ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PSYCHROTROPHIC SPORE FORMERS IN PAST-EURIZED MILK AND THEIR EFFECT ON KEEPING QUALITY OF STEAMED AND STERILIZED MILK

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN

DAIRYING

(QUALITY CONTROL)

TO

THE KURUKSHETRA UNIVERSITY, KURUKSHETRA

By

SHILPA NAGPAL

DIVISION OF DAIRY BACTE
NATIONAL DAIRY RESEARCH
(I. C. A. R.)
KARNAL (Haryana) INDIA

1987

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PSYCHROTROPHIC SPORE FORMERS IN PAST-EURIZED MILK AND THEIR EFFECT ON KEEPING QUALITY OF STEAMED AND STERILIZED MILK

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN

DAIRYING

(QUALITY CONTROL)

TO

THE KURUKSHETRA UNIVERSITY, KURUKSHETRA

By

SHILPA NAGPAL

DIVISION OF DAIRY BACTERIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL (Haryana) INDIA

1987

Registration No. 80-ap-380

Dedicated

TO

My Beloved Parents

Ashit & Shikha

Dr. T.S. Sudarsanam,
I.D.D.(Tech.), M.Sc. (Dairying), Ph.D.(Dairying)
Scientist S-2

DIVISION OF DAIRY MICROBIOLOGY,
NATIONAL DAIRY RESEARCH INSTITUTE,
(I.C.A.R.)
KARNAL-132 001 (HARYANA) INDIA.

Dated: 19 May, 1987

This is to certify that work reported in this dissertation entitled " ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PSYCHROTROPHIC SPORE FORMERS IN PASTEURIZED MILK AND THEIR EFFECT ON KEEPING QUALITY OF STEAMED AND STERILIZED MILK" was carried out by Miss Shilpa Nagpal under my guidance and direct supervision at this Institute in partial fulfilment for her M.Sc. Dairying (Quality Control) course.

(T.S. SUDARSANAM)

ACKNOWLEDGEMENTS

I feel unique pleasure in extending my sincere thanks, admiration and ineffable gratefulness to Dr. T.S. Sudarsanam, Scientist S-2 for his benevolent and sustained guidance, constant encouragement and constructive criticism during entire period of the investigation.

Honourable thanks are extended to the Director, Joint Director, Head of Dairy Microbiology Division, N.D.R.I., Karnal.

I owe profound thanks to Dr. D.N. Prasad, ex-Head of Dairy Microbiology Division, Dr. P.K. Aggarwal, Dr. Rattan Chand for sincere encouragement throughout the course of study.

Words fail to express my thanks to my colleagues and friends Aradhana, Archana, Veena for their kind help and cooperation. It would be remise of me, if I forget to mention the valuable inspiration, love, everlasting affection, encouragement given by Shikha and Ranju Khurana.

I gratefully acknowledge Mrs. Shakuntla Devi for her timely help.

Thanks are extended to Mr. Jagdish for his pains who has really given better shape of the wording written as Typist.

At least but not least I shall be failing if I donot express my profound feelings with regard and affection to my parents, brother Ashit and sister Shikha for their help, moral support and continuous encouragement throughout my time of study.

Shilpa

(SHILPA NAGPAL)

Dated
19/5/87

C O N T E N T S

.....

LIST OF TABLES

...Contd... LIST OF TABLES

.....

LIST OF FIGURES

......

CHAPTER I

INTRODUCTION

1. INTRODUCTION

The term 'psychrotrophs' is referred to those microorganisms which can multiply at a temperature of 7°C or less irrespective of their optimal growth temperature (International Dairy Federation, 1974). Eddy (1960) reported that psychrotrophs can grow well at 5°C or lower, but whose optimal growth temperature is 20-30°C.

Mostly the psychrotrophs are heat sensitive and are killed during pasteurization of milk hence, do not cause deterioration of milk if proper care is taken to avoid post-processing contamination. But psychrotrophic spore formers can survive heat treatments much higher than commercial pasteurization or even UHT treatment of milk. Psychrotrophic habitation is found in bacteria, actinomycetes, yeasts, fungi, algae and protozoa. Till recently, psychrotrophic bacteria were believed to occur among the genera *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Aerobacter* and *Bacillus* (Lawrence, 1967 and Von Bocklemann, 1970), but more recent report indicates the presence of psychrotrophic lactic acid bacteria in pasteurized milk (Sudarsanam and Srinivasan, 1982) and psychrotrophic spore formers in pasteurized milk (Uplacksh and Sudarsanam, 1986).

With the advent of UHT processing, aseptic packaging and improved methods to control post-pasteurization contamination, the psychrotrophic spore formers are emerging as the major spoilage organisms in heat processed milk and other dairy foods. Earlier, psychrotrophic strains of the geneus Bacillus were isolated from soil and water. But recent reports indicate psychrotrophic spore formers are found in both raw and pasteurized milk held for long periods at refrigeration temperature of 0 to 7^{o}C. Spores of psychrotrophic organisms resistant to pasteurization treatment and capable of subsequent outgrowth under refrigerated storage have been reported in pasteurized, sterilized, aseptically packaged products and in raw milk.

The psychrotrophs are very important group of micro-organisms in the dairy industry as they are both proteolytic and lipolytic in nature and the development of a large population of these microorganisms in milk or in milk products can lead to a serious deterioration of its quality (Thomas, 1970; Thomas and Druce, 1971; Thomas and Thomas, 1973)

Growth of psychrotrophic bacteria in pasteurized milk can cause flavour and colour defects and can also cause serious spoilage problems (Law, 1979). Most gram-negative psychrotrophic bacteria are readily eliminated by pasteurization (Witter, 1961). However, many spore formers produce heat-resistant extracellular proteinases (Law, 1979; Cousin, 1982; Fairbairn and Law, 1986) and lipases (Stewart <u>et al</u>., 1975; Law, 1979; Cousin, 1982) and can withstand pasteurization temperatures and remain active in products made from the heat treated milk (Downey, 1980; Cousin, 1982; Deeth and Fitz-Gerald, 1983; Fairbairn and Law, 1986). Proteinases are associated with gelation of UHT, sterilized milk (Law et al., 1977), whereas lipases can produce flavour defects associated with fat breakdown in UHT milk (Law et al., 1976; Law, 1979; Downey, 1980; Cousin, 1982).

During refrigeration the psychrotrophic spore formers influence over the quality of pasteurized milk and caused spoilage. Our present investigation has been taken up with a view to study the action of different species of the psychrotrophic spore formers in the keeping quality of pasteurized milk. Little information is available on the heat resistant proteolytic psychrotrophic spore forming microorganisms isolated from pasteurized milk. Hence, the present project deals with the following objectives:

1. Isolation of proteolytic psychrotrophic sporeforming Bacillus from pasteurized milk samples.
2. Characterization and identification of the isolates.
3. Their influence over the quality of the steamed and sterilized milks.

: : : : :

: : :

:

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Definition of Psychrotrophs

More than a decade ago psychrotrophs in milk were called psychrophiles and were reported to be destroyed by proper pasteurization (Andrews *et al*., 1953; Witter, 1961) Foster in 1887, reported the existence of these bacteria at 0°C. Stokes (1963), stated that the psychrophiles can be observed at 0°C within one week, although the temperature where growth was most rapid was closer to 30°C or lower. Schmidt-Nielsen was the first to name these organisms as psychrophiles in 1902.

Most of the literature published before 1960 probable refer to psychrotrophs rather than psychrophiles. The term psychrophile should be implied only to those organisms whose optimum temperature of growth or minimum temperature of generation is achieved by low temperature (Eddy, 1960; Kandler, 1966) whereas, these bacteria actually grow better at higher temperature generally above 20°C (Muller, 1903). The organisms are in fact, cold tolerant rather than cold loving.

Mosell suggested that the word 'psychrotrophic' should be used for microorganisms able to grow on solid media at $5^{o}C$ or below regardless of their optimum growth temperature (Eddy, 1960; Otte et al., 1979).

Morita (1975) has defined psychrotrophic bacteria as microorganisms having an optimum growth temperature of $15^{o}C$, a maximum growth temperature of $20^{o}C$ and a minimum growth temperature of $0^{o}C$ or below. Witter (1961) reported that the psychrophilic bacteria grow at a relatively rapid rate at or below $45^{o}F$ ($7.2^{o}C$). Eddy (1960), defined the psychrotrophic (cold thriving) for microorganisms able to multiply at or below $5^{o}C$ irrespective of their optimum growth temperatures. This term has now come into general use.

The term psychrophile, psychrotrophs and cryophile are still used interchangeably by Microbiologists; however, psychrotrophic will be used to identify microorganisms able to grow at $7^{o}C$ or less, regardless optimum temperature and psychrophiles will be used for microorganisms that conform to Morita's definition (Morita, 1975). Shehata et al. (1971) reported that psychrophilic spore formers grew well within 2 weeks at $0^{o}C$ but they grew faster at 20 to $25^{o}C$.

2.2 Types of Psychrotrophic Microorganisms

Psychrotrophs are both ubiquitous and numerous in nature and include yeasts, molds and bacteria (Stokes, 1966). Psychrotrophic bacteria include long or short rods, cocci or vibrio, gram positive or gram negative bacteria. Sporeformers or non sporeformers and aerobic, facultative anaerobic or anaerobic organisms.

The main psychrotrophic microflora are gram negative rods with *Pseudomonas* spp. comprising of 50% of the genera, *Ps. fluorescens* predominates and other species include *Ps. putida*, *Ps. fragi*, *Ps. aeroginosa* (Cousin and Bramley, 1981). *Alcaligenes*, *Aeromonas*, *Acinetobacter*, *Flavobacterium* and coliform accounts for most of the remaining genera of psychrotrophic gram negative bacteria, although psychrotrophic species of gram positive genera, *Micrococcus*, *Lactobacillus*, *Bacillus* and *Arthrobacter* have been reported in raw milk usually in smaller numbers than gram-negative bacteria (Cousin, 1982). More recent reports indicate the presence of psychrotrophic lactic acid bacteria in pasteurized milk (Sudarsanam and Srinivasan, 1982). Early literature reported that the psychrotrophic microorganisms are predominately gram-negative, non-spore forming, catalase positive rods (Ingraham, 1962; Stokes, 1963).

Early investigators concluded that psychrotrophic bacteria do not survive laboratory or commercial pasteurization (Thomas, 1958; Witter, 1961; Thomas and Druce, 1969; Luck, 1972). However, presence of gram positive bacteria has become more important in pasteurized milk and dairy products because of the spore forming nature and production of heat stable extracellular enzyme. Witter (1961) reported that the most of the psychrotrophs are heat sensitive in nature. ~~Carol~~ Credit _et al_. (1972) reported that 84% of the bacteria isolated from commercially pasteurized milk held at 4.5°C for 30 days were belonging to genus Bacillus and rest belonging to genera _Micrococcus_, _Microbacterium_, _Achromobacter_ and _Alcaligenes_.

Later on Stokes (1966) isolated psychrotrophic organisms from mud which were incredibly heat resistant . Because of the heat resistant nature of these microorganisms they have been linked with the possible spoilage of the dairy products (Grosskopf and Harper, 1969; Credit _et al_., 1972; Mikolajcik, 1978). Shehata and Collins (1971) reported the psychrotrophic spore formers in raw milk. Grosskopf and Harper (1974) reported psychrotrophic spore forming bacteria relating _Bacillus_ spp. in pasteurized milk. Sharma _et al_. (1984) reported psychrotrophic spore forming bacilli from raw milk. Malik and Mathur (1983) reported proteolytic psychrotrophs predominately _Pseudomonas_, _Flavobacterium_, _Alcaligenes_, _Micrococcus_ and _Staphylococcus_ spp. from raw milk, raw cream, ice cream mix, butter, flavoured milk and paneer. Kroll _et al_. (1984) also identified proteolytic psychrotrophic bacteria in raw milk as _Ps_. _fluorescens_, _Flavobacterium_, Streptococcus faecalis subsp. liquefaciens and Serratia sp. Chug and Cannon (1971) reported that 83.3% of raw milk samples contained 2 to 900 spores/ml. Later, Coghill (1982) observed the thermoduric psychrotrophic organisms isolated in raw and pasteurized milk belong to genera Bacillus, Pseudomonas and Corynebacterium which are probably due to post-pasteurization contamination. El-Bassiony et al. (1985) reported thermoduric psychrotrophs from laboratory pasteurized milk as 81.2% Bacillus sp., 12.5% as Micrococcus and 6.2% Streptococcus spp. and distribution of psychrotrophs in raw milk was 27.8%, Pseudomonas , 22.2% of Coliform, 14.4% Proteus, 13.3% Alcaligenes, 11.1% Bacillus, 5.6% Flavobacterium, 3.3% Micrococcus and 2.2% Streptococcus sp.

2.3 Temperature

2.3.1 Growth Temperature

Elliot and Michener (1965) reported that the optimum growth temperature for the psychrotrophs is 20 to 30°C, although they can grow at low temperature close to 0°C. The minimum growth temperature for psychrotrophic bacteria has been reported as -10°C (Ingraham and Stokes, 1959; Thomas, 1966). Kraft and Ray (1979) stated minimum growth temperatures as -10°C for most bacteria, -12°C for most yeasts and -18°C for most molds. The maximum growth temperature for the psychrotrophs is reported as 30°C with some having maxima of 37°C to 45°C (Ingraham and Stokes, 1959; Higoshi et al., 1975).

Normally growth at a low temperature is characterised by a long lag phase and a slow logrithmic phase (Greene and Jezeski, 1954). The lag phase is shortest at the optimum temperature and becomes increasingly longer as the temperature is lowered. Some microorganisms with longer generation times at a given temperature did not attain the same population levels as those for microorganisms with longer generation times (Greene, 1959).

2.3.2 Response to Low Temperature

The ability of microorganisms to grow at low temperature has been linked to cell preamiability and substrate uptake (Inniss, 1975; Mortis, 1975; Inniss and Ingraham, 1978; Herbert and Bhakoo, 1979). Moderate temperature change can alter physiology of permeability functions of microorganisms that grow at low temperatures.

Protein synthesis can be affected by temperature. The ability to carry out protein synthesis is linked to the ribosomal content of the cell (Inniss, 1975; Morits, 1975). Some researchers have postulated that the microorganisms synthesise increased amounts of enzymes to adjust to growth at low temperature in response to reduced enzyme activity (Elliot and Michener, 1965). Lipase production by _Ps_. _fluorescens_ in broth cultures in the range 4-30^{o}C was greatest at 8^{o}C whereas optimal growth was at 20^{o}C (Anderson, 1980). Others have also reported that increasing the temperature above 8°C had a depressing effect on lipase production by *Ps. fluorescens* (Alford and Elliot, 1960) and by *Ps. fragi* (Nashif and Nelson, 1953).

2.3.3 Temperature Adaptability

Azuma *et al*. (1962) reported that adaptability of bacteria to low temperature was poor. It was reported earlier that the ability of microorganisms to grow at low temperature is a specific property and are not able to adapt the low temperature. There is genetic evidence that a mesophile can be converted to a psychrotrophs by transduction or exposure to ultraviolet light and that a psychrotroph can be converted to a mesophile by exposure to ultraviolet light. However, Morita (1975) concluded that a true psychrophile cannot be made by these methods. Since psychrophiles contain more than one thermolabile enzyme, temperature adaptability of psychrotrophic pseudomonas species were studied using thermal gradient incubator and change observed suggested that adaptation depended on the microorganisms ability to metabolize substrate normally (Zachariah and Liston, 1973).

2.4 Source

Foster in 1892 isolated psychrotrophic bacteria in fresh and salt water in and on fish, in meat, milk, garden, soil, canal water, meadow water (Ingraham and Stokes, 1969).

Hence many investigators have confirmed that psychrotrophs are ubiquitous in nature. Most psychrotrophic organisms in milk and dairy products usually come from soil, water and vegetation (Thomas et al., 1966). Water is also a source of psychrotrophs, contaminating in milk and dairy products Pseudomonas, Achromobacter. Alcaligenes and Flavobacterium dominated the psychrotrophic flora in water with Chromobacteriu Bacillus and Coliforms in lesser number (Thomas, 1958; Thomas, 1966; Thomas et al., 1966). Morse et al. (1968) reported that the psychrotrophic count of water ranged from less than 10 to more than 10,000 m.o./ml.

Very few of the psychrotrophs found in milk have been isolated from air in clean milking parlors or dairies (Thomas et al., 1966). Poorly cleaned dairy farm and processing plant equipment probably constitute major source of microorganisms of raw milk with psychrotrophs (Thomas, 1958; Thomas, 1966). Thomas and Thomas (1977) reported that gram negative rods were dominant in rinses from equipment in milk plants.

Post pasteurization contamination of milk and dairy products generally results from improperly cleaned and sanitized equipments as well as from airborne contamination (Thomas and Druce, 1969).

2.5 Incidence

Milk produced under sanitary conditions usually contains 10% of the total microflora as psychrotrophs but milk produced under unsanitary conditions can contain more than 75% psychrotrophs (Thomas and Thomas, 1973). Johnston and Bruce (1982) reported that 83.8% Bacillus 9.9% coryneform group as thermoduric psychrotrophs in milk.

