated comparatively at high temperatures offered porous structure at low magnification SEM micrographs (Fig. 26). At higher magnification, the microstructure appeared complex in its organization. Micelles were found to be involved in

FIGURES 23-25. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{70}) STORED FOR 24 H AT 4-6C.

Fig. 23. General view of fractured coagulum of sample.

Fig. 24. SEM micrograph showing Streptococcal chain with covered surface (black arrow). Wide interspace resulting from casein micelles aggregation can be seen the background.

Fig. 25. TEM micrograph showing casein aggregates as seperate entities surrounded by fibrillar structures. Typical heart shaped (white arrow) and free floccules (black arrow) of denatured whey protein are spotted (X 4,000).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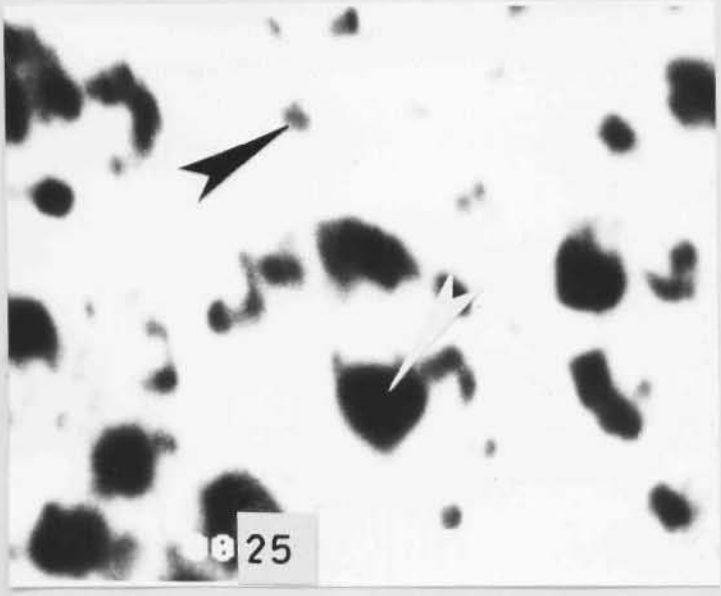
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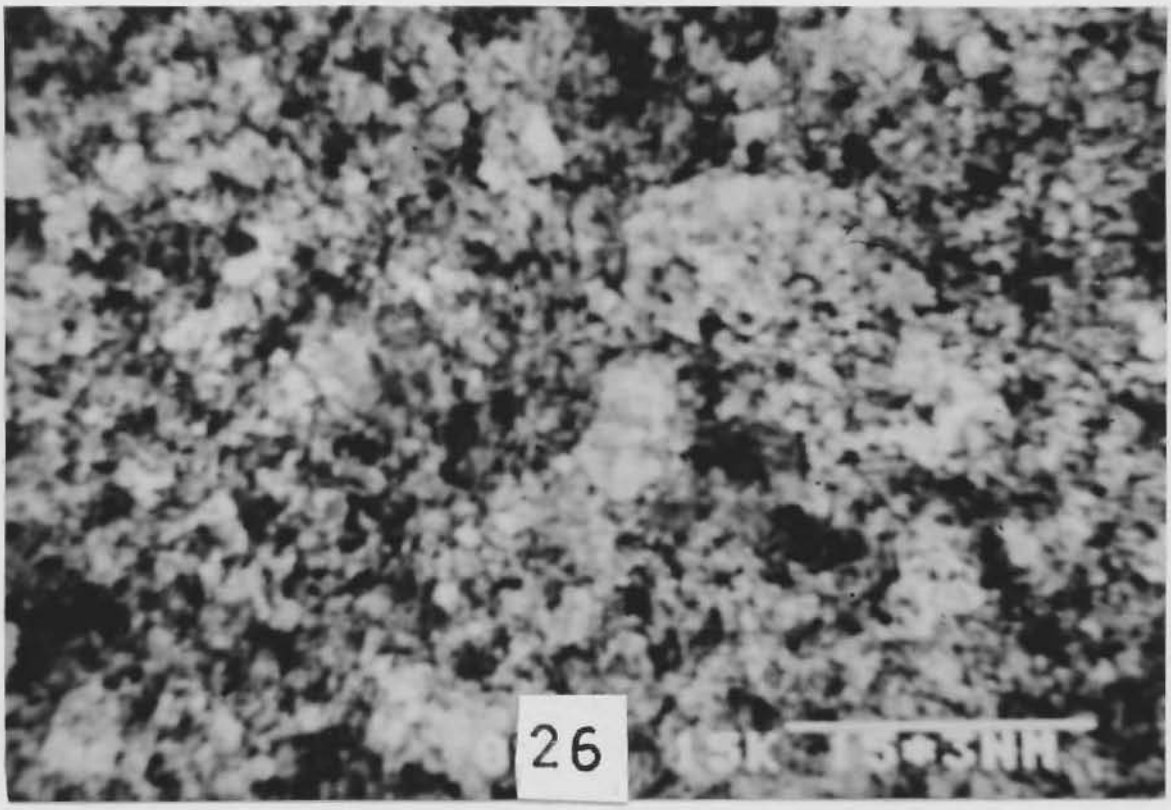
FIGURES 26-27. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{90}) STORED FOR 24 H AT 4-6C.

Fig. 26. Microstructure of fractured coagulum.

Fig. 27. SEM micrograph showing complex microstructure comprising both chains (white arrow) and aggregates (black arrow) of casein micellee; Bacterial surface shows high deposition of protein like material on it.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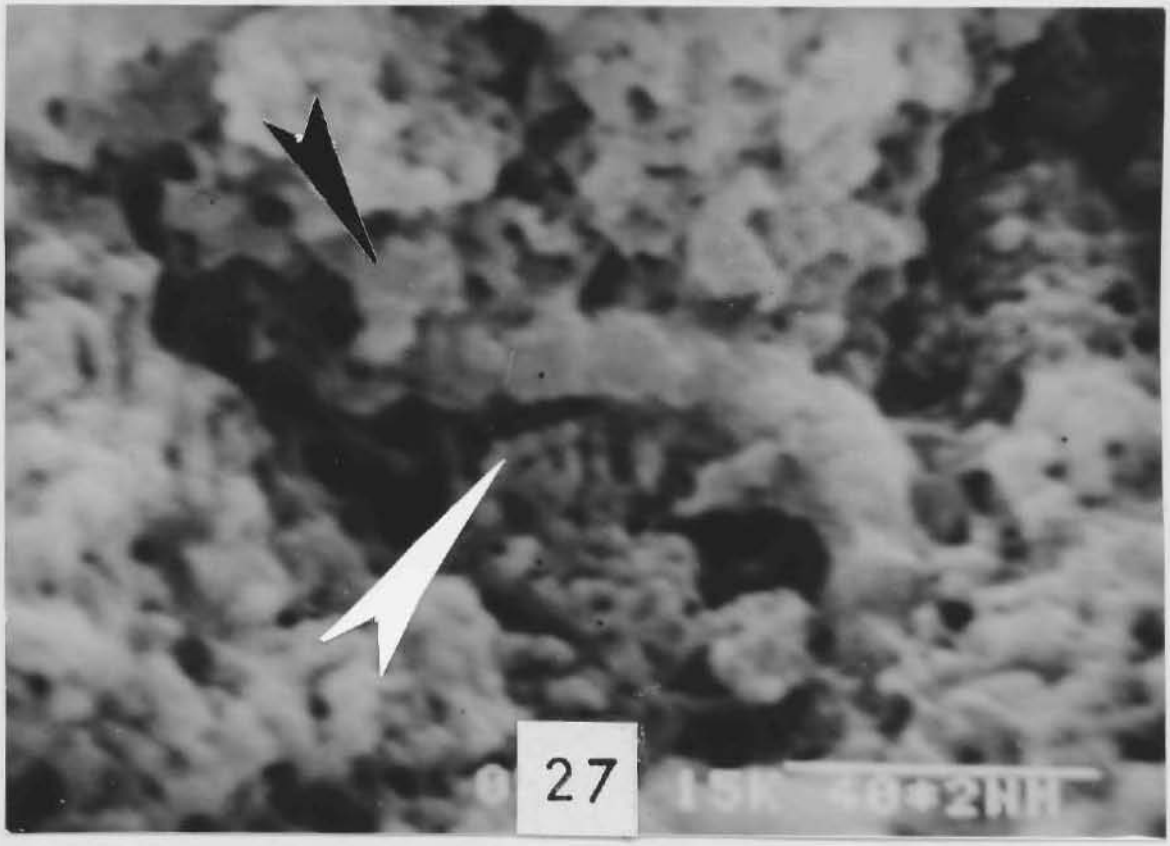

27

aggregation and chain formation as demonstrated in Fig. 27. The micelles are interlinked through surface to surface contact. Thread or tubular protein structures were not observed in the sample. Fine micelles of varied dimensions were also noted. Comparatively heavier deposition was seen on the surface of lactic streptococci in chain. Free and aggregated micelles with fuzzy boundaries were commonly observed in ultrathin sections under TEM study.

4.4.2.3 Yoghurt from buffalo milk preheated at 70C:

Moderately porous microstructure with irregularly distributed, deep pockets on the surface of coagulum was noticed (Fig. 28). Occasionally, pockets were seen occupied by smooth compact structures. Microbial surface appeared clean and microbes were of regular dimensions; Lactobacilli 720 nm and Streptococci 1000 nm in diameter (Figs 29,30). Casein micelles were mostly aggregated and free micelles measured 500-800 nm in diameter. The surface of unfractured coagulum was seen covered by globular aggregates.

TEM micrographs revealed the aggregated casein micelles placed discretely in the matrix. Free floccules of coagulated protein like structures were seen frequently apart from the casein micelles (Fig. 31).

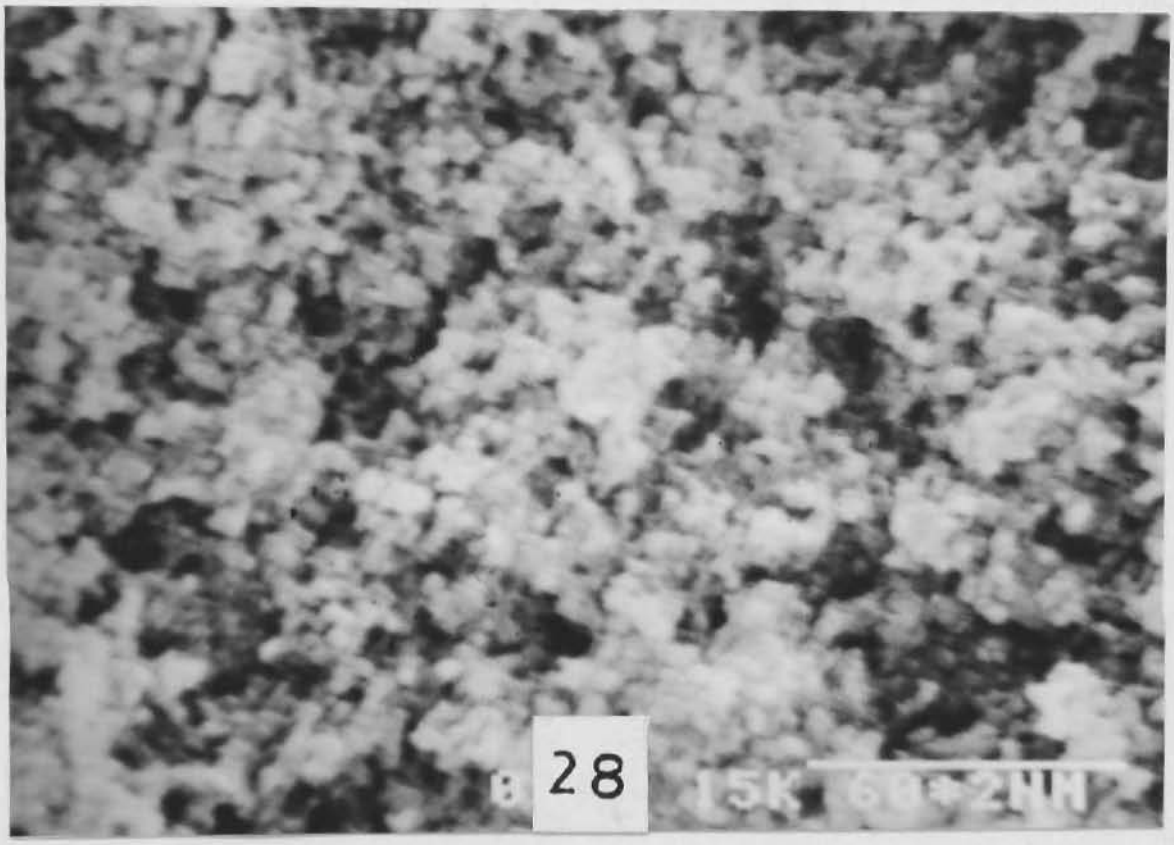
4.4.2.4 Yoghurt from buffalo milk heated at 90C:

Sample presented a highly porous microstructure equipped with uniformly distributed deep and big pockets.

FIGURES 28-31. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURT (B_{70}) STORED FOR 24 H AT 4-6C.

Fig. 28. Microstructure of coagulum showing aggregation of casein micelles.

Fig. 29. SEM micrograph showing inclusion bodies occupying the interspace.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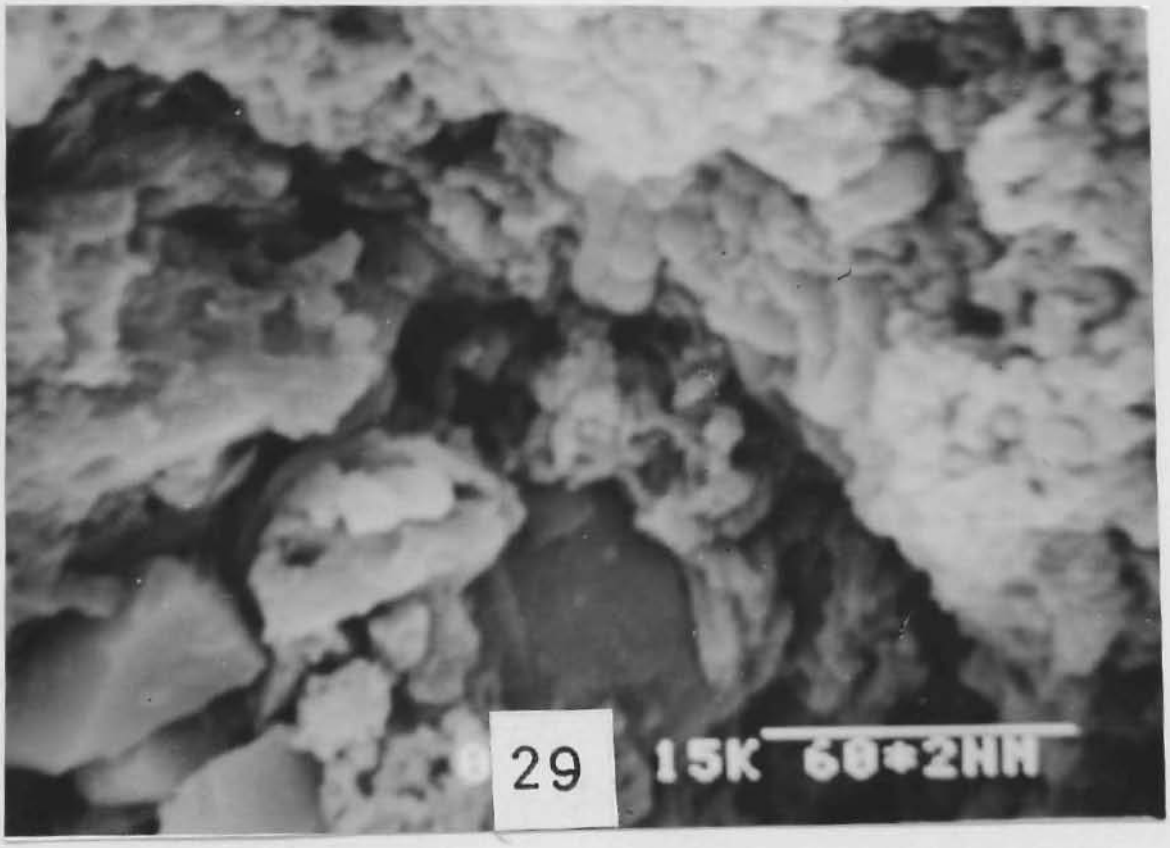
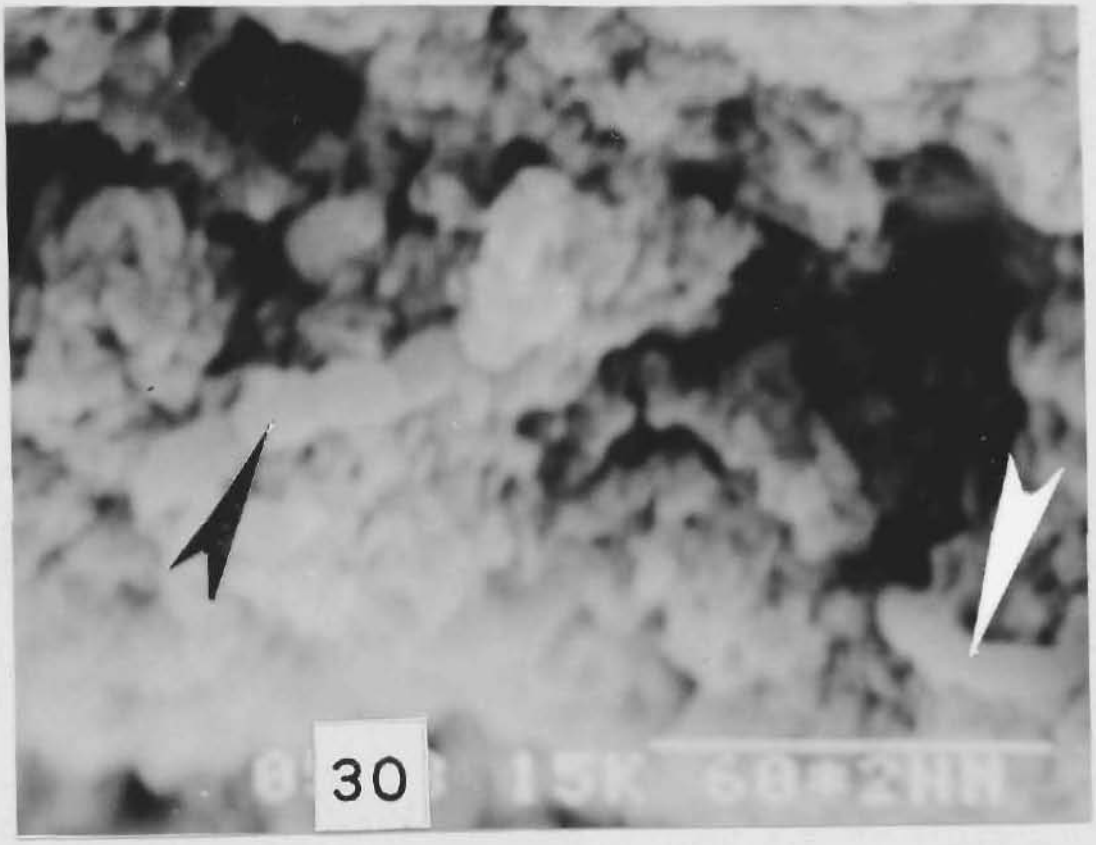

29
15K 60*2HN

Fig. 30. Smooth surface streptococcal cells in chain (black arrow) and covered surface lactobacillus (white arrow) are spotted in the microfield.

