MICROBIOLOGY OF CHEDDAR CHEESE MANUFACTURED BY USING MOLD RENNET (*Mucor pusillus*)

A DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
Master of Science
IN
DAIRYING
(DAIRY MICROBIOLOGY)
TO THE KURUKSHETRA UNIVERSITY
KURUKSHETRA

By
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KARNAL (Haryana) INDIA

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Registration No. 79-dk-61

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TO MY

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May 20 , 1982

I certify that the work reported in this Thesis entitled "MICROBIOLOGY OF CHEDDAR CHEESE MANUFACTURED BY USING MOLD RENNET (Mucor pusillus)" was carried out by Mr. R. Mallikarjuna Reddy under my guidance and direct supervision at this Institute, in partial fulfilment for the Degree of Master of Science in Dairying (Dairy Microbiology) of Kurukshetra University, Kurukshetra.

(S. NEELAKANTAN)

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R M Reddy
(R. MALLIKARJUNA REDDY)

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CHAPTER I

INTRODUCTION

INTRODUCTION

Cheese is a concentrated fermented dairy product. The quality of the cheese will mainly depend upon the starter culture, the type of precipitation by rennin action and the type of ripening. In fresh Cheddar cheese, about 3.2% fat of the milk is raised to 32% fat and about 2.3% protein to 23% and the water soluble compounds leak out with the whey. As a result lactose, soluble protein and water soluble salts are found in very low proportions than in original milk.

The fermentation pattern in Cheddar cheese is initiated with the inoculated starter bacteria and rennin coagulated milk curd. The development of lactic acid from lactose by streptococci starts early in the curd during cheddaring. During cheddaring the curd blocks are piled and repiled over a prolonged period and the gradual increase in the lactic acid production strip the calcium from the relatively inert dicalcium para-casein and transform into mono-calcium paracasein and free paracasein giving plastic properties to the resulting cheese. At salting, the peak acidity is reached and the lactic acid fermentation is mostly stopped. The resultant curd has sufficient substrate for enzyme action leading to proper flavour and texture development during ripening for 4 to 12 months at 2.2°C to 15.6°C at 85% relative humidity.

The quality of milk and skim milk, bacterial starter inoculum, the type of rennin, the standardized cheddaring time-table, milling, salting, and pressing influence the initial coagulation, acid development and texture of the fresh cheese and the hardening, waxing and the period, temperature and humidity during ripening influence the microbial and enzymic changes of the resultant Cheddar cheese its composition and quality.

The milk clotting enzyme, rennet is almost universally employed for the production of different varieties of cheeses, although pepsin and a commercial animal rennet under the trade name of 'Metroclot' have also been employed. The animal rennet is derived from the abomasum of young suckling calves, which are sacrificed for the extraction of the enzyme. Attempts have also been made to find a suitable substitute for animal rennet. Some of the factors that have prompted investigations on this subject are the non-acceptability of cheese made with animal rennet to the vegetarian populations in certain countries like India and Isreal, its accute shortage in traditional rennet- producing countries, and nonavailability of material at competitive prices. Hence, there is considerable scope for the development of a suitable rennet substitute of non-animal origin (plants or microorganisms). The chief plant materials which have been investigated for this purpose include Ficus carica, Carica papaya, Withania coagulans, pumpkin etc. Microorganisms that have been

investigated for this purpose includes : i) Bacteria : Bacillus subtilis K-26, K-12, etc., ii) Mold : Absidia ramosa, Endothia parasitica, Mucor pusillus Lindt, Mucor miehei, Rhizopus oligosporus, etc. A few commercial preparations derived from microbial sources are now available to a limited extent for use in the production of cheese. Although these preparations cannot be claimed to be entirely satisfactory, they can, to a considerable extent replace the calf rennet.

In the initial stages of manufacture of all kinds of cheeses, lactic acid formation by starter bacteria is essential. The type of organisms used in the starter is determined mainly by the heat treatment given to the curd during manufacture. If the curd is heated (cooked) only upto 38°C or slightly higher, as in Cheddar cheese, S. lactis or S. cremoris is used. The culture may consist of single strain, but more often it contains several strains of one or the other species. The main function of a starter is to produce acid at a steady rate from the moment it is added to cheese milk, continuously through the entire cheese making process and a short time afterwards until the accumulated acid, lactate ion and salt prevent further growth of starter organisms.

The number and types of microorganisms vary to some extent at different stages of cheese manufacture. The lactic acid bacteria grow well upto the beginning of ripening and are observed in billions/g

cheese in the initial stages of ripening. Although rapid multiplication occurs in the press, the highest bacterial count is observed initially during 2 to 5 days of ripening. In Cheddar cheese the predominant flora is Streptococcus lactis forming about 99% of the total. This decreases after 5 days. The growth of lactobacilli in Cheddar cheese mostly occur after complete utilisation of lactose. However, both streptococci and lactobacilli have significant role to play during ripening. The decrease in population of microorganisms during ripening is due to autolysis of cells. This autolysis helps in the release of intracellular enzymes associated with proteolytic, lipolytic and body textural changes in cheese.

Proteolysis in cheese is catalysed by proteolytic enzymes present in cheese and results in breakdown of casein into smaller peptides, amino acids and ammonia. Proteolytic enzymes include extracellular and intracellular or membrane bound proteases from the starter bacteria, and other bacterial enzymes, milk, coagulating agent, yeast and molds. Proteolysis in cheese influences flavour since free amino acids have typical flavour characteristics and contributes probably to the cheese flavour. Proteolysis can also cause certain defects especially bitterness.

Hydrolysis of fat occurs due to the action of lipases from lactic acid bacteria and microorganisms present in cheese.

Due to its action considerable amount of free fatty acids accumulates in cheese which imparts a characteristic flavour. Excessive hydrolysis of fat results in the development of rancid flavour in the cheese.

Lactic acid bacteria produce considerable amounts of flavour components such as carbonyl compounds, sulphur compounds, nitrogenous compounds, etc., which imparts a good flavour and aroma to the cheese. The chief flavour compounds in the cheese include acetoin, diacetyl, acetaldehyde, methyl-ethyl ketone, hydrogen sulphide, ammonia and free amino acids. For proper flavour and aroma cheese starter should contain flavour producing bacteria such as Streptococcus diacetylactis, Leuconostoc etc.

Since it has been reported that milk - clotting enzyme from Mucor pusillus Lindt is very much close to that of traditional calf rennet, an attempt has been made to prepare Cheddar cheese in 110 litres experimental cheese vat with Mucor rennet and LF culture.

The present study includes the following aspects :

i) Activity of LF- culture,

ii) Milk-clotting and proteolytic activities of mold rennet (Mucor pusillus Lindt) ;

iii) Changes in acidity, pH and lactic acid bacterial count during cheddaring.

iv) Microbiological changes during cheese ripening : Non-lactic, lactic acid bacteria, yeast and mold, proteolytic and lipolytic bacterial counts.

v) Chemical changes during cheese ripening : pH, titratable acidity, moisture, fat, protein, non-protein nitrogen, ammonia nitrogen, volatile fatty acids, diacetyl, proteolytic and lipolytic activities.

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CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

HISTORICAL :

The milk of domestic mammals has formed an important part of the food of human beings since prehistoric times. Since the milk would sour rapidly in warm climates, crude butter and cheese would have been the earliest dairy products. It was early recognised that cheese making is a convenient method of converting milk constituents into a product that keeps well, is less bulky, of higher nutritive value and both palatable and readily digestible.

Cheese was included in the offerings of the ancient Greeks and cheese making was a well established craft. The earliest efforts at cheese making was probably made by the Aryans in Central Asia. During the height of the Roman Empire, dairying and cheese making became important industries. From the time of the Roman Empire until about 1600, little advance was made in cheese making as an industry. Until about 1850, practically all cheese making appears to have been made only by individuals and the cooperative cheese making became based on the Cheddar method due to its easy making, transportability and keeping quality (Davis, 1963).

The period of 1860 - 1880 saw the introduction of the factory system for cheese making. Inspite of advances in cheese making from 1600 onwards there appear to have been no significant advance in cheese

technology until 1870 when a commercial rennet was prepared by Hansen in Denmark.

About 1900, five outstanding developments in cheese technology took place in Britain, U.S.A. and Denmark. These were : i) The use of titratable acidity measurements to control acidities in cheese making ii) Introduction of pure starter cultures of lactic streptococci iii) The pasteurization of cheese milk to destroy harmful microorganisms iv) Refrigerated ripening of cheese and v) Development of processed cheese.

Little advance was made from 1900 to 1945 with the exception of the discovery of bacteriophage in New Zealand in 1930's as the cause of starter failures. During the past three decades great advances have been made and are being made in mechanisation of cheese making processes and new methods of packaging in different countries (Davis, 1976).

The increase in milk production available for cheese making and the concomitant decrease in the availability of calf rennet has revived interest in other milk coagulating enzymes (Green, 1977). The suitability of rennet substitutes of vegetable and microbial origin for cheese manufacture has been tried (Dewane, 1960 ; Veringa, 1961 ; Babbar et al., 1965 ; Sardinas, 1969 ; Scott, 1973 ; Ryu ,1974; Martens and Naudts, 1976 ; Srinivasan, 1978 and Christensen,

1979). The use of suitable lactic starter cultures is essential for the development of body, texture and flavour characteristics of Cheddar cheese. The starter culture characteristics and action in cheese manufacture have been studied (Marth, 1963; Sharpe, 1979; Chapman and Sharpe, 1981). Microbiological and chemical changes brought about during ripening of Cheddar cheese are of great significance for the organoleptic acceptance by the consumers (Marth, 1963; Reiter and Sharpe, 1971, Sharpe, 1972; Wong 1978; Chapman and Sharpe, 1981). The synthesis of flavour compounds in Cheddar cheese has also been reported (Hiller and Jago, 1979 ; Law, 1981).

MILK CLOTTING ENZYMES :

The digestive enzyme, renin, derived from the stomach of suckling calves, lambs and goats has been used for many centuries to coagulate milk for cheese making. Besides, attempts are being made to prepare several milk clotting enzymes (rennet) of vegetable and microbial origin for the production of satisfactory cheese.

Rennet is a very powerful milk coagulant and its effect in cheese making takes place in three phases viz., i) an enzymic, destabilising phase, where the protective colloidal nature of K-casein is destroyed and para-K-casein is formed within the casein micells ; the change can proceed at low temperatures and is the basis of continuous methods of curd-making (Barridge, 1972); ii) a non-enzymic coagulating phase, which can proceed only at high temperature ; and

iii) a mainly proteolytic phase which takes place around pH 5.2 - 5.8 ; and includes the breakdown of the milk proteins to peptides. This last phase is essential for cheese ripening, and the peptides formed are utilized and further degraded by the starter cultures (Law and Sharpe, 1978).

Clotting and proteolytic activities : The important factor determining the suitability of a coagulant is the ratio of clotting and proteolytic activities. Most substitutes are more proteolytic than rennet relative to their clotting activity (Martens and Naudts, 1973). If the proteolytic activity is excessive, the yield of cheese and retention of fat by the curd may be diminished (Veringa, 1961; Ritter, 1970). Pig pepsin is exceptional in being less proteolytic than rennet during ripening (Green & Foster, 1974) but this also results in textural defects and slow flavour development (Maragoudakis et al., 1961 Melachouris and Tuckey, 1964). However, imbalance of clotting and proteolytic activities can some times be corrected by slight alteration of the manufacturing conditions. Clotting may be aided by the addition of $CaCl_2$ or the use of milk at a lower pH value, and it may be possible to manipulate factors affecting ripening by appropriate modifications in temperature and other technological factors during cheese making or ripening.

Contaminating Enzymes : Certain coagulants contain enzymes other than proteinases that may affect cheese-making or ripening

of activity, substrate specificity and mode of action. Although several microbial milk clotting preparations have been tried as rennet substitutes, only few of them are produced commercially. The crude preparations exhibiting milk clotting activities are invariably accompanied by proteolytic enzymes.

Almost all commercial rennet preparation have been claimed to give cheese of comparable quality. However, the yield of cheese has been reported to be slightly lower than the conventional cheese made using animal rennet. Protein losses in whey and increase in soluble nitrogen content during manufacture and ripening have been reported with many cheese preparations. (Kikuchi et al., 1968 a, b; Edeleten et al., 1969; Huig, 1969 Kikuchi and Toyoda, 1969; Maubois and Mocquot, 1969, Mickelman and Fish, 1970; Sannabhadti et al., 1970; Thomson et al., 1970; Apola et al., 1973; Brinkman and Dhiven, 1974; Carbone and Emalon, 1974; Morvai - Baoz, 1974; Reps et al., 1974 a, b; Stavlund and Kiermeier, 1974; Reps et al., 1975).

Kikuchi and Toyoda (1970) prepared Cheddar, Edam and Gouda cheeses using microbial rennets from Bacillus polymyxa (BR) and Mucor pusillus Lindt (MR) and found that the curd coagulant by MR and BR was a softer and more brittle at cutting than that of calf rennet (CR). However, after cooking no significant differences were observed between the curds. They further found that whey in

MR and BR vats was slightly turbid in each trial and cheese yields were lower than that from calf rennet.

A bitter taste was frequently found in cheeses made with crystalline enzymes. There was no difference in maturity index between MR and CR cheeses of the Cheddar type. It was found that the bitter taste in cheeses made with microbial rennets was not due to action of contaminant proteases, but was an inherent characteristic of those enzymes.

It has been reported that cheeses made with Mucor pusillus Lindt enzyme are virtually identical in all respects to those made with calf rennet. (Kyla - Siurola and Antila, 1970; Carbone et al., 1974; Ryu; 1974 Wigley, 1974; Kawano and Arima, 1975 ; Pien, 1976).

Reps et al., (1979) observed that milk clotting enzymes (Fromase, Rennilase and Maryne) from M. miehei had more deisrable milk-clotting properties than the preparations from Mucor pusillus and Endothia parasitica. It was also found that cheeses prepared with Mucor miehei enzymes had a comparable good quality with that of cheese with calf rennet (Wigley, 1974; Carimi, 1977; Birkkjaer and Thomsen, 1978; Reps et al., 1978). Krishnaswamy et al., (1976) observed slightly higher fat loss in whey in the cheese made with Rhizopus oligosporus enzyme that of with calf rennet. Both types of cheese developed mild flavour and aroma in 30 days without marked differences in body and texture. Rao and Mathur (1979) reported that

the milk clotting enzyme preparation from Bacillus subtilis K-26 can be used for the manufacture of Cheddar cheese.

Coagulating enzymes from plants : Some of the plants studied for coagulating enzymes include Ficus glomarata, F. religiosa and F. carica (Krishnamurthy and Subramanyan, 1948, 1949; Whitaker, 1959; Zukerman et al., 1961, 1963; Krishnaswamy et al., 1961), Withania coagulans (Kothavalla and Khubchandani, 1940; Narain and Singh, 1942; Dastur et al., 1948), Carica papaya (Dastur, 1949), Papain (Windland and Kosikowsky, 1946), Cynara cardunculus (Vieira and Barbosa, 1970) and pumpkin (Rebecca and Leibowitz, 1963). Although no detailed information is available in respect of the quality of cheese prepared using these enzymes, but the cheese made appear to develop bitter taste in varying degrees, bitterness decreasing in 3 - 6 months (Krishnaswamy et al., 1961). Vieira and Barbosa (1970) reported that the enzyme extract from the flowers of Cardoon (Cynara cardunculus) is satisfactory for soft bodied cheeses like Serra and Roquefort, although with the latter it causes some loss in yield.

STARTER CULTURE

Lactic acid bacteria are used as starters in cheese manufacture for initially fermenting lactose to mainly lactic acid. The lactic starters used in cheese making normally include streptococci, leuconostocs and lactobacilli. Selected strains

of these genera are used as combined cultures or as single strain cultures or as mixtures of single strain cultures (Holzapfel and Kriel, 1973; Legg, 1973; Frzinkyan et al., 1975; Kirov and Chamakov, 1975; Stevic et al., 1975 ; Mostert and Husmann, 1976; Gruev, 1977; Butkus et al., 1978; Dolezlek and Richterova, 1978; Limsowtin, 1978; Petterson, 1978; Sharpe, 1979). The type of starters used is responsible for the quality of cheese obtained (Bijok and Domanska Strzatk-Owska, 1970; Shapovalov et al., 1972; Dilanyans et al., 1976; Steffen et al., 1978). The importance of starter cultures and the acid developed by them on the characters like texture (Belovsov et al., 1976 and Hoglund et al., 1976) and flavour (Lowrie et al., 1973; Belovsov et al., 1976) have been extensively studied.