Muir _et_ _al_. (1978) observed that safe refrigerated storage was about 72 h at less than 8°C. But Grosskopf and Harper (1974) isolated _B_. _cereus_, _B_. _circulans_, _B_. _coagulans_, _B_. _lentus_, _B_. _licheniforms_, _B_. _macerans_, _B_. _megaterium_, _B_. _subtilis_, _B_. _pumilus_ in milk stored at 4°C/21-25 days. Ledford _et_ _al_. (1980) analysed 24 fresh (43 hr old) and 24 aged (held until sell by date) pasteurized milk samples and found correlation of log SPC with flavour score was (r = 0.80) when evaluated by trained panel.

Early researcher concluded that psychrotrophic bacteria do not survive laboratory or commercial pasteurization (Luck, 1972; Thomas, 1958; Thomas and Druce, 1969; Witter, 1961). Thomas and Druce (1969) reported that none of the psychrotrophic cultures isolated from milk and milk products refrigerated at 7°C or below survived pasteurization treatments, but some cultures isolated from milk and dairy products held at 8 to 10°C were heat resistant. Washam _et_ _al_. (1977) isolated heat resistant microorganisms from pasteurized milk. The sporeformers were identified as sp. of _Bacillus_ and non-sporeformers were identified as sp. of _Corynebacterium_ and _Arthrobacter_. Sudarsanam _et_ _al_. (1982) isolated and identified heat resistant psychrotrophic _S_. _lactis_-121,

S. cremoris-40 and S. lactis subsp. diacetylactis-128 from pasteurized milk. Davies (1975) reported survival of spore forming bacteria in the heat processing of milk. Mottar (1984) also studied heat resistance of psychrotrophic bacteria (Pseudomonas and Flavobacterium) with high extracellular proteinase activity. Coghill and Juffs (1979) reported that pasteurization probably promotes spore germination and vegetative cell multiplication proceeds. Heat treatment of the spores stimulates germination and subsequent outgrowth whereas, heat treatment of the milk affected the initial rate of active cell multiplication (Mikolajcik and Koka, 1968). Maxcy (1979) reported that freshly pasteurized milk did not contain gram negative bacteria and their presence in milk was a result of post pasteurization contamination. Thomas et al. (1960) also found that occurrence of psychrotrophs in pasteurized milk is due to post-pasteurization contamination. Later research had indicated that some gram negative bacteria may survive pasteurization temperature (Stadhouders, 1975). Yama et al. (1981) showed that Pseudomonas sp. secure most frequently in holding method of heat treatment. Oliveria and Parmelee (1976) found at least 20 psychrotrophs /ml in freshly pasteurized commercial and laboratory milk samples in United States.

Since most growth appeared after 7 days, the psychrotroph may have been injured and needed time to recover before multiplication began or to make adjustment to new heat treated environment. Higher temperature for short times were less effective in destroying the *Pseudomonas* sp. than were lower temperature for longer times because condition created in the milk at high temperature may favour recovery of injured cell. Whereas, Whang and Cho (1981) found that no psychrotrophs were present in UHT milk (treated at 135°C/2 sec). A double heating of milk at 80°C/10 min separated by aerobic incubation of 1 to 24 h did not reduce the spore loads of *B. cereus* and *B. subtilis*, because the germination rates varied for these species (Brown *et al*., 1979).

2.6 Significance

Milk produced under hygienic conditions does not often show a rapid increase in psychrotrophic bacterial content when held at temperature $\angle 5^{\circ}C$, but milk produced under unhygienic condition or heavily contaminated with psychrotrophs from poorly cleaned dairy and equipment usually shows a much more rapid increase (Thomas, 1960). The spoilage aspects of these organisms in pasteurized milk depends upon the types present, cell number, product shelf-life, storage temperature etc. (Washam *et al*., 1971). The levels of contamination by the psychrotrophic bacilli range from 25-35% (Grosskopf and Harper, 1969; Shehata and Collins, 1971; Johnston and Bruce, 1982) although contamination levels as high as 83% have been reported (Chug and Cannon, 1971).

Many investigators have shown that the temperature of cold storage is important than the initial concentration of psychrotrophic bacteria in determining the rapidity of off-flavour development.

Bacillus cereus is considered to be the most important of the sporeforming spp. found in milk. It produce 'bitty cream' defect in pasteurized milk (Cox, 1975; Davies, 1975), cause poisoning (Gilbert and Taylor, 1976).

Psychrotrophic Bacillus sp. isolated by Johnston and Bruce (1980) exhibited biochemical capabilities which would be of significance in milk spoilage, 84% hydrolysed casein, 77% were lecithinase positive, 83% were proteolytic in litmus milk, 57% hydrolysed cream and 8% fermented lactose

2.7 Enumeration of Psychrotrophs

Many methods which have been proposed for enumerating psychrotrophic microorganisms indicate a lack of agreement on the subject, since no precise definition for psychrotrophic bacteria exists enumeration procedures are difficult to establish (Donald et al., 1963). Presently, standard methods for the examination of dairy products recommends incubating plates at 7°C for 10 days (Marth, 1978). Hartley et al. (1969) concluded that no single method can determine everything that necessary to assess the microbial quality of milk and dairy products. Johnes (1971) concluded that a temperature of 32°C was too high for some psychrotrophs to grow and 7°C for 10 days was too long to wait for a count. Eleventh edition of Standard Method for Enumeration sanctioned optimal use of incubation time temperature (5-7°C/7-10 days). Baumann <u>et</u> <u>al</u>. (1963) stated that 2 degrees difference in incubation temperature, 3 days difference in incubation time can cause significant difference in psychrotrophic counts. However, higher counts were obtained at 7°C/10 days than at 5°C/7 days. Thomas (1969) proposed 25°C for 5 days for the total colony count of bacteria in refrigerated milk but 28-30°C for 3-4 days for routine purposes. Waes (1966) suggested holding the plates at 17°C for 16 hours followed by incubation at 7°C for 3 days. Luck (1972) reported that studies made by his coworkers with plate incubation at 27 or 32°C for 2 days gave a good indication of psychrotrophic count. Juffs (1970) noted a four day count for psychrotrophs (24 hours at 15°C followed by 72 hours at 5-7°C) and gave results comparable to the standard method of incubation plates at 7°C for 10 days. Luck and Hopkins (1975) recommended the following methods: 3 days at 15°C, 2 days at 15°C plus 1 day at 7°C and 1 day at 17°C plus 3 days at 7°C.

Lund and Sogaurd (1980) compared three methods of enumerating psychrotrophic counts using incubation periods of (i) 17°C for 17 h + 7°C for 3 days, (ii) 21°C for 25 h or (iii) 7°C for 10 days. He suggested that results of (i) are similar to the standard method (iii) Oliveria and

Parmelle (1976) suggested a rapid method for enumeration of psychrotrophs using incubation at 21°C for 25 h and found a good correlation (0.996) of this method with the standard psychrotrophic count method (7°C/10 days). Griffiths et al. (1980) used this rapid method (21°C/25 h) for enumerating psychrotrophs in double cream and compared the two methods of enumeration (6°C/14 days and 21°C/25 hours). A very high correlation ($r = 0.9$) was obtained between the methods.

Preliminary incubation of milk at temperature above 10°C for 18-24 hrs has been recommended as a modification to various methods (Druce, 1975). Johns (1971) recommended preincubation of 16 h at 17°C before microbiological testing of raw milk. Coelho and Coelho (1979) studied influence of cultures medium on accounting of psychrotrophic bacteria in milk and pasteurized liquid derivatives. They used standard plate, heart infusion, tryptone glucose extract, tripticase soya and Rogosa agar followed by tripticase soya, rogosa, tryptone glucose extract and standard plate agars.

Kathleen and Donvon (1958) described a selective medium for *Bacillus cereus* in milk without the prior use of a heat treatment. Medium having egg-yolk citrate, lithium chloride and polymyxin in the nutrient agar. Rowe and Gilmour (1983) used 3 media having minimal salt medium (1% NH_4Cl, 1% Na_2HPO_4, 0.2% KH_2PO_4, 0.05% $CaCo_3$ and 0.05% $MgSo_4$). Supplemented with 3% spray-dried sweet whey, 1% casein or three % whey +

1% casein, incubated at 7°C for 14 days and observed extracellular protease and lipase activities. Significant amounts of both enzymes were produced in medium containing whey + casein, but was low in medium supplemented with whey and absent from the medium supplement with casein.

2.8 Biochemical Changes

Milk serves as a complete and unsupplemented nutrient medium for growth and activity of psychrotrophic bacteria. Psychrotrophic organisms are important in milk because they are spoilage organisms in milk and milk products under prolonged storage at low temperature. These microorganisms biochemically alter the milk constituents (Ingraham and Stokes, 1959). Bacillus cereus was the dominating spoilage bacteria in market milk and milk products (Chopra et al.,1980)

Microbial enzymes produced by psychrotrophic bacteria cause undesirable changes in milk and milk products includes discoloration, fermentation of lactose, glucose and other sugars, proteolysis causing off-flavour and lipolysis (Bigalke, 1985).

Yan et al. (1985) and Gordon (1982) studied extracellular protease isolated from psychrotrophic spore formers which hydrolyse protein and fat.

Slight biochemical changes occur in early growth phase of psychrotrophs resulting in a lack of freshness or a stale taste and on subsequent cold storage, a variety of defects appears, development of off-flavour and odors due to proteolysis and lipolysis by psychrotrophs (Thomas, 1966; Thomas and Druce, 1969).

Psychrotrophic bacteria producing heat resistant lipase and proteinase cause an increase in free-fatty acids and free amino acids on storage at low temperature. Marshal (1982) studied the degradation on milk and milk products caused mainly by the action of bacterial enzymes like phospholipases, heat stable proteinases, hydrolase in milk.

Proteinase components are more important than lipase components on the flavor quality of milk (Christen _et al_., 1985). Psychrotrophic bacteria cause changes in stability of milk coagulation (Cousin and Marth, 1977). Psychrotrophic sporeformers cause blowing or sweet curdling of milk (Skrebkova, 1979). Phillips _et al_. (1981) found that the spoilage of cream on prolonged storage at low temperature was mainly due to _Bacillus_ spp. 63% of the isolates showed enzymatic action on milk proteins and fat and 46% showed phospholipase activity. _B. subtilis_ cause rapid proteolysis in pasteurized and boiled milk (Jayachandran _et al_., 1985).

Changes in milk proteins as a result of psychrotrophic growth or enzymatic action are important in the keeping quality of milk and milk products at refrigeration temperature. Jonathanp et al. (1984) found changes in casein micelles size by growth of psychrotrophic bacteria in raw skim milk. Release of various nitrogen components or degradation of individual protein fractions have been observed when studying proteolysis caused by psychrotrophic enzyme (McCaskey, 1967).

Ikonomov et al. (1970) found that at low temperature (7-10°C) *Streptococcus lactis* exhibited greater proteolytic activity than other cultures of lactic acid bacteria. Vanderzant and Moore (1955) found no correlation between psychrotrophic population and proteolytic activity. Juffs et al. (1968) reported that the proteolytic activity was not proportional to growth since the proteolytic activity peak was more pronounced at 3°C than 20°C. *Pseudomonas aeroginosa* was the most prolific producer of proteolytic enzyme. A large population was not required for production of significant amounts of heat resistant proteases (Adam et al., 1975). Williamson et al. (1962) and Maksimova (1970) observed a direct relationship between coagulation rate and proteolytic activity.

2.9 Control

Psychrotrophic spore formers are very important in the dairy industry. The control must focus on destruction of psychrotrophs or prevention of their growth in milk. Since proper cleaning and sanitizing of dairy equipments are important for production of milk with acceptable microbial quality. Control of psychrotrophic sporeformers should begin at farm level. Low temperature reduces the rate of microbial growth (Bodyfelt and Davidson, 1975) sporeformers in pasteurized milk have become more important because they are heat resistant and grown at refrigeration temperature. Mikolajcik and Koka (1968) demonstrated that the heat resistant Bacillus spores increased the rate of germination and outgrowth in milk. Spores of B. cereus were activated by pasteurization of milk and outgrowth was observed within a short time (Wilkinson and Davies, 1974). However, spores of B. subtilis and B. licheniformis demonstrated no outgrowth in pasteurized milk within 3 h. Fitz-Gerald et al. (1982) concluded that low temperature treatment are not the effective means of overcoming problem in dairy industry caused by heat-resistant bacterial lipases.

Franklin (1970) observed that the temperature co-efficient for change in a relation rate was greater for bacterial spore destruction than for chemical change, therefore, heating to a higher temperature for a shorter time should result in bacterial spore destruction . This principle is used for UHT processing of milk.

Brown et al. (1979) suggested variations of the tyndallization process as a means of destroying spore formers in milk. Shehata and Collins (1972) suggested a process in which milk was heated at 87.8°C for 20 sec, stored at 32°C for 4 h and reheated at 76.7°C for 20 sec. Since the number of spores was reduced by two or more log cycles, the shelf-life of milk could be increased by 2 to 3 days. Brown et al. (1979) observed no effect on B. cereus and B. subtilis by a double heat treatment of 10 min at 80°C separated by anaerobic incubation of 1 to 24 h at 30°C. Fairbairn and Law (1986) studied the different ways of controlling psychrotrophs in raw milk (refrigeration thermization, addition of preservatives, activation of inhibitory systems in milk, seeding with lactic acid bacteria) and concluded that low temperature storage ($\angle$4°C) and good hygienic practices are the best way of controlling numbers of psychrotrophs in raw milk. Heating milk at the farm for 10 sec. at 165°F and seeding the milk with lactic acid bacteria prior to refrigerated storage was suggested to control psychrotrophic bacteria (Honer, 1981). Bjork (1978), Bjork et al. (1979) observed that the lacto-peroxidase system is naturally occurring inhibitory system in raw milk that can reduce the bacterial content. The lactoperoxidase system which consists of lactoperoxidase, thiocyanate and hydrogen peroxide, inhibited lactic streptococci and gram negative rods, especially Pseudomonas (Bjork et al., 1975). Mistry and Kosikowsky (1985) studied the influence of potassium sorbate and hydrogen peroxide on psychrotrophic bacteria in milk and observed that the bacterial growth was retarded and the shelf life was increased with 0.2% potassium sorbate.

Various additives have been examined for their effectiveness in inhibitory psychrotrophic bacteria (Moustafa and Collins, 1969). Moustafa and Collins (1969) researched selected food additives for their bactericidal effect on Ps. fragi and found that only potassium sorbate was effective at a suitable concentration (0.3%) at pH 5.5. Similar results were reported for Ps. fluorescens, however, the concentration of sorbate and pH of the medium determined ultimate growth.

Shehata et al. (1985) studied the influence of sublethal shocks, L-cystine, L-asparagine and nisin on generation of psychrophilic bacillus spores in milk. The sporicidal effect of Nisin was enhanced when L-cystein was added prior to nisin, but reduced when L-asparagine was added. The sporicidal effect of nisin + L-cystein was in order B. basidus, B. licheniforms, B. pumilus, B. cereus.

2.10 Keeping Quality

Since multiplication rate and activity of bacteria are reduced at low temperature. Keeping quality of milk is improved by refrigerated storage. However, psychrotrophs multiply and become active at refrigeration temperature, causing spoilage of milk and dairy products. Many researchers have concluded that psychrotrophic contamination and keeping quality are closely related.

Off flavour have been detected organoleptically in milk in less than 5 days at 1 to 4.4°C. The most common defect observed were fruity and rancid flavor (Thomas et al, 1961; Thomas, 1966), B. circulans produces fruit/and rancid flavor and B. coagulans and B. laterospora produced fruity and unclean flavor (Shehata et al.,1971). Raw and pasteurized milk usually spoil when held at refrigerated temperature because of the action of psychrotrophs contamination (Elliot et al.,1974). The populations needed to cause detectable changes in milk varies among genera, among species and within a genus, but levels at which flavor changes occurred were similar at 6° and 20°C (Punch et al., 1965). Milk spoilage by psychrotrophs was reported in a range of population of $1x10^2$ to $1x10^7$/ml (Tekinson and Rothwell, 1974). Most psychrotrophs will produce a detectable flavor change when the population exceeds 1 million/ml according to Richter (1979). Patel and Blankenagel (1972) observed that milk still was acceptable 14 days after pasteurization with a count of $5x10^7$ organisms/ml. Low temperature has been an important control for prolonging the shelf life of pasteurized milk. Smith et al. (1972) observed that milk with high psychrotrophic counts usually had shorter shelf life than milk with low counts. But Lausten (1978) found that Pseudomonas, Acinetobacter and Bacillus spp. still remain dominant in milk at 7°C/10 days, Boyd et al. (1955) found that when storage temperature was lowered from 40°F to 33°F the keeping quality was found to be extended 11 to 14 days. Blank et al. (1984) studied the effect of cold storage and enrichment with psychrotrophic bacteria on quality of UHT milk. They concluded that the enrichment with psychrotrophs showed slight higher values of NPN, some free amino acids, HMF and viscosity due to proteolysis. However, microbial spoilage was dependent on the number of microorganisms contaminating the milk, length of lag phase of growth, rate of growth at storage temperature and type of microorganisms present. Lipolysis in stored milk was not closely related to the total concentration of psychrotrophic bacteria but lipolytic rancidity was not observed when pseudomonas counts was below $5x10^6$ c.f.u./ml (Muir et al., 1978). Finley et al. (1968) noted that as storage temperature increased, the keeping quality decreased, 81% of milk held at 0°C remained acceptable for over 3 weeks, but only 15% of that held at 7.2°C was acceptable for more than one week.

