STUDIES ON STRESS RESPONSE IN THERMOPHILIC STARTERS

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

BY

PRANGYA PARAMITA TRIPATHY

DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL - 132001 (HARYANA), INDIA

2006

Regn. No. 101010

Dedicated to
My Beloved Parents,
Life Partner,
Family
'n'
ALMIGHTY

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This is to certify that the thesis entitled, **"Studies on Stress Response in Thermophilic Starters"** submitted by **Prangya Paramita Tripathy** towards the partial fulfillment of the award of the degree of **Doctor of Philosophy in Dairy Microbiology** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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Major Advisor & Chairman
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(Prangya Paramita Tripathy)

तापजयी जामनों में तनावाधीन प्रतिक्रिया पर अध्ययन

सारांश

डेरी जामनों की नस्ल सुधार पर अन्वेषण हेतु 36 तापजयी जामनों के अति अम्लीय तथा शीत तनाव परिस्थितियों में उनके किण्विक व्यवहार की पड़ताल की गई। इनमें से तीन यथा- *लैक्टोबैसिल्लस डेलब्रुक्काई* संतति *बलगैरीकस* एन सी डी सी 307, *एल. एसिडोफिलस* एन सी डी सी 14 और *स्ट्रैप्टोकोक्कस थर्मोफिलस* एन सी डी सी 74 उनके निम्न पी हेच एवं निम्न ताप पर पनपने की प्रक्रियाओं के कारण चुना गया। एन टी जी उपचार तथा पराबैंगनी विकिरण से वांछित उत्परिवर्ति प्राप्त नहीं हुए। संवर्धांश की प्रारम्भिक पी हेच तथा तापमान में क्रमिक कमी लाने से कम पी हेच (4.3 पर *एल. डेलब्रुक्काई* प्रजाति *बलगैरीकस* के $^{4.3}$ पी हेच $_{307}$ व 4.0 पर *एल. एसिडोफिलस* के $^{4.0}$ पी हेच $_{14}$) तथा कम तापमान, 12^0 से. पर *एस. थर्मोफिलस* के ^{12}टी $_{74}$ नामक स्थाई उत्परिवर्ति प्राप्त हुए । ये उत्परिवर्ति सम्बद्ध अति अम्लीयता तथा कम तापमान पर पनपे तथा इन्होंने दुग्ध उत्पाद में अम्ल व अन्य विशेषताएं भी पैदा कीं। प्रकाश तथा पटेक्षण इलेक्ट्रान सूक्ष्मदर्शी द्वारा लैक्टोबैसिल्लसों में कोशिका आकार में कमी पाई गई तथा वृत्त की लम्बी कड़ियां टूट कर गुच्छे बन गए। तीनों उत्परिवर्तियों की प्रशीतन अवशीतन प्रक्रिया के चार चक्रों के उपरांत जीवित रहने की क्षमता में क्रमश: 20, 24 व 65 गुणा वृद्धि हुई। उत्परिवर्ति $^{4.0}$ पी हेच $_{14}$ अत्याधिक 2% लवण और 1.0 पी हेच पर भी मूल संवर्ध की अपेक्षा जीवित रहा। तीनों उत्परिवर्तियों ने अधिक प्रोटीन विखंडन व अम्ल उत्पादन क्षमता का प्रदर्शन किया। इस से नब्बे सहस्र डाल्टन के व्यूहाणुभार क्रम में अधिक प्रोटीन उत्पादन देखा गया। मूल तथा उत्परिवर्ति कोशिकाओं से प्राप्त प्ररसकों (प्रोटोप्लास्टस) के संयोग से बने पांच युतकोशा प्राप्त हुए। इन युत कोशिकाओं ने अधिक अम्ल व पित्त सहिष्णुता का प्रदर्शन किया जो एफ 3 $^{4.0}$ पी हेच $_{14}$-14 में सर्वाधिक थी। इस युतकोशा को योगर्ट में एक उपांगग के रूप में प्रयोग किया गया। पंद्रह दिन के संभरण पर इसकी जीवित कोशिकाओं की गिनती में केवल एक छेदा (लॉग) क्रम की कमी आई। एन सी डी सी 74 और उसकी 2, 4 व 6 घण्टे तक शीताघात की हुई कोशिकाओं की द्विविमा शिलषक (2D Gel) विद्युत संचरण करने पर 36 विभिन्न दमनित, प्रेरित और नई प्रोटीनों के चिन्ह प्राप्त हुए। दो विशिष्ट शीत अघात सम्बंधी चिन्ह देखे गए। चिन्ह क्रमांक 1 नई (12सहस्त्रडाल्टन, पी आइ4.1) तथा चिन्ह क्रमांक 3 प्रेरित (8सहस्त्रडाल्टन, पी आई 4.1) प्रोटीन थी।

Studies on Stress Response in Thermophilic Starters

ABSTRACT

To improve LAB for better biotechnological application 36 thermophilic cultures were studied for their fermentation behaviour under extreme acid and cold stress conditions. Three promising organisms, *L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14 and *S. thermophilus* NCDC 74 were selected based on their low pH (lactobacilli) and low temp (streptococci) growth kinetics. NTG treatment and UV irradiation did not produce desirable mutants. Gradual decrease in incubation temp and initial medium pH resulted in stable adapts (low pH 4.3 for strain NCDC 307, $^{4.3}pH_{307}$ and 4.0 for NCDC 14, $^{4.0}pH_{14}$ and low temp 12 ^{0}C for NCDC 74, $^{12}T_{74}$). These adapts grew at respective high acidity and low temp, produced acid and other attributes in the product. Light and scanning electron microscopy revealed loss of *Lactobacillus* cell size and chain breakage and clump formation in *Streptococcus*. Freeze thaw survivability increased by 20, 24 and 65 folds in $^{4.3}pH_{307}$, $^{4.0}pH_{14}$ and $^{12}T_{74}$ adapts respectively after four cycles. $^{4.0}pH_{14}$ adapts tolerated 2% bile salts and survived pH 1.0 more than the parents. All the three adapts exhibited increased proteolytic activity and acid production. Protein production increased in the 10 KDa to 90 KDa MW range. Genome shuffling by protoplast fusion resulted in five fusants, viz., $F1^{12}T_{74}$-2, $F1^{12}T_{74}$-13, $F1^{4.3}pH_{307}$-1, $F3^{4.0}pH_{14}$-6 and $F3^{4.0}pH_{14}$ -14 exhibiting high acid and bile tolerance; $F3^{4.0}pH_{14}$-14 gave the highest. This fusant was used as an adjunct in yoghurt. Its viable counts decreased only by one log during 15d storage. Strain NCDC 74 showed 36 repressed, induced or new proteins at 20^{0}C for 2, 4 and 6h by 2D gel electrophoresis. Of these two cold shock proteins (CSP) occurred as Spot 1 (new, 12 kDa, pI 4.1) and Spot 3 (induced, 8 kDa, pI 4.1) by peptide mass finger printing (PMF) technique.

LIST OF ABBREVIATIONS

ATR	=	Acid tolerance response
APS	=	Ammonium per sulphate
~	=	approximately
AU	=	Arbitrary Unit
Å	=	Aungstrom
cfu	=	colony forming units
Conc.	=	concentration
d	=	days
^{0}C	=	degree celsius
dil	=	dilution
dist	=	distilled
EDTA	=	Ethylenediaminetetraacetic acid
h	=	hours
IPG	=	Immobilized pH gradient strips
IAM	=	Iodoacetamide
IEF	=	Isoelectric focussing
LCMS	=	Liquid chromatography mass spectrometry
MALDI	=	matrix assisted laser desorption ionisation
µg	=	microgram
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
min	=	minute
NaCl	=	sodium chloride
NCDC	=	National Collection of Dairy Culture
NSS	=	Normal saline solution
OD	=	Optical density
OD_{650}	=	Optical density at 650nm
PAGE	=	Polyacrylamide gel electrophoresis
PMF	=	Peptide Mass fingerprinting
rpm	=	revolutions per minute
SDS	=	Sodium dodecyl sulphate
Sec	=	Second
Sol	=	solution
TEMED	=	Tetramethyl ethylene diamine
Temp	=	temperature
TFA	=	trifluoroacetic acid
TOF	=	time of flight
UTR	=	Untranslated region
VH	=	volthour
vol	=	volume

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Introduction

1.0 INTRODUCTION

Lactic acid bacteria play an instrumental role as starters in fermented dairy foods. These organisms constitute a heterogeneous group of microbes, that normally includes members of the genus *Lactococcus, Lactobacillus,* and *Streptococcus*. Many species of LAB are used in combination as essential biocatalytic agents to carry out biochemical reactions under a variety of controlled conditions that result in formation of enormous fermented dairy foods. When present in such foods, LAB function as biopreservatives, preventing microbial spoilage and control pathogenic microorganisms through acidification, competition for essential nutrients, and/or production of a variety of other inhibitory metabolites. They also play an important role in health maintainance and health promotion by contributing in the production of functional foods with a production of various bioactive peptides (e.g. ACE-inhibitory, Immunomodulating, Antimicrobial bioactive peptides etc.). These organisms have been given the 'generally regarded as safe' (GRAS) status because of their long history in human nutrition. Based on their optimum growth temperature, LAB starters are categorised as mesophilic and thermophilic. Thermophlilic lactic acid bacteria (TLAB) have traditionally been used for the manufacture of yoghurt and hard 'cooked' cheeses (Emmental, Gruyere, Parmigiano, Grana etc.), which all require an incubation of the milk at a relatively high temperature (40 ^{0}C or above) during their production process. Dahi produced in India during summer season is also a product primarily of TLAB. However, majority of commonly used LAB strains are not able to grow or survive at such high temperatures and those that are mainly used as thermophilic lactic dairy starters belong to the species, viz.,

Streptococcus thermophilus, *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or *lactis*.

The industrialization of food bio-transformations increased the economic importance of lactic acid bacteria, as they play a crucial role in the

development of the organoleptique and hygienic quality of fermented milk products. Therefore, the milk reliability of starter strains not only in terms of quality and functional properties (important for the development of aroma and texture) but also in terms of growth performance and robustness has become essential. Fermentation, the oldest and most sophisticated application of biocatalysis, represents a $30-50 billion world market. Every food industry including fermented foods is directly concerned with the physiologic state of microbial populations. The physiological state again is highly influenced by a variety of harsh conditions imposed on the organisms by industrial processes. For example, during the pre-production, production and post-production phases of dairy foods by microbial fermentations, LAB cultures get subjected to various stresses originating from both biotic and abiotic factors. Stress in TLAB may be induced by lyophilization or concentration, extreme temperature (heat, cold), salt (NaCl) treatment, rising lactic acid concentration with concomitant pH shift and nutrient deprivation (Somkuti and Steinberg, 1999). Besides the above factors, quality of milk also affects the bacterial growth. The residual chemicals, viz., detergents, antibiotics, xenobiotics and toxic microbial metabolites may also cause stress on the TLAB affecting their activity adversely (NDRI Annual Report, 1997-1998). These and other types of stress induced by production parameters may have deleterious effect on the survival of TLAB cultures and their biochemical performance at the desired levels. However, an important factor resulting in poor survival of LAB appears to be lactic acid produced during fermentation and storage. The survival rate decreases in most of the starters due to low pH, that affects the cellular metabolism and causes injury to the cells. Acid stress results in self-limiting the growth as a consequence of which further acid production is reduced (Lim *et al.*, 2001; Beales, 2004). This has serious repercussions in factory schedule and may cause huge economic losses. Hence the stress responses of LAB are of interest being of practical relevance.

The stress response studies have numerous potential applications in industry. For example

i) survival during long-term storage could be improved by modifying growth conditions during starter preparation such that cells are pre-adapted for the storage stress.

ii) selection procedure can be developed to isolate stress-resistant or multi-stress-resistant strains.

iii) the same approach could serve or be applied as a reference model for other dairy starter bacteria to develop their stress-resistant strains.

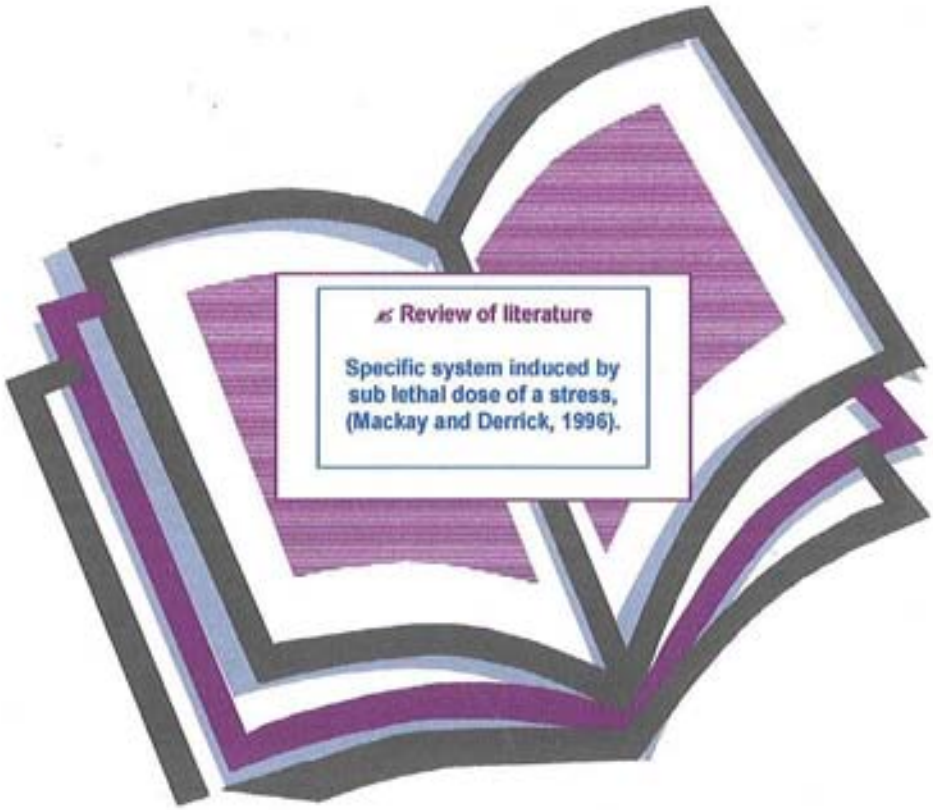
iv) it could be of interest to improve the viability of LAB in the final product, and thereby improve their potential probiotic effects.

v) stress response studies also play a significant role in quality assurance, as these robust strains survive and remain metabolically active under harsh conditions, thus inhibiting the growth of pathogenic organisms.

To assess the stress response in these organisms several approaches have been proposed. The genomic and transcriptomic approaches help to identify stress-related genes and their regulatory networks, whereas proteomic study enables us to know the expression at functional level. In addition to it, growth kinetics and morphological observations help to improve our knowledge regarding their behaviour. Therefore a combinatorial approach is an urgent need for better understanding the behaviour/adaptation mechanisms of lactic acid bacteria under harsh condtions (Tripathy *et al.*, 2004). While genes implicated in stress responses are numerous in LAB, the levels of characterization of their actual role and regulation differ widely between species. The functional observations of several stress proteins (for example, HS proteins, Csp, etc.) and of some of their regulators (for example, HrcA, CtsR) unveil even more striking differences that exist between LAB and classical model microorganisms. As in other bacteria, adaptive responses appear to be a usual mode of stress protection in LAB. A better understanding of the mechanisms of stress resistance should allow to understand the bases of the adaptive responses and cross-protection, and to rationalize their exploitation to prepare LAB for use in industrial processes (Guchte *et al.*, 2002).

Knowledge of the genetics of stress response in mesophiles e.g. *Lactococcus lactis* is already highly developed, but the reports dealing with TLAB have only appeared recently and research is mainly directed towards the cold- and acid- stress responses. Since understanding the mechanism of stress response may lead to the development of cultures with their improved capacity to survive and function under industrial production conditions, extensive work on stress response phenomena in TLAB needs to be carried out. The proposed research project is an attempt on these lines and was undertaken with the following objectives:

1. To analyze the viability of thermophilic lactic dairy starters under different stress conditions.
2. To identify plasmid(s) and protein(s) responsible for their stress tolerance.
3. To develop potential stable lactic strains by exploring natural gene transfer systems.



Review of Literature

2.0 REVIEW OF LITERATURE

Bacteria display an amazing capacity to survive and grow in extremely hostile environments. Entire group of organisms have adapted their life-styles to prefer these extreme environments (e.g. thermophiles, halophiles, acidophiles etc.). Even within a group, a very wide range of environmental parameters may be tolerated. For most, the tolerance can be pushed to maximum limits if the cell is provided sufficient opportunity to sense and adapt to a deteriorating condition.

Lactic acid bacteria (LAB) play an important role in the food industry because of their widespread application as starter cultures in many fermentation processes. The most commonly used thermophilic starters belong to three species, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subspecies *bulgaricus*. The thermophilic lactic acid bacteria (TLAB) used in the dairy industry are selected for their flavor, texture and acidification properties. During industrial processes TLAB are subject to stresses such as alteration in temperature, pH shifts, and stresses also occur during preservation, starvation, freezing and lyophilization. Hence the stress responses of TLAB are of interest and practical relevance. The genetic and physiological stress responses of the thermophilic lactic acid bacteria thus far have hardly been studied. As the knowledge of the mechanisms of resistance to stress could be used to improve the technology of starters and development of certain products (Guzzo *et al.*, 1994), it would be interesting to carry out an in depth study on stress responses of TLAB to develop stress tolerant strains.

2.1 SURVIVAL OF MICROORGANISMS UNDER DIFFERENT STRESS CONDITIONS

Bacteria live in habitats of frequently changing conditions and have evolved very sophisticated responses to adapt to environmental charges. The resistance encoded by defense systems can be divided into two classes: the

first comprises a specific system induced by a sub lethal dose of a chemical or physical stress, that permits survival against a challenge dose of the same agent (Mackay and Derrick, 1996). The second class of resistance comprises general systems, which prepare cells to survive against many different environmental stresses, without the need for cultures to have had prior exposure to particular stress (Flahaut *et al.*, 1996, Glaasker *et al.*, 1998; Lorca *et al.*, 1999; Pichereau *et al.*, 2000) (Fig 2.1).



Fig 2.1: Stress systems in Microorganisms

Both these systems have intensively been studied in several microorganisms under various stress conditions including some of the lactic acid bacteria. The literature pertinent to the subject is reviewed below.

2.1.1 Specific Systems/Phenotypic adaptation

Pretreatment of cells with mild stress exposure induces higher resistance to severe and lethal stress and the phenomenon is known as tolerance, phenotypic adaptation or habituation.

This phenomenon has been observed for various microorganisms under different stress conditions discussed below.

2.1.1.1 Cold- and heat-shock stress in LAB

Various degrees of freezing temperature are used in many scientific or industrial processes for technologic strain storage. Cells can be injured during freezing by endocellular ice crystal formation or dehydration, which

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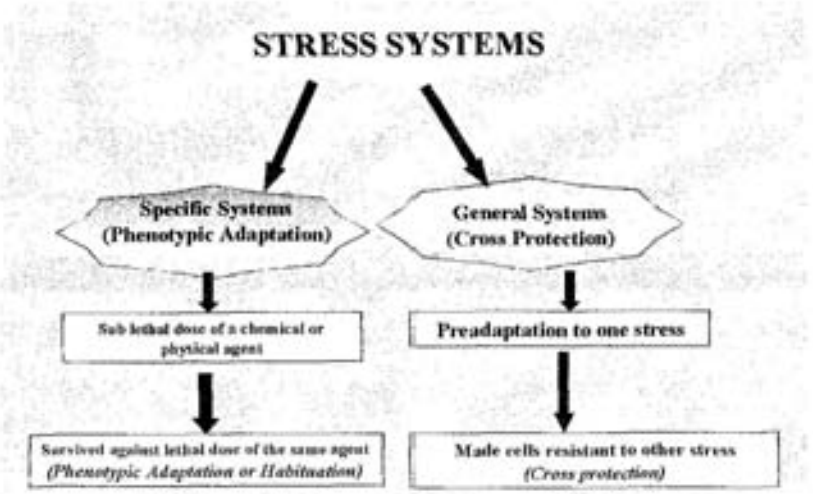


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corresponds to hypothermal and osmotic, stresses respectively (Mazur, 1984). Different types of chemical or physical pretreatment including sublethal stresses can generate cryotolerance. Cold pre-incubation at sub-optimal temperature has been shown to induce bacteria to adapt to freezing stress (Thammavong *et al.*, 1996; Panoff *et al.*, 1995). In this context Panoff *et al.* (1995) have reported the survival of *Lactococcus lactis* at low temperature. According to him survival of bacteria first grown at 30 ^{0}C to stationary phase and subsequently incubated at 4 ^{0}C for 28 days was 30 % whereas nearly 0.03 % of the culture survived when held at 30 ^{0}C throughout the experiment. Similarly, survival of their freezing thawing treatment was better (95 %) subsequent to preincubation at 8 ^{0}C for 48 h than without such adaptation which resulted in 75 % of surviving cells.

Low temperature adaptation is highly relevant from a practical point of view, since many LAB fermentations are initiated by the addition of frozen starter culture that should benefit from a high freeze thaw survival capacity. The post fermentation acidification taking place of low temperature in the synergistic yogurt fermentations of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* is a well-known, undesired property. This results in a product that contains too much lactic acid and is therefore unfit for consumption (de Vos, 1996).

Understanding the cold adaptation of *S. thermophilus* could provide the basis for targeted strain improvement to overcome post processing acidification and to increase the number of viable cells after freezing. In this connection, Wouters *et al.* (1999) reported that *S. thermophilus* adapted to freezing during a pretreatment at 20 ^{0}C thus resulting in approximately 1000 fold increased survival after four freeze thaw cycles compared to mid exponential phase cells grown at an optimal temperature of 42 ^{0}C.

From the foregoing review it may be inferred that, despite the importance of TLAB in food fermentations, only countable reports are available regarding the cold adaptation studies. Therefore further investigation needs to be carried out regarding the low temperature adaptation in this section of microorganisms.

Bifidobacteria are well known for their importance in infant gastrointestinal ecology. Understanding the basic stress responses at molecular level in these organisms is of current interest. With this aim, Schimdt and Zink (2000) recorded the specific stress response phenomena in *Bifidobacteria adolescentis* NCC251. The organism showed about 24- and 128- fold higher thermo tolerance at 55 ^{0}C for 10 and 20 mins respectively after prior subjecting the cells to adaptation at 47 ^{0}C for 15 mins (Fig 2.2).