The production of lactic acid by starter activity imparts a fresh acid flavour to curd cheeses, assists in the formation of rennet coagulum by causing shrinkage of the curd and moisture expulsion and promotes characteristic texture formation during cheese making. The low pH of fresh cheese curd (5.0 - 5.2) helps to suppress the growth of pathogenic and spoilage bacteria, and thus preserves the product. Lactic acid bacteria also produce traces of flavour and aroma compounds and their proteolytic and to a lesser extent, lipolytic activity aids the maturation of cheese.

In addition growth of lactic acid bacteria produces the low oxidation-reduction potential (Eh), necessary for the production of reduced sulfur compounds, such as methanthiol, which may contribute to the aroma of cheddar cheese (Manning et al., 1976)

In cheddar cheese making the most rapid increase in number of starter bacteria occurs during curd making. The average count of S. cremoris (NCDO 924) in 15 Cheddar cheese made over a 12 month period rose from 2×10^7 cfu/ml in milk 20 minutes after inoculation to 4×10^8 cfu/g in the curd at maximum scaled ($39^{\circ}C$) 2 hrs later, and to 1.4×10^9 cfu/g in the curd at seperation from whey 1 hr. 20 min later (Chapman and Sharpe, 1981).

Acid development by starter bacteria :

Acidity is one of the major factors in cheese making, both in the actual clotting of milk and in the mellowing of the curd. The starter organisms continue to grow and produce acid until the lactose completely is used up. It is apparent, therefore, that the final acidity reached in the cheese is determined by the amount of sugar left in the curd during making and this is in turn regulated by the moisture content.

Brown and Price (1934) observed an increase in the pH of Cheddar cheese ripened for about 24 months (5.05 - 5.58). Dolby (1941)

stated that a change in acidity at removal of whey produces a definite change in the quality of the mature cheese. Baron (1947) stated that the acidity at milling time also affected the properties of mature cheese. Pimblett (1962) was of the opinion that varying the period from removal of whey to the milling of curd from 1 hr - 3 hr had little influence on the final grade scores of cheese.

Fresh curd with excessive moisture will contain too much lactose and will most likely become sour. Reverse will be the case with too dry curd (Czulak et al., 1969). When acid development was rapid, the removal of calcium from the curd was sufficient to effect the physical properties and phosphorous loss was sufficient to affect the buffering capacity of cheese.

The acidity in whey has been shown to increase as a result of diffusion from the curd of buffer ions specifically the phosphate and citrate (Lewis, 1974). The acid development during cheese manufacture has been shown to control the initial moisture contents of cheese. The acidity has also been reported to effect both the hardness of the curd and the body of cheese in the course of ripening. The higher the acidity, the greater will be the hardness or in other words, the lower the moisture content (Stefanovic, 1974).

Titratable acidity of cheese increases due to lactic fermentation during early stages of ripening (Umemoto and Sato, 1975). El-Koussay et al., (1977) reported that the higher the acidity, the greater was the reduction in cheese yield. Harvey et al., (1977) reported the lactic acid content of Cheddar cheese of different ages ranging from 1.03 to 1.62%, and the sugars viz., lactose, galactose and glucose contents were 0.07 - 0.48%, trace to 0.12% and trace, respectively. Rao (1978) observed that faster acid development resulted in the lower initial moisture content of cheese.

Microbial changes during ripening :

Evans (1918) and Evan et al., (1919) reported that the microflora of ripened Cheddar cheese consisted of i) streptococci of the Streptococcus lactis type, ii) lactobacilli of the L. casei type, iii) streptococci other than those of the S. lactis type and iv) micrococci.

Hucker (1922) reported the presence of following organisms in Cheddar cheese : i) Sporeformers, ii) Gram-negative rods, iii) lactobacilli, iv) S. lactis v) cocci, vi) Streptococci other than S. lactis and vii) yeasts.

Sherwood (1939) studied the bacterial flora of New Zealand Cheddar cheese, which consisted almost exclusively of Lactobacillus plantarum, L. casei, betabacteria, betacocci and other lactic acid bacteria.

Alford and Frazier (1950) isolated and identified seven groups of micrococci on the basis of characteristics indicative of potential value in cheese ripening. These organisms were Micrococcus freudenreichii, M. caseolyticus and M. conglomeratus.

Dawson and Feagan (1957) used a mixed culture of S. lactis and S. cremoris in Cheddar cheese making. They observed that maximum number of S. lactis were reached at the half cheddaring stage and of S. cremoris at milling. The maximum count of S. lactis was retained until the cheese left the press, where S. cremoris count had already begun to decline. During ripening a high population of S. lactis was maintained for 8 wk after which a gradual decline took place. Cells of S. cremoris died out rapidly after 2 wk and almost completely disappeared by 8 wk. It was felt that the difference between S. lactis and S. cremoris in their survival in Cheddar cheese might influence growth of other microorganisms and, hence, affect flavour development in the cheese.

An examination of Rannell (1960) of stained sections of Cheddar cheese, demonstrated that streptococci generally occured singly and in small groups throughout the cheese, but that their distribution was quite irregular. Bacteria which developed in the cheese during ripening generally formed discrete microcolonies associated mainly with curd junctions.

The only bacteria found regularly in high numbers in Cheddar cheese were of the S. lactis type (Marth, 1963). These bacteria were thought to function, primarily, through production of lactic acid.

Ajaib Singh et al., (1967) found that the various types of microorganisms in bacterial rennet and control cheese appeared to consist largely of streptococci and lactobacilli, followed by acid-forming types, sporeformers, lipolytic and proteolytic bacteria in order of their predominance. Lactobacilli occured in more or less constant numbers in all cheese blocks. Yeasts and molds of control cheese always showed higher number than experimental blocks.

Cheddar cheeses were manufactured by conventional commercial methods except that the starter cultures used (S. cremoris strains 108, 104 and P_2 and S. lactis strains H1 and ML8) were adjusted to contain different proportions of proteinase-positive and proteinase -negative variants. Control cheeses were made with 100% proteinase positive strains. At salting cell densities in the curd were similar for control and experimental cheeses and were independent of the propertions of the two types of cells present (Mills and Thomas, 1980).

Effect of Salt on growth of Starter cultures :

The effect of salt on acid production in cheese curd by S. lactis and S. cremoris was investigated by Walter et al., (1958).

S. lactis was generally not inhibited by less than 1.6% salt or not significantly at 1.6% to 2.0% salt. S. cremoris however, was inhibited slightly by 1.4%, definitely by 1.6%, and almost completely by 2.0% salt. Mixture of cultures appeared to be more uniform in resistance to sodium chloride than single strains. Lactose utilisation and lactic acid production by starter organisms in Cheddar cheese were markedly affected by variation in salt-in-moisture (S/M) levels between 4 and 6% (Turner and Thomas 1980). In cheese with low S/M levels (about 4%), lactose was completely utilized in about 8 days and L-lactate was the major end product. In contrast, with high S/M levels (6%), Starter metabolism virtually stopped so that lactose concentrations were still high after several weeks storage. This residual lactose was utilized by non-starter bacteria and D-lactate was a major end product. In most of the cheese making trials, the non-starter flora comprised mainly of pediococci (Turner and Thomas, 1980). Growth rate of these organisms and rate of lactose utilization increased when the initial storage temperature of the cheese was raised. Starter bacteria appeared to play a major role in lactose utilization in Cheddar cheese after 1 day, only when the S/M level was low, or when relatively salt tolerant strains were used.

Effect of Milk-clotting enzymes on starter culture : There appears to be a direct relationship between the available nitrogen and or

proteolytic activity and the lactic acid production by various microorganisms (Anderegg and Hammer, 1929; Harriman and Hammer, 1931; Gorvia and Mabitt, 1956; Stadhouders, 1961 and Citti et al., 1965).

Lactic streptococci used as cheese starters require the activity of surface bound proteolytic enzymes for normal growth in milk. Strains lacking this activity, called as 'slow variant' grow to a limited extent in milk and do not produce significant amounts of acid during cheese making. Proteolytic activity thus plays an important role in starter activity. Therefore, it is imperative to examine the effect of proteolytic enzymes present in commercial rennets on the starter activity during cheese making.

Reiter and Oram (1962) observed that the growth of S. lactis strains which have less rigorous amino acid requirement than S. cremoris was stimulated by the presence of proteolytic enzymes in the commercial rennet preparations. Shovers and Bavisotto (1967) prepared short-hold Cheddar cheese with 100% animal rennet, 100% FDF (Fermentation Derived Rennet from Endothia parasitica) and observed no difference in acidity at 'cut' and at 'draw'. But the activities at 'pack' and at 'mill' were higher in the FDF cheese. The growth factors produced from the proteolytic activity of rennet markedly stimulate the acid production of some starters but have no effect on others(Pearce, 1969).

Nelson (1969) compared 'Emporase' (milk-clotting enzyme from Mucor pusillus Lindt), pepsin blend with Veal rennet and Veal-pepsin (Quick set) in the manufacture of American Cheddar cheese by using 1% level of starter. No differences in whey acidities at packing or at milling were observed among the three cheeses. Dolezalek (1973) studied the effect of various microbial rennets on starter activity of lactic acid bacteria and found them to be stimulatory to most of the starters tested.

Sannabhadti and Srinivasan (1976) reported that the acidity development during cheddaring with Absidia ramosa rennet was slightly faster than Meito rennet (from Mucor pusillus Lindt) and similar to animal rennet. Rao (1978) showed that the acid development was relatively faster in case of cheese made with crude or purified bacterial milk clotting enzyme (from B. subtilis K-26) than in animal rennet cheese when used either singly or in combination with calf rennet or swine pepsin. Pearce (1979) reported that activity of S. cremoris (20 strains) and S. lactis (8 strains) was generally stimulated by proteolytic enzymes present in comercial rennet. The extent of stimulation varied with source and type of milk. A three-fold increase in rennet concentration over that normally used in cheese making did not produce any additional stimulation, while 10-fold and 30-fold increases caused slight inhibition. He further suggested that rennet clearly has a differential modifying effect on starter acid production.

PROTEOLYSIS IN CHEDDAR CHEESE

Primary changes in cheese proteins consist of conversion into proteoses, peptones, peptides and finally to amino acids, while the secondary changes consist of further breakdown into amino acids.

The three main proteolytic agents in Cheddar cheese are : i) Milk coagulants ii) Milk protease and iii) Starter bacterial enzymes and non-starter bacterial enzymes (in aseptically made cheeses).

The extent of casein proteolysis is characteristic of the particular cheese variety being studied. In general, casein is initially hydrolysed to large peptides by milk coagulant and native milk enzymes, then to smaller peptides by bacterial or mold enzymes (Creamer, 1978).

i) Proteolysis due to milk coagulant :

The most obvious function of a cheese making coagulant is conversion of the liquid milk to a gel by proteinases.

Distribution of rennet in the Cheese curd : The coagulant is partly incorporated into the curd and survives throughout cheese-making into the ripening period and the residual coagulant contributes to proteolysis in the cheese during ripening (Lawrence et al., 1972 ;

Holmes and Ernstrom, 1973; Green and Foster, 1974). Holmes (1975) observed that at pH 6.6, 31% of rennet occured in curds and 72% in whey, while at pH 5.2 respective values were 86 and 17%. Distribution of Mucor pusillus protease was independent of pH with approximately 15% in the curds and 85% in the whey. During Cheddar cheese making 7 and 58% of the original rennet, 6 and 93% of the original rennet/peptin mix was active in the curds and whey, respectively at dipping. He further reported that after overnight pressing 6.3 and 4% of the rennet Mucor pusillus protease rennet/ pepsin mix respectively remained active in the cheese.

Nature of proteolysis by milk coagulant : While studying the nature of proteolysis by rennin in a large number of cheese samples, Tsugo and Yamuchi (1959) reported that rennin degrades β-casein only after long incubation period. Ledford et al. (1966) observed that α- casein was much more readily hydrolysed than β-casein at all pH values from 5.5 to 7.0. Ledford et al. (1968) showed that rennin degradation of α_s-casein is more pH dependent than β-casein at all pH values in the range 5.5 to 7.0. Its degradation at pH 6.0 was several times more than β-casein.

As the enzymes vary in their activity and specificity the rate and type of proteolysis and, thus, the development of body and flavour are dependent on the coagulant used (Naudts, 1969). Creamer et al. (1971) showed that three bonds in β-casein are appreciably

more sensitive, than the others to rennin proteolysis, and that these bonds are probably located near the C-terminus of the protein. Creamer and Richardson (1974) reported that the degradation of α_{s_1} - casein by rennet in Cheddar cheese proceeds via an initial change of the phenylalanine - 24-Valine-25 bond by rennet. They isolated two peptides, which are indistiguishable by amino acid analysis. Rennin was shown to act on a specific sensitive phenylalanine-phenylalanine bond in α_{s_1} - casein to produce a basic peptide containing residues 1 - 23 of the original protein (Hill et al., 1974). At pH 6.4 and 30°C the action was specific and rapid.

Cheddar cheese prepared with a mixture of Noury rennet (NF) from Mucor pusillus and swine pepsin after ripening for 43 and 189 days showed an increase of α_{s_1} casein breakdown when NF proportions in the coagulant increased as revealed by gel filtration and starch gel electrophoresis (Green and Stackpole, 1975).

Hofi et al., (1976) compared the proteolytic activities in cheeses made with different milk-clotting enzymes-Mucor miehei (M1), Endothia parasitica (M2) and Mucor pusillus Lindt (M3). The use of M3 and M2 in Ras cheese making enhanced protein break down as compared with calf rennet (R) or M1 cheeses. Cheese made with M3 showed the highest free amino acid content followed by R cheese, than M1 and M2 treatments which were almost the same. Mohram et al., (1976) studied proteolysis in cheese using mixtures of equal parts

of rennet + Mucor miehei (RM1), rennet + Endothia parasitica protease (RM2) or rennet + Mucor pusillus Lindt protease (RM3) enhanced protein breakdown more than RM1 imparted bitterness to Ras cheese which persisted until the 4th month and disappeared thereafter. Antila and Witting (1978) reported that proteolysis was more rapid and more flavour defects occured in the cheese made with the microbial rennets than with the animal rennet.

O'Keefee et al.(1978) showed that milk coagulant was primarily responsible for the formation of large peptides, while small peptides and free amino acids were produced principally by the starter possibly from coagulant produced peptides.

ii) Proteolytic activity of milk protease :

The existence of milk protease with casein was first demonstrated by Wharner and Polis (1945). Peterson (1960) attributed the minor role of proteolytic degradation in cheese ripening to the enzymes of milk including protease.

Carini and Bozzalati (1970) reported that proteolysis by natural milk protease involve the reduction of α_s and β-caseins and formation of products of low electrophoretic mobility. They reported the temperature range for milk protease activity to be 5 to 45°C. Noomen (1975) observed that both α_s and β- casein were degraded by milk protease. However, β-casein was degraded faster

than α_s-casein. Geo and Alais (1976) showed that milk protease hydrolysed β-casein preferentially.

Milk protease survives pasteurization and takes part in protein break down in milk and milk products (Jost et al., 1976). It is known that α_s - I appears during the ripening of various cheeses and the conversion of αS - casein to αS - I is due to the action of chymosin. However, Kaminogawa et al. (1978) showed that $\alpha S1$- I is produced not only by chymosin but by milk acid protease.

As the pH range in which this enzyme is active overlaps that of cheese ripening, it is considered that the enzyme may have some significance in proteolytic breakdown of casein during cheese ripening (Kamanigawa et al., 1978).

iii) Proteolysis due to starter culture :

Proteolysis by starter organisms is of paramount importance during cheese ripening. Investigations on proteolysis in cheese by starter organisms are complicated by the presence of rennet and as such conclusive results can not be obtained due to the lack of control of bacterial flora.

In general, the changes in proteins are believed to be brought by five main groups of bacterial enzymes namely : i) proteinase , ii) peptidase, iii) transaminase, iv) amino acid decarboxylase and v) deaminases.

Very little or no difference in the rate and amount of proteolysis by strains of S. lactis and S. cremoris was observed by Kelley (1932). According to Allen and Knoweles (1934), starter streptococci were responsible for protein breakdown during the initial stages of cheese ripening but later on, the lactobacilli were the predominant microflora in cheese. The role of lactobacilli in proteolysis during cheese ripening has been confirmed by Hammer (1935). Davis et al. (1937) showed that there was greater increase in non-protein nitrogen in skim milk culture of lactic acid bacteria containing sterile rennet, as compared to similar cultures without rennet or milk containing rennet alongwith starter.