Jackson (1978) reported that in addition to microbiological deterioration which affect the shelf life of milk are enzymatic and physical deterioration. White et al. (1979) inoculated raw milk with $1x10^5$ proteolytic psychrotrophs/ml and incubated the milks at 5 to 25°C for 24 h before pasteurization and storage at 4°C. After 14 days, this milk was unacceptable on the basis of flavor scores and high proteolysis values. Proteolytic value and acceptability do not always correlate well with the microbial population (Elliot *et al*., 1974). Aggarwal and Srinivasan (1986) studied the influence of *Bacillus cereus* on the keeping quality of pasteurized milk. *Bacillus* spp. are the potential spoilage organisms in milk and milk products (Magdoub *et al*., 1983). Langeveld *et al*. (1965) reported that some milk was spoiled by aerobic sporeformers strains of *Bacillus circulans* which fermented lactose, *B. cereus* strains have been implicated in bitty cream and sweet curdling defects of pasteurized milk (Choudhary and Mikolajcik, 1971; Cox, 1975). Overcast *et al*. (1974) also studied *Bacillus cereus* is the causative organism exhibiting sweet curdling within 10 days on refrigeration . The curd usually develops only on the bottom of the container of milk and may result from casein degradation (Choudhary and Mikolajcik, 1971).

: : : : :

: : :

:

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Collection and Analysis of Pasteurized Milk Samples

One hundred and ten samples of pasteurized toned milk in pouches were collected from Experimental Dairy of National Dairy Research Institute, Karnal. They were divided into two batches and stored at two different temperatures 7°C and 22°C respectively. The samples were anlaysed for psychrotrophic spore count, proteolytic psychrotrophic spore count.

Twenty buffalo milk samples were collected from the Cattle Yard and analysed for acidity, pH, COB, alcohol and proteolytic activity tests to study the action of isolates on steamed and sterilized milk.

3.1.1 Psychrotrophic spore count

Samples were heated to 80°C for 10 min in water bath and suitable dilutions of heat treated milk samples were plated on TDYA (Tryptone dextrose yeast extract agar). The plates were incubated at 22°C for 25 hrs (Lausten, 1981 and Luck et al., 1984) and 7°C for 10 days (I.D.F., 1981).

3.1.2 Proteolytic psychrotrophic spore count

Heat treated milk samples plated on TDYA fortified with reconstituted skim milk gave the proteolytic psychrotrophic spore count. The plates were incubated at 22°C for 25 hrs and at 7°C for 10 days (I.D.F., 1981; Lausten, 1981 and Luck *et al*., 1984). The colonies showing discrete zone of clearance around them were counted.

3.2 Isolation of Psychrotrophic Spore Formers

Typical colonies showing comparatively bigger zone of clearance on milk agar plates (TDYA fortified with 10% RSM) were selected. Spore formers were picked up randomly from other psychrotrophic bacteria by microscopic examination. Selected isolates were inoculated into BHI broth (Brain Heart Infusion Broth) for their enrichment. The tubes were incubated at 37°C for 24 hrs. Representative colonies of proteolytic spore formers were purified by repeated streaking on milk agar. Isolated colonies that appeared on incubation at 22°C for 48 hrs were transferred on to TDYA slants and maintained at 5°C in a refrigerator. The cultures were subcultured at monthly intervals.

3.3 Screening the Isolates for Protease Activity

The proteolytic psychrotrophic spore forming isolates were screened for the quantitative production of extracellular protease in a broth medium containing 1% tryptone and 0.25% yeast extract (pH 7.0) which supported both growth as well as enzyme production. Medium was inoculated at 2% level from 24 hr old broth cultures and incubated at 22°C for 48 hr. The cell free supernatant was obtained by centrifugation at 7000 rpm for 30 min and the protease activity in the supernatant was assayed.

3.4 Assay of Protease Activity

The protease activity was determined according to method of Keay and Wildi (1970) with some modifications.

3.4.1 Procedure

To one ml of the substrate (1% casein in 0.05M phosphate buffer, pH 7.0), equiliberated for 5 min at 37°C, added 1.0 ml of suitably diluted (1:5) culture supernatant previously equiliberated at the same temperature and after mixing, the reaction mixture was incubated at 37°C for 1 hr. The reaction was terminated by adding 2.0 ml of 0.4M trichloroacetic acid (TCA) and the mixture was further incubated at the same temperature for 10 min. The precipitated proteins were then filtered through Whatman No.1 filter paper and the tyrosine content in the filterate was determined colorimetrically. For blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as described above.

3.4.2 Determination of free tyrosine

Tyrosine content of TCA soluble filterate was determined by developing color with Folin's phenol reagent according to the method of Lowry et al. (1951) with some modifications.

3.4.2.1 Reagents:

A. Folin's phenol reagent: It was freshly prepared according to the method of Folin-Ciocalteau (1927) and diluted 1:1 with distilled water before use.

B. Sodium carbonate solution: A 0.4M solution of sodium carbonate was prepared in distilled water.

3.4.2.2 Procedure:

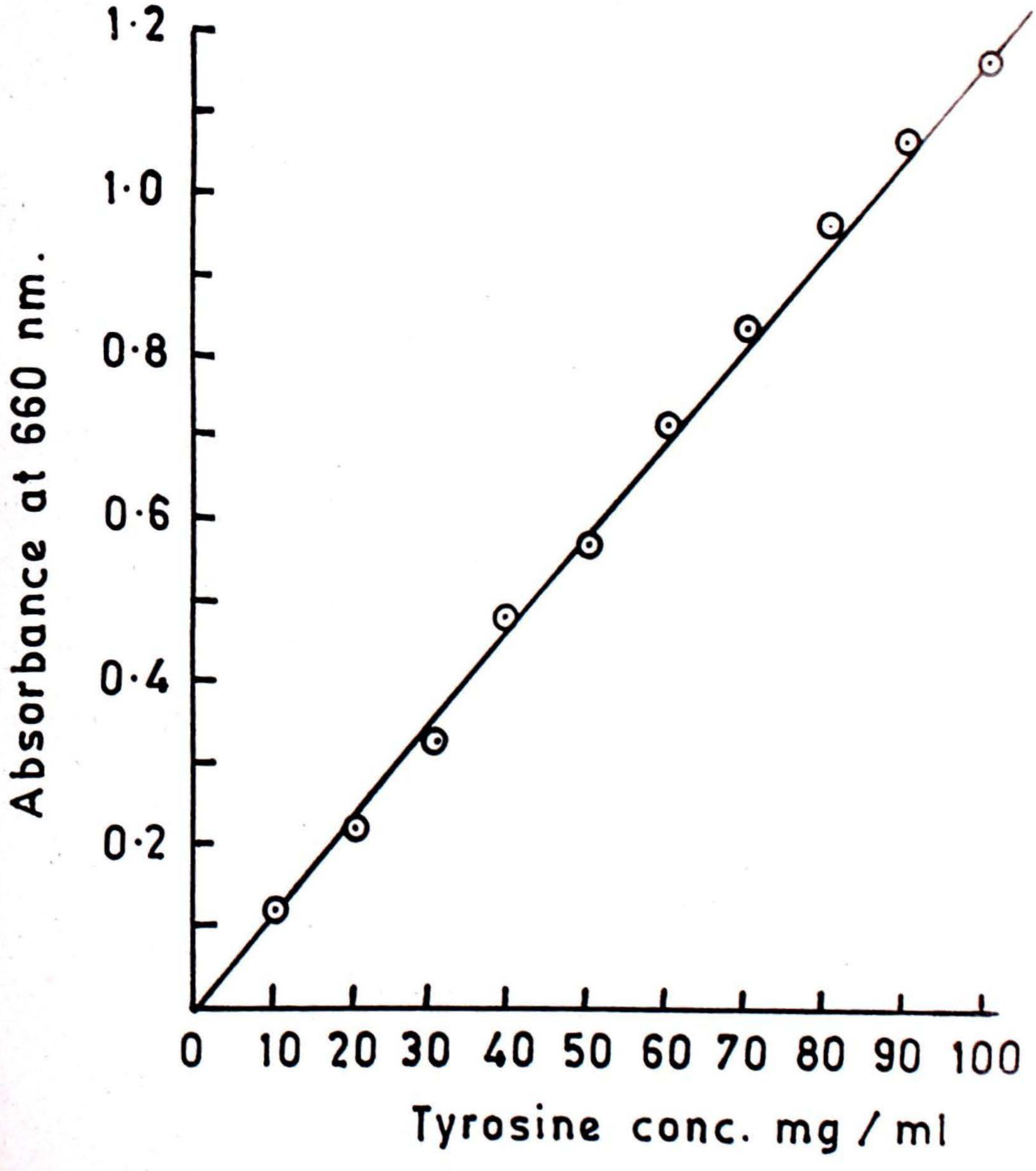
To one ml of filterate obtained from TCA precipitation, 5.0 ml of 0.4M sodium carbonate solution was added followed by 1.0 ml of 1N Folin's reagent and incubated at $37^{\circ}C$ for 20 min for color development. The intensity of blue color was measured at 660 nm in Elico Spectrocol Colorimeter.

3.4.3 Standard curve for tyrosine

A stock solution of L-tyrosine (BDH) was prepared by dissolving 100 mg in minimum quantity of 0.1N sodium hydroxide and making the volume to 100 ml with glass distilled water, thereby getting a concentration of 1000 g per ml. A series of dilutions were then prepared from stock solution to have solutions with tyrosine concentration ranging from 10 to 100 µg/ml. One ml aliquots of the tyrosine solutions were treated

Fig.1: Standard curve for tyrosine

FIG. 1. STANDARD CURVE FOR TYROSINE
(Keay and Wildi method)



as in 3.4.2.2 for developing color. The standard curve (Fig. 1) was drawn by plotting the optical density values against the tyrosine concentration.

3.4.4 Unit of activity

The protease activity of the enzyme on casein was expressed in terms of units, where a unit is defined as the amount of enzyme required to release TCA soluble fragments giving blue color equivalent to one µg of tyrosine under the conditions of the assay.

3.5 Identification of Psychrotrophic Spore Forming Isolates

All the selected isolates were identified according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

3.5.1 Media and reagents

The various cultures media and reagents used in characterization of the isolates and their composition are given below. All media, unless otherwise stated, were sterilized by autoclaving at 121°C and 15 psi for 20 min.

3.5.1.1 Tryptone dextrose yeast extract agar:

Tryptone	5.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	7.2 ± 0.1

3.5.1.2 Peptone broth:

Peptone	5.0 g
Potassium dihydrogen phosphate	5.0 g
Glucose	5.0 g
Distilled water	1000 ml
pH	7.0

3.5.1.3 Nitrate broth:

Peptone	5.0 g
Potassium nitrate	0.2 g
Distilled water	1000 ml
pH	7.0

3.5.1.4 Nutrient broth:

Peptone	5.0 g
Yeast extract	2.0 g
NaCl	5.0 g
Beef extract	1.0 g
Distilled water	1000 ml
pH	7.4

3.5.1.5 Gelatin agar:

Peptone	5.0 g
Beef extract	3.0 g
Gelatin	3.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.0

3.5.1.6 Acid mercuric chloride:

Mercuric chloride	12.0 g
Distilled water	80.0 ml
Conc. HCl	16.0 ml

3.5.1.7 Nutrient gelatin:

Gelatin	150.0 g
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
pH	7.0

3.5.1.8 Koser's citrate medium:

Sodium ammonium phosphate	1.5 g
KH_2PO_4	1.0 g
$MgSO_4$	0.2 g
Sodium citrate	2.5 g
Bromothymol blue	0.016
pH	6.8

3.5.1.9 Basal medium for sugar fermentation:

Peptone	10.0 g
Sodium chloride	5.0 g
Bromothymol blue (0.2% aqueous solutions)	12.0 g
Distilled water	1000 ml
pH	7.2-7.4

Ten per cent solution of different sugars were separately sterilized by millipore filter and added aseptically to the basal medium at 1% level before use.

3.5.1.10 <u>Starch agar:</u>

Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	5.0 g
Agar	16.0 g
Soluble starch	2.0 g
Distilled water	1000 ml
pH	7.5 - 7.6

3.5.1.11 <u>Lugol's iodine:</u>

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	1000 ml

The solution was diluted 1:5 with distilled water before use.

3.5.1.12 <u>Urea agar:</u>

peptone	1.0 g
Sodium chloride	5.0 g
Potassium dihydrogen phosphate	2.0 g
Agar	20.0 g
Distilled water	1000 ml

The ingredients of the medium were dissolved by heating and the pH adjusted to 6.8. After filteration, 1.0 g glucose and 6 ml of 0.2% phenol red solutions were added to the molten base which was then steamed for 1 hr and cooled to 50 to 55°C. Aqueous solution of urea was separately sterilized by membrane filteration and added aseptically to the basal medium at 20% level. It was then distributed aseptically into sterile tubes.

3.5.1.13 Tryptone broth:

Tryptone	10.0 g
Beef extract	3.0 g
Distilled water	1000 ml
pH	7.4

3.5.1.14 Kovac's reagent:

p-Dimethyl amino-benzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Conc. HCl	25.0 ml

The aldehyde was dissolved in alcohol by gently warming in a water bath about 50-55°C and then acid was added after cooling the solution. It was stored at 4°C in an amber colored bottle.

3.5.1.15 Hydrogen peroxide:

H_2O_2	3% aqueous solution

3.5.1.16 Nitrate test reagent:

i) Solution A:	α-Sulfanilic acid	0.4 g
	5N Acetic acid	50 ml
ii) Solution B:	L-Naphthylamine	0.25 g
	5N Acetic acid	50 ml

Equal volumes of solution A and B were mixed before use.

3.5.1.17 Skim milk agar:

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	7.5 ± 0.1

Skim milk powder (SMP) at 10% level was reconstituted and sterilized, the agar was cooled to 50°C, added reconstituted sterile skim milk at the rate of 10% v/v before plating.

3.5.1.18 Physiological saline (dilution blank):

Sodium chloride	9.0 g
Distilled water	1000 ml

3.5.1.19 0.1N NaOH:

Sodium hydroxide	4.0 g
Distilled water	1000 ml

3.5.1.20 Phenolphthalein solution:

Phenolphthalein	1.0 g
Ethyl alcohol	100 ml

3.5.1.21 Brain heart infusion broth:

Ready made BHI media(HI)	37.0 g
Distilled water	1000 ml

3.5.1.22 Litmus milk:

Skim milk	100 ml
Litmus solution	10 %

3.5.1.23 Kligler iron agar:

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
NaCl	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferric citrate	0.3 g
Sodium thiosulphate	0.3 g
Phenol red	0.05 g
Agar	12.0 g
Distilled water	1000 ml
pH	7.4 ± 0.2

3.5.2 Morphological characterization

The cultures of selected isolates were studied for shape of cells, motility, gram reaction and spore position by microscopic examination.

3.5.3 Cultural and biochemical characterization

The following tests were conducted for cultural and biochemical characterization of the selected isolates.

3.5.3.1 Catalase test:

Forty eight hr old TDYA slant cultures of the isolates incubated at 22°C were taken and 2-3 drops of 3% H_2O_2 solution was poured over the growth. Liberation of gas bubbles due to decomposition of H_2O_2 was taken as the positive catalase reaction.

3.5.3.2 Reaction in litmus milk:

Litmus milk tubes inoculated with the isolates were incubated at 22°C for 24 hr and observed for acid production, reduction of litmus, curdling and peptonization.

3.5.3.3 Acetyl methyl carbinol production:

The cultures were inoculated into glucose phosphate peptone water and incubated at 22°C for 24 hr. Three millilitres of 40% potassium hydroxide solution (KoH) and pinch of creatine was added to the tubes, mixed and kept for 30 min. A red coloration was indicative of acetyl methyl carbinol production.

3.5.3.4 <u>Nitrate reduction test</u>:

The isolates were inoculated into nitrate broth and incubated at 22°C for 24 hr. Reduction of nitrate to nitrite was indicated by the development of red color within a few minutes after the addition of 1.0 ml of nitrate test reagent to the culture.

3.5.3.5 <u>Gelatin hydrolysis</u>:

Hydrolysis of gelatin was examined both on gelatin agar and in nutrient gelatin tubes.

3.5.3.5.1 <u>Gelatin Agar</u>:- The cultures were streaked on the gelatin agar and incubated at 22°C for 24 hr. Observations were made for the clearing around the line of growth when acidified mercuric chloride solution was poured over the plates.

