

**PRODUCTION OF DIRECT VAT SET DAHI
CULTURES**

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CERTIFICATE

This is to certify that the thesis entitle “*Production of Direct Vat Set Dahi cultures*” submitted by *Ms. DEEPA, B.G.*, MDK 801 in partial fulfillment of the requirement for the award of degree of *Master of Technology (Dairy Science)* in **Dairy Microbiology** to the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work done by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, association ship, fellowship or other similar titles.

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Affectionately Dedicated
to My Beloved Parents

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INTRODUCTION

I. INTRODUCTION

Dairying has played a prominent role in strengthening of the India's rural economy. It has been recognized as an instrument to bring about socio-economic transformation. The white revolution has transformed India's dairy industry.

India ranks first in milk production with 110 million tonnes during the year 2010 and projected production is up to 141 million tonnes by the end of year 2015. The percentage of fluid milk conversion is 46 percent and remaining is utilized as milk products. Fermentation is one method of preserving the milk. Beneficial bacteria are used to bring about controlled fermentation and they include lactic acid bacteria such as lactococci, streptococci, pediococci, leuconostoc and lactobacilli. About 7.1% of milk produced in India is used in the preparation of dahi.

Dahi is a major fermented milk product which plays an important role in human diet in India and neighbouring countries. It is made from cow milk or buffalo milk or combination thereof where part of lactose has been converted into lactic acid by lactic acid bacteria. Dahi has the same nutritional value as that of milk from which it is made and easily digestible than milk, as 60% of lactose is fermented and protein is partially hydrolyzed by the starters used in the preparation. At home, Dahi is prepared by boiling of milk, cooling to room temperature, inoculating with previous batch of dahi and keeping at room temperature overnight. The microflora of dahi is not well defined usually a heterogenous group of organisms which include *Lactococcus lactis ssp. lactis*, *Lactococcus lactis ssp. cremoris*, *Lactococcus lactis ssp. diacetylactis*, *Streptococcus*

thermophilus, *Lactobacillus delbrueckii ssp. bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus plantarum* etc. (Shekar and Mariappan., 2007).

The traditional method for the production of starters is through propagation of liquid culture that involves various stages such as preparation of stock, mother, working and finally bulk culture. This method of propagation is material and time consuming along with labour oriented. 'Bulk starter' in liquid form was used to inoculate the milk in the manufacture of various fermented milk products such as dahi, yoghurt, buttermilk, cheese and other fermented products. Liquid cultures if not propagated properly may be contaminated with spore forming bacteria, coliforms and yeasts so on.

Over the past 10-15 years, the use of starter cell concentrates designated as either Direct Vat Set (DVS) or Direct Vat Inoculation (DVI) cultures have increasingly being used in order to overcome the problems encountered in liquid starters, particularly in small plants, to replace bulk starter in cheese and fermented milk manufacture. DVS can be used directly as inoculation in heat treated cooled milk in fermentation vat. The terms DVI and DVS are used interchangeably. In addition to these high activity cell concentrates, lower activity commercial cell concentrates have been used for many years to inoculate milk for bulk starter preparation, and in the manufacture of 'long set products' that require extended incubation. (Mullan, 2006).

Solid State Fermentation (SSF) technique has been widely used in the preparation of fermented foods, enzymes, organic acids, fungal biomass and enriched animal feeds which involves the controlled growth of microorganisms on solid substrate. Solid State

Fermentation (SSF) has been defined as “the fermentation process occurring in the absence of free water, so as to contain large number of viable cells in concentrated form”.

SSF cultures if produced under strict hygienic conditions may provide an alternative method for the production of DVI cultures. SSF processes generally employ a natural raw material which may be inert (paddy husk, wheat bran) or nutritive (dhals, grams) as carbon and energy source. Solid substrate (matrix), however, must contain enough moisture. Depending upon the nature of the substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads to relatively high water activity (a_w) on the solid or gas interface, in order to allow higher rate of biochemical processes. Low diffusion of nutrients and metabolites takes place in lower water activity conditions whereas compaction of substrate occurs at higher water activity. Hence, maintenance of adequate moisture level in the solid matrix along with suitable water activity are essential elements for SSF processes.

Solid substrates should have generally large surface area per unit volume. Smaller substrate particles provide larger surface area for microbial attack but pose difficulty in aeration or respiration due to limitation in inter-particle space availability. Larger particles provide better aeration or respiration opportunities but provide lesser surface area. In bioprocess optimization, sometimes it may be necessary to use a compromised size of particles for the reason of cost effectiveness (Pandey and ashok 2007).

None of the Indian companies are producing DVS cultures. Production of DVS culture through SSF technique may be a break through in starter culture propagation.

Keeping in view the above facts, an attempt has been made in the present investigation with the following objectives:

1. Characterization of the predominating microflora from commercially available dahi samples
2. Growth study of the selected isolates in Milk and Nutritive substrates
3. Preparation of important lactic cultures as direct vat set cultures
4. Evaluation and Shelf life study of direct vat set cultures

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Microorganisms are ubiquitous in nature. Based on their usefulness they have been grouped into beneficial and harmful. Beneficial bacteria are useful in the preparation of fermented foods. Lactic acid bacteria (LAB) are one among beneficial bacteria that are Gram positive, facultative anaerobes, acid tolerant and are widely used as starters in the manufacture of various fermented foods such as dahi, yoghurt, batter, sauerkraut etc.

The functions of lactic acid bacteria include production of lactic acid to impart a distinctive fresh and acidic aroma, contribution towards production of flavour for the fermented products, mild proteolytic and lipolytic changes which impart to the body and texture of the products and finally prevents the growth of spoilage organisms by acid production and by production of bacteriocins by certain lactic acid bacteria.

In the production of various fermented products, the use of starter cultures occupy the center stage. The quality of the finished product is therefore dependent on the activity and performance of starters used. Starters produced by conventional methods such as liquid culture, freeze dried, frozen culture, concentrated cultures etc. have been used by industry with various drawbacks. Continuous attempts are being made to improve the methodology for the production of starters for industrial application.

2.1 Dahi

PFA (1956) defined Dahi as a product obtained from pasteurized or boiled milk by souring naturally or otherwise, by harmless lactic acid or other bacterial cultures.

According to ISI (1973), Dahi is a product obtained by lactic fermentation of cow or buffalo milk or mixed milk through the action of single or mixed strains of lactic acid bacteria or by lactic fermentation accompanied by alcoholic fermentation of yeast.

Dahi is a fermented milk product made from cow milk or buffalo milk or combination of the two, where part of lactose has been converted into lactic acid by lactic acid bacteria. Dahi has the same nutritional value as that of milk from which it is made and easily digestible than milk, as 60% of lactose is fermented and protein partially hydrolyzed (Awan and Rahman, 2002)

Shekar and Mariappan (2007) found that microflora of dahi is heterogenous includes *Lactococcus lactis ssp.lactis*, *Lactococcus lactis ssp .cremoris*, *Lactococcus lactis ssp .diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii ssp. bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, lactose fermenting yeast etc.

Potential health or nutritional benefits are possible from some species of lactic acid bacteria like *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum*. Among these are: improved nutritional value of fermented milk, control of intestinal infections, improved digestion of lactose, control of colon cancer and control of serum cholesterol levels to reduce the risk of cardiovascular diseases. These benefits are the result of growth and action of starter cultures used in the manufacture of cultured foods such as Dahi, Yoghurt etc. The production of fermented milks is based on the use of starter cultures, such as lactic acid bacteria that initiate rapid acidification of heat treated, cooled milk (Ali, 2010).

2.2 Nutritional and therapeutic properties of Dahi:

Dahi is the rich source of calcium and protein just like milk. Apart from benefits of milk dahi contributes additional health benefits compared to milk (Sarkar *et al.*, 1996).

1. The enzymes produced by the starter cultures continue to work in the intestines degrading the particular substrates that help in stimulating the growth of intestinal microflora.
2. Helps lactose sensitive persons to avoid flatulence and diarrhea.
3. Helps in preventing cancer of colon
4. Helps to replenish intestine with good bacteria following a treatment with antibiotics

Kaup (1988) recommended dahi for the lactose intolerant individuals because of the reduced lactose content. Besides this, lactic acid also helps in the absorption of calcium and phosphorous in the intestine. Dahi is an excellent source of protein and meets the minimum daily protein requirement of an adult and this protein is highly digestible as most of the protein is in the pre-digested form.

Dahi is an age old indigenous fermented milk product in India and has managed its popularity in Indian diet despite changing lifestyle, food habits and fulfills nutrition (Khurana and Kanawjia, 2007).

Hariom *et al.* (2007) prepared Dahi by using *Lactobacillus acidophilus* and *Lactobacillus .casei*, that helped in the production of free fatty acids and conjugated linoleic acid. Increased content of conjugated linoleic acid confer nutritional and therapeutic value for dahi.

Acceptable quality of Dahi could be obtained with the application of acid producing and flavour (primarily diacetyl) producing organisms and adopting a two-stage fermentation. Biotechnological innovations suggested inclusion of certain probiotic and beneficial bacteria for further enhancement in the dietetic properties of traditional Dahi (Sarkar *et al.*, 1996).

2.3 Enumeration of lactic acid bacteria:

Vinderola and Reinheimer (2000) enumerated lactobacilli from curd sample by using MRS agar and the sample showed count of $5.76 \log_{10} \text{cfu/g}$.

Lactic acid bacteria were enumerated from dahi after plating well diluted (10^5) 0.1 ml of sample on de Mann Rogosa Sharpe agar (MRS) and incubated anaerobically in anaerobic jar at 35°C for 48hrs (Esayas *et al.*, 2003)

Cheriguene *et al.* (2007) enumerated lactic acid bacteria from goat's milk by plate count technique, MRS agar was used for the enumeration of pediococci and leuconostoc, while M17 agar was used for the enumeration of lactococci and Rogosa agar for lactobacilli. Plates were incubated at their optimum temperature anaerobically.

Lactic acid bacteria were enumerated from curd showed log viable count of lactococci of 7.87, leuconostoc of 3.20 and lactobacilli of 5.45 per gram by selective plating technique by using Neutral Red Chalk Lactose Agar, Sucrose Agar and Rogosa Agar respectively (Pradeep, 2007).

2.4 Isolation and Characterization of lactic acid bacteria:

Sarkar *et al.* (1996) isolated lactococci, leuconostoc and pediococci from curd, chhana, whey, cheese, silage, rotten vegetables available in local markets of India.

Esayas *et al.* (2003) isolated the lactic acid bacteria from dahi samples. The colonies (10 numbers) were randomly selected from countable MRS agar plate of dahi sample. The colonies were purified by successive streaking on appropriate agar media (MRS) before being subjected to characterization. The colonies (5 numbers) with different morphology from each plate were transferred to MRS broth, incubated for about 12 hours and maintained in the refrigerator at 4⁰C . The isolates were grouped as lactic acid bacteria after examining for their colony morphology, cell morphology, Gram's reaction, catalase reaction and Gas production from glucose fermentation.

The use of lactic acid bacteria (LAB) in foods has a long history and most strains are considered as commensal microorganisms with no pathogenic potential. Their presence in intestinal epithelium of human gastro intestinal tract, and their traditional use in fermented foods and dairy products with significant health benefits, attest to their safety. The natural habitat of lactic acid bacteria are milk, fruits and vegetables especially those which undergo natural fermentation (Breidt, 2007).

Subramanian and Pavan kumar ora (2007) isolated 75 isolates belonging to genus *Lactobacillus*, from 150 samples of buffalo milk collected from the various sources during the different seasons of the year. These isolates were identified to the species level by subjecting them to a battery of standard physiological and biochemical tests. Accordingly, the *Lactobacillus* species such as *Lactobacillus casei* (21.05%),

Lactobacillus plantarum (24.21%), *Lactobacillus acidophilus* (10.53%), *Lactobacillus bulgaricus* (15.79%), *Lactobacillus lactis* (12.63%) and *Lactobacillus helveticus* (15.79%) were identified.

Borpuzari *et al.* (2007) identified 10 strains of *Lactobacillus acidophilus* selected out of a total of 273 strains of lactobacilli isolated from market sour curd samples.

Leuconostoc spp. of 65 numbers of dairy origin were isolated from 10 raw milk samples. The physiological and biochemical traits of each isolate were studied and characterized. The species identified were *Leuconostoc dextranicum*, *Leuconostoc mesentriodes*, *Leuconostoc paramesenteroides*, *Leuconostoc lactis* (Subramanian and Pavan kumar Ora, 2007).

Sang *et al.* (2007) characterized lactic acid bacteria isolated from commercial yoghurt beverages. All the strains isolated were identified as *Lactobacillus paracasei*, *Lactobacillus helveticus*, *Lactobacillus casei ssp. casei* and *Lactobacillus paracasei* using an API 50 CHL kit.

Rashid *et al.* (2007) obtained 266 strains of lactic acid bacteria obtained from 28 dahi sample from different areas of Bangladesh. The isolates were identified as *Lactobacillus fermentum*, *Lactobacillus delbrueckii ssp bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophilus*, *Leuconostoc mesentrioides ssp. lactis*, *Lactococcus lactis ssp. lactis*, *Lactococcus raffinolactis* and *Pediococcus oentosaceus* based on morphological, physiological and biochemical characteristics and sugar fermentation test and 16S r-RNA analysis.

Lactic acid bacterial isolates of 55 numbers were obtained from 2 traditional Malaysian fermented foods Tapai and Tempoyak and from fresh goat milk. Among 55 isolates, 16 were homofermentative. Lactobacilli strains (2 numbers) both isolated from Tapai, produced higher biomass as well as higher lactic acid yields (Mohd Adnan and Tan, 2007).

Soomro *et al.* (2007) isolated 116 numbers of lactic acid bacteria from market dahi from Rawalpindi. The characterization of isolates revealed that the abundant species were *Lactobacillus delbrueckii ssp. bulgaricus* (28 isolates) followed by *Streptococcus thermophilus* (20 isolates) and others were *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus .helveticus* based on morphological and physiological characteristics.

A total of 218 lactic acid bacterial isolates were obtained from the predominant microflora of the selected cheeses and were identified as Lactococci (98), Leuconostoc (56), mesophilic lactobacilli (54), pediococci (8) and enterococci (2). Among 218 isolates 84 of the isolates produced mainly malty, spicy or sulfide flavours in pasteurized whole milk and were not characterized further. Some good producers of diacetyl - acetoin in milk (more than 100 mg/l) were found among a total of 129 lactic acid bacteria selected, which were less acidifying and proteolytic (Garabal *et al.*, 2008).

Kahala *et al.* (2008) characterized *Lactococcus lactis ssp. lactis* and *Lactococcus lactis ssp. cremoris* and aroma producers *Lactococcus lactis ssp diacetylactis* and *Leuconostoc mesentroides ssp. mesenyteroides* as starters from the Villi. They optimized starter cultures for industrial scale manufacture of Villi.

Jayalalitha *et al.* (2009) collected 35 samples of raw milk from different areas of Chennai city. Raw milk samples were used to isolate and identify the incidence of lactobacilli. MRS agar, LBS agar with streak plate method was found to be good for isolating *Lactobacillus* from raw milk. A total of 46.15% of isolates were identified among which 27.55% were *Lactobacillus delbrueckii ssp. bulgaricus* and 18.6% were *Lactobacillus acidophilus*.

Dahi samples (35 numbers) were collected from the local market of Rawalpindi and isolated 69 numbers of lactic acid bacteria. Out of 69 isolates, 26% were *Streptococcus thermophilus* followed by 22% as *Lactobacillus delbrueckii ssp. bulgaricus*, 16% were *Lactobacillus acidophilus*, 9% were *Lactococcus lactis ssp lactis*. (Mehmood *et al.*, 2009).

Lactic acid bacteria were enumerated from yoghurt sample using Elliker and MRS agar used for the isolation of lactobacilli. M17 agar was used for the detection of streptococci. Plates were examined after 48 and 72 hrs of incubation *Lactobacillus delbrueckii ssp. bulgaricus* were enumerated by surface spreading of 0.1 ml sample on Elliker agar medium (Dragana and Gordana, 2009).

A characterization study carried out by Forouhandeh *et al.* (2010) revealed 37 Lactobacilli isolates from different kinds of traditional and local cheeses and yoghurt of Besmenj zone in Iran. Among the 37 isolates, 69% were *Lactobacillus rhamnosus*, 15% were *Lactobacillus .paracasei* and 16% were *Lactobacillus fermentum* based on Bergey's Manual of Systematic Bacteriology.

Mahantesh *et al.* (2010) isolated lactic acid bacteria from the samples of dahi and cucumber and characterized them based on biochemical and molecular methods. These isolates were assigned to the genera lactobacilli, pediococci and weissella.

2.5 Growth study of selected lactic acid bacterial isolates on different media:

2.5.1 Growth study of selected isolates in milk:

Wong and Yalet (1999) studied the growth of *Lactococcus lactis ssp. lactis*, *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* in nonfat milk medium and observed that rapid increase in viable count to 10^8 cfu/ml when cells were added at the beginning of growth of lactic acid bacteria. The cells were inactivated slowly when added after 24 hrs and rapidly when added after 72 hrs of lactic acid bacterial growth.

Commercial utilization of bifidobacteria, lactobacilli and streptococci depend on their rapid growth and acidification of milk as well as their acid and oxygen tolerance. *Lactobacillus acidophilus* was isolated and identified from fermented milk. The pH of the milk gradually decreased from 6.7 to 4.1 after 24 hrs of incubation at 37°C. The maximum rate of lactic acid production was obtained with mixed culture at 45°C (Miloud and Ahmed , 2006).

Meshref *et al.* (2007) conducted the growth study of *Bifidobacterium infantis* and *B. angulatum* by inoculation into the milk and incubated at 37°C for 16 hrs. The bacterial counts were conducted at the beginning and every 4 hours and upto 16 hrs, the viable counts ranged from 8.12 to 8.56 log₁₀cfu/ml.

Lactococcus lactis ssp .*lactis*, *L. euconostoc mesentriodes* ssp .*mesentroides*, *Lactobacillus viridescens* when inoculated individually to sterile skim milk at their optimum growth temperature, curdled the milk at 24 hrs of incubation. At every 6 hrs, the samples drawn showed peak in the DMC at 24 hrs of incubation that ranged between 8.13 to 8.31 log₁₀cells/ml (Pradeep, 2007).

Prabha *et al.* (2008) studied the activity of *L. acidophilus* in sterile skim milk and found DMC of 8.36 log₁₀cells/ml at 24 hrs of incubation at 37⁰C.

2.5.2 Growth study of selected isolates on solid substrate:

Schaffner and Benchat (1986) experimented on suitability of aqueous plant seed extract to serve as substrates for fermentation by lactic acid bacteria. The bacteria investigated were *Lactobacillus heleveticus*, *Lactobacillus casei*, *Lactobacillus .delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *Streptococcu .thermophilus*. The aqueous extracts of cowpea, peanut, soybean and sorghum were used as substrates for nutritionally superior sour tasting product containing 20 – 25 grams lactic acid/kg cereal. Sensory qualities improved and shelf life were improved due to protective action of lactic acid.

Yoghurt like product was prepared by fermentation of rice with lactic acid bacteria *Lactobacillus plantarum*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* singly or in various combinations. The product inoculated with all the three bacteria was superior to other products (Shin, 1989).