Peterson et al. (1948) believed that in ripening of Cheddar cheese, there is an active protease largely of bacterial origin and this enzyme enhanced the ripening process. They demonstrated that the initial enzyme content of cheese is relatively low but it increased steadily as the cheese ripened and this could only be due to production of enzymes by microorganisms growing during the ripening period. Morgan and Nelson (1951) reported that the proteolytic enzymesof S. lactis liberate simple peptides and amino acids from milk protein in amounts which are proportionate to the occurrence of such amino acids in the protein.

Baribo and Foster (1952) compared the relative efficiencies of proteolytic enzymes extracted from one year-old cheese with that produced by S. lactis and concluded that a portion of the protein

breakdown in cheese might be due to starter streptococci. Rennet was thought to provide a part or all the rest of the proteolytic activity. Vanderzant and Nelson (1953) showed that protease-peptone fraction of milk proteins is most suceptible to proteolysis and casein may not be the most readily hydrolysable protein in milk by S. lactis. Yamamoto et al. (1953) reported that cheese made with lactobacilli as starters gave a more uniform type of cheese.

Stadhouders (1961) noted that the time of cheese ripening could be shortened by the presence of excessive amounts of proteolytic enzymes of streptococci. Protein hydrolysis of streptococci depends upon two factors namely, proteolytic activity per cell and the total number of streptococci present in cheese.

To investigate the action of rennet and lactic acid bacteria in the cheese making process, Yamamoto and Yoshitake (1962) prepared starter cheeses and cheeses in which the starter was replaced by lactic acid. Fifteen amino acids were detected after one month in the cheese with starter but only three amino acids were found in the cheese without the starter thereby implying that the amino acids are formed by the starter and not by the rennet.

Reports on the optimum conditions for proteolysis by intracellular and extracellular proteases of starter streptococci showed the optimum range of pH for these enzymes to be 6.0 to 8.5

(Vedhera and Boyd, 1963; Willamson et al., 1964; Sato and Nakashima, 1965; Sato and Ohima, 1966) and very slight or no activity at pH 5.0 (Sasaki and Nake, 1959). Vedhera and Boyd (1963) reported that S. lactis possesses a proteinase system with an optimum activity at pH 5.5. Vedamuthu et al. (1966) could find significant differences in the rate of protein degradation in cheeses made using commercial starters, two of which produced defective cheese and one produced a normal cheese.

Enzymic activity and amino acid production in cheese indicated that proteolysis was mainly due to starter enzymes and that it proceeded at a constant rate throughout the cheese ripening (Dulley, 1974). The residual rennet in the cheese maintained its clotting activity throughout ripening. Lee (1974) analysed 640 lbs of Cheddar cheese and reported that water soluble protein was increased by 6.07, 4.73, 8.24, 16.80 and 25.34% on the 1st, 2nd, 7th, 30th and 90th day of ripening, respectively.

Green and Foster (1974) claimed the behaviour of bacterial proteinases in cheese to be similar to that of rennet. On contrary Gripon et al. (1977) reported that enzymes of lactic acid bacteria produce mainly free amino acids unlike rennet that only produces peptides. Visser (1977) showed that the proteolytic action of rennet must stimulate the starter bacteria significantly to liberate amino acids in cheese. In normal asceptic cheeses prepared with

rennet and starter, far higher levels of amino acid nitrogen were accumulated than in corresponding asceptic rennet free cheese (ARF). Another interesting observation was that in ARF cheese, distinct amounts of soluble nitrogen were produced giving evidence that starter bacteria are capable of attacking paracasein in cheese and converting it to soluble products, independently of the breakdown products of rennet action.

Nieuwoudt (1977) felt that total soluble nitrogen content can be used to assess the ripeness of cheddar cheese. He further reported that soluble nitrogen and free tyrosine contents increased logarithmically with ripening time (up to 8 months) and were greater at 16°C than at 8°C. Park et al. (1978) observed total nitrogen content in cheese immediately after manufacture and in waxed and unwaxed cheeses after 6 months ripening was 22.10, 26.00 and 35.52%, respectively.

Deaminases, transaminases, and decarboxylases degrade or modify amino acids of peptides (Desmazeud, 1978). Thompson (1980) observed a greater degree of protein hydrolysis in cheddar cheese made with S. cremoris ATCC 14365.

LIPOLYSIS IN CHEDDAR CHEESE :

Hydrolysis of milk fat catalyzed by enzymes was earlier considered as a major problem in Dairy Industry because of the production of rancid flavour. This defect in milk, cream and other dairy products has been applied in recent years as a useful, controlled process for the development of desirable flavours in various dairy products. The role of free fatty acids in imparting characteristic flavour to various dairy products gradually became apparent as technologists evaluated and studied the chemistry of several food flavours.

Free fatty acids as one of the chemical compounds are also responsible, in part, for the flavour in cheddar cheese. These fatty acids may arise through activity of lipase indigenous to milk (if not destroyed), microbial lipase, or lipases from other sources introduced into milk at the time of cheese manufacture.

Limited studies have been carried out on the use of lipolytic microorganisms in Cheddar cheese manufacture. Mattick and Hiscox (1939) ascribed high levels of fatty acids present in cheese to the presence of large numbers of non-lactic bacteria. Hammer (1940) reported that rancid flavour disappeared from cheeses made from milk to which butyric acid had been added. Disappearance of flavour resulted from the activity of the microorganisms which can attack sodium butyrate.

Babel and Hammer (1945) found an increase in water-soluble and water-insoluble volatile acids as well as cheese fat acidities to raw milk cheese than in pasteurized milk cheese. Peterson et al. (1948) reported that cheese lipase which is active at pH 5.0 become prominant after 5 - 20 days of ripening and resulted from bacterial activity. They further reported that volatile fatty acids particularly acetic, butyric acids were substantially higher in raw milk than in pasteurized milk cheese. The presence of free butyric, caproic, caprylic and capric acids in aged cheese has been attributed to the action of intracellular bacterial lipase in cheese fat. Hammer (1948) indicated that the organism metabolising sodium butyrate may be a varient of Streptococcus lactis.

Tests by Tuckey et al. (1948) demonstrated that rancid cheese resulted from the use of Geotrichum candidum in combination with lactic starter culture. Studies by Hood et al. (1949 a, b) showed that cheese became rancid when milk was inoculated with lipolytic bacteria at a higher rate of inoculum. Peterson and Johnson (1949) reported that the lactobacilli cultures isolated from normal cheddar cheese produced intracellular lipases which were active at pH 5.0 and 6.0. These ensymes hydrolysed butter fat and liberated n-butyric, caproic and caprylic acids. Kannan and Basu (1957) have indicated that lipase with an optimum pH of 8.6 showed little initial acidity in cheese, but the ensyme activity declined

after 110 days. Lipase activity has been found to be more vigorous at all stages of ripening.

Harper (1959) has attributed to the development of flavour in aged cheese to free fatty acids. Ohren and Tuckey (1964) reported that Cheddar cheese flavour was related to a balance of free fatty acids and acetate.

Fryer et al. (1967) have shown that some lactic acid bacteria are lipolytic in nature. Such observations have also been confirmed by using asceptic vat technique in the cheese making process by Reiter et al. (1967). Chander et al. (1973) also found the lipolytic nature of some lactic cultures. Stadhouders and Vering (1973) studied the importance of starter bacteria for fat hydrolysis during cheese ripening. Starter bacteria or their diesterases hydrolyze triglycerides to liberate lower chain fatty acids for improved flavour. These are capable of hydrolyzing fairly rapidly to mono and diglycerides. Iwask and Kosikowski (1973) reviewed the potential of microbial lipases to the increased quantities of free fatty acids and the final flavour of cheese. Arnold et al. (1975) noted the relationship between lipolysis and flavour which involves microbial lipase and esterases. Umemoto and Sato (1975) obtained increased amount of free fatty acid during different stages of cheese ripening. On the contrary, Deeth and Fitzerald (1975) noted inverse relation between level of lipolysis

and flavour score. The flavour score attributable to the increased amounts of free-fatty acids was responsible for down grading of some local cheeses.

Jaunsyms (1976) determined the fatty acids chromatographically in Cheddar and Dutch cheeses. The contents of low-molecular weight volatile fatty acids ($C_4 - C_{10}$) were 6.6% for the former and 5.98% for the latter cheese and those of polyunsaturated fatty acids were 0.74 - 4.0%. No correlation was found between contents of free fatty acids and taste or flavour of the cheese (Kowaleswska, 1977).

Paulson et al. (1979) observed that fatty acids with chain length C_4 were released in all cheeses containing Propionibacterium shermanii and L. bulgaricus. The organisms used in the cheese production had relatively little influence on the neutral volatiles that were produced.

Effect of Milk Coagulant on Microbial lipases :

The milk coagulant used for cheese preparation will affect the activity of the starter lipases. Brandl (1970) found that animal rennet and Endothia parasitica rennet caused no inhibition of butter fat hydrolysis in a staphylococcal culture, but the Mucor pusillus did. At pH 5.5 and 6.5, subtilisin and B. subtilis crude proteinase resulted in a definite inhibition of lipase activity. He reported that Mucor pusillus rennet showed a lipolytic activity at pH 5.5 to

8.1, where as no lipolytic activity was observed in calf rennet and Endothia parasitica rennet.

FLAVOUR DEVELOPMENT DURING RIPENING OF CHEDDAR CHEESE :

Flavour changes in Cheddar cheese are brought by the biochemical activity of microflora present in cheese during various stages of ripening. A number of chemical compounds are released during such changes and the relative proportions of cheese compounds are important in imparting the desired flavour characteristics to the cheese. The various flavour-contributing components in cheese can be broadly grouped as follows : i) carbonyl compounds, ii) nitrogenous compounds iii) sulphur compounds iv) fatty acids and their derivaties and v) other flavour compounds.

Carbonyl compounds :

The carbonyl compounds present in Cheddar cheese can be further classified into acidic and neutral-carbonyls. Acidic carbonyl compounds that are present in Cheddar cheese are oxaloacetic; oxalosuccinic, glyoxylic, α- ketoisocaproic pyruvic, α-ketoglutaric, α- acetolactic acid (Brandsauter and Nelson, 1956 ; Bassette and Harper, 1958 ; Kristofferson and Gould, 1959; Harvey and Walker (1959)).

The neutral carbonyl compounds present in cheese are considered to be degradation products of acidic compounds. The carbonyl compounds belonging to this category in Cheddar cheese include : diacetyl (Calbert and Price, 1948; Bassette and Harper, 1958; Day and Keeney, 1958; Patton et al., 1958 ; Bassette et al., 1967) butyraldehyde (Dacre, 1955); acetaldehyde and acetone (Bassette and Harper, 1958; Day and Kenney, 1958; Patton et al., 1958 ; Kristofferson and Gould, 1959; Harvey and Walker, 1959, 1960; Day et al., 1960 Lindsay and Day, 1965); acetyl methyl carbinol (Bassette and Harper, 1958; Day and Kenney., 1958; Harvey and Walker, 1959; Harvey., 1960; Keenan et al., 1967) methyl ethyl ketone (Scarpellino and Kosikowski, 1958; Scarpellino, 1961); 3-hydroxy butanone (Patton et al., 1958) ; butanone-2 (Day and Kenney, 1958; Patton et al., 1958; Day et al. 1960; Harvey and Walker, 1959, 1960); Pentanone-2 ; heptanone-2 ; Undecanone-2 and nonanone-2 (Day and Kenney, 1958; Harvey and Walker, 1959, 1960; Day et al 1960); tridecanone-2 (Day and Kenney, 1958; Harvey and Walker, 1959); formaldehyde (Day and Kenney, 1958; Kristofferson and Gould, 1959; and Day et al., 1960); 3-methyl thiopropanol and 3-methyl butanol (Day and Keeney, 1958; Day et al., 1960). propionaldehyde (Day et al., 1960); ethanol (Keenan et al., 1966; Bassette et al., 1967).

Galbert and Price (1948) showed that the threshold value for diacetyl in cheese is about 0.5 ppm, which imparts the typical flavour and aroma to Cheddar cheese. Calbert and Price (1949) found diacetyl in all lots and kinds of cheese examined. The majority of the lots contained less than 0.05 mg of diacetyl per 100 g of cheese. The diacetyl content of Cheddar cheese ranged from 0.016 to 0.335 mg per 100 g of cheese. A small quantity of diacetyl probably contributes to the typical flavour of Cheddar cheese. They reported that the majority of lots of Cheddar cheese with an excellent flavour had a diacetyl content of less than 0.05 mg per 100 g. Larger amounts of diacetyl than this frequently appear to be associated with flavour defects.

Kristofferson and Gould (1959) could not establish a definite relationship between flavour and amounts of the individual carbonyl compounds although samples of cheese with greatest fluctuations in the level of carbonyl compounds during ripening showed highest intensity of the desirable flavour. According to Kosikowski (1959) and Wolin (1961), some of the carbonyl compounds in ripened cheese are said to be derived from casein.

Harper (1965) reported that other compounds produced by the growth of S. lactis in skim milk were glyoxylate, succinate, alpha - acetolactate, oxalosuccinate, oxaloacetate and glutamate.

Nitrogenous compounds :

After about four weeks or so of ripening, peptide hydrolysis takes place and extent of breakdown appears to be sufficient also to make the cheese pliable. The breakdown products of proteins provide the background to the cheese flavour and give the bulk to the cheese although this background cannot be truly termed as the cheese flavour. At one time or other, practically all the amino acids have been detected in cheese and their decomposition products such as tyramine, cadaverine, putrescine, histamine, tryptamine and ammonia have been implicated in particular flavours (Marth, 1967).

Sulphur compounds :

The presence of hydrogen sulphide in cheddar cheese has been observed by a number of investigators (Kristoffersen and Gould, Kirstoffersen et al., 1960; Walker, 1959). Generally, higher levels of this compound were detected in raw milk than in pasteurized cheese. Kristoffersen and Gould (1959, 1960) suggested that intensity of Cheddar flavour was more closely related to the free fatty acids and hydrogen sulphide than to any other combinations of compounds.

Mey (1965) considered the hydrogen sulphide content of Cheddar cheese, [illegible]

greater fluctuation during ripening (6 ug were found in a 4 months and a 10 months old sample, 170 ug in an 8 months old sample).

Fatty acids and their derivatives :

Suzuki et al. (1910) observed that volatile fatty acids were formed increasing amounts during the ripening period ; acetic and propionic acids developed after lactose disappeared and reached their maximum level at three months, after which a decrease was noted, whereas butyric acid and caproic acid concentrations continually increased; formic acid was detected in cheese only after it was five and one-half months old and valeric acid was never observed. It was concluded by these investigators that lactates probably were the principal sources of acetic and propionic acids.

Mattick and Hiscox (1939) related levels of fatty acids in cheese to its microflora and concluded that higher volatile acid content was associated with the presence of high numbers of nonlactic bacteria.

Sheuring and Tuckey (1947) while studying fat constants of milk fat from ripening Cheddar cheese noted a rise in acid number during ripening. It was an indication of fat hydrolysis.

Other flavour compounds :

Several other compounds have been isolated which may contribute to flavour directly or through formation of different compounds.

Examples are ethyl and secondary butyl alcohols (Dacre, 1955; Patton et al., 1958). Kristoffersen (1962) demonstrated that uric acid was present in and influenced the body of Cheddar cheese. In ripened cheese ethanol varied in concentration in an unsystematic way (Manning, 1978).

Hucker and Marguart (1926) observed that addition of large numbers of S. lactis cells to milk before cheese making had no effect on subsequent flavour development. Kelley (1933) while conducting similar experiments, concluded that S. cremoris produced more flavour and aroma in cheese during early stages of ripening. Tests by Perry (1960) indicate that flavour differences in finished cheese are, in part, determined by whether S. lactis or S. cremoris was used as a starter.

Ajit Singh et al. (1976) showed that the number and types of bacteria present in cheese influence the formation of flavour compounds. Law and Sharpe (1977) reported that starter enzymes are not directly responsible for formation of flavour compounds, but that flavour compounds are formed by non-enzymic (or atleast non-microbial) reactions which take place only in cheeses containing starter; conditions necessary for flavour development are a supply of flavour precursors (from enzymic breakdown of lactose, protein

and fate of milk), a low pH and Eh. Factors affecting flavour in ripened cheeses are enzymes, microorganisms, manufacturing process, and ripening (Kristoffersen, 1972).

Starter enzymes added as lysozyme - treated cells produced flavour precursors (free amino acids) but not flavour compounds (Law and Sharpe, 1972).

The mechanism of flavour compounds formation in fermented dairy products can involve either their viable microflora directly, the enzymes of dead microbial cells, or non-enzymic reactions controlled by the conditions of low pH and Eh created by the lactic acid bacteria (Law, 1981).