3.5.3.5.2 <u>Nutrient gelatin</u>:- Stab inoculation of the isolates were made in nutrient gelatin tubes and incubated at 22°C. At different intervals during incubation, the tubes were removed from incubator, held at 4°C for 30 min and observed for the liquefaction of gelatin.

3.5.3.5 <u>Tolerance to sodium chloride</u>:

The isolates were inoculated in nutrient broth containing 5% sodium chloride. The tubes were incubated at 22°C for 24 hr and observed for growth.

3.5.3.7 Citrate utilization:

A loopful of the culture was inoculated into Koser's citrate medium and incubated at 22°C for 24 hr. Growth in medium was indicative of the utilization of citrate as the sole carbon source by the organism and color changes from lighter green to blue.

3.5.3.8 Fermentation of sugar:

The cultures were inoculated into different tubes of the basal medium containing 1% of the following sugars: fructose, mannitol, arabinose, xylose, raffinose, sucrose, maltose, lactose, dextrose, salicin. After incubation at 22°C for 24 hr, the tubes were examined for acid and gas production as indicated by the change in color of the indicator from blue to yellow and accumulation of gas in Durham tube.

3.5.3.9 Hydrolysis of starch:

The isolates were streaked on starch agar plates and after incubation at 22°C for 24 hr. The plates were flooded with Lugol's Iodine. Appearance of clear area around the colonies against a blue black background were observed.

3.5.3.10 Urease production:

The production of urease by the isolates was examined using urea agar. The slants were streaked with the culture and incubated at 22°C for 24 hr. Presence of red color indicated hydrolysis of urea.

3.5.3.11 Indole production:

The cultures were inoculated into tryptone broth tubes and incubated at 22°C for 24 hr. Development of a pink color in amyl alcohol layer on addition of Kovac's reagent was taken as positive test.

3.5.3.12 H_2S production:

The production of H_2S by the isolates was examined using Kligler iron agar. The slants were streaked with the cultures and incubated at 22°C for 24 hr. Presence of black color indicated production of H_2S. Red slope and yellow butt shows ~~sugar~~ glucose fermentation and yellow slope and yellow butt shows lactose hydrolysis.

3.6 Keeping Quality Test

Following keeping quality tests were performed on steamed milk (steamed for 1 hr) and sterilized milk (autoclaved at 121°C and 15 psi for 20 minutes), inoculated the isolates in the above milks and incubated at 22°C and at 7°C.

3.6.1 Clot-on-Boiling test (COB):

Five ml milk was taken in test tube and heated in water bath at boiling temperature for 5 to 6 min. Removed the tubes from the water bath and rotated them in an almost horizontal position, examined the film on the sides of the tube for any precipitated particles. The formation of clot constitutes a positive COB test.

3.6.2 Alcohol test

Five ml of milk was taken in a test tube and 5 ml of 95% ethyl alcohol was added, shaken well and examined the film of milk on the sides of the tube for any flakes. The formation of flakes indicate a positive alcohol test.

3.6.3 Acidity test:

Titratable acidity of milk is due to the growth of organisms, increased with the elapse of time was determined by titrating 10 ml of milk sample with N/10 NaOH using phenolphthalein as indicator. The results were expressed as per lactic acid (ISI, 1960).

3.6.4 pH

pH was determined by Global Digital pH meter.

3.6.5 Proteolytic activity

The protease activity was determined according to method of Keay and Wildi (1970) with some modification. The method is same as used in 3.4.1 Assay of protease activity.

: : : : :

: : :

:

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The data obtained in the present study shows the isolation, identification and characterization of psychrotrophic sporeformers from pasteurized toned milk and their effect on keeping quality of sterilized and steamed milks. The data presented are in the form of tables 1 to 16 and figures 1 to 5.

4.1 Bacteriological Examination of Pasteurized Milk

The data obtained from the analysis of 110 samples of pasteurized toned milk for psychrotrophic spore count and proteolytic psychrotrophic spore count are presented in summarized form in Tables 1 and 2. Pasteurized toned milk samples in pouches were stored at 7°C for 10 days and at 22°C for 24 hrs prior to analysis.

4.1.1 Psychrotrophic spore count

When the pasteurized toned milk was analysed after storage at 7°C for 10 days, on further incubation of plates at 22°C for 24 hrs, 43 samples showed the psychrotrophic spore count in the range of 500-1000/ml, in 4 samples ranged from 100-500/ml, 8 samples ranged from 1000-1500/ml and 11 samples falls in the range of 1500/ml to 2000/ml and 44 samples indicated the psychrotrophic spore count >2000/ml.

Table 1: Distribution of psychrotrophic spore count and proteolytic psychrotrophic spore count in pasteurized toned milk

Type of sample	Psychrotrophic spore count				Proteolytic psychrotrophic spore count			
	Incubation Temperature				Incubation Temperature			
	22°C*		7°C**		22°C*		7°C**	
	Range/ml	No. of samples	Range/ml	No. of samples	Range/ml	No. of samples	Range/ml	No. of samples
Pasteurized toned milk stored at 22°C for 24 h	1-500	0	1-1000	6	Nil	0	Nil	0
	501-1000	12	1001-5000	29	1-25	14	1-50	5
	1001-1500	17	5001-10000	28	26-50	47	51-100	39
	1501-2000	22	>10000	47	51-75	9	101-200	57
	2001-5000	48			76-100	39	201-300	9
	>5000	11			>100	1	>300	6

* Plates incubated for 24 hr;

** Plates incubated for 10 days

Table 2: Distribution of psychrotrophic spore count and proteolytic psychrotrophic spore count in pasteurized toned milk

Type of sample	Psychrotrophic spore count – Incubation temperature				Proteolytic psychrotrophic spore count – Incubation temperature			
	$22^{o}C$*		$7^{o}C$**		$22^{o}C$*		$7^{o}C$**	
	Range/ml	No. of samples	Range/ml	No. of samples	Range/ml	No. of samples	Range/ml	No. of samples
Pasteurized toned milk stored at $7^{o}C$/10 days	Nil	0	Nil	0	Nil	11	Nil	0
	1 - 50	0	1 - 50	0	1 - 25	57	1- 25	3
	50 -100	0	51 -100	0	26 - 50	17	26- 50	52
	101-500	4	101-500	0	51 - 75	12	51- 75	25
	501-1000	43	501-1000	1	76 -100	9	76-100	30
	1000-1500	8	1001-1500	24	>100	4	>100	0
	1501-2000	11	1501-2000	37				
	>2000	44	>2000	48				

* Plates incubated for 24 hr;

** Plates incubated for 10 days

When same milk was incubated at 7°C for 10 days, 48 samples showed the psychrotrophic spore count >2000/ml. In 37 samples counts ranged from 1501-2000/ml, in 24 samples from 1001 to 1500/ml and in one sample ranged from 501-1000/ml (Table 1).

During pasteurization treatment most of the cells were destroyed and the cell fragments might recupe during preincubation period and those cells need time to repair themselves and multiply hence at 7°C/10 days, counts were more (Sudarsanam and Srinivasan, 1982; Uplacksh and Sudarsanam, 1986). These findings were tallying with the findings of the above authors. When the pasteurized toned milk was analysed, after storage at 22°C for 24 hrs, on incubation at 22°C for 24 hrs, 12 samples showed spore count ranged from 501-1000/ml, 17 had counts ranged from 1001-1500/ml, 22 samples indicated spore count ranged from 1500 to 2000/ml and 48 samples ranged from 2001-5000/ml and 11 samples showed even more than 5000/ml. On incubation at 7°C for 10 days, when same milk was analysed for psychrotrophic spore counts, counts were higher as compared to the count of 22°C for 24 hrs. 47 samples showed spore counts >10000 and in 28 samples data ranged from 5001-10,000 spore count, 29 samples count ranged from 1001 to 5000 per ml and 6 samples showed counts ranged from 1-1000/ml.

Bishop and White (1985) reported that a preincubation at 21°C for 14 hrs was best. Chug and Cannon (1971) stated that 83.3% of the raw milk samples contained 2 to 900 spores/ml. El-Bassiony _et al_. (1985) reported that counts obtained with incubation at 21°C for 25 hrs were slightly higher than those obtained with incubation at 7°C for 10 days ranged from 42,000 to 24 millions/ml (mean 9,52,000/ml) for psychrotrophs.

Mikolajcik and Simon (1978) examined 109 raw milk samples and noted that 13% of the milk heated at 80°C for 12 units contained psychrotrophic spore count of 10 or more/ml. After storage at 7°C/7 days 58% of the sample had psychrotrophic spore count 10 or more/ml and an average of 340 psychrotrophic spore/ml. Johns (1971) reported that psychrotrophic bacterial count ranged from 40 to 4,00,000/ml of milk. Lausten (1978) compared incubation for 25 hr at 21°C with incubation for 10 days at 7°C and there was a high correlation 0.968 (7°C) and 0.984 (22°C) between shelf life and increase in psychrotrophic spore count (Luck _et al_., 1980).

4.1.2 Proteolytic psychrotrophic spore count

Pasteurized toned milk when stored at 7°C for 10 days and then analysed for proteolytic psychrotrophic spore count on incubation at 7°C for 10 days and 21°C for 24 hr showed difference in counts at both the temperatures. 14 samples have proteolytic psychrotrophic spore counts in the range of 1-25 per ml, 47 samples ranged from 26-50, 9 ranged from

51-75, 39 ranged from 76-100 and 1 sample had counts more than 100. Same sample when incubated at 7°C for 10 days showed spore counts ranged from 1-50 in 5 samples, 51-100 in 39 samples, 101-200 in 51 samples, 201-203 in 9 samples and more than 300 in 6 samples. Some proteolytic spore formers grew well at 22°C and some need longer time to grow. At low temperature the proteolytic counts increased as compared to the temperature at 22°C for 24 hrs.

Proteolytic psychrotrophic spore count in milk stored at 7°C for 10 days was inoculated with the isolated spores and incubated at 22°C for 24 hr. 11 samples showed no count, 57 samples ranged from 1-25 counts/ml, 17 samples showed range from 26-50 counts/ml, 12 samples showed 51-75 counts/ml and 9 samples denoted 76-100 counts/ml and only 4 samples showed counts more than 100. But when incubated at 7°C for 10 days, proteolytic psychrotrophic spore count ranged from 1-25 in 3 samples, ranged from 26-50 in 52 samples and from 51-75 in 30 samples and none have more than 100 counts/ml.

4.2 Isolation of Psychrotrophic Sporeformers

Typical colonies of psychrotrophic sporeforming bacteria exhibiting clear zone around them on tryptone dextrose milk agar plates and other sporeforming colonies on tryptone dextrose yeast agar were randomly selected and purified by repeatedly streaking on the same agar (Fig.1, 2).

Fig.2: Purification of selected psychrotrophic spore forming isolates

A. Streak plate technique

A total number of 66 such colonies were isolated from 11 pasteurized toned milk samples.

In a similar study, Coghill and Juffs (1979) isolated psychrotrophic sporeformers in 52 out of 107 pasteurized milk samples. Malik and Mathur (1983) isolated 65 proteolytic psychrotrophic non-sporeforming bacteria from [illegible] samples of raw milk. In a similar study, Sharma et al. (1984) also isolated 65% proteolytic sporeforming bacilli from [illegible] samples of raw milk.

4.3 Screening of the isolates for protease activity

All the sixty six isolates of psychrotrophic sporeformers were quantitatively screened for their protease production in a broth medium containing [illegible] tryptone and [illegible] yeast extract (pH 7.0). Protease activity in the cell free extract obtained after centrifuging the 24 h old culture inoculated the sporeformers at [illegible] level and incubated at [illegible] for 48 hr, was assayed using casein as the substrate (Table 3). Maximum enzyme activity of 300 units was exhibited by the isolate No.46 and the minimum [illegible] units by the isolate No.[illegible]. The distribution of the proteolytic psychrotrophic isolates on the basis of enzyme activity is presented in Table 4. Thirty three isolates constituting 50% of the total 66 isolates exhibit enzyme activity between 151-200 units and one isolate constituting 1.5% of the total exhibited enzyme activity of 300 units per ml.

Table 3: Protease activity of psychrotrophic spore forming isolates

Isolate Number	Enzyme activity (units/ml)	Isolate Number	Enzyme activity (units/ml)	Isolate Number	Enzyme activity (units/ml)
1	245	23	150	45	300
2	200	24	160	46	200
3	160	25	150	47	195
4	150	26	145	48	170
5	130	27	145	49	160
6	160	28	150	50	180
7	120	29	150	51	160
8	120	30	150	52	200
9	120	31	150	53	180
10	110	32	145	54	150
11	170	33	180	55	160
12	160	34	180	56	200
13	100	35	160	57	160
14	145	36	230	58	160
15	130	37	210	59	180
16	220	38	170	60	195
17	160	39	160	61	180
18	180	40	170	62	195
19	145	41	145	63	200
20	150	42	120	64	180
21	150	43	220	65	200
22	120	44	220	66	110

Table 4: Distribution of psychrotrophic spore forming isolates according to their enzyme activity

Enzyme activity (units/ml)	No. of isolates	Total isolates (%)
<50	0	0
51-100	1	1.5
101-150	25	38
151-200	33	50
201-250	6	9
251-300	1	1.5
>300	0	0

Proteolytic changes brought about by the psychrotrophs sporeformers in milk have been reported by several workers (Yan et al., 1985 and Gordon, 1982). Sharma et al. (1984) reported that the protease activity of 50 psychrotrophic sporeforming isolates ranged from 20 to 480 units/ml. 24% of the total isolates exhibited enzyme activity in the range of 51-100 units/ml while 2% had protease activity of more than 300 units/ml.

4.4 Identification of the Isolates

4.4.1 Microscopic examination

All the sixty six isolates were found to be motile, rod shaped, sporeforming bacilli with ability to form central spores (Table 5).

4.4.2 Cultural and biochemical characteristics

All the cultural and biochemical characteristics of all the 66 isolates are presented in Table 5. All the isolates grew well at 22^{o}C when grown on tryptone dextrose yeast agar. All the isolates were aerobic, catalase positive and were negative for indole, H_2S and AMC production. Forty-eight percent of the isolates showed proteolysis when incubated in litmus milk, 80% of isolates liquified gelatin, 48% hydrolysed starch, 55% reduced nitrate to nitrite and 88% could grow in basal medium containing

Fig.3: Selected psychrotrophic spore forming isolates showing protease activity

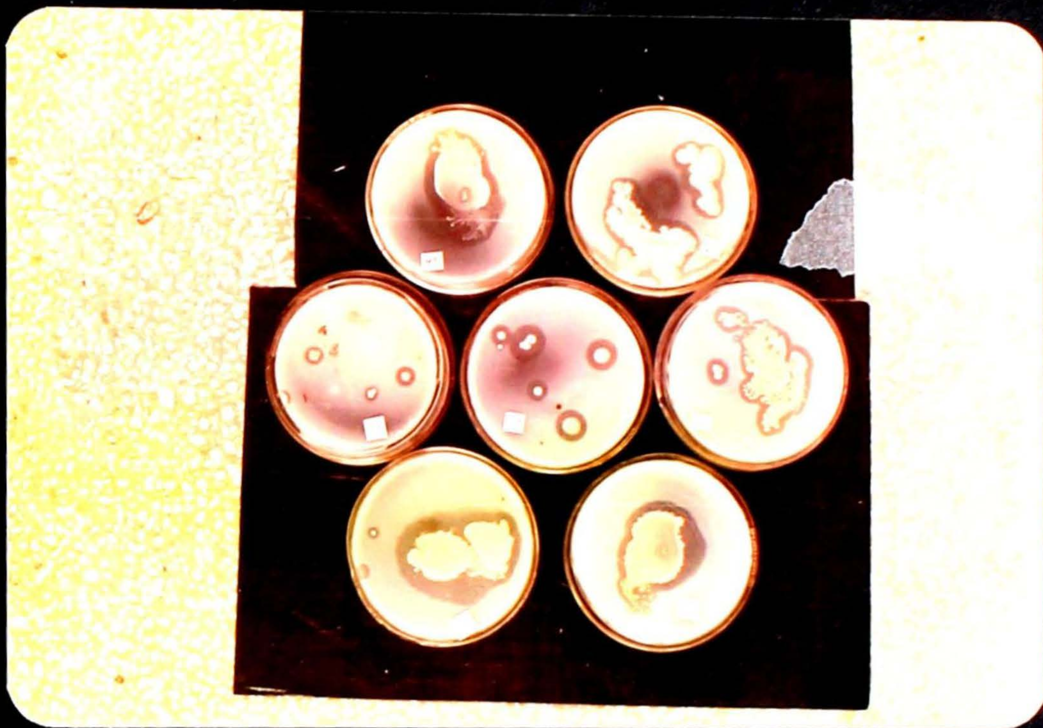


Table 5: Morphological, cultural and biochemical characteristics of psychrotrophic sporeforming isolates

Isolate No.	Shape	Motility	Colony characteristics	Spore position	Catalase	Urease	AMC	H_2S	Nitrate	Indole	Starch	Gelatin Stab.	Gelatin agar	NaCl 5%	Citrate	Litmus milk
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Rods	+	Large round white	C	+	−	−	−	−	−	−	+			−	+
2	Rods	+	Large smooth yellow	C	+	−	−	−	−	−	−	+	+	+	−	+
3	Rods	+	Small round smooth	C	+	−	−	−	−	−	+	−	−	−	−	−
4	Rods	+	Large yellow spreading	C	+	−	−	−	−	−	−	+	+	+	−	+
5	Rods	+	Large round	C	+	−	−	−	+	−	−	+	+	+	+	+
6	Rods	+	Spreading irregular yellow	C	+	−	−	−	−	−	+	−	−	+	+	+
7	Rods	+	Large round band	C	+	−	−	−	+	−	+	+	+	+	−	−
8	Rods	+	Large round	C	+	−	−	−	+	−	+	+	+	+	−	−
9	Rods	+	Small sround smooth	C	+	−	−	−	−	−	−	−	−	+	−	−
10	Rods	+	Large spreading yellow	C	+	−	−	−	+	−	+	−	+	−	−	+
11	Rods	+	Large round white	C	+	−	−	−	+	−	+	+	+	−	−	+
12	Rods	+	Large round white	C	+	+	−	−	−	−	−	+	+	+	−	−
13	Rods	+	Large spreading	C	+	−	−	−	+	−	−	−	+	+	+	−
15	Rods	+	Large round	C	+	−	−	−	−	−	+	−	+	+	−	+
16	Rods	+	Small round	C	+	−	−	−	+	−	−	−	−	+	−	−
17	Rods	+	Large spreading	C	+	−	−	−	+	−	+	+	+	+	+	+
19	Rods	+	Large spreading yellow	C	+	−	−	−	−	−	−	−	+	+	+	−

Contd.....