Chickpea and blackgram dhal, very well supported the growth of *Lactobacillus acidophilus* with counts reaching upto 9.32 log₁₀cfu/g with addition of skim milk powder (Vanisri, 1995).

Solid state fermentation is defined as growth of microorganisms on solid materials with out the presence of free liquid (Lopez and Henry, 1998).

Prabha (1999) obtained maximum viable count of 9.10 log₁₀cfu/g when fermented dried (20⁰C for 20 hours) blackgram dhal using *Bifidobacterium* spp was used as substrate with 1 : 0.8% of moisture, 1% of skim milk powder and MnSO₄ of 0.005% . .

Kabede *et al.* (2007) studied effects of temperature (7^o, 15^o, 25^o & 37^oC) on the growth of lactic acid bacteria and yeasts in ‘Sethemi’, South African naturally fermented milk. Lactic acid bacterial counts decreased rapidly at 37^oC, from 2nd day to 5th day, while the numbers remained stable at 25^oC. pH of the milk was decreased with higher temperature than at 7^oC and 15^oC of incubation. The temperature of 25^oC was found to be ideal for producing fermented milk with high lactic acid bacterial counts, low pH and a visually acceptable coagulum.

Lactobacillus strains (2) and *Lactococcus* strain (1) were selected to study their growth and lactic acid production. The *Lactobacillus* strains, both isolated from fermented milk product ‘Tapai’, produced higher amounts of cells and lactic acid from glucose as compared to the *Lactococcus* strain isolated from fresh goat’s milk (Ahmad *et al.*, 2007).

Zhao *et al.* (2008) developed novel, SSF method, using a netting bag bioreactor for probiotic cultures. A high yield of biomass (1.2×10^{11} cfu/g dry substrate) was obtained using this method. The netting bag method was more cost-effective as well as time and space saving. The material cost was low. Thus, it was suggested that netting bag SSF can be applied widely to produce probiotic bacteria in developing areas.

Dieghri-Hocine *et al.* (2007) studied that Chick pea based medium appeared specially efficient for the growth of plant isolate of *Lactobacillus plantarum* with 1.28 \log_{10} cfu/g higher than that obtained on MRS medium.

2.6 Propagation of lactic acid bacteria using different types of cultures:

Zahoor *et al.* (2003) isolated *Lactobacillus delbrueckii ssp. bulgaricus* from indigenous source and preserved by three different method namely, on agar slope method, under oil method and liquid form using MRS medium. Oil method was found to be the best method for preservation and maintance of culture.

The industrial exploitation of lactic acid bacteria (LAB) as starter cultures depends strongly on the preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and activity. Freeze-dried preparations exhibit advantages relative to preparations made with other techniques in terms of long-term preservation, coupled with convenience in handling, storage, marketing and application. Degrees of survival of LAB cultures was high during freeze drying and subsequent storage (Ana *et al.*2004).

Effect of lyoprotectants on survival of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus sakei* after freeze drying was investigated. For freeze-dried *L.sakei*, the survival in skim milk and soy milk was greater than that in trehalose , the highest number of *L. lactis* ssp. *lactis* survivors was found in mixed lyoprotectants containing 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, 8.73 g skim milk and 79.36 g soyamilk with distilled water (1:1). The number of *L. lactis* and *L. sakei* survivors in these formulations was increased by 11.69-20.15% and 9.51-18.15% respectively (Suree Nanasombat and Niracha Sriwong, 2007).

Evaluation of 16 freeze dried cultures that included lactic acid bacteria, 8 of *Lactobacillus plantarum*, 3 of *Lactobacillus pentosus*, 2 of *Weissella paramesenteroides*, 2 of *Lactobacillus fermentum* and 1 of *Leuconostoc mesenteroides* ssp. *mesenteroides* were carried out. Their ability to tolerate the freeze-drying process was assessed by dehydration in a glycerol solution of increasing concentration, followed by staining with two fluorescent markers. The technological characteristics identified after the freeze-drying process, were a high cell concentration or high survival rate. The ability of the freeze-dried strains to recover their acidification activity was evaluated through the determination of the pH, titratable acidity (% lactic acid/g dry weight) and cell count over 24 hrs on MRS broth (Yao *et al.*, 2009).

Barbara P. Keogh (2002) concluded that the effect of freezing and storage in liquid nitrogen on 13 strains of lactic streptococci. Cultures were frozen in droplet form and collected in mesh bag. Viable counts were performed before and after freezing in liquid nitrogen and after storage in liquid nitrogen. There was no decrease in viable count

or loss in activity of the cultures due to freezing and storage. Frozen cultures of some strains showed a shorter lag period after inoculation into milk than control cultures.

2.7 Preparation of lactic cultures as direct vat set cultures:

Cultures for direct inoculation offer significant advantages in the production of fermented milks all over the world. The Direct Vat Set (DVS) cultures are convenient for the dairy manufacturer and ensure consistency in the final fermented product, provide a high texture and mouth feel, something that is not normally achievable by using bulk starters (Elsborg, 1997).

2.7.1 Definitions of Direct Vat Set (DVS) dahi culture:

Direct Vat Set cultures for fermented milk products are blends of defined and controlled single strains that have been selected for their performance in the final product (Honer, 1995).

Direct Vat Set culture is a highly concentrated and standardized freeze dried dairy culture used for direct inoculation of milk (Deepak and Didriksen, 2007).

According to Krishan and Gandhi (2009), Direct Vat Set cultures are the highly concentrated dairy cultures of single as well as mixture of defined strains. The cell count in the DVS culture are 10^9 - 10^{10} cfu/g.

2.7.2 Advantages of Direct Vat Set dahi cultures:

Mullan (2006) has mentioned advantages of DVS culture. He suggested that

- Centralized concentrate production enables a manufacturer to establish a team of technical experts and to develop the necessary technology and protocols to produce a quality product.
- Detailed quality control tests can be performed on each batch of concentrate and only batches meeting the manufacturer's specification are released for factory use.
- No incubation or sub-culturing was required at the factory. This reduced the probability of phage or other forms of contamination occurring since all the factory-staff had to do is to thaw the concentrate or open

Hunger and Peterson (1992) reported that development of new fermented milk product which contained viable bacteria of human origin. These cultures were available as DVI cultures for fermented milk production.

Two successive cell-recycle bioreactors were used for the production of concentrate starter cultures (Boyaval *et al.*, 1992).

A range of direct-vat-set single-strain cultures, HR (Hansen rod) and HC (Hansen coccus) cultures, introduced by Chr. Hansen Laboratory (Milwaukee, USA) was described. The cultures could be used in various combinations by cheese manufacturers to produce a range of customized cheeses (Anon, 1993).

Reiner (1993) produced DVS (direct Vat Set) cultures as concentrated starter cultures (approx. 10^{11} cfu/g) in frozen or freeze dried form which were suitable for direct inoculation into processed milk. Use of DVS cultures in the manufacture of sour milk product was discussed with reference to: choice of bacteria for DVS cultures; properties

of suitable bacteria; advantages of DVS cultures over traditional culture systems; development of YO-FLEX yoghurt cultures for specific flavour and viscosity; and manufacture of probiotic sour milk products using DVS cultures was possible.

Reiner (1995) produced Direct Vat Set cultures, from specific, single organisms with tailored properties, are gaining ground. Advantages of using DVS cultures including their ability to be applied in direct inoculation and culturing, making them easy to use and eliminate the need for an in-house culture unit; the fact that they were made from defined strains. Various DVS-cultures, divisible into types with no gas production and those with aroma and/or gas formation, were produced by Chr. Hansen for production of fermented dairy products.

Concentrated cultures were produced by two methods Viz, mechanical separators and diffusion culture techniques or dialysis (Portner and Markl, 1998).

Stanley (1998) used DVI cultures of *Streptococcus thermophilus* blended with DVI mesophilic lactococci for the manufacture of cheddar cheese.

Concentration method used by the commercial manufactures consisted of two stage concentration before freeze drying or freezing (Hoir *et al*, 1999).

Stokes *et al*. (2001) used DVS culture of *Streptococcus thermophilus* to counteract failure due to *lactococcal* phage in DVS cultures.

Starter concentrates were produced by batch and continuous method. Batch method of starter concentration was by centrifugation while continuous method required

diffusion culture technique. The starter concentrate could be used as DVS culture that reduced subculturing time, labour and required less inoculum level compared to liquid culture. Starter concentrates were efficiently used in the preparation of dahi, cheese and other fermented milk products in Dairy Industry (Hansen, 2002).

Mixture of *Lactococcus lactis ssp. lactis* and *L. lactis ssp. cremoris* were used frequently in white cheese making. White cheese samples were manufactured from milk using 1 of 3 different commercial direct vat starter cultures or a lyophilized starter culture and ripened at $4 \pm 1^\circ\text{C}$ for 90 days. Composition, titratable acidity and ripening indices of the cheese samples were determined on the 2nd, 30th, 60th and 90th days of ripening (Dagdemiir, 2003).

Ganina *et al.* (2005) identified 20 strains of lactic acid bacteria and bifidobacteria that could be recommended for inclusion in DVS dahi starters in conjunction with appropriate encapsulation technology.

Among the recent innovative technologies in preservation of starter cultures, Direct vat Set culture is a promising method and rapidly finding role in the manufacture of fermented milk products such as Dahi, Yoghurt and Kefir (Krishan Kumar and Gandhi, 2009).

2.8 Evaluation of shelf life study of Direct Vat Set cultures:

Shamanova and korobochka. (2000) produced a probiotic milk product using DVS cultures and fermented at $30 \pm 2^\circ\text{C}$ for 12 to 14 hrs or at $35\text{-}37^\circ\text{C}$ for 6 to 8 hrs. The resulting product was of creamy consistency with pH of 4.4 – 4.5. The lactic acid bacteria

of more than 10^7 and bifidobacterial counts of 10^6 cfu per ml were obtained and the shelf life was less than or equal to 7 days at $4 \pm 2^\circ\text{C}$.

Fermented dairy products prepared by using DVS starter cultures from Chr. Hansen. The products prepared were Yoghurt, Kefir and Russian fermented milk product Tvorog with extended shelf life was noticed (Snyatkovskii *et al*, 2004).

Yadav *et al.* (2007) studied that dahi prepared with DVS cultures of lactococci and 2 adjunct probiotic cultures of *Lactobacillus acidophilus* and *Lactobacillus casei*. The survival of bacterial species was affected during storage of dahi at 7°C , in which viable counts increased up to day 2 and gradually decreased from day 2 to day 8. The quality of refrigerated dahi was assessed by a panel of trained judges. The pH of the dahi significantly decreased over the storage period.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

Dahi is a popular fermented milk product in India. Microflora of dahi is considered as the mixed microflora including lactococci, streptococci, leuconostoc and lactobacilli. For the preparation of dahi, it requires inoculation of active culture and setting time within 16 to 18 hrs of incubation or lower. Maintenance of the activity, requires various starter propagation protocol. Liquid culture should be maintained as stock, mother, working and bulk cultures. Since liquid culture maintenance is cumbersome and as direct vat set cultures are gaining importance, the growth of starter cultures on edible solid substrates may be promising with regard to ease and convenience in handling during the preparation of fermented milk product. Various materials used and methods followed in the present study are presented in this chapter.

3.1 Lactic cultures

The lactic cultures were isolated from the domestic and market dahi samples. The isolates were characterized by using various biochemical test as per the Bergey's Manual of Systematic Bacteriology.

3.2 Bacteriological media and diluents

3.2.1 Arginine broth (Harrigan, 1998)

| | |
|--------------------------------|------|
| Tryptone | 5.0g |
| Yeast extract | 2.5g |
| D-glucose | 0.5g |
| Dipotassium hydrogen phosphate | 2.0g |

| | |
|------------------------|--------|
| L-arginine monohydrate | 3.0g |
| Distilled water | 1000ml |
| pH | 7.0 |

All the ingredients were dissolved in distilled water, pH adjusted to 7.0, distributed into sterile test tubes and autoclaved at 121⁰C for 15 min.

3.2.2 Edible solid substrate medium (Prabha, 1999)

| | |
|--|------|
| Black gram dhal | 100g |
| Reconstituted skim milk powder (9% TS) | 1.0g |
| Tomato juice | 10ml |
| Potable water | 70ml |

Reconstituted skim milk, tomato juice and potable water were added to black gram dhal soaked for 15 min and sterilized for 121⁰C for 30 min, as black gram dhal showed higher number of spore formers.

3.2.3 Gibson's semi-solid medium (Harrigan, 1998)

a) Nutrient agar with additives

| | |
|---------------------------|-------|
| Peptone | 10.0g |
| Beef extract | 10.0g |
| Sodium chloride | 5.0g |
| Yeast extract | 2.5g |
| D-glucose | 50.0g |
| Manganese sulphate (0.4%) | 10ml |

| | |
|-----------------|--------|
| Agar-Agar | 15g |
| Distilled water | 1000ml |
| pH | 6.5 |

The ingredients were dissolved except D-glucose and agar by steaming, adjusted the pH to 6.5, then added D-glucose and agar, dissolved by heating and one ml was transferred to sterile test tubes and were sterilized at 121⁰C for 15 min.

b) Reconstituted skim milk

| | |
|-----------------------------|-------|
| Skim milk (9% total solids) | 800ml |
|-----------------------------|-------|

Skim milk powder of 9g was added to 100ml of lukewarm water, mixed thoroughly, transferred to sterile conical flask and sterilized by autoclaving at 121⁰C for 15 min. After sterilization, 4 ml of sterile skim milk was transferred aseptically to the sterile test tube containing 1 ml of molten sterile nutrient agar with additives and mixed thoroughly, cooled to 50⁰C. The culture of 0.1 ml was inoculated and contents were mixed thoroughly and incubated at optimum temperature of culture.

c) Nutrient agar for sealing (Harrigan, 1998)

| | |
|-----------------|--------|
| Peptone | 10.0g |
| Beef extract | 10.0g |
| Sodium chloride | 5.0g |
| Agar-Agar | 15.0g |
| Distilled water | 1000ml |
| pH | 7.2 |

Nutrient agar was prepared by dissolving all the ingredients by steaming except agar-agar, pH was adjusted to 7.2 agar was added, steamed again and sterilized by autoclaving at 121⁰C for 15 min. Sterile molten nutrient agar was used to seal the tube.

3.2.4 Litmus milk (Harrigan, 1998)

| | |
|---|--------|
| Reconstituted skim milk (9% total solids) | 1000ml |
| Litmus solution (4%) | 10ml |

Litmus powder of 4 g was weighed, transferred to clean pestle and mortar, triturated. Portion by portion of distilled water was added, mixed and filtered. Prepared litmus solution of 10 ml was added to 1000 ml of reconstituted skim milk and sterilized at 121⁰C for 15 min.

3.2.5 de Mann, Rogosa, Sharpe (MRS) fermentation medium (de Mann *et al.*, 1960)

a) Basal medium

| | |
|---|--------|
| Peptone | 10.0g |
| Yeast extract | 5.0g |
| Tween 80 | 1.0g |
| Dipotassium hydrogen phosphate | 2.0g |
| Sodium acetate | 5.0 |
| Triammonium citrate | 2.0g |
| Magnesium sulphate (MgSO ₄ .7H ₂ O) | 0.2g |
| Manganese sulphate (MnSO ₄ .4H ₂ O) | 0.05g |
| Chlorophenol red | 0.004g |

| | |
|-----------------|--------|
| Distilled water | 1000ml |
| pH | 6.5 |

The ingredients were dissolved in distilled water by steaming, pH adjusted to 6.5 and then sterilized at 121⁰C for 15 minutes after dispensing into 10ml quantity to saline tubes containing Durham's tubes.

b) Sugar solution:

Sugar solution of 10% was prepared by dissolving 10g of sugar in 100 ml of distilled water and filter sterilized by using membrane filter into sterile conical flask.

c) Final sugar fermentation medium

To the 10 ml of basal medium (a) 1ml of sugar solution was added to saline tube containing Durham's tubes, that gave final concentration of sugar as 1%.

3.2.6 Neutral Red Chalk Lactose Agar (Harrigan, 1998)

| | |
|-----------------------------------|--------|
| Peptone | 3.0g |
| Meat extract | 3.0g |
| Yeast extract | 3.0g |
| Lactose | 10.0g |
| Calcium carbonate, precipitated | 1.5g |
| Neutral red (1% aqueous solution) | 5ml |
| Agar-Agar | 15g |
| Distilled water | 1000ml |
| pH | 6.8 |

All the weighed ingredients except lactose, neutral red and agar were transferred to the distilled water dissolved by steaming, adjusted the pH to 6.8 and then lactose, neutral red and agar were added, transferred to one third of the capacity of conical flask and sterilized at 121⁰C for 15 min.

3.2.7 Normal saline (Harrigan, 1998)

Sodium chloride of 8.5 g was dissolved in 1000ml of distilled water, distributed in 9 ml and 99 ml aliquot in test tubes and diluent bottles respectively and were autoclaved at 121⁰C for 15 min.

3.2.8 Phosphate buffer (Harrigan, 1998)

| | |
|--------------------------------|--------|
| Potassium dihydrogen phosphate | 34.0g |
| Distilled water | 1000ml |

Stock solution of phosphate buffer was prepared in 500 ml of distilled water, adjusted the pH to 7.2 using 0.1N sodium hydroxide solution and made up to 1000 ml with distilled water. To prepare working solution, 1.25 ml of stock phosphate buffer solution was taken and made up to 1000 ml with distilled water. Distributed in 9 ml and 99 ml of quantities and sterilized by autoclaving at 121⁰C for 15 min.

3.2.9 Reconstituted skim milk

Commercially available Nandini skim milk powder was procured and reconstituted by dissolving 9 g in 100 ml of distilled water to give 9% total solids (TS), distributed in test tubes and sterilized at 121⁰C for 15 min by autoclaving.

3.2.9.1 Rogosa Agar [Harrigan, 1998]

| | |
|--|--------|
| Tryptone | 10.0g |
| Yeast extract | 5.0g |
| D-glucose | 20.0g |
| Tween-80 | 1.0g |
| Potassium dihydrogen phosphate | 2.0g |
| Ammonium citrate | 2.0g |
| Sodium acetate, hydrated | 25.0g |
| Glacial acetic acid | 1.32ml |
| Magnesium sulphate , hydrated (MgSO ₄ .2H ₂ O) | 0.575g |
| Maganese sulphate, hydrated (MnSO ₄ .2H ₂ O) | 0.14g |
| Ferrous sulphate, hydrated | 0.034g |
| Agar-Agar | 5.0g |
| Distilled water | 1000ml |
| pH | 5.4 |

Dissolve all the ingredients in distilled water and dissolved by steaming except D-glucose. Then added glucose and agar-agar adjusted pH to 5.4, distributed in sterile conical flasks as required and sterilized at 121⁰C for 15 min.

3.2.9.2 Sucrose agar (Harrigan, 1998)

| | |
|--------------------------------|-------|
| Tryptone | 10.0g |
| Yeast extract | 5.0g |
| Dipotassium hydrogen phosphate | 5.0g |

| | |
|---------------------|--------|
| Triammonium citrate | 5.0g |
| Sucrose | 50.0g |
| Agar-agar | 15.0g |
| Distilled water | 1000ml |
| pH | 6.5 |

All the ingredients except sucrose, agar- agar were dissolved in distilled water by steaming, adjusted pH to 6.5, then sucrose and agar- agar were added, dissolved, distributed as required and autoclaved at 121⁰C for 15 min.