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

Cheese starter culture, lactic Fermenti (1-964) was obtained from Hansen's Laboratory, Denmark. Rennets used were, Fungal rennet from Mucor pusillus Lindt, from Mourty Lab, Holland (B. No. C.H. 4521); Hansen rennet or animal rennet from Hansen's Laboratory, Denmark and Bacterial rennet from Bacillus subtilis K-26 and Pseudomonas fragi from D.B. Division, N.D.R.I., Karnal. Cow Milk and skim milk used for cheddar cheese manufacture were obtained from Experimental Dairy, N.D.R.I., Karnal. Chemicals used were of analytical grade obtained from Sigma, Sarabhai M. Chemicals, B.D.H. and Polypharm.

METHODS

1.0 Purity and Activity of LF-culture.

1.1 Purity of LF-culture :

The LF culture was grown in yeast dextrose agar tubes and 24 hrs old culture was observed for its Gram reaction and morphology. (APHA, 1967).

1.2 Acid production :

1.2 Acid production by LF - culture was tested according to the method of Horral - Ellicker (1947).

Reagents :

1) N/9 sodium hydroxide (NaOH)

ii) Phenolphthalein solution (0.1% in alcohol)

Procedure :

Three ml of 18 - 24 hrs old culture was transferred to 100 ml of sterilized milk, and incubated at 37°C for 3½ hrs. Ten ml of the above culture was transferred to a 50 ml beaker and titrated against N/9 NaOH to the phenolphthalein end point. Activity of the culture was expressed as percentage of lactic acid using the following formula.

$$\text{lactic acid}(\%) \quad \frac{\text{Volume of N/9 NaOH} \times 100 \times 0.09}{\text{Volume of sample}} \times \frac{N}{9}$$

Activity of the starter was graded according to Horrat Hllicker (1947) as follows :

Titratable acidity	Grade
More than 0.4%	Active
0.3 - 0.4%	Satisfactory
Less than 0.3%	Inactive

1.3 Test for flavour production by starter culture :

Reagents :

i) 40% Potassium hydroxide (KOH)

ii) Creatine powder.

Procedure :

Two ml of 18 - 24 hrs old cultures was taken into a test tube along with 2 ml of 4% LSB, followed by a pinch of crystalline powder. Contents were mixed and kept undisturbed at room temperature for 30 min and the formation of pink coloured ring was observed. Flavour compounds like acetoin, diacetyl and acetaldehyde, in the presence of potassium hydroxide, results with creatine to form a pink coloured ring on the top of the liquid column.

2.0 Milk clotting and proteolytic activities of yeasts

2.1 Milk clotting activity :

The milk clotting activity was assayed according to the method of Sen and Kothari (1978).

(a) Substrate : Reconstituted skim milk (10%) sterilized with 0.01 M $CaCl_2$ was used as substrate. It was pre-warmed at 37°C for use the assay to use.

(b) Procedure : The substrate was distributed in 10 ml quantities into test tubes and placed in water bath at 30°C for 5 min. Then 1 ml of the enzymatic solution was added and mixed well in the above test tube. The time taken for the first appearance of the flakes of coagulated casein was accurately determined with the help of a stop watch.

(c) Unit of activity : The milk clotting activity was expressed in terms of units, where a unit is defined as the amount of enzyme required to clot 10 ml of the reconstituted skim milk at 30°C in 60 sds (60/t, where 't' is the clotting time in sds).

2.2 Proteolytic activity :

Proteolytic activity was determined by the method of Keay and Wildis (1970).

(a) Substrate : Casein was used as substrate for the assay of proteolytic activity. It was prepared from fresh raw cow skim milk by isoelectric precipitation at pH 4.5 with acetic acid. The precipitated casein was seperated from the acid whey by passing through muslin cloth, thoroughly mixed with sufficient acetone in a mixer and filtered through a Buchner funnel using suction. The process was repeated twice with acetone and finally with ether, the solvent being removed under vacuum. The casein thus obtained was dried over calcium chloride at room temperature and stored in a stoppered glass bottle in a cool and dry place until use.

One per cent solution of the casein was prepared, by dissolving 2.5 g in minimum amount of 0.1N NaOH using a magnetic stirrer. After adjusting the reaction to pH 6.0 with 0.1N HCl, the

final volume was made upto 250 ml with 0.2 M phosphate buffer. (pH 6.0). When not used fresh, the solution was kept in refrigerator (4 - 6°C) with a thin layer of toluene on top to act as preservative.

To 1.0 ml of the above filtrate, 5.0 ml of sodium carbonate solution was added, followed by 1.0 ml of Folin's reagent (1.0 N). After mixing thoroughly the tubes were incubated at 37°C for 20 min and the intensity of blue colour developed was measured at 660 nm. is an Elico Spectrocol colorimeter model CL-23. Proteolytic activity of the rennet was expressed in units (mg tyrosine released per ml 1% rennet solution).

Standard curve for tyrosine :

A stock solution of L-tyrosine (Sigma) was prepared by dissolving 100 mg in 20 ml 0.1N NaOH and making up the volume to 100 ml with distilled water, thus getting a concentration of 1000 ug per ml. A series of dilutions were accurately prepared from the above stock solution to give tyrosine concentrations, ranging from 20 to 200 ug/ml.

One ml of aliquots of the different concentration of tyrosine solutions were assayed for proteolytic activity as described earlier.

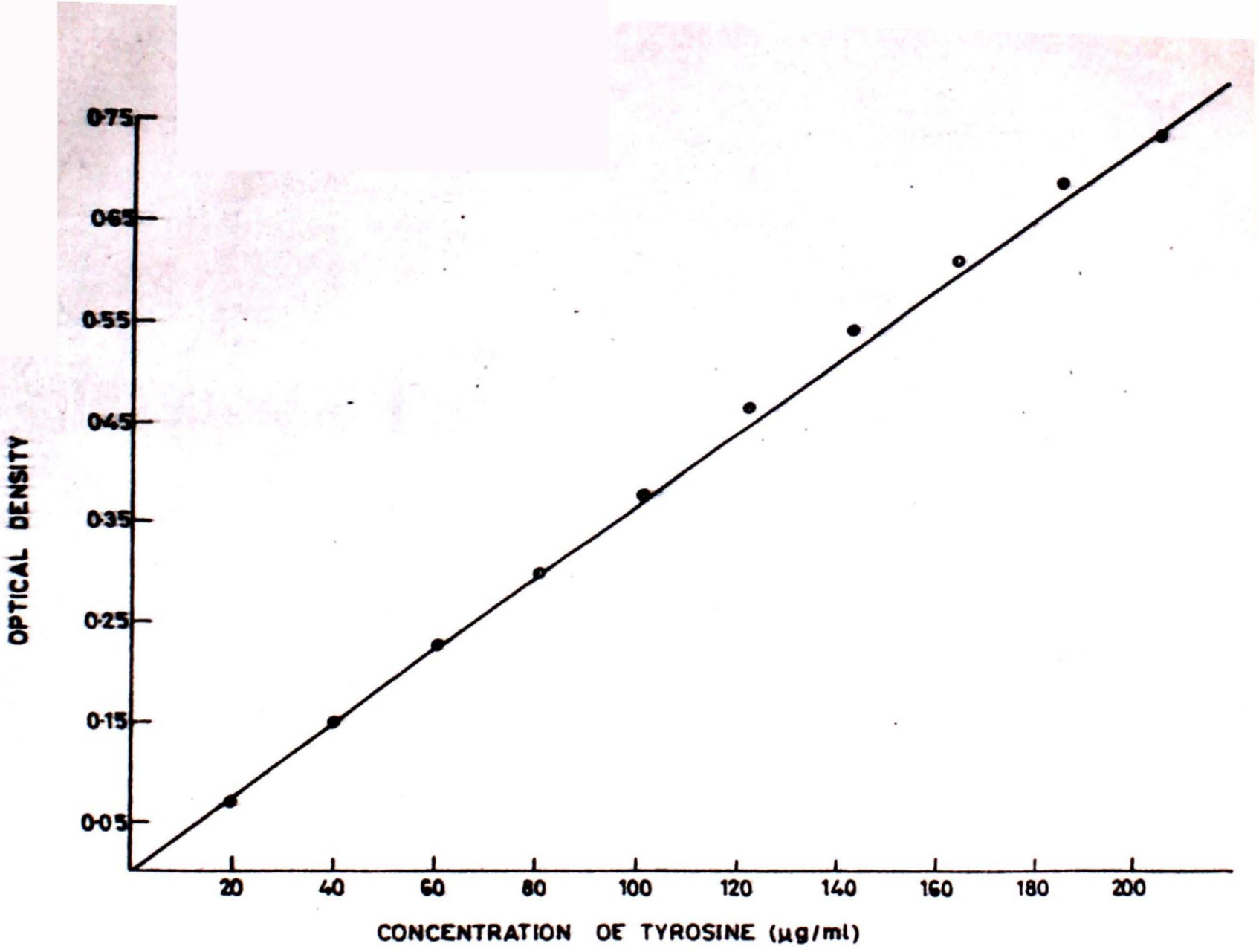

FIG. 1. STANDARD CURVE OF TYROSINE FOR PROTEOLYTIC ACTIVITY.

The standard curve was drawn by plotting the optical density values against the tyrosine concentration (Fig. 1).

3.0 Effect of rennet on lipolytic activity of Pseudomonas fragi

Pure culture of Pseudomonas fragi was used as the test organism for comparing the extent of lipolysis in tributyrin agar, in the presence of 50 ppm concentration of mold, bacterial and animal rennets and no rennet (control). Tributyrin agar was distributed in 19 ml quantities in test tubes and sterilized. After cooling to 48°C, One ml of the required concentration of the rennet and one ml of diluted cell suspension of P. fragi were added, thoroughly mixed and plated immediately. The plates were incubated at 37°C for 24 hrs. The diameter of the lipolytic zones around isolated colonies were measured in cm.

Tributyrin Agar :

Peptone	5.0 g
Yeast extract	3.0 g
Tributyrin	2.0 ml
Agar	15.0 g
Distilled water	1000 ml
pH	7.5

4.0 Cheese making schedule

Standardization of milk : Cow milk was tested for fat and casein contents (ISI, 1960) and was standardized with skim milk to the casein/fat ratio of 0.7 to reduce fat losses in whey and to get maximum yield of cheese. The standardized cow milk consisted of 96 litres cow milk and 14 litres of skim milk.

Pasteurization of milk : One hundred and ten litres of the standardized cow milk was pasteurized at 65°C for 30 min, and cooled to 30°C in an experimental cheese vat.

Ripening of milk : To the cooled pasteurized milk (at 30°C) in a cheese vat, 1.5% of 18 hrs old LF culture was added, with thorough mixing.

Setting the milk : After the starter action for 15 min 1.2 g of the Noury rennet as 10% solution in distilled water was added and mixed throughly to clot the milk in 30 min.

Cutting the curd : After checking the curd with thermometer, curd was cut into cubes, using wider wire cheese knives. The cut curd was stirred at a low speed for 5 min for separation of whey.

Cooking the curd : Using steam in the cheese vat jacket, cooking was started, to increase the temperature gradually to peak temperature of 39°C in one hr, with steady agitation.

Draining the whey : At the end of cooking, agitation was stopped to permit the curds to settle. Then the curds were pushed through the whey to the end of the vat with a curd rake. After inserting the strainer, the valve was opened to drain off the whey. During run-off, part of the jacket was filled with warm water to keep the vat warm for cheddaring.

The whey and curd samples were collected periodically for analysis of pH titratable acidity, lactic acid bacterial count and residual rennet activity.

Cheddaring the curd : When the curd appeared to be about one inch below the surface of the whey, they were trenched over the length of the vat. Trenched curd was permitted to mat for 15 min following complete whey removal. The two trenched curd columns were cut longtitudinally down the middle of curd columns with a large bread knife and then horsontally at approximately 10 inches intervals. Curd blocks were left undisturbed for 15 min at one inch apart from each other. Then the blocks were turned over. This was repeated twice at 15 min intervals. Then the individual blocks were piled, and double blocks were turned for every 15 min until the end of cheddaring period so that new surfaces were exposed.

The titratable acidity of the clear whey, as it flowed from the vat gate exit at the beginning, middle and end of the cheddaring

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period or at different time intervals was determined. When a titrable acidity of about 0.38% in the clear whey reached, the cheese blocks were removed for milling.

Milling the curd slabs : The flattened out curd slabs were fed into a milling machine that was suspended over the cheese vat, and chopped into pieces. The curds were turned and spread with a stainless steel hand fork during milling.

Salting the curd : Common salt (NaCl) at 3% concentraction was sprinkled over the curd. Salt was applied in three lots, allowing them for uniform mixing.

Hooping and pressing : At the bottom of the metal mould or hoop, one muslin cap cloth was placed. Then salted curd cubes were filled in the metal hoop and was covered with the cloth. The steel rim was fitted into the metal mould. The mould was inserted for pressing at 20 p.s.i. gauge pressure and pressed over night.

Drying the cheese : Cheese block was properly labelled and kept at 60 per cent relative humidity for 2 days.

Curing : The whole cheese block was paraffined at 245°F (118.3°C) for 6 sds, and placed in curing room at 85% relative humidity for ripening for 10 months. During ripening cheese samples were collected

and analysed for microbial counts, chemical parameters and enzyme activities.

5.0 Microbial analysis of cheese

Sampling of Cheddar cheese and determination of its microbial contents was done according to the methods of APHA (1960).

5.1 Lactic acid bacterial count :

Suitable dilutions of cheddar cheese homogenates were plated out using yeast dextrose agar.

Yeast dextrose agar

Yeast extract	0.5%
peptone	0.5%
Dextrose	1.0%
Agar	2.0%
pH	6.8

5.2 Non-lactic bacterial count :

Suitable dilutions were plated with nutrient agar and incubated at 37°C for 24 - 48 hrs.

Nurtient agar

Peptone	0.5%
Beef extract	0.3%

Agar	2.0%
pH	7.0

5.3 Proteolytic bacteria : Sterile skim milk (10%) was added to nutrient agar, and used for plating. Colonies producing a clear zone due to proteolysis were counted.

5.4 Lipolytic bacteria : The lipolytic bacteria were counted by plating on tributyrin agar according to the procedure of Franklin and Sharpe (1963).

5.5 Yeast and mold count : Potato dextrose agar was used for enumeration of yeasts and molds (APHA - 1960). The plates were incubated at 22°C for 5 to 7 days.

Potato dextrose agar

Extract from 200 g Potato	500 ml
Glucose	20.0 g
Agar Agar	20.0 g
Distilled Water	500 ml
pH	3.5

pH adjustment : One ml of sterile 10% tartauric acid was added to 100 ml of PDA before pouring into the plates.

6.0 Chemical analysis of cheese

6.1 pH : Ten grams of cheese sample was ground with 10 ml of distilled water in a mortar with a pestle. pH of the cheese paste was measured using the Global digital pH meter (ISI, 1960).

6.2 Titratable acidity :

Ten gram of Cheddar cheese sample was ground with 20 ml of distilled water in a mortor with a pestle. Then this slurry was titrated against N/9 NaOH to the phenolphthalein end point. The titratable acidity was expressed as percentage lactic acid (ISI, 1960).

6.3 Moisture :

One gram of cheese sample was taken in a moisture dish and kept in an oven at 100°C for 24 hrs and weighed for constant weight. Then the moisture percentage was calculated (ISI, 1960).

6.4 Fat :

Three grams of ground cheese was taken, in a cheese cup and inserted in cheese butyrometer. Ten ml of 95% concentrated Sulphuric acid was added to the butyrometer and the contents were mixed well. For proper mixing, the butyrometer was kept in hot water for 20 min. One ml of amyl alcohol was added to the butyrometer and butyrometer was closed with rubber bung and thoroughly mixed. The butyrometer was placed in a centrifuge and centrifuged for 5 min at maximum

speed, and the fat per cent was noted (ISI, 1977).

6.5 Proteolytic degradation in cheese :

The proteolytic breakdown in cheese was measured following the method of Vakaliers and Price (1957).

Reagents :

a. 0.5 M Sodium citrate.

b. 1.41 N HCl.

Procedure

Ten grams of cheese was blended with 40 ml of 0.5 M Sodium citrate solution and about 40 ml of distilled water in a warring blender for 7 min at high speed. The total volume of the above solution was made upto 250 ml with distilled water. One hundred ml of the above solution was adjusted to pH 4.5 with 1.41 N HCl. The total volume of the solution was then made upto 125 ml, filtered and the filtrate was subsequently used for the estimation of proteolytic activity as described earlier (2.2).