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
20	Rod	+	Small round	C	+	+	-	-	-	-	-	-	-	+	-	-
21	Rod	+	Small round	C	+	+	-	-	-	-	-	-	-	+	+	-
22	Rod	+	Small round yellow	C	+	+	-	-	-	-	-	+	-	+	-	-
23	Rod	+	Large thin round	C	+	+	-	-	+	-	+	+	-	+	+	-
24	Rod	+	Large spreading irregular	C	+	-	-	-	+	-	-	+	+	+	-	-
25	Rod	+	Large round	C	+	-	-	-	+	-	+	+	+	-	-	+
26	Rod	+	Small round	C	+	±	-	-	+	-	-	+	-	+	+	+
27	Rod	+	Large round	C	+	+	-	-	+	-	-	+	+	+	+	+
28	Rod	+	Large round smooth yellow	C	+	-	-	-	+	-	+	+	+	+	-	-
29	Rod	+	Large irregular spreading	C	+	±	-	-	+	-	-	+	-	+	-	-
30	Rod	+	Large round yellow	C	+	-	-	-	+	-	+	+	+	+	-	+
31	Rod	+	Large round creamish	C	+	-	-	-	+	-	+	+	+	+	-	+
32	Rod	+	Large round white	C	+	±	-	-	-	+	-	+	+	-	-	+
33	Rod	+	Large irregular yeloow	C	+	-	-	-	-	-	-	+	+	-	-	-
34	Rod	+	Large spreading	C	+	+	-	-	-	-	-	+	+	+	+	-
35	Rod	+	Large spreading	C	+	-	-	-	-	-	+	+	+	-	+	+
36	Rod	+	Large round	C	+	-	-	-	-	-	+	+	+	-	-	-
37	Rod	+	Small round	C	+	-	-	-	-	-	+	+	+	-	-	-
38	Rod	+	Small round orange	C	+	+	-	-	-	+	+	+	+	+	-	-
39	Rod	+	Large smooth yellow	C	+	±	-	-	-	+	-	+	-	+	+	-
40	Rod	+	Large smooth white	C	+	+	-	-	-	-	+	+	+	+	+	-
41	Rod	+	Large spreading white	C	+	+	-	-	-	+	-	+	+	-	-	+

...Contd... Table 5

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
42	Rod	+	Large spreading white	C	+	–	–	–	–	–	–	+	+	–	–	–
43	Rod	+	Large spreading white	C	+	+	–	–	–	–	–	+	+	–	–	+
45	Rod	+	Large smooth yellow	C	+	–	–	–	–	+	–	–	+	–	+	–
46	Rod	+	Large smooth creamish	C	+	–	–	–	–	+	–	+	+	+	–	–
47	Rod	+	Large spreading white	C	+	+	–	–	–	–	–	–	–	+	–	–
48	Rod	+	Large round yellow	C	+	–	–	–	–	–	+	+	+	–	–	–
49	Rod	+	Small spreading white	C	+	–	–	–	–	+	–	–	+	–	–	+
50	Rod	+	Large starlike white	C	+	–	–	–	–	–	+	+	+	–	–	–
52	Rod	+	Large smooth round white	C	+	–	–	–	–	–	–	+	+	+	+	–
54	Rod	+	Pin point white	C	+	±	–	–	–	+	–	+	+	–	+	–
55	Rod	+	Large spreading yellow	C	+	+	–	–	–	+	–	+	+	+	+	+
56	Rod	+	Large spreading	C	+	+	–	–	–	+	+	+	+	+	+	+
57	Rod	+	Pin point white	C	+	–	–	–	–	+	–	+	+	+	+	+
58	Rod	+	Small round	C	+	–	–	–	–	+	–	+	+	+	–	–
59	Rod	+	Small round	C	+	±	–	–	–	+	–	+	+	+	–	+
60	Rod	+	Small round	C	+	–	–	–	–	+	+	+	+	+	–	+
61	Rod	+	Small round	C	+	–	–	–	–	+	+	+	+	+	–	+
62	Rod	+	Large smooth yellow	C	+	–	–	–	–	+	+	+	+	+	+	–
63	Rod	+	Small round	C	+	–	–	–	–	+	+	+	–	+	–	+
64	Rod	+	Small round	C	+	–	–	–	–	–	+	+	+	+	–	+
65	Rod	+	Small round	C	+	–	–	–	–	–	–	+	+	+	–	+
66	Rod	+	Small rod	C	+	–	–	–	–	–	+	+	–	–	–	+

Table 5a: Biochemical characteristics of psychrotrophic sporeforming isolates: carbohydrate utilization

Isolate Number	Fructose	Mannitol	Arabinose	Xylose	Raffinose	Sucrose	Maltose	Lactose	Dextrose	Salicin
1	2	3	4	5	6	7	8	9	10	11
1	+	±	+	±	-	-	-	-	+	-
2	+	±	+	±	-	-	-	-	+	-
3	x	x	x	x	x	x	x	x	x	x
4	+	±	+	+	-	-	-	+	-	-
5	+	+	+	-	-	+	+	-	+	-
6	+	+	+	+	±	-	-	+	-	-
7	-	+	+	+	-	+	-	+	-	-
8	-	+	+	-	-	-	-	+	-	-
9	-	+	-	-	-	-	+	-	+	-
10	±	-	+	+	-	+	+	+	-	-
11	-	+	+	-	±	-	-	-	+	-
12	-	+	+	-	-	-	-	-	+	-
13	-	±	+	±	-	-	+	+	+	-
15	±	-	+	±	-	-	+	-	-	-
16	±	+	+	±	-	-	-	+	+	-
17	±	±	+	-	-	-	+	+	+	-
19	±	±	+	-	-	-	-	-	+	-

Contd....

...Contd... Table 5a

Isolate Number	Fructose	Mannitol	Arabinose	Xylose	Raffinose	Sucrose	Maltose	Lactose	Dextrose	Salicin
20	−	±	+	+	−	−	−	−	+	−
21	−	±	+	+	−	−	−	−	+	−
22	−	±	+	±	−	−	−	+	−	−
23	−	±	+	±	−	−	−	−	+	−
24	+	±	+	−	−	−	−	+	−	−
25	+	±	+	±	±	−	+	−	+	−
26	−	±	+	+	−	−	−	−	+	−
27	−	+	+	±	−	−	−	−	+	−
28	−	+	+	+	±	−	−	+	−	−
29	−	+	−	+	−	+	−	−	+	−
30	+	+	+	±	−	+	−	+	−	−
31	+	−	−	−	+	+	+	−	+	−
32	−	−	+	−	+	+	−	−	+	−
33	−	−	+	±	±	−	−	−	+	−
34	±	±	−	−	−	+	−	−	+	−
35	−	±	+	+	−	−	−	−	+	−
36	±	−	−	±	−	−	−	−	+	−
37	−	−	−	+	−	−	−	−	+	−
38	+	+	+	+	±	+	−	+	−	−
39	+	+	+	+	±	−	+	+	+	−
40	−	−	−	±	−	+	+	−	+	−
41	−	±	+	±	−	+	+	+	−	−

Contd....

Isolate Number	Fructose	Mannitol	Arabinose	Xylose	Raffinose	Sucrose	Maltose	Lactose	Dextrose	Salicin
42	-	±	-	+	-	+	+	-	+	-
43	-	-	-	+	-	+	-	+	-	-
45	-	±	+	+	-	+	-	+	-	-
46	-	+	+	+	-	+	-	+	-	-
47	-	+	+	±	-	+	-	+	-	-
48	-	±	-	-	-	-	-	+	-	-
49	+	+	+	+	-	+	-	+	+	-
50	-	-	-	±	-	-	-	+	-	-
52	+	+	+	-	-	+	+	-	+	-
54	-	±	+	+	-	-	+	+	+	-
56	+	-	-	-	-	-	-	-	+	-
56	+	-	-	-	-	-	-	-	+	-
57	-	-	-	+	-	+	-	+	-	-
58	+	+	+	+	+	+	+	+	-	-
59	+	+	+	+	+	+	+	+	+	-
60	+	+	+	+	+	+	+	+	-	-
61	+	+	+	+	-	+	+	+	-	-
62	+	+	+	+	+	+	+	-	+	-
63	+	+	+	+	+	+	-	+	-	-
64	+	+	+	+	-	+	-	+	-	-
65	+	+	+	+	-	+	-	+	-	-
66	+	-	-	-	-	-	-	-	+	-

5% salts. Of the various carbohydrates tested for the utilization by the isolates, glucose was utilized by 58% and arabinose by 73% xylose by 73%, mannitol by 75%, fructose by 50%, raffinose by 23%, sucrose by 45%, lactose by 52% and maltose by 28%. 28% of the isolates utilized citrate as the sole source of carbon and 28% produced urea.

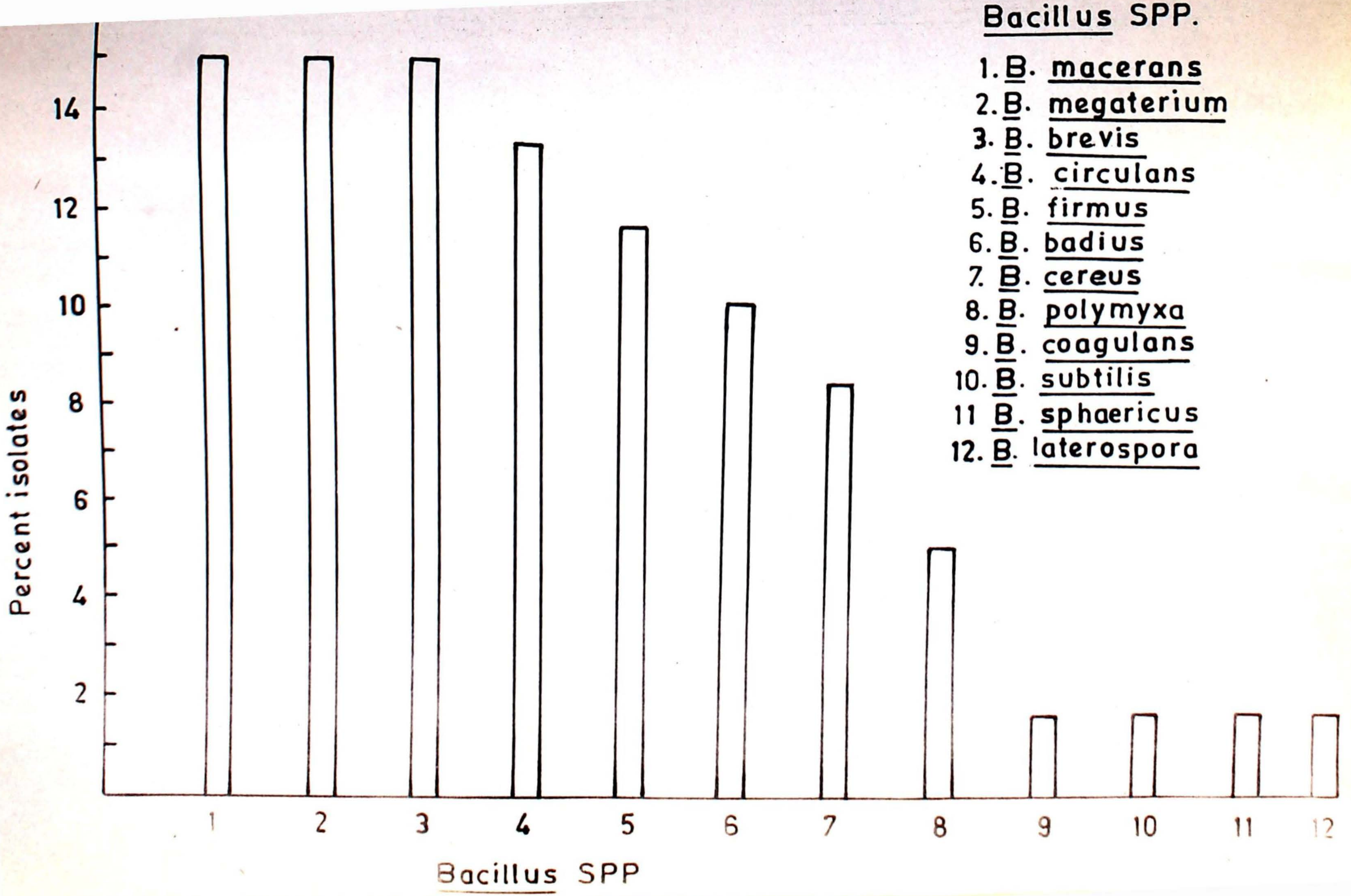
The spore producing types were placed in the genus Bacillus because they were all aerobic sporeforming, rod shaped organisms showed catalase positive characters. On the basis of the results of various morphological, cultural and biochemical tests (Table 5), all the isolates were assigned into 12 groups of Bacillus spp. (Table 6). Out of 60 isolates, 9 isolates were identified as B. marcerans, B. megaterium, and B. brevis, 8 were B. circulans, 7 were B. firmus, 6 were B. badius, 5 were B. cereus, 2 were B. polymyxa and one each B. coagulans, B. subtilis, B. sphaericus and B. laterospora.

Coghill and Juffs (1979) isolated 23 Bacillus spp. from pasteurized milk and identified 15 as B. cereus, 3 B. megaterium, 2 B. coagulans, 2 B. licheniformis and 1 B. firmus. Credit et al. (1972) isolated 84% of the bacteria belonging to genus Bacillus from pasteurized milk. Overcast and Atmaram (1974) also isolated psychrotrophic strain of B. cereus from pasteurized milk. Shehata et al. (1983) isolated 756 Bacillus from raw milk as: B. subtilis

Table 6: Identification of psychrotrophic spore forming isolates

Isolate Number	Identity	Total isolates
38, 47, 58, 59, 60, 61, 63, 64, 65	*B. macerans*	9
1, 2, 4, 28, 30, 32, 45, 49, 54	*B. megaterium*	9
12, 13, 19, 20, 21, 33, 34, 40, 52	*B. brevis*	9
6, 10, 11, 24, 35, 41, 42, 43	*B. circulans*	8
7, 8, 16, 25, 26, 31, 46	*B. firmus*	7
9, 15, 36, 37, 48, 66	*B. badius*	6
5, 27, 55, 56, 57	*B. cereus*	5
23, 39, 62	*B. polymyxa*	3
22	*B. coagulans*	1
17	*B. subtilis*	1
50	*B. sphaericus*	1
29	*B. laterospora*	1

Fig.4: Distribution of psychrotrophic spore forming Bacilli



Bacillus SPP.
1. B. macerans
2. B. megaterium
3. B. brevis
4. B. circulans
5. B. firmus
6. B. badius
7. B. cereus
8. B. polymyxa
9. B. coagulans
10. B. subtilis
11 B. sphaericus
12. B. laterospora
Percent isolates
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
Bacillus SPP

(42.5%), B. megaterium (34.8%), B. circulans (4.9%), B. cereus (4.6%) and only 47% isolates were psychrotrophic belonging to B. cereus (20), B. pumilus (15), B. badius (6), B. licheniformis (5) and B. firmus (1). Chug and Cannon (1971) isolated psychrotrophic sporeforming bacteria from raw milk and identified as B. firmus (46.3%), B. megaterium (23%), B. brevis (15.7%) and the remainder belonged to B. coagulans, B. polymyxa, B. macerans, B. circulans and B. cereus. Sharma et al. (1984) isolated 50 strains from raw milk of which 36% were B. cereus, 20% B. polymyxa, 14% each B. laterospora and B. circulans, 10% B. pumilus, 4% B. subtilis and 2% were B. coagulans.