3.2.9.3 Yeast Glucose Lemco Broth (YGB) [Harrigan, 1998]

| | |
|-----------------|--------|
| Peptone | 10.g |
| Meat extract | 10.0g |
| Sodium chloride | 5.0g |
| D-glucose | 5.0g |
| Yeast extract | 3.0g |
| Distilled water | 1000ml |
| pH | 7.0 |

The ingredients except D-glucose were dissolved in distilled water by steaming, adjusted pH to 6.5, D-glucose was added, dissolved, distributed as required and autoclaved at 121⁰C for 15 min.

3.2.9.4 Yeast Glucose Lemco Agar (YGA) [Harrigan, 1998]

| | |
|-------------|--------|
| YGB | 1000ml |
| Agar – Agar | 15g |

Agar – Agar of 15 g was weighed, transferred to 1000ml of YGB, steamed and transferred to conical flask to one third of capacity. The medium was sterilized by autoclaving at 121⁰C for 15 min.

3.3 Reagents and Stains**3.3.1 Borax methylene blue (Harrigan, 1998)**

| | |
|---------------------------|-------|
| Methylene blue | 1.0g |
| Borax (Sodium metaborate) | 0.1g |
| Distilled water | 100ml |

Borax was dissolved in distilled water. In pestle and mortar, methylene blue was powdered and dissolved in a portion of water, slowly transferred the remaining portion of water, filtered and stored in an amber coloured bottle.

3.3.2 Catalase test reagent (Harrigan, 1998)

Commercially available hydrogen peroxide (30%) was diluted to obtain 3% solution and prepared freshly whenever needed.

3.3.3 Crystal violet

| | |
|-----------------|-------|
| Crystal violet | 0.5g |
| Distilled water | 100ml |

Crystal violet was powdered in a mortar, transferred portion by portion of water, mixed, filtered and stored in an amber coloured bottle.

3.3.4 Gram's iodine (Harrigan, 1998)

| | |
|------------------|-------|
| Potassium iodide | 2.0g |
| Iodine crystals | 1.0g |
| Distilled water | 300ml |

Potassium iodide was dissolved in distilled water, then added iodine crystals dissolved, filtered and stored in amber coloured bottle.

3.3.5 Phenolphthalein indicator (Harrigan, 1998)

| | |
|-----------------|-------|
| Phenolphthalein | 1.0g |
| Ethyl alcohol | 110ml |
| Distilled water | 200ml |

Phenolphthalein was dissolved in ethyl alcohol 0.1N sodium hydroxide solution was added drop by drop till faint pink colour appeared. The volume was made up to 200 ml with distilled water.

3.3.6 Safranin (Harrigan, 1998)

| | |
|-----------------|-------|
| Safranin | 0.25g |
| Distilled water | 100ml |

Safranin was powdered in a mortar, transferred portion by portion of water, mixed, filtered and stored in an amber coloured bottle.

3.4 Maintenance of Lactic acid bacteria

Stock lactic acid cultures were maintained in a yeast glucose agar stabs (0.75% of agar) and subcultured once in 21 days. Working lactic cultures were maintained in a yeast glucose broth and sterile skim milk, which were stored in refrigerator and subcultured once in a week.

3.5 Isolation of Lactic acid bacteria

Domestic and market samples of curd were collected in a sterile sample bottle. Sample of 11g were weighed separately under aseptic condition on to the sterile aluminum foil and transferred to 99ml of diluent bottle and mixed by rotating by placing on working bench to get 10^{-1} dilution and using this, required dilutions were prepared. Appropriate dilutions were transferred to four sets of Petri plates. To the first set of Petri plate sterile, molten Neutral Red Chalk Lactose Agar (NRCLA) maintained at 50°C was added, to the second set of Petri plates, Yeast Glucose Agar (YGA) was added, to the third set of Petri plate, Sucrose Agar (SA) was added, to the fourth set of Petri plate, Rogosa Agar (RA) was added, mixed thoroughly and allowed to solidify. First dilution was lab pasteurized ($63^{\circ}\text{C}/30\text{min}$), cooled to room temperature and required dilutions were prepared using sterile pipettes, 1ml quantity of required dilutions were transferred to label Petri plates and sterile molten YGA maintained at 50°C was poured, mixed thoroughly and incubated at 37°C . Neutral Red Chalk Lactose Agar, Sucrose Agar plates were incubated in anaerobic jar at 30°C for 24 - 48hours. Yeast Glucose Agar, Rogosa Agar plates were incubated in anaerobic jar at 37°C for 24 - 48hours. After incubation pink coloured colonies in NRCLA plates, all the colonies in YGA plates and glistening

colonies on Sucrose Agar, all the colonies on Rogosa Agar were counted. The counts were expressed as colony forming units per gram (cfu/g) by multiplying average count with dilution factor and then converted into \log_{10} cfu/g of sample. The colonies were selected based on Harrison's disc. The isolates were purified by streaking on YGA for 3 times and purified isolates were maintained in Yeast glucose broth and stabs containing 0.75% of agar.

3.6 Preliminary Identification of isolates

3.6.1 Simple staining

Smear was prepared by loop of broth culture of 18 hrs old with the help of sterile inoculation loop on to the clean slide, the smear was air dried, heat fixed by low flame and flooded with borax methylene blue for 5 min. Air dried stained smear was viewed under 100X objective of bright field microscope, which revealed the cell morphology.

3.6.2 Gram's staining

Smears prepared from 18 hours old broth cultures were examined for Gram's staining reaction as per the standard procedure described by Harrigan (1998). Gram's staining helped in declaring the isolates as Gram positive or negative.

3.6.3 Catalase test

A drop of 18 hrs old broth culture was placed using sterile Pasteur pipette on a clean slide and a drop of fresh hydrogen peroxide (3%) was mixed with the culture. The presence of catalase was indicated by the liberation of free oxygen as gas bubbles by effervescence.

3.6.4 Litmus milk reaction

Litmus milk was inoculated with young broth culture and incubated at 30⁰C or 37⁰C for 2 days based on the optimum temperature of isolate. Acid, Reduction and Coagulation were observed and taken as positive for litmus milk reaction and reported as ARC.

3.6.5 Gas from glucose

Sterile Gibson's semi solid medium of 1 ml with 4 ml sterile reconstituted skim milk was inoculated with 0.1 ml young broth culture mixed thoroughly. After mixing, the molten nutrient agar at 50⁰C was poured to give a layer 1/2 cm above the surface of the medium. The tube was then incubated at 30⁰C for lactococcal and leuconostoc isolates and at 37⁰C for *Streptococcus thermophilus* and lactobacilli up to 24 - 48 hrs. The gas production during fermentation was indicated by disruption of the agar seal as well as the presence of bubbles in the medium, and reported as production of CO₂ from glucose indicating hetero fermentative characteristic of culture. No gas in medium indicated the homofermentation characteristic with only lactic acid production by the culture.

3.7 Biochemical tests

3.7.1 Ammonia from arginine

Arginine broth was inoculated with young broth culture at 1% level incubated anaerobically at optimum temperature (30⁰C/37⁰C depending on the lactic culture) for 24- 48 hrs. If turbidity is noticed in the test tubes result was positive for ammonia production.

3.7.2 Diacetyl in milk

Skim milk of 10 ml was inoculated with 0.1 ml of young culture of lactococci and incubated at 30⁰C for 24 hrs. Potassium hydroxide (40%) of 0.5 ml and pinch of creatine was added and held at room temperature for 30 min., pink colour within 30 min indicated diacetyl production.

3.7.3 Growth at various temperatures

Sterile yeast glucose broth was inoculated at 1% with young culture of lactococci and incubated anaerobically in BOD set to 40⁰C and 50⁰C separately. Isolates of lactobacilli at 1% were inoculated separately to sterile yeast glucose broth and incubated anaerobically in BOD set to 15⁰C and 45⁰C for 24-48 hrs. Turbidity in the broth was taken as positive for the test in both the cultures.

3.7.4 Growth at various concentration of sodium chloride

Sterile yeast glucose broth tubes containing 3%, 4% and 6.5% of sodium chloride, were inoculated at 1% of young broth test cultures of lactococci and leuconostoc and incubated anaerobically at 30⁰C for 24 to 48 hrs. Turbidity in the broth was taken as positive for the test.

3.7.5 Dextran Production from Sucrose

Young broth culture of leuconostoc was streaked on the Sucrose agar plate and slime production on agar indicated positive for the test.

3.7.6 Survival at 55⁰C and 63⁰C

Young broth cultures of *Leuconostoc* and *Streptococcus thermophilus* were inoculated separately into sterile yeast glucose broth at 1% and subjected to 55⁰C and 63⁰C for 30 min respectively and incubated at their optimum growth temperature. The presence of turbidity in yeast glucose broth indicated positive result

3.7.7 Sugar fermentation tests

Sugar fermentation tests were carried out by inoculating 1% of the young broth cultures into sterile sugar fermentation medium and incubated at optimum growth temperature for 24 to 48 hrs. Turbidity, yellow and gas production in the Durham's tubes of the fermentation medium indicated positive for the test.

3.8 Identification key for the identification of Lactococci (Harrigan, 1998)

| Biochemical tests | <i>L.lactis</i> ssp. <i>lactis</i> | <i>L.lactis</i> ssp. <i>cremoris</i> | <i>L.lactis</i> ssp. <i>diacetylactis</i> |
|------------------------------|------------------------------------|--------------------------------------|---|
| Gram's reaction | + | + | + |
| Catalase test | - | - | - |
| Litmus milk reaction | ARC | ARC | ARC |
| CO ₂ from glucose | - | - | - |
| Growth at 40 ⁰ C | + | - | ± |
| Growth at 50 ⁰ C | - | - | - |
| Growth in 4% NaCl | + | - | ± |
| Ammonia from arginine | + | - | ± |
| Diacetyl in milk | - | - | + |

3.8.1 Identification key for *Streptococcus thermophilus* (Parker and Duerden, 1990)

| Biochemical test | <i>Streptococcus thermophilus</i> |
|--------------------------------------|--|
| Gram's reaction | + |
| Catalase test | - |
| Litmus milk reaction | ARC |
| CO ₂ from glucose | - |
| Survival at 63 ⁰ C/30 min | + |
| Growth at 45 ⁰ C | + |
| Growth at 6.5% NaCl | - |
| Glucose fermentation | + |
| Lactose fermentation | + |
| Ammonia from arginine | - |

3.8.2 Identification key for *Leuconostoc* (Parker and Duerden, 1990)

| Biochemical test | <i>L.mesenteroides</i> <i>ssp. dextranicum</i> | <i>L.mesenteroides</i> <i>ssp.mesenteroides</i> | <i>L.mesenteroides</i> <i>ssp. paramesenteroides</i> | <i>L. lactis</i> | <i>L.mesenteroides</i> <i>ssp. cremoris</i> |
|---------------------------------------|---|--|---|------------------|--|
| Gram's reaction | + | + | + | + | + |
| Catalase test | - | - | - | - | - |
| Litmus milk reaction | ARC | ARC | ARC | ARC | ARC |
| CO ₂ from glucose | + | + | + | + | + |
| Dextran production from sucrose | + | + | - | - | - |
| Acid from sucrose | + | + | + | + | - |
| Acid from trehalose | + | + | + | - | - |
| Acid from Arabinose | - | + | + | - | - |
| Survival at 55 ⁰ C/ 30 min | - | - | - | + | - |
| Growth in 3% salt | + (slow) | + | + (slow) | + (slow) | - |
| Growth in 6.5% salt | - | + (slow) | + (slow) | - | - |

3.8.3 Key for the identification of Lactobacilli (Scardovi, 1986)

| Biochemical tests | <i>L.delbrueckii</i> ssp. <i>bulgaricus</i> | <i>L. acidophilus</i> | <i>L. fermentum</i> | <i>L. hilgardii</i> | <i>L. helveticus</i> | <i>L. plantarum</i> |
|------------------------------|--|-----------------------|---------------------|---------------------|----------------------|---------------------|
| Gram's reaction | + | + | + | + | + | + |
| Catalase test | - | - | - | - | - | - |
| Litmus milk reaction | ARC | ARC | ARC | ARC | ARC | ARC |
| CO ₂ from glucose | - | - | + | + | + | + |
| Growth at 15 ⁰ C | - | - | - | + | - | + |
| Growth at 45 ⁰ C | + | + | + | - | + | V |
| Ammonia from arginine | + | - | + | - | - | - |
| Fermentation of Salicin | + | - | - | - | - | - |
| Fermentation of Mellibiose | - | + | + | - | - | - |

3.9 Screening of isolates based on their activity in sterile skim milk

All the isolates were inoculated at 2% level in 10 ml of sterile skim milk tubes. Isolates were selected based on setting time, Direct Microscopic Count and titratable acidity.

3.9.1 Growth study of selected isolates in sterile skim milk and on solid substrate

Selected isolates were inoculated into sterile skim milk and black gram dhal as solid substrate with additives 1% skim milk powder, 10% of tomato juice and 70% moisture. The setting time, Direct Microscopic Count and titratable acidity in terms of % lactic acid was determined for every 6 hrs up to 48 hrs in sterile skim milk and only Direct Microscopic Count in solid state fermented cultures.

3.9.2 Preparation of mixed dahi culture

Selected isolates were mixed in the ratio of 0.15 % : 0.3 % : 0.5% : 1 % = Leuconostoc : Lactococci : Lactobacilli: *Streptococcus thermophilus* respectively to prepare dahi based on the DMC results of the domestic and market dahi samples.

3.9.3 Growth study of Direct Vat Set Culture

Combination of isolates were inoculated at 1% into sterile black gram dhal medium (solid substrate medium) and incubated at 30⁰C. At every 6 hrs of incubation, DMC was determined up to 48 hrs. Based on the results of DMC, the period that showed a good biomass was selected for further study.

3.9.4 Production of DVS culture by SSF technique

Sterile black gram dhal medium was inoculated with liquid dahi culture, mixed thoroughly and incubated at 30⁰C for a period that yielded a good biomass determined by DMC. After optimum incubation period, fermented dhal was spread on a sterile Petri plate aseptically, dried in BOD incubator at 20⁰C for 24 hrs and packed in polythene pouches and stored in refrigerator and ambient temperature (24⁰C)

3.9.5 Preparation of Dahi

The whole milk of Nandini homogenized milk was taken from the market, heat treated to 85⁰C for 30 min and cooled and divided into three parts inoculated with 2% of liquid culture and 0.5% of refrigerated DVS culture and room temperature stored DVS culture respectively. The inoculated milk was transferred to 50 ml sterile polypropylene cups and incubated at 30⁰C/18-20 hrs.

Homogenized milk (Distributed in 3 parts)



Heat treat at 85⁰C/30 min



Cooled to room temperature



Inoculation to each part of heat treated with
2% of liquid culture, 0.5% DVS dahi culture



Incubated at 30⁰C/16 hrs.



Stored at refrigeration temperature



Dahi

3.9.6 Analysis of Dahi

3.9.6.1 Sensory Analysis

The sensory analysis was carried out by serving samples of dahi with control (prepared using liquid culture) and experimental dahi samples (prepared using DVS culture stored at room temperature and refrigeration temperature) to a panel of judges with score card (9 point hedonic scale for Dahi) to judge the quality of product, with respect to colour and appearance, body and texture, flavour and overall acceptability (appendix). The scores given by panel of judges were then statistically analyzed.

3.9.6.2 Determination of Titrable Acidity (IS:SP:18, part II,1981)

Acidity of dahi was determined by taking exactly 10g of well mixed sample in a conical flask to which 10 ml of boiled, cooled water was added and mixed thoroughly. Phenolphthalein indicator of 5 drops was added and titrated against standardized N/10 sodium hydroxide (NaOH) and the rundown of NaOH was multiplied by 0.09 and expressed as percent Lactic acid.

3.9.6.3. Direct Microscopic count (DMC) [Harrigan, 1998]

Samples to be analyzed were mixed well and diluted to 1:10 by using normal saline. Sample of 0.01 ml (10 μ l) was transferred using a breeds pipette onto a breeds slide and smear was prepared by spreading evenly in the marked area of 1sq.cm. The smear was fixed using ethanol for 2 min, dipped in xylene for 5 min to defatened and finally stained by using borax methylene blue for 5 min, then observed under the oil immersion objective and organisms were counted in each of the field. The average number of

organisms per field was calculated and multiplied by microscopic factor determined by using stage micrometer and dilution factor and expressed as number of organisms per gram of the sample.

3.9.6.4 Shelf life study of DVS culture of dahi

The packed DVS cultures were stored at refrigeration temperature and room temperature separately. Viability of refrigeration stored DVS culture was checked at every week up to 3 months, by subjecting to viable counts using YGA (Total bacterial count), NRCLA (lactococci), YGA (*Streptococcus. thermophilus*, selected by subjecting 1:10 dilution for pasteurization), Sucrose agar (leuconostoc) and Rogosa agar (lactobacilli). Similarly viability of room temperature stored DVS culture was checked at every 3 days up to 3 months for the microbiological parameters similar to refrigeration stored DVS culture.

RESULTS

IV. RESULTS

Direct Vat Set (DVS) Dahi culture is a concentrated culture that can be used for the direct inoculation of milk. It is a combination of different strains of culture that are technically blended to achieve the desired functionality in the final fermented milk product. DVS dahi cultures are available in the form of frozen and freeze dried pellets. The study was conducted for the production of DVS dahi culture through SSF technique and results of the experiments are presented in this section.

4.1 Enumeration of lactic acid bacteria from dahi samples:

Enumeration of lactic acid bacteria from the domestic and market dahi samples of Bengaluru were carried out and details regarding dahi samples were enumerated (Table 1a & 1b). Both the domestic and market samples were analyzed using Yeast glucose Agar (YGA) for total lactic acid bacterial count and for streptococci, Neutral Red Chalk Lactose Agar (NRCLA) for lactococci, Sucrose Agar (SA) for leuconostoc and Rogosa Agar (RA) for lactobacilli and their numbers were enumerated. It may be seen that log counts of lactococci ranged from 0 to 6.45 (Table 2 & Fig. 1). It was noticed that log count of streptococci was 0 and 4.70 and leuconostoc viable count ranged from 0 to 4.50, where as lactobacilli ranged between 0 and 5.50 \log_{10} cfu/g of dahi samples. Total lactic acid bacterial viable counts in dahi samples D₁ – D₁₀ ranged from 6.32 to 7.04.