Fig. 31. TEM picture showing the aggregation of micelles at low magnification (X 2,000).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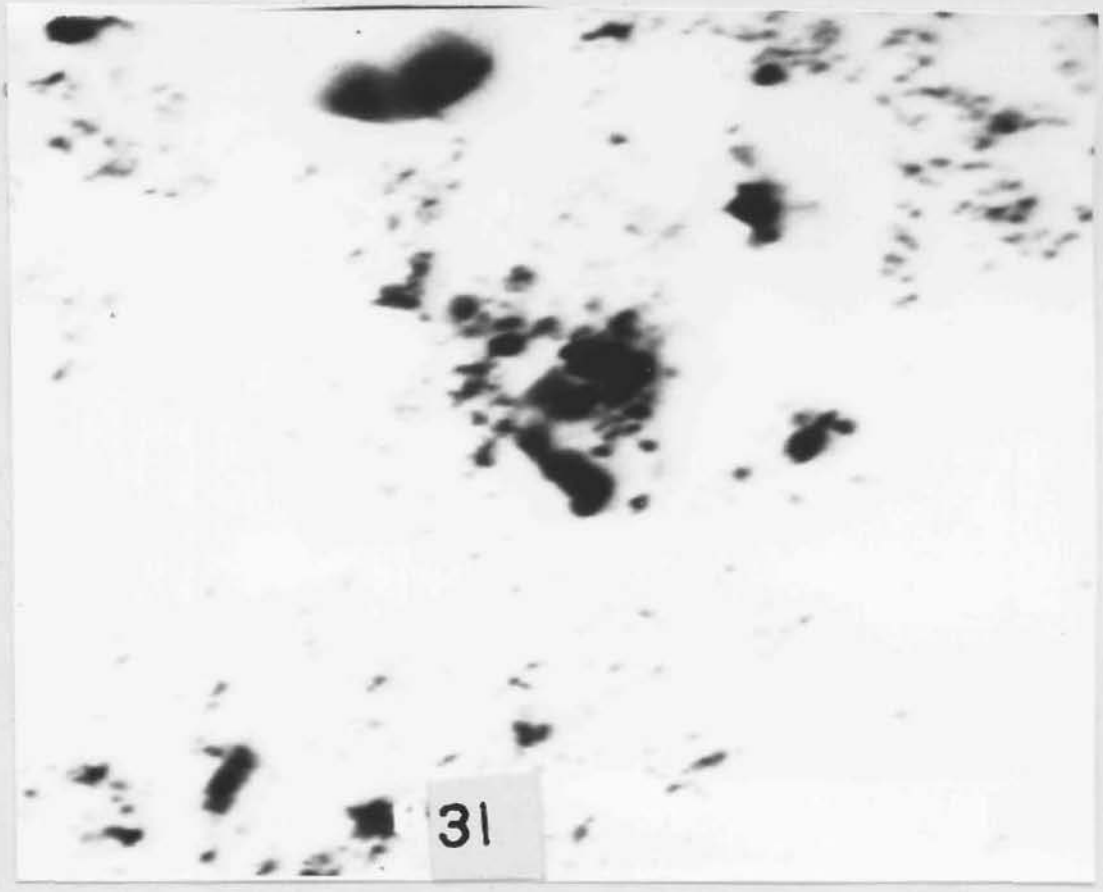

31

Micelles arranged in chains contributed to the network of casein matrix. As compared to fresh buffalo milk yoghurt, a tendency of aggregation among casein micelles in addition to chain formation was noticed. The sample was found quite rich in lactic acid bacteria. The bacteria with clean surfaces were localised mostly in pocket (Figs. 32,33,34). Streptococci and lactobacilli measured 1,000 nm and 720 nm in diameter respectively. Smooth spherical inclusion bodies were found settled in pockets as shown in Fig. 33.

The ultrathin sections through coagulum revealed and highly dense compact micelles arranged in linear fashion (Fig. 35). Casein micelles showed short hair like projections on the surface at higher magnification and interlinking among these micelles through these structures was clearly visible in Figs. 36,37. Long thread like protein structures were missing in this sample.

4.4.3 Yoghurts after 72 h of storage

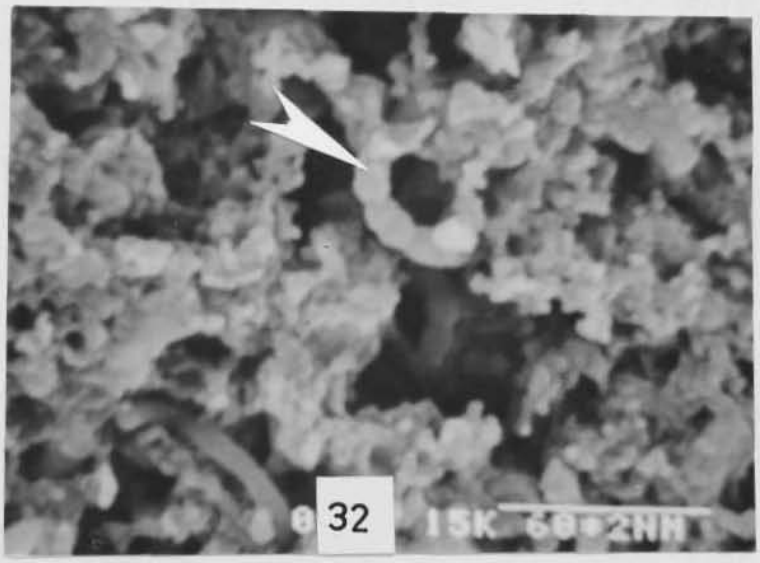
Electron microscopic studies of Yoghurts prepared from cow and buffalo milks stored for 72 h were conducted to observe the changes in microstructure corresponding to this period of storage.

FIGURES 32-37. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURT (B_{90}) STORED FOR 24 H AT 4-6C.

Fig. 32. Microstructure of coagulum showing deep pockets and clean surfaced lactic acid bacteria in these pockets. (streptococci- white arrow; lactobacillus- black arrow).

Fig. 33. Besides yoghurt microflora, lumps (black arrow) are seen entrapped in the matrix. Typical chain of casein micelles (white arrow) is visible.

Fig. 34. Crosslinking of micelles chains resulting in typical pockets formation is documented in this micrograph.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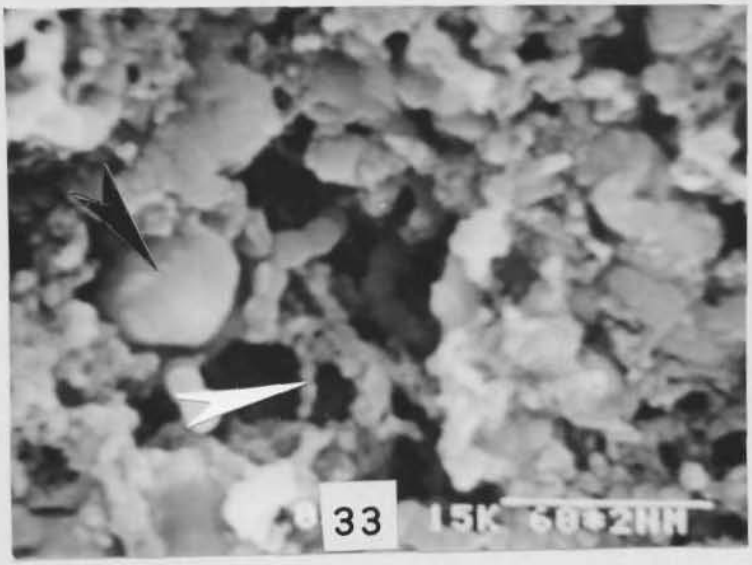
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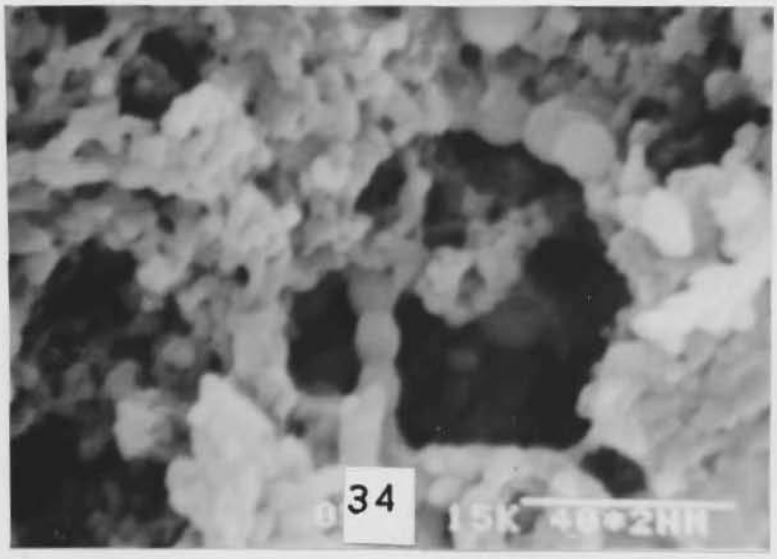
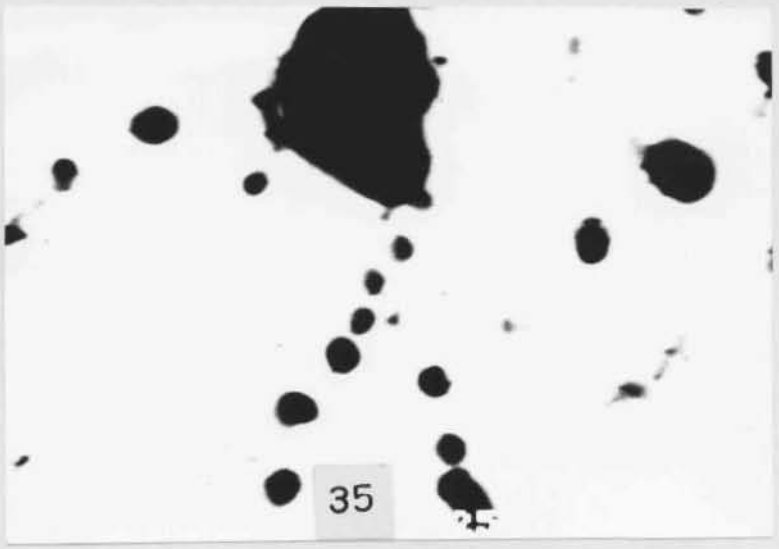

34

Fig. 35. TEM micrograph showing compact casein micelles and aggregates arranged in a linear fashion (X 2,000).

Fig. 36. Magnified view of casein micelles in chain (X 5,000).

Fig. 37. Interlinking of casein micelles and aggregates through short hair like structures of heat induced complex present on the surface of the micelles as revealed by TEM (X 4,000).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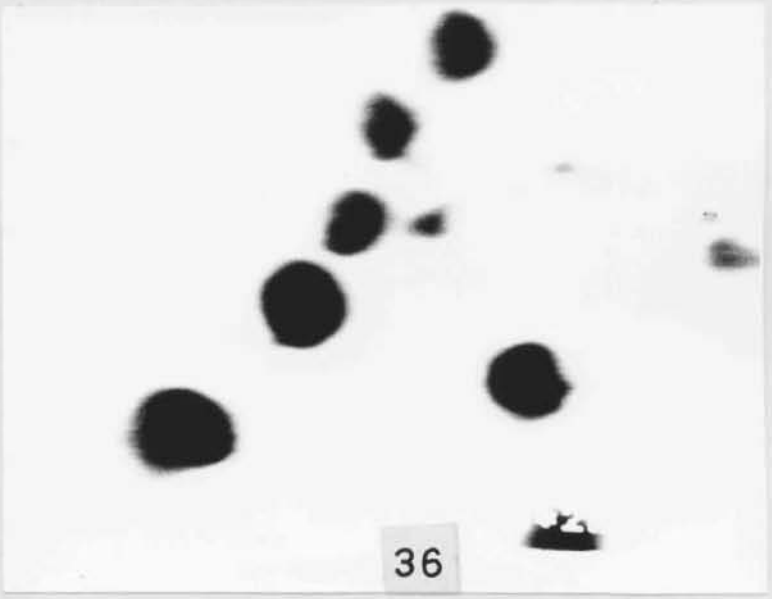
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37

4.4.3.1 Yoghurt from cow milk preheated at 70C:

Irregular microstructure could be visualised even at low magnification (Fig. 38). Large inclusion bodies and flakes like structures made their appearance in the coagulum and globular aggregates were seen sandwitched between these structures (Fig. 39). Casein micelles measured between 400-600 nm in diameter.

Casein micelles were seen clustered in large aggregates as shown in transmission micrographs. Micelles are seen distinctly joined by loosely arranged fibrillar structures (Figs. 40,41). Streptococcal cells could be spotted with the dimension of 1200 nm. Figure 42 shows how the casein micelles are fused to form a continuous chain leaving cavities in between the matrix.

4.4.3.2 Yoghurt from cow milk preheated at 90C:

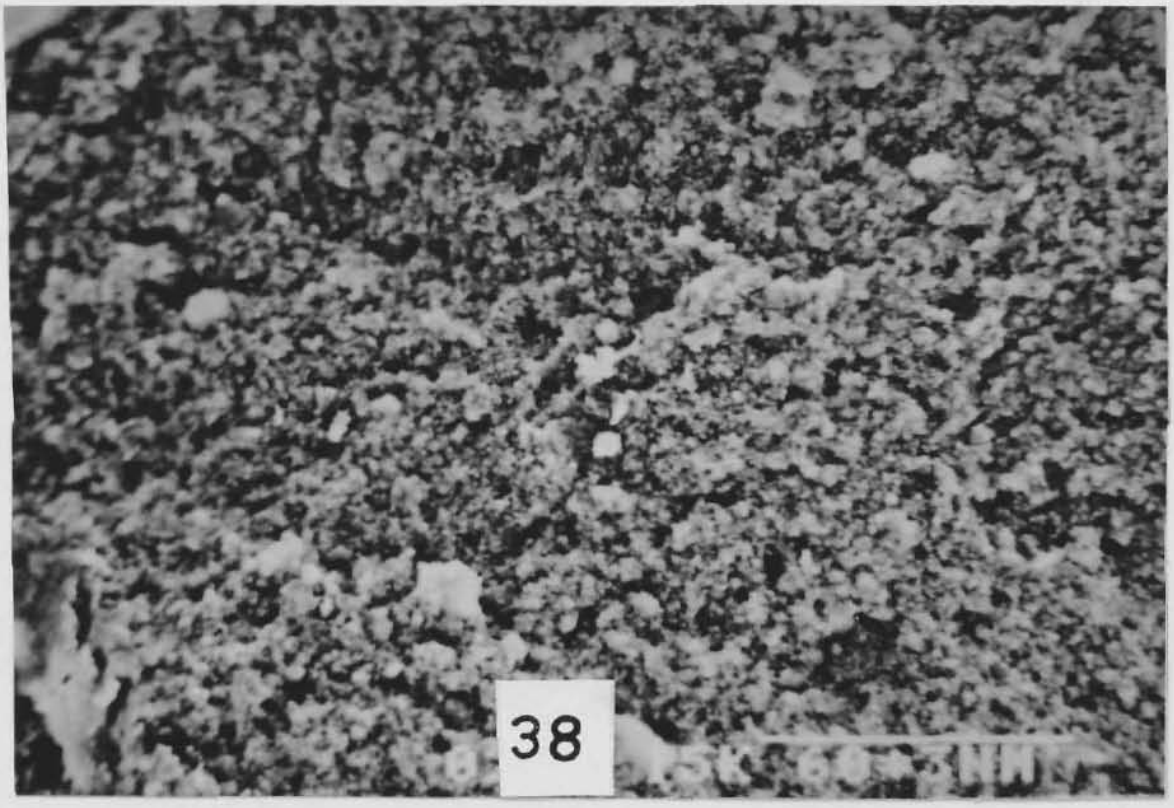
SEM studies revealed a compact and irregular microstructure of the sample. Pockets were not that frequent in this sample. Flocculated casein micelles and smooth lumps dominated the matrix (Fig. 43). Heavy uneven deposition on bacterial surfaces was seen and streptococci and lactobacilli measured 1200 nm and 800 nm in diameter respectively (Figs. 44, 45).

Casein micelles of comperatively very small size in clusters and in linear chains were shown in TEM micrographs (Figs. 46,47). Unfused micelles showed short

FIGURES 38-42. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{70}) STORED FOR 72 H AT 4-6C.

Fig. 38. Microstructure of coagulum showing high aggregation of casein micelles. Numerous big lumps are visible even at this low magnification.

Fig. 39. SEM micrograph showing sheets of compact casein and long flakes. Loosely aggregated globular clusters and sandwitched in between (black arrow).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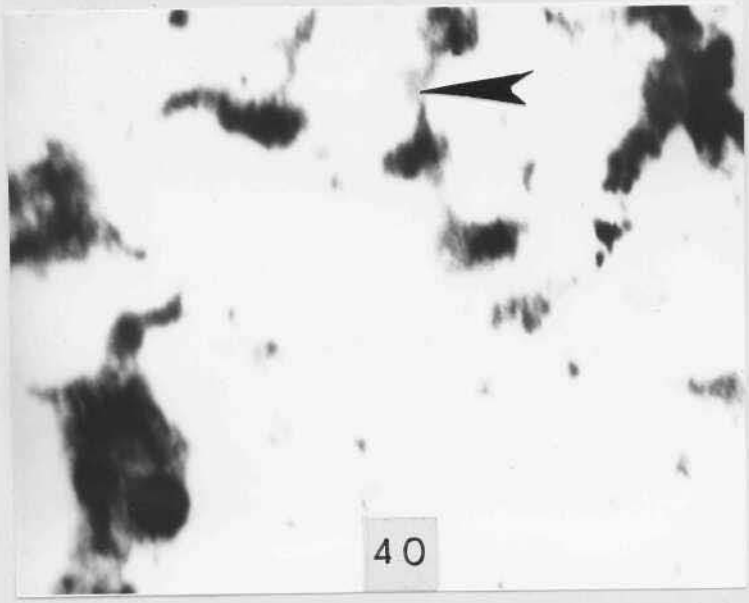
FIGURES 43-47. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{90}) STORED FOR 72 H AT 4-6C.

Fig. 43. Microstructure of coagulum showing extensive fusion and casein micelles and lumps formation (black arrow).

Fig. 44. SEM micrograph showing lactobacillus cell with heavy deposition on the surfaces.

Fig. 45. Streptococcal chain with heavy deposition on the surface of cells; lumps are seen in the background.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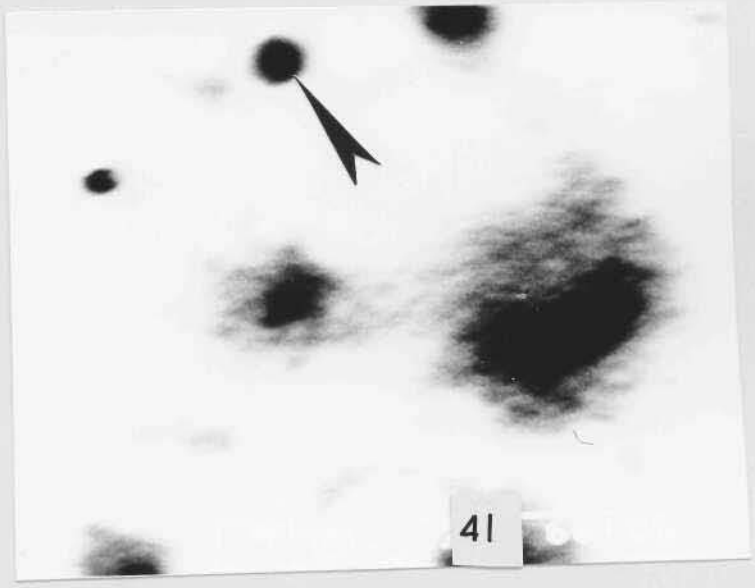
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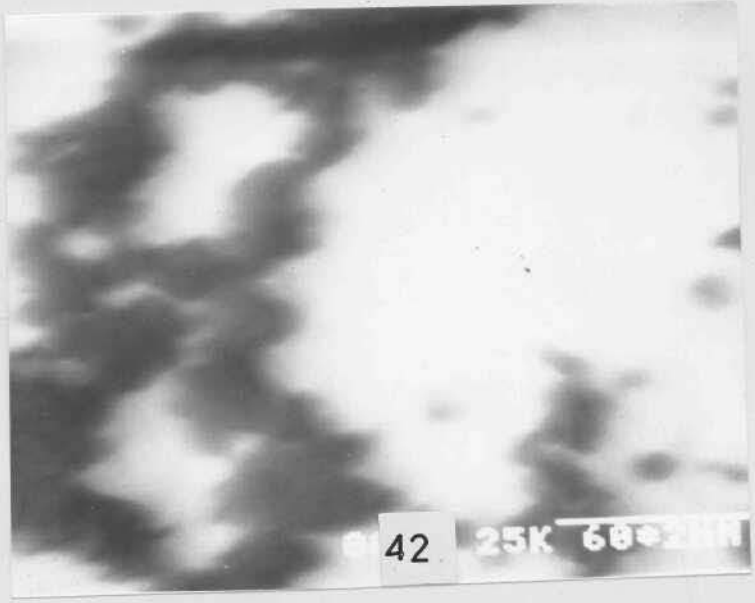
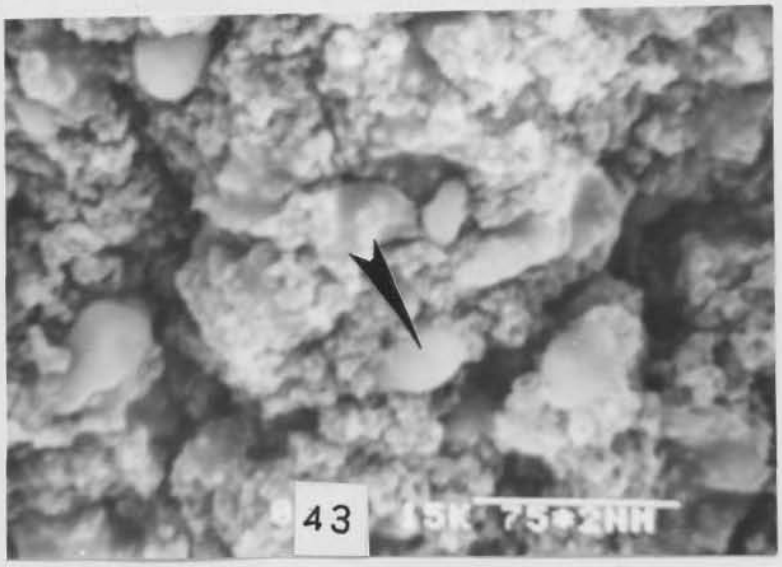

42
25K

FIGURES 43–47. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{90}) STORED FOR 72 H AT 4–6C.