4.5 Keeping Quality Tests

Twelve isolates, one from each group of Bacillus were studied in detail for their effect on the keeping quality of steamed and sterilized whole milk samples. One whole milk lot was steamed for one hour and other lot was sterilized by autoclaving at 121°C, 15 psi for 20 minutes. Data presented in Table 7 to 16. Regarding the keeping quality the samples were inoculated with the selected isolates stored at 7°C for 10 days at 22°C for 24 to 36 hrs. The quality assessment was done on the basis of stability test: heat stability test (COB) and alcohol stability test.

4.5.1 Stability test

4.5.1.1 Clot-on-Boiling test (COB):

In case of sterilized whole milk due to faster growth *B. cereus*, *B. firmus*, *B. circulans* and *B. macerans* at 22°C showed COB test positive only after 10 hrs of incubation while their growth at 7°C was slow and showed COB test positive only after 10 days. But *B. coagulans*, *B. polymyxa*, *B. brevis*, *B. megaterium* and *B. badius* showed COB test negative at 22°C after 24 hr storage and at 7°C for 10 days. More or less similar results were obtained in case of steamed whole milk with the exception of *B. polymyxa* and *B. brevis* which grew faster at 7°C and gave COB positive after 3 days (Table 7 and 8).

B. cereus, *B. firmus*, *B. circulans* and *B. macerans* are the important spoilage organisms at 22°C and *B. polymyxa* is important spoilage organism at 7°C. The above results correlate with the findings of Aggarwal and Srinivasan (1986). Aggarwal and Srinivasan (1986) reported that pasteurized milk inoculated with *B. cereus* show COB positive after 14 hrs. Similarly, Jayachandran *et al*. (1985) reported that COB test was positive for *B. subtilis* after 12 hr incubated at 22°C in boiled and pasteurized milk.

Table 7: Heat stability tests for selected isolates of psychrotrophic spore formers in sterilized whole milk* stored at 22°C and 7°C (COB)

Isolates	Storage temperature (22°C)				Storage temperature (7°C)					
	Period of incubation (hrs)				Period of incubation (days)					
	0	10	18	24	0	1	3	5	7	10
B. cereus	-	+	+	+	-	-	-	-	-	+
B. firmus	-	+	+	+	-	-	-	-	-	-
B. circulans	-	+	+	+	-	-	-	-	+	+
B. brevis	-	-	-	-	-	-	-	-	-	-
B. subtilis	-	-	+	+	-	-	-	-	+	+
B. coagulans	-	-	-	-	-	-	-	-	-	-
B. laterospora	-	-	-	+	-	-	-	+	+	+
B. polymyxa	-	-	-	-	-	-	-	-	-	-
B. megaterium	-	-	-	-	-	-	-	-	-	-
B. badius	-	-	-	-	-	-	-	-	-	-
B. sphaericus	-	-	+	+	-	-	-	-	-	-
B. macerans	-	+	+	+	-	-	-	-	-	+

* Sterilized by autoclaving at 121°C, 15 psi for 20 minutes.

Table 8: Heat stability tests for selected isolates of psychrotrophic spore formers in steamed whole milk* stored at 22°C and 7°C (COB)

Isolate	Storage temperature (22°C) Period of incubation (hrs)			Storage temperature (7°C) Period of incubation (days)					
	0	24	36	0	1	3	5	7	10
B. cereus	-	+	+	-	-	-	-	-	+
B. firmus	-	+	+	-	-	-	-	-	-
B. circulans	-	+	+	-	-	-	-	-	-
B. brevis	-	-	+	-	-	+	+	+	+
B. subtilis	-	-	-	-	-	-	-	-	-
B. coagulans	-	-	+	-	-	-	-	-	-
B. laterospora	-	+	+	-	-	-	-	-	-
B. polymyxa	-	-	-	-	+	+	+	+	+
B. megaterium	-	+	+	-	-	-	-	-	-
B. badius	-	-	+	-	-	-	-	-	-
B. sphaericus	-	+	-	-	-	-	-	-	-
B. macerans	-	+	+	-	-	-	-	-	-

* Steamed for one hour

4.5.1.2 Alcohol test:

Alcohol test was positive for most of the isolates within 24 hr of storage at 22°C (Table 9 and 10). In case of sterilized milk alcohol test was positive for B. cereus, B. circulans, B. subtilis even within 24 hr of storage at 7°C. It was probably due to the formation of acid and activation of metabolites by psychrotrophic bacteria in milk which disturbs the salt balance and leads to flake formation while treating with alcohol. Similar results were obtained in case of steamed milk but B. polymyxa and B. sphaericus could not disturb the salt balance when it was stored at 22°C for 36 hrs.

4.5.2 Acidity

Data presented in Table 11 and 12 showed the effect of selected isolates on development of acidity in steamed and sterilized milks when stored at 7°C for 10 days, showed a slow increase in acidity. Maximum acidity was shown by B. macerans (0.44%) at 7°C after storage for 10 days and minimum was shown by B. coagulans (0.27%) at the same temperature of storage. But on storage at 22°C there was a rapid increase in acidity. Maximum attained by B. macerans (0.54% as L.A.) and minimum (0.29% L.A.) by B. badius and B. polymyxa.

But when steamed milk was stored at 7°C for 10 days maximum acidity (0.67%) attained by B. polymyxa and minimum

Table 9: Alcohol stability tests for selected isolates of psychrotrophic spore formers in sterilized whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C)				Storage temperature (7°C)					
	Period of incubation (hrs)				Period of incubation (days)					
	0	10	18	24	0	1	3	5	7	10
B. cereus	-	+	+	+	-	+	+	+	+	+
B. firmus	-	+	+	+	-	-	-	-	-	-
B. circulans	-	+	+	+	-	+	+	+	+	+
B. brevis	-	-	-	+	-	-	-	-	+	+
B. subtilis	-	+	+	+	-	+	+	+	+	+
B. coagulans	-	-	-	-	-	-	-	-	-	+
B. laterospora	-	+	+	+	-	-	-	+	+	+
B. polymyxa	-	-	+	+	-	-	-	-	-	-
B. megaterium	-	-	-	-	-	-	-	-	+	+
B. badius	-	-	+	+	-	-	-	-	-	-
B. sphaericus	-	+	+	+	-	-	-	-	-	-
B. macerans	-	+	+	+	-	-	+	+	+	+

* Sterilized by autoclaving at 121°C, 15 psi for 20 minutes

Table 10: Alcohol stability tests for selected isolates of psychrotrophic spore formers in steamed whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C)			Storage temperature (7°C)					
	Period of incubation (hrs)			Period of incubation (days)					
	0	24	36	0	1	3	5	7	10
B. cereus	-	+	+	-	+	+	+	+	+
B. firmus	-	+	+	-	-	+	+	+	+
B. circulans	-	+	+	-	-	-	-	-	+
B. brevis	-	+	+	-	+	+	+	+	+
B. subtilis	-	-	+	-	-	-	-	+	+
B. coagulans	-	-	+	-	-	-	-	+	+
B. laterospora	-	+	+	-	-	-	-	-	-
B. polymyxa	-	-	-	-	-	-	-	-	+
B. megaterium	-	+	+	-	+	+	+	+	+
B. badius	-	+	+	-	+	+	+	+	+
B. sphaericus	-	-	-	-	-	-	-	-	-
B. macerans	-	+	+	-	-	-	-	-	-

* Steamed for one hour

Table 11: Developed acidity exhibited by selected isolates of psychrotrophic sporeformers in sterile whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C) Period of incubation (hrs) 0	10	18	24	Storage temperature (7°C) Period of incubation (days) 0	1	3	5	7	10
B. cereus	0.18	0.37	0.38	0.42	0.18	0.28	0.29	0.30	0.30	0.32
B. firmus	0.18	0.38	0.43	0.45	0.18	0.23	0.24	0.25	0.29	0.30
B. circulans	0.18	0.38	0.41	0.44	0.18	0.23	0.26	0.28	0.29	0.36
B. brevis	0.18	0.36	0.39	0.41	0.18	0.21	0.23	0.24	0.27	0.31
B. subtilis	0.18	0.30	0.40	0.42	0.18	0.21	0.27	0.27	0.27	0.30
B. coagulans	0.18	0.32	0.33	0.36	0.18	0.23	0.23	0.23	0.24	0.27
B. laterospora	0.18	0.27	0.29	0.32	0.18	0.24	0.27	0.27	0.27	0.28
B. polymyxa	0.18	0.27	0.29	0.29	0.18	0.23	0.26	0.26	0.30	0.36
B. megaterium	0.18	0.29	0.29	0.33	0.18	0.23	0.23	0.29	0.29	0.30
B. basidus	0.18	0.27	0.27	0.29	0.18	0.22	0.26	0.26	0.29	0.31
B. sphaericus	0.18	0.32	0.36	0.40	0.18	0.24	0.28	0.31	0.32	0.33
B. macerans	0.18	0.34	0.45	0.54	0.18	0.27	0.29	0.29	0.35	0.44

* Sterilized by autoclaving at 121°C, 15 psi for 20 minutes

Table 12: Developed acidity exhibited by selected isolates of psychrotrophic sporeformers in steamed whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C)			Storage temperature (7°C)					
	Period of incubation (hrs)			Period of incubation (days)					
	0	24	36	0	1	3	5	7	10
B. cereus	0.18	0.26	0.34	0.18	0.26	0.29	0.31	0.34	0.37
B. firmus	0.18	0.45	0.61	0.18	0.31	0.31	0.32	0.34	0.40
B. circulans	0.18	0.32	0.34	0.18	0.29	0.30	0.30	0.31	0.31
B. brevis	0.18	0.27	0.29	0.18	0.24	0.32	0.32	0.37	0.55
B. subtilis	0.18	0.32	0.34	0.18	0.27	0.30	0.31	0.31	0.34
B. coagulans	0.18	0.30	0.32	0.18	0.28	0.31	0.31	0.34	0.37
B. laterospora	0.18	0.27	0.29	0.18	0.22	0.29	0.29	0.32	0.34
B. polymyxa	0.18	0.27	0.32	0.18	0.32	0.36	0.40	0.41	0.67
B. megaterium	0.18	0.40	0.45	0.18	0.29	0.31	0.31	0.34	0.36
B. badius	0.18	0.30	0.34	0.18	0.30	0.32	0.33	0.33	0.34
B. sphaericus	0.18	0.27	0.30	0.18	0.28	0.32	0.32	0.34	0.34
B. macerans	0.18	0.45	0.59	0.18	0.29	0.31	0.32	0.34	0.35

*Steamed for one hour

by *B. circulans* (0.31%) but storage at 22°C for 36 hrs maximum acidity (0.61%) attained by *B. firmus* and minimum (0.29%) by *B. brevis*.

Jayachandran *et al*. (1985) reported that acidity of boiled milk inoculated with *B. subtilis* increased from 0.14 to 0.31 (% L.A.) when incubated at 30°C for 24 hrs

4.5.3 pH

Changes of pH in steamed and sterilized milk on storage at 7°C and 22°C due to the inoculation of selected isolates of psychrotrophic sporeformers are presented in Table 13 and 14. There was a slight change in pH at 7°C after 10 days storage of both steamed and sterilized milk. In sterilized milk decrease in pH from 6.7 to 6.2 by *B. laterospora* and from 6.7 to 6.4 by *B. megaterium* were noted. In steamed milk there was a slow decrease in pH from 6.7 to 6.6 and 6.7 to 6.3 in *B. brevis* and *B. firmus* respectively at 7°C after 10 days. The isolates caused a rapid decrease in pH at 22°C in both steamed and sterilized milk. In case of sterilized milk, *B. macerans* exhibited maximum reduction in pH at 22°C after 24 hr ranged from 6.6 to 5.2 and in the case of steamed milk maximum reduction in pH was from 6.7 to 5.25 by *B. firmus*.

4.5.4 Proteolytic activity

Data presented in Table 15 and 16 show the effect of selected isolates on proteolytic changes in steamed

Fig. 5: Standard curve for tyrosine

FIG. 5. STANDARD CURVE FOR TYROSINE
(Keay and Wildi method)

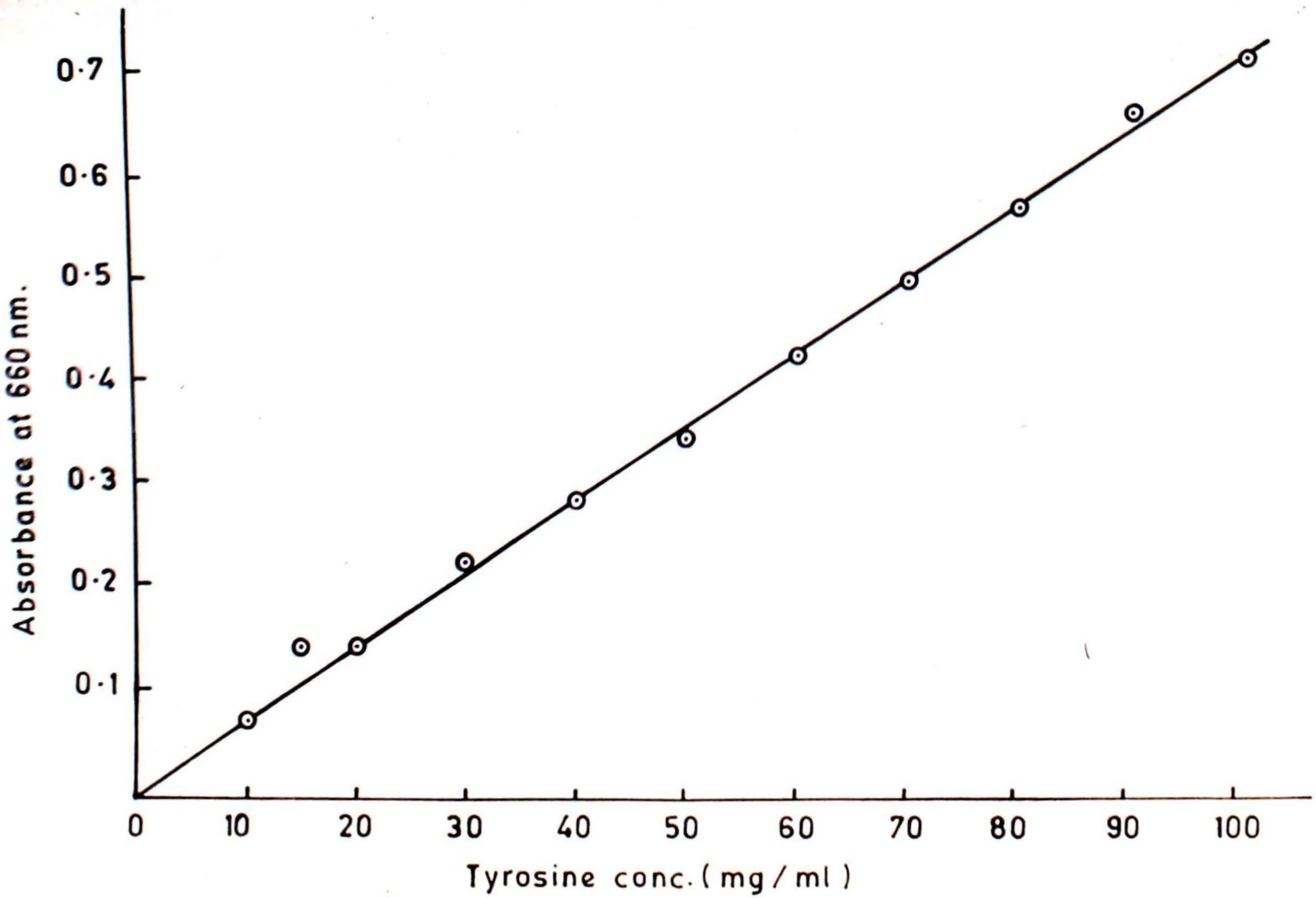


Table 13: Changes in pH exhibited by selected isolates of psychrotrophic spore formers in sterilized whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C)				Storage temperature (7°C)					
	Period of incubation (hrs)				Period of incubation (days)					
	0	10	18	24	0	1	3	5	7	10
B. cereus	6.6	6.37	6.33	6.30	6.7	6.44	6.40	6.40	5.95	6.40
B. firmus	6.6	6.20	6.03	5.64	6.7	6.48	6.45	6.41	6.40	6.40
B. circulans	6.6	6.31	6.20	6.15	6.7	6.47	6.46	6.40	6.40	6.40
B. brevis	6.6	6.43	6.29	6.29	6.7	6.51	6.50	6.45	6.40	6.38
B. subtilis	6.6	6.30	6.20	6.16	6.7	6.46	6.46	6.44	6.40	6.38
B. coagulans	6.6	6.43	6.30	6.28	6.7	6.50	6.50	6.46	6.40	6.40
B. laterospora	6.6	6.44	6.42	6.39	6.7	6.50	6.47	6.40	6.39	6.20
B. polymyxa	6.6	6.53	6.50	6.50	6.7	6.51	6.44	6.43	6.42	6.34
B. megaterium	6.6	6.53	6.50	6.50	6.7	6.52	6.51	6.50	6.50	6.44
B. badius	6.6	6.47	6.40	6.35	6.7	6.50	6.48	6.45	6.44	6.40
B. sphaericus	6.6	6.38	6.32	6.30	6.7	6.48	6.47	6.43	6.43	6.40
B. macerans	6.6	6.29	5.68	5.20	6.7	6.54	6.41	6.28	6.30	6.30