Table 1a : Area of Domestic Dahi samples collected from in and around Bengaluru

| Details of the domestic sample | | Quantity of sample |
|--------------------------------|--------------------|--------------------|
| Sample | Area of collection | 100g |
| D ₁ | Hebbal | |
| D ₂ | Siddlaghatta | |
| D ₃ | Chikkaballapura | |
| D ₄ | Banashankari | |
| D ₅ | Bagepalli | |

Table 1b : History of Dahi samples collected from local markets of Bengaluru

| Sample | Brand name | Details of the market sample | | |
|-----------------|-----------------|------------------------------|--------|-------|
| | | Mfd. Date | Weight | Price |
| D ₆ | Nandini premium | 20-2-2009 | 200g | Rs.8 |
| D ₇ | Nandini | 20-5-2009 | 200g | Rs.8 |
| D ₈ | Nilgiris | 7-10-2009 | 200g | Rs.13 |
| D ₉ | Nestle | 20-10-2009 | 400g | Rs.25 |
| D ₁₀ | Britania | 28-12-2009 | 200g | Rs.15 |

Table 2 : Enumeration of Lactococi, *Streptococcus thermophilus*, Leuconostoc and Lactobacilli from Domestic and Market Dahi samples

| Sample | Total lactic acid bacterial count | Lactococci | <i>S. thermophilus</i> | Leuconostoc | Lactobacilli |
|------------------------|---------------------------------------|------------|------------------------|-------------|--------------|
| Domestic sample | Count (log₁₀ cfu/g) | | | | |
| D ₁ | 6.44 | 4.44 | - | 2.00 | - |
| D ₂ | 6.32 | 4.22 | - | - | 2.10 |
| D ₃ | 7.04 | 6.25 | - | - | - |
| D ₄ | 7.04 | 6.32 | - | 2.48 | 5.14 |
| D ₅ | 6.69 | 6.38 | - | - | 5.50 |
| Market samples | | | | | |
| D ₆ | 6.41 | 5.36 | - | - | 3.40 |
| D ₇ | 6.45 | 6.00 | - | 4.50 | 4.66 |
| D ₈ | 6.70 | - | - | - | 3.50 |
| D ₉ | 6.80 | 5.80 | 4.70 | - | - |
| D ₁₀ | 6.50 | 2.00 | 3.00 | 1.50 | 4.10 |

Note: Total Lactic Acid Bacterial Count – YGA plates incubated at 37⁰C/24-48hrs

Lactococci -NRCLA plates incubated at 30⁰C/24-48hrs

Leuconostocq -Sucrose Agar plates incubated at 30⁰C/24-48hrs

Lactobacilli -Rogosa Agar plates incubated at 37⁰C/24-48hrs

Streptococcus thermophilus -I dilution was pasteurized, plated using YGA & incubated at 37⁰C/24-48hrs

All the plates were incubated in anaerobic jar.

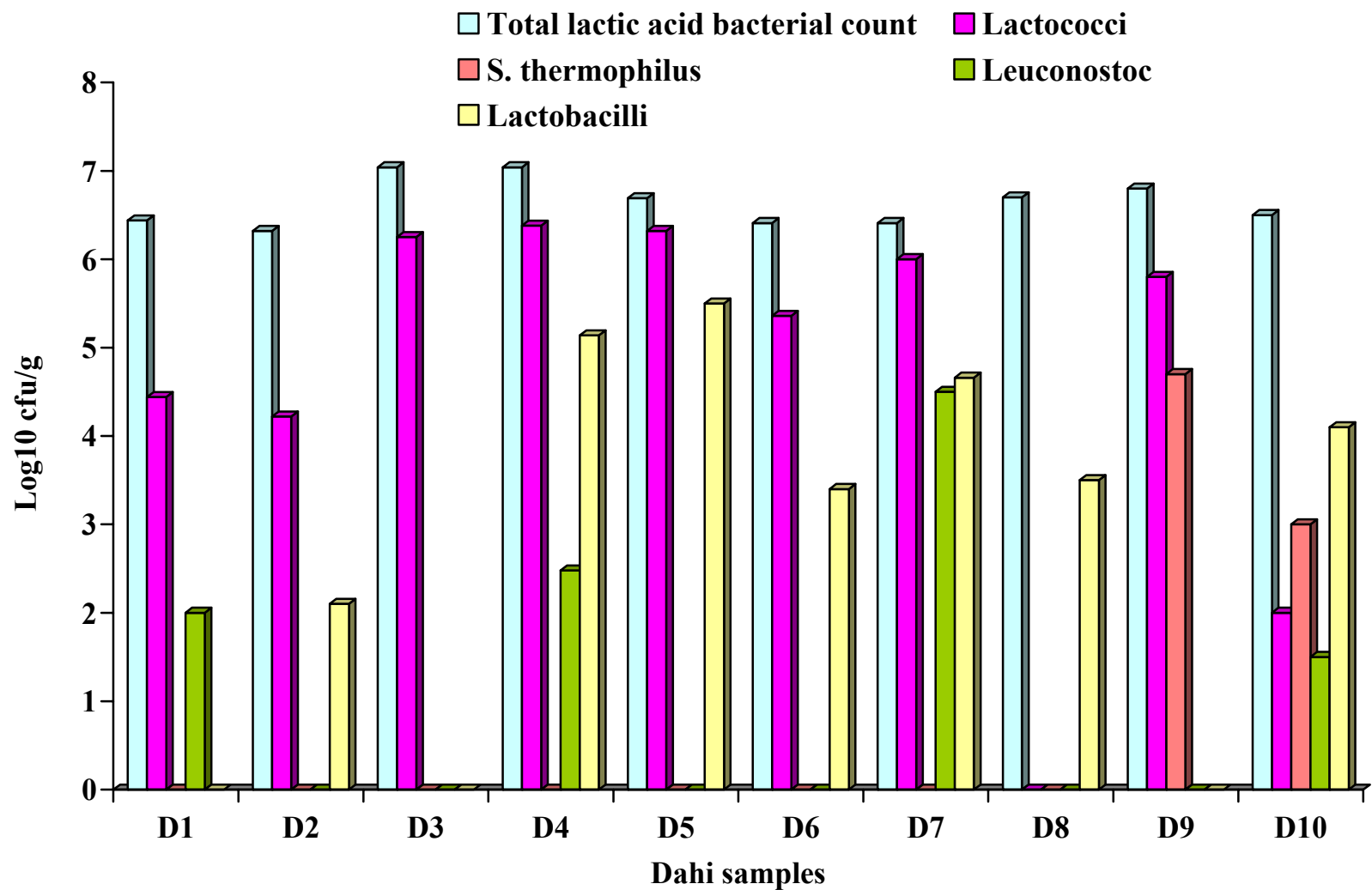


Fig. 1 : Enumeration of lactic acid bacteria in domestic and market Dahi samples

4.1.1 Isolation of lactic acid bacterial isolate:

After enumeration of lactic acid bacteria from dahi samples, colonies were selected based on and colony morphology and characteristics of colonies were noted. Selected isolates were individually transferred to yeast glucose broth. Based on Gram's reaction, isolates were declared Gram positive cocci or rods and purified by streaking onto Yeast Glucose Agar three times. Pure cultures were maintained in the Yeast Glucose Agar stabs containing 0.75% of agar. Based on the colony characteristics on selective media and cell morphology all these isolates were grouped upto genus level (Table 3 & Fig. 2). Isolates (9 numbers) picked from the NRCLA plate were circular, smooth, pinpoint and pink coloured. Isolates obtained from YGA plates (7 numbers) were smooth, creamy obtained from yeast glucose agar plate. Isolates of 18 numbers picked from the Sucrose Agar plate were opaque and glistening. Isolates (38 number) obtained from Rogosa agar plates were subsurface and oval shaped.

Table 3 : Distribution of lactic acid bacterial isolates in Dahi samples

| Identity | Number of isolates obtained from different dahi Samples | | | | | | | | | |
|-------------------------------|---|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|-----------------|
| | D ₁ | D ₂ | D ₃ | D ₄ | D ₅ | D ₆ | D ₇ | D ₈ | D ₉ | D ₁₀ |
| Lactococci | 9 | | | | | | | | | |
| <i>L. lactis ssp.lactis</i> | 6 | - | - | 1 | - | - | - | - | - | - |
| <i>L. lactis ssp.cremoris</i> | 2 | - | - | - | - | - | - | - | - | - |
| Streptococci | 7 | | | | | | | | | |
| <i>S. thermophilus</i> | - | - | - | - | - | - | - | - | 4 | 3 |
| Leuconostoc | 18 | | | | | | | | | |
| <i>L.lactis</i> | - | - | - | - | - | - | 4 | - | - | - |
| Unidentified | 6 | - | - | 4 | - | - | - | - | - | 4 |
| Lactobacilli | 38 | | | | | | | | | |
| <i>L.fermentum</i> | - | 5 | - | 2 | 3 | 4 | - | 5 | - | 3 |
| <i>L.hilgardii</i> | - | - | - | 4 | - | 2 | - | - | - | 4 |
| <i>L. acidophilus</i> | - | - | - | - | - | - | 4 | - | - | 2 |

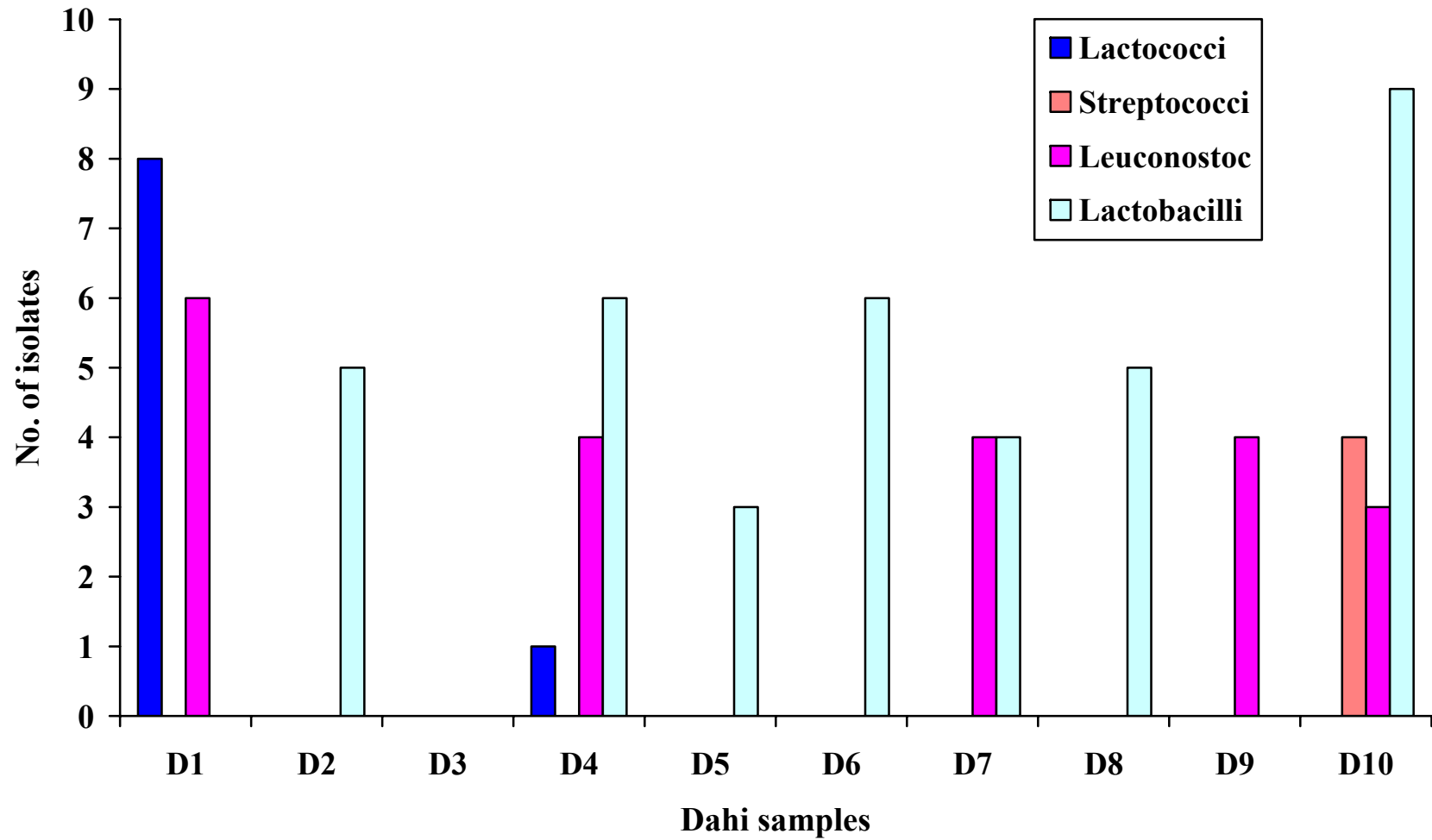


Fig. 2 : Number of isolates in dahi samples

4.1.2 Identification of lactic acid bacterial isolates obtained from Dahi samples:

Purified lactic acid bacterial isolates (72 numbers) obtained from Dahi samples were subjected to preliminary tests such as simple staining, Gram's staining, catalase test and litmus milk reaction. All the 72 lactic isolates were Gram positive, catalase negative and curdled the milk and reduced the litmus, giving the indication of lactic nature of the isolates. These preliminary tests identified the isolates to genus level.

4.1.2.1 Identification of lactococcal isolates:

All the lactococcal isolates (9 numbers) obtained from Dahi samples were homofermentative as they did not produce CO₂ from glucose (Table 4). These isolates were subjected to specific biochemical tests such as growth at 40⁰ and 50⁰C, growth in 4% NaCl, diacetyl in milk and ammonia from arginine for species level identification. Based on the results of biochemical tests, 7 of the isolates were identified as *L.lactis ssp.lactis* and 2 of them were identified as *L.lactis ssp.cremoris*.

4.1.2.2 Identification of *Streptococcus thermophilus* isolates:

The isolates of *S.thermophilus* (7 numbers) obtained from Dahi by selective plating through lab pasteurization were homofermentative as they did not produce CO₂ from glucose due to their heterofermentative nature (Table 5). The isolates showed growth at 45⁰C and fermented glucose and lactose.

Table 4 : Identification of lactococcal isolates obtained from Dahi samples

| Isolate No. | Growth at | | Growth in 4% NaCl | Diacetyl in milk | Ammonia from arginine | Identity |
|---|-----------|------|-------------------|------------------|-----------------------|------------------------------|
| | 40°C | 50°C | | | | |
| Lc1, Lc2, Lc4, Lc5, Lc6, Lc7, Lc9 (7 numbers) | + | - | + | - | + | <i>L.lactis ssp.lactis</i> |
| Lc3, Lc8 (2 numbers) | - | - | - | - | - | <i>L.lactis ssp.cremoris</i> |

Note : All these isolates were Gram positive cocci, Catalase negative, ARC in Litmus milk, No CO₂ produced from glucose.

Table 5 : Identification of *Streptococcus thermophilus* isolates obtained from Dahi samples

| Isolate No. | Growth at 45°C | Growth at 6.5 % salt | Fermentation of Sugar | | Ammonia from arginine | Identity |
|--|----------------|----------------------|-----------------------|---------|-----------------------|-----------------------|
| | | | Glucose | Lactose | | |
| St1, St2, St3, St4, St5, St6, St7 (7 numbers) | + | - | + | + | - | <i>S.thermophilus</i> |

Note : All these isolates were Gram positive cocci, Catalase negative, ARC in Litmus milk, No CO₂ produced from glucose.

4.1.2.3 Identification of leuconostoc isolates:

Total number of 18 isolates of leuconostoc selected, produced CO₂ from glucose due to their heterofermentative nature (Table 6) and were subjected to specific biochemical tests such as dextran from Sucrose Agar; fermentation of arabinose and sucrose; survival at 55⁰C/30 min and growth in 3% NaCl and 6.5% NaCl for species level identification. Only 4 out of 18 isolates were identified as *L.lactis* based on the results of biochemical test and remaining 14 of the isolates were unidentified, as the results of biochemical tests did not match with the identification key of leuconostoc.

4.1.2.4 Identification of lactobacilli isolates:

Among the 38 lactobacilli isolates, 32 were heterofermentative as they produced CO₂ during utilization of glucose (Table 7), remaining 6 isolates were homofermentative as they did not produce CO₂ from glucose and were subjected to series of specific biochemical tests such as growth at 45⁰C, 15⁰C, ammonia from arginine and fermentation of salicin, mellibiose for species level identification. Based on the results of specific biochemical tests, 22 isolates were identified as *L .fermentum*; 10 isolates as *L.hilgardii* and remaining 6 were identified as *L.acidophilus*.

4.2 Screening of lactic acid bacterial isolates for their activity in skim milk:

All the 72 lactic acid bacterial isolates were inoculated into the sterile skim milk to observe the setting time (Table 8), based on the shorter setting time, isolates were selected for further study. Isolates of lactococci and leuconostoc curdled the milk at 16-30 hrs and 18 hrs at 30⁰C respectively. *Streptococcus thermophilus* and lactobacilli curdled the sterile skim milk with incubation period of 18 – 24 hrs at 37⁰C.

Table 6 : Identification of leuconostoc isolates obtained from Dahi samples

| Isolate No. | Dextran from sucrose | Acid from sugar | | Survival at 55 ⁰ C/30min | Growth in | | Identity |
|--|----------------------|-----------------|---------|-------------------------------------|-----------|----------|--------------------------|
| | | Arabinose | Sucrose | | 3%Nacl | 6.5%Nacl | |
| Leu1, Leu2, Leu3, Leu4 (4 numbers) | - | - | + | + | + | - | <i>Leuconosto lactis</i> |
| Leu5, Leu6, Leu7, Leu8, Leu9, Leu10 (6 numbers) | - | + | - | + | + | + | Unidentified |
| Leu11, Leu112, Leu13, Leu14 (4 numbers) | - | - | + | - | - | - | |
| Leu15, Leu16, Leu17, Leu18 (4 numbers) | - | + | - | + | + | + | |

Note: All these isolates were Gram positive cocci, Catalase negative, ARC in Litmus milk, CO₂ produced from glucose.

Table 7 : Identification of lactobacilli isolates obtained from Dahi samples

| Isolates No. | Gas from glucose | Growth at 45 ⁰ C | Growth at 15 ⁰ C | Ammonia from arginine | Fermentation of sugar | | Identity |
|---|------------------|-----------------------------|-----------------------------|-----------------------|-----------------------|------------|----------------------|
| | | | | | Salicin | Mellibiose | |
| Lb1, Lb2, Lb3, Lb4, Lb5, Lb6, Lb7, Lb8, Lb9, Lb10, Lb11, Lb12, Lb18, Lb25, Lb26, Lb27, Lb28, Lb34, Lb35, Lb36, Lb38, Lb39 (22 numbers) | + | + | - | + | - | + | <i>L.fermentum</i> |
| Lb13, Lb14, Lb15, Lb16, Lb17, Lb29, Lb30, Lb31, Lb32, Lb33 (10 numbers) | + | - | - | + | - | - | <i>L.hilgardii</i> |
| Lb19, Lb20, Lb21, Lb22, Lb23, Lb24 (6 numbers) | - | + | - | - | + | + | <i>L.acidophilus</i> |

Note : All these isolates were Gram positive rods, Catalase negative, ARC in Litmus milk, *L.acidophilus* (6 numbers) did not produce CO₂ from glucose, while other 32 isolates produced CO₂ from glucose.