Fig. 43. Microstructure of coagulum showing extensive fusion and casein micelles and lumps formation (black arrow).

Fig. 44. SEM micrograph showing lactobacillus cell with heavy deposition on the surface.

Fig. 45. Streptococcal chain with heavy deposition on the surface of cells; lumps are seen in the background.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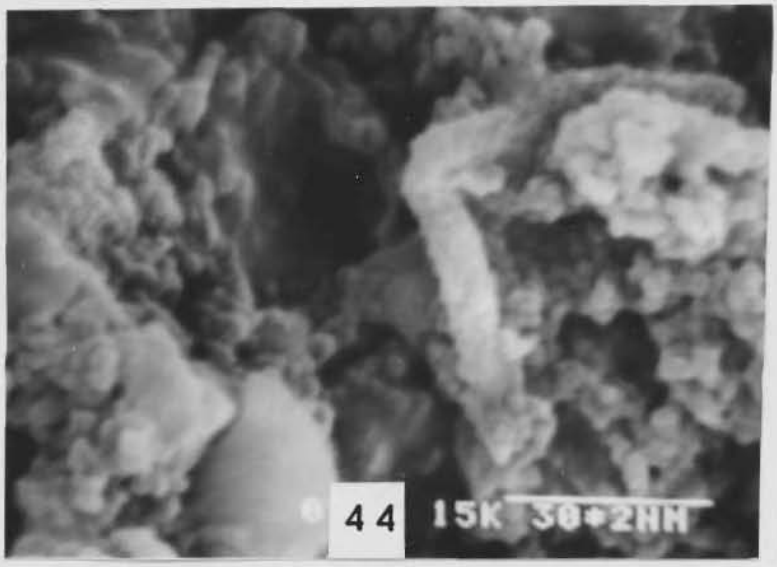

44
15K 30*2MM

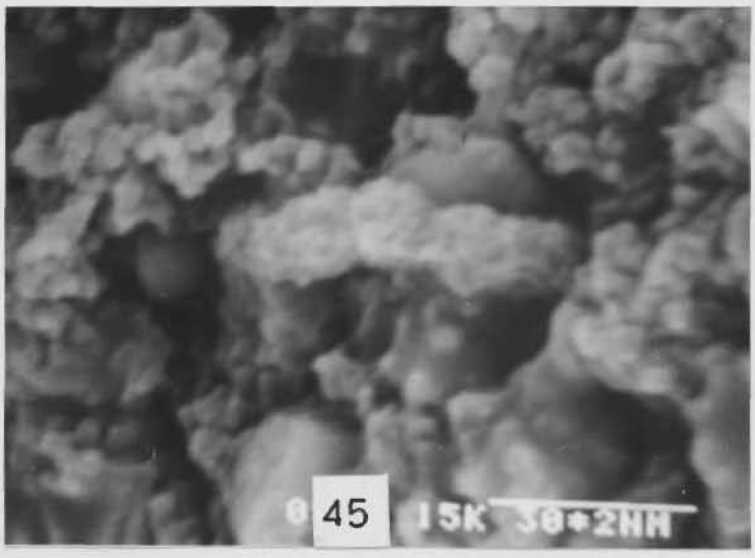

45
15K 30*2MM

Fig. 46. Small spherical casein micelles free and in chains as shown in TEM micrograph (X 2,000).

Fig. 47. Compact micelles of high density showing linearity. Short projections (black arrow) are seen on the surface joining to adjacent micelle surface (X 4,000).

projections or fibriller structure on the surface as revealed by TEM micrographs at higher magnification. Joining between two micelles through these fibres could be easily seen while aggregates of protein material was not prevalent.

4.4.3.3 Yoghurt from buffalo milk preheated at 70C:

An ill defined microstructure characterised the sample. Flake-like structures were seen concentrated mostly in pockets. Casein micelles failed to maintain regular structures and rendered the flocculated appearance along with loose globular clusters. Coral shaped structure made the appearance in the microstructure of this sample (Figs. 48,49). Streptococci were unusually large in size measuring 1800 nm in diameter.

TEM micrographs presented the casein micelles highly aggregated into loose clusters. Less electron dense protein structures in form of long strands appeared in the micrographs (Fig. 50). Free aggregates of these structures were also seen prevalent in the microstructure.

4.4.3.4 Yoghurt from buffalo milk preheated at 90C:

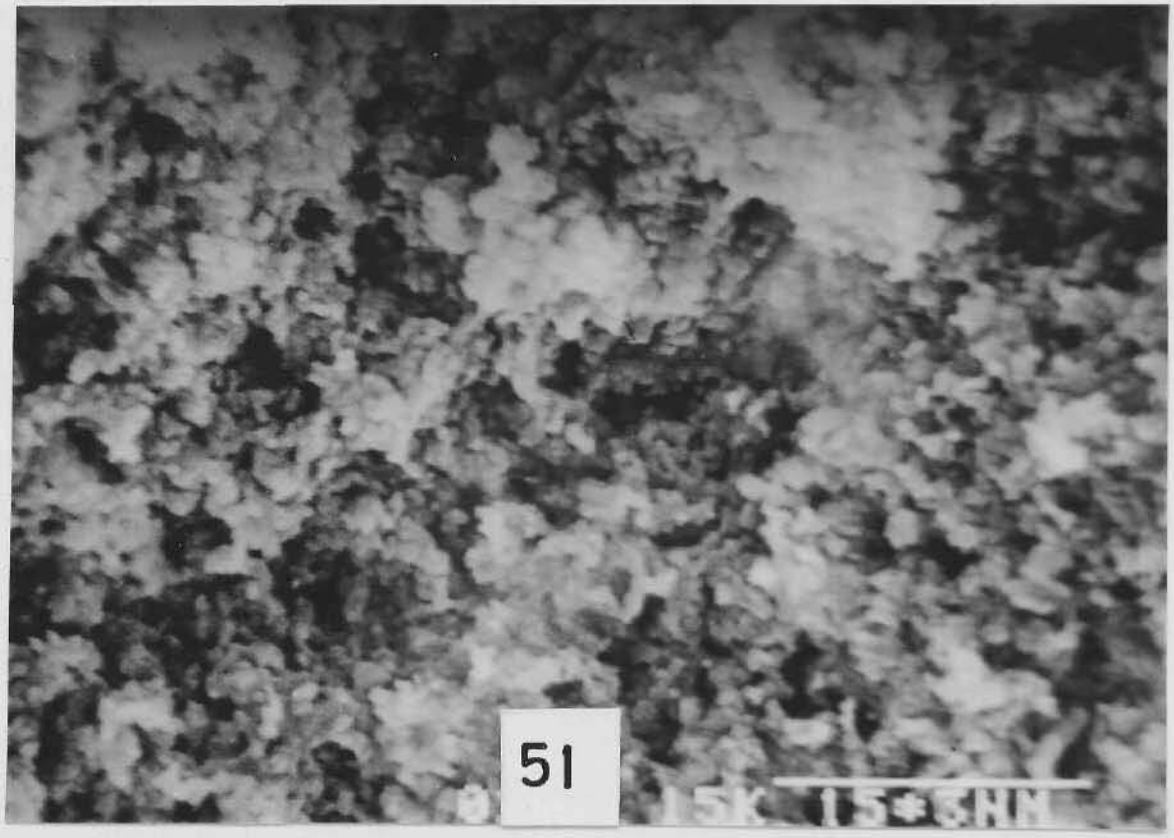
As compared to other samples stored for similar period, this seemed to preserve the organised microstructure somewhat better (Fig. 51). Both shallow and deep pockets were seen uniformly distributed. Similer to previous

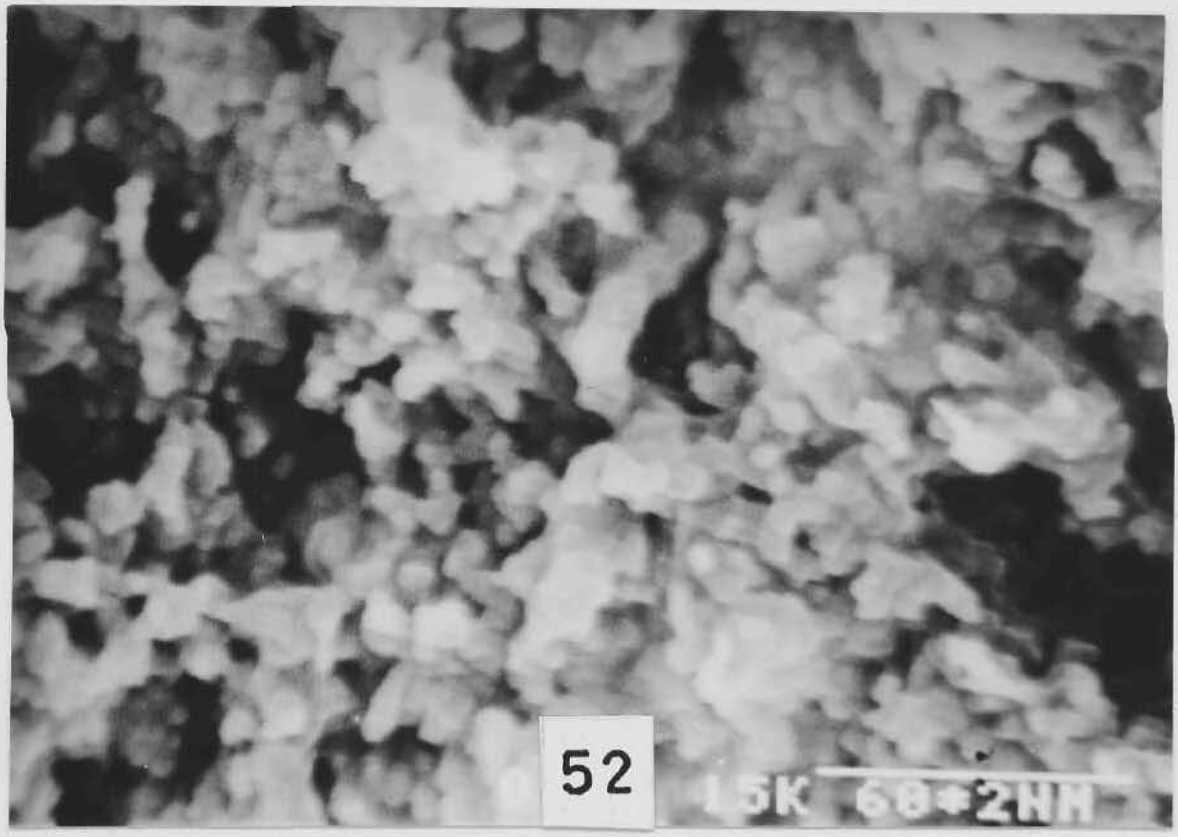
FIGURES 48–50. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURT (B_{70}) STORED FOR 72 H AT 4–6C.

Fig. 48. Microstructure of coagulum at low magnification.

Fig. 49. Micrograph showing coagulated globular structures aggregates (white arrow) and large flakes occupying the interspace.

Fig. 50. Ultrastructure of loose aggregates of casein micelles and flocculated whey protein structure prevailing the matrix (X 4,000).

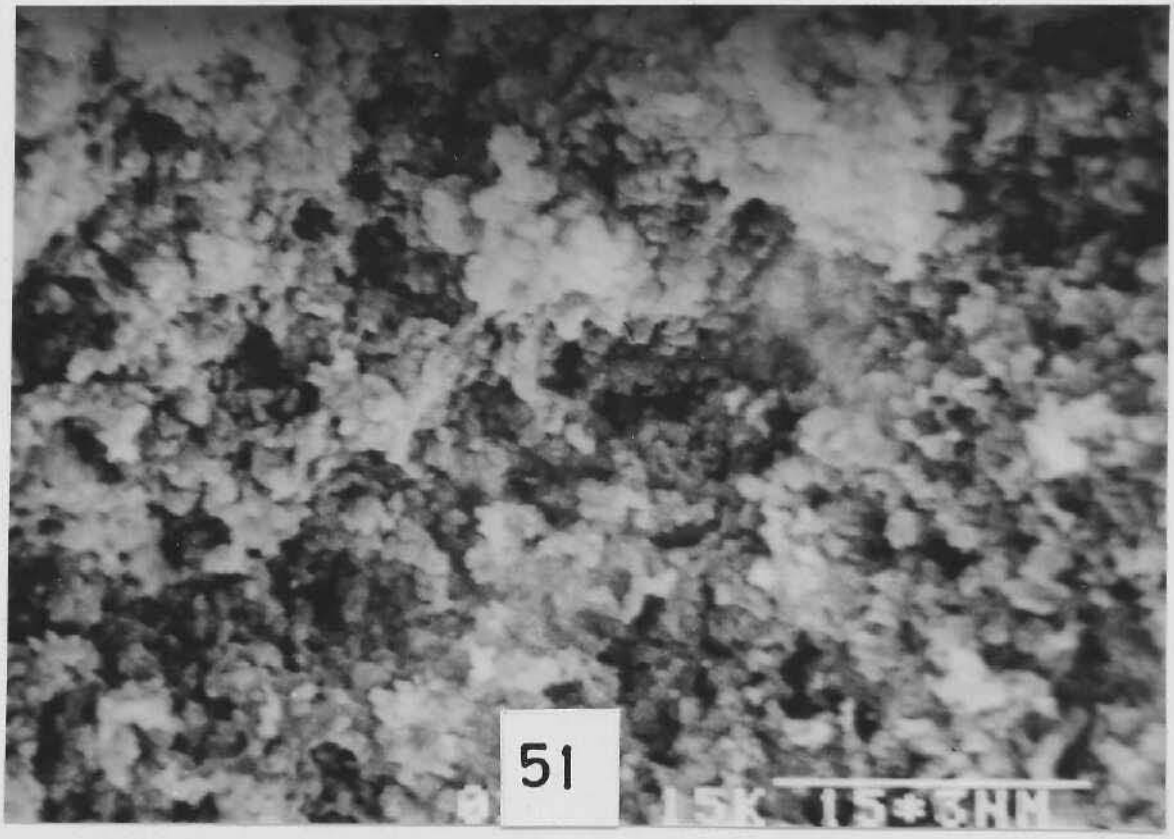

51
15K 15*3NM


52
15K 60*2NM

FIGURES 51-54. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURT (B_{90}) STORED FOR 72 H AT 4-6C.

Fig. 51. SEM micrograph depicting microstructure of coagulum at low magnification.

Fig. 52. Casein micelles in chains forming the network of microstructure. A short chain of Streptococci can also be seen.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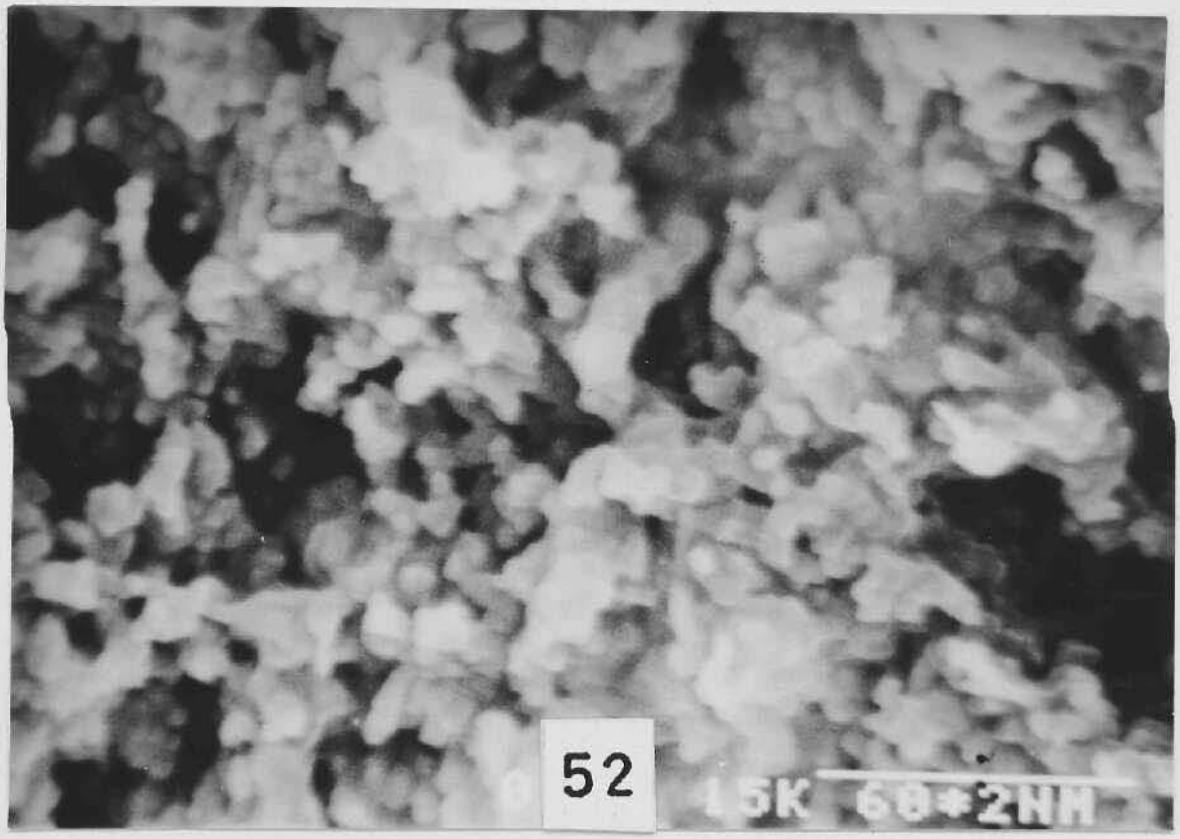

52

buffalo milk yoghurt samples, casein micelles were seen arranged in chains and thus forming a linear structure. The surface of yoghurt microflora continued to show structure less deposition (Fig.52).

TEM micrograph showed that micelles were of very smaller size arranged in a linear fashion (Figs. 53,54). On close examination, outline of micelles were found distinct bearing hairy projections. However, aggregation of micelles was not rare in this sample.

4.4.4 Yoghurts after storage of 120 h

Yoghurts stored for 120 h at low temperature were studied under SEM and TEM to visualise the effect of prolonged storage on microstructure.