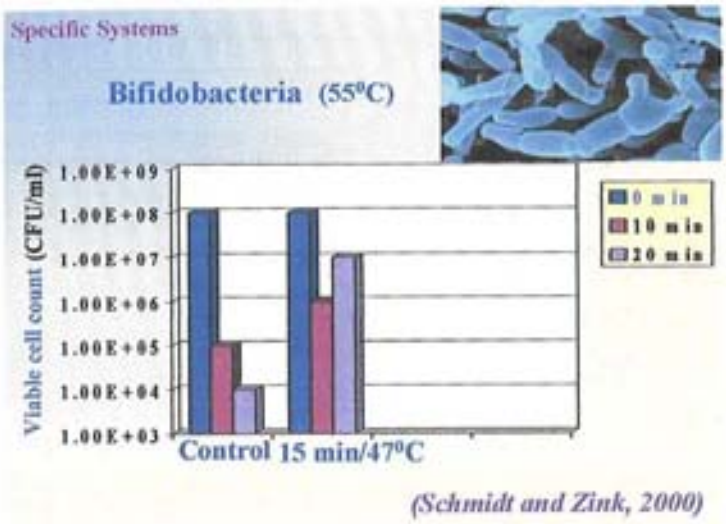


Fig 2.2: Specific stress system in *Bifidobacteria adolescentis* NCC251 (Schmidt and Zink, 2000)

A major challenge associated with the application of probiotic cultures in functional foods is the retention of viability during processing. Earlier it has been shown that the destruction of bacteria during heat stress and spray drying can not only be ascribed to a thermal effect, but also to a non-thermal drying effect caused by the loss of bound water at the cell surface (Daemen and vander Stege, 1982). To address this problem, the potential of environmental adaptation as a survival mechanism enabling a human derived probiotic *Lactobacillus* strain to withstand heat stress and spray drying was recently investigated by Desmond *et al.*, (2002). Heat adapted (52 ^{0}C for 15 min) *L. paracasei* NFBC exhibited 300- and 700- fold greater thermo tolerance (survival at a lethal temperature of 60 ^{0}C) compared with controls, in MRS broth and reconstituted skim milk (RSM) respectively.

2.1.1.2 Cold and heat-shock stress other than LAB

In recent years, modifications in the brewing process have induced the unusual emergence of strictly anaerobic bacteria including *Pectinatus*, which is frequently encountered. This gram-negative, beer spoilage bacterium causes due to sulphuric compounds and volatile acids (Haikara *et al.*, 1981; Haikara, 1991). Under more drastic environmental conditions, bacterial cells can also change their physiological state and concomitantly become more resistant against severe lethal stress. Boiling of wort and pasteurization of beer are common technologies to prevent microbial contamination. With this aim, heat resistance in three strains of *Pectinatus* has been studied (Watier *et al.*, 1995). In another interesting report by Flahaut *et al.* (2000) compared between two species of *Pectinatus*. They observed that the thermal adaptation for a period of one hour at 40 ^{0}C *(P. cerevisiiphilus)* and that of 41 ^{0}C *(P. frisingensis)*, induced tolerance against heat challenge (49 and 50 ^{0}C for *P. cerevisiiphilus* and *P. frisingensis* respectively). Survival of *P. cerevisiiphilus* and *P. frisingensis* adapted cells was respectively; 3400- and 790- fold higher than control.

Enterococcus faecalis is a natural member of the human and animal intestinal flora and therefore is present in high concentration in feces and largely expelled into the external environment. Able to survive a wide range of pH, heating and high salt concentrations, *Enterococcus faecalis* copes with moderate stress conditions and can be recovered in water and food products. This bacterium is an indicator of fecal contamination in water and its detection can be used to indicate the presence of pathogenic bacteria (Ostrolenk *et al.*, 1947; Burton, 1949). The study of *Enterococcus faecalis* response to several individual stresses was undertaken to elucidate as to how this bacterium could survive in harsh conditions and cope with some processes used to control food borne pathogens and to characterize its adaptive response to nutritional and physicochemical fluctuations (Rince *et al.*, 2000). It was observed that an adaptation at 50 ^{0}C induced a 6000-fold increase of heat tolerance at 62 ^{0}C in *Enterococcus faecalis* ATCC19433.

2.1.1.3 Osmotic Stress

In general living organisms are adapted to function in a rather limited set of physiological conditions, and significant deviations will immediately trigger stress mechanisms. One such environmental factor, which has often been overlooked, is humidity or water concentration. An environment of high solute concentration is always low in water activity, and as the cytoplasmic membrane is freely permeable to water. this situation will lead to subsequent dehydration and cessation of growth unless the organism has the means to adapt and respond to such a low water environment. Generally, the internal osmotic pressure in bacterial cells is greater than that of the surrounding environment and so pressure is exerted outward on the cell wall; this is called turgor pressure (Gutierrez and others 1995). Therefore, to survive variations in osmotic pressure of the surrounding medium, the bacterial cells must maintain turgor. If the a_w of the environment is reduced, the cells would lose water to ensure that restoration of the osmotic equilibrium across the cell occurred. If the reduced aw is due to the increase in solutes unable to be transported across the cell membrane, then a temporary loss in turgor pressure would occur. The bacteria then raise their internal solute levels (compatible solutes), resulting in an increase in internal osmotic pressure and restoration of turgor pressure (Figure 2.3). This is termed osmoregulation (Gutierrez et. al., 1995). Therefore, osmoregulation is initiated after the reduction in turgor of the cell following loss of cell water or an increase in the external solute concentration that cannot cross the membrane.

At high osmolarities, bacteria have evolved to accumulate high cytoplasmic levels of a certain class of solutes (compatible solutes) that do not interfere too seriously with the functioning of cytoplasmic enzymes (Gutierrez and others 1995). The internally accumulated solutes allow continued activity of cytoplasmic enzymes at lower water activities (Booth and others 1994). Osmoregulation also regulates the activity of porins, transport proteins, and potassium in the cell membrane (Gould 1989). Bacterial compatible solutes are accumulated either by de novo biosynthesis

(endogenous osmolytes, such as glutamate, proline, ectoine, trehalose, and sucrose) or by uptake from the environment (exogenous osmolytes such as glycine betaine) (Csonka and Hanson, 1991). The compatible solutes produced internally are highly soluble, pH neutral, and are usually end product metabolites. They can include sugars from the breakdown of carbohydrates, amino acids from protein degradation, and cations such as K+. Examples include betaine, trehalose, glycerol, sucrose, proline, choline, carnitine,mannitol, glucitol, and ectoine. The cell membrane is selectively permeable to them, allowing the cytoplasmic pool to be determined by the external osmotic pressure (Galinski 1995; Gutierrez and others 1995). The preferred exogenous bacterial-compatible solutes are glycine betaine, which is found in higher plants, and the amino acid proline. In many foods, however, peptides are more readily available than free amino acids and hence peptides have become an important source of both nutrients and compatible solutes (Amezega and others 1995). Peptides have also been shown to play an important role in the adaptation of *L. monocytogenes* to osmotic stress (Amezega and others 1995).

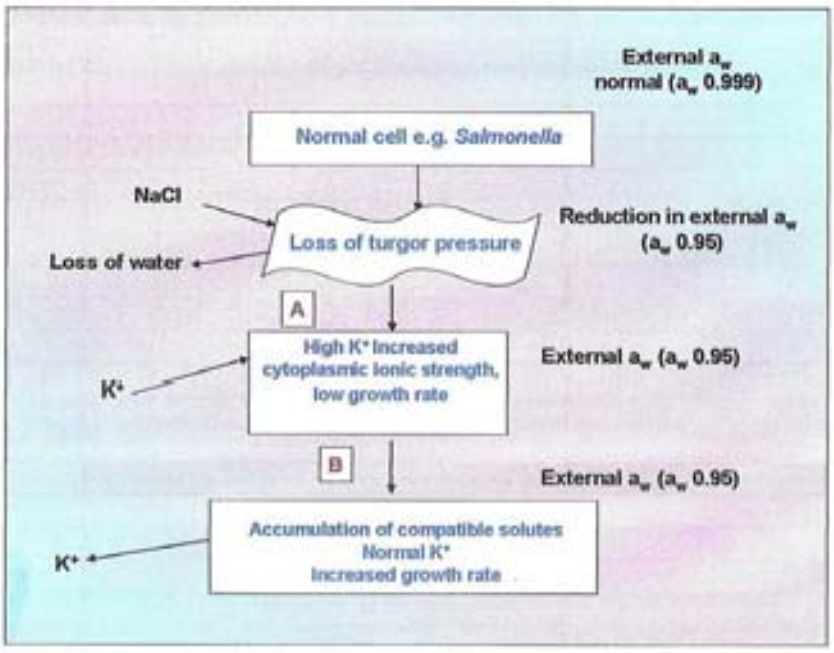


Fig 2.3: Osmoregulation: 'A' as the bacterium loses water, cytoplasmic level of K+ increases . 'B'. This triggers enzymes such as glutamate dehydrogenase to form glutamate from a ketoglutamate. As the glutamate levels increase, water starts to reenter the cell and growth resumes (**Beales, 2004**).

(endogenous osmolytes, such as glutamate, proline, ectoine, trehalose, and sucrose) or by uptake from the environment (exogenous osmolytes such as glycine betaine) (Csonka and Hanson, 1991). The compatible solutes produced internally are highly soluble, pH neutral, and are usually end product metabolites. They can include sugars from the breakdown of carbohydrates, amino acids from protein degradation, and cations such as K+. Examples include betaine, trehalose, glycerol, sucrose, proline, choline, carnitine,mannitol, glucitol, and ectoine. The cell membrane is selectively permeable to them, allowing the cytoplasmic pool to be determined by the external osmotic pressure (Galinski 1995; Gutierrez and others 1995). The preferred exogenous bacterial-compatible solutes are glycine betaine, which is found in higher plants, and the amino acid proline. In many foods, however, peptides are more readily available than free amino acids and hence peptides have become an important source of both nutrients and compatible solutes (Amezega and others 1995). Peptides have also been shown to play an important role in the adaptation of *L. monocytogenes* to osmotic stress (Amezega and others 1995).

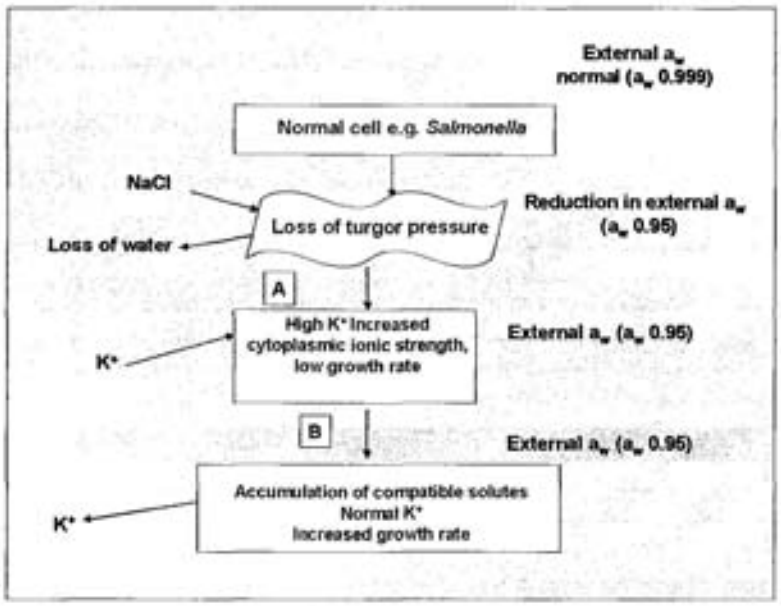


Fig 2.3: Osmoregulation: 'A' as the bacterium loses water, cytoplasmic level of K+ increases . 'B'. This triggers enzymes such as glutamate dehydrogenase to form glutamate from a ketoglutamate. As the glutamate levels increase, water starts to reenter the cell and growth resumes (**Beales, 2004**).

A most remarkable work by Cayley *et al.* (1991) has demonstrated with *Escherichia coli* as a model organism, that how osmotic changes in environment affect cytoplasmic volume and growth rate. Surprisingly, the amount of bound water in the cytoplasm was relatively independent of the osmolarity (0.4 ml/g dry weight), approximately 205 % of total water in fully hydrated cells. Therefore the immediate effect of high osmolarity was shown to be a reduction in the volume of free water. From their data they deduced a linear relationship between cytoplasmic volume and growth rate (Cayley *et al.*, 1991).

In recent years, several reports have become available regarding the osmotic stress response phenomenon in microorganisms. In one such experiment, the adaptation of *L. alimentarius* to environmental stresses was studied extensively by Lemay *et al.* (2000). When the organism was first grown in 8 % NaCl and then transferred to MRS broth containing 50 – 55 mM citric acid or 100 – 110 mM gluconic acid, growth was observed after one day (OD_{600} = 0.4 – 0.5) which increased 0.6 to 0.8 after three days. However, only limited growth was observed when the condition was reversed.

A preconditioning (e.g 30 min) of logarithmic cells with 0.1 % bile salts resulted in a 300 fold and 21 fold protection against 0.3 % and 0.4 % bile salts respectively in case of *Bifidobacterium* species (Schimdt and Zink, 2000).

In response to high osmolality, bacteria accumulate compatible solutes (betaine, proline), which restore turgor and stimulate growth (Csonka and Epstein, 1996). Membrane-embeded compatible solutes transporters have been extensively characterized at the genetic and biochemical levels in *Escherichia coli*, *Salmonella typhimurium* and in *Bacillus subtilis*. These transporters are activated upon osmotic shift (Poolman and Glaasker, 1998) but molecular mechanisms of their activation are still unchacterised. They are potentially sensitive to the modifications of membrane composition in response to osmotic stress. Such systems can be considered as osmosensors (Wood, 1999). Growth of *Lactococcus lactis* was shown to be inhibited at high salt concentrations, but growth under these conditions was stimulated considerably by the presence of betaine in the medium (Guillot *et al*, 2000).

Lactococcus lactis subsp. *cremoris* NCDO763 accumulate glycine-betaine (betaine) when submitted to an osmotic stress with NaCl. Betaine transport activity not only increased with the extent of the osmotic up shock, but also with growth temperature, and supplementation of the medium by Tween 80. Fatty acid analysis of the lipid fraction of the *Lactococcus lactis* NCDO763 revealed significant modification of the fatty acid composition of the membrane when cells are submitted to the osmotic stress, high temperature or Tween-80 medium supplementation. The main modification in *L.lactis* membrane fatty acid composition in response to high osmolality is the increase of Cyclopropane Fatty Acid (CFA) Δ C19:0, where as unsaturated/ saturated ratio remains unchanged (Guillot *et al,* 2000).

In another report, the behaviour and state of the cell envelopes from *Bacillus subtillis* cultures grown in Luria Bertani (LB) medium with and without 1.5 M NaCl were compared under hypertonic conditions, the hydrophobicity of the cultures was found to increase. The phospholipids and fatty acid composition showed important differences: a higher cardiolipin (CL) content [at the expense of Phosphatidyl glycerol (PG)], and a higher unsaturated and straight chain fatty acid content resulted (Lopez *et al,* 2000).

2.1.1.4 Low pH (Acid Stress)

A lesser-known yet extremely important adaptation involves the bacterial response to extremes of pH as an example, *Escherichia coli* and *Salmonella typhimurium* managed to grow in conditions (minimal medium) ranging between pH 5 and 8.5, which represents more than a 3000-fold range in H^+-ion concentration. These organisms can also survive from pH 4 to pH 9 (a 100,000-fold range) for extended periods of time. However, if the cells are first allowed to adapt to moderate acid or alkaline conditions, before testing their limits, one finds they can survive over a 1000,000-fold range of H^+-ion concentrations. This capability can be very important to organisms both in natural or pathogenic situations where pH conditions can fluctuate dramatically (Renberg *et al,* 1993).

2.1.1.4.1 *Salmonella typhimurium, E. coli* and *Shigella flexneri*

An inducible acidification tolerance response (ATR) was first identified in *S.typhimuriun* by Foster and Hall (1990). The basic discovery was that cells grown in minimal medium at pH 7.7 to pH 5.8 (a mild acid stress for one generation) enabled survival at an extreme acid challenge of pH 3.3.

The second pH dependent system is called stationary phase ATR (Lee *et al*, 1994). Stationary phase cells will induce a very effective acid tolerance when they are exposed to a pH below 4.5 for one or two hour. A stationary phase ATR effectively protects cells from pH 3.0 acid challenges for several hours.

In addition to these two pH dependent ATR systems, there is a pH independent, Rpo-S dependent stationary phase acid resistance mechanism that was observed in when compared in log phase versus stationary phase cells at pH 7 (Lee *et al.*, 1994).

In a separate study, a comparison of ATR of *E. coli* and *S. flexneri* revealed that *Shigella* had neither log phase nor stationary phase ATR systems and that *E. coli*, although it had a log phase system, was constitutively acid tolerant in stationary phase (Hall *et al*, 1995) (Fig 2.4).

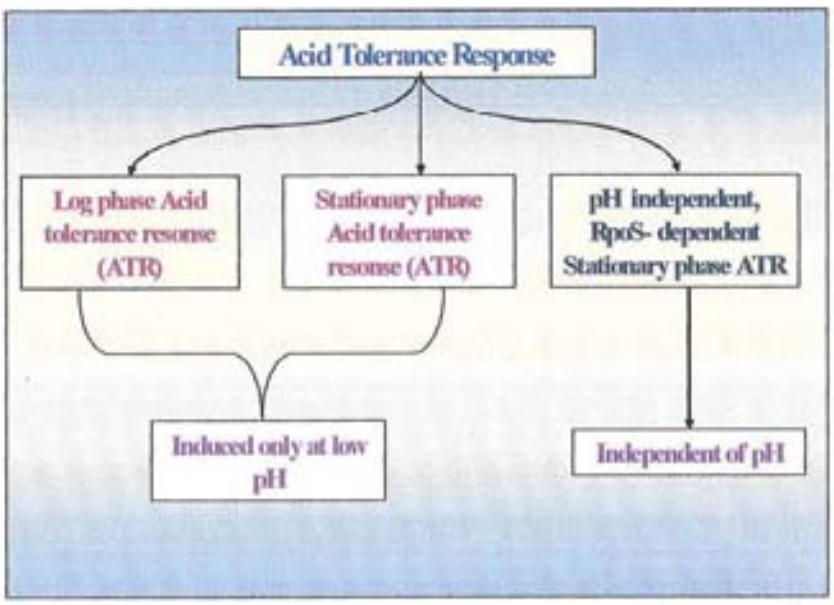


Fig 2.4: Classification of acid tolerance response (ATR) in bacteria

2.1.1.4.1 Salmonella typhimurium, E. coli and *Shigella flexneri*

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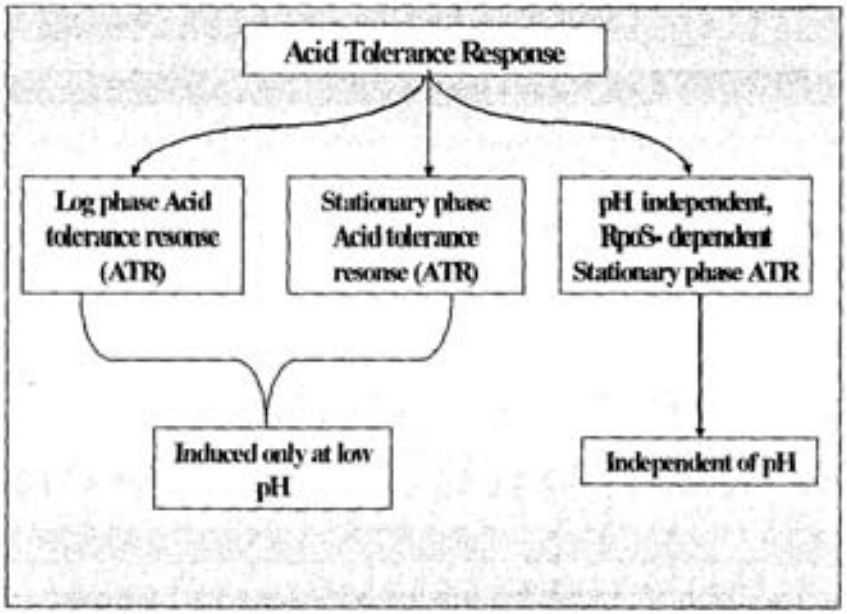


Fig 2.4: Classification of acid tolerance response (ATR) in bacteria

2.1.1.4.2 *Streptococcus mutans*

Adaptation to low pH by *S. mutans* was shown in that cells obtained from cultures grown in pH initial medium pH 5.0 exhibited a lower pH drop than did cells prepared from pH 7.0 cultures. This was due to a shift in the pH optima of glucose uptake and glycolysis as well as an increase in the F_1F_0 H^+-translocating ATPase (Belli and Marquis, 1991).

2.1.1.4.3 *Lactic acid bacteria*

Lactic acid bacteria are gram-positive bacteria, regarded as generally-regarded-as-safe (GRAS) status. They are used in food fermentations, ubiquitous gastric commensals, and as potentially beneficial probiotic bacteria. The acidic environments encountered in food and in the gastrointestinal tract provide a significant survival challenge for these diverse organisms. For example, the preferred delivery vehicles for probiotic cultures are yogurts and fermented milks, both of which present an acid challenge. Subsequently the bacteria will need to survive the highly acidic gastric juice if they are to reach the small intestine in a viable state (Lee and Selminen,1995; Henriksson *et al.*, 1999). Hence, acid tolerance is accepted as one of the desirable properties used to select potentially probiotic strains. Thus, it is timely to consider the mechanisms used by this group of gram-positive organisms to protect themselves from the challenge posed by low pH environments and comment on the strategies by which they can be improved. While reviewing the acid resistance mechanism in gram-positive bacteria, Cotter and Hill, (2003) suggested various mechanisms that are responsible for the survival of these organisms in acidic environments (Fig 2.5). These include F_1F_0-ATPase proton pump, a combination of constitutive and inducible strategies which result in the removal of protons (H^+), alkanization of the external environment, changes in the composition of the cell envelope, production of general shock proteins and chaperones, expression of transcriptional regulators, and responses to changes in cell density.These mechanisms counter the negative impact of a reduction in cytoplasmic pH,which can include loss of activity of the relatively acid-sensitive glycolytic enzymes

(which severely affects the ability to produce ATP) and structural damage to the cell membrane and macromolecules such as DNA and proteins.