Table 8 : Screening of lactic acid bacterial isolates based on their activity in skim milk

| Isolates | Setting time (hrs) |
|--|---------------------------|
| Lactococcal isolates | |
| <i>L.lactis ssp.cremoris</i> Lc8 <i>L .lactis ssp.lactis</i> Lc1, Lc2 | 24 |
| <i>L .lactis ssp.lactis</i> Lc3, Lc4, Lc5, Lc6, Lc7, Lc9 | 30 |
| Leuconostoc isolates | |
| <i>L.mesentriodes ssp.lactis</i> Leu1, Leu2, Leu3, Leu4 Unidentified isolates Leu5, Leu6, Leu7, Leu8, Leu9, Leu10, Leu11, Leu12, Leu13, Leu14, Leu15, Leu16, Leu17, Leu18 | 18 |
| Streptococcus thermophilus isolates | |
| St1, St2, St3, St4, St5, St6 | 18 |
| St7 | 24 |
| Lactobacilli isolates | |
| <i>L.fermentum</i> Lb7, Lb8, Lb9, Lb10, Lb18, Lb25, Lb26, Lb27, Lb28, Lb34, Lb35, Lb36, Lb37, Lb38, Lb39. <i>L.hilgardii</i> Lb13, Lb14, Lb15, Lb16, Lb17 <i>L.acidophilus</i> Lb19, Lb20 | 18 |
| <i>L.fermentum</i> Lb1, Lb2, Lb3, Lb4, Lb5, Lb6, Lb11, Lb12, Lb21, Lb22, Lb23, Lb24 <i>L.hilgardii</i> Lb29, Lb30, Lb31, Lb32, Lb33 | 24 |

4.3 Activity of lactic acid bacterial isolates in sterile skim milk:

L.lactis ssp.lactis Lc1 & Lc2 and *L.lactis ssp.cremoris* Lc8 curdled the milk at 24 hrs of incubation at 30⁰C (Table 9) and DMC ranged from 8.08 to 8.31 log₁₀/g and acidity ranged between 0.35 to 0.44% lactic acid. Other isolates showed curdling of milk at 30hrs of incubation.

The isolates (6 numbers) of *Streptococcus thermophilus* St1 – St6 curdled the milk at 18 hrs of incubation with DMC ranged from 8.21 to 8.69 log₁₀/g and titratable acidity was determined between 0.41 to 0.69% lactic acid (Table 10).

All the leuconostoc isolates irrespective of their identity set the milk at 18hrs. The DMC observed from 8.20 to 8.90 log₁₀/g with acidity of 0.29 to 0.47 % lactic acid. Among the isolates Leu1 and Leu6 showed highest DMC (Table 11).

All the isolates of the lactobacilli (25 numbers) irrespective of their identity as *L.fermentum*, *L.hilgardii* and *L.acidophilus* set the milk at 18 hrs of incubation at 37⁰C with DMC ranging between 7.97 to 8.91 log₁₀/g and acidity produced by the isolates ranged between 0.32 to 0.96 % lactic acid (Table 12). *L.fermentum* Lb8 and Lb35 showed highest DMC of 8.77 and 8.91 log₁₀/g respectively.

4.4 Growth study of selected lactic acid bacterial isolates in sterile skim milk :

The selected isolates of lactic acid bacteria were inoculated into sterile skim milk and incubated at optimum temperature (30⁰C or 37⁰C depending on the isolates), for every 6 hrs the sample were drawn up to 48 hrs. The setting time of milk DMC and titratable acidity were recorded.

Table 9 : Activity of lactococcal isolates in sterile skim milk

| Isolate No. | Identity | DMC (log₁₀/g) | Setting time (hrs) | Titrateable Acidity (% LA) |
|--------------------|------------------------------|---------------------------------|---------------------------|-----------------------------------|
| Lc1 | <i>L.lactis ssp.lactis</i> | 8.31 | 24 | 0.44 |
| Lc2 | <i>L.lactis ssp.lactis</i> | 8.20 | | 0.39 |
| Lc8 | <i>L.lactis ssp.cremoris</i> | 8.08 | | 0.35 |

Note: Incubation temperature was at 30⁰C.

Table 10 : Activity of *Streptococcus thermophilus* isolates in sterile skim milk:

| Isolate No. | DMC (log₁₀/g) | Setting time (hrs) | Acidity (% LA) |
|--------------------|-------------------------------------|-------------------------------|---------------------------|
| St1 | 8.67 | 18 | 0.67 |
| St2 | 8.40 | | 0.48 |
| St3 | 8.69 | | 0.69 |
| St4 | 8.21 | | 0.41 |
| St5 | 8.50 | | 0.55 |
| St6 | 8.52 | | 0.58 |

Note: Incubation temperature was at 37⁰C.

Table 11 : Activity of leuconostoc isolates in sterile skim milk

| Isolate No. | Identity | DMC (log₁₀/g) | Setting time (hrs) | Acidity (%LA) |
|------------------------|--------------------------------|-------------------------------------|-------------------------------|--------------------------|
| Leu₁ | <i>L.lactis</i> (4 numbers) | 8.76 | 18 | 0.41 |
| Leu ₂ | | 8.20 | | 0.37 |
| Leu ₃ | | 8.21 | | 0.30 |
| Leu ₄ | | 8.20 | | 0.31 |
| Leu ₅ | Unidentified (14numbers) | 8.57 | | 0.39 |
| Leu₆ | | 8.90 | | 0.47 |
| Leu ₇ | | 8.30 | | 0.36 |
| Leu ₈ | | 8.24 | | 0.30 |
| Leu ₉ | | 8.25 | | 0.29 |
| Leu ₁₀ | | 8.32 | | 0.30 |
| Leu ₁₁ | | 8.40 | | 0.36 |
| Leu ₁₂ | | 8.46 | | 0.33 |
| Leu ₁₃ | | 8.28 | | 0.29 |
| Leu ₁₄ | | 8.30 | | 0.32 |
| Leu ₁₅ | | 8.39 | | 0.37 |
| Leu ₁₆ | | 8.48 | | 0.38 |
| Leu ₁₇ | | 8.35 | | 0.39 |

Note: Incubation temperature was at 30⁰C.

Table 12 : Activity of lactobacilli isolates in sterile skim milk

| Isolate No. | Identity | Setting time (hrs) | DMC (log ₁₀ /g) | Acidity (% LA) |
|-------------|-----------------------|--------------------|----------------------------|----------------|
| Lb7 | <i>L. fermentum</i> | 18 | 8.34 | 0.72 |
| Lb8 | | | 8.77 | 0.92 |
| Lb9 | | | 8.10 | 0.50 |
| Lb10 | | | 8.60 | 0.56 |
| Lb18 | | | 8.56 | 0.70 |
| Lb25 | | | 8.53 | 0.89 |
| Lb26 | | | 8.67 | 0.81 |
| Lb27 | | | 8.76 | 0.90 |
| Lb28 | | | 8.34 | 0.71 |
| Lb34 | | | 8.54 | 0.78 |
| Lb35 | | | 8.91 | 0.96 |
| Lb36 | | | 8.43 | 0.64 |
| Lb37 | | | 8.52 | 0.75 |
| Lb38 | | | 8.26 | 0.50 |
| Lb39 | | | 8.65 | 0.81 |
| Lb13 | <i>L. hilgardii</i> | 7.97 | 0.40 | |
| Lb14 | | 8.41 | 0.81 | |
| Lb15 | | 8.30 | 0.76 | |
| Lb16 | | 8.20 | 0.35 | |
| Lb17 | | 8.22 | 0.33 | |
| Lb19 | <i>L. acidophilus</i> | 8.09 | 0.32 | |
| Lb20 | | 8.32 | 0.50 | |
| Lb21 | | 8.49 | 0.69 | |
| Lb22 | | 8.28 | 0.57 | |
| Lb23 | | 8.26 | 0.52 | |

Note: Incubation temperature was at 37⁰C.

L. lactis ssp. lactis (Lc1 and Lc2) when grown in skim milk curdled the milk at 24 hrs. The DMC increased with simultaneous increase in titratable acidity up to 24 hrs of incubation at 30⁰C. DMC in case of Lc1 ranged between 6.01 to 8.43 log₁₀/g with acidity of 0.16 to 0.36. while DMC of Lc2 ranged between 6.13 to 8.03 log₁₀/g with acidity of 0.16 to 0.33% lactic acid (Table 13 and Fig. 3). The stastical analysis indicated significant difference between *L. lactis ssp. lactis* Lc1 and Lc2 with respect to DMC.

S. thermophilus isolates (St1, St3) were grown in the sterile skim milk and curdled the milk at 18 hrs of incubation at 37⁰C. The DMC of St1 ranged between 6.02 to 8.49 log₁₀u/ml while DMC of St3 ranged from 6.04 to 8.76 log₁₀/g (Table 14 and Fig. 4). Initial titratable acidity was 0.16 and reached 0.30 in case of St1 and 0.41 by St3 at 24 hrs of incubation. The stastical analysis indicated significant difference between *S. thermophilus* St1 and St3 with respect to DMC.

Skim milk was curdled by *Leuconostoc lactis* (Leu1) and *Leuconostoc sp. leu6* at 18 hrs when incubated at 30⁰C. The DMC increased with simultaneous increase in titratable acidity from 0.16 to 0.30 during 0 to 18 hrs of incubation in both the isolates. The DMC ranged between 6.04 and 8.21 log₁₀/ml at 0 to 24 hrs of incubation and 6.02 to 8.57 log₁₀/g (Table 15 and Fig. 5) respectively at 0 to 18 hrs of incubation in case of leu1 and leu6 respectively. The stastical analysis indicated significant difference in DMC with respect to *Leuconostoc lactis* Leu1 and *Leuconostoc sp. Leu6*.

Table 13 : Growth study of *L. lactis ssp. lactis* Lc1 and Lc2 in sterile skim milk

| Time (hrs) | DMC (log ₁₀ /ml) | Acidity (%LA) |
|------------------|-----------------------------|---------------|
| LC1 | | |
| 0 | 6.01 | 0.16 |
| 6 | 6.32 | 0.18 |
| 12 | 6.57 | 0.21 |
| 18 | 7.78 | 0.24 |
| 24 | 8.43 | 0.36 |
| 30 | 8.03 | 0.28 |
| 36 | 7.70 | 0.26 |
| 42 | 7.43 | 0.27 |
| 48 | 6.97 | 0.25 |
| CD ≥ 0.05 | 0.2 | - |
| LC2 | | |
| 0 | 6.13 | 0.16 |
| 6 | 7.07 | 0.19 |
| 12 | 7.23 | 0.21 |
| 18 | 7.74 | 0.27 |
| 24 | 8.03 | 0.33 |
| 30 | 7.95 | 0.32 |
| 36 | 7.61 | 0.32 |
| 42 | 7.29 | 0.32 |
| 48 | 6.97 | 0.32 |
| CD ≥ 0.05 | 0.07 | - |

Note: Incubation at 30⁰C for up to 48hrs

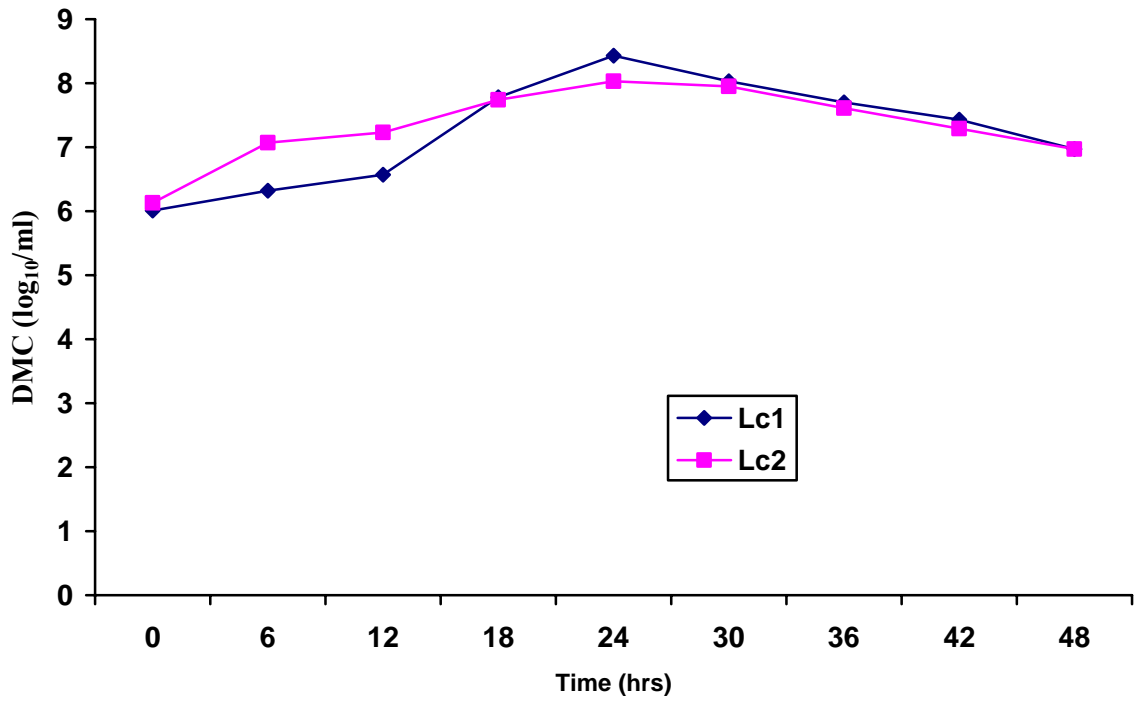


Fig. 3 : Growth study of *Lactococcus lactis ssp. lactis*, Lc1 and Lc2 in sterile skim milk

Table 14 : Growth study of selected *Streptococcus thermophilus* in sterile skim milk

| Time (hrs) | DMC (log₁₀/ml) | Acidity (%LA) |
|-----------------------|--------------------------------------|--------------------------|
| St1 | | |
| 0 | 6.02 | 0.16 |
| 6 | 7.78 | 0.21 |
| 12 | 7.99 | 0.23 |
| 18 | 8.49 | 0.30 |
| 24 | 8.41 | 0.30 |
| 30 | 7.91 | 0.30 |
| 36 | 7.72 | 0.30 |
| 42 | 7.25 | 0.30 |
| 48 | 6.79 | 0.30 |
| CD ≥ 0.05 | 0.05 | - |
| St3 | | |
| 0 | 6.04 | 0.16 |
| 6 | 7.42 | 0.27 |
| 12 | 7.81 | 0.37 |
| 18 | 8.76 | 0.41 |
| 24 | 8.18 | 0.40 |
| 30 | 7.92 | 0.40 |
| 36 | 7.63 | 0.40 |
| 42 | 7.14 | 0.40 |
| 48 | 6.80 | 0.40 |
| CD ≥ 0.05 | 0.2 | - |

Note: Incubated at 37⁰C for up to 48hrs.

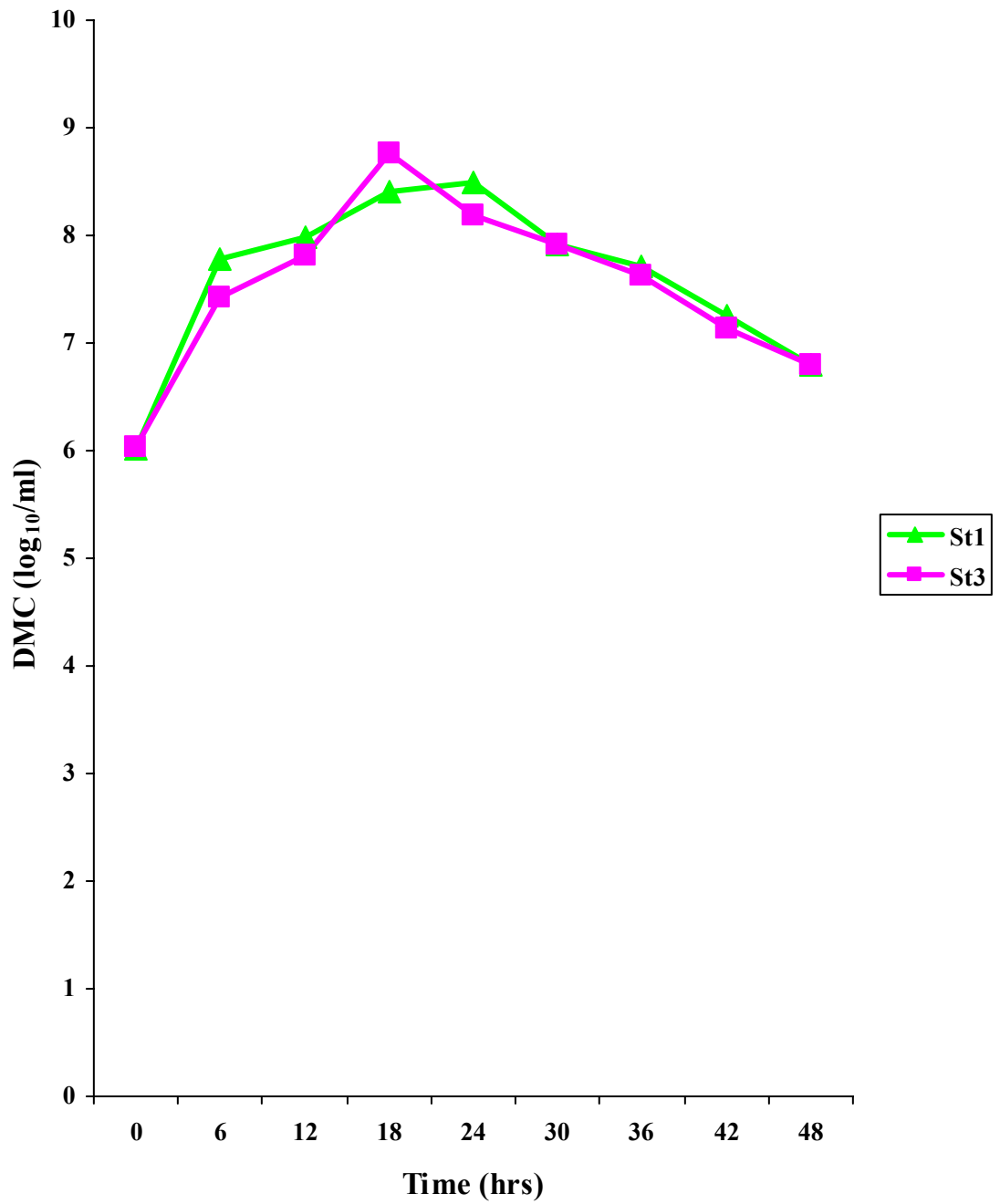


Fig. 4 : Growth study of *Streptococcus thermophilus* , St1 and St3 in sterile skim milk

Table 15 : Growth study of *Leuconostoc lactis* Leu1 and *Leuconostoc* sp. Leu6 in sterile skim milk

| Time (hrs) | DMC (log₁₀/ml) | Acidity (%LA) |
|------------------------|----------------------------------|----------------------|
| Leu₁ | | |
| 0 | 6.04 | 0.16 |
| 6 | 7.28 | 0.18 |
| 12 | 8.02 | 0.19 |
| 18 | 8.44 | 0.22 |
| 24 | 8.21 | 0.30 |
| 30 | 8.08 | 0.26 |
| 36 | 7.89 | 0.24 |
| 42 | 7.44 | 0.23 |
| 48 | 7.16 | 0.25 |
| CD ≥ 0.05 | 0.1 | - |
| Leu₆ | | |
| 0 | 6.02 | 0.16 |
| 6 | 7.14 | 0.19 |
| 12 | 7.99 | 0.20 |
| 18 | 8.57 | 0.30 |
| 24 | 8.21 | 0.22 |
| 30 | 7.84 | 0.23 |
| 36 | 7.43 | 0.21 |
| 42 | 7.17 | 0.22 |
| 48 | 6.82 | 0.23 |
| CD ≥ 0.05 | 0.2 | - |

Note: Incubation at 30⁰C for up to 48hrs.