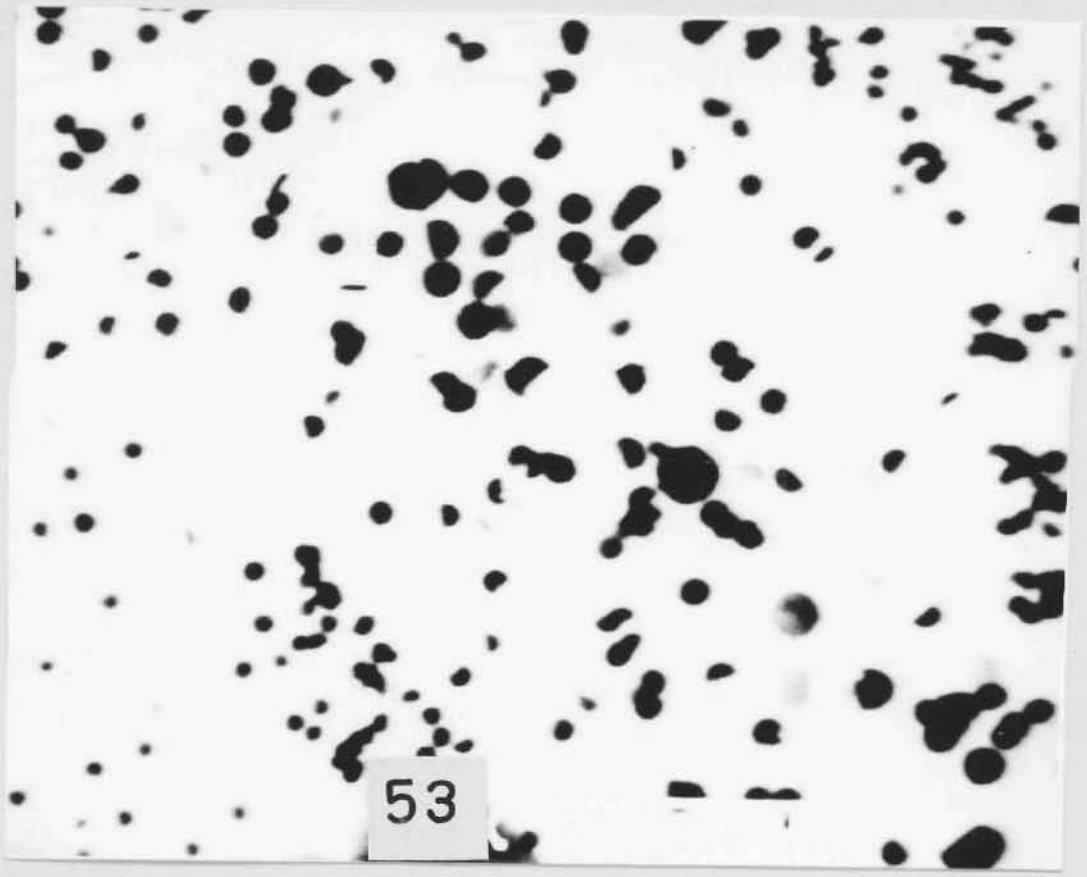
4.4.4.1 Yoghurt from cow milk preheated at 70C:

Clusters of casein micelles and could be observed as usual with flat sheet like structures of compacted casein dominating the microstructure of unfractured coagulum (Figs. 55,56). Both clean cells (diameter 730 nm) and unclean cells (diameter 730-860 nm) of lactobacillus were observed in fractured specimens. Streptococci with diameter 1500 nm showed considerable deposition on the surface (Fig.57).

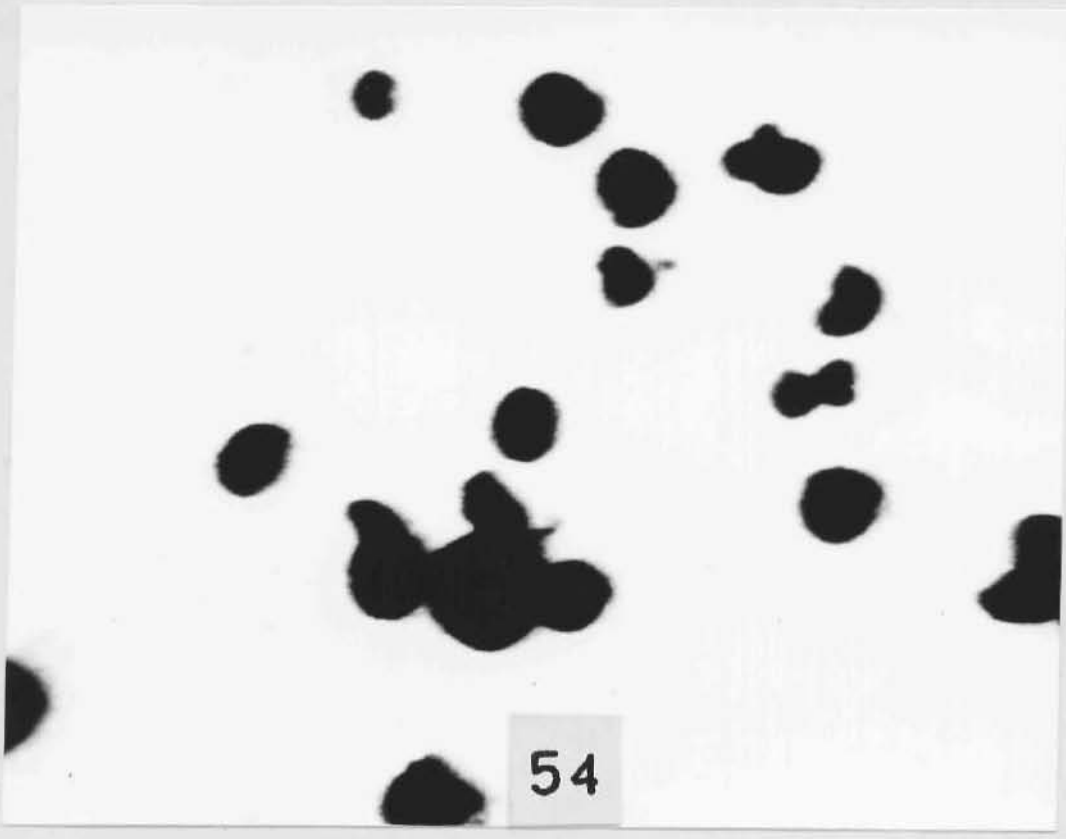
In TEM micrographs, micelles were seen rather separate entities of irregular shapes. Free floccules of less

Fig. 53. Small casein micelles with comparatively smooth outline are seen mostly forming the chains as revealed by TEM at low magnification (X 2,000).

Fig. 54. Ultrastructure of casein micelles (X 5,000). No floccules of denatured whey protein are seen around the micelles.



53



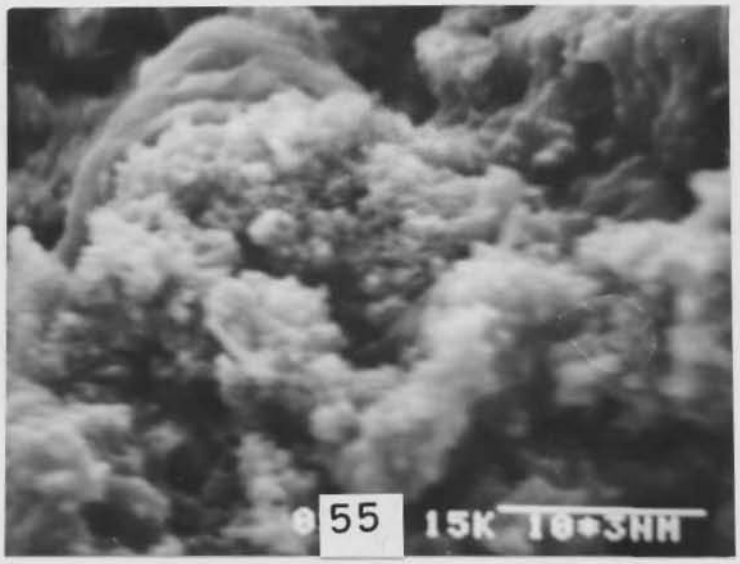
54

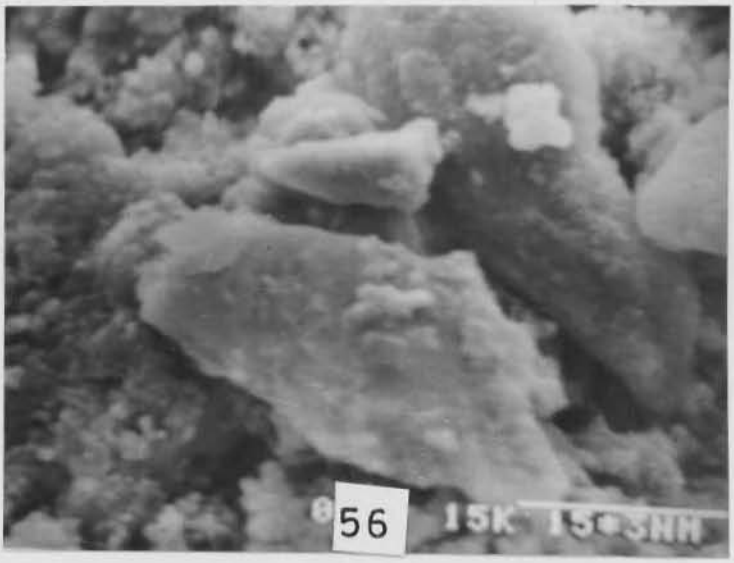
FIGURES 55–59. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{70}) STORED FOR 120 H AT 4–6C.

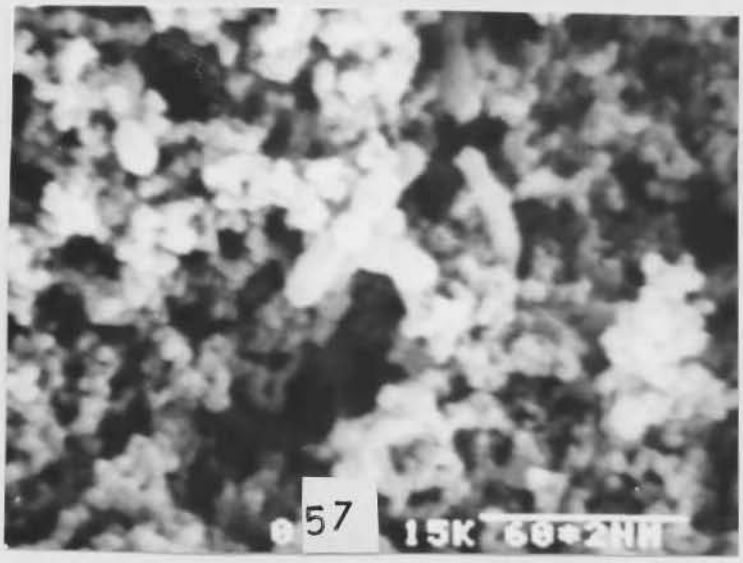
Fig. 55. Compact microstructure of coagulum.

Fig. 56. Large compact sheet like structures presumably of protein dominating the microstructure of unfractured coagulum. Aggregates of globular structures are seen around these sheets.

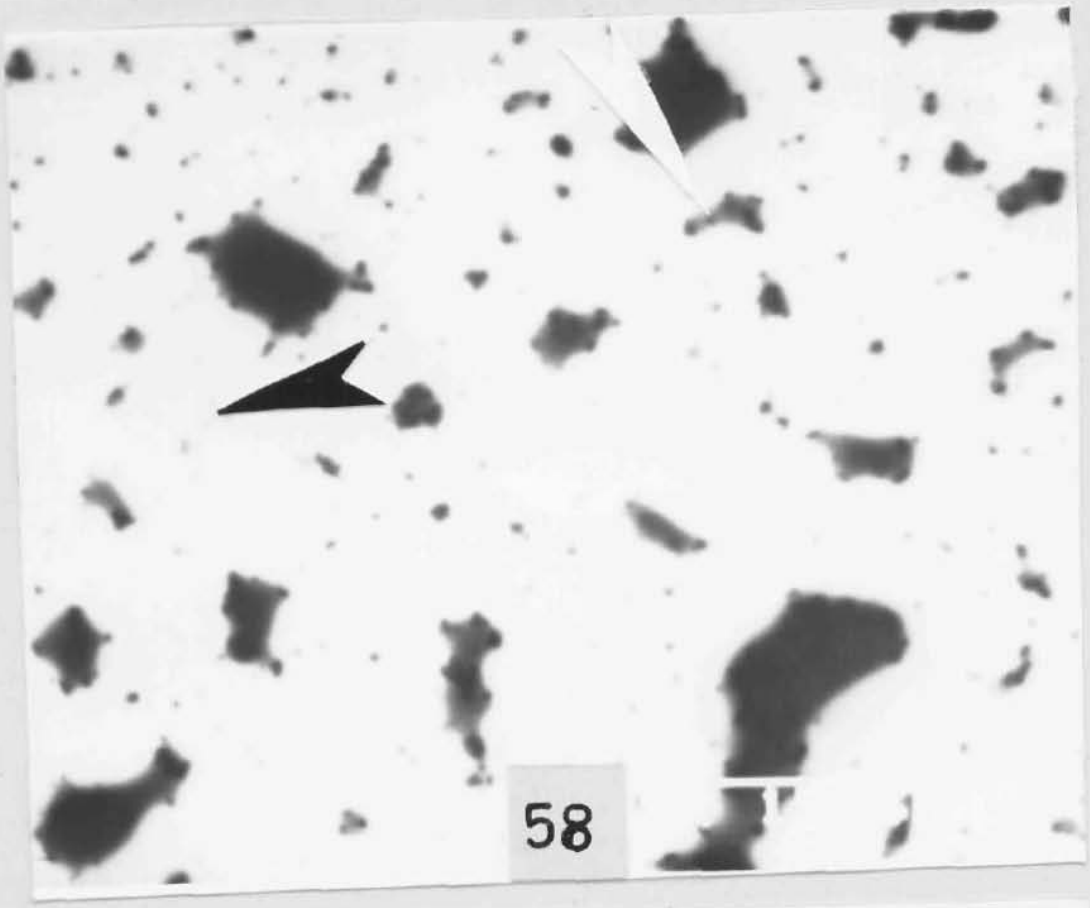
Fig. 57. Microstructure of fractured specimen showing lactic acid bacteria.


55
15K 10.3MM

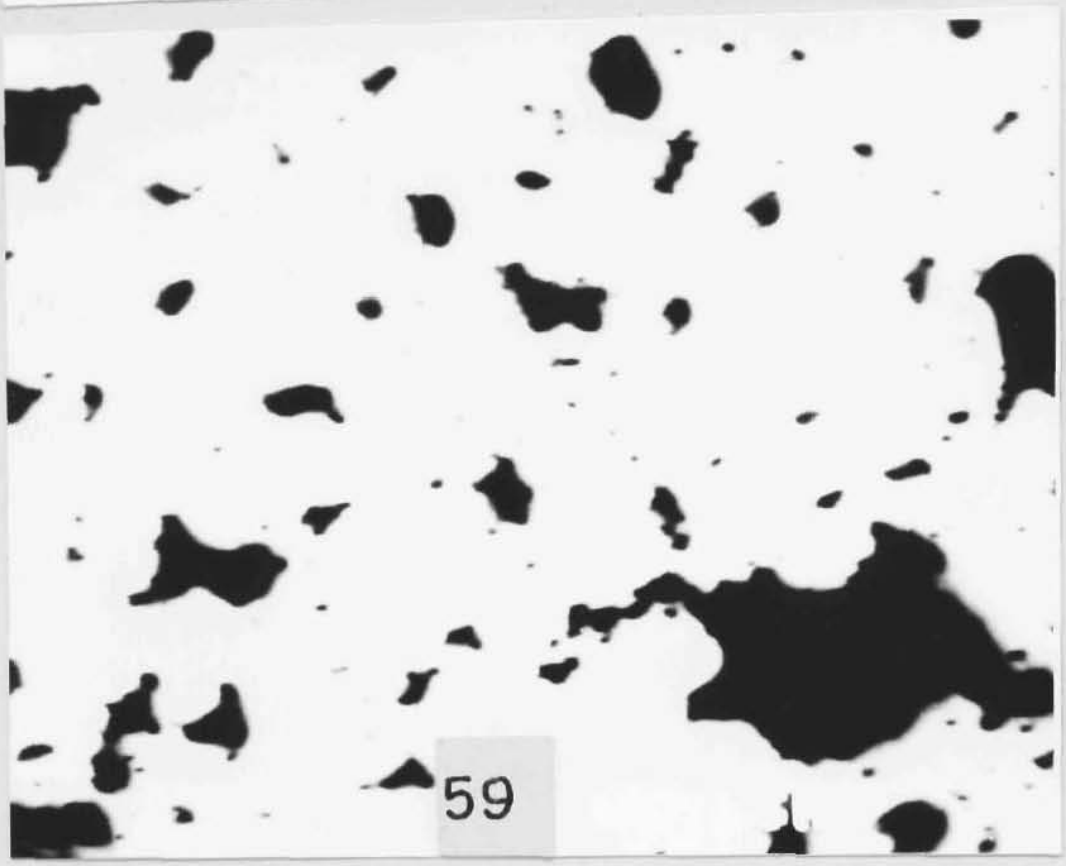

56
15K 15.3MM


57
15K 6.6.2MM

Figs. 58, 59. TEM micrographs showing casein micelles aggregates of irregular sizes. Threads of denatured whey protein attached to these aggregates (white arrow) and in form of free floccules (black arrow) are frequently seen (X 2,000).



58



59

electron density were found distributed throughout the matrix (Figs. 58,59).

4.4.4.2 Yoghurt from cow milk preheated at 90C:

This sample continued to present the complex microstructure at this interval of storage. Smooth spherical inclusion bodies appeared localised on the surface of coagulum (Fig.60). The lactic acid bacteria with clean and unclean surfaces were generally spotted in interspaces (Figs. 61,62). Pockets were not so well demarcated and seemed to fused into big interspaces in the coagulum. Micelles were seen both in free and aggregated form as revealed by TEM. Micelles managed to maintain their integrity in clusters and free micelles measured 600-1700 nm in diameter. Fibrilee joining the casein eggregates were commonly observed in different microfields (Figs. 63,64,65).

4.4.4.3 Yoghurt from buffalo milk preheated at 70C:

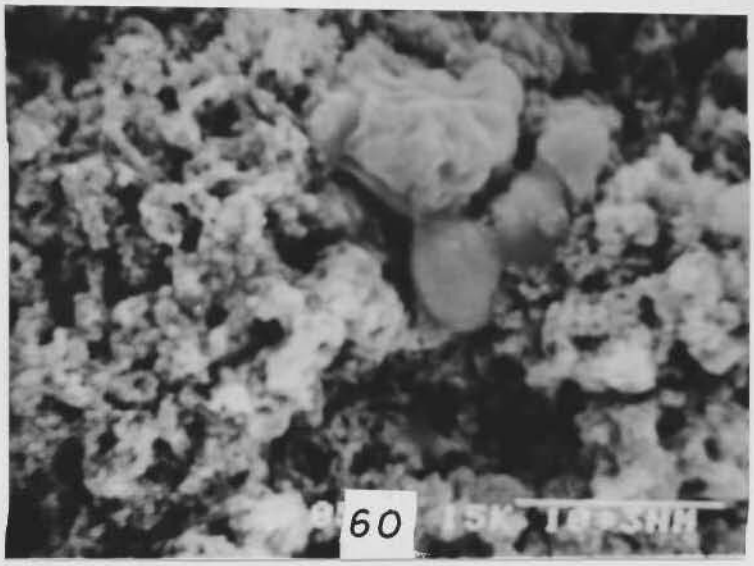
Fractured coagulum offered a porous microstructure with large interspaces resulting presumably by distortion of pockets distributed in the coagulum. Lactic acid bacteria can be spotted in coagulum even at low magnification in Fig.66. Small globular structures could be seen in unfractured specimen. Clean surfaced streptococci and lactobacilli measuring 1,000 nm and 720 nm in diameter respectively were observed in pocket (Fig.67). Diplococci with covered surfaces measured 1200 nm in diameter. Lumps and sheet like structures

FIGURES 60-65. ELECTRON MICROSCOPY OF COW MILK YOGHURT (C_{90}) STORED FOR 120 H AT 4-6C.

Fig. 60. SEM micrograph showing lumps alongwith casein aggregates in unfractured coagulum.

Fig. 61. Streptococci (black arrow) and lactic acid bacteria (white arrow) spotted on the pockets of coagulum.