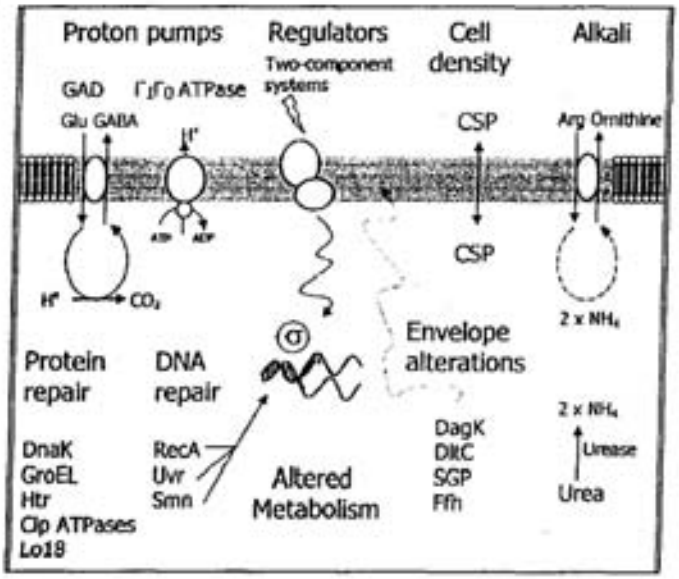


Fig. 2.5: Graphical presentation of the mechanisms of acid resistance available to gram-positive bacteria. (These have been divided into eight categories, and a number of examples are demonstrated. (i) Proton pumps such as the F1F0ATPase or that utilized by the GAD system bring about an increase in internal pH. (ii) Proton repair involving chaperones, proteases, and heat shock proteins results in the protection of proteins or their degradation if damaged. (iii) DNA damaged as a consequence of a low internal pH can be repaired through the excision of errors or the restarting of stalled replication forks. (iv) The involvement of regulators such as 2CSs and sigma factors can induce minor or global responses. (v) Cell density affects cell-to-cell communication. (vi) Cell envelope alterations can protect cells by changing architecture, composition, stability, and activity. (vii)The production of alkali by the ADI or urease system increases the internal pH of the cell. (viii) Metabolic properties can be altered) **(Cotter and Hill, 2003).**

The three main systems involved in lactococcal pH homeostasis are the ADI system, the H^+- ATPase proton pump, and the GAD system. LABs are also capable of inducing an ATR in response to mild-acid treatment (Hartke *et al.* 1996; O'Sullivan et al., 1997; Rallu *et al.*, 1996). The systems induced by this response include pH homeostatic, protection, and repair mechanisms. Acid adaptation of *Lactococcus lactis* subsp. *cremoris* strains MG1363 and 712 both require de novo protein synthesis (Rallu et. al., 1996), as does *Lactobacillus casei* (all oral LABs) (Ma *et al.*, 1997). In contrast, acid adaptation in *L. lactis* subsp. *lactis* strain IL 403 is reported to be independent of protein synthesis (Hartke *et al.*, 1996), although induction of UV-inducible proteins and heat shock proteins is observed. Heat shock proteins are also

induced following acid adaptation of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lim *et al.*, 2000).

Lactic acid bacteria produce lactic acid during fermentation processes. It is important to note that lactic acid is an weak undissociated organic acid and can cross the membrane. At cytoplasmic pH, it dissociates and, thus poses a stronger stress to cells at a given extracellular pH than (Fig 2.6), for example, hydrochloric acid (Kashket, 1987).

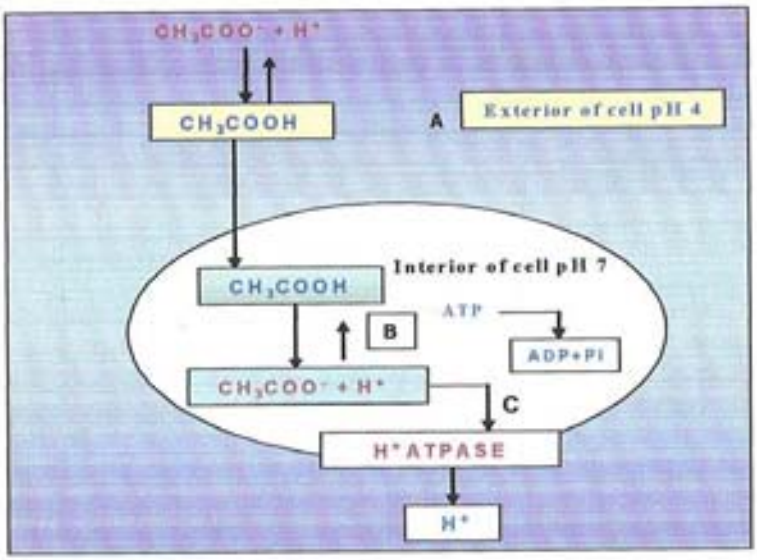


Fig.2.6: **Interaction of weak acids on the microbial cell**. A). Exterior of the cell favours undissociated weak acid; B). On entering the cell, the interior of the cytoplasmic pH favours dissociation of the acid and the acid dissociates, thus possess stronger stress to the cells; C). Proton pumps remove excess H^+ ions and use energy

L. lactis resisted pH 4.5 (with HCl) in minimal medium, but its viability rapidly decreased by incubation at pH 4.0. Cells could survive this low pH when adapted to a sub lethal pH 5.5 for only 15 min in a defined medium adjusted with both HCl and lactic acid (Hartke *et al.,* 1996). Acid adaptation was shown to be chloramphenicol sensitive in *L .lactis* subsp. *cremoris* MG1363 but chloramphenicol independent in *L. lactis* subsp. lactis IL1403 (Hartke *et al.,* 1996).

Working in the same line De Angelis *et al.* (2001) documented the acid stress response in *Lactobacillus sanfranciscensis* CB1 (an important sourdough lactic acid bacterium). The organism resisted low pH (up to 3.2

when adjusted with lactic acid and up to 3.4 when adjusted with HCl), after initial exposure of the cells to sub lethal acidic conditions (i.e. pH 5.0 for one hour).

The acid-stress response in thermophilic lactic acid bacteria is currently gaining attention. To this end very recently Lim *et al* (2001) recorded acid-adaptation phenomena in *Lactobacillus delbrueckii* subsp. *bulgaricus*. As per their finding, adapted cells (30 min incubation at pH 4.75) were ~250 fold more tolerant to lethal acid stress (30 min at pH 3.6) than non adapted ones. the acid tolerance response in lactic acid bacteria including TI AB have been demonstrated by several workers and presented in Table 2.1.

Table 2.1 Acid tolerance response in LAB

LAB	Adaptation period	Tolerance level	References
Lactococcus lactis	pH 5.5/15 min (HCl & Lactic acid)	pH 4.0	Hartke *et al.*, 1996
Lactobacillus sanfranciscensis CB1	pH 5.0/1h (HCl & Lactic acid)	pH 3.2, pH 3.4	De Angelis *et al.*, 2001
Lactobacillus delbrueckii subsp. *bulgaricus*	pH 4.75/30 min (HCl)	pH 3.6	Lim *et al.*, 2001

2.1.1.5 Starvation

Exhaustion of an essential nutrient limits growth of a culture, which then enters the stationary growth phase. A major cause of growth restriction in natural environments is starvation for one or several essential nutrients. Bacteria have developed different survival strategies. Some bacteria, such as *Bacillus* species or *Clostridium* species undergo major differentiation programmes leading to the formation of highly resistant endospores. The majority of bacterial genera however do not show such drastic morphological changes upon starvation. Stationary phase cells of the gram-negative bacterium *Escheichia coli* assume characteristic cell morphology (Hengge-Aronis, 1993). The Gram-positive bacterium *Staphylococcus aureus* showed a decrease in cell size after 25 days under glucose starvation (Watson *et al* 1998). In a recent study by Kim *et al.* (2001), the survival response of

Lactococcus lactis during long-term (2 year) glucose starvation revealed significant smaller cell size than the parent cells.

However cells of gram-positive bacterium *Enterococcus faecalis* do not significantly shrink during glucose starvation or incubation in an oligotrophic environment (Hartke *et al.*, 1998). Starved cells usually show a general stress response mechanism and will be reviewed later on.

2.1. 2 General Stress Response (Cross Protection)

Pretreatment with a mild acid stress makes cells resistant to a lethal challenge with the same stress condition, as has been discussed for a number of conditions. Moreover, preadaptation to one stress condition can render cells resistant to other stress-imposing conditions. This is of relevance for cells in environments where they can be exposed to combinations of stress conditions. The best example of such a multiple stress resistance was that obtained in stationary phase. Also exposure to UV-light ($100J/m^2$) conferred cross protection against a treatment with heat, acid, or ethanol and to a lesser extent, against H_2O_2 (Hartke *et al.*, 1995).

Pretreatment with low salt showed an effect on the induction of thermo tolerance for *Bacillus subtilis* but was significantly less effective than a preceding mild heat stress (Volker *et al.*, 1992). Bile salt and acid adapted *Enterococcus faecalis* was reported to show a better survival to thermal acid stress (Flahaut *et al.*, 1996). Cross-protection against heat was also induced by salt stress as demonstrated recently for *Bifidobacterium adolescentis* NCC251 and *Bifidobacterium longum* NCC481 (Schimdt and Zink, 2000). Lorca *et al.* (1999) reported the cross resistance of different kinds of environmental stress in *Lactobacillus acidophilus* CRL639.

The metabolic reprogramming leads to a cellular state of enhanced resistance to a greater number of stress conditions, compared to that in exponentially growing cells (Giard *et al.*,1996; Hartke *et al.*, 1998). For example, *E. faecalis* cells starved the energy source glucose developed strong cross protection against such diverse treatments as heat, ethanol, acid, osmotic, oxidative, sodium hypochlorite or bile salt stresses (Giard *et al.*,

1996). Under complete starvation established by incubation in tap water *Enterococcus faecalis* cells become additionally to the heat, acid and sodium hypochlorite stresses, significantly more resistant towards UV_{254}nm irradiation (Hartke *et al.*, 1998). Interestingly when *Lactococcus lactis* was kept under glucose starvation for two years, the resultant isolates showed a higher resistance against the bile salt and H_2O_2 stresses compared to the parent (Kim *et al.*, 2001).

Most pH-stress protection systems include a mechanism for sustaining cytoplasmic pH and many inducible systems offer cross-protection to other stresses. Acid-adapted cells of *Salmonella typhimurium* indicated increased resistance to heat and salt tolerances (Leyer and Johnson, 1993; Lee *et a* 1994). Very recently De Angelis *et al.* (2001) described the cross-protection phenomenon in acid-adapted cells of *Lactobacillus sanfranciscensis* CB1. The organism responded a greater survival rate in presence of 5 % NaCl and a temperature of 10 ^{0}C after initial exposure to sub lethal acidic conditions.

2.2 EFFECT OF ENVIRONMENTAL STRESS ON PLASMIDS OF LACTIC ACID BACTERIA

The ability of *Lactococci* to metabolize lactose, other sugars and citrate etc., is not a stable characteristics. Attempts in using commercially plasmid encoded properties of LAB attracted the attention towards the instability of these plasmids, a number of plasmid borne genes encoding important commercial characteristics of *Lactococci* have been cloned successfully (deVos, 1996).but the production of these plasmid encoded gene products and their utilization through bacterial cultures in fermented foods is mainly dependent on the stability of plasmids under varied fermentation conditions which further depends on a number of factors. Physiological as well as genetic factors can alter the ability of a host to retain a plasmid (Primrose *et al.*, 1984; Wouters *et al.*, 2000). So, various methods to determine the instability of plasmids have been designed, mathematical models to predict the nature of plasmid and its retention in a cell were constructed (Kim *et al* 1984). The important environmental factors that affect plasmid stability are discussed below.

2.2.1 Temperature

Horng *et al.* (1991) revealed the temperature sensitivity of Lac+ plasmids in *L. lactis* subsp. *lactis* and *L.lactis* subsp. *cremoris*. At higher temperature *L.lactis* plasmid was more unstable than *L. cremoris* plasmid. The region responsible for the temperature sensitive phenotype in *L.lactis* LMO230 was tentatively localized to a 1-2 kbp Cla1-Hind III fragment which was distinct from the replication region of pSK 11L (a lactose plasmid of *L. lactis* subsp. *cremoris*SK11 cloned in *L.lactis* LMO230. This suggested that the closely related *L. lactis* and *L.cremoris* subspecies behaved differently under different temperatures regarding the maintenance of plasmids. Bassit *et al.* (1995) showed that in *L. lactis* subsp. *diacetylactis* maximal diacetyl production occurred at 18^{0}C, which was 1.7 times more than diacetyl produced at 30^{0}C while acetoin production remains unchanged. In a recent report Somkuti and Steinberg (1999) demonstrated the presence heat stress protein genes (*hsp*) was found to be located on plasmids ranging from 2.8kb to 11kb in size, while *hsp* was present in a 2.5 kb plasmid in *L.lactis* subsp. *cremoris*.

2.2.2 Low pH

Differences in response to different pH have been observed as some plasmids are sensitive to alkaline pH and others to acidic pH. Loss of plasmids associated with lactose metabolism from strains of *L. lactis* subsp. *lactis* was enhanced when Foucand *et al.* (1990) transferred the cells to various buffer solutions like sodium succinate buffer (pH 6), phosphate buffer and citrate buffer before plating them on M-17 agar. Working on similar lines, Sinha (1991) investigated the effect of environmental stress caused by acidic pH in low nutrient media on the loss of plasmids from strains C-2 and ML3 of *L. lactis* subsp. *lactis* and ML1 and SC607 of *L. lactis* subsp. *cremoris* strains when grown overnight (16-18h) in skim milk and unbuffered medium (M17) at 32^{0}C and subsequently held at that temperature for extended periods (≥ 96 h). Lac variants were obtained from each strain in milk and M17 broth. Plasmid profiles when compared with their parental Lac+ strains showed loss of one or more plasmid bands. None of the lac mutants showed loss of

smaller plasmids (< 5 Mda) indicating that the smaller plasmids in Lactococci are more stable under these conditions than larger plasmids (>10 M Da) Gupta *et al.* (1992) had studied the effect of pH on lactococcin production in *L.lactis* subsp. *lactis* 484. M17G broth at pH 6.0 resulted in lesser accumulation of Lactococcin when the *L .lactis* was cultured in it for maximal incubation period i.e., 24 h as compared to lactococcin produced in M17 broth set at pH 7.0. Increase in pH to 7.5 of broth medium resulted in no accumulation lactococcin even after 24 h.

2.2.3 Concentration of Nutrients and Sodium Chloride

Change of fermentable sugar from lactose to glucose in medium for few generations led to loss of lactose metabolizing activity (Smith *et al.*, 1992 and Sinha, 1991). The ability of some strains of *L. lactis* subsp.*diacetylactis* to utilize citrate was unaffected by cultivation for more than two weeks on citrate free medium containing glucose or lactose as fermentable carbohydrate. This made Smith *et al.* (1992) to demonstrate for the first time that the plasmid of citrate utilizing strains is very stable. Reduction in the plasmid number becomes a strategy used by *L. lactis* to conserve energy during glucose starvation (Kim *et al.*, 2001). Compared to parent cells which normally possess four plasmids, one-year-old isolates possessed a maximum of two plasmids, and all of the two-year-old isolates possessed only one plasmid. What was interesting was the fact that, 12-kb plasmid possessed by all of the two year isolates and by the one year old TCT isolate, did not correspond in size to any of the original plasmids. From these results it is clear that the factors, which influence plasmid stability, are several and varied and no single limitation affected all plasmids tested.

2.3 PROTEOMIC ANALYSIS OF LAB UNDER DIFFERENT STRESS CONDITIONS

The term proteome describes the complete set of proteins expressed by the genome in an organism (analogues to 'Genome', which is defined as the full nucleic acid component of a species). However although the DNA sequence of the genome allows us to deduce which proteins might be made, it can not tell us what proteins are actually expressed in any given sample,

whether they undergo post-translational modifications (e.g. phosphorylation, glycosylation etc.) or anything about their functions. Analysis of the protein component of a cell or tissue provides this information. With the advent of molecular biology tools combined with bioinformatics in recent years stimulate researchers and scientists to work in this area. Utilization of these techniques in bacterial stress response study had generated plethora of information about the various stress proteins and relevant ones are reviewed here.

2.3.1 High Temperature

When organisms as diverse as bacteria, plants or animals are suddenly shifted to a temperature significantly higher than normal growth temperature or are subjected to certain other stresses the synthesis of heat shock proteins is rapidly induced (Lindquist and Craig, 1988; Neidhardt *et al.*, 1984). By utilizing one- and two-dimensional electrophoresis methods, it had been observed that approximately 17 proteins were induced when the temperature was shifted from 28 to 42^{0}C in case of *Escherichia coli.* The most characterized of the *Escherichia coli* heat shock proteins are DnaK and GroEL, both of which have functioned at normal growth temperature as well as heat shock temperatures. One reported function of GroEL is to act as a protein chaperone (Golobinoff *et al.*, 1989). Exploiting the same technique with minor modification Whittaker and Batt (1991) characterized heat-shock response phenomenon in *Lactococcus lactis* subsp. *lactis.* The enhanced synthesis of approximately 13 proteins was observed in cells labeled with ^{35}S upon heat shock at 42 ^{0}C. Of these heat shock-induced proteins, two appeared to be homologs of GroEL and DnaK, based on their molecular weights and reactivity with antiserum against the corresponding *Escherichia coli.* GroEL protein is a member of the highly conserved hsp60 or cpn family. Its functions as a molecular chaperonin has already been demonstrated (Ellis, 1987). In *Escherichia coli* the adenosine triphosphate dependent folding of many newly synthesized polypeptides by GroEL needs the participation of the so called cochaperonin GroES (Zeilstra *et al.*, 1991). GroES was induced by heat treatment at 42 ^{0}C and 50 ^{0}C in *Lactococcus lactis* subsp. *lactis* (Hartke *et al.*, 1997). GroES was also induced by an acid shift from pH 7 to

pH 5.5 and by UV_{254} nm irradiation, reported by the same author. They concluded that as compared to GroEL, GroES showed significantly higher induction under all stress situations. DnaK and GroEL proteins were also found to be common between bile salts, acid and heat treatment in *Enterococcus faecalis*, thus demonstrated a cross-resistance mechanism (Flahaut *et al.*, 1996). While investigating the heat shock response phenomenon in the thermophilic lactic acid bacterium *Streptococcus thermophilus*, Auffray *et al.* (1995) reported that at least 22 heat shock proteins were induced, after temperature up shift from 42 ^{0}C to 58 ^{0}C, by pretreating the cells at 52 ^{0}C for 30 min. Among these proteins, two related to DnaK and GroEL were clearly over expressed during this stress.

2.3.2 Low Temperature

2.3.2.1 Mechanism of cold shock response

The cold-shock response is a physiological response of living cells to temperature downshift, which is considered to be essential for cells to survive at low temperatures. A cell from a higher eukaryote lives in homeostasis and is thus buffered against sudden changes in the environment. In contrast, a bacterial cell has to react immediately when exposed to unfavourable external conditions, through such responses as entering the stationary or sporulation phase. The major hurdle for bacterial cells is that translation, which consumes the lion's share of the total cellular energy, needs to be rapidly switched off, yet the translational apparatus must be preserved so that when the extracellular conditions improve, the ribosomes can be quickly re-activated for protein synthesis to take advantage of this situation. In a recent work, Wilson and Nierhaus (2004) provided a first detailed insight into a bacterial translation mechanism designed to cope with cold shock conditions by analysing combined structural and biochemical analysis of an essential stress protein in *Escherichia coli*. Cold shock occurs when bacteria are exposed to sudden downshifts in temperature, and is characterized by the expression of a well-defined set of proteins, which include helicases, nucleases and a plethora of nucleic acid−binding proteins such as the so-called cold shock family of proteins (Csps) (Weber *et al.*, 2003; Inouye and Phadtare, 2004; Gualerzi *et*

al., 2003). In contrast to stress conditions like heat shock, no cold-specific σ-factor has so far been identified, suggesting that the cold shock response is predominantly post-transcriptional (Inouye and Phadtare, 2004). Consistent with this notion, many of the proteins up-regulated in *E. coli* during cold shock are associated with the translational apparatus, such as the trigger factor, a ribosome-binding protein chaperone, CsdA, an ATP-dependent RNA helicase-like protein, RbfA, an RNA-binding protein implicated in ribosome assembly, and the translation initiation factors IF1 and IF3 (Weber *et al.*, 2003; Inouye and Phadtare, 2004; Gualerzi *et al.*, 2003). In *E. coli*, a cold shock induced ribosome-binding protein factor that inhibits translation suggested that a more active process may be operating (Weber *et al.*, 2003). In *E. coli*, this protein factor is encoded by the gene *yfi*A, and termed RaiA (ribosome-associated inhibitor) or protein Y (PY) (Agafonov *et al.* 1999 and 2001). The solution structures of PY (Ye *et al.*, 2002; Rak *et al.*, 2002) as well as of a PY homolog from *Haemophilus influenza* (Parsons *et al.*, 2001) revealed that the protein have a globular domain comprising two α-helices and four β-sheets, followed by a flexible C-terminal tail. PY has been shown to bind to small (30S) ribosomal subunits and 70S ribosomes, which are stabilized against dissociation, but no binding to large (50S) ribosomal subunits has been detected (Agafonov *et al.* 1999).

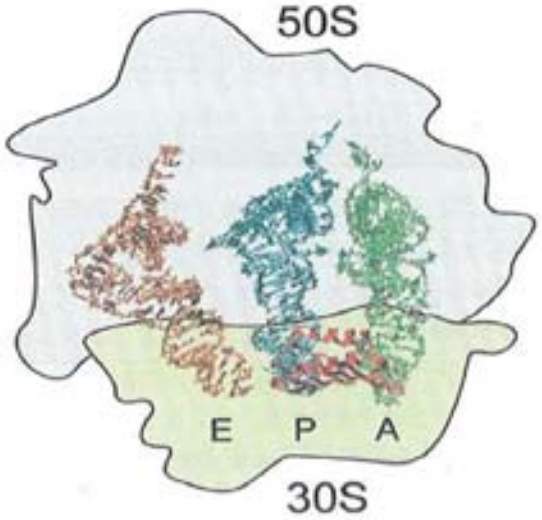


Fig 2.7: The binding site of protein Y on the ribosome overlaps with A- and P-site tRNA. The relative position of protein Y (red) to A- (green), P- (blue) and E-site (orange) tRNA is shown on the background of a schematic representation of a 70S ribosome, composed of 30S and 50S subunits as indicated **(Wilson and Nierhaus, 2004).**

During cold shock, or upon entry into stationary phase, PY associates with ribosomes, where it has been proposed to interfere with elongation by decreasing the accuracy of translation and preventing the binding of aminoacyl-tRNAs to the A site of the ribosome. However, the exact mechanism by which PY regulates translation and protects the ribosomes during cold shock has remained unclear (Agafonov *et al.* 2001; Ye *et al.*, 2002; Rak *et al.*, 2002; Parsons *et al.*, 2002; Maki*etal.*,2000).