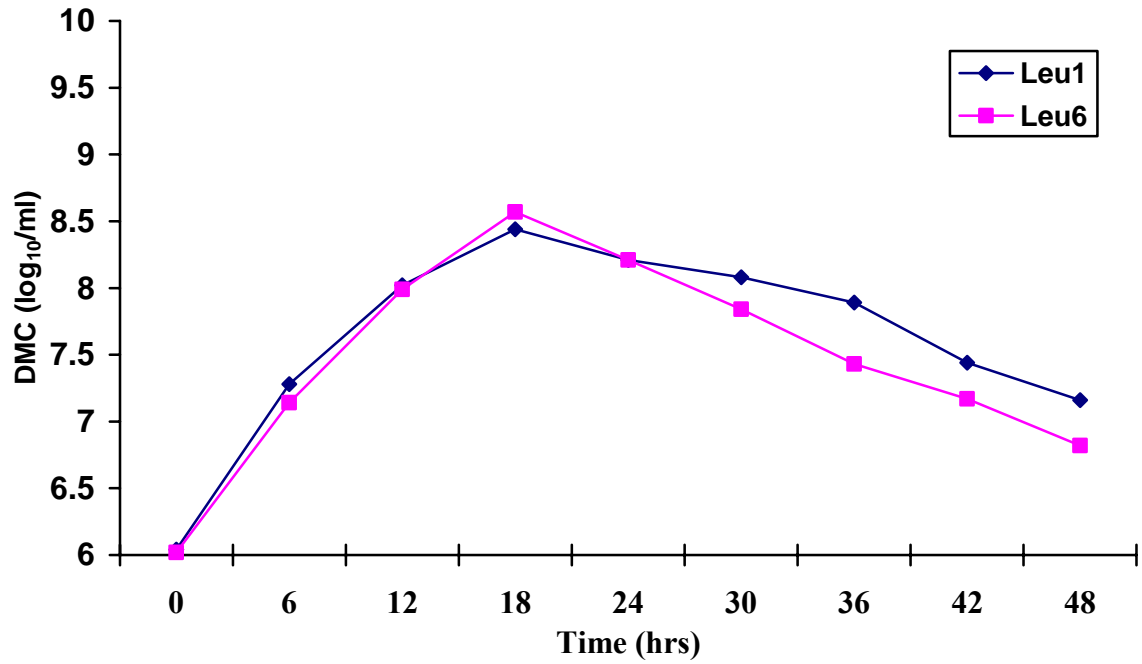


Fig. 5 : Growth study of *Leuconostoc lactis*, Leu1 and unidentified isolate Leu6 in sterile skim milk

L. fermentum (Lb8, Lb35) when grown in sterile skim milk curdled the milk at 18 hrs at 37⁰C with DMC ranging between 6.03 to 8.52 log₁₀/g and 6.02 to 8.46 log₁₀/g respectively. Both the isolates increased acidity from initial 0.16% to 0.63% and 0.59% in case of Lb8 and Lb35 respectively (Table 16 and Fig. 6). The stastical analysis indicated significant difference between *L. fermentum* Lb8 and Lb35 with respect to DMC.

4.5 Growth study of lactic acid bacterial isolates on solid substrate:

Blackgram dhal was used as edible solid substrate to carry out solid state fermentation along with additives like skim milk powder (1%), tomato juice (10%) and moisture of 70%. The medium was sterilized and inoculated with the selected isolates and incubated at their optimum temperature either at 30⁰C or 37⁰C depending on culture. At every 6 hrs interval and upto 48 hrs the samples were drawn and DMC was determined.

L. lactis ssp lactis when grown on black gram dhal showed DMC ranging from 6.03 to 9.46 log₁₀/g and *L. lactis ssp lactis* (Lc1) showed highest count of 9.46 log₁₀/g at 24 hrs of incubation at 30⁰C. *S.thermophilus* St1, St3 showed DMC of 6.01 to 9.28 and 6.04 to 9.45 log₁₀/g respectively from 0 to 24 hrs of incubation at 37⁰C.

Leuconostoc lactis (Leu1) and *Leuconostoc* sp. (Leu6) showed DMC of 9.10 and 9.38 log₁₀/g respectively at 24 hrs of incubation at 30⁰C. *L.fermentum* (Lb8 and Lb35) showed highest DMC of 9.29 and 9.24 log₁₀/g at 24 hrs of incubation at 37⁰C respectively (Table 17).

Table 16 : Growth study of *Lactobacillus fermentum* Lb8 and Lb35 in sterile skim milk

| Time (hrs) | DMC (log₁₀cfu/ml) | Acidity (%LA) |
|-------------------|-------------------------------------|----------------------|
| Lb8 | | |
| 0 | 6.03 | 0.16 |
| 6 | 7.39 | 0.28 |
| 12 | 7.86 | 0.59 |
| 18 | 8.52 | 0.63 |
| 24 | 8.16 | 0.63 |
| 30 | 7.78 | 0.63 |
| 36 | 7.33 | 0.62 |
| 42 | 7.03 | 0.62 |
| 48 | 6.92 | 0.62 |
| CD ≥ 0.05 | 0.08 | - |
| Lb35 | | |
| 0 | 6.02 | 0.16 |
| 6 | 7.46 | 0.19 |
| 12 | 7.85 | 0.43 |
| 18 | 8.46 | 0.59 |
| 24 | 8.18 | 0.58 |
| 30 | 7.99 | 0.51 |
| 36 | 7.57 | 0.57 |
| 42 | 7.10 | 0.57 |
| 48 | 6.84 | 0.57 |
| CD ≥ 0.05 | 0.1 | - |

Note: Incubation at 37⁰C for up to 48 hrs.

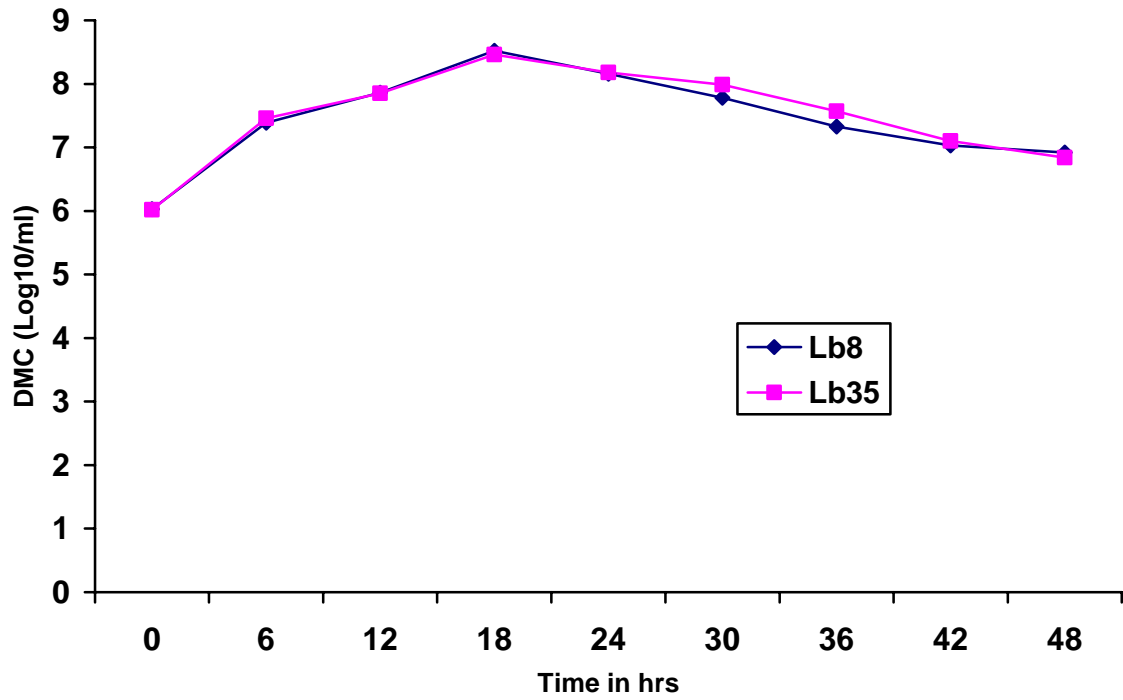


Fig. 6 : Growth study of *Lactibacillus fermentum*, Lb8 and Lb35 in sterile skim milk

Table 17 : Growth studies of isolates on solid substrate medium

| Isolate | Incubation period in hrs. | | | | | | | | |
|----------------------------------|----------------------------|------|------|------|-------------|------|------|------|------|
| | 0 | 6 | 12 | 18 | 24 | 30 | 36 | 42 | 48 |
| | DMC (log ₁₀ /g) | | | | | | | | |
| <i>L.lactis ssp.lactis</i> (Lc1) | 6.03 | 7.14 | 8.24 | 8.80 | 9.46 | 8.44 | 8.08 | 7.66 | 7.01 |
| <i>L.lactis ssp.lactis</i> (Lc2) | 6.01 | 7.53 | 8.36 | 8.64 | 9.34 | 8.59 | 8.19 | 7.82 | 6.92 |
| <i>S. thermophilus</i> (St1) | 6.01 | 7.47 | 8.00 | 8.83 | 9.28 | 8.54 | 8.29 | 7.93 | 7.36 |
| <i>S. thermophilus</i> (St3) | 6.04 | 7.23 | 7.82 | 8.60 | 9.45 | 8.50 | 8.12 | 7.86 | 7.29 |
| <i>L. lactis</i> (Leu1) | 6.03 | 7.37 | 7.92 | 8.88 | 9.10 | 8.45 | 8.05 | 7.66 | 7.19 |
| Unidentified sp. (Leu6) | 6.01 | 7.39 | 8.24 | 8.93 | 9.38 | 8.64 | 8.96 | 7.67 | 7.05 |
| <i>L. fermentum</i> (Lb8) | 6.04 | 7.82 | 8.19 | 8.82 | 9.29 | 8.88 | 8.44 | 8.02 | 7.69 |
| <i>L. fermentum</i> (Lb35) | 6.02 | 7.96 | 8.50 | 8.71 | 9.24 | 8.77 | 8.12 | 7.89 | 7.42 |

Note: -Solid substrate consists of Black gram dhal, skim milk powder (1%), tomato juice (10%) and moisture (70%)

-Growth study was carried out at every 6hr of interval for up to 48 hrs.

-Isolates in solid substrate medium were incubated at their optimum

Temperature

4.6 Setting time of Dahi culture at different temperature:

As dahi culture is heterogeneous in nature, based on DMC of dahi samples procured, the lactic cultures were combined at 0.15%, 0.35%, 0.5%, 1%, of *Leuconostoc lactis*, *Lactococcus lactis ssp.lactis*, *Lactobacillus fermentum* and *Streptococcus thermophilus* respectively and inoculated into sterile whole milk for their performance at different temperatures such as 20⁰C, 22⁰C, 25⁰C, 27⁰C and 30⁰C. At 30⁰C, Set I (*Leuconostoc* sp. Leu6: *Lactococcus lactis ssp lactis* Lc1 : *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3) culture curdled the milk at 6 hrs only while Set II (*Leuconostoc lactis* Leu1: *Lactococcus lactis ssp lactis* Lc2 : *Lactobacillus fermentum* Lb35: *Streptococcus thermophilus* St1) took 8 hrs, hence set I dahi culture was used in further studies (Table 18).

4.7 Growth study of mixed Dahi culture in sterile skim milk:

Mixed Dahi culture was prepared by combination of *Leuconostoc* sp. Leu6: *Lactococcus lactis ssp lactis* Lc1 : *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3 at the ratio of 0.15 %: 0.3 %: 0.5%: 1%. The growth of mixed Dahi culture was observed by inoculating to sterile skim milk and incubated at 30⁰C for 0 to 48 hrs. The setting time was noticed at 18 hrs but peak DMC was noticed at 24 hrs of incubation i.e., 8.68 log₁₀/g with acidity of 0.65% LA. The stastical analysis indicated significant difference in the growth of mixed dahi culture at different hours of incubation (Table 19).

Table 18 : Setting time of mixed culture at different temperature

| Temperature of incubation (0 ⁰ C) | Dahi culture | |
|--|---------------------------|-----------------------------|
| | Lc1+St3+Leu6+Lb8 Set I | Lc2+St1+Leu1+Lb35 Set II |
| | (Setting time in hrs) | |
| 20 | 24 | 36 |
| 22 | 8 | 12 |
| 25 | 8 | 12 |
| 27 | 8 | 12 |
| 30 | 6 | 8 |

Note: Combination was done at the ratio of 0.15 % : 0.3 % : 0.5% : 1 % = *Leuconostoc lactis*: *Lactococcus lactis ssp lactis* : *Lactobacillus fermentum*: *Streptococcus thermophilus*

Table 19 : Growth study of Dahi Culture in sterile skim milk at 30⁰C

| Time (hrs) | DMC (log₁₀cfu/g) | Acidity (%LA) |
|-------------------|------------------------------------|----------------------|
| 0 | 6.04 | 0.19 |
| 6 | 7.40 | 0.31 |
| 12 | 7.81 | 0.42 |
| 18 | 8.37 | 0.50 |
| 24 | 8.68 | 0.65 |
| 30 | 8.47 | 0.60 |
| 36 | 8.27 | 0.60 |
| 42 | 7.90 | 0.55 |
| 48 | 7.72 | 0.55 |
| CD ≥ 0.05 | 0.09 | - |

Note: Dahi culture-Isolates were combined in the ratio of 0.15 % : 0.3 % : 0.5% : 1 % =
Leuconostoc sp. Leu6: *Lactococcus lactis* ssp *lactis* Lc1 : *fermentum* Lb8:
Streptococcus thermophilus St3

4.8 Growth study of mixed Dahi Culture on sterile black gram dhal medium:

Leuconostoc sp. Leu6: *Lactococcus lactis ssp lactis* Lc1 : *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3 were grown on sterile black gram dhal medium and incubated at 30⁰C for 48 hrs. The samples were drawn at every 6 hrs up to 48 hrs and DMC was determined.

DMC of mixed Dahi culture ranged from 6.02 to 9.42 log₁₀/g (Table 20). It is indicated that significant difference existed on incubation period on mixed Dahi culture at 30⁰C .

4.8.1 Sensory attributes, acidity and DMC of Dahi prepared from SSF Dahi culture or Direct Vat Set (DVS) Dahi culture:

Dahi was prepared using 2% of liquid culture (C), 0.5% of refrigerated DVS dahi culture and ambient DVS dahi culture and after inoculation of culture, incubated at 30⁰C (Table 21) and served to panel of judges. Judges gave maximum score to dahi prepared from the refrigerated DVS dahi culture compared to ambient SSF culture. The stastical analysis indicated significant difference between liquid culture, refrigerated DVS dahi culture and ambient DVS culture

Table 20 : Growth study of Mixed Dahi Culture on sterile black gram dhal medium at 30⁰C

| Time (hrs) | DMC (log₁₀cfu/g) |
|-------------------|------------------------------------|
| 0 | 6.02 |
| 6 | 7.25 |
| 12 | 8.44 |
| 18 | 8.80 |
| 24 | 9.42 |
| 30 | 8.51 |
| 36 | 8.01 |
| 42 | 7.87 |
| 48 | 7.35 |
| CD ≥ 0.05 | 0.1 |

Note: Solid substrate consists of Black gram dhal, skim milk powder (1%), tomato juice (10%) and moisture (70%).

- Dahi culture in solid substrate medium was incubated at 30⁰C for up to 48 hrs.

Table 21 : Sensory attributes, acidity and DMC of Dahi prepared using SSF Dahi culture or Direct Vat Set (DVS) Dahi culture.

| Dahi cultures | Sensory score out of 9 points | Acidity (%LA) | DMC (log₁₀cells/ml) |
|----------------------|--------------------------------------|----------------------|---------------------------------------|
| C | 7 | 0.61 | 8.20 |
| CR | 8 | 0.67 | 8.51 |
| CRT | 6 | 0.58 | 8.25 |
| CD ≥ 0.05 | 0.8 | - | - |

Note : C: Liquid culture, CR: Refrigerated DVS dahi culture, CRT: ambient DVS dahi Culture incubated at 30⁰C for 18 hrs.

4.5.1 Storage study of solid state fermented or DVS Dahi culture by selective plating procedure at ambient temperature and refrigeration temperature :

Viability of DVS dahi culture was carried out that which was stored at ambient temperature at every three days up to 60 days (Table 22 and Fig. 7). Initial viable count of DVS culture i.e, total lactic counts stored at ambient temperature (24⁰C), was 9.75 and after 9th day the counts were 8.10, on 26th day the viable counts were 6.96 and on 48th day of storage it was 6.01 and on 60th day it was 5.12, similarly trend was noticed in viable counts of *Streptococcus thermophilus* and lactobacilli, the initial counts were 9.48 and 9.19 and reduced to 4.23 and 4.52 respectively. The statistical analysis indicated significant difference in viability of storage DVS dahi culture at ambient temperature and refrigeration temperature. The lactococci and leuconostoc behaved in a similar manner with respect to viability, initial counts were 8.98 and 9.19 that reduced to 3.86 and 3.49 log₁₀ cfu/g of DVS culture.

Storage study of DVS culture in refrigeration temperature, revealed that total lactic counts, initially 9.75 reduced to 9.40 on 70th day of storage and similar trend was noticed on lactococci, streptococci and lactobacilli. The initial viable counts in case of lactococci and leuconostoc were 8.98 and 5.47 respectively (Table 23 and Fig. 8).

Table 22 : Storage study of DVS Dahi culture by selective plating procedure at ambient temperature

| Storage period (Days) | TLABC | Lactococci | Streptococci | Leuconostoc | Lactobacilli |
|-----------------------|-------------------------|------------|--------------|-------------|--------------|
| | log ₁₀ cfu/g | | | | |
| Initial | 9.75 | 8.98 | 9.48 | 9.19 | 9.45 |
| 3 | 8.99 | 8.54 | 8.98 | 8.81 | 8.94 |
| 6 | 8.71 | 8.12 | 8.62 | 8.10 | 8.60 |
| 9 | 8.10 | 7.81 | 8.05 | 7.94 | 8.02 |
| 12 | 7.85 | 7.37 | 7.60 | 7.44 | 7.74 |
| 16 | 7.41 | 7.20 | 7.11 | 7.08 | 7.25 |
| 19 | 7.21 | 7.18 | 7.04 | 6.88 | 7.09 |
| 23 | 7.01 | 6.81 | 7.00 | 6.67 | 6.98 |
| 26 | 6.96 | 6.65 | 6.58 | 6.31 | 6.85 |
| 29 | 6.87 | 6.16 | 6.92 | 6.20 | 6.77 |
| 32 | 6.76 | 5.93 | 6.83 | 6.11 | 6.64 |
| 35 | 6.51 | 5.80 | 6.76 | 6.01 | 6.49 |
| 38 | 6.35 | 5.47 | 6.27 | 5.99 | 6.28 |
| 42 | 6.21 | 5.26 | 6.12 | 5.62 | 6.04 |
| 45 | 6.19 | 5.02 | 5.94 | 5.10 | 5.90 |
| 48 | 6.01 | 4.87 | 5.64 | 4.89 | 5.79 |
| 51 | 5.81 | 4.43 | 5.38 | 4.56 | 5.58 |
| 54 | 5.64 | 4.29 | 4.89 | 4.13 | 5.13 |
| 57 | 5.53 | 4.08 | 4.65 | 3.81 | 4.89 |
| 60 | 5.12 | 3.86 | 4.23 | 3.49 | 4.52 |
| CD ≥ 0.05 | 0.05 | 0.1 | 0.07 | 0.05 | 0.1 |

Note: Viability of DVS Dahi culture was carried out by selective plating procedure.