Fig. 62. Beside casein aggregation, chain formation of micelles is also evident (white arrow). Lactic acid bacteria showing both clean and deposited surface at higher magnification.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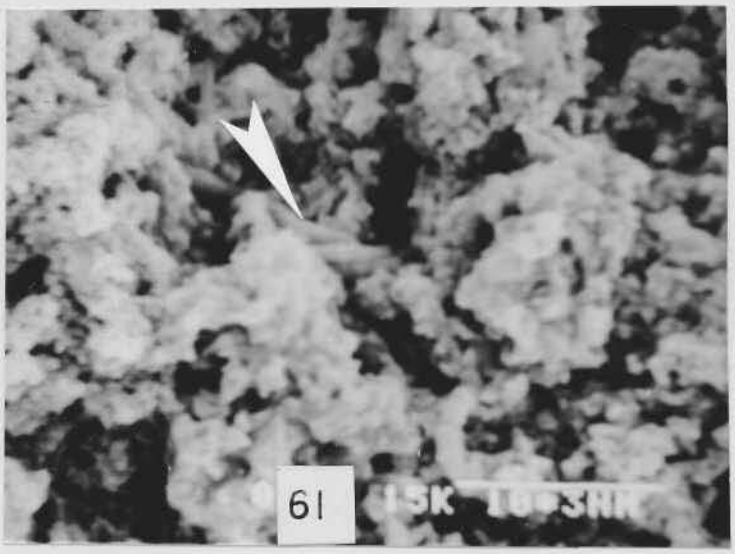

61

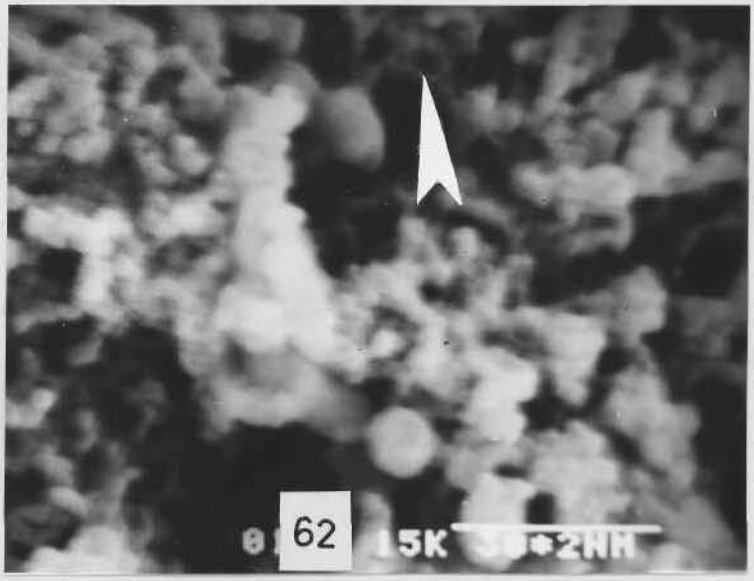
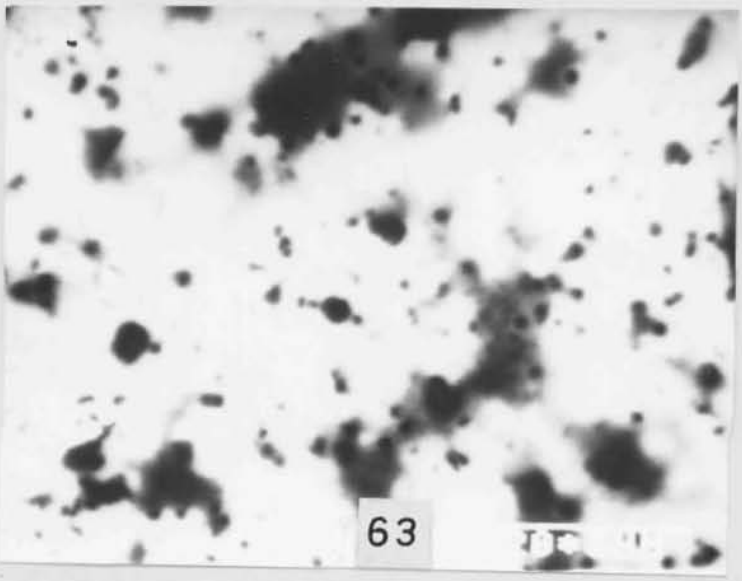

62

Fig. 63. A section through yoghurt coagulum showing ultrastructure of casein micelles (X 1,500).

Fig. 64. Micelles are seen surrounded by comparatively more electron dense thread like structures in this sample (X 4,000).

Fig. 65. Micelles joined through afore-said fibriller structures as seen at high magnification (X 10,000).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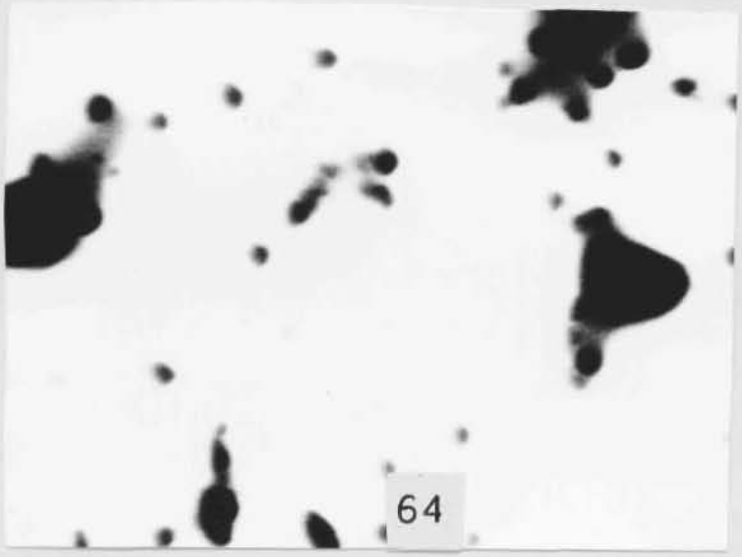
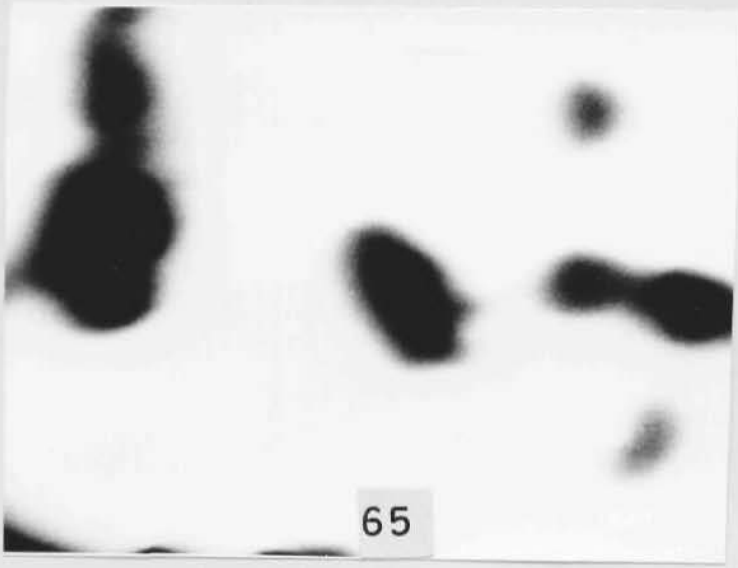

64

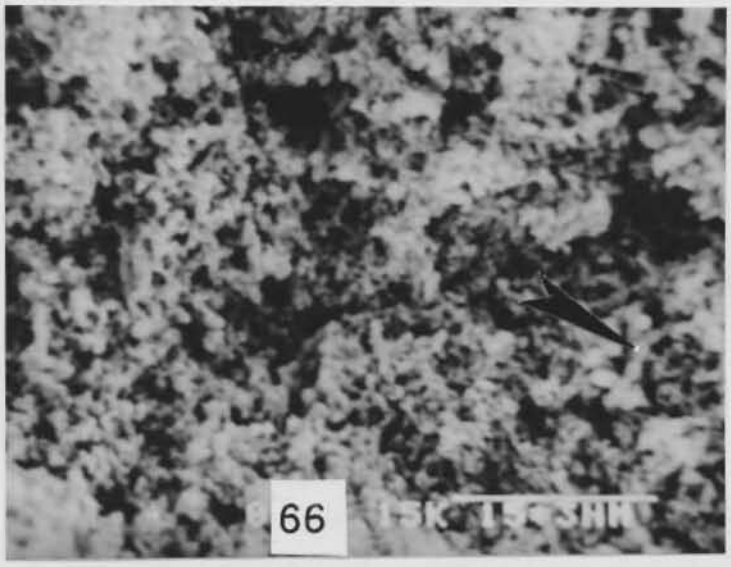

65

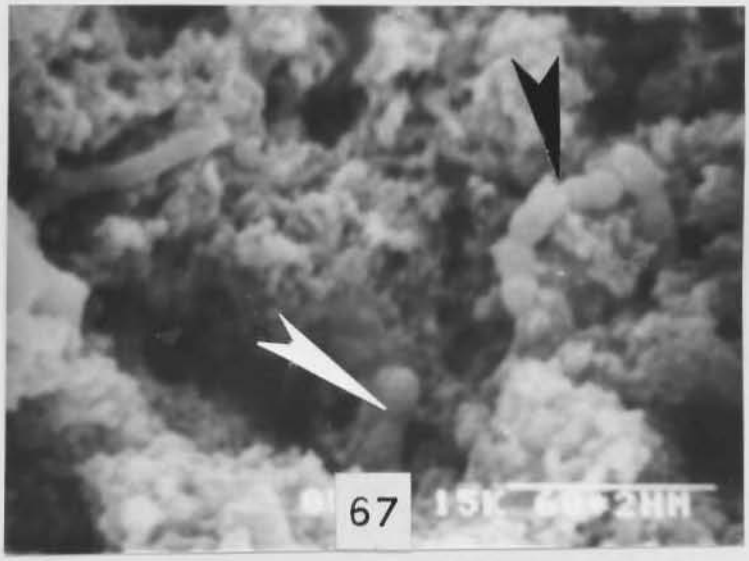
FIGURES 66–70. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURTS (B_{70}) STORED FOR 120 H AT 4–6C.

Fig. 66. Large interepaces in the microstructure as shown under SEM. Yoghurt microflora can be spotted even at low magnification (black arrow).

Fig. 67. Micrograph showing lactic acid bacteria with clean (black arrow) and covered surface (white arrow).

Fig. 68. Sheet like protein structures surrounded by tiny globular structures observed in unfractured coagulum.


66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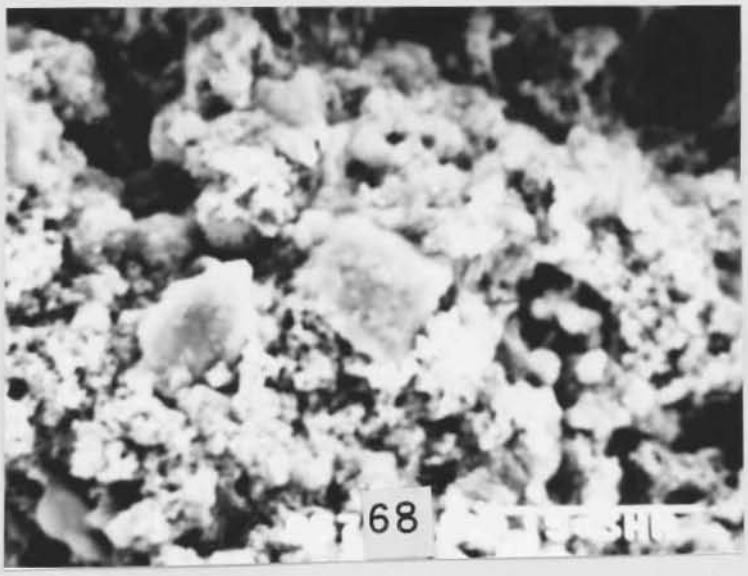

68

were found in the pockets frequently (Fig.68). Presence of big aggregates of small micelles and pockets in the coagulum was evidenced in TEM micrographs. The protein like material interconnecting these aggregates and in the form of free aggregates dominated the microstructure (Fig.69,70).

4.4.4.4 Yoghurt from buffalo milk heated at 90C:

This sample offered highly porous structure, numerous pockets were distributed uniformly. The long chains of micelles, interwoven and interconnected presented an organised network (Fig. 71). Sample even at this stage of storage, continued to support the growth of lactic acid bacteria to a great extent. Streptococci with clean surfaces measured 1000 nm, while streptococci with covered surfaces measured 1200-1400 nm in diameter (Figs.72,73). Short lactobacillus cell of 6.4 μ in length was spotted at this stage of storage. The compact micelles arranged in linear fashion were observed at lower magnification in TEM micrographs (Fig. 74). Micelles joined through short fibriller structures could be seen in Fig.75. In addition to this, a cluster of smooth surfaced spherical bodies with dimension of 1200 nm indicated the presence of streptococci (Fig.76).

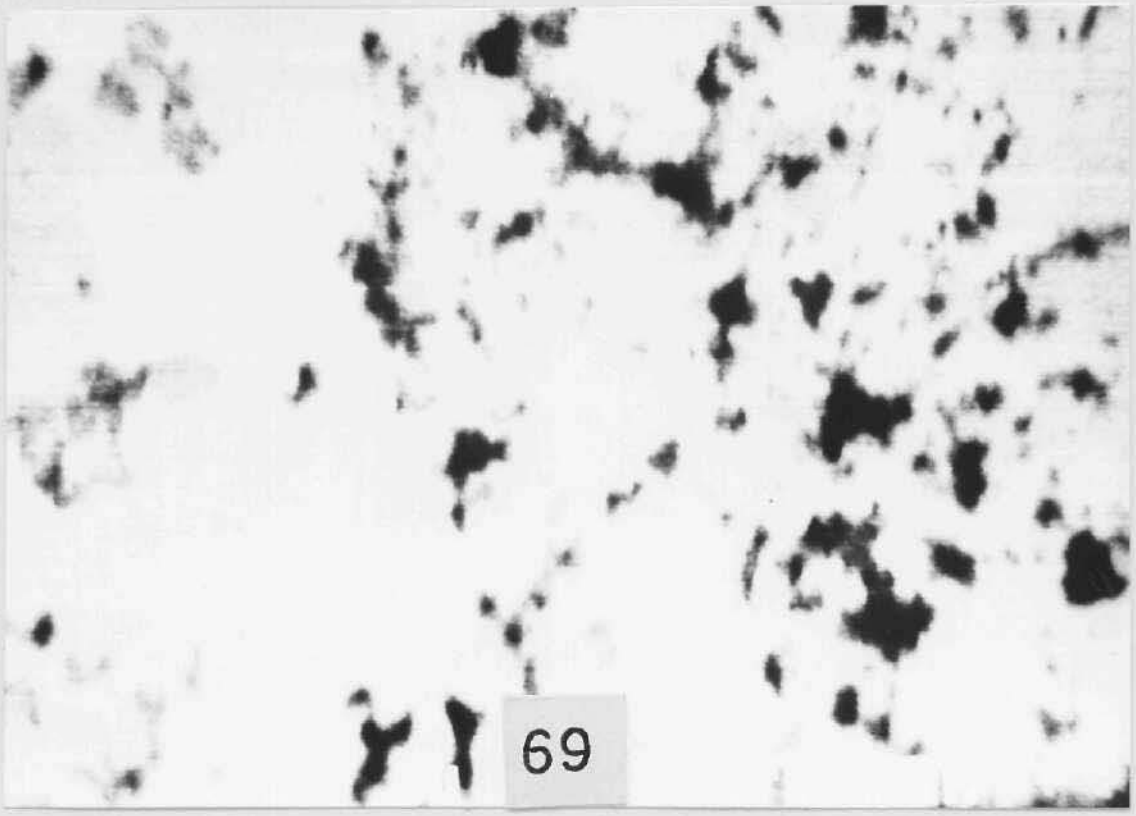
4.5 APPLICATION OF SEM TECHNIQUES IN YOGHURTS

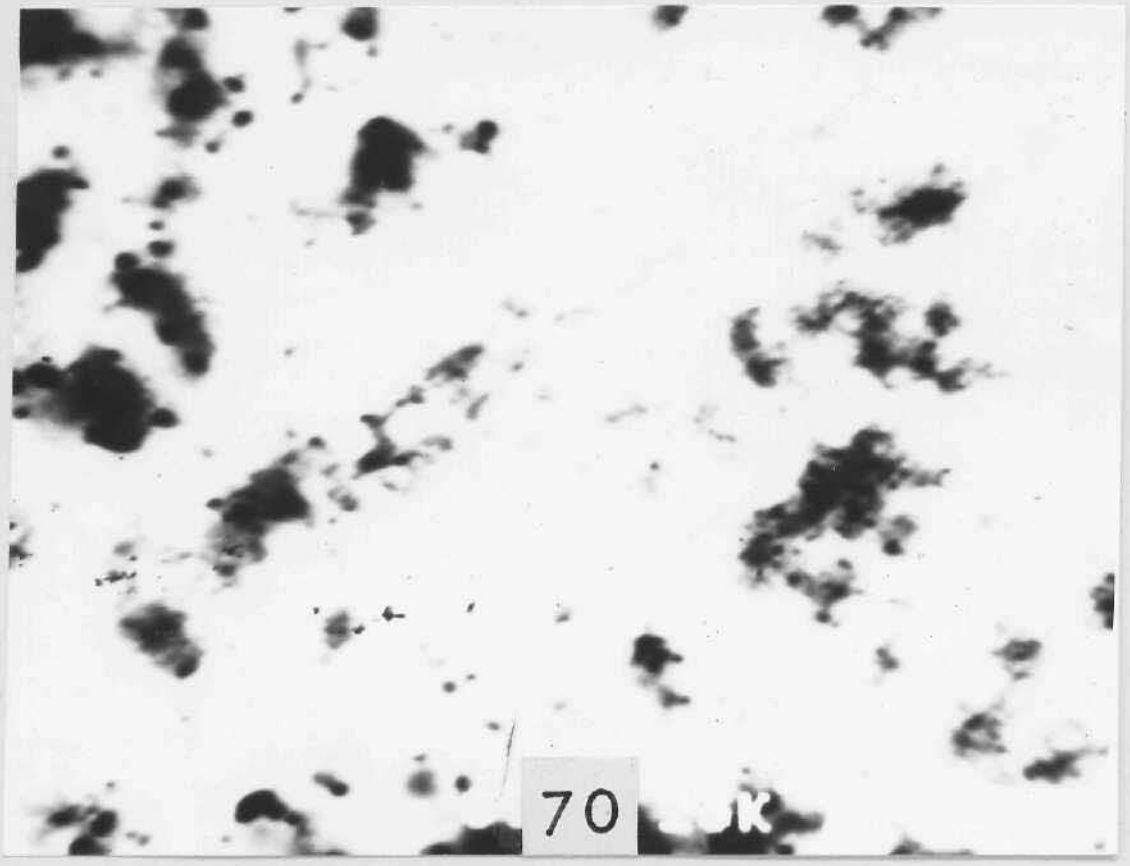
4.5.1 Double fixation versus single fixation of Yoghurts coagulum

To see the suitability of single fixation in gluteraldehyde for yoghurts, yoghurts at 24 h interval of storage were preserved in glutaraldehyde and switched over

Fig. 69. Discrete entities of casein micelles interlinked by denatured whey protein structures as revealed by TEM (X 4,000).

Fig. 70. Loosely aggregated casein clusters surrounded by denatured whey protein floccules (X 2,000).


69

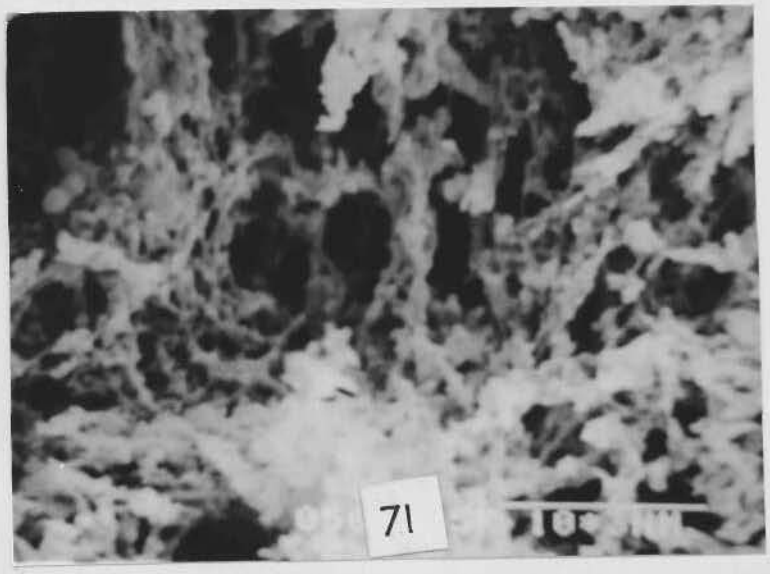

70

FIGURES 71-76. ELECTRON MICROSCOPY OF BUFFALO MILK YOGHURT (B_{90}) STORED FOR 120 H AT 4-6C.