* Sterilized by autoclaving at 121°C, 15 psi for 20 minutes

Table 14: Changes in pH exhibited by selected isolates of psychrotrophic sporeformers in steamed whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C)			Storage temperature (7°C)					
	Period of incubation (hrs)			Period of incubation (days)					
	0	24	36	0	1	3	5	7	10
B. cereus	6.7	6.43	6.42	6.7	6.6	6.6	6.6	6.5	6.5
B. firmus	6.7	5.60	5.25	6.7	6.6	6.6	6.5	6.3	6.3
B. circulans	6.7	6.27	6.13	6.7	6.6	6.6	6.5	6.5	6.5
B. brevis	6.7	6.47	6.37	6.7	6.7	6.6	6.6	6.6	6.6
B. subtilis	6.7	6.43	6.31	6.7	6.6	6.6	6.6	6.6	6.6
B. coagulans	6.7	6.45	6.34	6.7	6.7	6.6	6.6	6.6	6.6
B. laterospora	6.7	6.50	6.46	6.7	6.6	6.6	6.6	6.6	6.4
B. polymyxa	6.7	6.57	6.40	6.7	6.7	6.6	6.6	6.6	6.5
B. megaterium	6.7	5.80	5.40	6.7	6.6	6.6	6.6	6.6	6.5
B. badius	6.7	6.39	6.00	6.7	6.6	6.5	6.5	6.5	6.5
B. sphaericus	6.7	6.48	6.25	6.7	6.6	6.5	6.5	6.5	6.5
B. macerans	6.7	5.54	5.27	6.7	6.5	6.5	6.5	6.5	6.5

*Steamed for one hour

Table 15: Proteolytic activity exhibited by selected isolates of psychrotrophic sporeformers in sterilized whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C) Period of incubation (hrs)				Storage temperature (7°C) Period of incubation (days)					
	0	10	18	24	0	1	3	5	7	10
	Proteolytic activity (units/ml)									
B. cereus	0	57	107	282	0	40	42	48	57	64
B. firmus	0	70	85	182	0	42	43	46	50	52
B. circulans	0	70	117	282	0	43	46	54	70	92
B. brevis	0	57	65	74	0	29	29	34	42	68
B. subtilis	0	68	115	282	0	35	43	50	60	68
B. coagulans	0	60	64	85	0	33	41	45	54	68
B. laterospora	0	64	77	83	0	42	43	46	57	68
B. polymyxa	0	64	64	65	0	45	48	54	62	68
B. megaterium	0	68	70	83	0	42	40	45	57	62
B. badius	0	70	77	85	0	40	43	50	85	88
B. sphaericus	0	70	88	97	0	43	43	48	65	74
B. macerans	0	85	129	282	0	35	50	55	74	78

* Sterilized by autoclaving at 121°C, 15 psi for 20 min.

Table 16: Proteolytic activity exhibited by selected isolates of psychrotrophic sporeformers in steamed whole milk* stored at 22°C and 7°C

Isolates	Storage temperature (22°C) Period of incubation (hrs)			Storage Temperature (7°C) Period of incubation (days)					
	0	24	36	0	1	3	5	7	10
	Proteolytic activity (units/ml)								
B. cereus	0	70	115	0	42	42	45	48	50
B. firmus	0	85	115	0	28	45	46	50	64
B. circulans	0	115	120	0	45	48	50	57	60
B. brevis	0	45	50	0	42	42	45	52	62
B. subtilis	0	78	129	0	45	50	55	56	57
B. coagulans	0	46	50	0	42	45	50	57	67
B. laterospora	0	46	64	0	40	40	45	57	62
B. polymyxa	0	42	60	0	62	62	65	68	72
B. megaterium	0	48	84	0	54	56	61	64	64
B. badius	0	45	50	0	57	58	60	62	62
B. sphaericus	0	46	54	0	43	48	48	50	50
B. macerans	0	100	100	0	28	31	50	54	54

*Steamed for one hour

and sterilized milks on storage at 7°C and 22°C. At low temperature there was a slow proteolytic change in both steamed and sterilized milk stored at 7°C and 22°C. Maximum enzyme activity of 92 units/ml was exhibited by *B. circulans* after 10 days of storage at 7°C. But the proteolytic changes were fast at 22°C after 24 hr. Maximum enzyme activity exhibited by *B. cereus*, *B. circulans* and *B. subtilis* (282 units/ml) and minimum by *B. brevis* (72 units/ml) and *B. polymyxa* (65 units/ml) in sterile milk.

Changes in milk protein as a result of psychrotrophic growth due to enzymatic action showed importance in the keeping quality of milk at refrigeration temperature. Release of various nitrogen components or degradation of individual proteins fractions were observed when studying proteolysis caused by enzymes produced by psychrotrophs (McCaskay, 1967). The enzyme protease attack on casein and whey protein leading to bitter flavour and coagulation of milk.

: : : : :

: : :

:

CHAPTER 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

5.1 The present investigation deals with the isolation, identification and characterization of psychrotrophic sporeforming bacteria from pasteurized milk and their effect on keeping quality of steamed and sterilized milk stored at $22^{\circ}C$ and $7^{\circ}C$ for 24 hr and 10 days.

5.2 On incubation at $22^{\circ}C$ for 24 hr the psychrotrophic count and proteolytic spore count ranged from 2001 to 5000/ml in 44% samples and 26-50/ml in 43% samples respectively, when the milk was stored at $22^{\circ}C$ for 24 hr. However, on storing the milk at $7^{\circ}C$ for 10 days, the respective counts were found to be 4000 in 40% samples and 1-25 in 52% samples.

5.3 On incubation at $7^{\circ}C$ for 10 days the psychrotrophic spore count and proteolytic psychrotrophic spore count ranged from 10,000 in 42% samples and 101 to 200 in 46% samples when milk was stored at $22^{\circ}C$ for 24 hr. However, on storing at $7^{\circ}C$ for 10 days, the respective counts in milk were found to be >2,000 in 43% sample, 26-50 in 47% samples.

5.4 From the 110 samples of milk 66 psychrotrophic sporeforming colonies showing discrete zone of clearance around them on the milk agar and on TDYA were selected for further studies.

5.5 The protease activity of the isolates ranged from 100 to 300 units/ml, 50% of the isolates have enzyme activity ranged from 151-300 units/ml while 38% exhibited enzyme activity in the range of 101-150 units/ml.

5.6 Based on morphological, cultural and biochemical characteristics all the psychrotrophic spore forming isolates were grouped into twelve Bacillus spp. Out of 60 isolates 15% were B. macerans, B. megaterium, B. brevis, 10% B. badius, 8% were B. cereus, 5% were B. polymyxa and 2% were B. coagulans, B. subtilis, B. laterospora and B. sphaericus. 11% were B. firmus, 12.5% were B. circulans

5.7 On incubation of these isolates in sterilized milk medium at 7°C for 10 days the acidity of milk samples were increased maximum in case of B. macerans (0.44% as L.A.) and at 22°C for 24 hr storage, maximum acidity was attained by B. macerans (0.54% L.A.). On storage of the milk at 7°C for 10 days after inoculated with the isolates, maximum acidity was exhibited by B. polymyxa (0.67% L.A.) and on storage at 22°C maximum acidity was attained by B. firmus (0.61%).

5.8 Change in pH was very little at 7°C for 10 days storage in both steamed and sterilized milk. But on storage at 22°C for 24 hr the maximum decrease in pH was 6.7 to 5.25 by B. firmus in steamed milk and 6.6 to 5.2 by B. macerans in sterilized milk.

5.9 B. cereus, B. firmus, B. circulans and B. macerans showed positive heat stability test in both the milks and alcohol stability test was positive for all the isolates stored for 24 hr at 22°C.

5.10 A significant increase in proteolytic activity was found in case of B. cereus, B. circulans and B. subtilis (282 units/ml) at 22°C after 24 hr storage in the case of sterile milk whereas in the case of steamed milk increase in proteolytic activity was comparatively less. Maximum enzyme activity was attained by B. subtilis (129 units/ml) at 22°C after 24 hr. Whereas the increase in proteolytic activity at 7°C for 10 days storage was very less compared to storage at 22°C for 24 hr in both the milks.

5.11 This investigation clearly reveals that the occurrence of psychrotrophic spore forming bacilli in pasteurized milk is of great importance to the dairy industry. The proteolytic activity of these bacilli causes the spoilage of milk and milk products. From the above observations it is observed that these isolates *B. cereus*, *B. circulans* and *B. subtilis* are the most important strains among the psychrotrophic sporeformers which cause the change in chemical quality of the steamed and sterilized milk.

: : : : :

: : :

:

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, D.M.; J.T. Barach and M.L. Speck (1975)
"Heat resistant protease produced by psychrotrophic bacteria of dairy origin".
J. Dairy Sci., 58: 828-834.

Aggarwal, P.K.; Srinivasan, R.A. (1986)
"Influence of Bacillus cereus on the keeping quality of pasteurized milk".
Indian J. Dairy Sci. 39: 95-97.

Alford, J.A & L.E. [illegible] ([illegible]) Food Research 25 [illegible]

Andrews, R.H. and O.W.Kaufman (1953)
"The destruction of psychrophilic bacteria in milk by HTST pasteurization based on thermal death times studies". J. Dairy Sci. 36: 570-571.

Azuma, Y.S.; S.B. Newton and L.D. Witter (1962)
"Production of psychrophilic mutants from mesophilic bacteria by ultraviolet irradiation".
J. Dairy Sci. 45: 1529-1530.

Baumann, D.P. and Reinbold, G.W. (1963)
"The enumeration of psychrotrophic microorganisms in dairy products".
J. Milk Food Technol. 26: 351-356.

Bergey's Manual of Determinative Bacteriology (1974)
Ed. R.E. Buchanan and N.E. Gibbons. 8th edn.
Waverly Press, Baltimore.

Bigalke, D. (1985)
"Biochemical changes in dairy products caused by growth of psychrotrophic microorganisms. 1. Microbial enzymes". Dairy and Food Sanitation, 5: 345-346.

Bishop, J.R. and C.H. White (1985)
"Estimation of potential shelf life of pasteurized fluid milk utilizing bacterial numbers and metabolites".
J. Food Prot. 48: 663-667.

Bjork, L. (1978)
"Antibacterial effect of lactoperoxidase system on psychrotrophic bacteria in milk".
J. Dairy Res. 45: 109-118.

Bjork, L.; C.G.Rosen; V. Marshal and B. Reiter (1975)
"Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria".
Appl. Microbiol. 30: 199-204.

Bjork, L.; O, Claesson and W. Schulthes (1979)
"The lactoperoxidase/thiocyanate/hydrogen peroxide system as a temporary preservatives for raw milk in developing countries".
Milchwissenschaft, 34: 726-729.

Blank, B.; E. Fluckiger, M. Ruegg; G. Steiger (1984)
"Effect of cold storage and enrichment with psychrotrophic bacteria on quality of UHT milk".
Dairy Sci. Abstr. 48: 3371.

Bodyfelt, F.W. and W.D. Davidson (1975)
"Temperature control. A procedure for profiling temperature of dairy products in stores".
J. Milk Food Technol. 38: 734-737.

Boyd, J.C.; C.K. Smith and G.M. Trout (1955)
"The role of psychrophilic bacteria in keeping quality of commercially pasteurized and homogenized milk".
J. Milk Food Technol. 18: 32.

Brown, J.V.; R. Wiles and G.A. Prentice (1979)
"The effect of a modified tyndallization process upon the sporeforming bacteria of milk and cream".
J. Soc. Dairy Technol. 32: 109.

Chopra, P.; A. Singh and M.S. Kalra (1980)
"Occurrence of Bacillus cereus in milk and milk products". Indian J. Dairy Sci., 33: 248-252.

Choudhary, A.K. and E.M.Mikolajcik (1971)
"Bacilli in milk. 2. Sweet curd formation".
J. Dairy Sci. 54: 321-325.

Christen, G.L.; and W.L. Whang (1985)
"Composition of heat resistance of lipase and protease".
J. Dairy Sci. 68 (Suppl.): 260-261.

Chug, B.H. and R.V. Cannon (1971)
"Psychrotrophic sporeforming bacteria in raw milk supplies". J. Dairy Sci. 54: 448.

Coelho, D.T. and E.B.B. Coelho (1979)
"Influence of culture medium on counting of psychrotrophic bacteria in milk and pasteurized liquid derivatives".
Dairy Sci. Abstr. 43: 7721.

Coghill, D. (1982)
"Studies on thermoduric psychrotrophic bacteria in South East Queensland Dairy Products".
Aust. J. Dairy Technol. 37: 147-148.

Coghill, D. and H.S. Juffs (1979)
"Incidence of psychrotrophic sporeforming bacteria in pasteurized milk and cream products and effect of temperature on their growth".
Aust. J. Dairy Technol. 34: 150-153.

Cousin, M.A. (1982)
"Presence and activity of psychrotrophic microorganisms in milk and dairy products. A review".
J. Food Prot. 45: 172-207.

Cousin, M.A. and E.H. Marth (1977)
"Psychrotrophic bacteria cause changes in stability of milk to coagulation by rennet or heat".
J. Dairy Sci. 60: 1042.

Cox, W.A. (1975)
"Problems associated with bacterial spores in heat treated milk and dairy products".
J. Soc. Dairy Technol. 28: 59-68.

Credit, C.; R. Hedeman; P. Heywood and D. Westhoff (1972)
"Identification of bacteria isolated from pasteurized milk following fefrigerated storage".
J. Milk Food Technol. 35: 708.

Davies, F.C. (1975)
"Heat resistance of Bacillus species".
J. Soc. Dairy Technol. 28: 69-78.

Deeth, H.C.; C.H. Fitz-Gerald and A.F. Wood (1983)
"Lipolysis and butter quality".
Aust. J. Dairy Technol. 34: 146-148.

Downey, W.K. (1980)
"Review of progress of dairy science: Flavor impairment from pre- and post manufacture lipolysis in milk and dairy products".
J. Dairy Res. 47: 237-252.

Eddy, B.P. (1960)
"The use and meaning of the term 'psychrotrophic'".
J. Appl. Bacteriol. 23: 189-190.

El-Bassiony, T.A.; Aboul-Khier, F.A.; Moustafa, M.K. and Ahmed, A.A. (1985)
"Psychrotrophic bacteria in raw and pasteurized milk".
Assiut. Vety. Med. J. 14: 115-119.

Elliot, R.P. and H.D. Michener (1965)
"Factors affecting the growth of psychrotrophic microorganisms in foods. A review".
USDA Technical Bulletin No.1320.

Elliot, J.A.; D.B. Emmons and A.R. Yates (1974)
"The influence of the bacterial quality of milk on the property of dairy products. A review". Cand. Inst. Food Sci. Technol. J. 7: 32-39.

Fairbairn, D.J. and B.A. Law (1986)
"Proteinases of psychrotrophic bacteria: their production, properties, effects and control". J. Dairy Res. 53: 139-177.

Finley, R.D.; H.B. Warren and R.E. Hargrove (1968)
"Storage stability of commercial milk". J. Milk Food Technol. 31: 382-387.

Fitz-Gerald, C.H.; C.H. Deeth and D.M. Ganguli (1982)
"Low temperature inactivation of lipase from psychrotrophic bacteria". Aust. J. Dairy Technol. 37: 51.

Folin, O. and Ciacalteau, V. (1927)
"Colorimetric procedure for amino acids". In methods in Enzymology. S.P. Colowick and Nov. Kaplan, eds. Vol.3: 467-477. Academic Press, New York, Cited: Spies, J.R. (1957).

Franklin, J.G. (1970)
"Spores in milk: Problems associated with UHT processing". J. Appl. Bacteriol. 33: 180-191.

Gilbert, R.J. and Taylor, A.J. (1976)
"Bacillus cereus food poisoning." In agriculture afisheries and food. Ed. Skinner, F.A. & earr, J.G. Society for Applied Bacteriology Symposium series No.4, pp. 197-213. London: Academic Press. Cited: Langeveld (1973).

Gordon. (1982)

Greene, V.W. (1959)
"Influence of temperature history on the response of psychrophiles to different incubation temperature". J. Dairy Sci. 42: 1097-1099.

Greene, V.W. and J.J. Jezeski (1954)
"Influence of temperature on the development of several psychrophilic bacteria of dairy origin". Appl. Microbiol., 2: 110-117.