Total Lactic Acid Bacterial Count – YGA plates incubated at 37⁰C/24-48hrs

Lactococci - NRCLA plates incubated at 30⁰C/24-48hrs

S.thermophilus - I dilution was pasteurized, plated using YGA & incubated at 37⁰C/24-48hrs.

Leuconostoc - Sucrose Agar plates incubated at 30⁰C/24-48hrs

Lactobacilli - Rogosa Agar plates incubated at 37⁰C/24-48hrs

All the plates were incubated in anaerobic jar.

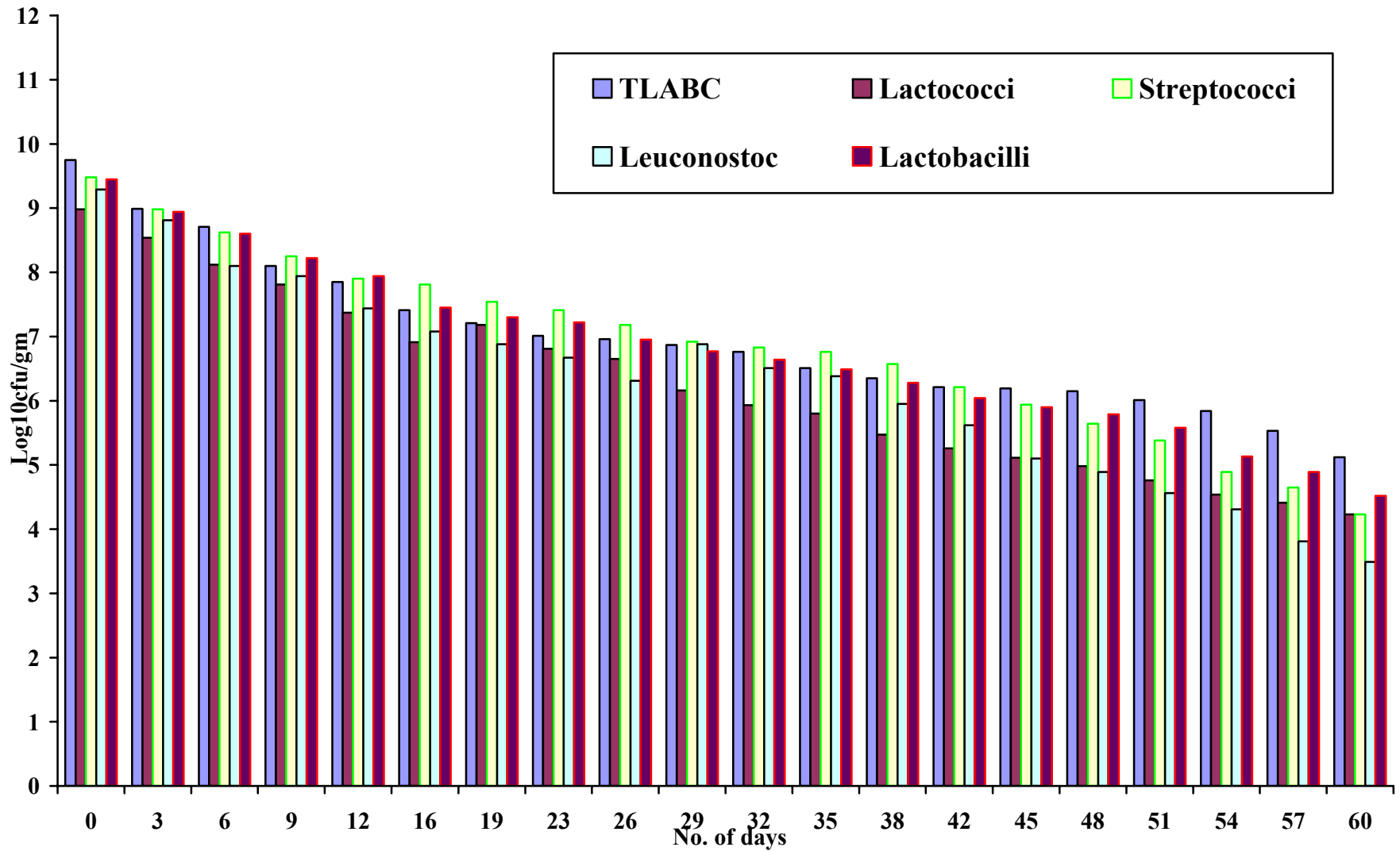


Fig. 7 : Viable count of DVS culture at ambient temperature

Table 23 : Storage study of DVS Dahi culture by selective plating procedure at refrigeration temperature

| Storage period (Days) | TLABC | Lactococci | Streptococci | Leuconostoc | Lactobacilli |
|-----------------------|-------------------------|------------|--------------|-------------|--------------|
| | log ₁₀ cfu/g | | | | |
| Initial | 9.75 | 8.98 | 9.48 | 9.19 | 9.45 |
| 7 | 9.32 | 8.74 | 9.35 | 9.11 | 9.31 |
| 14 | 9.10 | 8.52 | 9.02 | 8.90 | 9.00 |
| 21 | 8.80 | 8.15 | 8.61 | 8.34 | 8.12 |
| 28 | 8.65 | 7.87 | 8.50 | 7.94 | 7.94 |
| 35 | 8.31 | 7.41 | 8.21 | 7.48 | 7.55 |
| 42 | 7.91 | 6.98 | 7.80 | 7.12 | 7.14 |
| 49 | 7.67 | 6.52 | 7.41 | 6.87 | 6.92 |
| 56 | 7.36 | 6.10 | 7.18 | 6.21 | 6.54 |
| 63 | 6.82 | 5.80 | 6.78 | 5.81 | 6.12 |
| 70 | 6.40 | 5.49 | 6.34 | 5.47 | 5.79 |
| CD ≥ 0.05 | 0.1 | 0.2 | 0.14 | 0.21 | 0.14 |

Note: Viability of DVS Dahi culture was carried out by selective plating procedure.

Total Lactic Acid Bacterial Count – YGA plates incubated at 37⁰C/24-48hrs

Lactococci - NRCLA plates incubated at 30⁰C/24-48hrs

S.thermophilus - I dilution was pasteurized, plated using YGA & incubated at 37⁰C/24-48hrs.

Leuconostoc - Sucrose Agar plates incubated at 30⁰C/24-48hrs

Lactobacilli - Rogosa Agar plates incubated at 37⁰C/24-48hrs

All the plates were incubated in anaerobic jar.

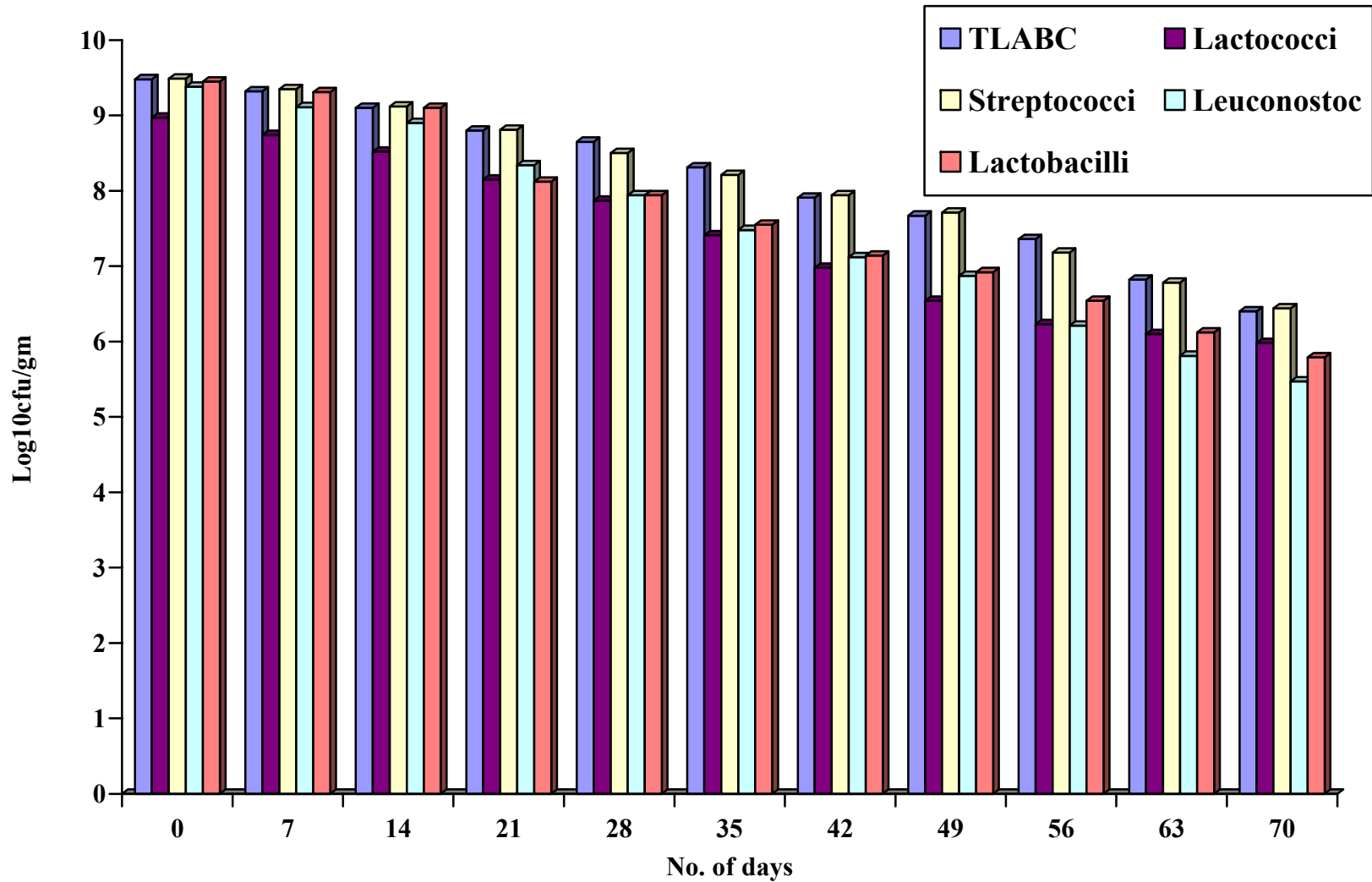


Fig. 8 : Viable count of DVS culture at refrigeration temperature

DISCUSSION

V. DISCUSSION

Dahi is an important fermented milk product which play a major role in human diet. Microflora of dahi is considered as heterogenous group with cocci and rods. Dahi is prepared by using liquid culture, dried or frozen cultures. These lactic cultures which are propagated by various methods have their own limitations. Liquid culture maintenance is cumbersome and requires prior preparation. Microbial contamination may be a problem normally encountered in liquid cultures propagation. Dried and frozen cultures show lower viability. To overcome these problems and to reduce risk with respect to quality, starter concentrates in the form of Direct Vat Set cultures and their preservation slowly came to existence. The present investigation was undertaken to know the facts regarding production of starter concentrates by Solid State Fermentation Technique and to use as DVS Dahi culture for the production of Dahi. In this chapter all the results obtained are well discussed.

5.1 Enumeration of lactic acid bacteria:

5.1.1 Sample collection:

Dahi samples were collected from local houses and market of Bengaluru, five from the house in and around the Bengaluru and five market samples such as Nandini premium, Nandini, Nilgiris, Nestle and Britannia. Sample collection was done as reported by Mehmood *et al.*,(2009) and Mahantesh *et al.* (2010) in Dahi samples.

5.1.2 Enumeration of lactic acid bacteria from domestic and market Dahi samples

All the samples plated showed the predominance of lactococci followed by lactobacilli except D₉. D₈ only showed the presence of lactobacilli. An overall average D₃ and D₄ showed total viable log count of 7.04. D₅ showed highest log count of lactococci 6.38. D₉ and D₁₀ only showed streptococci log count of 4.70 and 3.00 respectively. Only 4 samples showed leuconostoc log count that ranged from 1.50 to 4.50.

Pradeep (2007) enumerated lactic acid bacteria from curd that showed viable log count of lactococci, leuconostoc and lactobacilli 7.87, 3.20 and 5.45 respectively by selective plating technique using Neutral Red Chalk Lactose Agar, Sucrose Agar and Rogosa Agar respectively.

5.2 Isolation of lactic acid bacteria

A total of 72 isolates of lactic acid bacteria were obtained from 10 samples of Dahi. Out of 72 isolates, 9 were lactococci, 7 were *S. thermophilus*, 18 numbers were leuconostoc and 38 numbers were lactobacilli. Majority of lactococcal isolates were picked from D₁ Dahi sample. *S. thermophilus* isolates were obtained from sample D₁₀. Majority of leuconostoc isolates of 4 numbers were picked up from D₇ sample. The lactobacilli isolates of 38 numbers were obtained from all the samples except D₁, D₃, and D₉.

Lactococcus lactis ssp. *lactis* (6) was the major species of lactococcus noticed from D₁ sample followed by *L.lactis* ssp. *cremoris*. All the 7 isolates were *S. thermophilus*; while the leuconostoc species identified were *Leuconostoc lactis* (4) from

D₇ sample and 14 isolates were unidentified as the characteristics did not match with the characteristics mentioned in the Bergey's Manual of Systematic Bacteriology. Among 38 isolates of lactobacilli, 22 numbers belonged to *L. fermentum* followed by *L.hilgardii* (14) and remaining 2 were *L. acidophilus*. In a similar type of study by Borpuzari *et al.* (2007), 10 strains of *L. acidophilus* were identified among total of 273 strains of lactobacilli from market sour curd samples.

Soomro *et al.* (2007) revealed abundant species of *L. delbrueckii ssp. bulgaricus* (28 isolates) followed by *S. thermophilus* (20 isolates), out of 160 strains of lactic acid bacteria from market dahi obtained from Rawalpindi.

5.3 Screening of lactic acid bacterial isolates in sterile skim milk:

All the lactic acid bacterial isolates were inoculated into sterile skim milk in order to screen them for their activity at their optimum temperature. *L. lactis ssp. lactis* Lc1, Lc2 and *L. lactis ssp. cremoris* Lc8 showed setting time of milk at 24 hrs of incubation at 30⁰C compared to other isolates of *L. lactis ssp. lactis* Lc3, Lc4, Lc5, Lc6, Lc7, Lc9 which took 30 hrs at 30⁰C to set the milk.

S. thermophilus isolates St1, St2, St3, St4, St5, St6 curdled the milk at 18 hrs of incubation at 37⁰C. St7 took 24 hrs to curdle the milk. All the 18 isolates of leuconostoc *Leuconostoc lactis* (4) and *Leuconostoc sp.*(14) set the milk at 18 hrs at 30⁰C.

All the isolates of lactobacilli curdled the milk between 18 to 24 hrs of incubation at 37⁰C. *L. fermentum* (15), *L.hilgardii* (5) and *L.acidophilus* (2) curdled the milk at 18

hrs while *L. fermentum* (12) and *L. hilgardii* (5) curdled the milk at 24 hrs of incubation at 37⁰C.

5.4 Activity of lactic acid bacterial isolates

Based on the screening studies, the lactic acid bacterial isolates were selected, that curdled the milk at the earliest. In case of *L. lactis ssp. lactis* Lc1, Lc2 and *Lactococcus lactis ssp. cremoris* Lc8 curdled the milk at 24 hrs of incubation at 30⁰C. Lc1 and Lc2 showed higher log count of 8.31 and 8.20 with titratable acidity of 0.44 and 0.35 % lactic acid respectively compared to other isolate Lc8 hence the isolates Lc1 and Lc2 were selected for further growth studies in milk and on solid substrate medium.

S.thermophilus (6) curdled the milk at 24hrs of incubation at 37⁰C. Among *S.thermophilus*, St1 and St3 showed more DMC of 8.67 and 8.69 log₁₀/g with acidity of 0.69 and 0.67% LA respectively compared to other isolates and both were used in further growth studies.

Leuconostoc lactis, Leu1 and *Leuconostoc* spp. Leu6 showed higher DMC of 8.76 and 8.90 log₁₀/g with titratable acidity of 0.41 and 0.47 respectively compared to other 15 isolates of *Leuconostoc* species. These 2 isolates were selected for growth studies.

L. fermentum Lb8 and Lb35 showed higher DMC of 8.77 and 8.91 log₁₀/g with titratable acidity of 0.92 and 0.96% lactic acid respectively when compared to other 16 isolates of lactobacilli.

Prabha *et al.* (2008) studied the activity of *L. acidophilus* in skim milk with DMC of 8.36 log₁₀/g.

5.5 Growth study of lactic acid bacterial isolates:

5.5.1 Growth study of lactic acid bacterial isolates in sterile skim milk:

The selected isolates of *L. lactis ssp. lactis* Lc1, Lc2 were grown in sterile skim milk at 30⁰C. At every 6 hrs of interval the samples were drawn for determining DMC and acidity. It was noticed that at 24 hrs the skim milk was set showing highest log counts of DMC 8.43 incase of Lc1 and acidity of 0.30% and DMC of Lc2 was 8.03 log count with acidity of 0.33%. Further DMC and acidity started reducing and 24 hrs was considered as ideal incubation time for the growth of lactococci.

The selected isolates of *S. thermophilus* St1, St3 were grown in sterile skim milk at 37⁰C. At every 6 hrs of interval, the samples were drawn for determining DMC and acidity. It was noticed that at 18hrs the skim milk was set showing highest DMC of 8.49 of St1 and acidity 0.30% and DMC of St3 was 8.76 with acidity of 0.41%. Further DMC and acidity started reducing after 18 hrs of incubation and hence 18 hrs. was considered as ideal incubation time for *S. thermophilus*.

The selected isolates of *Leuconostoc lactis* Leu1 and *Leuconostoc* species Leu6 were grown in sterile skim milk at 30⁰C. It was noticed that at 24 hrs of incubation, the inoculated sterile skim milk was set showing highest DMC of 8.21 incase of Leu1 and acidity 0.30% and DMC of Leu6 was 8.57 with acidity of 0.30%. In later incubation period, DMC and acidity started reducing. Ideal incubation time for leuconostoc was 24 hrs at 30⁰C.

The selected isolates of *L. fermentum* Lb8, Lb35 were grown in milk at 37⁰C. At every 6 hrs of interval the samples were drawn for determining DMC and acidity. Incubation period of 18 hrs was ideal for both the isolates of *L. fermentum* as the skim was set with highest DMC of 8.52 and 8.46 log₁₀/g with acidity of 0.63 and 59% respectively. The ideal incubation time for *L. fermentum* was 18hrs.