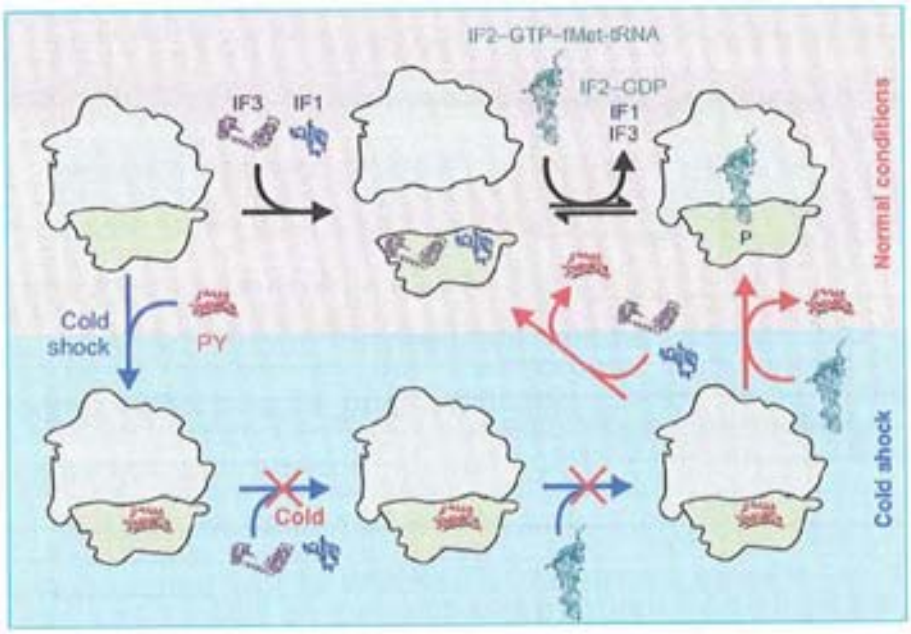


Fig 2.8: Scheme for protein Y–mediated regulation under cold-shock conditions. Under normal growth conditions (top panel, shaded red), run-off 70S ribosomes from translation are dissociated by the combined action of IF1 (blue) and IF3 (purple). In the presence of IF2, the ternary complex containing the initiator tRNA, IF2–GTP–fMet-tRNA, is delivered to the ribosome. Subunit joining leads to dissociation of the initiation factors, leaving the initiator tRNA at the P site (P_i complex). A decrease in temperature produces a cold shock (blue arrow) as seen in the bottom panel (shaded blue), which leads to upregulation in expression of cold-shock proteins such as protein Y (PY, red). PY binds to run-off 70S ribosomes at a position overlapping the A and P sites (see Fig 2.7), preventing the binding of the initiation factors IF1 and IF3 and therefore the dissociation of the 70S ribosomes into its subunits. PY also prevents binding of mRNA and tRNA to the 70S ribosome, thus preventing translation initiation. When temperatures return to normal, PY has a lower affinity for the ribosome than tRNA and initiation factors, allowing ribosomes to follow the alternative pathway into active translation (red arrows). **(Wilson and Nierhaus, 2004).**

By reviewing the work regarding the structural and functional approach to this protein Wilson and Nierhaus (2004) suggested a scheme for PY-mediated regulation during cold-shock conditions (Fig 2.7 and Fig 2.8). Further the work of Vila-Sanjurjo *et al.* (2004) adds further weight to the suggestion that PY may operate in several bacteria, chloroplasts and cyanobacteria under a variety of different stress conditions.

2.3.2.2 Identification of cold shock protein in bacteria including TLAB

The cold-shock response is a physiological response of living cells to temperature downshift, which is considered to be essential for cells to survive at low temperatures. A total of 17 cold shock proteins (Csps) have been identified by two-dimensional electrophoresis following a temperature downshift from 37 to 10 ^{0}C (Jones *et al.*, 1992) in *E. coli*. CspA is the major cold-shock protein in *Escheichia coli* and its production reaches more than 10 % of total cellular protein synthesized during the cold shock response (Goldstein *et al.*, 1990). Recently Jiang *et al.* (1996) proposed that CspA functions as an RNA chaperone to prevent the formation of secondary structures in RNA molecules at low temperature. Such a function may be crucial for efficient translation of mRNAs at low temperatures and may also have an effect on transcription. In a variety of bacteria, families of CSPs, consisting of three to nine members, have been described of which CspA in *Escherichia coli* (CspA ^{E}) and CspB in *Bacillus subtilis* (CspB ^{B}) are the best characterized (Graumann *et al.*, 1998; Wouters *et al.*, 1998 and Yamanaka *et al.*, 2000). CspA^{E} and CspB^{B} are capable of binding to single-stranded DNA and RNA, and based on these characteristics, several functions for CSPs have been suggested, such as transcriptional activators (Brandi *et al.*,1994; Jones *et al.*, 1992 and La Teana *et al.*,1991), RNA chaperones that facilitate the initiation of translation (Graumann *et al.*, 1997; Jiang *et al.*,1997), and freeze-protective proteins (Willimsky *et al.*, 1992). Recently it has been shown that CSPs regulate the expression of cold-induced genes as antiterminators (Bae *et al.*, 2000). Regulation of *csp* genes takes place at several levels, and for CspA^{E} it was shown that cold-shock induction is achieved at the transcriptional level (Goldenberg *et al*, 1997 and Tanabe *et al.*,1992) as well

as at the level of mRNA and protein stablilty (Brandi *et al.*, 1996; Fang *et al.*, 1997 and Goldenberg *et al.*, 1996).

For *L. lactis* MG1363, a family of five *csp* genes has been identified. The *L.lactis* chromosome was found to contain two sets of two tandemly located and cold-inducilble *csp* genes (*cspA/cspB* and *cspC/cspD*) and a single, constitutively expressed *cspE* gene (Wouters *et al.*, 1998). By using *L. lactis* strains specifically overproducing the respective CSPs, it was found that these proteins protect against freezing and might be involved in the regulation of (non-7-kDa) cold-induced proteins (CIPs) (Wouters *et al.*, 2000).

As opposed to mesophilic lactic acid bacteria, cold shock response in TLAB is gaining less attention. Only two reports were available regarding these group of microorganisms. Low-temperature adaptation and cryoprotection were studied in the thermophilic lactic acid bacterium *Streptococcus thermophilus* CNRZ302. *Streptococcus thermophilus* actively adapts to freezing during a pretreatment at 20 ^{0}C, resulting in an approximately 1,000-fold increased survival after four freeze-thaw cycles compared to mid-exponential phase cells grown at an optimal temperature of 42 ^{0}C. By two-dimensional gel electrophoresis several 7-kDa cold-induced proteins were identified, which are the major induced proteins after a shift to 20 ^{0}C. Sequence analysis revealed *csp*-like sequences that were up to 95 % identical to those of *csp* genes of *Streptococcus thermophilus* ST1-1, *Streptococcus dysgalactiae, Streptococcus pyogenes,* and *Lactococcus lactis* (Wouters *et al.,* 1999). In another study Perrin *et al.* (1999) identified a 21.5 kDa new cold shock protein along with 7.5 kDa major cold shock protein by two dimensional electrophoresis. Thus it may be noted that ample of scope remain to be elucidated regarding the cold shock response in these group of firmicutes. Keeping this in mind in the present study an attempt was made to explore the cold shock response in TLAB.

2.3.3 Low pH

Important changes in protein pattern have also been observed during acid adaptation of *Escherichia coli* (Heyde and Portalier, 1990; Hickey and

Hirschfeld, 1990), *Salmonella typhimurium* (Foster, 1991; Foster, 1993 and Hickey and Hirschfeld, 1990), and *Aeromonas hydrophila* (Karem *et al.*, 1994). The adapted cells are protected from acid death relative to unadapted cells by several orders of magnitude (Foster and Hall, 1990; Goodson and Rowbury, 1989). This inducible survival mechanism is referred to as the acid tolerance response (ATR) (Foster and Hall, 1990). The ATR is dependent on protein synthesis in *E. coli* and *S. typhimurium* (Foster, 1991, Karem *et al.*, 1994, O'Hara and Glenn, 1994; Raja *et al.*, 1991). The synthesis of some acid-inducible proteins overlaps with other global response systems (Foster, 1991; Heyde and Portalier, 1990). The lactic acid tolerance response (LATR) of the lactic acid bacterium *Lactococcus lactis* subsp. *lactis* was reported by Hartke *et al.*, (1996). A dramatic increase in survival to a severe acid stress (pH 3.9) was obtained by preexposing the cells for 30 min to a mildly acid shock at pH5.5. Whole-cell protein extract analysis revealed that during the acid tolerance response 33 polypeptides were induced over the level of naïve cells. Among these are the major heat shock proteins DnaK and GroEL. The acid-stress response in thermophilic lactic acid bacteria is currently gaining attention and countable reports are available till recently. In *Lactobacillus delbrueckii* subsp. *bulgaricus*, three highly induced proteins have been identified, GroES, GroEL and DnaK. They were also responsible for heat stress response in this organism (Lim *et al.*, 2001). The one- and two-dimensional protein patterns of *Streptococcus thermophilus* PB18 in the exponential and stationary phases of growth were analysed. One-dimensional SDS-PAGE showed that a 16 kDa protein was over expressed in stationary phase as well as 2 hour after an acid shock, and that it was not expressed when the bacteria reached the stationary phase in medium with limiting lactose concentrations (5 or 10gl/1), in which the pH (5.5) was not as acid as in control cultures (pH 4.7, lactose 20 g/l). The results supported the idea that this protein was expressed in response to the acidic environment and not in response to growth phase (Gonzalez-Marquez *et al.*, 1997). *Lactobacillus sanfranciscensis* CB1, an important sourdough bacterium can withstand low pH after initial exposure to sub lethal acidic conditions (De Angelis *et al.*,

2001). An increased level of expression of GrpE was observed in acid adapted and acid tolerant mutants as compared to non-adapted cells.

Different proteins expressed during acid, low temp and heat stress are presented in Table 2.2.

Table 2.2 Proteins expressed during low temp, heat and acid stress

Organisms	Treatment	Proteins expressed	References
E. coli	28 to 42 ^{0}C	DnaK and GroEL	Lindquist & Craig, 1988
L. lactis subsp. *lactis*	37 to 42 ^{0}C	Homologues of DnaK and GroEL	Whittaker & Batt, 1991
L. lactis subsp. *lactis*	42 and 50 ^{0}C	Gro ES	Hartke *et al.*, 1997
L. lactis ssp. *lactis*	pH 7.0 to pH 5.5,UV_{254nm}	Gro ES	Hartke *et al.*, 1997
E. faecalis	Bile salt, acid and heat	GroEL and DnaK	Flahaut *et al.*, 1996
S. thermophilus	42 to 58 ^{0}C	DnaK and GroEL	Auffray *et al.*, 1995
L. lactis MG1363	30 to 10 ^{0}C	CSP (7-KDa)	Wouters *et al.*, 2001
S. thermophilus CNRZ 302	42 to 20 ^{0}C	CSP (7-KDa)	Wouters *et al.*, 1999
	42 to 20 ^{0}C	CSP (7.5 kDa) and new protein 21.5 kDa	Perrin *et al.*, 1999
L. delbrueckii subsp. *bulgaricus*	pH 6.0 to pH 4.75	GroES, GroEL & DnaK	Lim *et al.*, 2001
S. thermophilus PB18	pH 5.0	ASP (16 kDa)	Gonzalez Marquez *et al.*, 1997
L. sanfranciscensis CB1	pH 3.2	GrpE	De Angelis *et al* 2001

2.4 GENOMIC IMPROVEMENTS IN LACTIC ACID BACTERIA

As the knowledge of genetics and molecular biology of lactic acid bacteria has advanced rapidly the genetic improvement of these industrially important group of bacteria are revolutionized. The strain construction relies

on our ability to transfer genes among the starter strain and to use recombinant DNA technique to localize important genes. A variety of gene transfer systems include, both the natural recombination systems like conjugation, transfection, transduction, transformation as well as induced gene transfer techniques such as protoplast fusion and electroporation.

2.4.1 Conjugation

Conjugation implies the process of gene transfer in which a donor cell, in close physical contact with a recipient cell may transfer a replica of genetic information to the recipient cell (Freifelder, 1983). A number of metabolic properties have been conjugally transferred in *Lactococci.* Conjugal transfer of Lac^+ phenotype in *Lactococci* was first reported simultaneously by two group of workers (Gasson and Davies, 1979 and Kempler and McKay, 1979). Other traits include Dip^+ (Diplococcin producing ability) in *L.lactis* subsp.*cremoris* (Davey and Pearce, 1982); Nis^+ (Nisin producing ability), Suc^+ in *L.lactis* subsp.*lactis* (Gasson, 1984). Some industrially important phenotypes namely proteinase activity (Prt^+), ropiness and antibiotic resistant (Ery^r, Str^r) have also been successfully transferred to various lactococcal recipient strains (Gasson and Davies, 1980; Neve *et al.*, 1987). Recently Akcelik (1993) reported the conjugative mobilization of heat-sensitive phage resistance (Hsp+) plasmid between *L. lactis* subsp.*lactis* strains.

It is thus evident that conjugation is perhaps one of the most efficient gene transfer systems in mesophilic *Lactococci* and recently researches are directed towards thermophilic lactic acid bacteria. However, there is a need to make it more efficient as the frequency of transfer of desirable traits is extremely low in most of the cases.

2.4.2 Transformation and Transduction

Besides conjugation, the method of transformation and tansduction are widely used for development of strains in lactic acid bacteria. For example, *Streptococcus thermophilus* ATCC19258, *Lactobacillus delbrueckii* subsp. *bulgaricus,* T-11 and *L .lactis* subsp.*lactis* IL1403 were transformed with

alpha-amylase gene (*amyA*) from *Streptococcus bovis* 148 using a wide host-range vector, and all the transformants secreted alpha amylase successfully (Satoh *et al.*, 1997). Davey and Heap (1993) reported a successful case of transduction in *L.lactis* subsp.*cremoris*. An induced phage 2204-T from an arginine-positive (able to hydrolyse arginine) *Lactococcus lactis* var. *lactis* 2204 L strain was used in transduction experiments with Lactococcus lactis var. cremoris 2204 (an arginine-negative strain containing the chloramphenicol resistance plasmid pFX3). Arginine-positive transductants were isolated at a low frequency (<10-10 arginine-positive isolates per initial recipient cell). Hybridization experiments with labelled DNA from phage 2204-T showed the presence of the temperate phage (2204-T) genome in total DNA of the transductant 2204 LT strains. No hybridization was observed with DNA from the arginine-negative strain 2204. A mitomycin C-treated derivative of 2204 LT that was arginine negative also showed no hybridization, indicating that the prophage had been eliminated. The presence of the chloramphenicol resistance plasmid pFX3 in both transductants and mitomycin C-cured derivatives was demonstrated by hybridization, confirming the origin of the arginine-positive isolates as derivatives of 2204. A standard electroporation procedure was used to introduce a range of Gram-positive plasmid vectors into 9 industrial strains of *Streptococcus thermophilus*. All the strains were transformable with at least 2 of the plasmids assessed, but electrotransformation frequencies depended on both the strain and the nature of the transforming DNA. Small rolling circle (RC) plasmids were electroporated at high frequency into a wide range of strains with efficiencies of 102-105 transformants/μg of transforming DNA. The presence of these plasmids did not affect doubling times during growth in broth, and they were extremely stable in slow milk-acidifying strains, with 85-100% of transformants retaining the selective markers over 105 generations. Vectors were less stable in fast-growing cultures. Of the 3 theta-type plasmids assessed, only pIL253 was electroporated at low frequency into some slow-growing strains. The presence of this plasmid caused a 40 % increase in doubling time and it

was lost from cells at a rate of 3% per generation. Attempts to alter the proteolytic status of slow-acidifying strains of *S. thermophilus* by the introduction of heterologous proteinase genes were also described. It is concluded that electroporation is a simple, efficient and reproducible means of introducing DNA into *S. thermophilus* and, in combination with the development of stable, endogenous plasmid vectors with food grade markers, would be invaluable in the genetic manipulation of this microorganism (O'-Sullivan and Fitzgerald, 1999). An important consideration in the genetic manipulation of lactic acid bacteria is whether or not strains retain their normal industrial properties after transformation with plasmid DNA. A survey of plasmid types in various lactic acid bacteria showed that pE194-type rolling circle (RCR) plasmids were present in 24 % of 70 commercial *Lactococcus lactis* cheese starters but not in any of 38 strains representing 9 species of *Lactobacillus*. By contrast, theta plasmids showing homology to the pCI305 rep gene were present in most (though not all) lactococcal strains and not in any of the lactobacilli. Plasmids not belonging to either group were also present in some *L. lactis* and *Lactobacillus* strains. Six selected *L. lactis* subsp. *cremoris* strains were transformable with pFX3 (a 4.5 kb pE194-type rolling circle vector), yet 4 of these strains had lower or zero transformation frequencies with pCI3340 (a 5.7 kb pCI305-based theta vector), possibly due to plasmid incompatibility. However, all transformants maintained full milk-coagulating activity. In addition to *L. lactis*, strains of *L. garviae, Enterococcus faecalis [Streptococcus faecalis]* and *Lactobacillus reuteri* were able to be transformed with pCI3340, making this a potentially useful vector for these species (Cambourn *et al.*, 1998). Lactose and galactose uptake and phospho-beta-galactosidase activity in *Pediococci* that had been transformed with a *Lactococcus lactis* lactose plasmid were investigated. Lactose-positive transformants, *Pediococcus acidilactici* SAL and *Pediococcus pentosaceus* SPL-2, accumulated [14C]lactose at a rate greater than the *Lactococcus lactis* control. Phospho-beta-galactosidase activity was higher in transformants than in Lactococcus lactis. [3H] galactose uptake indicated that a wild-type

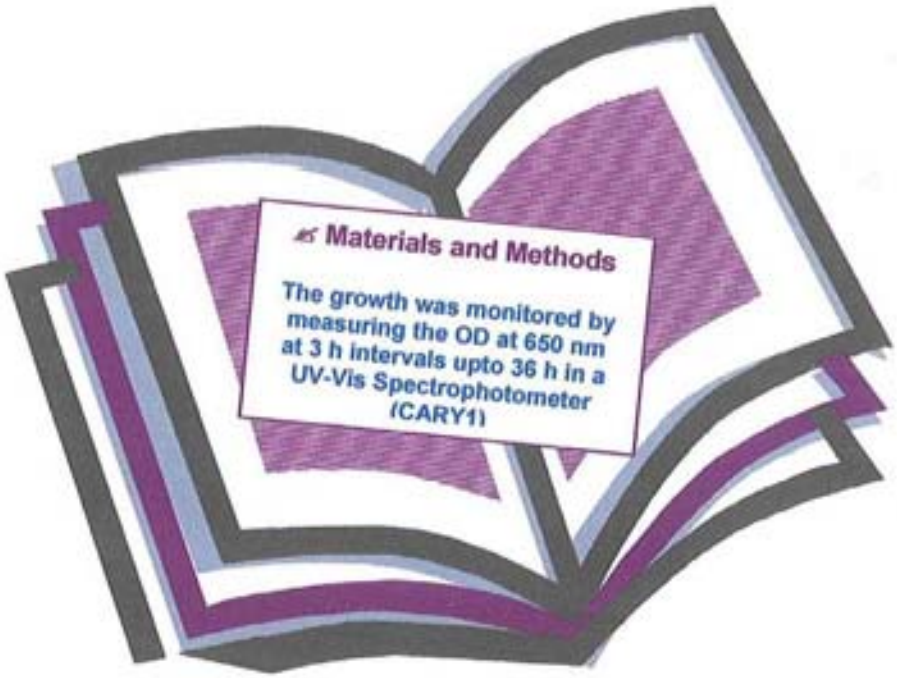
galactose transport system and the introduced lactose phosphotransferase system both functioned in galactose uptake by *Pediococcus* spp. transformants. Significantly lower amounts of free galactose were detected in milk cultured with *Lactobacillus helveticus* LH100 and SAL or SPL-2 than in milk cultured with a LH100 plus *Streptococcus thermophilus* TA061 control starter (Caldwell *et al.*, 1998). *Streptococcus salivarius* var. *thermophilus* (*Streptococcus thermophilus*) is a homofermentative, thermophilic lactic acid bacterium, used in dairy starter cultures. Despite its widespread and long-term use, its molecular biology and genetics have only recently started to be investigated. This study reports the isolation and characterization of a cryptic, 3-kb plasmid, which was converted into an *Escherichia coli-S. thermophilus* shuttle vector. Using this and other plasmids, transformation was optimized and used to integrate non-replicative plasmids into the bacterial genome. The resulting cointegrates were able to amplify. Resolution of the cointegrates was used for gene-replacement by introducing an in vitro-generated deletion into a genomic-located structural gene. By the same mechanism, a heterologous, promoter-less marker gene was inserted onto the genome between the permease and beta-galactosidase gene of the lactose operon. It was thereafter expressed as a functional part of the operon and followed lactose regulation. The control region of the lactose operon was investigated by analyzing promoter up and down mutants (Mollet *et al.*, 1993). The microflora of traditional yoghurt consists of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Using 2 promiscuous plasmids, conjugational pAMBETA1 and non-conjugational pNZ18, both bacterial species were analysed for their abilities to participate in gene transfer by conjugation, transduction or transformation. While no transfer into *L. delbrueckii* subsp. *bulgaricus* was observed under any of the conditions tested, *S. thermophilus* was able to act as recipient or donor in conjugation and transduction. Transduction even occurred in yoghurt. Transformation rates observed for *S. thermophilus* were low and may be at least partly attributed to the presence of surface-associated nuclease activities.

Segregational stability of pAMBETA1 was shown to depend on environmental factors and on the host bacteria harboring the plasmid (Heller *et al.*1996).