Fig. 71. Highly porous microstructure of coagulum.

Fig. 72. Microcolony of streptococci localised in the pockets of three dimensional network of casein.

Fig. 73. A long streptococcal chain showing deposition on the surface at higher magnification.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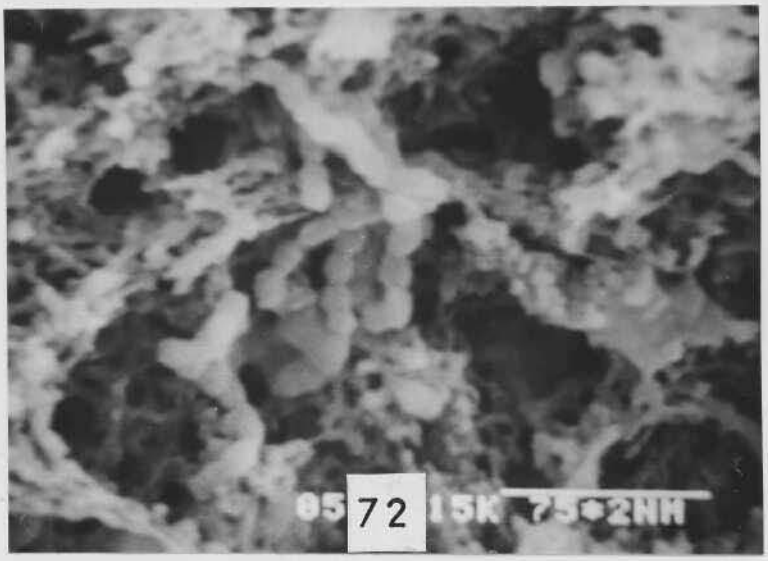

72

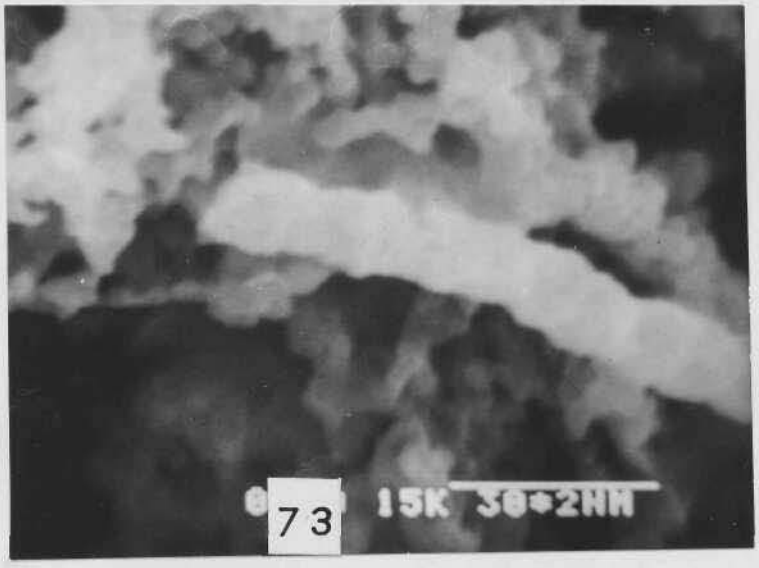
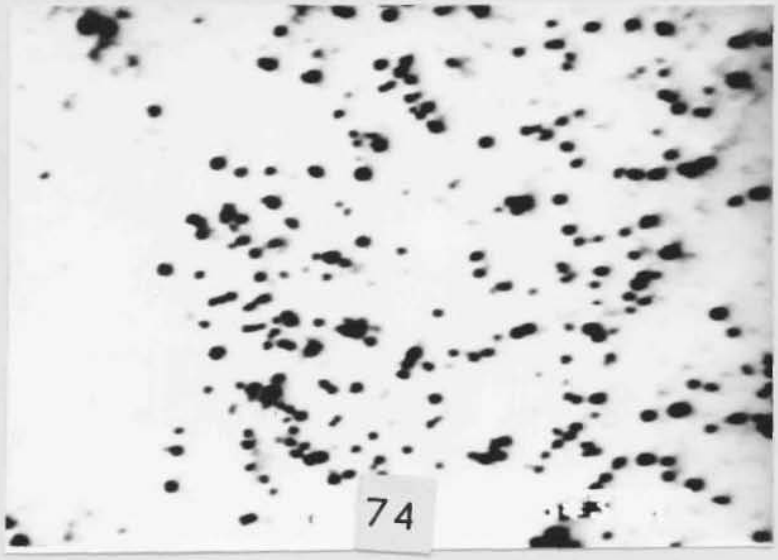

73
15K 30*2NM

Fig. 74. TEM micrograph showing small casein micelles in chains distributed in the matrix (X 2,000).

Fig. 75. Hairy projections stretching between two casein micelles as shown at higher magnification (X 2,000).

Fig. 76. A section through typical microcolony of streptococci observed under TEM (X 10,000).



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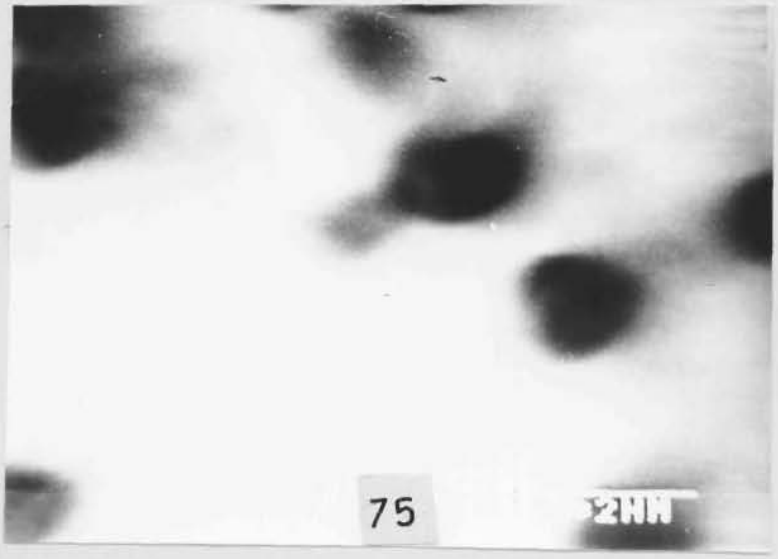
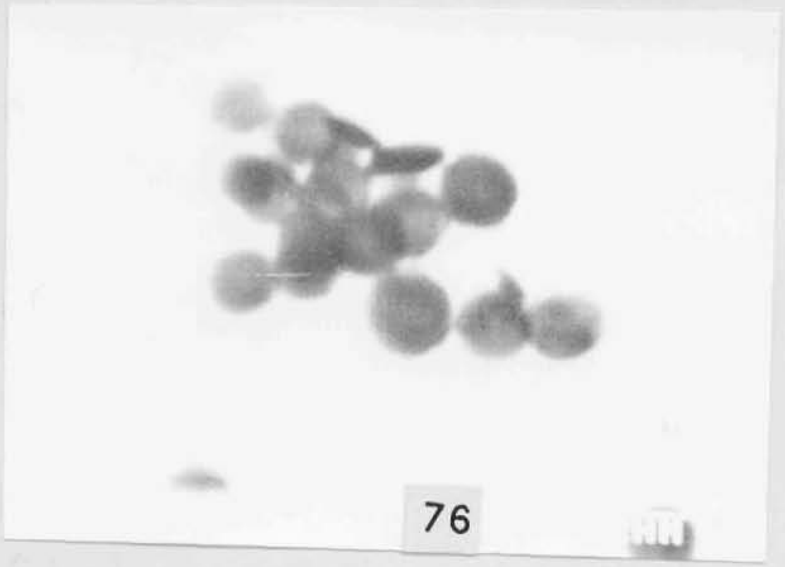


75



76

directly to conventional air-drying. The reason for choosing Yoghurts at this particular interval of storage was that it was presumed that microstructure of coagulum is stabilized by this time.

All the samples - cow and buffalo milk yoghurts prepared from milk heated at 70C and 90C presumed the microstructure comparable to doubly fixed samples. The microstructure of buffalo milk yoghurts was found more porous as compared to that of cow milk yoghurts (Fig. 77). The distribution and size of tpockets and interspaces varied accordingly. Casein micelles of various shapes like typical heart, spherical, elliptical were observed in cow milk yoghurts. The tendency of uniformly well preserved lactic acid bacteria particularly streptococci to get colonised in pockets and interspaces in the coagulum was frequently seen in various micrographs (Fig. 78,79). Besides long cells, small lactobacillus cells could also be spotted. Not only casein and microflora, single fixation was found equally efficient in preserving the inclusion bodies in the coagulum (Fig.80).

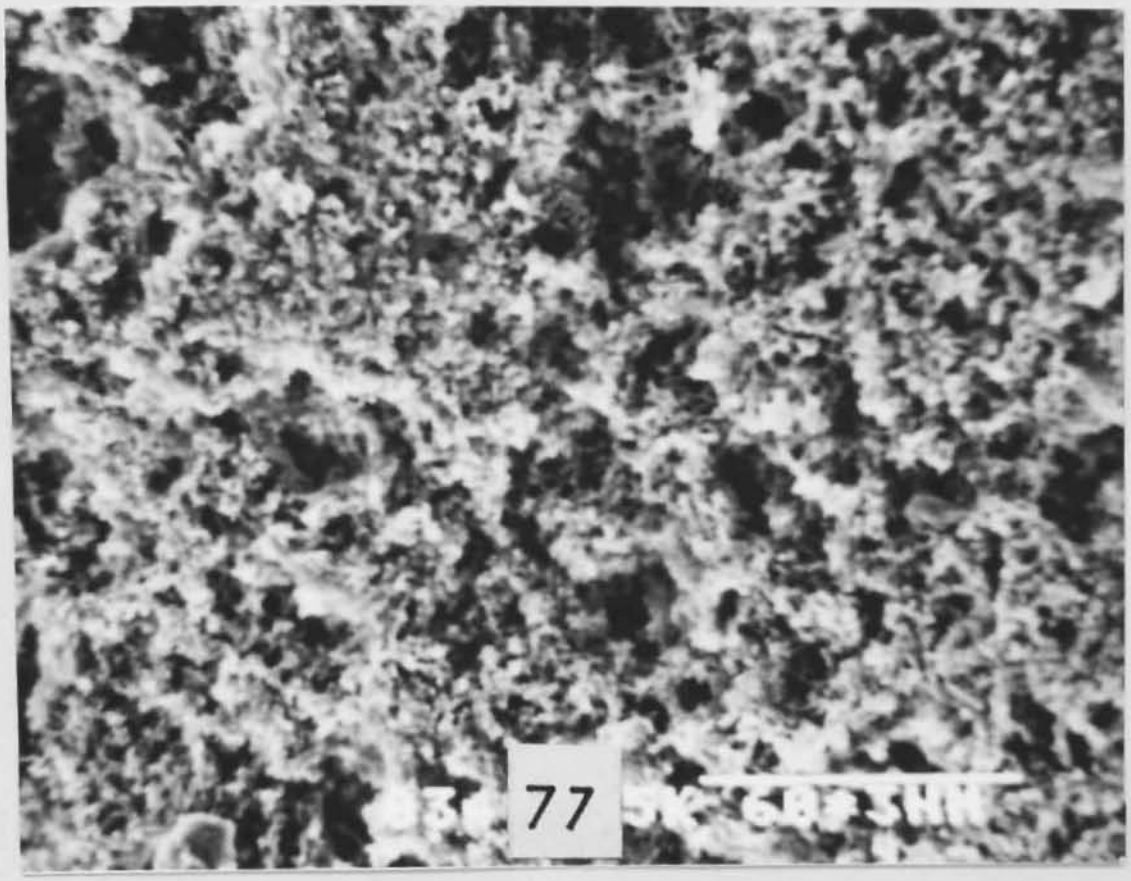
4.5.2 Air-drying versus Freeze-drying of Yoghurts

For SEM, freeze-drying was employed as an alternative to air-drying, for the purpose of drying the yoghurt specimens after fixation. Yoghurt samples were doubly fixed in glutaraldehyde followed by osmic acid and subsequently subjected to freeze-drying in a lyophilizer.

FIGURES 77–80. SCANNING ELECTRON MICROSCOPY OF SINGLY FIXED AIR–DRIED SAMPLES OF COW AND BUFFALO MILK YOGHURTS STORED FOR 24 H AT 4–6C.

Fig. 77. Well preserved Microstructure of B_{70} sample observed at low magnification.

Fig. 78. Clean surfaced streptococci in chains (black arrow) and small rod of lactobacillus (white arrow); casein network is seen in the background.


77

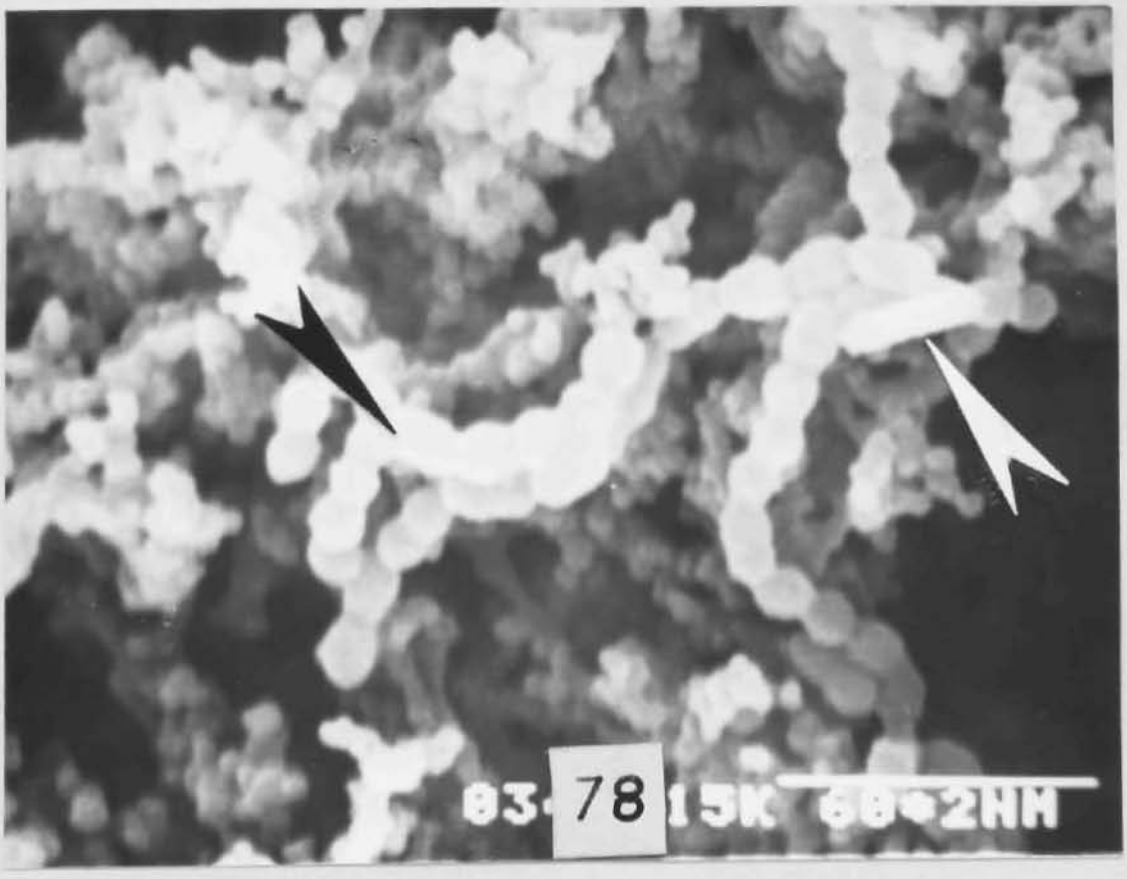
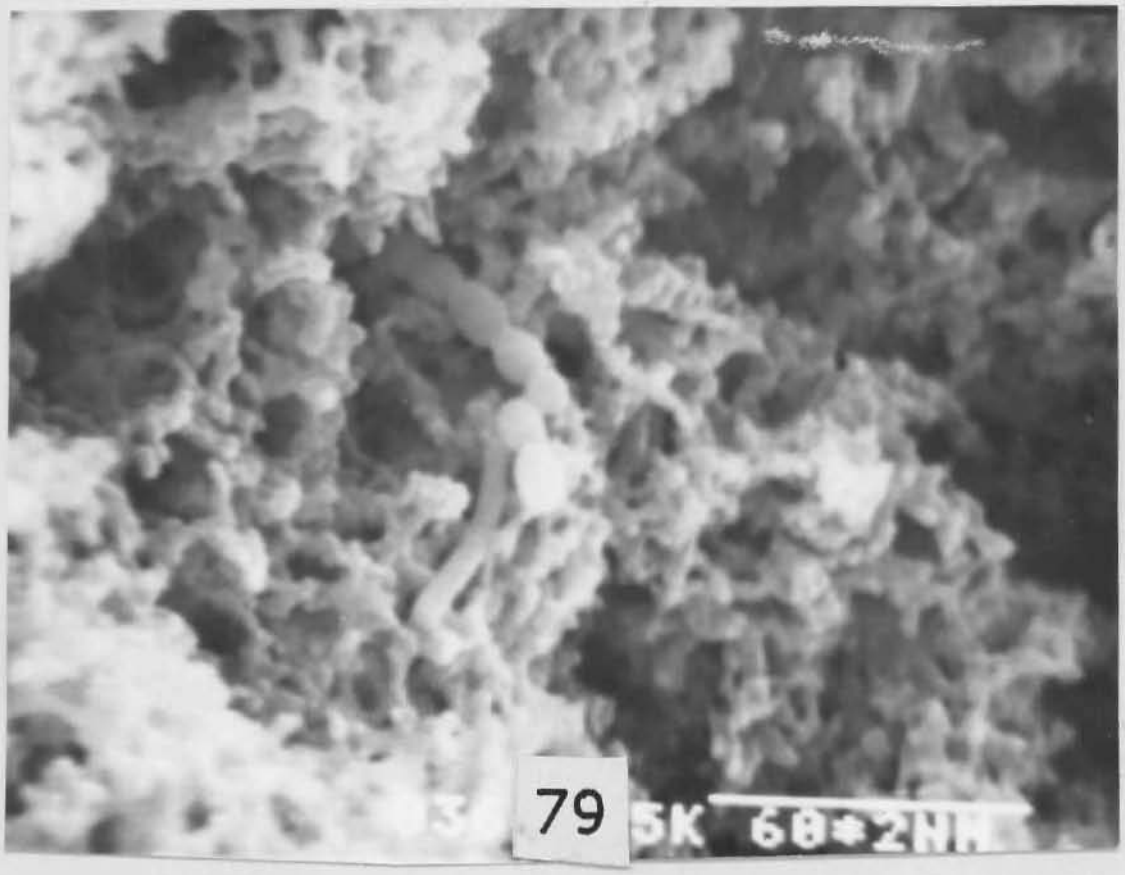
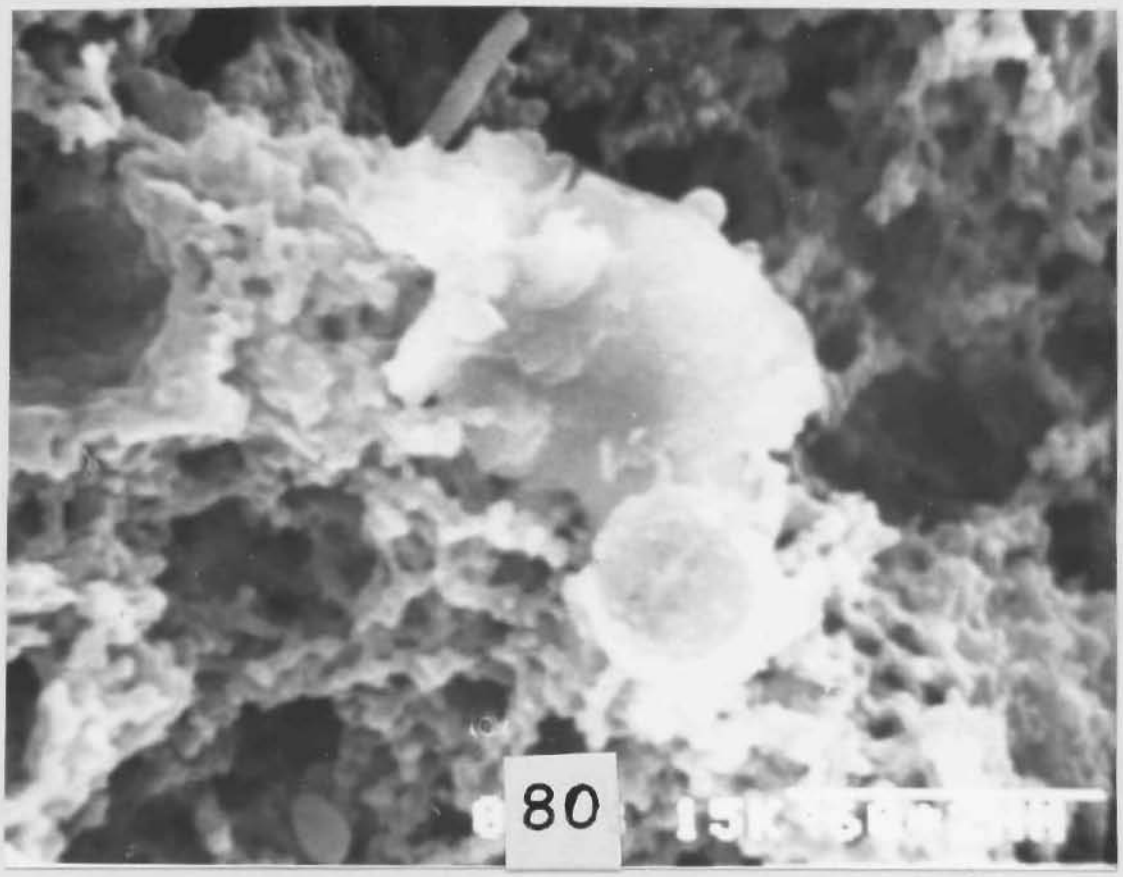

03-78 15K 60*2MM

Fig. 79. Three dimensional network of casein micelles entrapping lactic acid bacteria.