6.5.2 Total nitrogen :

The cheese sample was taken in 0.2 g lot and digested with concentrated sulphuric acid, sodium sulphate and copper sulphate and then the nitrogen was determined by alkaline distillation, (Micro-Kjeldahl method) (ISI, 1960).

6.5.3. Non-protein Nitrogen :

Five grams cheese was taken and a paste was made with 10 ml distilled water (40°C) in a pestle and mortar. To this, 25 ml of 5% trichloro acetic acid solution was added. Total volume was made upto 50 ml and filtered. Two ml of filtrate was taken for nitrogen determination (ISI, 1960).

6.5.4. Protein nitrogen :

It is calculated by the difference of total nitrogen and non-protein nitrogen.

6.5.5. Free ammonia :

Two grams of cheese was dissolved in 25 ml of distilled water (40°C), filtered and the filtrate was mixed with 40% NaOH and directly distilled (ISI, 1960).

6.6.0 Lipolytic activity

Lipolytic activity of the cheese was determined according to the modified copper-soap method of Shipe et al., (1980).

Reagents :

i) Copper reagent :

Five ml of triethanol amine was mixed with 10 ml of 1M aqueous $Cu(NO_3)_2 . 3H_2O$. This mixture was dilyted to 100 ml with

saturated sodium chloride solution. Then the pH of this solution was adjusted to pH 8.3 with 1N sodium hydroxide. Then the mixture was stored in dark at room temperature for use.

ii) Colour reagent :

Sodium diethyl dithio carbonate solution (0.5%) in n-butanol.

iii) Solvent :

Mixture of chloroform, heptane and methanol in the ratio of 50:49:1 (V/V/V).

Procedure :

In a glass stoppered tube, 0.25 g cheese was taken and mixed with 0.25 ml of distilled water with the help of glass rod. Then 0.1 ml of 0.7 N hydrochloric acid was added and contents were mixed in a Vortex shaker. Then 2 ml of copper reagent was added and contents were again mixed in a Vortex shaker. Then 6.0 ml of solvent mixture was added and the sample was shaken for 30 min in a rotary Ebubach shaker at 240 rpm. The contents were centrifuged for 10 min at 3800 - 3900 rpm in an International centrifuge. Then 3.5 ml of the solvent layer was transferred to a test tube containing 0.1 ml colour reagent. Contents were mixed and the intensity of colour developed (Yellow colour) was measured at 440 nm in a spectrocol colorimeter.

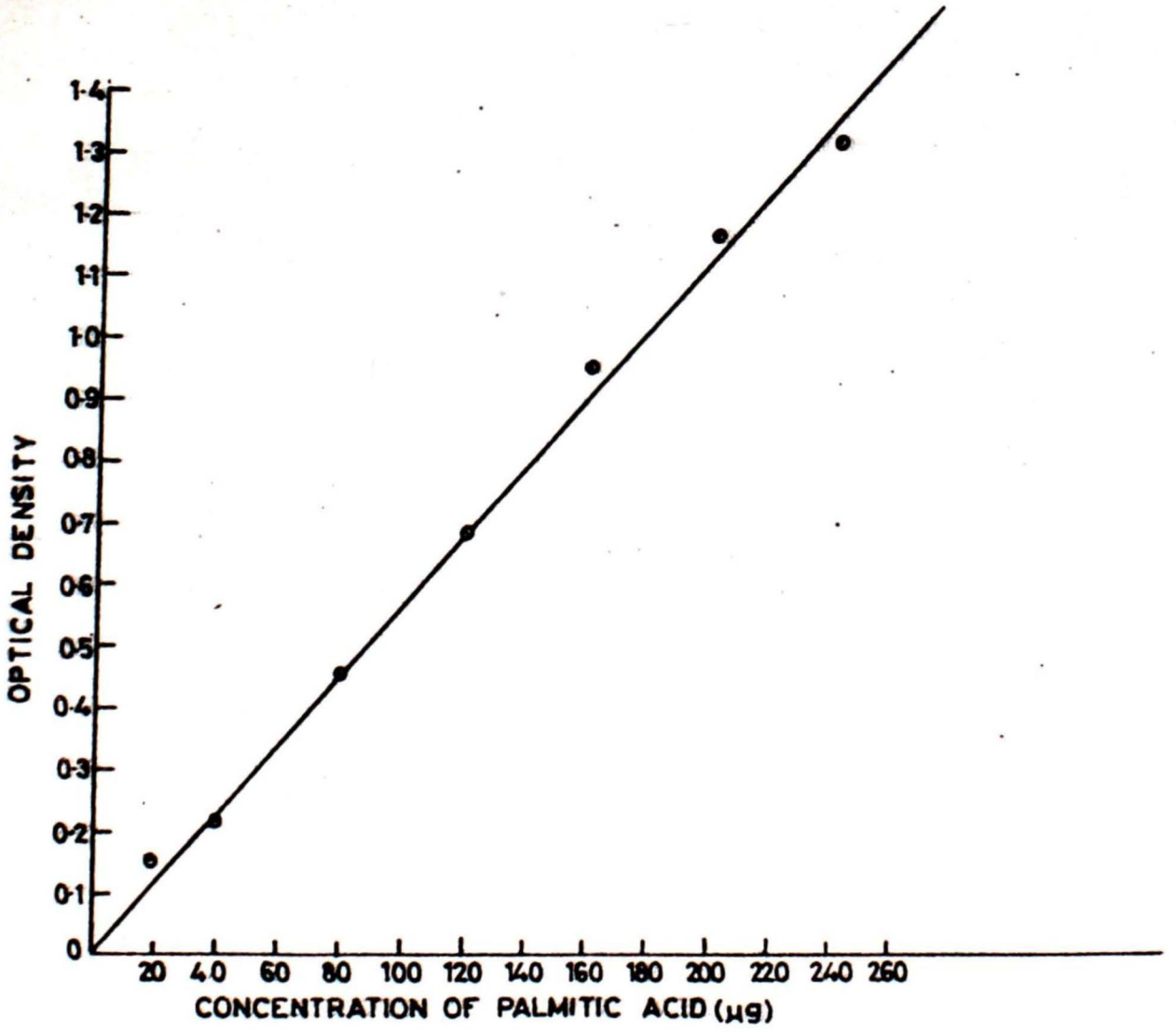


FIG. 2. STANDARD CURVE OF PALMITIC ACID FOR LIPOLYTIC ACTIVITY.

Preparation of the standard curve :

Weighed quantity of palmitic acid was dissolved in chloroform-heptone-methanol (CHM) solvent mixture and volume was made upto 250 ml. Then 1.0, 2.0, 3.0, 4.0 and 5.0 ml lots of the solution was made upto 100 ml with CHM solvent. The blank control was used constituting 0.5 ml of skim milk. The standard solution with the blank was used for lipolytic standard curve (Fig. 2).

6.7.1. Volatile acidity

Volatile acidity of Cheddar cheese was estimated by the method of Hempenious and Liska (1968).

Fifty grams of the cheese sample was ground with 10 ml distilled water and transferred to a 200 ml Kjeldahl flask and 3.0 ml of 1N sulphuric acid was added. The flask was closed with a rubber cork with the wash bottle type of fitting. The longer tube dipping into the sample was connected to the condensor. Then 100 ml of the distillate was collected and titrated against 0.1 N NaOH and the volatile acidity is expressed as ml of 0.1 N NaOH per 50 g of the cheese.

6.7.2. Diacetyl :

Diacetyl content in cheese was estimated according to the method of Pack et al. (1964).

Reagents :

1) Buffered hydroxylamine hydrochloride :

(a) 33.0 g of K_2HPO_4 was dissolved in 100 ml of distilled water.

(b) 11.0 g of hydroxylamine hydrochloride was dissolved in 250 ml of distilled water.

(c) 35.0 g of sodium acetate was dissolved in 100 ml of distilled water.

Solutions, a, b and c were mixed in the ratio of 2:4:1.

2) Acetone phosphate :

29.0 g of K_2HPO_4 was dissolved in distilled water and 40 ml of pure acetone was added. The solution was made upto 200 ml with distilled water and stored in a coloured bottle and kept in the refrigerator.

3) Alkaline tartarate solution :

Saturated potassium sodium tartarate (100 g/100 ml of distilled water) was mixed with concentrated NH_4OH in the ratio of 22 : 3.

4) $FeSO_4$ $7H_2O$ solution :

5.0 g of $Fe\ SO_4 . 7H_2O$ was dissolved in 100 ml of 1% H_2SO_4.

5) Tween - 80.

Procedure :

Twenty grams of the cheese was transferred to a test tube fitted with a trap containing 1 ml of buffered hydroxylamine. The test tube was immersed in a water bath at 65°C and flushed with nitrogen gas for 1½ hrs. The hydroxylamine trap was then disconnected and the tip of the connecting tube was rinsed into the trap, with a few drops of 33% $K_2\ HPO_4$.

Then, 2 - 3 ml of Tween - 80 was added to check frothing during nitrogen flushing.

The hydroxylamine trap was then immersed in a water bath at 75°C. After 10 min. 0.5 ml of acetone phosphate was added. The mixture was cooled and 1.5 ml of alkaline tartarate and 0.1 ml of $FeSO_4$ solution were added. The contents were thoroughly mixed at each stage. The final volume was made upto 5.0 ml with the addition of 33% $K_2\ HPO_4$. Pink colour was developed and it was measured at 530 mu in the Spectrocol Colorimeter.

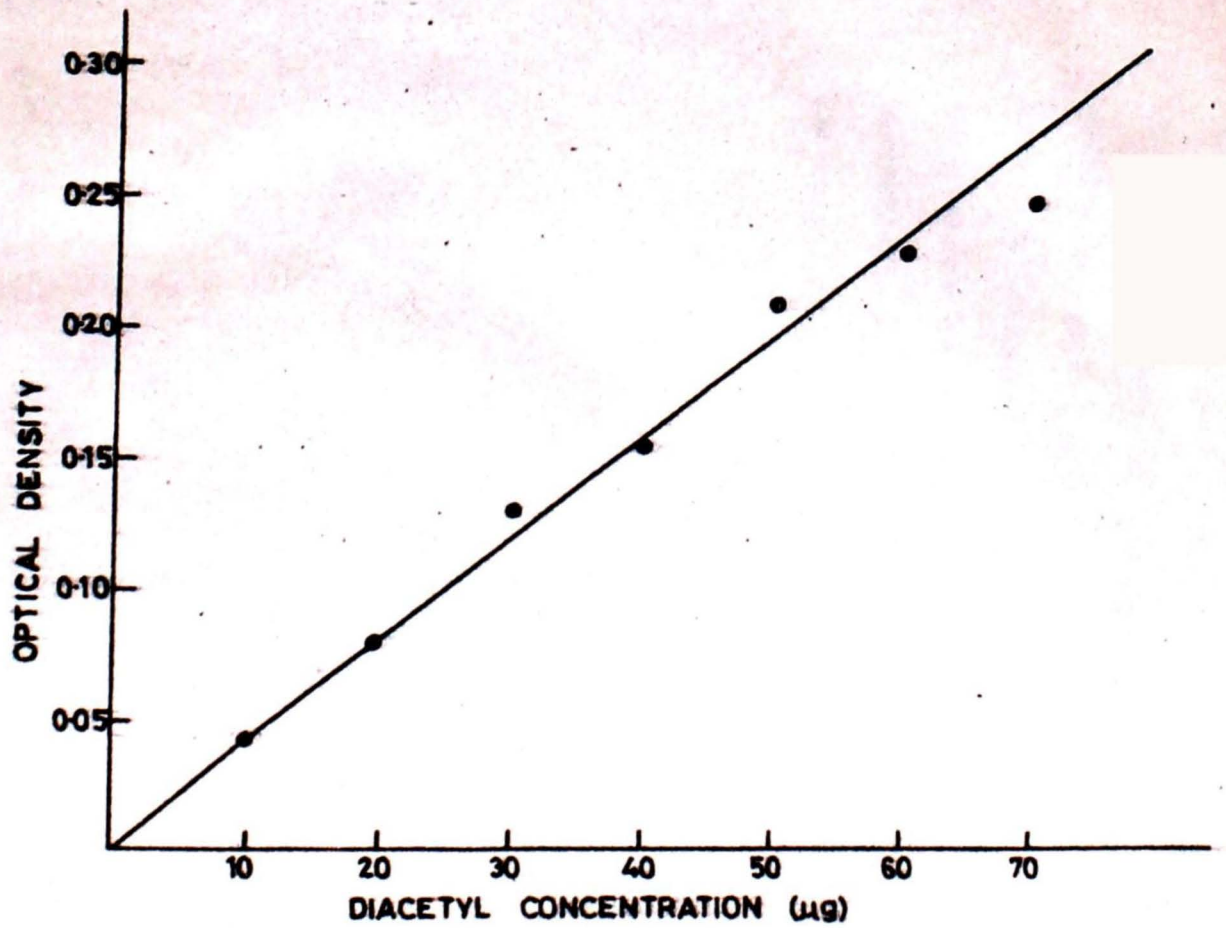
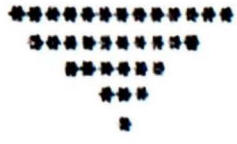


FIG. 3. STANDARD CURVE OF DIACETYL.

The concentration of diacetyl was determined by comparison with a standard curve of dimethyl glyoxime or diacetyl. Blanks used to Zero the colorimeter were sterile, uncultured non fat milk treated exactly as the samples being tested for diacetyl. The solution of glyoxime or diacetyl was prepared to give 100 ug diacetyl per milliliter. Different concentrations of diacetyl solutions were treated as sample and standard curve was plotted (Fig. 3).



CHAPTER IV

RESULTS

RESULTS

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1.0 Activity and flavour production by LF-culture :

Microscopic examination of 24 hrs old LF-culture smear stained for Gram reaction, showed gram positive cocci arranged in chains.

1.1 Activity :

When LF culture was inoculated in skim milk and incubated at 37°C for 3½ hrs, it produced 0.37% lactic acid. The grade of activity of the culture was found to be satisfactory.

1.2 Flavour production :

When the LF - culture was tested for flavour production for 30 min at room temperature, a slightly pink coloured ring formation was observed on the top of the liquid column indicating thereby, slight flavour production.

2.0 Milk - clotting and proteolytic activities of rennet :

The milk - clotting and proteolytic activities of Noury rennet from Mucor pusillus Lindt, Hansen's animal rennet and bacterial rennet from Bacillus subtilis K-26 are presented in Table - 1.

Table - 1 Milk-clotting and proteolytic activities of different milk clotting enzymes.

S.No.	Milk Coagulant	Milk-clotting activity (units).	Proteolytic activity (ug tyrosine)	MCA/PA
1.	Noury Rennet (Mucor pusillus Lindt)	3.334	120	0.028
2.	Hansen's animal rennet	3.158	68	0.046
3.	Bacterial rennet (B. subtilis K-26)	4.000	190	0.021

MCA Unit : Amount of enzyme required to clot 10 ml of milk at $30^{\circ}C$ in 60 sds.