2.4.3 Protoplast Fusion

Protoplast fusion is a genetic exchange in which protoplasts from two phenotypically distinct strains are indued to fuse by fusogenic agent like PEG to form recombinants containing genetic information from both strains. Protoplast fusion in bacteria was established in the year 1976 by Fodor and Alfoldi, and Schaeffer *et al.* The same was subsequently applied to *L.lactis* subsp. *lactis* derivatives successfully by Gasson (1980), who demonstrated recombination of the chromosomal markers like maltose utilization (Mal^+) and Str^+, as well as plasmid markers (Lac^+ and Ery^+) among *L.lactis* subsp. *lactis* 712 derivatives. The frequency of chromosomal recombination ranged from 3.0×10^{-5} to 7.1×10^{-5}, while the frequency of plasmid transfer ranged from 1.9×10^{-3} to 3×10^{-3} recombinants per minority parent strain regenerant. Intergeneric protoplast fusion has also been reported by various workers (Cocconcelli *et al.*, 1986 and Reed, 1987). Cocconcelli *et al.*(1986) demonstrated that when *L. lactis* subsp. *lactis* SH 4174 and *Lb .reuteri* DSM 20016 protoplasts undergo fusion by exposures to PEG, plasmid pAM β–I and trehalose fermenting ability were transferred into *Lb. reuteri* at the frequency of 8×10^{-4} to 1×10^{-5} and 8.5×10^{-5} respectively per regenerated *Lb. reuteri* protoplasts. The possibility of producing an improved Lactobacillus strain by the fusion of *L. casei* and *L. acidophilus* was investigated by Yeeh *et al.* (1996). The metabolism of maltose, lactose, mannose and galactose, including galactosidase activity, was examined in 8 fusants of *L. casei* and *L. acidophilus*. Four fusants metabolized maltose and lactose simultaneously. Two fusants had greater acid producing capabilities than *L. casei* or *L. acidophilus*. In 2 other fusants, beta-galactosidase activity was increased by 7.6-8% compared with that in *L. acidophilus*. Two fusants had phospho-beta-galactosidase activities of 800 and 548 nmol mg protein-1 min-1 which were similar to those in *L. casei* (400 nmol mg protein-1 min-1). It was concluded that fusion between *L. casei* and *L. acidophilus* produces stable fusants with

improved characteristics compared with *L. casei* or *L. acidophilus*. Current methods for the improvement of industrial microorganisms range from the random approach of classical strain improvement (CSI) to the highly rational methods of metabolic engineering. Although CSI is robust, it is time and resource intensive. Rational approaches are information and tool intensive, and rely on models that simplify biological systems. All aspects of cell physiology are inherently complex; however some are more readily addressable rationally than others when the appropriate genetic tools are available. Environmental tolerance, a particularly complex and poorly understood phenotype, is not an obvious target for rational engineering. In this context, Patnaik *et al.* (2002), suggested protoplast fusion could be an effective tool to manipulate industrial microorganisms. They identified shuffled strains of *Lactobacillus* that produced threefold more lactic acid than the wild type at pH 4.0. Thus genome shuffling by protoplast fusion seems broadly useful for the rapid evolution tolerance and other complex phenotypes in industrial microorganisms.



Materials and Methods

3.0 MATERIALS AND METHODS

3.1 TEST CULTURES

For the present investigation all the following 36 thermophilic lactic acid and four other test bacteria were obtained from National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, NDRI, and Karnal. The composition of media and reagents utilised in the present study has been given in Annexure I.

Organism	NCDC strain No.
Lactobacillus acidophilus	
1	11
2	13
3	14
4	15
5	16
6	195
7	291
Lactobacillus delbrueckii subsp. *bulgaricus*	
1	4
2	8
3	9
4	26
5	27
6	184
7	253
8	277
9	281
10	285
11	293
12	304
13	305
14	307
15	308

Streptococcus thermophilus	
1	74
2	75
3	76
4	80
5	158
6	175
7	177
8	199
9	217
10	218
11	295
12	303
13	311
14	312
Escherichia coli	136
Bacillus cereus	66
Bacillus subtilis	240
Staphylococcus aureus	105

3.2 MAINTENANCE AND PROPAGATION OF CULTURES

All the thermophilic lactic acid bacterial cultures were maintained in skim milk and chalk litmus milk media in test tubes. The cultures were activated by propagation in skim milk at 42 ^{0}C, every fortnight.

3.2.1 Purity of the Culture

Purity of all the cultures was determined by Gram staining.

3.3 ANALYSIS FOR THE VIABILITY OF THERMOPHILIC LACTIC ACID BACTERIA (T LAB) UNDER STRESS

The following stress conditions of temp and pH were employed for preliminary screening of the lactic acid bacterial strains listed above.

Table 3.1 Preliminary screening of T LAB at different stress levels

Stress Type	Stress levels for growth
Temp (^{0}C)	10, 15, 20, 25 and 42 (Normal temp)
pH	3.5, 4.0, 4.5, 5.0 and 6.5 (Normal pH)

3.3.1 Temperature of Incubation

For study of the effect of temp on growth and survivability of the test organisms, inoculated MRS and LM17 media tubes/small flasks were stored at different degrees of temp as per Table 3.1 in BOD incubators, etc.

3.3.2 Initial Medium pH

The pH of MRS and LM17 media was adjusted to values ranging from 3.5 to 6.5 with NaOH or 50 % lactic acid (w/v) as per requirement using a pH meter (water proof pH scan BNC microprocessor based pocket pH meter, Eutech Instruments, Singapore). Lactic acid and NaOH were sterilized separately and pH of the media was adjusted aseptically. Initially a small vol (e.g. 10 ml) of each medium was taken and to it NaOH or lactic acid was added and the pH was measured. Later on each medium pH was adjusted directly adding calculated amount of the sterile acid or alkali aseptically.

3.3.3 Growth Measurement

Standardized inoculants were prepared by growing the cultures overnight in MRS and LM17 broths (for *Lactobacillus* species and *S. thermophilus*, respectively) to exponential phase (OD at 650 nm = 0.4 to 0.5, ~ 10^7 cells/ml). The growth media in 200 ml flasks in duplicate were inoculated at the rate of 1% and incubated at 42 ^{0}C for a period of 36 h. The growth of the organisms was monitored by measuring the OD at 650 nm of the resultant culture at 3 h intervals upto 36 h in a UV-Vis Spectrophotometer (CARY1). At the same time cell counts were determined by standard plate count method. The plates were incubated for 48 h at 42 ^{0}C and the numbers of cfu were counted.

For all the ten stress culture conditions and the 36 cultures under test a total of 360 growth curves were obtained. The DMFit curve fitting programme

designed by Baranyi (IFR, Norwich, UK) was used for the log counts data fit applying Baranyi function (Baranyi and Roberts, 1994). Estimation of growth rate (μ_{max}/h) was derived from the resultant programme.

The cultures/ strains those showed best growth at lower temp and lower pH ranges were taken for further study. Out of the 36 cultures, two cultures were thus selected (*L. delbrueckii* subsp. *bulgaricus* NCDC 307 and *L. acidophilus* NCDC 14) for low pH studies and one (*S. thermophilus* NCDC 74) for low temp studies.

3.4 STRESS TOLERANCE RESPONSE

Before attempting to improve the selected TLAB with respect to their behaviour acid tolerance response (ATR) in lactobacilli and cold shock response in *S. thermophilus* NCDC 74 were determined.

3.4.1 Acid Stress Tolerance Response (ATR)

3.4.1.1 Acid stress tolerance response of lactobacilli

Acid tolerance response was determined as per Hartke *et al.* (1996). Log phase cells were harvested at OD_{650} of 0.541 for *L. delbreueckii* subsp. *bulgaricus* NCDC 307 and at 0.987 for *L. acidophilus* NCDC 14, respectively. Cells were resuspended in MRS broth, which prior to use was adjusted to pH 4.0 to 6.0 with 50 % lactic acid. The cells were exposed at each test pH for 30 min. Exposure of the cells at normal medium pH was used as control. For experiments with chloramphenicol (Sigma Chemical Co., USA), cells were incubated with 100 µg/ml of antibiotic during the 30 min treatment. In all the cases the resultant cells were subjected to acid challenge at pH 3.5.

For studying the kinetics of acid resistance in lactobacilli, the organisms were grown at pH 4.0 (NCDC 14) and pH 4.5 (NCDC 307) for up to 60 min with 10 min intervals. The organisms after each interval were subjected to acid challenge at pH 3.5. Three trials were conducted in each case.

The survival (%) and tolerance factor were calculated as per following formulae:

$$\text{Survival (\%)} = \frac{\text{Cfu at end of the treatment}}{\text{Cfu before treatment}} \times 100$$

Tolerance factor = Survival (%) of treated cells/ Survival (%) of control cells

3.4.2 Cold stress tolerance response of *S. thermophilus* NCDC 74

3.4.2.1 Cold shock treatment

S. thermophilus NCDC 74 was cultured at 42 ^{0}C in LM17 broth containing lactose (10 g/l). For cold shock treatments, five 60 ml aliquots of culture were prepared in LM17 medium until mid exponential phase (OD_{650} = 0.464, 6h), after which cells were centrifuged at 4000 rpm for 10 min, and the pellet was resuspended in the same vol of pre cooled (25, 20, 15, and 10 ^{0}C) broth supplemented with 100 μg chloramphenicol/ml (to arrest new protein formation) in separate flasks in duplicate. The flasks were incubated at 42 ^{0}C (for control) and the specified degrees of temp up to 30 h, measuring the OD_{650} at every 5 h intervals.

3.4.2.2 Freeze thaw survivability

To study the freeze thaw survivability mid log phase (OD_{650} = 0.464) *S. thermophilus* cells and those at 2, 4, and 6 h after cold shock at 10, 15 and 20 ^{0}C were frozen in LM17 broth. One ml aliquots were spun down for 5 min at 13,000 rpm and resuspended in 1 ml of fresh LM17 medium. The suspensions were subsequently frozen at –20 ^{0}C for 24 h and then thawed for exactly 4 min at 30 ^{0}C in a water bath. This cycle of freezing and thawing was repeated four times. The cfu were first before freezing and after thawing in each consecutive cycle were determined by spread plating appropriate decimal dil on LM17 agar after 2 d at 42 ^{0}C. The survival (%) and tolerance factor was calculated as per following formulae:

$$\text{Survival (\%)} = \frac{\text{Cfu at end of the treatment}}{\text{Cfu before treatment}} \times 100$$

Tolerance factor = Survival (%) of adapted cells/ Survival (%) of control cells

3.4.2.3 Determination of critical temperature for growth and growth rate

The optimum and critical temp of *S. thermophilus* NCDC 74 was derived from DMFit version 2.0. The growth rate (μ_{max}/h) also was derived from this programme.

3.5 DEVELOPMENT OF T-LAB CAPABLE OF GROWING UNDER ACID AND COLD STRESS

3.5.1 Mutation

3.5.1.1 Induction of mutation by physical agents

3.5.1.1.1 Ultraviolet irradiation

The irradiation source was mineral light lamp model UVS-12 consisting of a twin 4-watt germicidal tube with built-in UV filter to give almost entirely UV rays of a ~ 2537Å wavelength. The light source which faced downwards was fixed to an adjustable stand. The base plate of the stand was suitably modified to hold a magnetic stirrer. The light source with magnetic stirrer was housed in a specially made wooden cabinet fitted with a 15 watt germicidal tube with provision for suitable ventilation on the top. The 15 watt tube was used for sterilization of the chamber prior to irradiation. The power source for the irradiation chamber was connected through a voltage stabilizer to have controlled speed of stirrer and also to ensure uniform UV energy output per unit area per unit time.

3.5.1.1.1.1 Measurement of UV dose

Ultraviolet light energy falling on per unit area per unit time was measured when kept at a specific distance i.e. ~4 inch.

3.5.1.1.1.2 Preparation of samples for irradiation

Log phase cultures were harvested by centrifugation of 5,000 rpm for 20 min (Kubota 6800, Germany) washed twice with phosphate buffer (0.02M, pH 7.0); and resuspended in the same buffer. The OD was adjusted to 0.5. All the above operations were carried out under aseptic conditions.

3.5.1.1.1.3 Irradiation of sample

20 ml suspension was transferred to 100 mm petridish and agitated for five min with a magnetic stirrer by sterilized teflon-coated bar magnet. After 5

min of stirring an aliquot of the sample, before irradiation was transferred to 9 ml of buffer in a test tube. The sample in the petridish was exposed to ultraviolet light with constant stirring. Samples were removed at 10, 15, 20, 25 and 30 min intervals by closing the metal shutter and transferred to appropriate dilution blanks. Irradiation experiments were carried out in dark to avoid photo reactivation.

3.5.1.2 Induction of mutation by chemical agents

3.5.1.2.1 N-methyl-N-nitro-N' nitrosoguanidine (NTG)

A sol of NTG was prepared at a conc of 200 μg/ ml and filter sterilized. It was added to each of the inoculated broth tubes to give a final conc of 400 μg/ml. Aliquots were withdrawn at 30 min intervals upto a period of 150 min. Appropriate dil were then plated in LM17 and MRS agar for streptococci and lactobacilli respectively. After incubation of the petridishes for 48 h at 42 ^{0}C, the number of survivors were counted. Individual colonies were examined for pH tolerance and temperature respectively.

3.5.2 Adaptation

3.5.2.1 Acid adaptation

Exposure of the organism to pH values below the growth range was considered the acid stress. Ability of the strains of *Lactobacillus acidophilus* NCDC 14 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307 to grow at various pH levels (3.5 to 6.5) adjusted with lactic acid in MRS medium was investigated. Taking clue from the acid stress tolerance response (ATR) studies pH 5.0 and pH 4.5 were chosen as the starting point for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307 and *Lactobacillus acidophilus* NCDC 14 respectively for adaptation to grow at lower pH.

The experimental protocol for these organisms was standardized after many trials. It consisted of a 4 % inoculum into tube/flask of broth at a given pH. After incubation subculturing was done at the same pH to determine growth. To confirm stability, passaging for 3 more times was done before 4 %

inoculation into broth at next lower pH. Where the growth was not observed in the subculturing, the next higher pH was considered to be the lowest adapted pH where the growth could sustain. The protocol was outlined in Fig 3.1 and Fig 3.2.

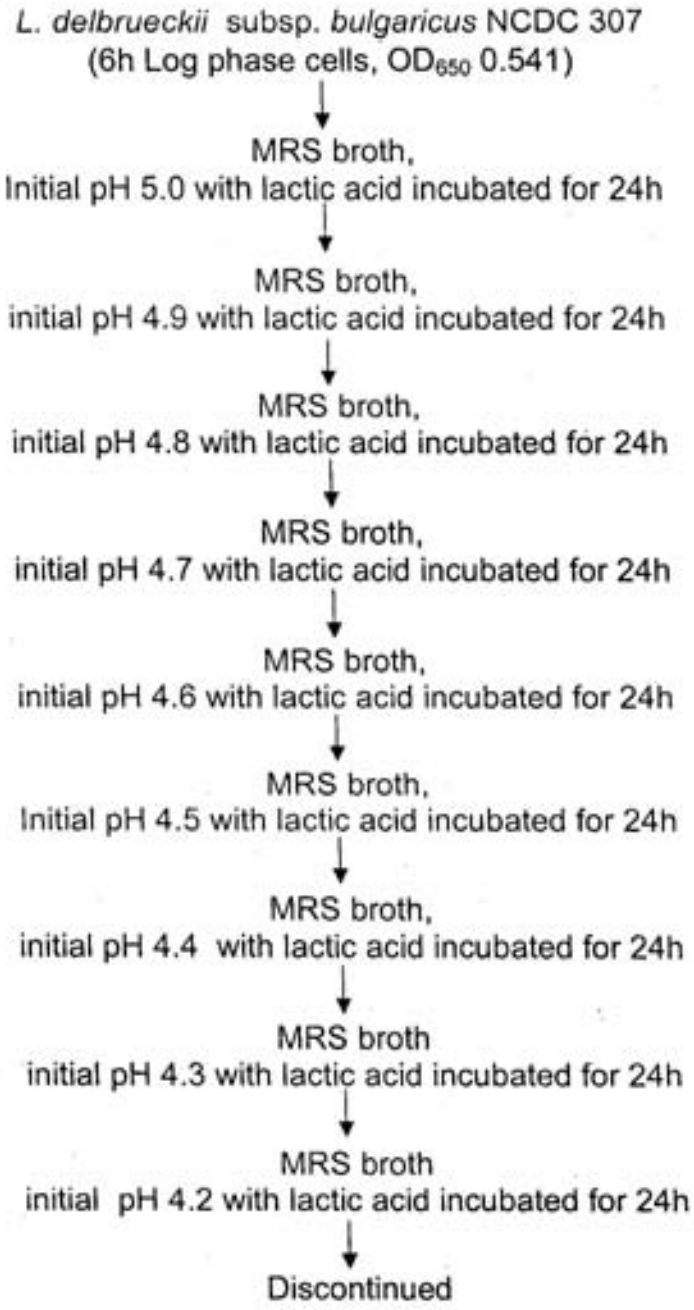


Fig. 3.1: Protocol for acid adaptation in *L. delbrueckii* subsp. *bulgaricus* NCDC 307

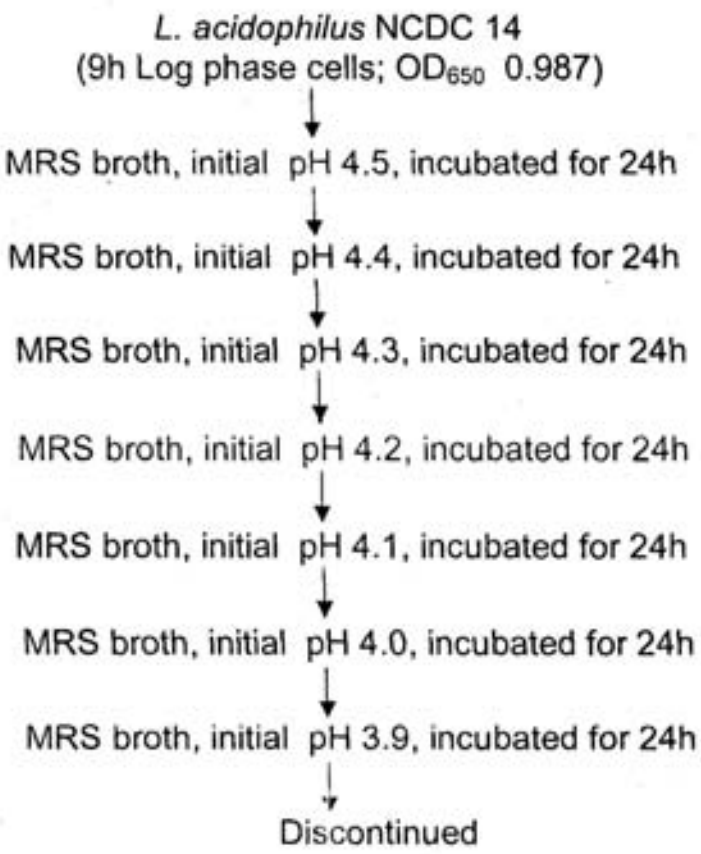


Fig. 3.2: Protocol for acid adaptation in *L. acidophilus* NCDC 14

Isolation of the stable adapts/mutants was done as per O'Driscoll *et al.* (1996) with some modification. The culture grown at the lowest two pH levels was centrifuged, washed twice in normal saline solution (NSS) and pour plated in MRS agar, adjusted to pH 4.2 and 4.3 for *L. delbrueckii* subsp. *bulgaricus* NCDC 307; pH 4.0 and 3.9 for *L. acidophilus* NCDC 14 at 42 ^{0}C for 48 h. Individual survivors were further grown in MRS broth at pH 6.5 and subsequently subjected to five repeated treatments to increase the tolerance at the pH 4.3 and pH 4.0 for *L. delbrueckii* subsp. *bulgaricus* NCDC 307 and *L. acidophilus* NCDC 14 respectively. For further studies, the stable adapts/ mutants were maintained at freezing temperature in MRS broth adjusted to the pH giving stable adapts.

3.5.2.2 Cold adaptation

For adaptation studies a temp slightly above the critical one was chosen as the starting point. In the present investigation initially the cultures were grown at 20 ^{0}C and the mid-log phase (OD_{650} = 0.524) was obtained

after 48 h of incubation. The experimental protocol for the organism to grow stably at lower temp finalized after many trials was as follows. A 4% inoculation was made into broth tube/flask and incubated at 20 ^{0}C for 48 h. It was subcultured two times. It was inoculated in fresh broth and incubated at 18 ^{0}C for 48 h before again subculturing twice. This cycle was repeated every time lowering the incubation temp by 2 ^{0}C till a growth was obtained. Where no growth was achieved, the nearest higher temp used was considered to be the lowest adapted temp.

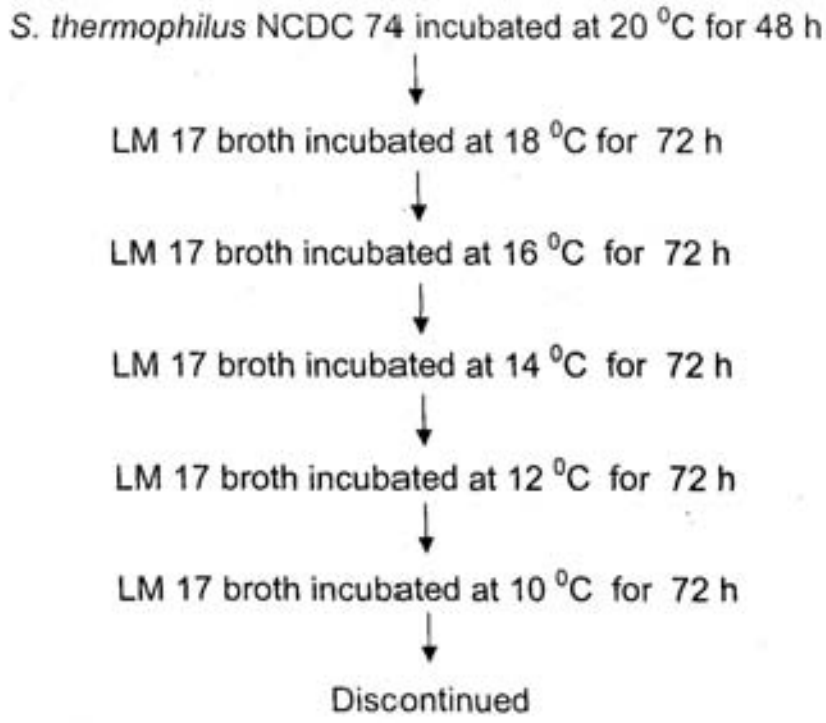


Fig. 3.3: Protocol for cold adaptation in *S. thermophilus* NCDC 74

The culture was not found to be stable at 10 ^{0}C, but was stable at 12 ^{0}C. The later was maintained at freezing temp for further studies.