Fig. 80. Smooth surfaced big inclusion bodies dominating the microstructure. Numerous pockets are seen in the background.


79
5K 60*2NM


80

Freeze-drying resulted in a flocculated microstructure in case of yoghurt coagulum as visible at low magnification (Fig.81). Casein micelles were seen either aggregated or stretched into sheet like structures both in fresh and stored samples. In this type of arrangement, regular pocket formation was not observed (Fig. 82).

Freeze-drying was found quite efficient in preserving the microflora of yoghurts- fresh and stored samples. Streptococcal chains with both clean and covered surface were frequently seen in fresh and stored yoghurts (Fig. 83). At higher magnification, diplococci cells were seen united to form the chain and the terminal cell of the chain was found comparatively bigger in size. No connecting material between bacterial cell and casein matrix was seen (Fig.84). Micro-colonies of lactic acid bacteria were well preserved in stored sample for 120 h (Fig. 85). Streptococci of higher dimension measuring 1300 nm in diameter was observed apart from long chains of lactobacilli, individual lactobacillus cell with diameter and length of 960 and 6400 nm respectively could also be spotted (Fig. 86). Connecting material between cell and casein matrix around was found missing in freeze-dried samples too.

The holes formation, a phenomenon alien to air-dried samples, was frequently observed in SEM micrographs obtained from samples dried through this technique.

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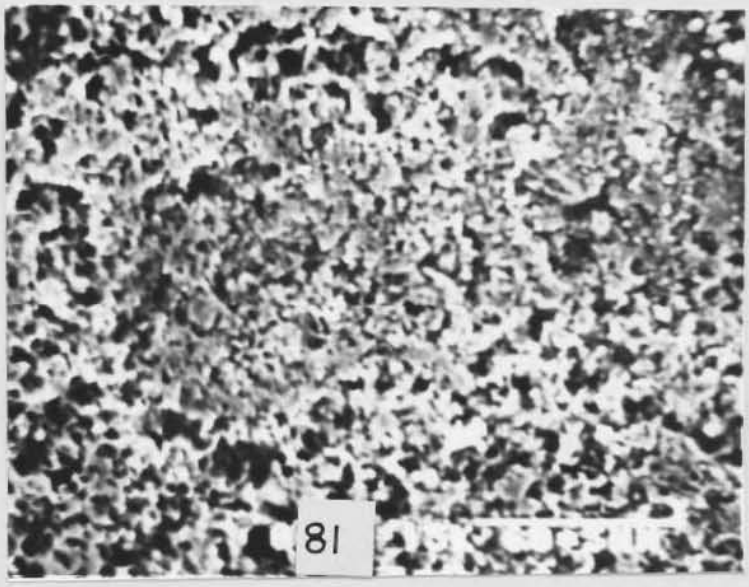
FIGURES 81-86. SEM OF FREEZE-DRIED COW AND BUFFALO MILK YOGHURTS.

Fig. 81. Flocculated microstructure of fresh buffalo milk yoghurt (B_{90}).

Fig. 82. Closed microstructure resulting from the stretching of casein into continuous sheet like structures in cow milk Yoghurt (C_{90}) stored for 24 h.

Fig. 83. Flocculated microstructure showing a chain of lactobacilli with deposition on the surface.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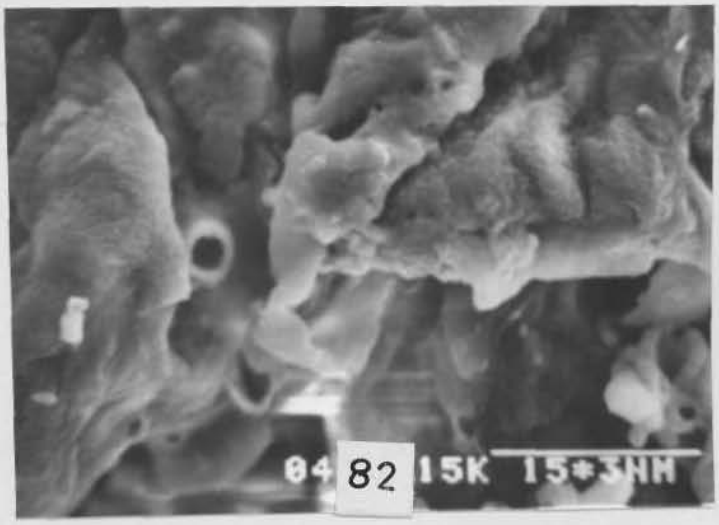
04 82 15K 15*3MM

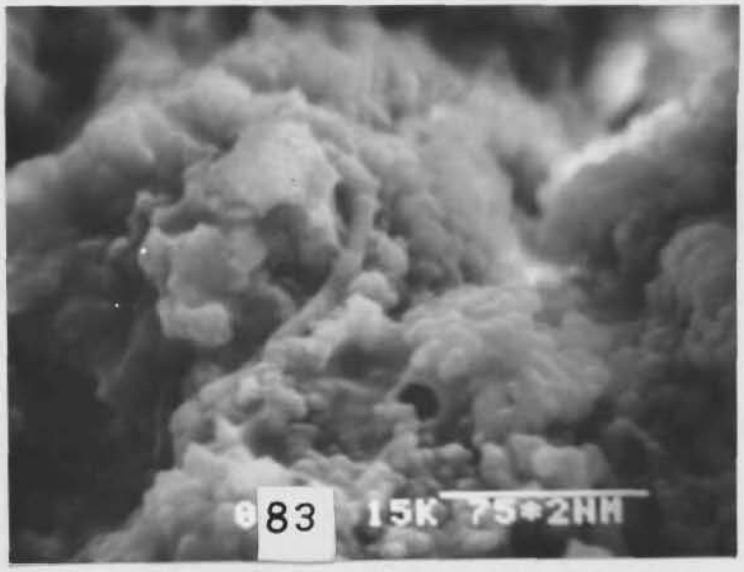
083 15K 75*2MM

Fig. 84. Smooth surfaced streptococci in fresh buffalo milk yoghurt (B_{70}) shown at higher magnification. Terminal cell is bigger in size.

Fig. 85. Lactobacilli and streptococci seen colonised in pockets and on the surface of coagulum, as shown in cow milk yoghurt (C_{90}) stored for 120 h at refrigeration temperature.

Fig. 86. SEM micrograph showing no connection between small, individual rod of lactobacillus and surrounding flocculated casein matrix in buffalo milk yoghurt (B_{90}) stored for 120 h at 4–6C.


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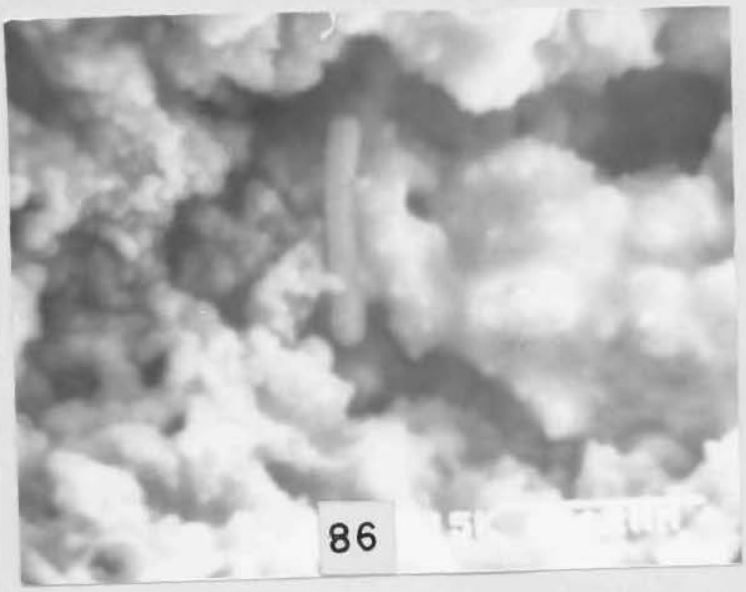
86

CHAPTER 5

DISCUSSION

5. DISCUSSION

Scanty informations are available in literature on the microstructure of Yoghurt prepared from fresh whole milk especially buffalo milk. Hence, Yoghurts were prepared from both cow and buffalo milks pre-heated at two different ranges of temperature , 70 and 90C for 30 minutes as holding time. These two temperatures are widely opted for treatment of milk in dairy industry before going for actual manufacture of the product. An attempt was made to determine the pH of gelation, acid content and viscosity of the finished product and to correlate these properties with microstructure of coagulum revealed by electron microscopy. The study was further extended to Yoghurts stored upto 120 h at low temperature.

5.1 pH MEASUREMENT OF MILK GELS DURING YOGHURT PREPARATION

The pH of milk gels from cow and buffalo whole milks pre-heated at 70C and 90C for the holding period of 30 minutes was measured till the completion of setting of coagulum.

It was apparent from the results that initiation as well as completion of gelation was earlier in cow and buffalo milks pre-heated at 90C than milks pre-heated at 70C. It was further observed that the pH values corresponding to curdling point and setting point of coagulums were lower in gels

from milk heated at 90C as compared to milk heated at 70C. From these results, it can be concluded that acid development was more in milks heated at 90C than those heated at 70C.

This difference in pH and acidity development in milk gels associated with different pre-heat treatments can be attributed to thermal decompsotion of lactose, dephosphorylation of casein and displacement of calcium-phosphate equilibrium among other factors. The involvement of all these factors in lowering of pH and more acid production in heated milk has been recognised earlier by Parry et al. (1974). The magnitude of impact of these factors may vary with the degree of severity. Under the light of these observations, variations in pH of gels from milks heated at 70 and 90C can be clearly understood.

Kalab et al. (1976) observed that skim milk heated at higher temperature (90C) without any holding time, gelled at higher pH. This variation can be explained on the basis of observation made by Fox and Morrissey (1977) that acidity development from lactose decomposition and dephosphorylation is significant only after prolonged heating of milk. Moreover, the role of fat present in whole milk used in the study can not be altogether ignored.

5.2 MEASUREMENT OF VISCOSITY AND JUDGEMENT OF YOGHURTS QUALITY

The viscosity of Yoghurt gives the fair idea of consistency and firmness of finished product and thus carries

significant impact on consumer value of it. The results obtained through viscosity measurement and judgement of quality showed that high heating of milk resulted in firm curds, free of syneresis from both cow and buffalo milks. Sample B_{90} was found two-times firmer than sample B_{70} and about 40-times than C_{70}. The off-flavour observed in cow milk yoghurt (C_{70}) can be associated with high whey content of the sample as has been reported in Yoghurt from milk fortified with whey protein by Modler et al. (1983). It is evident that buffalo milk yoghurt excels over cow milk yoghurt in terms of consistency and quality of coagulum (Table 2) and observations are obviously in favour of Yoghurt production from buffalo milk particularly in Asian countries. The superiority of heating of milk at 80-90C for the holding period of 30 minutes over pasteurization and UHT treatment was endorsed by Stadhouders and Hassing (1973). Labropoulos et al. (1981a) also observed that Yoghurt prepared from milk heated at 82C for 30 minutes was firmer than Yoghurt prepared from milk heated at 63C for 30 minutes and UHT treated milk. It has been established that whey protein denaturation and disulphide bonds formation between these protein and K-casein results in firm curd formation on heating of milk (Lyster, 1970; Parry et al., 1974 and Cremer et al., 1978). Labropoulos et al. (1981b) proposed that a specific change manifested in whey protein during heating of milk above 80C for 30 minutes contributes towards formation of firm structure.

This contention gets the support from the recommendations of different groups of workers for heating of cow and buffalo milks at 80–90C for 30 minutes to prepare Yoghurt (Storgards, 1964; Humphreys Plunkett, 1969; Rasic and Kurmann, 1978; Davies et al., 1978; Huhn et al., 1981; Dolezalk and Vokacova, 1981; Modler et al., 1983 and Modler and Kalab, 1983).

5.3 ACIDITY MEASUREMENT OF FRESH AND STORED YOGHURTS

The acidity of fresh and stored yoghurts was taken as an index of activity of starter culture and coagulum-stability at fresh stage and during storage. The acidity of various samples thus was estimated in terms of percent of lactic acid. The results suggested that during storage, acidity development in yoghurts from cow and buffalo milk pre-heated at 90C was negligible, whereas there was a substantial increase in acid content of samples from milks heated at 70C. After 120 h of storage, cow and buffalo milk yoghurts (C_{70} and B_{70}) showed an increase of 30.0 and 33.3 percent respectively in acid content over corresponding fresh samples.

It can be explained from the results obtained that yoghurts prepared from milk given high heat treatment especially B_{90} sample were found more stable in behaviour during the storage than yoghurts from milks pre-heated at 70C.

As far as the acceptability of the product is concerned, buffalo milk yoghurts comperatively have attained

the acidity more in the range acceptable commercially than cow milk yoghurts after storage of 120 h. Nevertheless, cow milk yoghurts had not become unpalatable outrightly after the same period of storage. Borrequio et al. (1981) and Modler et al. (1983) have considered the Yoghurt samples of good quality stored for one to two weeks at low temperature. However, in a tropical environment like India, it is hardly advisable to store the Yoghurt for longer period in view of severity of temperature fluctuation and economics involved with storage at low temperature.

5.4 STUDY OF MICROSTRUCTURE OF FRESH AND STORED YOGHURTS AS REVEALED BY ELECTRON MICROSCOPY

The associative action of yoghurt starter culture results into acidification of skim or whole milk and thus forming the typical yoghurt gel. In contrast to fluid milk, the microstructure of Yoghurt appears as a three-dimensional structure formed by fusion and aggregation of casein micelles into multiple chains. The similar microstructure of milk gels has been reported earlier by various workers (Kalab et al., 1973; Kalab and Emmons, 1975 and Kalab et al., 1976). The lactic acid bacteria and whey is found entrapped in the protein network. There are several factors influencing and further contributing to the microstructure of finished product. The factors taken into consideration in the present study include type of milk, pre-heat treatment of milk, pH of coagulation and finally storage at refrigeration temperature.

To understand the modus operandi of all these variables during development of microstructure, the electron microscopy has been employed to study the microstructure of yoghurts from cow and buffalo milks.

5.4.1 Fresh yoghurts

5.4.1.1 Casein microstructure in cow and buffalo milk yoghurts:

The microstructure of cow milk yoghurts was observed as a three-dimensional spongy network of clustered and aggregated casein micelles. The big interspaces were of common occurrence in sample C_{70}, while these were restricted in dimension by pockets formation in C_{90}. Kalab (1979) extensively studied the yoghurt microstructure and the pocket formation. The contention forwarded by him that pockets are formed as a result of bacterial action is untenable. Rather it appears more convincing on the basis of observations that fusion of micelles gives rise to pocket formation when bacteria are entrapped during wheying. In C_{70}, micelles were seen either free or in clusters, whereas a tendency of interlinking among micelles in addition was found existing in C_{90}. In comparison, the free micelles in C_{70} were bigger in size (average diameter 400 nm) than those of C_{90} (average diameter 350 nm).

It can be concluded from the results that aggregation of micelles was higher in cow milk yoghurt (C_{70})

than C_{90}. Carroll et al. (1971) postulated that size of casein micelles is influenced by several factors. This included the interaction of heat denatured whey proteins with micelles, deposition of insoluble serum protein on micelles and increase in calcium content leading to calcium bridging among micelles on heating of milk. Hence, it is logical to comprehend that big micelles formation in yoghurt gel is a temperature dependent phenomenon closely associated with whey protein denaturation. Whey protein is likely to be denaturated at both the pre-heat temperatures used in the present study, the difference anticipated only in the degree of denaturation. further, denaturation of whey protein is expected to be somewhat low at 70C. Moreover, denaturation to extent of 99 percent at 80C for 30 minutes has been experimentally proved (Humphreys and Plunkett, 1969). Creamer et al. (1978) and Labropoulos et al. (1981a,b) asserted that formation of a heat induced complex between micellar and non-micellar protein accounts for the presence of small micelles at higher temperature treatment. The wider interspaces observed in the C_{70} may be due to the casein aggregation leading to fewer chains and thus forming a more open structure (Kalab et al., 1976). The resultant soft coagulum from this type of microstructure is expected to be virtually vulnerable towards syneresis. The comparable situation is found to exist in sample C_{70}.

The unfractured coagulum of C_{70} was frequently seen covered with loose aggregates of tiny globular structures.

These structures were not found in fractured coagulum of the same sample and were altogether missing in both fractured and unfractured coagulum of C_{90} sample. More experimental evidences are therefore needed before any conclusion is drawn about the nature and origin of these globular aggregates.

The informations obtained from ultrathin sections revealed that casein micelles were loosely arranged into big aggregates of irregular shapes surrounded by fine thread like structures. Similar structures were observed in heated skim milk by Creamer et al. (1978), and Kalab et al. (1976). Davies et al. (1978) visualised these structures as prominent appendages present on the surface of casein micelles in yoghurt gel prepared from milk heated at 90C. All these workers were of the view that these thread like structures are in fact, the heat induced complex formed by the interaction of β-lactoglobulin and K-casein.

These whey protein structures appeared as flocculated protein in the form of long strands between casein micelles and thus separating the micellar aggregates as individual entities in sample C_{70}. Much comparable microstructure has been observed by Modler and Kalab (1983) in yoghurt from skim milk fortified with whey protein. It will be relevant to point out that yoghurt sample C_{70} has been found to be the weakest coagulum having maximum whey content on the basis of viscosity values and visual observation.

In case of sample C_{90}, rather compact, small and spherical micelles were seen surrounded by short hair like projections. There appeared a distinct linearity among these micelles but connecting strands were found missing between some micelles at certain points. Various models suggested by different workers depicting the sectioning through three-dimensional network of milk gels can easily explain this discontinuity among micelles in a chain (Kalab and Harwalkar, 1974; Kalab *et al.*, 1976). The long strands of flocculated protein were less prevalent in the sample but, free aggregates of these structures were frequently observed. The microstructure is similar to that of yoghurt prepared from skim milk pre-heated at 88C for 30 minutes and fortified with casein in form of caseinate by Modler and Kalab (1983).

Studies on microstructure of Yoghurt using buffalo milk has been taken for the first time. Microstructure of buffalo milk yoghurts was found moderately porous in sample B_{70} highly porous in sample B_{90}. Pockets were shallow in former sample whereas deep and regular pocket formation was observed in latter sample. Casein micelles were also found free, inter-linked and aggregated into clusters in sample B_{70} and measured about 300 nm in diameter. The micelles were observed extended into tubular structures and chains formed by joining of these structures into a highly organised network of protein matrix in B_{90}.