Bacterial rennet had high milk-clotting (4.000 units) and proteolytic activities (190 ug tyrosine) followed by mold rennet (3.334 units and 120 ug tyrosine) and animal rennet (3.158 units and 68 ug tyrosine). The ratio of milk-clotting activity to proteolytic activity was more for animal rennet (0.046) followed by mold (0.028) and bacterial (0.021) rennets. Hence, it was noted that the mold rennet was intermediary in its milk-clotting and proteolytic

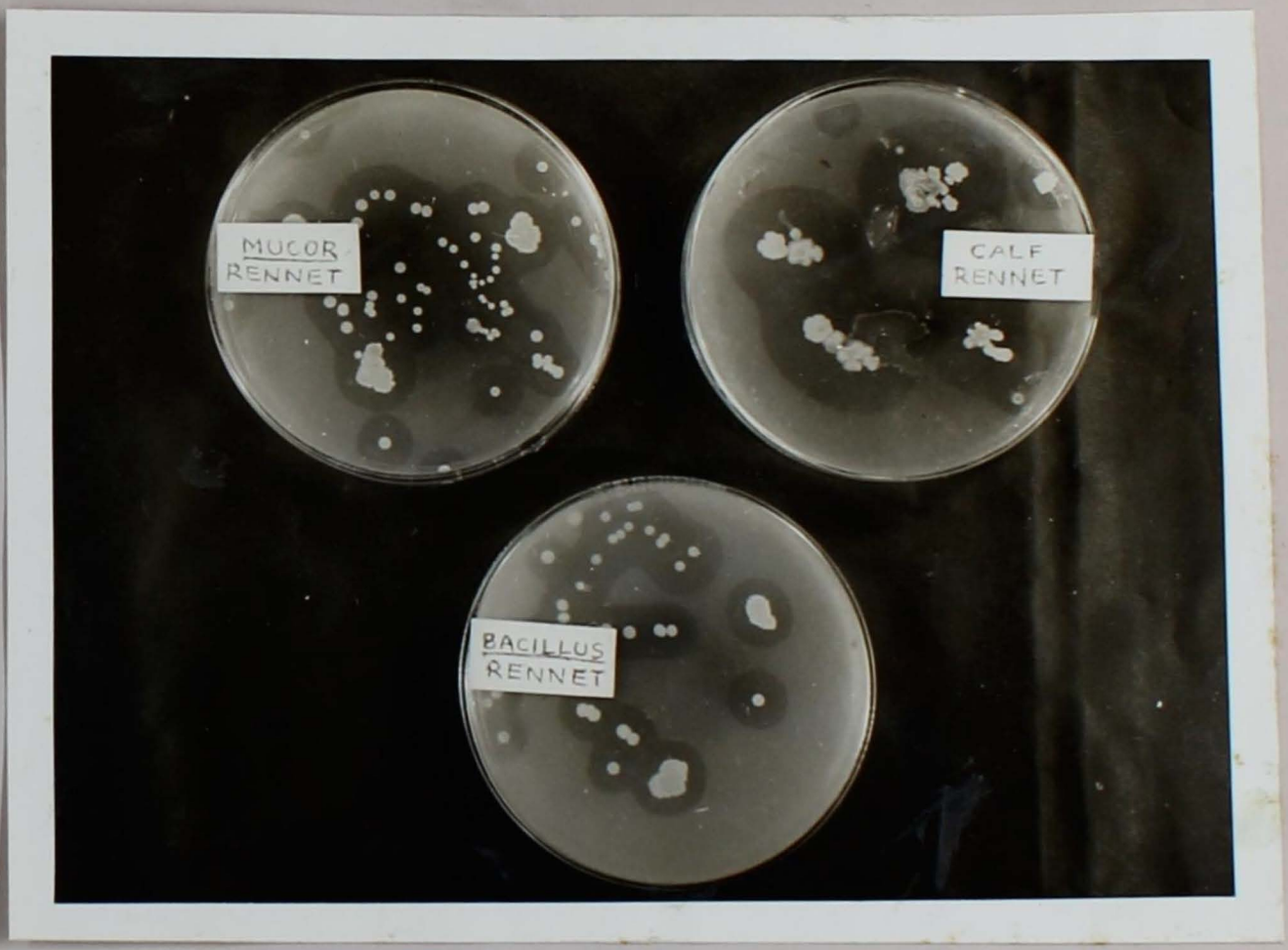
activities as compared to that of animal and bacterial rennets.

3.0 Effect of rennet on lipolytic activity of Pseudomonas fragi :

The effect of rennet on the lipolytic activity of Pseudomonas fragi is shown in Fig.4. The extent of lipolysis with the incorporation of 50 ppm rennet was of the same order with both mold and bacterial rennet, wherein the lipolysis zone had a diameter of 1.2 cm and comparable to control. In the case of animal rennet the lipolysis zone with 50 ppm rennet incorporation was 1.8 cm. Hence, it was noticed that both mold and bacterial rennet had inhibitory effect on the lipolytic activity of the bacteria and on the extent of lipolysis.

4.0 Cheddar cheese making Schedule :

The schedule of Cheddar cheese preparation using standardized cow milk, 1.5% starter culture (LF) and Mucor pusillus rennet , the cheddaring time-table and ripening are presented in Table - 2. One hundred and ten litres of standardized milk yielded 13.8 kg of green cheese, and after milling, salting and pressing the Cheese yield was 11.4 kg. The time taken for cheddaring was 5 hrs and a titratable acidity of 0.38% lactic acid and pH of 5.36 were noted in the whey at the end of cheddaring.



Effect of rennet on lipolytic activity of Pseudomonas fragi.

Table - 2 Cheddar cheese manufacturing time-table.

S.No.	Processing step	Time		Period	
		From	To	hr.	min.
1.	Pasteurization of Standardized milk (63.5°C).	10.00AM	10.30AM	0 -	30
2.	Cooling of milk to 30°C.	10.30AM	11.00AM	0 -	30
3.	Starter action (1.5%)	11.00AM	11.15AM	0 -	15
4.	Rennet action	11.15AM	11.45AM	0 -	30
5.	Cooking	11.45AM	12.45 Noon	1 -	00
6.	Cheddaring	12.45 Noon	5.45 PM	5 -	00
7.	Milling & Salting (Yield of green cheese was 13.8 kg and salt added was 414 g).	5.45PM	6.00 PM	0 -	15
8.	Pressing (Pressed cheese wt. 11.4 kg).	-	-	24 -	00
9.	Hardening (at 8°C)	-	-	48 -	00
10.	Ripening (at 8°C)	-	-	(10 months).	

(110 litres of standardized milk comprising of 96 lites of cow milk and 14 litres of skim milk was used to maintain casein/fat ratio to 0.7).

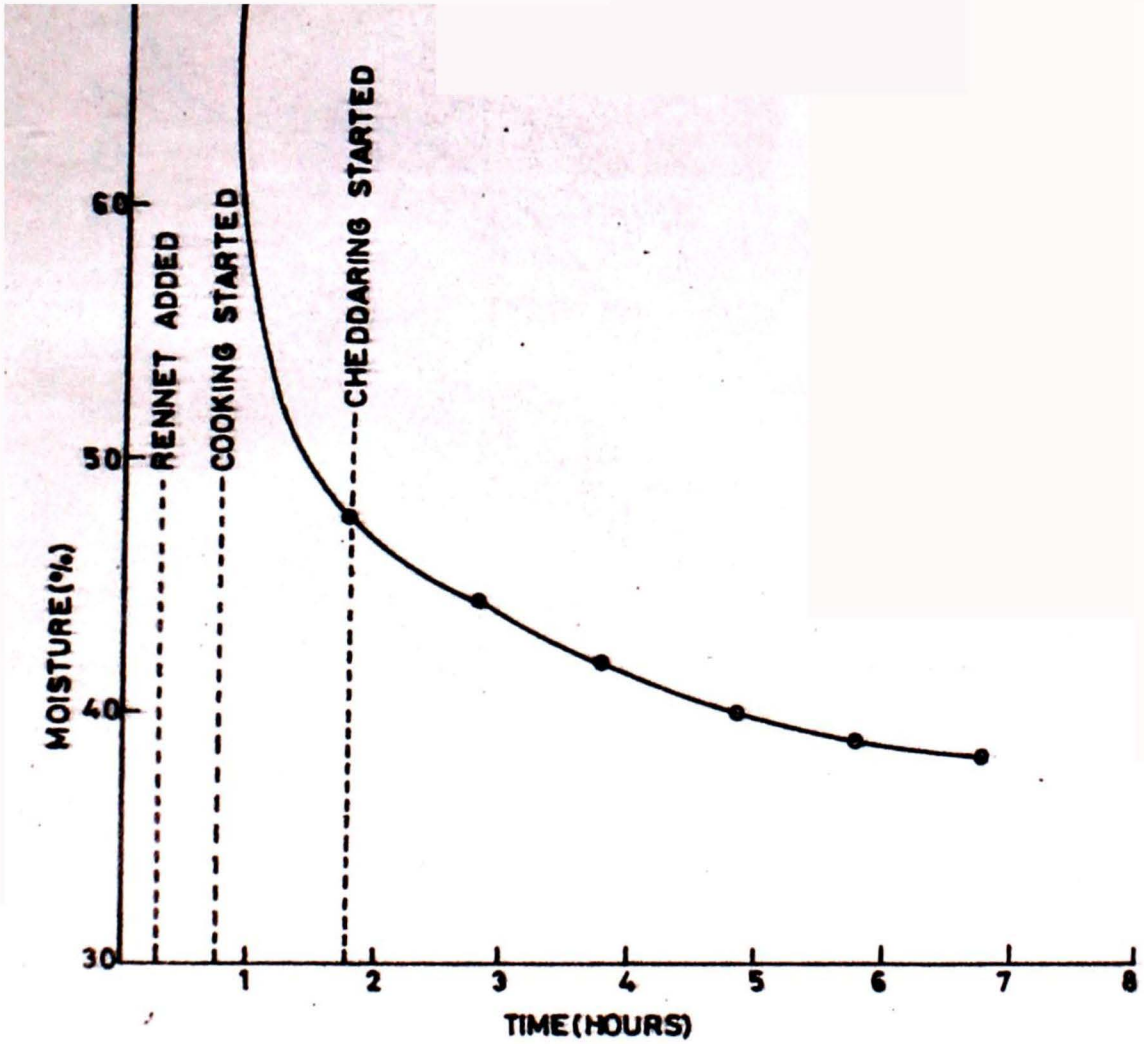


FIG.5a. EXPULSION OF MOISTURE FROM CHEESE CURD DURING INITIAL PREPARATION AND CHEDDARING OF CHEESE.

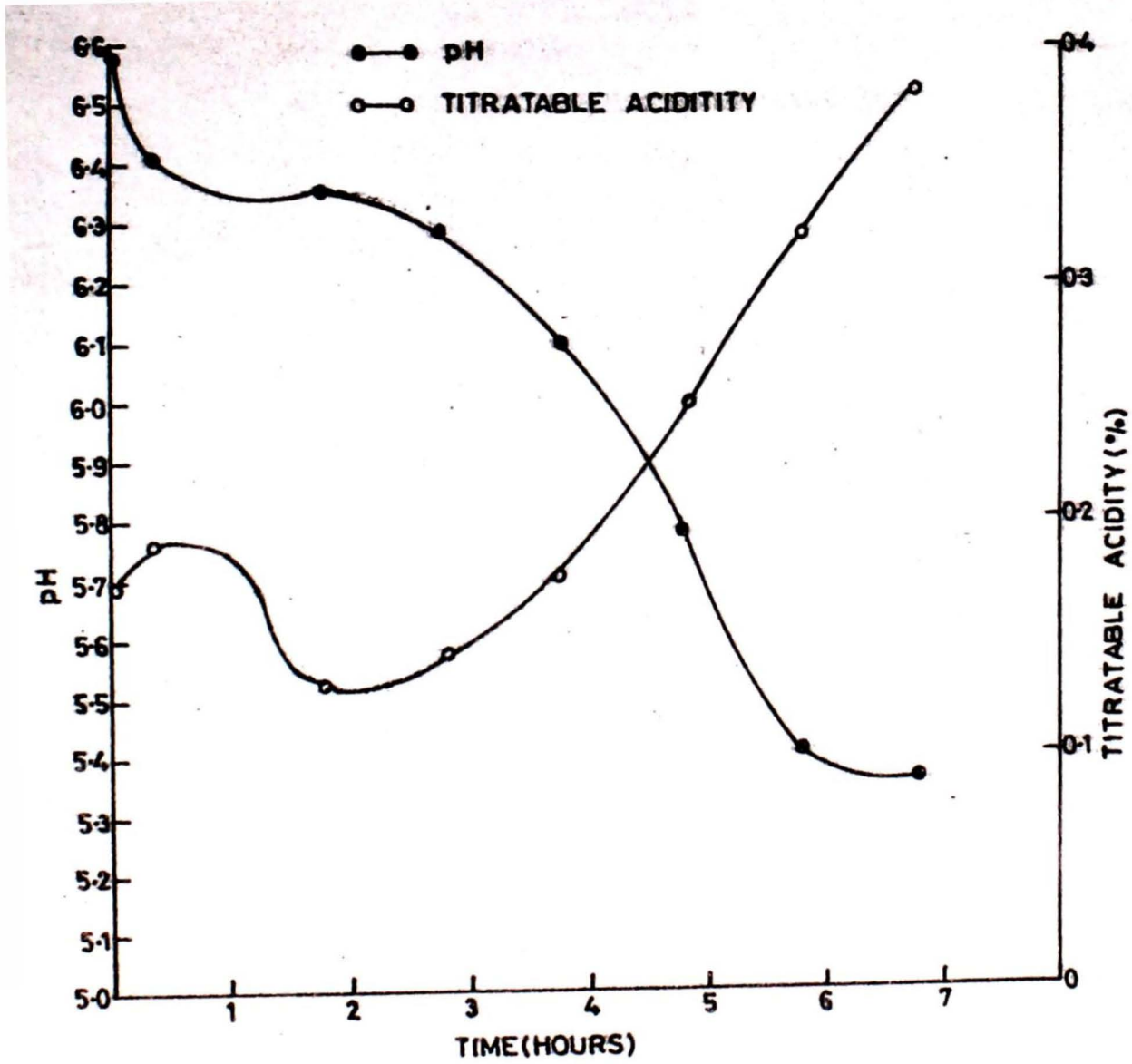


FIG. 5 b. CHANGES IN pH AND TITRATABLE ACIDITY DURING INITIAL PREPARATION AND CHEDDARING OF CHEESE.

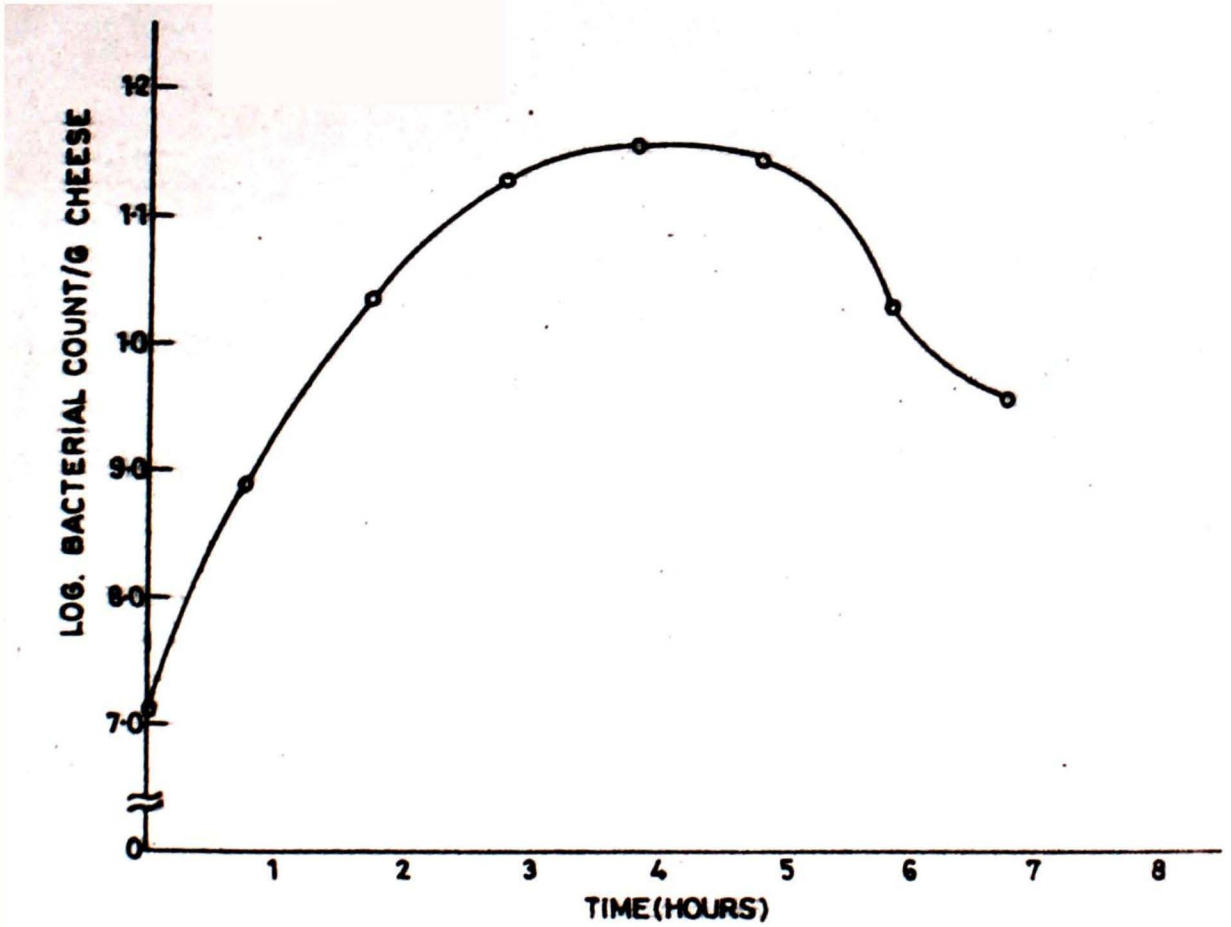


FIG.5c. GROWTH OF STARTER BACTRIA (LF) DURING INITIAL PREPARATION AND CHEDDARING OF CHEESE.