3.5.3 Nomenclature of the Improved T-LAB

The cultures obtained after completion of the experiments for development/ selection of stress tolerant cells, the resultant cultures were trivially rechristened for further studies. The cultures which yielded desired adapts/ mutants were christened as follows:

Table 3.2 **Nomenclature of the adapts**

T-LAB	Strain designation of Parent	Strain designation of Adapts
Lactobacillus acidophilus NCDC 14 (adapted at low pH)	C_{14}	$^{4.0}pH_{14}$
Lactobacillus delbrueckii subsp. *bulgaricus* NCDC 307 (adapted at low pH)	C_{307}	$^{4.3}pH_{307}$
Streptococcus thermophilus NCDC 74 (adapted at low temp)	C_{74}	$^{12}T_{74}$

3.6 CHARACTERISTICS OF ADAPTED CELLS

3.6.1 Gross Cell Morphology

3.6.1.1 Light microscopy

Parent and adapted cells of *L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14 and *S. thermophilus* NCDC 74, were gram stained, observed through light microscope (Olympus Model BX50F-3, Japan) and photographed.

3.6.1.2 Electron microscopy

Parent and adapted cells of *L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14 were examined under scanning electron microscope.

3.6.1.2.1 Procedure

3.6.1.2.1.1 Sample preparation

Parent cultures of the lactobacilli (*L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14) were grown in 60 ml of MRS broth up to mid log phase. The adapts were grown in 100 ml of MRS broth (pH 4.3 and 4.0, respectively) for 18 to 20 h. All the cultures were centrifuged at 13,000 rpm for 15 min at 4 ^{0}C in a Kubota 6800 (Japan) centrifuge. Then the cell pellets were washed twice using 0.1M cacodylate buffer, pH 7.2.

3.6.1.2.1.2 Fixation

Samples were transferred to labeled micro beakers containing 2.5 % buffered glutaraldehyde fixative. The beakers were covered with aluminium

foil and kept at 4 ^{0}C for 2 h. Glutaraldehyde was carefully removed using a pasteur pipette. Again, all the samples were doubly fixed in 2% osmic acid at 4 to 6 ^{0}C for 2 h. These solutions were replaced gradually from one to another in the same micro beaker.

3.6.1.2.1.3 Dehydration

After fixation the samples were subjected to dehydration as per the schedule given below:

Concentration of Mixture	Duration
30% ethanol	5 -10 min in 4 ^{0}C
50% ethanol	5 -10 min in 4 ^{0}C
70% ethanol	5 -10 min in 4 ^{0}C
90% ethanol	5 -10 min in 4 ^{0}C
Absolute Ethanol/ Acetone	30 -45 min in 4 ^{0}C or 1 h at room temp.

3.6.1.2.1.4 Air drying

For air drying the samples were further processed two times through acetone for 20 min each time and the dried samples were then mounted on aluminium stubs coated with copper strips. The stubs were kept overnight in desiccator.

3.6.1.2.1.5 Coating of specimens

3.6.1.2.1.5.1 Gold coating

The aluminium stubs were coated with gold at approximately 100 - 200 Å thickness on Hitachi IB-3 ion coater. The ion current was kept at 6 mA at fine vaccum of 0.05 – 0.07 torr for 2 to 4 min.

3.6.1.2.1.6 SEM observation

The aluminium stubs loaded with specimens were placed in the specimen holder and inserted into the Hitachi S-405A Scanning Electron Microscope under vaccum. The SEM was operated at 15 kV using secondary electron mode. Specification and scale of Hitachi S-405A Electron Microscope are given in Annexure – III.

3.6.1.2.1.7 *Electron micrography*

3.6.1.2.1.7.1 *Processing of photographic film*

The observations were recorded as electron micrographs on OR W0 35 mm perforated film (40 ASA) with the help of attached camera assembly. The film was developed in Kodak D 19 film developer for 8 - 10 min at 20 ^{0}C and fixed in acid hardening fixer (Agfa - 301) for 10 - 15 min. The film was thoroughly washed in running tap water and dried in air.

3.6.1.2.1.7.2 *Printing of electron micrographs*

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

3.6.1.2.1.8 *Analysis of electron micrographs*

Electron micrographs were visually observed with all precision and accuracy. The measurement of individual cells of control and treated organisms was made from a large number of micrographs using precision scale. Average values of all such observations were recorded and further interpreted.

3.6.2 Plasmid Analysis

The plasmid isolation was carried out following the modified protocol of Sullivan and Klaenhammer (1993).

3.6.2.1 Isolation of Plasmid

Parent cultures of *Lactobacillus* spp. were grown in 10 ml of MRS broth overnight at 37 ^{0}C. The adapts were grown in 50 ml of MRS broth adjusted to pH 4.3 and pH 4.0 respectively, inoculated at the rate of 2 % inoculum and incubated overnight at 42 ^{0}C. The parent and adapted cultures of *Streptococcus thermophilus* were grown at 42 ^{0}C overnight in 10 and 50 ml LM17 broth and inoculated at the rate of 1% and 2%, respectively.

Cells were harvested by centrifugation at 10,000 rpm for 10 min at 5 ^{0}C. The pellets were resuspended in 200 µl of sol I containing 30 mg/ml of lysozyme (lysozyme separately weighed 0.03 g and suspended in 1 ml of Sol I) and incubated at 37 ^{0}C in water bath for 15 min. 400 µl of Alkaline SDS (Sol II) was added and mixed immediately by several quick inversions. The mixtures were incubated at room temp for 7 min. Then 300 µl of ice cold 3 M sodium acetate (Sol III) was added and mixed immediately by repeated inversions followed by incubation at room temp for 5 min. The mixtures were then centrifuged at 4000 rpm for 15 min at room temp. The supernatant was transferred to a new eppendorf tube and an aliquot of 650 µl isopropanol (room temp) was added. The samples were mixed well and centrifuged for 15 min at 4 ^{0}C in eppendorf microfuge. The supernatant was discarded and pellet was resuspended in 320 µl of sterile dist water. An aliquot of 250 µl of 7.5 M ammonium acetate containing 0.5 mg/ml of ethidium bromide was added and mixed in equal vol of chloroform: iso-amyl alcohol, mixed well, followed by centrifugation at maximum speed for 5 min at room temp. Then the upper layer was transferred to new eppendorf tube carefully and again extracted with chloroform: iso amyl alcohol. The upper phase was removed to new tube and an equal vol of isopropanol (-20 ^{0}C) was added and kept at room temp for 30 min or at -20 ^{0}C overnight. The samples were centrifuged at 12,000 rpm for 15 min at 4 ^{0}C. The supernatant was discarded and the pellet was washed with 70 % ethanol, dried using speed vac concentrator (Martin Christ) and resuspended in 20 µl of TE buffer. The samples were incubated at 63 ^{0}C for 10 min and electrophoresed.

3.6.2.2 Agarose gel electrophoresis

Plasmid DNA samples were electrophoresed on the agarose gel (0.7%) by following the procedure of Sambrook *et al.* (1989).

3.6.2.2.1 Procedure

Agarose (0.7%) was made by dissolving 0.35 g in 50 ml or 1.1 g in 150 ml of 1X TAE buffer (pH 8.0) in microwave oven. The surface was leveled and molten agarose was cooled to 37 ^{0}C, ethidium bromide was added at a final

conc of 0.1µg/ml and poured in casting tray of midi gel using appropriate comb (8 wells). After complete setting of gel the comb was taken out carefully and mounted the plate in submarine gel electrophoresis system (Bio Rad).The electrophoresis tanks were filled with 1XTAE buffer to cover the gel to a depth of about 1 mm. The DNA samples were mixed with 1X TAE with 5 µl of tracking dye and were loaded slowly into the slots of submerged gel using a micropipette. Electrophoresis was carried out at 80 volts for 45 min to one h. The electrophoresed gel was examined under UV trans illuminator (302 nm; Photodyne) and were photographed using a Polaroid camera (Photodyne) loaded with Polaroid type 55 and 57 sheet film (MP4 system).

3.6.3 Protein Profiling

The comparison of protein profiling of parent and adapted cells was initially tried with Laemelli (1970) method with modifications as described below. Before proceeding for electrophoresis the preparation of protein samples and quantitation were described.

3.6.3.1 Sample preparation

Log phase cells of parent (120 ml) and adapted cells (240 ml) were centrifuged at 13,000 rpm/15 min in 4 ^{0}C in a Kubota 6800 refrigerated centrifuge. Then the cell pellets were washed twice in 50 mM sodium phosphate buffer (pH 7.0). Two to three ml of sonication buffer (pH 7.0) was added to each cell pellet and kept at -20 ^{0}C overnight. The pellets were thawed to room temp and sonicated on ice using sonicator (Branson Sonic Power Company, Danbury, Comnecticut) for a period of 4 to 5 min (30 sec burst with one min gap and cooling it on ice in between).

3.6.3.2 Quantitation/estimation of protein

3.6.3.2.1 Assay procedure

For this purpose Bradford (1976) method was followed. An aliquot of 0.3 ml of protein sol was mixed with 3 ml of dye. The absorbance was read at 595 nm against a reagent blank in which protein sol was substituted with 50 mM phosphate buffer. The absorbance was read at 595 nm after 5 min (not

were cold shocked for 2, 4 and 6 h at 20 ^{0}C. Log phase cells grown at 42 ^{0}C were taken as control. The samples were removed after 2, 4 and 6 h of cold shock, washed twice in 50 mM phosphate buffer (pH 7.0). Samples were resuspended in sonication buffer (pH 7.0) (4 to 5 ml) and kept at 20 ^{0}C overnight. The pellets were thawed to room temperature and sonicated on ice for a period of 4 to 5 min (30 sec burst with one min gap and cooling it on ice in between). The sonicated samples were centrifuged (5000 rpm/15 min). The supernatant were taken for protein estmation.

3.6.3.4.2 Quantitation/estimation of protein

Estimation of protein was performed by Bradford (1976) method. The procedure was essentially same as described in section 3.6.3.2.1.

3.6.3.4.3 Tricine SDS-PAGE

Tricine SDS PAGE method described by Schagger and Jaggow (1987) were performed to separate proteins of low molecular weight.

3.6.3.4.3.1 Protocol

3.6.3.4.3.1.1 Gel preparation

Prepared the separating gel and poured it immediately to a height of 3 cm into the gel cassette with 0.5 mm spacers. After complete setting of the separating gel, spacer gel was layered over the polymerized gel. Finally stacking gel was layered over the polymerized spacer gel and the comb was placed into the stacking gel. After polymerization of the gel the comb was removed carefully.

3.6.3.4.3.1.2 Sample preparation

Before loading the sample onto the gel, sample sol. was mixed with sample buffer in the ratio of 1:2 (in case of 3X buffer) and 1:5 (in case of 6X buffer). The resultant mixture was heated to 100 ^{0}C for 5 min. Similarly molecular weight markers were prepared. Low and high molecular weight markers were procured from Bangalore Genei Pvt. Limited.

3.6.3.4.3.1.3 Electrophoresis

The reservoir buffers were properly chilled and poured into the electrophoretic unit. 20 µl of each heat treated sample was loaded to different

wells in the gel. The upper tank buffer was carefully layered over the sample and the lower chamber was filled with lower tank buffer to the level where it just touched the bottom of the gel. Electrophoresis was carried out at 4 ^{0}C at a constant voltage of 40 V for 6 h.

3.6.3.4.3.1.4 Fixation

After electrophoresis the gel was dipped in fixing sol and kept at 60 ^{0}C for 20 min.

3.6.3.4.3.1.5 Staining

At the end of fixation step, the gel was kept in staining sol for 40 - 45 min.

3.6.3.4.3.1.6 Destaining

After staining for 40 to 45 min., the gel was transferred to the destaining sol (10% glacial acetic acid) at room temp for 2 h with renewal of destaining sol after every 30 min. During destaining the gel was never swirled to avoid diffusion of protein bands.

3.6.3.4.4 2D-Gel electrophoresis

The samples were lyophilized, protein was estimated by Bradford (1976). The samples were clean up by 2D-clean up kit (Amersham biosciences), to remove salts, nucleic acids etc., to avoid streaking. High resolution 2D PAGE were followed as per standard procedure O' Farrell (1975). IPG strips were rehydrated using rehydration buffer. 200 µg of sample was mixed with 100 µl of rehydration buffer. It was loaded on IPG strips (17 cm, pH gradient range 4-7, Biorad). Overnight rehydration was done at 20 ^{0}C. Isoelectric focusing (IEF) was carried out (PROTEAN IEFCELL, BIORAD) in a three step process with a total final voltage of 50,000 VH.

1st Step	-	500 V /1h
2nd step	-	1000 V/1h
Final step	-	4000 V/2.5 h

The IEF was carried out overnight at 20 ^{0}C.

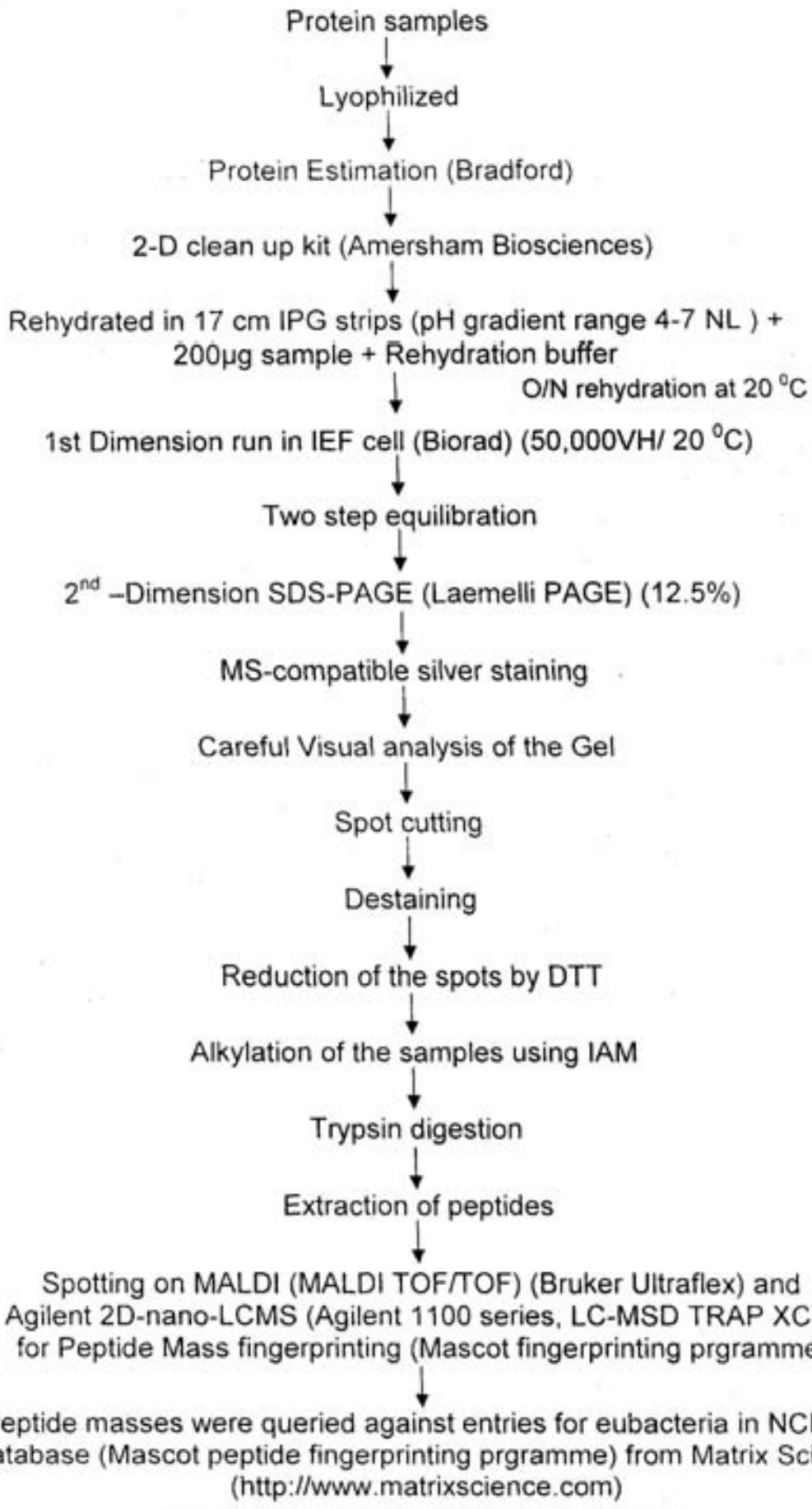


Fig. 3.5: Protocol for proteomic analysis

3.6.3.4.4.1 Equilibration

Prior to running the second dimension, it is necessary to equilibrate the IPG strips in SDS-containing buffers. The two step equilibration also ensures that cysteines are reduced and alkylated, which minimizes or eliminates vertical streaking that may be visible after straining of the second dimension gels. Equilibration buffer I contains DTT which reduces sulfhydryl groups, while equilibration buffer II contains iodoacetamide which alkylates the reduced sulfhydryl groups. The second dimension SDS PAGE was carried out as per standard procedure in 13 % gel (Laemelli, 1970). Molecular weights and isoelectric points were calibrated by using comigrating standards (Amersham Biosciences). The gels were silver strained by MS compatible silver staining as per Blum *et al.* (1987).

3.6.3.4.4.2 Analysis of gels

Careful visual analyses of scanned gel pictures were done to differentiate spots in control and cold shocked cells.

3.6.3.4.5 Peptide mass finger printing (PMF) by matrix assisted laser desorption ionization (MALDI) – time flight (TOF) mass spectrometry and 2D Nano LCMS

In gel tryptic digestion of two dimensional protein spots was performed as follows. Gel pieces were washed twice in 25 mM ammonium bicarbonate buffer (pH 8.0) containing 50 % (vol/vol) acetonitrile prior to vacuum drying. Rehydration was done with 50 mM ammonium bicarbonate buffer (pH 8.0) containing 0.02 g of sequencing grade modified porcine trypsin (Promega, Madison, Wis.)/l. Trypsin digestion was performed for 18 h with a thermomixer (Eppendorff, Hamburg, Germany) at 37 ^{0}C and 500 rpm and stopped by addition of 0.4% (vol/vol) trifluoroacetic acid. Resulting peptides were recovered in the supernatant and spotted directly onto a MALDI plate and also loaded into 2D-Nano LCMS. All spectra were recorded in positive reflector mode and with a delayed extraction of 130 ns and a 62 % grid voltage. The spectra were calibrated by using an external calibration mixture. Peptide masses were queried against entries for eubacteria in the NCBInr database

by using the Mascot peptide mass fingerprinting program from Matrix science (http://www.matrixscience.com). For a match to be considered, a minimum of three matching peptides were required.

3.6.4 Biochemical Characteristics

The parent cultures and the adapts were examined for their ability to ferment lactose for acid production and proteolytic activity in milk. A freshly grown milk culture (16 h) of the test organism was inoculated at the rate of 1% in sterilized 11% reconstituted non-fat dry cow milk (NFDCM) and incubated at 42 ^{0}C. After 16 h, the inoculated milk samples were analysed for developed acidity and proteolytic activity.

3.6.4.1 Developed acidity (ISI:1981)

The curdled milk samples diluted by mixing 10 g of thoroughly mixed curd with 10 ml of dist water. The mixtures were titrated against N/9 NaOH using phenolphthalein (0.1%) as indicator. The values were expressed as percent lactic acid as per the following formula:

% Lactic acid=		9VN/W
V	=	Vol of N/9 NaOH used
N	=	Normality of NaOH
W	=	Weight of curd samples.

3.6.4.2 Proteolytic activity (Hull, 1947; Lowry et al., 1951)

3.6.4.2.1 Procedure

Two ml of 12.5 % TCA was added to 1 ml of the curdled milk sample. After thorough mixing, the samples were allowed to settle at room temp for 15 min. The samples were filtered through Whatman No. 42 filter paper to get clear filtrates. Five ml of alkaline carbonate reagent was added to 1ml of each 1:10 diluted filtrate. The mixture was kept for 10 min. It was followed by the addition of 0.5 ml of Folin-Ciocalteau reagent. The samples were mixed well and allowed to proceed for the reaction for 30 min at room temperature. The blue colour developed was measured at 660 nm using Spectrophotometer (Pye Unicam SP-30 UV, UK). The activity was calculated by "reading off" the standard tyrosine curve and expressed in equivalent amount of tyrosine.

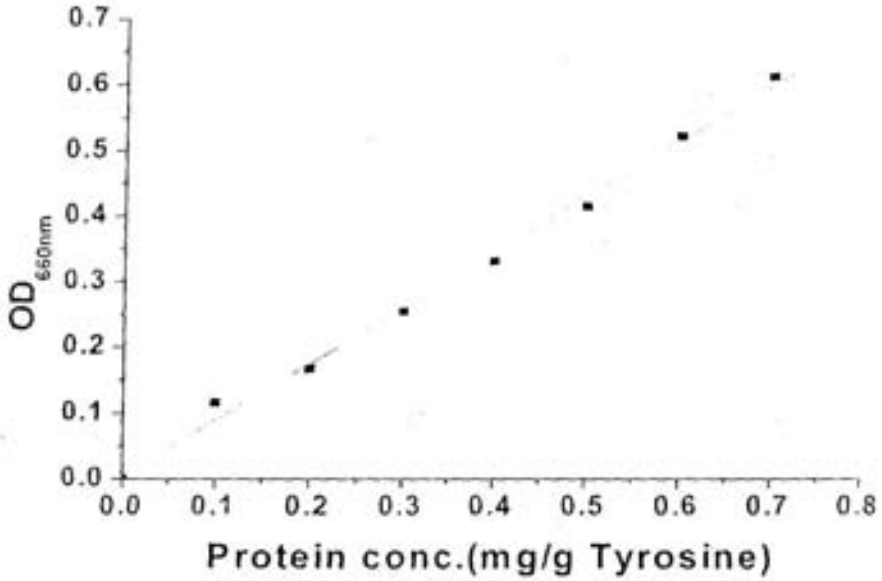


Fig. 3.6 Standard curve for estimation of proteolytic activity

3.6.5 Freeze-Thaw Survival Capacity

One ml broth cultures of parents and adapts were spun down for 5 min at 13, 000 rpm and resuspended in 1 ml of fresh MRS or LM 17 media. The suspensions were subsequently frozen at –20 ^{0}C for exactly 24 h and then thawed for exactly 4 min at 30 ^{0}C in a water bath. The number of cfu was determined just before freezing and after thawing in four consecutive cycles of freeze-thaw challenges (24 h freeze periods and thawing for 4 min at 30 ^{0}C) by spread plating appropriate decimal dilutions on LM17 and MRS agar after 2 d incubation at 42 ^{0}C. In a separate set of experiments chloramphenicol (100 μg/ml) was added during adaptation.