A close observation of SEM micrographs of B_{70} and B_{90} samples revealed that microstructure of these samples varied considerably. In buffalo milk yoghurt (B_{70}), loose aggregates of globular structures of very small dimensions as compared to casein micelles in term of size, were seen sandowitched between casein micelles and aggregates (Fig.15). It may be noteworthy to mention that similar structures has also been noted in cow milk yoghurt (C_{70}). On the other hand, in B_{90} sample, there existed no such aggregates and casein micelles were seen linked together through tubular structures. In addition, flocculated free cotton like structures could be spotted on the surface of coagulum in B_{90} (Figs. 18,20).

On the basis of these observations, it can be proposed that loose aggregates and tubular structures reported in B_{70} and B_{90} respectively are truly the structural modifications of specific milk constituent manifested by pre-heat temperature variation. Modler and Kalab (1983) reported the flocculated whey protein material connecting the casein micelles in yoghurt prepared from skim milk fortified with whey protein and pre-heated at 88C for 30 minutes. It is very likely that denatured whey protein may attain the form of minutes globular structure in both cow and buffalo milks pre-heated at 70C, whereas at 90C, the denatured whey protein probably is flocculated into bridging material between casein micelles and remains as free floccules at some places. The cotton like aggregates observed in the B_{90} appear to be these free floccules.

Modler and Kalab (1983) also anticipated the presence of these free flocculas in microstructure and expressed the possibility of being washed out during preparation of samples for SEM.

At ultrastructural level, irregular micelles appeared as more electron dense separate entities interspaced by less electron dense thread like structures of whey protein in sample B_{70} (Fig. 16). Flocculated protein material was seen mostly free in the matrix. In sample B_{90}, micelles appeared compact, electron dense and spherical in shape and were arranged in linear chains. The micelles were found to join in to chains through short hair like projections as observed by Kalab et al. (1976) in Yoghurt from milk heated at higher temperature. It can, therefore, be assumed that whey protein behaves differently in terms of its association with casein micelles depending on the distribution of whey protein in the microstructure and pre-heat temperature. Flocculated protein material in the close proximity of micelles may be involved in the formation of hair like projections thus extended to other micelles. In contrast, whey protein placed far apart from micelles exists as free long strands coiled into aggregates.

5.4.1.2 Distribution pattern and structure of lactic acid bacteria in Yoghurt coagulum:

Fermentation of milk into Yoghurt is carried out by mixed culture of S. thermophilus and L. bulgaricus.

As such, presence of any microorganisms other than yoghurt culture in coagulum will be considered as contaminant. Distribution pattern and structure of these lactic acid bacteria in coagulum are likely to exert their influence on the quality of product. Generally, yeast and mold have been found as contaminants during storage of Yoghurt (Burreguio et al., 1981).

Lactic acid bacteria in the present study were seen distributed throughout the coagulum either on the surface or in pockets as isolated long chains. However, pockets in protein matrix seemed to provide more suitable environment for their multiplication. Streptococci were spotted either in small chains, sometimes diplococci or in long chains established into microcolonies but lactobacilli in chains were not prevalent in the fresh stage. The presence of bacterial chains of unusual length indicates that milk supports the growth of lactic acid bacteria to a greater extent as a natural medium comparable to other nutritional media. Numerically, streptococci seemed to outnumber the lactobacilli in distribution at this stage.

Regarding morphology of lactic acid bacteria in coagulum, these bacteria were interestingly found in two different situations. Some cells were found smooth, while other existed with ocovered surface in the coagulum. The smooth cells of streptococci measured 1.0 μ in diameter, whereas cell with covered surface were found 1.2μin diameter.

For smooth and covered lactobacillus cell, the diameter recorded were 0.72 μ and 0.8 μ respectively. Ruegg et al. (1981) reported the dimensions of streptococci and lactobacilli as 0.94 and 0.78μrespectively in Gruyere cheese.

The observation of lactic acid bacteria with covered surface was the unusual feature of present study. The bacterial surface was seen covered with fine deposition of protein like structures. The extent of deposition on the surface seemed to be higher in streptococci and amounted to about 20 percent increase in diameter of cell. In case of lactobacilli, the deposition was observed comperatively of lower magnitude to the extent of about 10 percent. From these observations, it may be possible to conclude that surface of streptococcal cells is more susceptible to the typical deposition as compared to lactobacillus cells. These may be explained on the basis of having either specific affinity between bacterial surface and protein like material or involvement of surface structure favourable to such deposition. Experimental evidences show that smooth surface of lactic streptococci when grown in nutritional broth appears undulated when observed at high magnification. Bladen and Stephan (1964) also observed similar convoluted and uneven surface structure in case Velionella spp. Hence, there is a possibility that surface structure of streptococci is associated with phenomenon of deposition.

fibres in yoghurt microstructure only when certain stabilizer and 'ropy' starter cultures were used. In the present study this possibility is totally ruled out, because neither any stabilizer nor any ropy culture has been incorporated.

5.4.1.3 Inclusion bodies:

Inclusion bodies refers to the structure of unusual shapes observed in the microstructure of coagulum. These structures results from modification of milk constituents accompanied by any change in the environment of microstructure during gel development. The composition of milk, manufacturing process and post-manufacturing operations play important role in the formation of inclusion bodies.

In addition to regular structural components of microstructure, smooth crystals and sheet like structures were observed in the coagulum of yoghurts from cow and buffalo milks (Figs. 14,20). The smooth crystallin structures probably are lactose present in coagulum. Kalab (1979) has reported similar structures of lactose in heat coagulated whey curd. It is well known that the growth of crystal decide the size and shape of lactose, depend upon the humidity environment and thus is the index of water content of microstructure. Sheet like structures observed only in the unfractured coagulum. were not of regular structures and resulted perhaps due to compaction of casein into sheets. The role played by drying methods employed for the sample preperation for SEM in the formation of these protein structures can be considered.

However, the presence and structure of these typical bodies could not be ascertained at ultrastructural level.

5.4.2 Stored yoghurts

5.4.2.1 Yoghurts stored for 24 h at 4-6C:

Except for few alterations, the microstructure of Yoghurts stored for 24 h at refrigeration temperature was observed similar to that of fresh samples. It was comparatively more open in terms of spatial configuration. Micelles were seen highly aggregated and thus leaving interspace wider than before as shown in Figure 24. Free micelles prevalent in fresh samples, became rare structures at this stage. However, yoghurt sample B_{90} was found to preserve the structure to the best possible extent (Figs.32-34). The aggregation of micelles may be associated with lowering of pH due to increase in acidity. There was no conspicuous change in the microstructure at ultrastructural level too. Domination of whey protein floccules was observed in low heated milk yoghurts (Figs.25, 31) while linearity and interlinking among compact casein micelles through short hair like structures were clearly visible in high heated milk yoghurts (Fig. 35-37).

Clean surfaced lactic acid bacteria were observed in addition to bacteria with covered surface. It shows that lactic acid bacteria are still actively growing at this stage of storage and clean surfaced bacteria represent the newly divided cells. The deposition of protein like material it seems, is

restricted to old cells wherever present in the coagulum. Occasionally, an increase in deposition was observed on the surface as supported by higher dimension of these bacteria (1500-1800 nm in diameter).

5.4.2.2 Yoghurts stored for 72 h at 4-6C:

There was no unusual change observed in the microstructure of yoghurts stored for 3 days at low temperature. A progressive increase in micellar aggregation accompanied by collapsing of pockets into wide interspaces was the common feature of all the samples except B_{90}. Streptococcal cells showed heavy deposition of proteinous material even upto 80 percent at some places. Inclusion bodies as flakes and/or sheets were observed as regular structures of the matrix (Figs 39, 43,39).

5.4.2.3 Yoghurts stored for 120 h at 4-6C:

The microstructure of yoghurts after storage of 120 h at refrigeration temperature continued to exhibit similar structures as observed earlier. Out of all the samples studied aggregation among casein micelles was maximum at this stage, virtually resulted in wide interspaces forming a more open structure. Under these circumstances, the coagulum of yoghurts is vulnerable towards syneresis. Contrary to these observations, yoghurt from buffalo milk pre-heated at 90C seemed to behave stable during the storage. A highly porous microstructure characterized by numerous pockets, the chains of micelles and lactic acid bacteria could be observed at this stage in various SEM micrographs (Fig.71-73). The stability

in structure of B_{90}, could be attributed to the stability in acidity of samples during storage. On the basis of qualitative observations, it was possible to deduce that casein micelles had undergone reduction in size slightly at this stage of storage. Kalab et al. (1976) observed the similar shrinkage effect among casein micelles in yoghurt prepared from fresh and dried milks after the storage of one week.

So far as the microbial distribution is concerned, both clean and covered surfaced lactic acid bacteria coexisted in the coagulum. The presence of small rod with clean surface affirmed the notion of growth of starter bacteria even at the late stage of storage. Streptococci and lactobacilli appeared to be distributed in the ratio of nearly 1:1 throughout the coagulum. Both TEM and SEM observations confirmed the microcolony formation by these bacteria particularly in B_{90} sample. The microcolony formation by smooth streptococcal cells can be easily evidenced in TEM micrograph (Fig.76).

5.5 APPLICATION OF SEM TECHNIQUES IN YOGHURTS

5.5.1 Double fixation VERSUS single fixation of Yoghurt

In most of the electron microscopic studies of Yoghurt, coagulum fragmented into small pieces has been fixed doubly in glutaraldehyde and osmic acid for SEM (Kalab, 1975; Kalab et al., 1976; Kalab, 1978; Davies et al., 1978; Modler and Kalab, 1983). Following the same technique, the coagulum was fixed doubly in the present study too. However, an attempt was made to evaluate the single fixation of the product in

buffered glutaraldehyde for its suitability towards SEM study of Yoghurt. Yoghurts stored for 24 h at 4-6C were used for this purpose.

Surprisingly, the single fixation in glutaraldehyde was found very efficient in preserving the microstructure of yoghurts from cow and buffalo milks. Figures 71-80 show the microstructure of yoghurt as revealed by single fixation of samples followed by air drying. The results obtained are comparable to those of doubly fixed samples. At some places, single fixation was found to excel over double fixation. For instance, surface of casein micelles and distribution of pockets were more clearly visible in this case. In the light of these findings, single fixation in 2.5 percent buffered glutaraldehyde for 3 h at low temperature can be considered as a less expensive, quick and equally efficient method for fixation of Yoghurt for SEM study.

5.5.2 Air-drying VERSUS Freeze-drying of Yoghurt

Preparation of Yoghurt for SEM like any other biological specimen consists of fixation, dehydration and coating with conductive materials like gold, palladium, platinum, and their alloys. The process of dehydration is considered an important step as it contributes to the final results in an effective way. Besides, air-drying, freeze-drying and critical point drying are the methods employed for the dehydration of dairy products (Kaleb, 1979, 1981).

Freeze-drying was employed as an additional method for drying yoghurt sample in the present study. Fresh and stored yoghurts fixed doubly in glutaraldehyde and osmic acid prior to freeze-drying, presented in general, a flocculated microstructure. Casein micelles were found highly aggregated or stretched into sheet like continuous structures in fresh and stored samples. Pockets were seen masked or deformed in this type of microstructure. In certain cases, hole formation was observed, the reason is not clearly understood from the available evidence. However, these may be artifacts formed during freeze-drying.

Microflora of Yoghurt on the other hand could be seen efficiently preserved in freeze dried samples. Microcolonies of lactic acid bacteria were depicted very well in the SEM micrographs of these samples (Fig.85). The surface of these bacteria both covered and clean could be visualised as shown in figures 84 and 86.

From these observations, it could be visualised that freeze-drying is not a suitable method of dehydration for SEM study particularly for yoghurt prepared from whole milk. In this study, the details are observed so far as, protein microstructure is considered. It is quite probable that fat present in the whole milk interferes during drying thereby masking the casein structure. Similar observation was also made by Kalab (1978) in case of Cheddar cheese where fat was retained in the curd. Lactic acid bacteria, it seems remain unaffected in the presence of fat. Hence, there are reasons to believe that

freeze-drying is not fully suitable for whole milk yoghurts, while air-drying has been proved an economic, less time consuming and highly efficient dehydration method for SEM. Kalab (1977 & 1978) has also found air-drying a reliable technique of dehydration for SEM studies of Cheese and Yoghurt.

The present investigation was confined to Yoghurts from fresh whole milks of cow and buffalo origins for observation of internal structure. Most of the findings were restricted to SEM study. Attempt was made to corroborate the SEM observations by TEM study but poor accessibility to sophisticated Transmission Electron Microscope proved a limitation. However, it is encouraging to admit that this is the first ever systematic electron microscopic study of a dairy product carried out in India. A relationship between microstructure and manufacturing conditions of Yoghurt including type of milk has successfully been worked out. The informations are comperatively of more significence in context of buffalo milk yoghurt. The investigation can be extended to yoghurt prepared from different formulations using stabilizers of indigenous origin for quality improvement. In addition, various problems associated with the body of coagulum encountered in dairy industry can be overcome. From technique point of view, electron microscopy can also be employed to visualise the microstructure of other fermented and non-fermented products being manufactured in our country.

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CHAPTER 6

SUMMARY

6. SUMMARY

The main features of the investigation comprised the preparation of Yoghurts from fresh cow and buffalo whole milks pre-heated at different temperatures for 30 minutes and subsequently study of microstructure of fresh and stored yoghurts. Attempts were made to see the relationship between microstructure and quality of yoghurt coagulum. Salient findings are highlighted as follows:

6.1 Yoghurts prepared from fresh, unfortified cow and buffalo whole milks pre-heated at 70C and 90C for 30 minutes were of acceptable quality.

6.2 pH measurement during yoghurt preparation showed that initiation as well as completion of gelation was earlier in cow and buffalo milks pre-heated at 90C than milks pre-heated at 70C. The pH values corresponding to curdling point and setting points of coagulums were lower in gels from milk heated at 90C than those heated at 70C.

6.3 On the basis of judgement of yoghurt quality and viscosity measurement, yoghurts were graded in descending order as follows: Yoghurt from buffalo milk pre-heated at 90C(B_{90}), yoghurt from buffalo milk pre-heated at 70C (B_{70}), yoghurt from cow milk pre-heated at 90C (C_{90}) and yoghurt from cow milk pre-heated at 70C (C_{70}).

6.4 During storage at refrigeration temperature, the percent increase in acidity content of different samples after 5 days of storage over fresh B_{70}, C_{70}, B_{90} and C_{90} was 33.3, 30.0, 9.4 and 6.0 respectively. Yoghurts from high heated milk were found consistent in behaviour during storage.

6.5 In general, the microstructure of yoghurt as revealed by SEM appeared as a typical 3-dimensional network of protein matrix. Casein micelles were seen fused to form chains and subsequently, the interconnection and cross linking of these micelle chains were found to result in spongy network of protein matrix. Lactic acid bacteria and inclusion bodies were observed entrapped in this system.

6.6 In Yoghurt sample C_{70}, casein micelles were observed in form of spherical and cylindrical shapes measuring 360-900 nm in diameter (average diameter, 400 nm). Micelles were seen mostly fused in to loose aggregates as illustrated by TEM studies. These aggregates were interlinked by less electron dense thread like structures of denatured whey protein. Free floccules of coagulated whey protein were also spotted frequently in this sample.

6.7 The microstructure of C_{90} sample appeared porous, characterized by large and deep pockets. Casein

micelles were both aggregated and interlinked as well into chains and were of comperatively smaller in size with average diameter, 350 nm. At ultra-structural level, compact clusters of casein micelles interlinked and surrounded by short hair like structures were observed but floccules of denaturated whey protein were not prevalent in this sample of cow milk yoghurt.

6.8 The coagulum of B_{70} was presented moderately porous and micelles were found, isolated, interlinked and aggregated. Size of micelles showed considerable variation (250-1350 nm); the average size micelle measured 300 nm in diameter. Loose aggregates of micelles were seen surrounded and interlinked by long fibrils of denaturated whey protein as shown in TEM micrographs. Besides, free aggregates of denatured whey protein were also present in the background.

6.9 Buffalo milk yoghurt 'B_{90}' was characterized by the highly porous microstructure. The deep pockets were found distributed throughout the coagulum. The micelles were seen regularly interconnected through tubular structures into long chains. These chains formed the lattice for further cross linking and thus resulted into a well organised network of protein matrix as revealed by SEM. TEM analysis showed that the micelles were rather compact and highly electron

dense. Short hair like projections of heat induced complex were present on the surface of micelles. Micelles were found interlinked through these structures. Free floccules of denatured whey protein were rare in this case.

6.10 In addition to these regular structures, lactose crystals and inclusion bodies resulting from the modification of casein were observed in different yoghurt gels. However, their presence could not be ascertained at ultrastructural level.

6.11 Lactic acid bacteria were seen distributed throughout the coagulums of different yoghurts both in micro-colonies and isolated. Morphologically clean surface bacteria and cells with covered surfece were spotted in the microstructure.

6.12 Clean surface streptococci and lactobacilli measured 1.0 μ and 0.7 μ in diameter.

6.13 Higher dimensions were recorded for bacteria with covered surface. Streptococci showed 20–80 percent and lactobacilli 10 percent increase in size in terms of diameter.

6.14 The covered surface bacteria were presumed to be old cells with external deposition of denatured whey protein like material.

6.15 Except minor alterations, there was no fundamental difference observed in the microstructure of yoghurts during storage upto 120 h.

6.16 Aggregation of casein micelles found to increase with progress of storage period. On the contrary, buffalo milk yoghurt B_{90} was able to maintain the microstructure even after storage of 5 days.

6.17 Single fixation of yoghurt coagulum in 2.5 percent buffered glutaraldehyde for 2-3 h reproduced the results comparable to those of double fixation in glutaraldehyde (2.5 percent) and osmic acid (2.0 percent).

6.18 For whole milk yoghurts, air drying was found more suitable than freeze -dyring for the purpose of dehydration during sample preparation for scanning electron microscopy.

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CHAPTER 7

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7. BIBLIOGRAPHY

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ANNEXURE I

SPECIFICATIONS OF HITACHI S-405 A SCANNING ELECTRON MICROSCOPE

Resolution	70 Å	
Magnification range	30 150,000x	
Electron optical system	Filement :	Pre-centered hairpin type filament
	Accelerating voltage:	5, 15, 25 KV
	Lens system:	3-lens system
	Objective lens aperture:	Moveble aperture (0.1, 0.2 mm dia. openings)
Specimen stage	X-traverse .....0 20 mm (continuous)	
	Y-traverse .... 0 10 mm (continuous)	
	Tilting angle .. -20 +70° (continuous)	
	Rotation angle .. 360° (continuous)	
	Z-traverse	5, 15 mm (semi-fixed); working distance (W.D.)
	Specimen size ..	60 mm dia. x 1 mm H(max.) 15 mm dia. x10 mm H(max.)
Display unit	CRT ... (afterglow type 150 x 135 mm) x 1	
	Scanning speed (Raster scan):	
	for viewing ...	0.08 sec (rapid scan) 0.5 sec 10 (30) sec
	for photo-graphing: ...	50 (150) sec Parenthesized figures indicate the scan speed with SIGNAL SELECTOR (X-RAY) depressed.

Contd....

....Contd...

Scanning mode	Slow scan
	Photo scan
	Reduced area rapid scan
Image mode	*Focus monitor*
	Stigmator monitor
	Dynamic focus
Evacuating system	Type ..... Automatic valve control
	Vacuum gauge .. Pirani gauge
	Ultimate vacuum . 5×10^{-6} Torr
	Vacuum pump ....DP 400 l/sec x 1,
	RP 160 l/ *min* x 1
	Evacuating time . About 2 min

ANNEXURE-II

NANOMETER/MICRON MARKER SCALE FOR HITACHI S-405 SEM

Magnification	Marker position	Microns (u)
X 100	30 x 4 NM	300
X 150	20 x 4 NM	200
X 200	15 x 4 NM	150
X 300	10 x 4 NM	100
X 400	75 x 3 NM	75
X 500	60 x 3 NM	60
X 1,000	30 x 3 NM	30
X 1,500	20 x 3 NM	20
X 2,000	15 x 3 NM	15
X 3,000	10 x 3 NM	10
X 4,000	75 x 2 NM	7.5
X 5,000	60 x 2 NM	6.0
X 6,000	50 x 2 NM	5.0
X 7,500	40 x 2 NM	4.0
X 10,000	30 x 2 NM	3.0
X 15,000	20 x 2 NM	2.0
X 20,000	15 x 2 NM	1.5
X 30,000	10 x 2 NM	1.0
X 40,000	75 x 1 NM	0.75
X 50,000	60 x 1 NM	0.60