In a similar type of growth study conducted by Pradeep (2007), *Lactococcus lactis ssp.lactis*, *Leuconostoc mesentriodes ssp.mesentroides*, *Lactobacillus. viridescens* when inoculated individually to sterile skim milk at their optimum growth temperature, curdled the milk at 24hrs of incubation. At 24 hrs. of incubation, all the cultures showed peak in the DMC that ranged between 8.13 to 8.31 log₁₀cells/ml.

5.5.2 Growth study of lactic acid bacterial isolates on solid substrate medium:

Individually selected isolates of *L. lacis ssp. lactis* Lc1, Lc2, *S. thermophilus* St1, St3, *Leuconostoc lactis* Leu1 and *Leuconostoc spp. leu6*, *L. fermentum* Lb8, Lb35 were inoculated in to sterile black gram dhal with 1% of skim milk powder, tomato juice (10%) and 70% moisture and incubated at optimum temperature of 30⁰ or 37⁰C depending on the isolates. At every 6 hrs of interval the sample was taken and DMC was determined. It was interesting to note that all the isolates grew well at 24 hrs of incubation and counts were 9.46 and 9.34 log₁₀/g for *L.lactis ssp.lactis* Lc1, Lc2, 9.28 and 9.45 log₁₀/g for *S. thermophilus* St1, St3, 9.10 and 9.38 log₁₀/g for *Leuconostoc lactis*, Leu1 and *Leuconostoc sp. Leu6*, 9.29 and 9.24 log₁₀/g for *L. fermentum* Lb8, Lb35 respectively. On solid substrate medium, one log count was noticed in all the lactic

cultures compared to submerged fermentation i.e. in skim milk. The highest DMC was noticed in Lc1, St3, Leu6 and Lb8.

On par with the above study, Dieghri-Hocine *et al.* (2007) also found chick pea based solid medium as a good medium for *Lactobacillus plantarum* with 1.28 log cfu/gm higher than in submerged fermentation in skim milk fermentation.

5.5.3 Setting time of sterile skim milk using mixed lactic cultures:

When the mixed culture of Set I containing *Leuconostoc* sp. Leu6: *Lactococcus lactis ssp lactis* Lc1 : *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3 were and Set II with *Leuconostoc lactis* Leu1: *Lactococcus lactis ssp lactis* Lc2 : *Lactobacillus fermentum* Lb35: *Streptococcus thermophilus* St1 were mixed in the proportion of 0.15 % : 0.3 % : 0.5% : 1 % respectively and inoculated into sterile skim milk and incubated at different temperatures, the shorter setting time of 6 hrs was noticed in case of Set I mixed Dahi culture, while Set II took 8 hrs at 30⁰C. Shorter setting time is ideal for the preparation of Dahi that reduces spoilage microflora.

5.5.4 Growth of mixed Dahi culture in sterile skim milk and solid substrate medium:

Set I containing *Leuconostoc* sp. Leu6: *Lactococcus lactis ssp lactis* Lc1: *Lactobacillus fermentum* Lb8: *Streptococcus thermophilus* St3 in the ratio of 0.15 % : 0.3% : 0.5 % : 1% was inoculated into sterile skim milk and at every 6 hrs of interval at 30⁰C samples were drawn and subjected to DMC and acidity. Maximum of 8.68 was obtained at 24 hrs of incubation with acidity of 0.65% and later the DMC as well as

acidity started decreasing, incubation period of 24 hrs was ideal for performance of dahi cultures.

Mixed Dahi culture was grown on black gram dhal medium as solid substrate at 30⁰C . For every 6 hrs of interval the sample was taken and subjected to DMC, at 24 hrs of incubation Dahi culture showed highest log count of 9.42.

Wong and Yalet (1999) found increase in viable count of *L.lactis ssp. lactis*, *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* in nonfat milk medium to about 10⁸ cfu/ml at 24 hrs of incubation.

5.6 Solid State Fermented (SSF) culture or DVS Dahi culture

SSF cultures yielded more counts because of more surface area compared to liquid culture. DVS Dahi culture obtained by solid state fermentation technique on black gram dhal medium was dried at 20⁰C for 18hrs and used as Dahi culture. The culture was inoculated at 0.5% to bulk that was equivalent to 2% of liquid culture. After inoculation of sterile milk with DVS Dahi culture, milk was allowed to stand for 2 min and then transferred to retail cups and incubated at 30⁰C to avoid the entry of dhal into actual product.

The SSF cultures were stored at ambient temperature (24⁰C) as well as at refrigeration temperature (7⁰C). Dahi prepared using both types of SSF cultures were given to judges for determining sensory attributes. It was interesting to note that SSF culture preserved at refrigeration temperature performed better and was liked by judges with maximum score than SSF culture preserved at ambient temperature. DVS cultures

available commercially as starter concentrates and as frozen (using liquid nitrogen - 196⁰C) or freeze dried. None of the Indian companies are producing it.

Prabha (1999) obtained maximum viable count of 9.10 log₁₀cfu/g by SSF fermentation when black gram dhal was used as substrate with 0,8% of moisture, 1% of skim milk powder and MnSO₄ of 0.005%, after fermentation and dried at 20⁰C.

Krishan Kumar and Gandhi (2009) reported that among the recent innovative technologies in preservation of starter culture, Direct vat Set culture is promising method and rapidly finding role in the manufacture of fermented milk products such as Dahi, Yoghurt and Kefir.

5.7 Effect of Storage temperature on the viability of DVS Dahi culture:

DVS Dahi cultures were stored at ambient temperature and refrigeration temperature and then enumerated for survivors. At room temperature enumeration of total lactic acid bacterial count, lactococci, streptococci, leuconostoc and lactobacilli were carried out for every 3 days up to 2 months. The count decreased slightly after 3 days but on 6th day tremendous decrease of 1 log count was noticed in almost all the counts. The counts decreased by one more log by the end of 23rd day and counts stabilized slowly by 26th day onwards, again the decrease in log number was noticed after 51days of storage and finally the count at the end of 60th day of ambient temperature storage was recorded as 4 log compared to initial count of 9.75 in total lactic acid bacterial count. Leuconostoc and lactococci behaved in a similar manner with reduction in viable count by 5.70 and 5 log numbers from initial count on 60th day of storage of DVS culture at ambient temperature. The reduction trend in viability was comparable among streptococci and

lactobacilli, accounting for 5 log numbers nearly 5 log numbers was noticed at ambient temperature storage on 60th day of ambient temperature storage.

At refrigeration temperature enumeration of total lactic acid bacterial count, lactococci, streptococci, leuconostoc and lactobacilli was carried out for every 7 days up to 2 months. The decrease in viable count was slight after 7 days but on 21st day tremendous decrease of one log number was noticed in almost all the counts. The counts decreased by one more log by the end of 49th day and counts stabilized and again decrease in one log number was noticed after 63rd day of storage and finally the count at the end of 70th day of refrigeration temperature storage was recorded as 3 log compared to initial count of 9.75 in total lactic acid bacterial count. Lactococci, streptococci, leuconostoc and lactobacilli behaved similarly with regard to viability in DVS Dahi cultures stored at refrigeration temperature. The reduction in viability ranged between 3-3.8 log numbers at the end of 70th day of storage.

Reports regarding the effect of storage temperature on viability of DVS Dahi culture are scanty. A report on use of DVS culture in increasing shelf life of product is given by Snyatkovskii et.al.(2004), who found extended shelf life in yoghurt, kefir and Tvorog (Russian Fermented milk product) which incorporated DVS starter cultures from chr.Hansen.

SUMMARY

VI. SUMMARY

Starters are the group of active and desirable microorganisms capable of bringing about desirable changes in the milk product through the process of fermentation. These are carefully selected microorganisms that are deliberately added to milk to initiate and accomplish carry out the desired fermentation in the production of fermented milk products. In other words starters bring about the specific changes in the appearance, body and texture. The traditional method for the production of starters is through liquid culture that involves various stages such as preparation of stock, mother and working cultures and finally bulk culture. This method of propagation is time consuming and labour oriented. Over the past 10-15 years, Direct Vat Set (DVS) cultures, are increasingly being used in order to overcome the problems encountered in liquid starters, particularly in small plants, to replace bulk starter in cheese manufacture. DVS can be used directly in fermentation vats to produce fermented milk products.

The present investigation was aimed at the production of Direct Vat Set Dahi cultures by using characterized lactic acid bacteria obtained from domestic and market Dahi samples, by screening of the isolates for their activity in skim milk, their growth phase on black gram dhal as solid state medium was studied and further incorporation as Dahi inoculum for the preparation of Dahi and finally storage stability of DVS culture at ambient and refrigeration temperature, was determined.

6.1 Dahi samples of 5 each were collected from the domestic and local markets of Bengaluru. The brands of the market Dahi included Nandini premium, Nandini, Nilgiris, Nestle and Britannia. Samples collected were serially diluted and

enumerated for total lactic acid bacterial count, lactococci, streptococci, leuconostoc and lactobacilli counts using Yeast Glucose Agar, Neutral Red Chalk Lactose Agar, selection by lab pasteurization of first dilution and plated using Yeast Glucose Agar, Sucrose Agar, and Rogosa Agar respectively.

- 6.2 The domestic Dahi samples contained total lactic acid bacterial count that ranged from 6.32 to 7.04 $\log_{10}\text{cfu/g}$ on YGA, lactococcal count ranged from 4.22 to 6.38 $\log_{10}\text{cfu/g}$ on NRCLA, leuconostoc count on Sucrose Agar ranged from 0 to 2.48, while lactobacilli count ranged between 0 to 5.50 $\log_{10}\text{cfu/g}$ on Rogosa Agar. None of domestic samples showed the presence of *Streptococcus thermophilus*. Samples D₃ and D₄ showed more count of total lactic acid bacteria 7.04 $\log_{10}\text{cfu/g}$. none of the samples showed all the lactic counts.
- 6.3 The Market samples contained total lactic acid bacterial count ranged from 6.41 to 6.80 $\log_{10}\text{cfu/g}$ on YGA, lactococcal count ranged from 0 to 6.45 $\log_{10}\text{cfu/g}$ on NRCLA, streptococcal count on YGA selected by lab pasteurization was between 0 and 4.70 $\log_{10}\text{cfu/g}$ leuconostoc count ranged from 0 to 4.50 $\log_{10}\text{cfu/g}$ on Sucrose Agar, while lactobacilli count ranged between 0 to 4.66 on Rogosa Agar. Market sample D₉ of Nestle brand showed highest total lactic acid bacterial count of 6.80 $\log_{10}\text{cfu/g}$. Higher counts of lactococci, leuconostoc and lactobacilli were noticed in D₇ (Nandini market sample). D₉ dahi sample from Nestle showed higher *Streptococcus thermophilus* count. D₁₀ sample from Britannia showed all the lactic counts.

- 6.4 Total number of 72 lactic acid bacterial isolates were obtained from the countable plate of Dahi samples. Preliminary tests such as simple staining, Gram's staining, catalase test and litmus milk reaction identified the lactic isolates upto genus level and series of biochemical tests were carried out for species level identification. Among 72 isolates, 9 were lactococci, out of them 7 were *L.lactis ssp. lactis* and 2 were *L.lactis ssp. cremoris*. *S. thermophilus* isolated accounted for 7 numbers, 18 isolates were leuconostoc among them 4 were *Leuconostoc lactis*, remaining 14 could not be speciated as the biochemical test results did not match with identification key developed for *Leuconostoc* spp. Lactobacilli isolates of 38 numbers were characterized as *L.fermentum* (22 numbers), *L. hilgardii* (10 numbers) and *L. acidophilus* (6 numbers).
- 6.5 All the identified isolates were screened for their activity in sterile skim milk. In lactococci *L.lactis ssp. lactis* (Lc1, Lc2) were selected for further study because they showed highest DMC of 8.31 and 8.20 log₁₀/g with 24 hrs of setting time respectively. In *S.thermophilus* St1, St3 were selected for study that showed highest DMC of 8.67 and 8.69 log₁₀/g with setting time of 18 hrs. Among leuconostoc isolates, *Leuconostoc lactis* Leu1 and *Leuconostoc* sp. Leu6 were selected because of highest DMC of 8.76 and 8.90 log₁₀/g. *L. fermentum* Lb8, Lb35 were selected that showed DMC of 8.77 and 8.91 log₁₀/g with setting time of 18 hrs respectively.
- 6.6 Growth study of *L.lactis ssp. lactis* Lc1 and Lc2, *Streptococcus thermophilus* St1 and St3, *L.lactis* Leu1, *Leuconostoc* sp. Leu6 and *L.fermentum* Lb8 and Lb35 in skim milk were carried by inoculating 1% of culture and for every 6 hrs of interval

upto 24 hrs, the samples were drawn for determining DMC and titratable acidity. *L.lactis ssp.lactis* Lc1 showed highest DMC of 8.43 log₁₀/g at 24 hrs. *S. thermophilus* St3 showed highest DMC of 8.76 log₁₀/g with setting time of 18 hrs. *Leuconostoc* species Leu6 showed highest DMC of 8.57 log₁₀/g with setting of 18 hrs. *L. fermentum*, Lb8 showed highest count of 8.52 with setting time of 18 hrs. In all the selected lactic isolates higher titratable acidity was noticed at the period where cultures showed highest DMC.

6.7 *L.lactis ssp. lactis* Lc1 and Lc2, *Streptococcus thermophilus* St1 and St3, *Leuconostoc lactis* Leu1, *Leuconostoc* sp. Leu6, *L.fermentum* Lb8 and Lb35 were grown on black gram dhal as solid substrate medium at the optimum temperature of the isolates for 48 hrs. For every 6 hrs up to 48 hrs the samples were drawn for determining DMC. Highest DMC were observed in *L.lactis ssp.lactis* Lc1, *Streptococcus thermophilus* St3, *Leuconostoc* sp. Leu6 and *Lactobacillus fermentum* Lb8 with log count of 9.46, 9.45, 9.38 and 9.29 log₁₀ /g at 24 hrs of incubation respectively.

6.8 Mixed Dahi culture was prepared by combining selected lactic cultures at the ratio of 0.15 % : 0.3 % : 0.5% : 1 % = Leu6 (*Leuconostoc* sp.) : Lc1(*Lactococcus lactis ssp. lactis*) : Lb8 (*Lactobacillus fermentum*): St3 (*Streptococcus thermophilus*)as Set I and Leu1 (*Leuconostoc lactis*): Lc2 (*Lactococcus lactis ssp. lactis*) : Lb35 (*Lactobacillus fermentum*): St1(*Streptococcus thermophilus*) as Set II based on the DMC observation of dahi samples procured and inoculated into sterile homogenized skim milk and incubated at different temperatures. Set I set the milk

at 6 hrs of incubation while set II showed setting time of 8 hrs at 30⁰C. Set I was selected as mixed dahi culture as it showed shorter setting time for further studies.

- 6.9 Mixed Dahi culture was inoculated to sterile skim milk and incubated at 30⁰C to study the growth at every 6 hrs of interval upto 48 hrs. higher DMC of 8,68 log with 0.65% lactic acid was observed 24 hrs of incubation.
- 6.10 Mixed dahi culture was grown on sterile black gram dhal with 1% of skim milk powder, 10% of tomato juice and 70% moisture as solid substrate medium at 30⁰C for 48 hrs. DMC was highest at 24 hrs of incubation accounting for 9.42 log₁₀/g. Incubation period of 24 hrs was considered ideal for the growth of mixed dahi culture in order to obtain good biomass.
- 6.11 Solid state fermented (SSF) Dahi culture or DVS Dahi culture was prepared by inoculating liquid culture of 2% level on to black gram dhal medium as solid substrate and incubated at 30⁰C for 24 hrs as it yielded higher DMC, dried at 20⁰C in BOD incubator for 18 hrs. The total lactic count of dried DVS culture was 9.75 log₁₀/g.
- 6.12 Dahi was prepared by using 0.5% of DVS Dahi culture which was stored at refrigeration and ambient temperature and 2% of liquid Dahi culture. The study revealed that dahi prepared by using refrigerated DVS Dahi culture was best accepted by the panel of judges.
- 6.13 The effect of storage temperatures (ambient and refrigerated) on the viable count of DVS Dahi culture was studied by subjecting the ambient temperature stored DVS

culture for viability at every 3 days upto 60 days and similarly the viability of refrigerated stored DVS culture was determined at every 7 days upto 70 days. The total lactic count decreased to 2 log by 19th day in case of ambient temperature stored DVS culture while 2 log reduction in refrigerated stored DVS culture was noticed at 49th day compared to initial count. Viable lactic count of ambient temperature stored DVS culture decreased at faster rate compared to refrigerated DVS culture. Reduction of 4.5 log numbers in lactic count was noticed at the end of storage period (60 days) in ambient temperature stored DVS culture while the reduction in viability of lactic cultures was 3.5 log in refrigerated stored DVS culture. More reduction in the viability of leuconostoc was observed compared to lactococci, streptococci and lactobacilli in ambient temperature stored DVS culture. The viability of streptococci decreased more in case of refrigerated stored DVS culture compare to lactococci, leuconostoc and lactobacilli.

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ABSTRACT

VIII. ABSTRACT

Dahi is an important fermented milk product having heterogeneous lactic microflora which play a major role in human diet. Liquid culture is normally used in the preparation of Dahi where microbial contamination with sequential propagation of liquid culture are the noted limitations. Dried and frozen cultures show lower viability and in order to overcome these problems, Direct Vat Set cultures are slowly introduced in the commercial sector. The present study was carried out to develop Direct Vat Set Dahi cultures through solid state fermentation technique. Dahi samples of 5 each were collected from houses and local markets of Bengaluru. That showed total lactic bacterial viable count ranging from 5.36 to 7.04. Among 72 lactic isolates obtained, lactobacilli predominated (38) followed by leuconostoc (18); lactococci (9) and streptococci (7). The lactic cultures that set the milk with higher direct microscopic counts in sterile skim milk were *Leuconostoc* sp. Leu6: *L. lactis* ssp. *lactis* Lc1: *L. fermentum* Lb8: *S. thermophilus* St3 especially at the ratio of 0.15 %: 0.3 %: 0.5%: 1 %. The growth of mixed dahi culture on sterile black gram dhal with 1% skim milk powder, 10% tomato juice and 70% moisture as solid substrate medium showed 9.42 log₁₀cfu/g at 24 hrs of incubation. SSF Dahi culture or DVS Dahi culture at ambient and refrigeration temperature revealed viable count reduction in total lactic count by 4.5 log and 3.5 log by the end of 70 days and 60 days of storage respectively. Dahi prepared from refrigerated stored DVS culture scored better than Dahi prepared out of ambient temperature stored DVS culture.

APPENDIX

APPENDIX**SENSORY SCORE CARD OF DAHI**

Name of the judge:

Date:

Name of the product:

Judges are requested to assess the product in terms of general acceptability on a 9 point hedonic scale.

Score system:

| | |
|--------------------------|---|
| Like extremely | 9 |
| Like very much | 8 |
| Like moderately | 7 |
| Like slightly | 6 |
| Neither like nor dislike | 5 |
| Dislike slightly | 4 |
| Dislike moderately | 3 |
| Dislike very much | 2 |
| Dislike extremely | 1 |

| Characteristics | Sample code | | | |
|--------------------------|--------------------|--|--|--|
| Colour and acceptability | | | | |
| Body and texture | | | | |
| Flavour | | | | |
| Overall acceptability | | | | |

Remarks if any:

Signature