3.6.6 Probiotic Properties

3.6.6.1 In vitro tolerance to simulated pH of the Human Stomach (Clark *et al.*, 1993)

Dist water was taken in a beaker and reagent grade hydrochloric acid (35.8 – 36.5 %, Qualigens, India) was added drop by drop, to obtain solutions having pH values of 1.0, 2.0 and 3.0. Sterile double glass dist water (pH 6.4) served as control. The solutions were prepared in vol of 100 ml each, transferred into separate, screw-capped bottles (Borosil), autoclaved at 121

^{0}C (1 atm or 1.1 kg per sq cm) for 20 min and stored at room temp until needed. Stored solutions were thoroughly mixed and 10 ml of each pH solution was transferred aseptically into a sterile test tube. One ml of stock cultures raised in MRS broth (pH 6.5 for parents, pH 4.3 and pH 4.0 for adapts of lactobacilli) and LM17 broth for *S. thermophilus*), containing ~ 10^9 cfu/ml were then added to the four pH solutions (1.0, 2.0, 3.0 and 6.4). Contents of the tubes were mixed. One ml of culture from each tube was taken immediately (time, 0 h) and appropriate decimal dilutions prepared using 9 ml blanks of 0.1% peptone. Pour plating was done using MRS agar (pH 6.5) for parent culture, MRS agar (adjusted to pH 4.3 and pH 4.0) for adapts of lactobacilli and LM 17 agar for streptococci. The inoculated test pH sol media were incubated at 42 ^{0}C. One ml of culture was removed from each of these tubes after 1, 2 and 3 h, and the plating procedure repeated. The plates were incubated at 42 ^{0}C for 72 h after which, the colony forming units were counted and recorded.

3.6.6.2 *In vitro* tolerance to Simulated Bile Concentrations of the Human Small Intestines (Clark and Martin, 1994)

Bile salts (Hi-media, India) sol with 1.0 and 2.0 per cent conc were prepared using double glass dist water, sterile double glass dist water served as control (0%). The sol were prepared in vol of 100 ml, transferred into screw-capped bottles (Borosil), autoclaved at 121 ^{0}C (1 atm or 1.1 kg per sq cm), and stored at room temp until required.

The sol were mixed well and 10 ml of each was transferred into a sterile test tube. One ml of stock culture raised in MRS broth (pH 6.5 for parents, pH 4.3 and pH 4.0 for adapts of lactobacilli) and LM17 broth for *S. thermophilus*, containing ~ 10^9 cfu/ml were then added to the bile sol (0, 1.0 and 2.0 %). The contents of the tubes were mixed. One ml of culture was removed from each tube immediately (0 h) and used to prepare dil series; 9 ml blanks of 0.1 percent peptone were used for this purpose. The bile sol containing culture were incubated at 42 ^{0}C. Pour plating was done using MRS agar (pH 6.5) for parent culture, MRS agar (adjusted to pH 4.3 and pH 4.0) for

adapts of lactobacilli and LM 17 agar for streptococci. The plates were incubated at 42 ^{0}C for 72 h. One ml of culture was again removed from each tube after 3 and 12 h, and the pour plating procedure repeated. After incubation was complete, colony counts were recorded.

3.6.6.3 Cell Surface Hydrophobicity

Two methods namely adhesion to hexadecane and salting out aggregation test were carried out to assess cell surface hydrophobicity.

3.6.6.3.1 Adhesion to hexadecane

The method of Rosenberg *et al.* (1980) with slight modifications was adopted to measure the cell surface hydrophobicity.

3.6.6.3.1.1 Protocol

The bacterial cells grown at 42 ^{0}C for 18 h, were harvested by centrifugation, washed twice with PUM buffer and re-suspended in PUM buffer. The absorbance was adjusted to 0.8 to 0.9 at 610 nm using spectrophotometer (UV-Vis Spectrophotometer, CARY1).

Hexadecane, 0.4 ml was added to 2.4 ml of bacterial suspension in acid washed test tubes and incubated at 37 ^{0}C for 10 min. The two phases were mixed, under controlled conditions for 2 min at full speed. The tubes were kept at 37 ^{0}C for 1h for phase separation. The aqueous phases were separated with a micropipette and the light absorbance of the aqueous phase was measured at 610 nm. The fraction of adherent cells was taken as percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared to that of original suspension.

$$\text{Percent cell hydrophobicity} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

3.6.6.3.2 Salting out aggregation test (SAT)

This method was adopted from Lindahl *et al.* (1981). Sodium phosphate (0.002M, pH 6.8) was used to dilute a solution of 4 M $(NH_4)_2\ SO_4$ (pH 6.8 adjusted with NH_4OH) and was further diluted using sodium phosphate (0.0002 M, pH 6.8). Serial dil were made to get ammonium

sulphate concentrations ranging from 3.0 to 0.2 M with gradients of 0.2M per dilution and the solutions ranging from 0.20 to 0.02 M differing by of 0.02 M. When necessary the pH was adjusted to pH 6.8 using NH_4OH. A bacterial suspension of 25 ml (5×10^9 cells/ ml) in 0.002 M phosphate buffer, pH 6.8 was mixed with equal volume of salt solutions on the depression of a concavity glass slide. The bacteria and salt solution mixture was gently mixed and kept for five min at 20 ^{0}C. Visual examination was conducted against black background for separation of bacterial aggregates.

3.6.6.4 Antimimicrobiocity

3.6.6.4.1 Agar well and disc assay

The indicator organism was grown for 16 to 18 h at their optimum growth temperature. 100 µl of the culture was transferred to 5 ml nutrient broth and incubated for four hours so that all the cells should be in the log phase. 50 µl of this culture was transferred to soft agar, kept for 15 min and then poured onto the base plates already prepared and kept at low temp and allowed for half an hour for proper solidification. The soft agar was allowed to solidify to generate a potential mat of the indicator bacteria. The plates were refrigerated at 5 ^{0}C for an hour before wells were punched of the agar with the cork borer. The bottom of the wells sealed and 100 µl of the supernatant was transferred. The plates were once again refrigerated at 5 ^{0}C for 3 - 4h to facilitate the diffusion of antibacterial compounds and then were incubated at 37 ^{0}C for 18 h. A clear zone of 1mm or greater extending laterally from the edge of wells was considered positive inhibition. In case of disc assay instead of punching wells, sterile filter paper disc was placed on refrigerated indicator lawn and 20 µl of culture supernatant were spotted on the discs.

3.6.6.4.2 Spot on lawn assay

Here 5 µl of the culture supernatant was directly applied on the indicator lawn instead of filter paper discs and examined for presence or absence of zones of inhibition. The inhibitory activity was expressed in terms of AU.

3.6.6.4.3 Definition of arbitrary unit (AU)

One unit of antibacterial activity was defined as the amount required to produce an inhibitory zone of 1.0 mm surrounding the well (5 mm) when 10^6 organisms of indicator strain were spread on the agar plate (92 mm diameter).

3.7 GENOME SHUFFLING OF SELECTED CULTURES BY PROTOPLAST FUSION

Genome shuffling was carried out by protoplast fusion method.

3.7.1 Protoplast Formation and Regeneration

Protoplasts of selected strains of *Streptococcus thermophilus* NCDC 74 and *Lactobacillus* spp. (*L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14) and their adapts were prepared.

The method of Fujita *et al.* (1983) for *Streptococcus thermophilus* and that of Cocconcelli *et al.* (1986) for *Lactobacillus* spp. were followed with some modifications. All the reagents are given in the appendices (Annexure-i)

3.7.1.1 Protoplast formation

3.7.1.1.1 Lactobacilli

Parent *L. delbrueckii* subsp. *bulgaricus* NCDC 307 and *L. acidophilus* NCDC 14 cells were cultured overnight in MRS broth at 42 ^{0}C in 50 ml screw cap test tubes. Adapts were cultured overnight in 100 ml MRS broth at their respective pH (i.e. pH 4.3 for *L. delbrueckii* subsp. *bulgaricus* NCDC 307 adapts and pH 4.0 for *L. acidophilus* NCDC 14 adapts). Parents were inoculated at the rate of 2 % to 30 ml of prewarmed MRS broth for control. A 4 % inoculum of each adapt was similarly used. Parents were grown for 4 h at 42 ^{0}C to an OD_{650} of 0.6 (approx $1x10^8$cfu) and the adapts for 12 h at 42 ^{0}C. Then all the four cultures were ice chilled, harvested by centrifugation (Kubota 6800, Japan) at 6,000 rpm for 10 min and washed twice with LBP at 4 ^{0}C. The cells were resuspended in 5 ml of LBP containing 10 mg/ml lysozyme and 30 µg/ml mutanolysin. The suspensions were incubated at 42 ^{0}C for varying periods ranging from 0 - 120 min and checked for the formation of protoplasts (spherical cells) under phase contrast microscope. The enumeration of

number of osmotically resistant colonies before and after their treatment for protoplast formation was carried out for the purpose of calculation of frequency of protoplast transformation.

3.7.1.1.2 *Streptococcus thermophilus*

For *S. thermophilus* parent and adapted cultures were maintained in LM17 medium. The procedure for protoplast formation was essentially same as described above for lactobacilli except that the buffers used were Tris HCl for washing and THMS for protoplast formation. The progress of protoplast formation of the test cultures was judged by microscopic examination.

3.7.1.2 Protoplast regeneration

3.7.1.2.1 Protoplast regeneration in lactobacilli

The protoplasts of lactobacilli were centrifuged at 6000 rpm, resuspended in equal vol of lactobacillus protoplast buffer (LBP) and plated on fortified MRS agar. The regenerated colonies were counted after 5 d of incubation at 32 ^{0}C in an anaerobic jar (BBL, Gas Pack Anaerobic system). Osmotically fragile and infragile cells were determined by making dilution of the incubation mixture in THMS buffer and dist water respectively and plating onto LM17 lactose agar.

The procedure is same as described for *S. thermophilus* except that the lactobacillus protoplast buffer was used for dilution and fortified MRS agar medium was used.

3.7.1.2.2 Protoplast Regeneration in S. thermophilus

The procedure was same as described for lactobacilli except that the THMS buffer was used for dilution and fortified LM 17 agar was used.

The percent regeneration calculated as follows:

$$\text{Percent regeneration} = \frac{\text{Cfu on regeneration medium - Cfu on MRS or LM 17}}{\text{Initial cell no}} \times 100$$

3.7.1.3 Protoplast fusion and regeneration of fusants

Attempts were made to fuse the protoplasts of parents and the respective adapts of lactobacilli as well as streptococci. The method followed

was essentially same as that of Patnaik *et al.*, 2002). Equal vol of protoplasts formed as in section 3.7.1.1 from corresponding populations were mixed, centrifuged and resuspended in 50 ml of lactobacillus protoplast buffer (LBP) for lactobacilli and THMS buffer for streptococci. Nine vol of 60 % PEG 6000 were mixed in 5 ml LBP or THMS and incubated for six min at room temp. The fused protoplast preparation thus obtained was centrifuged, washed with LBP or THMS, resuspended in 100 ml LBP or THMS respectively and serial dilutions were plated in RM and incubated anaerobically at 37 ^{0}C regenerated on RM at 37 ^{0}C. The plates were scraped to generate a pooled fusion of library. The formation of protoplasts, their fusion and subsequent regeneration was repeated 3 times in case of *L. acidophilus* whereas in *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* it was repeated only once using the pooled regenerated cells from one fusion being the inoculum for the subsequent protoplast culture.

Samples from each of the pooled fusion libraries (F1-F3) were saved fro latter analysis. Non-shuffled controls were prepared by the recursive formation and regeneration of protoplasts without exposure to PEG.

3.7.1.4 Analysis of fusants

3.7.1.4.1 Probiotic properties

The parameters studied were the same as described in the section of 3.6.6. for the parents and their respective adapts.

3.7.1.4.2 Freeze thaw survival capacity

The procedure followed for determining freeze thaw survival capacity of the fusants was same as described in the section of 3.6.5.

3.7.1.4.3 Cell morphology

Cell morphology of fusants was studied by light microscopy.

3.8 Freeze Drying of selected cultures for submission to NCDC

3.8.1 Freeze-Drying of Selected Parent and Adapted Cultures

Method followed was as per Sinha *et al.* (1972) with slight modifications for freeze drying of the parent, adapts and fusants of lactobacilli and streptococci.

3.8.1.1 Preparation of cell suspension

Cells of parent, adapts and fusants of the rods and cocci (*L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14, and *S. thermophilus* NCDC 74) were subclutured twice in MRS and LM17 broth respectively. The final subculture was done at the rate of 2 % in 50 ml (in case of parent) and 100 ml (in case of adapts and fusants) of respective broths and incubated at 42 ^{0}C. The cultures obtained at the end of logarithmic phase were then centrifuged, washed twice in normal saline and resuspended in 3 ml of sterile reconstituted skim milk in each case.

3.8.1.2 Freeze drying procedure

Aliquots (0.2 ml) of the cell suspension prepared as described above were transferred to freeze drying ampoules (100 x 6 mm). Freeze drying of the cell suspensions was carried out in Edward's centrifugal freeze drier (Model 5 PS) in two stages, namely primary freeze drying at 20 – 25 μ Hg for 3 h followed by secondary drying for 1 h. The tubes were sealed under vacuum at 20 to 25 μHg towards the end of secondary drying. The ampoules were stored in a deep freeze at -20 ^{0}C. Sufficient ampoules were prepared for analysis as well as submission to NCDC.

3.9 APPLICATION OF DEVELOPED THERMOPHILIC LACTIC ACID BACTERIAL STRAINS FOR FERMENTED MILK PRODUCT FORMATION

A preliminary study was made to ascertain the usefulness of the developed cultures in milk product formation. After the adapts and fusants developed from the test thermophilic LAB, were evaluated regarding their various probiotic attributes, the best one among them was selected for formation of a fermented milk product.

3.9.1 Preparation of Fortified Yoghurt Product

From the previous experiments it was found that the fusants of *L. acidophilus* NCDC 14 (C_{14}) i.e. F3 $^{4.0}pH_{14}$ – 14 proved to be most promising.

The method for preparing yoghurt containing sufficient (>10^5 cfu/g) viable cells of *L. acidophilus* NCDC 14 and F3 $^{4.0}pH_{14}$ – 14. Hence, these fusants were studied further. Buffalo milk was standardized to 4.5 % fat, heat treated to 90 ^0C for 5 minute and cooled rapidly to room temp. Then it was divided into four groups and inoculated as follows:

Group I	Standard yoghurt prepared with NCDC-144 culture (1%).
Group II	NCDC 144 (1%) + C_{14} (1%)
Group III	NCDC 144 (1%) + $^{4.0}pH_{14}$ (2%)
Group IV	NCDC 144 (1%) + F3 $^{4.0}pH_{14}$ - 14 (2%)

Higher inocula of the developed cultures were taken for their compatible growth in the final product. The seeded milk was distributed into food-grade, 100 ml ice-cream cups with lids (sterilized by hot water dipping, followed by UV-irradiation) upto the brim and incubated at 42 ^0C until setting (7-8h). Freshly prepared probiotic yoghurt conformed to the recommendations laid down for probiotic foods (Lee and Salminen, 1995) with viable counts of 7.0 to 7.4 log cfu of *L. acidophilus* NCDC 14 and F3 $^{4.0}pH_{14}$ – 14 per g of product.

3.9.2 Analysis of Yoghurt

The yoghurt products prepared as above were analyzed for the following parameters.

3.9.2.1 Titratable acidity

It was determined according to the Bureau of Indian Standards (ISI, 1960).

3.9.2.2 Enumeration of viable organisms

Ten g aliquots of freshly prepared yoghurt products were drawn after mixing the contents of the containers thoroughly. Appropriate dilutions were made in sterile 0.1% buffered peptone water. The media used for enumeration of different lactic acid bacteria were as follows.

Table 3.3 Media for enumeration of parents, fusants and adapts in yoghurt

Organisms enumerated	Agar
S. thermophilus	M 17 (pH 6.5)
L. bulgaricus	MRS (pH 6.5)
L. acidophilus NCDC 14 (C_{14})	Salicin (pH 5.0)
Adapts of C_{14} ($^{4.0}pH_{14}$)	MRS (pH 4.0)
Fusants of C_{14} (F3 $^{4.0}pH_{14}$ – 14)	MRS (pH 4.0)

3.9.2.3 Sensory evaluation

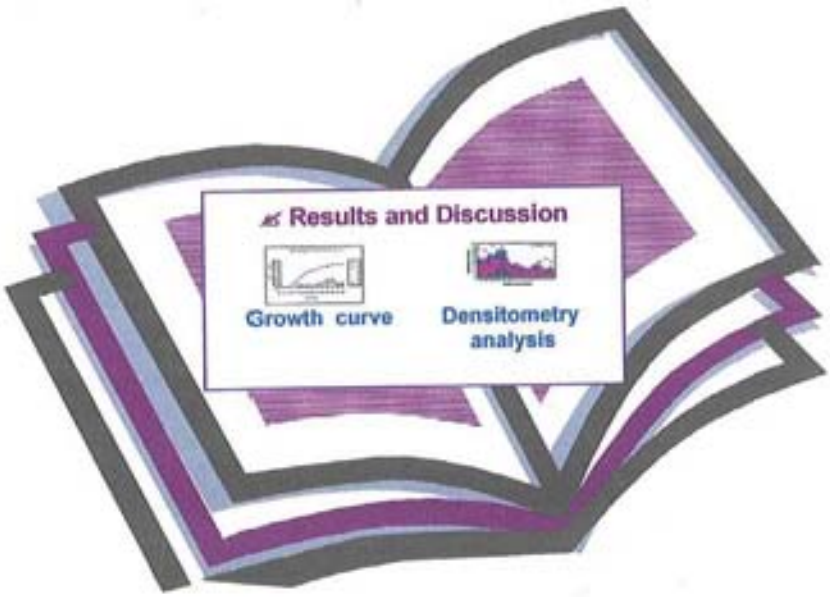
Fresh samples of standard and fortified yoghurt prepared in sterile ice cream cups as above were subjected to sensory evaluation by a panel of eleven experts. A 100 point score card (Annexure II) was used for the purpose of evaluation. Paired t-test was used to test the means (sensory scores) of two samples which are not-independent. Guidelines given by the Bureau of Indian Standards (ISI, 1971a, b) were followed strictly during sensory evaluation.

3.9.2.4 Storage studies

Control and fortified yoghurt products prepared as described in sub section 3.9.1 above were stored for up to 15 d at refrigeration temperature (4 to 7 ^{0}C). The samples were removed and analysed for their physical, microbiological and sensory attributes at 0, 5, 10 and 15 d as described in sub section 3.9.2.

3.10 DATA ANALYSES

The resultant data was analysed statistically by various statistical software e.g. Origin 6.1, DMFit 6.0, MS Excel 2003 etc. The 1D PAGE were analysed by Non linear dynamics software.



Results and Discussion

4.0 RESULTS AND DISCUSSION

The resultant data obtained by the conduct of present investigation has been presented in the form of tables, figures, graphs, histograms and photographs. All the results indicated therein represent the average values of replicate trials.

4.1 ANALYSIS FOR THE VIABILITY OF THERMOPHILIC LACTIC ACID BACTERIA (TLAB) UNDER ACID AND TEMPERATURE STRESS

One of the objectives of the present investigation was to analyse the viability of TLAB under cold and acid stress conditions. All the 36 TLAB of the genera (*Lactobacillus* and *Streptococcus*) available with the NCDC, NDRI, Karnal were screened.

4.1.1 Growth Measurement

For all the ten atmospheric conditions studied as applied to the 36 lactic cultures under test, a total of 360 growth curves were obtained from the resultant OD (optical density) and total viable cell counts (log cfu). The data pertaining to the same has been presented in Fig 4.1 to 4.71. The correlation coefficients calculated from OD and cell counts (log cfu) of each culture have been shown in the Tables 4.1, 4.3 and 4.5. Tables 4.2, 4.4 and 4.6 depict the growth rate (μ_{max}/h) of all the cultures after fitting the log cfu counts in DMFit curve fitting programme designed by Baranyi and Roberts (1994).

Fig 4.1 to 4.7 depict the growth curves (from $O.D_{650}$ and log cfu values) of *L. acidophilus* cultures at varying pH. All the strains grew well at pH 6.5 and pH 5.0., Growth was slow with a longer lag time at pH 4.5. No apparent growth occurred at pH 4.0 and pH 3.5. Table 4.1 demonstrated growth rate of all the cultures computed from DMFit programme version 2.0. *L. acidophilus* NCDC 14 showed higher growth rate at pH 5.0 and 6.5 (Table 4.2). The correlation coefficient ($r^2 > 0.8$) at three pH values was equally good as compared to other cultures (Table 4.1). Growth curves of all the *L. delbrueckii* subsp. *bulgaricus* strains at different pH have been given at Fig 4.15 – 4.29.

All the strains grew well at pH 5.0 and 6.5. However, strains NCDC 307 and NCDC 308 grew with a longer lag time at pH 4.5 which was ~9h in the former and ~15h in the latter. *L. delbrueckii* subsp. *bulgaricus* NCDC 307 showed overall growth rate of 0.05, 0.80, 0.63 at pH 4.5, 5.0 and 6.5 respectively (Table 4.4). The correlation coefficient (r^2) between OD_{650} and log cfu values of this strain was higher than 0.8 at all the pH levels (Table 4.3).