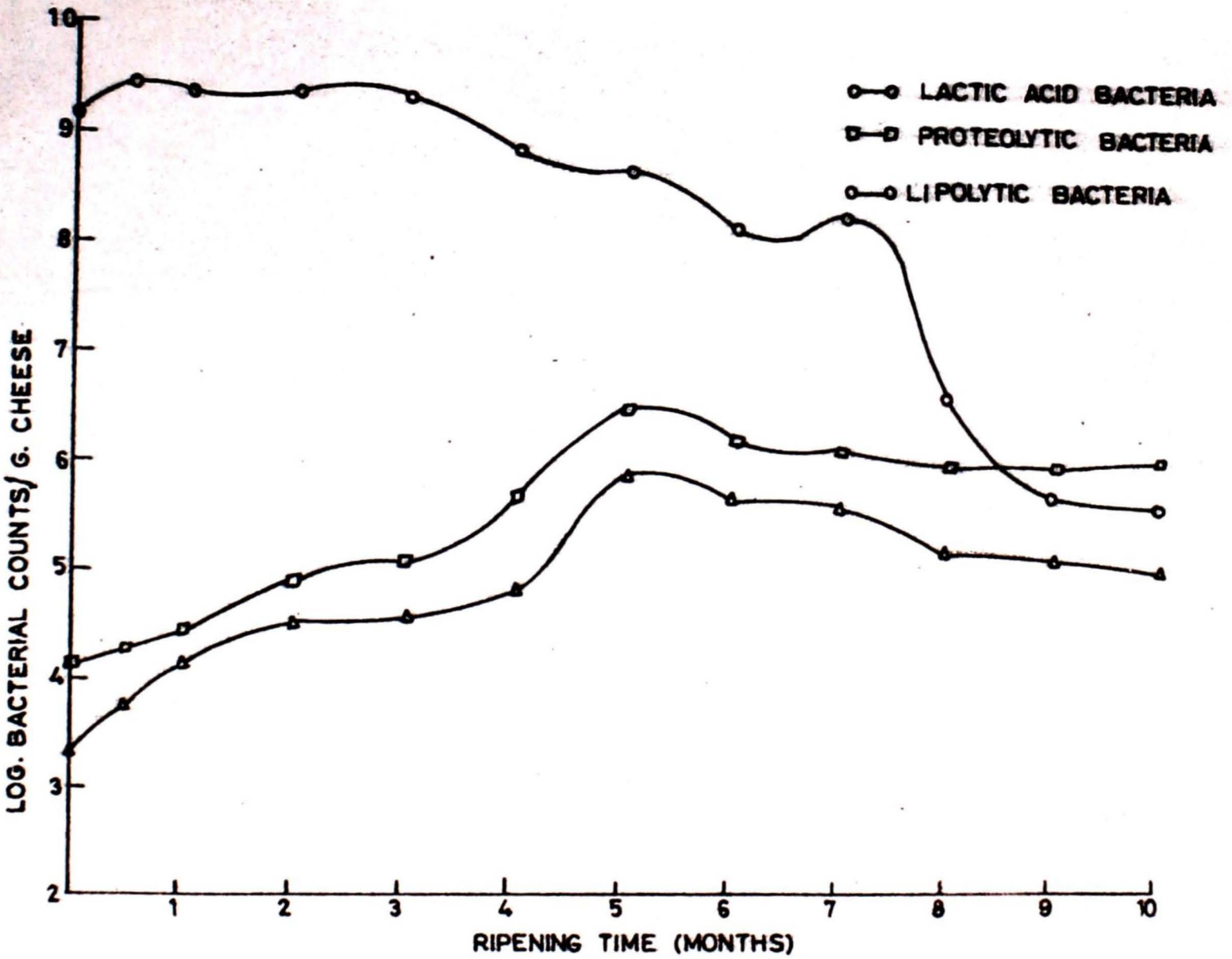


FIG. 6. INCIDENCE OF PROTEOLYTIC, LIPOLYTIC AND LACTIC ACID BACTERIA DURING CHEESE RIPENING.

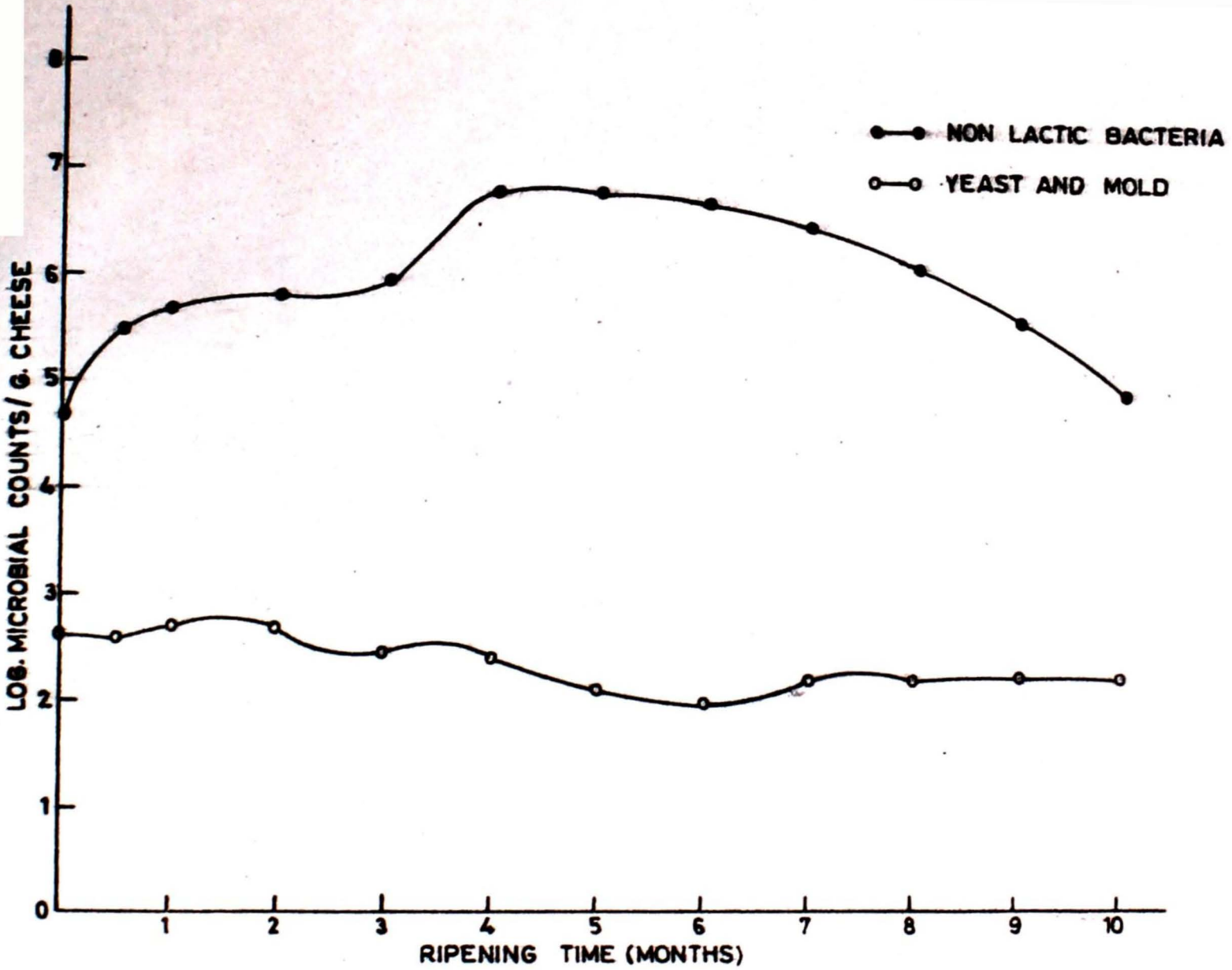


FIG.7. INCIDENCE OF NON-LACTIC BACTERIA AND YEAST AND MOLD DURING CHEESE RIPENING.

count of Cheddar cheese during ripening did not show much variation and ranged from 1.2 to 6.0 x 10^2 cfu/g cheese (Fig. 7).

7.0 Chemical analysis of cheese :

7.1 pH : Cheddar cheese had a pH of 5.30 at the beginning of ripening, after which the pH decreased to 5.06 during the first five months of ripening. In the later five months of ripening, Cheddar cheese showed a gradual increase in the pH to 5.30 (Table - 3). Cheddar cheese prepared with fungal rennet showed a pH of 5.30 at 10 months of ripening, whereas cheese with animal rennet showed slightly higher pH i.e. 5.43 at same stage of ripening (Table - 7).

7.2 Titratable acidity :

The titratable acidity during ripening of Cheddar cheese increased from 0.9% lactic acid at the beginning to 2.03% lactic acid at 10 months of ripening (Table-3). Animal rennet cheese had a titratable acidity of 2.10% lactic acid at 10 months of ripening (Table - 7).

7.3 Moisture :

As the period of ripening progressed, the moisture content of fungal rennet cheese decreased from the initial 37.75% to that of 34.0% at 10 months ripening (Table-3). Animal rennet cheese had a

slightly high moisture content (36.07%) at 10 months ripening stage than that of fungal rennet cheese (Table-7).

Table - 3 Chemical composition of Cheddar cheese during ripening.

Age/Months	Moisture (%)	pH	Titratable acidity(%)	Fat (%)
0	37.75	5.30	0.9	34.2
½	37.50	5.25	1.08	34.1
1	36.80	5.24	1.20	34.0
2	36.70	5.20	1.28	33.8
3	36.50	5.17	1.31	33.5
4	36.50	5.10	1.40	33.2
5	36.50	5.06	1.50	33.1
6	36.00	5.12	1.63	33.1
7	35.60	5.25	1.75	33.1
8	35.00	5.30	1.82	33.0
9	34.60	5.35	1.95	33.0
10	34.00	5.40	2.03	32.9

7.4 Fat Content :

Fat content of cheese during ripening slightly decreased from initial value of 34.2% to 32.9% at 10 months ripening (Table-3).

7.5 Proteolytic degradation in cheese :

7.5.1 The proteolytic activity of cheese at different stages of ripening is presented in Table-4. Cheddar cheese prepared with fungal rennet had 0.850 mg tyrosine per g cheese at the beginning of ripening, which increased appreciably to 5.720 mg tyrosine per g cheese at 10 months ripening (Table-4). In animal rennet cheese 5.0 mg tyrosine per g cheese was observed at 10 months ripening (Table-7).

7.5.2 The changes in different nitrogen compounds during ripening of Cheddar cheese are presented in Table-5. Total nitrogen of cheese remained more or less constant throughout the ripening period i.e. 6.28 to 6.46%, though there was a slight increase during 10 months ripening. As the ripening time advanced, there was a decrease in protein nitrogen and a steady increase of non-protein and ammonia nitrogen. At the end of 10 months ripening the non-protein and ammonia nitrogen contents of cheese were 1.50 and 0.42% as compared to 0.12 and 0.18% at the beginning of ripening, respectively. Cheddar cheese manufactured with calf rennet had 1.22% non-protein nitrogen and 0.268% ammonia nitrogen, which were slightly lesser than that of fungal rennet cheese (Table-7).

Table-4 Proteolytic and lipolytic activities of Cheddar cheese during ripening.

Age/Months	Proteolytic activity (mg tyrosine/g cheese)	Lipolytic activity (ug palmitic acid/g cheese)
0	0.850	38.0
½	0.950	40.2
1	1.520	45.20
2	2.450	50.25
3	2.875	55.60
4	3.620	65.00
5	3.850	78.56
6	4.044	98.62
7	5.000	109.68
8	5.437	123.44
9	5.625	131.00
10	5.720	137.12

Table-5 Changes in nitrogen constituents during Cheddar cheese ripening.

Age/Months	Total nitrogen (%)	Protein nitrogen (%)	Non-protein nitrogen (%)	Ammonia nitrogen (%)
0	6.28	5.66	0.62	0.180
½	6.35	5.47	0.88	0.190
1	6.39	5.39	1.00	0.210
2	6.38	5.33	1.05	0.243
3	6.40	5.20	1.20	0.250
4	6.39	5.18	1.22	0.260
5	6.39	5.11	1.28	0.275
6	6.40	5.07	1.32	0.290
7	6.41	5.03	1.38	0.320
8	6.40	5.00	1.40	0.340
9	6.42	4.97	1.45	0.380
10	6.46	4.94	1.50	0.420

7.6 Lipolytic activity :

There was increase in the lipolytic activity of the cheese during ripening from 38 to 137.12 ug palmitic acid per g cheese at the beginning and end of ripening, respectively (Table-4). At the end of 10 months ripening calf rennet cheese showed a lipolytic activity of 960 ug palmitic acid per g cheese, which was seven fold higher than Mucor rennet cheese (Table-7).

7.7 Flavour compounds :

7.7.1 Diacetyl : The diacetyl content of fresh cheese at the beginning of ripening was 0.85 ppm which decreased to 0.25 ppm at the end of 10 months ripening (Table-6). There was marked decrease in the diacetyl content up to 2nd month of ripening, after which there was only a slight decrease. The diacetyl content of animal rennet cheese and fungal rennet cheese at the end of 10 months ripening was more or less similar (Table-7).

7.7.2 Volatile acidity : The volatile acid content of cheese during ripening is presented in Table-6. The volatile acidity increased from 2.10 to 7.42 ml of N/10 NaOH per 50 g cheese during initial and final stages of ripening. The volatile acidity of 10 months ripened animal rennet cheese was 8.05 as compared to 7.42 ml of N/10 NaOH per 50 g mold rennet cheese (Table-7).

Table-6 Flavour constituents of Cheddar cheese during ripening.

Age/Months	Diacetyl (ppm)	Volatile acidity ml of N/10 NaOH/50g cheese
0	0.850	2.10
½	0.700	2.75
1	0.550	3.60
2	0.450	3.90
3	0.300	4.15
4	0.280	4.40
5	0.280	4.75
6	0.275	5.60
7	0.275	5.90
8	0.275	6.60
9	0.260	7.25
10	0.250	7.42

Table-7 Comparison of chemical composition, enzyme activity and organoleptic score of Cheddar cheese made by using mold (*M. pusillus* Lindt) and calf rennet. (10 months ripened)

Constituent	Mold rennet	Calf rennet
Moisture(%)	34.00	36.07
pH	5.40	5.43
Titratable acidity (%)	2.03	2.10
Fat (%)	32.90	30.60
Total nitrogen (%)	6.46	6.01
Protein nitrogen (%)	4.94	4.79
Non-protein nitrogen (%)	1.50	1.22
Ammonia nitrogen (%)	0.42	0.26
Proteolytic activity (mg tyrosine/g cheese)	5.72	5.00
Lipolytic activity (ug palmitic acid/g cheese)	137.12	960.00
Diacetyl (ppm)	0.250	0.225
Volatile acidity (ml of N/10 NaOH/50 g cheese)	7.42	8.05
Organoleptic Score		
Flavour (45)	37.37	37.50
Body & Texture(30)	25.75	27.25
Finish (15)	15.00	15.00
Colour (10)	9.37	9.37
Total (100)	87.49	89.12

7.8 Organoleptic evaluation of Cheddar cheese :

The Cheddar cheese made from both mucor rennet and calf rennet was compared after 10 months ripening. The body, texture and flavour of both the cheeses were comparable and organoleptically acceptable. There was no bitterness or rancidity in the cheeses. The organoleptic score of the mold and calf rennet cheeses are given in Table-7. Mold and calf rennet cheeses had a total organoleptic score of 87.49% (ranged 84 - 90%) and 89.12% (ranged 86 to 90.5%), respectively.

CHAPTER V

DISCUSSION

DISCUSSION

Manufacture of a good quality Cheddar cheese from cow milk depends on the use of an active starter culture, suitable rennet, appropriate cheddaring time-table and desirable changes during controlled ripening. The starter bacteria used in Cheddar cheese normally include single or mixed strains of streptococci or LF culture, which have the ability to produce lactic acid slowly and steadily from the lactose substrate. Eventhough, the calf rennet has been widely used as the coagulating enzyme for cheese in many countries, attempts have also been made to find rennet substitutes of microbial and plant origin(Ryu, 1974; Christensen, 1979). The cheddaring time table has to be standardised for appropriate acid production, formation of a good body and texture and extent of green curd recovery. The cheese after salting, pressing, hardening and waxing is kept for ripening in the cold store (8°C) over a period of 8 to 10 months for the development of desirable chemical and microbiological changes, so that the ripened cheese is organoleptically acceptable. Hence, in the present study the Cheddar cheese was prepared by using LF culture, Mucor pusillus rennet and the microbial and chemical changes during cheddaring and ripening have been reported.