Griffiths, M.W.; J.D. Phillips and D.D. Muir (1980)
"Rapid plate counting technique for enumeration of psychrotrophic bacteria in pasteurized double cream". J. Soc. Dairy Technol. 33: 8-10.

Grosskopf, J.C. and W.J. Harper (1969)
"Role of psychrophilic sporeformers in long life milk".
J. Dairy Sci. 52: 897.

Grosskopf, J.C. and W.J. Harper (1974)
"Isolation and identification of psychrotrophic sporeformers in milk". Milchwissenschaft 29: 467-470.

Hartley, J.C.; E.R. Vedamuthu and G.W. Reinbold (1969)
Bacteriological methods for evaluation of raw milk quality. A review II. Bacterial tests cited to measure milk quality".
J. Milk Food Technol. 32: 4-15.

Herbert, R.A. and Bhakoo (1979)
"Microbial growth at low temperatures".
In A.D. Rusell and R. Fuller (eds). Cold tolerant microbes in spoilage and environment. Academic Press, New York. p.1-16.

Higoshi, H.; S. Hamada and Doi (1975)
"Studies on the growth temperature of psychrotrophic bacteria: growth at various incubation temperature of psychrotrophic pseudomonas originated from raw milk and raw meat.
Jap. J. Vety. Sci. 37: 165-177.

Honer, C. (1981)
"Raw milk psychrotrophs and the importance of their control".
Dairy Record. 82: 104D, 106, 108.

I.D.F. (1974)
I.D.F. (1981)
"Enumeration of psychrotrophic microorganisms, colony count technique at 6.5°C".
I.D.F. Standards, 101, 1981 (Provisional).

Ikonomova, L.; Stefanova-Kondrateuko, M. (1970)
"Lactic acid fermentation at low temperature".
Vet. Med. Nauki. Sof. 7: 45-54.
Cited: Dairy Sci. Abstr. 33: 2015.

Ingraham, J.L. (1962)
"Newer concepts of psychrophilic bacteria.
In proceedings low temperature microbiology symposium. 1961. Campbell Soap Company, Caniden, New Jersey, p. 41-56.

Ingraham, J.L. and J.L. Stokes (1959)
"Psychrophilic bacteria".
Bacteriological Review (1959) 23: 97-108.

Innis, W.E. (1978)
Interaction of temperature and psychrophilic microorganisms. Ann. Rev. Microbiol. 29: 445-465.

Innis, W.E. and J.L. Ingraham (1978)
"Microbial life at low temperatures: Mechanisms and molecular aspects".
In D.J. Kushner (ed.) Microbial life in extreme environments. Academic Press, New York, p.73-104.

Indian Standard Institution (1962)
Methods of test for dairy industry.
IS: 1479 (Part III), I.S.I., New Delhi.

Jackson, A.L. (1978)
"Short shelf-life milk products".
J. Soc. Dairy Technol. 31: 80-90.

Jayachandran, T.; P.S. Krishnamurthi and N. Natrajan (1985)
"Study on biochemical changes by spores of Bacillus subtilis in pasteurized milk and boiled milk".
Cheiron . 14: 138-142.
Cited: Dairy Sci. Abstr. 47: 7842.

Johns, C.K. (1971)
"Bacteriological testing of milk for regulatory purpose - usefulness of current procedures and recommendations for change. II. Bacteriological testing of raw milk for regulatory purposes."
J. Milk Food Technol. 34: 173-180.

Johnston, D.W. and Bruce, J. (1982)
"Incidence of thermoduric psychrotrophs in milk produced in the West Scotland".
J. Appl. Bacteriol. 52: 333-337.

Jonathanp, B.F. and E. H. Marth (1984)
"Changes in size of casein micelles caused by growth of psychrotrophic bacteria in raw skim milk".
J. Food Prot. 47: 16-19.

Juffs, B.H.S. (1970)
"A four day count for psychrotrophs".
Aust. J. Dairy Technol. 25: 30-32.

Juffs, H.S.; A.C. Hayward and H.W. Doelle (1968)
"Growth and proteinase production in Pseudomonas spp. cultivated under various conditions of temperature and nutrition."
J. Dairy Research 35: 385-393.

Kandler, O. (1966)
"Zur Defiuction der" "Psychrophileu" Bacterieu".
Milchwissenschaft 21: 257-260.

Kathleen, O. Donvon (1958)
A selective medium for _Bacillus cereus_ in milk.
J. Appl. Bacteriol. 21: 100-103.

Keay, L. and Wildi, B.S. (1970)
"Protease of genus _Bacillus_ 1. Neutral protease.
Biotech. Bioeng. 12: 179-212.

Kraft, A.A. and C.R.Ray (1979)
"Psychrotrophic bacteria in foods".
Food Technol. 33: 66-71.

Kroll, S.; B. Kellerer; M. Busse; and M.Klostermeyer (1984)
"Proteolytic psychrotrophs of raw milk. 1. Identification on the basis of physiological characteristics".
Milchwissenschaft 39: 538-540.

Langeveld et al (1965)

Lausten, K. (1978)
"Development of aerobic psychrotrophs in whole and skim milk".
Cited: Dairy Sci. Abstr. 43: 1638.

Law, B.A. (1979)
"Reviews of the progress of dairy science: Enzyme of psychrotrophic bacteria and their effect on milk and milk rpdoucts".
J. Dairy Res. 46: 573-588.

Law, B.A.; M.E. Sharpe and H.R. Chapman (1976)
"The effect of lipolytic gram-negative psychrotrophs in stored milk on the development of rancidity in cheddar cheese".
J. Dairy Res. 43: 459-468.

Law, B.A.; A.T. Andrews and M.E. Sharpe (1977)
"Gelation of UHT sterilized milk by protease from a strain of _Pseudomonas fluorescence_ isolated from raw milk".
J. Dairy Res. 44: 145-148.

Lawrence, R.C. (1967)
"Microbial lipases and related esterases Part II. Estimation of lipase. Recent work concerning their effect on dairy products".
Dairy Sci. Abstr. 29: 59-70.

Ledford, R.A.; Senyk, G.F.; Shipe, W.F. and D.K. Bandler (1980)
"Bacteriological evaluation of fresh and aged market milk". J. Dairy Sci. 63(Suppl.) : 13.

Lowry, O.H.; Rosebrough, M.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with the Folin Phenol reagents". J. Biol. Chem. 193: 265-275.

Luck, H. (1972) "Bacteriological quality tests for bulk cooled milk. A review". Dairy Sci. Abstr. 34: 101-102.

Luck, H. and F. Hopkins (1975) "Comparison of different plate count tests for determining the number of psychrotrophs". South African J. Dairy Technol. 7: 89-94.

Lund, R. and H. Sogaurd (1980) "Psychrotrophs and liquid milk production. 1. Comparison of 3 methods for determining psychrotroph count in raw milk". Cited: Dairy Sci. Abstr. 45: 5783.

Magdoub, M.N.L.; A.E.Shehata; El-Samragy, Y.A. and A.A. Hassan (1983) "Growth parameter of psychrotrophic Bacillus spp. isolated from raw milk". Asian J. Dairy Research 2: 97-105.

Maksimova, A.K. (1970) "Intensification of production of culture milk". Moloch. Prom. 27, 32. Cited: Dairy Sci. Abstr., 33: 3676.

Malik, R.K. and D.K. Mathur (1983) "Isolation and identification of protease producing psychrotrophic bacteria from dairy products in India". J. Soc. Dairy Technol. 36: 76-78.

Marshall, R.T. (1982) "Relationship between the bacteriological quality of raw milk and the final products. A review of basic information and practical aspects". Cited: Dairy Sci. Abstr. 44: 8638.

Marth, E.H. (1978) Standard methods for the examination of dairy products. 14th edn. American Public Health Association, Washington, D.C.

Maxcy, R.B. (1967) "Nature and growth response of the microflora of pasteurized milk". J. Milk Food Technol. 30: 213-218.

McCaskey, T.A. (1967)
"Microbial and enzymatic activity in raw milk held at low temperature".
Diss. Abstr. 4046B.

Mikolajick, E.M. (1978)
"Psychrotrophic sporeformers: A possible keeping quality problem in market milk".
American Dairy Review 40: 34A 34D.

Mikolajcik, E.M. and Koka, M. (1968)
"Bacilli in milk. Spore germination and growth".
J. Dairy Sci. 51: 1579.

Mikolajcik, E.M. and Simon, M.T.(1978)
"Heat resistant psychrotrophic bacteria in raw milk and their growth at 7°C".
J. Food Prot. 41: 93-95.

Mistry, V.V. and Kosikowsy, F.V. (1985)
"Influence of potassium sorbate and hydrogen peroxide on psychrotrophic bacteria in milk".
J. Dairy Sci. 68: 605-608.

Morita, R.Y. (1975)
"Psychrotrophic bacteria".
Bacteriol. Rev. 39: 144-167.

Morse, P.H. ; H. Jackson; C.H. McNaughton; A.G. Legatt; G.B. Landerkin and C.K. Kokns (1968)
"Investigation of factors contributing to the bacterial count of bulk tank milk. 1. Influence of two day storage and effect of results of preliminary incubation".
J. Dairy Sci. 51: 1182-1187.

Mottar (1981)
Moustafa, H.H. and E.B. Collins, (1969)
"Effect of selective food additives on growth of *Pseudomonas fragi*".
J. Dairy Sci. 52: 335-340.

Muir, D.D.; Kelly, M.E. and J.D. Phillips (1978)
"The effect of storage temperature on bacterial growth and lipolysis in raw milk".
J. Soc. Dairy Technol. 31: 203.

Miller (1903)
Nashif, S.A. and F.E. Nelson (1953)
The lipase of *Pseudomonas fragi*. II. Factors affecting lipase production.
J. Dairy Sci. 36: 481-488.

Oliveria, J.S. and Parmelee, C.E. (1976)
"Rapid enumeration of psychrotrophic bacteria in raw and pasteurized milk".
J. Milk Food Technol. 39: 269.

Otte, I.; A. Tolle and G. Hahn (1979)
Zur Analyses der Mikroflora von Milch and Milch produkten. 2. Miniaturisierte Primae tests zur Bestimmung der Gattung.
Milchwissenschaft, 34: 152-156.

Overcast, W.W. and K. Atmaram (1974)
"The role of Bacillus cereus in sweet curdling of fluid milk".
J. Milk Food Technol. 37: 233.

Patel, G.B. and B. Blankenagel (1972)
"Bacterial counts of raw milk and flavor of milk after pasteurization and storage".
J. Milk Food Technol. 36: 146-157.

Phillips, J.D.; Griffiths, M.W. and Muir, D.D. (1981)
"Growth and associated enzymatic activity of spoilage bacteria in pasteurized double cream".
J. Soc. Dairy Technol. 34: 113-118.

Punch, J.D.; J.C. Olson, Jr. and E.L. Thomas (1965)
Psychrophilic bacteria. III. Population levels associated with flavour or physical change in milk".
J. Dairy Sci. 48: 1179-1183.

Richter, R. (1979)
"Psychrotrophic bacteria and shelf life".
American Dairy Rev. 41: 50J.

Rowe, M.T.; and Gilmour, A. (1983)
"Nutritional factors affecting extracellular enzyme production by Pseudomonas fluorescence 1352".
Milchwissenschaft. 38: 705-707.

Sharma et al (1984)

Shehata, T.E. and E.B. Collins (1971)
"Isolation and identification of psychrotrophic species of Bacillus from milk.
Appl. Microbiol. 21: 466-469.

Shehata, A.E.; Magdoub, M.N.I., Sultan, N.E. and El-Samragy, Y.A. (1983) Aerobic mesophilic and psychrotrophic sporeforming bacteria in buffalo's milk.
J. Dairy Sci. 66: 1228.

Shehata, A.E.; Magdoub, M.N.I.; El-Samragy, Y.A. and Sultan, N.E. (1985)
"Influence of sub-lethal heat-shock, L-cysteine, L-asparagine and nisin on generation of psychrotrophic Bacillus spores in milk".
Egy. J. Dairy Sci. 13: 137-148.

Skerbkova, E.L. (1979)
"Sporeforming bacteria causing defects in evaporated milk".
Cited: Dairy Sci. Abstr. 44: 3090.

Smith, K.L.; L.E. Hull; G.B. Lane and A.J. Baggot, Jr. (1972)
"Keeping quality of milk exposed to high temperature as experienced during transport in automobiles.
J. Milk Food Technol. 35: 588-590.

Stadhouders, J. (1975)
Microbes in milk and dairy products.
An ecological approach. Neth. Milk Dairy J. 29: 104-126.

Stewart, D.B.; J.G. Murray and S.D. Neill (1975)
"Lipolytic activity of organisms isolated from refrigerated bulk milk".
Int. Dairy Fed. Annual Bull. 86: 38-50.

Stokes, J.L. (1963)
"General biology and nomenclature of psychrophilic microorganisms. P. 187-192.
In N.E. Gibbons (Ed.) Recent Progress in Microbiology.
University of Toronto Press, Canada.

Stokes, J.L. and M.L. Redmond (1966)
"Quantitative ecolony of psychrophilic microorganisms"
Appl. Microbiol. 14: 74-78.

Sudarsanam, T.S. and R. A. Srinivasan (1982)
Studies on psychrotrophic lactic acid bacteria in pasteurized milk with special reference to proteolytic activity.
Ph.D. Thesis: Submitted to Kurukshetra University.

Tekinson, O.C. and Rothwell, J. (1974)
"A study of the effect of storage at 5°C on the microflora of heat-treated market cream".
J. Soc. Dairy Technol. 27: 57.

Thomas, S.B. (1958)
"Psychrophilic microorganisms in milk and dairy products. Part II. Dairy Sci. Abstr. 20: 449-469.

Thomas, S.B. (1966)
"Source, incidence and significance of psychrotrophic bacteria in milk".
Milchwissenschaft. 21: 270-271.

Thomas, S.B. (1970)
"Psychrotrophic microorganisms in market cream. A review".
Dairy Ind. 35: 79-84.

Thomas, S.B. and R.G. Druce (1969)
Psychrotrophic bacteria in refrigerated milk. Part III. Dairy Ind. 34: 501-505.

Thomas, S.B. and B.F. Thomas (1973)
"Psychrotrophic bacteria in refrigerated bulk collected raw milk . Part I."
Dairy Ind. 38: 11-15.

Thomas, S.B. and B.F. Thomas, (1977)
"The bacterial content of milking machine and pipeline milking plant. A review".
Dairy Ind. Int. 42: 7-12.

Thomas, S.B.; Griffiths, M.J. and Foulkes, J.B. (1960)
"Psychrophilic bacteria in pasteurized milk"
Dairy Engineering. 77: 438-445.

Uplacksh, V.K. and Sudarsanam, T.S. (1986)
"Studies on psychrotrophic spore formers in pasteurized milk with reference to its keeping quality". M.Sc. Dissertation Submitted to Kurukshetra University.

Vanderzant, W.C. and A.V. Moore (1955)
The influence of certain factors on the bacterial counts and proteolytic activities. of several psychrotrophic organisms.
J. Dairy Sci. 38: 743-750.

Von Bockelmann, I. (1970)
Lipolytic and psychrotrophic bacteria in cold stored milk. XVIII Inter. Dairy Congress.
IE: 106 (Abst).

Waes, G. (1966)
Some observations on the enumeration of psychrotrophic bacteria in milk.
Dairy Ind. 21: 282-285.

Washam, C.J.; H.C. Olson, and E.R. Vedamuthu (1977)
Heat-resistant psychrotrophic bacteria isolated from pasteurized milk".
J. Food Prot. 40: 101-108.

Whang, D.W. and Cho, J. (1981)
"Psychrotrophic bacteria in milk"
Korean J. Vet. Publ. Hlth. 5: 11-14.

White, C.H.; M. Bulthaus, and R.T. Marshall (1978)
Defects caused by adding protease producing psychrotrophs to raw milk.
J. Dairy Sci. 62 (Suppl.1): 46 Abstr.

Wilkinson, G. and F.L. Davies (1974)
Some aspects of the germination of Bacillus cereus in milk. Pages 153-159. In A.N. Barker, G.W. Gould, and J. Wolf (eds) Spore Research 1973. Academic Press, New York.

Williamson, W.T.; Tove, S.B. and Speck, M.L. (1962)
"Proteolysis and curd tension in milk associated with accelerated starter culture growth".
J. Dairy Sci. 45: 164.

Witter, L.D. (1961)
"Psychrotrophic bacteria. A review".
J. Dairy Sci. 44: 983-1015.

Yamamoto, K.; Sato, H. and Akatsu, K. (1981)
"Distribution of psychrotrophic organisms in commercial milk in glass bottles and in paper package."
J. Food Hygiene Soc. Jap. 22: 162-166.

Yan, L.; Lanelois, B.E.; Oleary, J. and Hicks, C.L. (1985)
"Purification and characterization of four extracellular proteases isolated from raw milk psychrotrophs".
J. Dairy Sci. 68: 1323-1336.

Zachariah, P. and J. Liston, (1973)
Temperature adaptability of psychrotrophic Pseudomonas. Appl. Microbiol. 26: 437-438.

.....