Microscopic examination of LF-culture after gram staining indicated the presence of gram positive cocci, arranged in chains.

The LF-culture appeared to be a mixed culture of Streptococcus spp., on the basis of cell size, arrangement and chain length. Single strain culture or mixture of single strains of Streptococcus lactis, S. cremoris and S. lactis sub sp. diacetylactis have been used for satisfactory production of cheese (Dutta et al., 1971 ; Holzapfal and Kriel, 1973; Legg, 1973; Frzinkyan et al., 1975; Kirov and Chamakov, 1975; Stevic et al., 1975; Mostert and Husmann, 1976; Gruev, 1977; Butkus et al., 1978; Dolezalek and Fichtervoa, 1978; Limsowtin, 1978; Petterson, 1978; Sharpe, 1979).

The LF-culture was found to be satisfactorily active with the production of 0.37% lactic acid as per the grading of Horral and Kllicker (1947). Hence, it is evident that the culture tested can produce sufficient lactic acid during cheese manufacture and ripening, which is of a paramount importance in cheese production.

Lactic acid bacteria produce traces of flavour and aroma compounds, apart from the production of lactic acid (Manning et al., 1976). A slightly pink coloured ring formation by the culture in the flavour production test, suggests the presence of low level of flavour compounds in the culture. These flavour compounds normally include diacetyl, acetoin, acetaldehyde, etc. The presence of flavour compounds in low concentration in the culture indicate that these compounds might be produced by the LF culture.

Milk-clotting and proteolytic activities of Mucor pusillus rennet, Hansen's animal rennet and bacterial rennet (B. subtilis K-26) have been compared and it was noted that the bacterial rennet showed high milk-clotting and proteolytic activities followed by fungal and animal rennets (Table-1). Rao and Mathur (1979) attributed the milk-clotting activity of bacterial rennet from B. subtilis K-26 to the enzyme like rennin or bacterial protease present in the crude enzyme preparation. Khan et al. (1979) demonstrated the presence of two major acid proteases in rennet from Mucor pusillus Lindt, one of which had significant rennin-like activity, whilst the other lacked it. Milk-clotting activity of Moury rennet can be attributed to the presence of this rennin-like enzyme. Milk-clotting activity of these rennets can be attributed to the destruction of phenylalanine-methionine bond in k-casein, and the destruction of the stabilizing action of k-casein against precipitation. Though, these three milk-clotting enzymes differ in their milk-clotting activity, they are believed to show same specific action on k-casein (Yu et al., 1968).

Proteolytic activities of the three rennets tested can be attributed to the presence of proteases. Rao and Mathur (1979) ascribed the proteolytic activity of bacterial rennet to the presence of proteases in the crude preparation. The proteolytic activity of Moury rennet can be attributed to the presence of

2 to 4 acid proteases (Green and Stackpoole, 1975; Edelsten and Jensen, 1970; Khan et al., 1979).

Milk-clotting and proteolytic activities of Mucor pusillus Lindt protease were 3.334 units and 120 ug tyrosine, respectively, and were reportedly closer to the values obtained by Iwasaki et al. (1967). Mucor protease and bacterial rennet were found to be more proteolytic than that of calf rennet (Mickelsen and Fish, 1970, Rao and Mathur, 1979).

The ratio of milk-clotting to proteolytic activity of Mucor rennet is nearer to that of calf rennet than bacterial rennet. Iwasaki (1967) also found that Mucor rennet had closer milk-clotting to proteolytic activity ratio to that of calf rennet. As the Noury rennet showed closer milk-clotting to proteolytic activity ratio to that of calf rennet, it may be expected that the yield of cheese and retention of fat by the curd may be more or less similar to that of calf rennet. If the proteolytic activity of rennet is more or milk-clotting to proteolytic activity ratio is less than that of calf rennet, the yield of cheese and retention of fat by the curd may be diminished (Veringa, 1961; Reitter, 1970). But the bacterial rennet exhibited excessive proteolytic activity and less ratio of milk-clotting to proteolytic activity than calf rennet.

The bacterial and mold rennet caused inhibition of lipolytic activity of Pseudomonas fragi in tributyrin agar, whereas the calf rennet did not show any such inhibition. Brandl (1970) reported that the animal rennet and Endothia parasitica rennet caused no inhibition of butter fat hydrolysis in a staphylococcal culture, but the Mucor pusillus rennet did and at pH 5.5 and 6.5, Bacillus subtilis crude proteinases resulted in a definite inhibition of lipase activity. The action of the rennet on the extent of lipolysis might influence the butter fat hydrolysis during the ripening of the cheddar cheese.

The yield of cheddar cheese manufactured with Mucor pusillus Lindt rennet is 10.36%, which is closer to that of 10 - 11% yield with calf rennet. This can be attributed to the similar milk-clotting to proteolytic activity ratio of Mucor and calf rennets.

After 15 min LF-culture activity (1.5%) in pasteurized milk, the titratable acidity of milk increased from 0.17% to 0.19% lactic acid and the pH decreased from 6.57 to 6.40. This increase in titratable acidity is possibly due to the growth of LF-culture in the milk and lactic acid in the inoculum. It is a well known fact that the starter bacteria utilize lactose and produce lactic acid. During cheddaring, increase in titratable acidity is positively

correlated with the multiplication of starter bacteria (Figs. 5 b, c).

The moisture was removed gradually in the cooked curd initially from 47.95 to 38.50% at the end of cheddaring. The increase in acidity of cheese curd results in the removal of whey or moisture from the curd during cheddaring. If the acid production is faster, the removal of whey is faster, which results in the yield of hard cheese (Dolby, 1941). The time taken for cheddaring was five hrs with a titratable acidity in whey of 0.2% lactic acid in the cheese prepared with Rowry rennet. This acid production was gradual during 5 hrs time. Hence, the final quality of cheese obtained was not hard, rather it had sufficient moisture (38.5%) to give proper body and texture to the Cheddar cheese.

Starter culture (LF) multiplies in the cheese curd at a rapid rate, within 2 hrs 45 min, after inoculation, and then attained a stationary phase followed by decline phase. The decrease in number of starter bacteria at the latter stage might be possibly due to reduction in the level of lactose, a readily available nutrient, reduction in the moisture content of cheese curd, etc.

At the end of cheddaring, cheese curd had elastic or rubbery texture. This elastic property of the cheese curd can be attributed

to the presence of monocalcium paracasinate. Casein is said to exist in rennet curd as diacalcium paracasinate. When lactic acid is formed by the starter bacteria, it reacts with diacalcium paracasinate to give mono calcium paracasinate and subsequently free paracasein, which imparts elastic properties to the cheese curd (Foster et al., 1955).

During ripening, the lactic acid bacteria initially increased in numbers upto 3rd month, after which a decline in number was observed (Fig. 7). During the course of cheese ripening, the lactic acid content in cheese also increased. The possible factors attributable to the decline of growth of lactic acid bacteria are reduction in the level of lactose and increase of lactic acid production (1.50 to 2.03%). The lactic acid produced by the starter bacteria in the initial stages of cheese ripening affected the growth of those bacteria in the latter period of cheese ripening (Davis, 1965). Dawson and Feagan (1957) reported that the S. lactis grew to maximum level in first 8 weeks of ripening, after which it showed a gradual decline in the growth.

Since the Moury rennet (Mucor pusillus Lindt) showed considerably higher proteolytic activity(120 ug tyrosine) , this milk coagulant might have caused some proteolysis of cheese. Rennet

hydrolyzed proteins primarily to smaller peptides, which underwent further breakdown to free amino acid level by starter bacteria (Lee, 1974, O'Keefee et al., 1978).

Proteolytic bacterial count increased initially up to 5 months of ripening and further showed no increase in number. The bacteria having proteolytic activity can utilize protein as main nutrient in the cheese and degrade them into water soluble nitrogen compounds like free tyrosine and ammonia.

The breakdown of protein or increase in the amount of breakdown products can be correlated with the growth of proteolytic bacteria. The proteolytic bacteria possess intracellular and extracellular proteases which bring about proteolytic degradation in cheese (Vadhera and Boyd, 1963; Dulley, 1974). Proteolytic bacteria releases extracellular proteases during the first five months of ripening and intracellular proteases due to autolysis of cells in the later stage of cheese ripening. These extracellular and intracellular proteases act on the partially degraded peptides formed due to the rennet action (Peterson et al., 1948; Vadhera and Boyd, 1963; Williamson et al., 1964). It can also be stated that these enzymes can directly degrade the milk protein into smaller compounds (Visser, 1977).

The decrease in protein nitrogen of Cheddar cheese can be attributed to the proteolytic breakdown by rennet, starter bacteria and other proteolytic organisms. The increase in non-protein nitrogen,

tyrosine and ammonia can be correlated with the hydrolytic activity of the rennet, extracellular and intracellular proteases during cheese ripening.

Accumulation of ammonia is possibly due to deamination of free amino acids by deaminases produced by starter bacteria (Desmazeud, 1978). The decrease in the pH from 5.30 to 5.06, is attributable to the production of lactic acid, whereas, the increase in pH from 5.06 to 5.40 to the accumulation of ammonia in the Cheddar cheese.

Due to the action of deaminases on free amino acids, ammonia and free fatty acids are said to be liberated (Desmazeud, 1978). The increase in the volatile acids can be partially attributed to the release of these free fatty acids by the action of deaminases.

Mold rennet cheese showed comparable levels of non-protein nitrogen, tyrosine and ammonia with that of animal rennet cheese. High levels of non-protein nitrogen (1.50%), tyrosine (5.720 mg/g cheese) and ammonia (0.420%) in mold rennet cheese than that of animal rennet cheese (1.22%, 5.0 mg per g and 0.268%, respectively), indicates much greater proteolytic breakdown in mold rennet cheese with LF- culture than that of animal rennet cheese.

Lipolysis in cheese is said to be brought about by starter bacteria. The lipolytic bacteria in cheese showed greater increase in number in first five months of cheese ripening and later they did

not grow much. Bacterial lipase hydrolyze the fat in cheese only after 15 - 30 days or so of ripening (Davis, 1965; Peterson et al., 1948). The decline in the growth of lipolytic bacteria in the second half of the cheese ripening period can be attributed to the lactic acid production and to the fact that lactic starter or lipolytic bacteria can not proliferate much when free fatty acids, especially lower chain fatty acids, are present in sufficiently high concentrations.

As a result of bacterial lipase action, free fatty acids are released continuously throughout the ripening period, from 38.0 to 137.12 ug of palmitic acid per g cheese. Lipolytic bacteria could hydrolyze milk fat, during ripening from triglycerides to di-and monoglycerides and further to free fatty acids(Stadhouders and Veringa, 1973). Increase in volatile acids in cheese can be attributed to the release of short chain fatty acids by microbial lipases (Iwasaki and Kosikowski, 1973; Umemoto and Sato, 1975).

Mold rennet cheese at 10 months ripening stage showed the lipolytic activity of 137.12 ug palmitic acid per g cheese, whereas, animal rennet cheese showed quite high lipolytic activity (960.00 ug palmitic acid per g cheese). Low lipolytic activity of fungal rennet cheese can be attributed to the inhibitory effect of this rennet on microbial lipase, as shown in Fig.4. As it was noted that 28.8% of rennet is present in cheese, a considerable effect on microbial

lipase is expected. Brandl (1970) reported that rennet from Mucor pusillus Lindt could show inhibitory action on microbial lipase, apart from its low lipolytic activity.

Diacetyl is one of the major flavour components in both animal and fungal rennet cheeses (Calbert and Price, 1948; Bassette and Harper, 1958; Day and Keeney, 1958; Patton et al., 1958). Calbert and Price (1948) reported that 0.5 ppm of diacetyl is the threshold in Cheddar cheese to give proper flavour. In fungal and animal rennet cheeses, almost comparable quantities of diacetyl i.e. 0.250 ppm and 0.225 ppm in cheese respectively, were observed at the end of 10 months ripening. The diacetyl might have contributed to the Cheddar cheese flavour. A decrease in the diacetyl content from 0.85 ppm to 0.30 ppm in first three months of ripening might be due to reduction of diacetyl to acetyl methyl carbinol by starter bacteria. Diacetyl in the fungal rennet cheese might have been produced by the starter inoculum.

Volatile acidity in the cheese was 2.10 ml of N/10 Sodium hydroxide/50 g cheese at the start of ripening, which increased gradually to 7.42 ml of N/10 NaOH/50 g cheese at 10 months of ripening. The volatile acids are produced by proteolysis (deamination) (Desmazeud, 1978), lipolysis (Harper, 1959; Stadhouders and Veringa, 1973) and lactose metabolism (Suzuki et al., 1910) by starter bacteria.

Both animal and fungal rennet cheeses had comparable volatile acidity contents (8.05 and 7.42 ml, respectively). Since the lipolytic activity of fungal rennet cheese is very much less, the volatile acids might have been produced by proteolysis i.e. due to deamination of free amino acids. Considerably high proteolytic activity and high amounts of ammonia in fungal rennet cheese suggest that sufficiently high amount of free fatty acids might have been produced due to proteolysis in fungal rennet cheese.

The yeast and mold count was low in cheese during ripening (1.2 to 6.0 x 10^2 cells/g). The fungal population also might have contributed partly to the proteolytic and lipolytic breakdown in Cheddar cheese during ripening.

The non - lactic acid bacteria occurred in appreciably high numbers in Cheddar cheese during the first four months of ripening and decreased further. Mattick and Hiscox (1939) ascribed high levels of fatty acids present in cheese to the presence of large numbers of non-lactic bacteria. Alford and Frazier (1950) emphasised that non-lactic bacteria, especially micrococci might be involved in the ripening of cheese.

The organoleptic evaluation of the mold rennet cheese ripened for 10 months was comparable to that of calf rennet cheese. The mold rennet cheese did not show any rancidity or bitterness. The cheeses made with Mucor pusillus rennet have been reported to be identical in all respects to those made with calf rennet (Wigley, 1974; Pien, 1976). Hence, it is concluded that a good quality Cheddar cheese can be manufactured using Mucor pusillus rennet and LF - culture with standardised techniques of cheddaring and ripening.

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CHAPTER VI

SUMMARY

SUMMARY
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Cheddar cheese was prepared using LF-culture and Noury rennet from Mucor pusillus Lindt and ripened for 10 months. The mold rennet had the milk-clotting and proteolytic activities of 3.334 units and 120 ug tyrosine/ml of 1% rennet solution, respectively. The LF culture produced 0.37% lactic acid in skim milk. It was found that during cheddaring, 28.8% of the total rennet added to standardised milk was trapped in curd and the rest in whey. The mold rennet exhibited inhibitory effect on lipase of Pseudomonas fragi.

During the first two hours of cheddaring, the lactic acid bacteria multiplied in logarithmic rate reaching a population of 3.2×10^{11} cells/g curd, and 0.38% lactic acid was produced in the cheese curd during 5 hrs of cheddaring. The moisture expulsion in the curd was gradual after cooking and the salted curd had a moisture content of 38.32%.

During ripening, the lactic acid bacteria increased in number initially for 15 days and showed a further decline in the growth. Lactic acid was produced gradually throughout the ripening period from 0.90 - 2.03%. The number of proteolytic and lipolytic bacteria increased appreciably during 5 months of ripening of Cheddar cheese and further slightly decreased. Non-lactic bacteria showed appreciable growth in first four months of ripening after which they decreased in number. Yeast and mold count was less and did not increase much in number during ripening of cheese.

There was gradual increase in the proteolytic activity during ripening which ranged from 0.850 to 5.720 mg tyrosine/g cheese. Mold rennet cheese showed high amount of proteolytic breakdown than that of calf rennet cheese after 10 months of ripening. Mold rennet cheese showed good extent of protein breakdown, with the accumulation of non-protein nitrogen and ammonia in the cheese, which was higher than that of animal rennet cheese. Mold rennet cheese at 10 months ripening had a lipolytic activity of 137.12 ug palmitic acid/g, as compared to 960.00 ug palmitic acid/g animal rennet cheese.

Both mold and calf rennet cheeses showed similar amount of volatile acids and diacetyl after 10 months ripening. As the ripening period advanced, volatile acids content increased in mold rennet cheese, which ranged from 2.10 to 7.42 ml of N/10 NaOH/50 g cheese. The mold rennet cheese after 10 months ripening had a comparable body, texture and flavour to that of calf rennet cheese. It did not show any off-flavours like bitterness or rancidity.

It is concluded that Cheddar cheese can be prepared satisfactorily with the microbial rennet from Mucor pusillus Lindt with 10 months ripening period and the ripened cheese was organoleptically acceptable and comparable to calf rennet cheese.

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CHAPTER VII

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