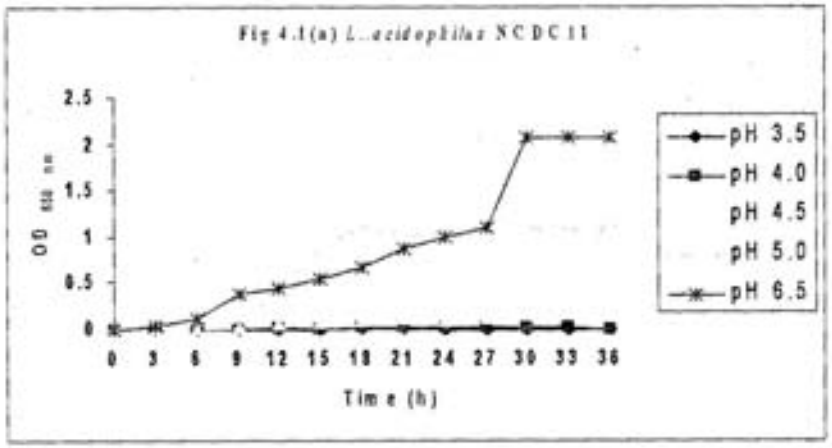
Table 4.1: Correlation coefficient (r^2) of *L. acidophilus* strains ($OD_{650\ nm}$ versus Log cfu) at different pH and temp (^{0}C)

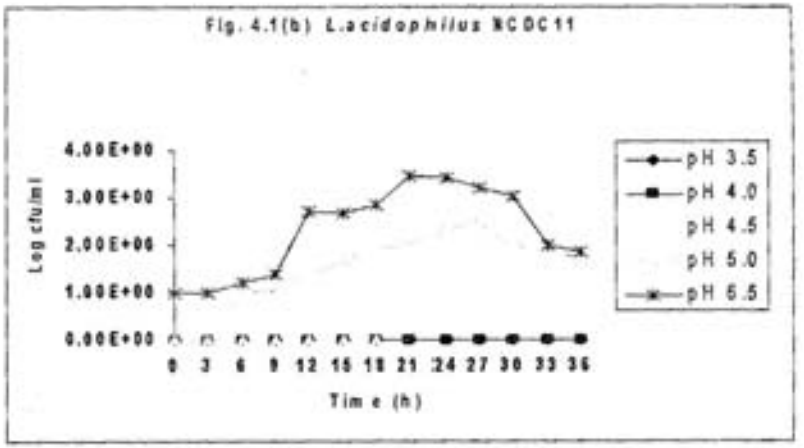
L. acidophilus strain no.	pH			Temp. (^{0}C)		
	4.5	5.0	6.5	20	25	42
NCDC 11	0.7129	0.8816	04030	0.7129	0.8816	0.4030
NCDC 13	0.8600	0.9421	0.7702	0.2844	0.6429	0.7702
NCDC 14	**0.8857**	**0.8617**	**0.9018**	0.8071	0.4849	0.9018
NCDC 15	0.5955	0.1431	0.3877	0.00	0.9124	0.3877
NCDC 16	0.2490	0.2490	0.6713	0.00	0.2498	0.1980
NCDC 195	0.2767	0.1848	0.4723	0.00	0.078	0.4723
NCDC 291	0.00	0.2041	0.9248	0.00	0.2921	0.9248

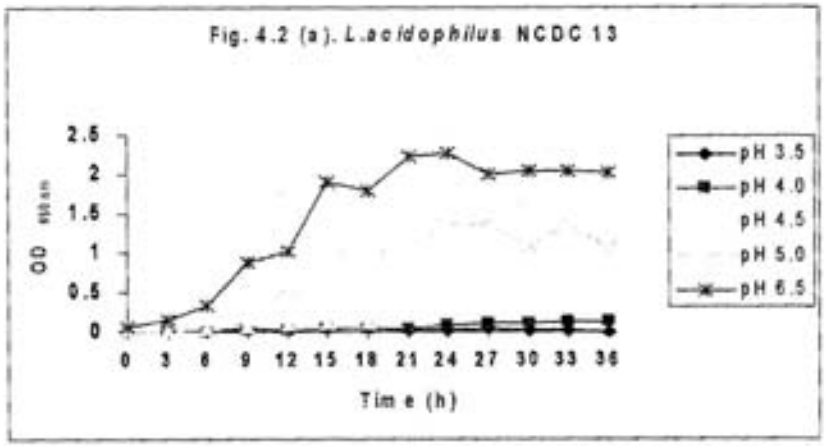
Table 4.2: Growth rate (μ_{max} / h) of *L. acidophilus* strains at different pH and temp (^{0}C)

L. acidophilus strain no.	pH			Temp (^{0}C)		
	4.5	5.0	6.5	20	25	42
NCDC 11	0.10	0.11	0.53	0.001	0.11	0.53
NCDC 13	0.10	0.27	0.30	0.001	0.01	0.30
NCDC 14	**0.047**	**0.71**	**0.72**	0.001	0.049	0.72
NCDC 15	0.012	0.13	0.075	0.00	0.001	0.075
NCDC 16	0.008	0.009	0.11	0.00	0.009	0.11
NCDC 195	0.00	0.00	0.16	0.00	0.047	0.16
NCDC 291	0.00	0.001	0.15	0.00	0.07	0.15

Fig 4.1 - 4.7: Growth curves of *L. acidophilus* strains at different pH (a: $OD_{650\ nm}$; b: Log cfu/ml)







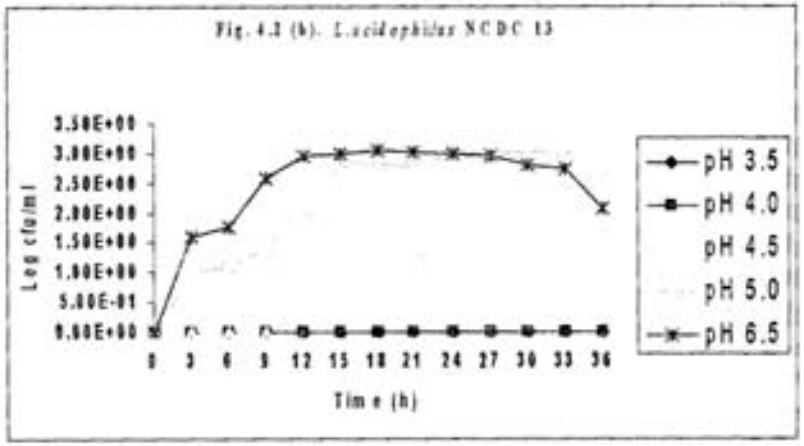


Fig. 4.3 (a). *L.acidophilus* NCDC14

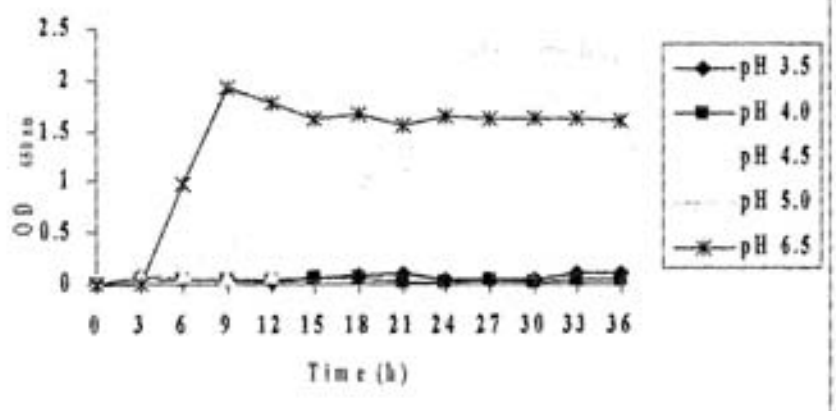


Fig. 4.3 (b). *L.acidophilus* NCDC 14

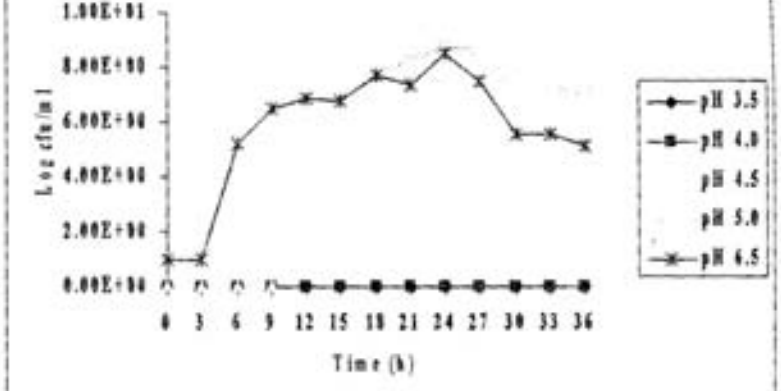


Fig.4.4 (a). Growth curve *L.acidophilus* NCDC15

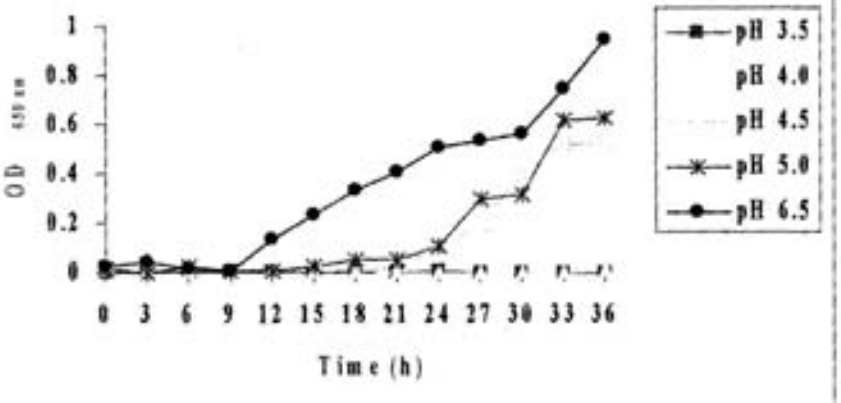
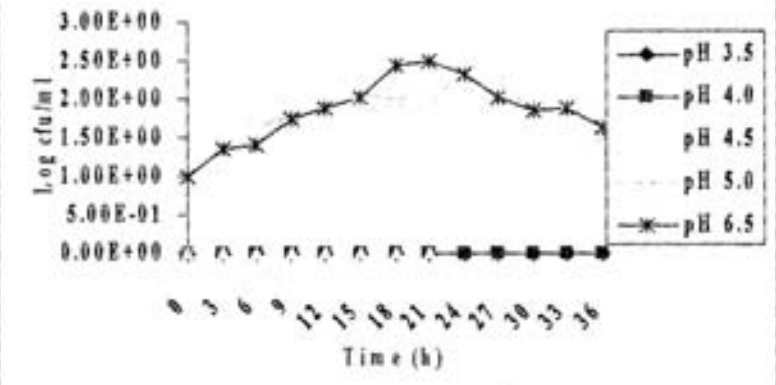
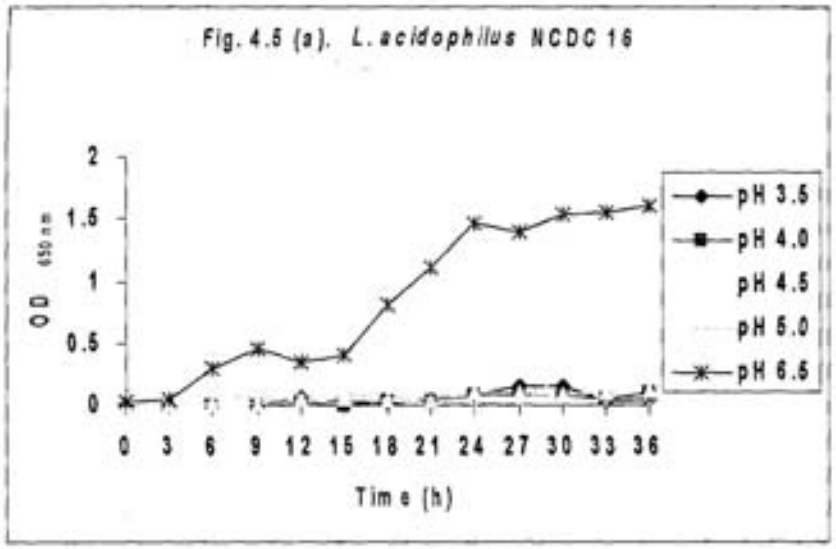
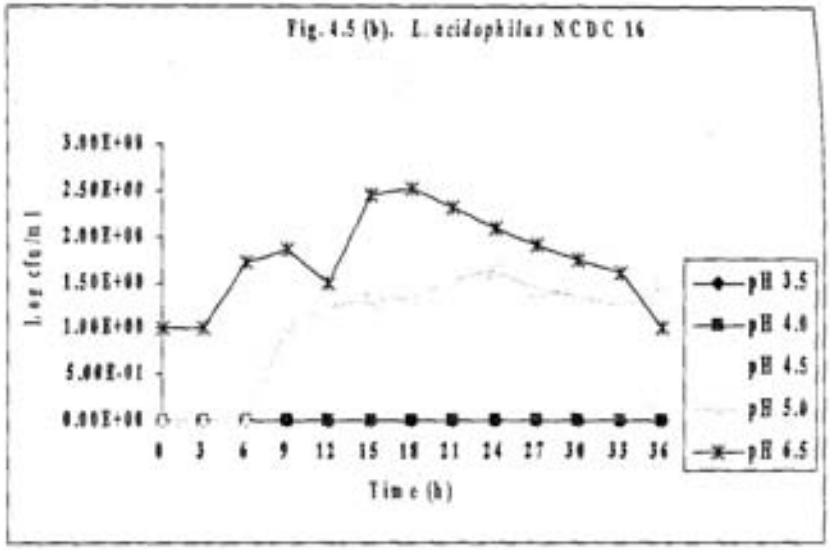
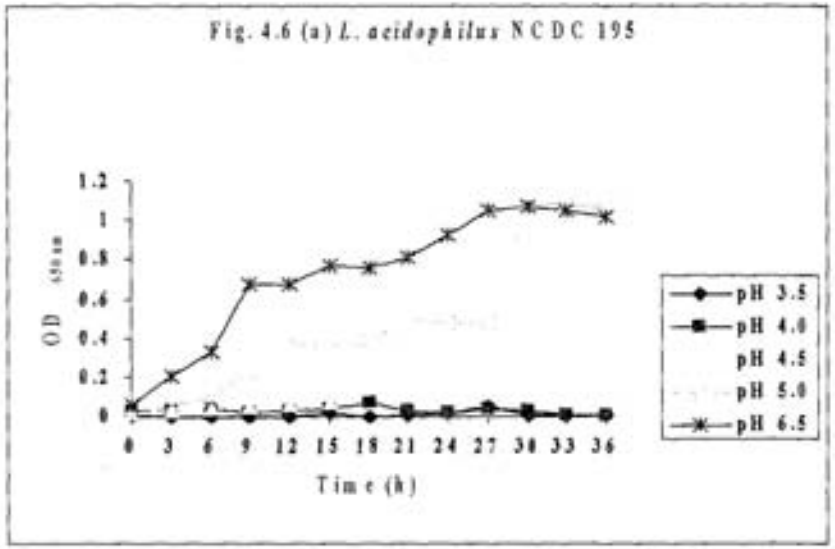


Fig.4.4 (b). *L.acidophilus* NCDC 15




Fig. 4.5 (a). L. acidophilus NCDC 16
OD 650 nm
2
1.5
1
0.5
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig. 4.5 (b). L. acidophilus NCDC 16
Log cfu/ml
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig. 4.6 (a) L. acidophilus NCDC 195
OD 650 nm
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5

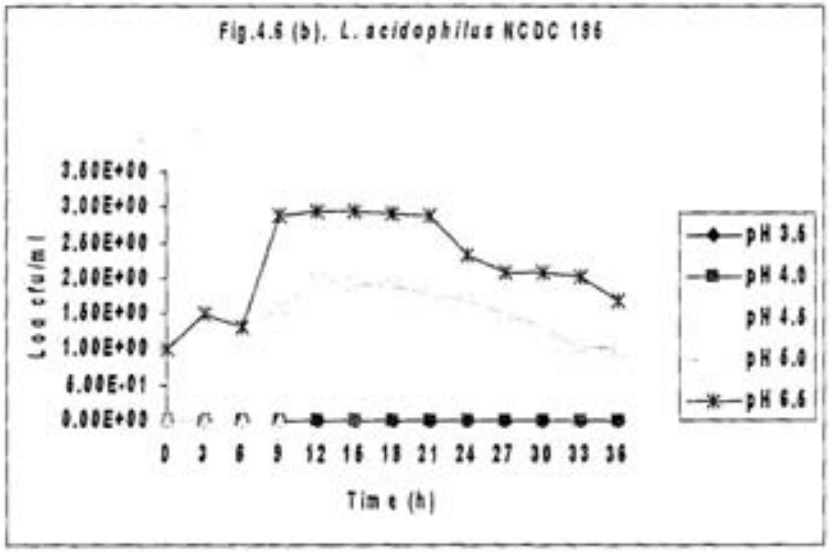

Fig.4.6 (b). L. acidophilus NCDC 195
Log cfu/ml
3.50E+00
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5

Fig. 4.7 (a). *L. acidophilus* NCDC 291

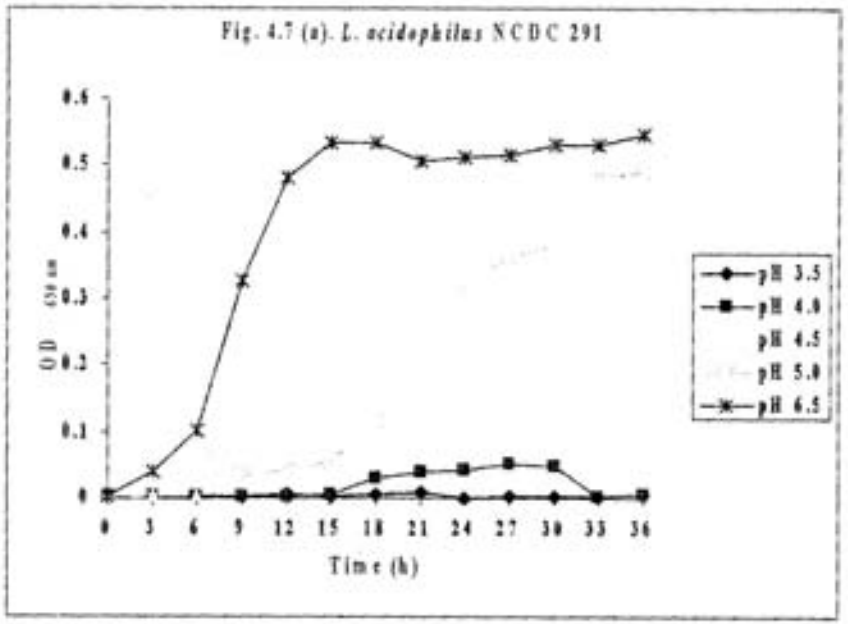


Fig. 4.7 (b). *L. acidophilus* NCDC 291

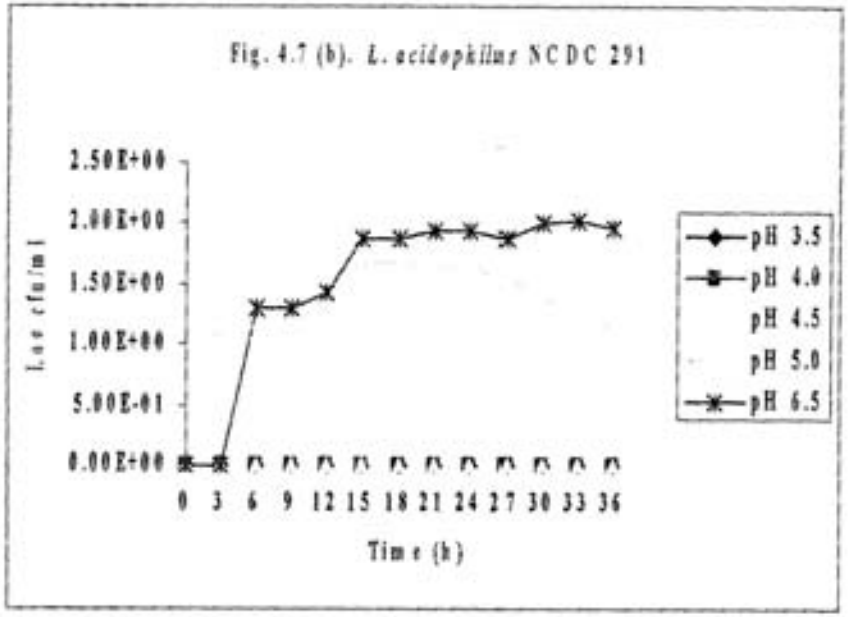
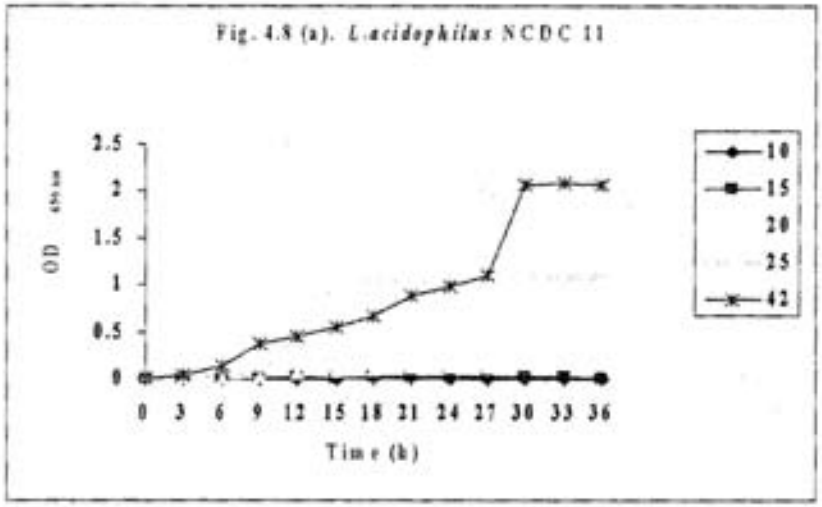
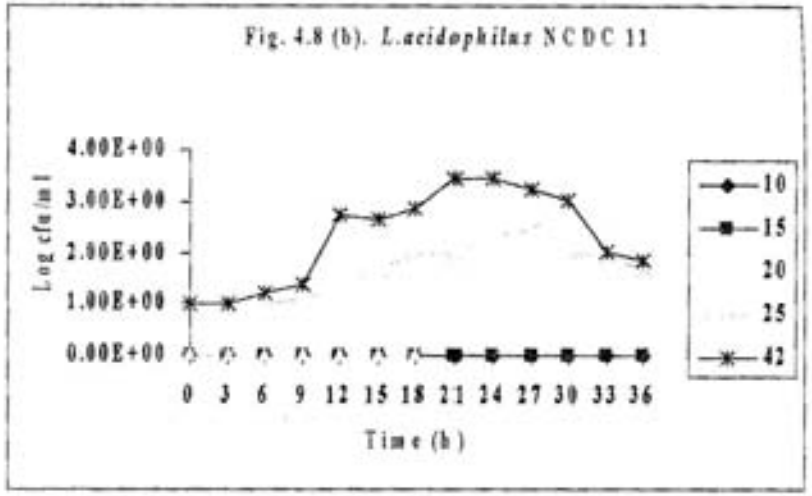
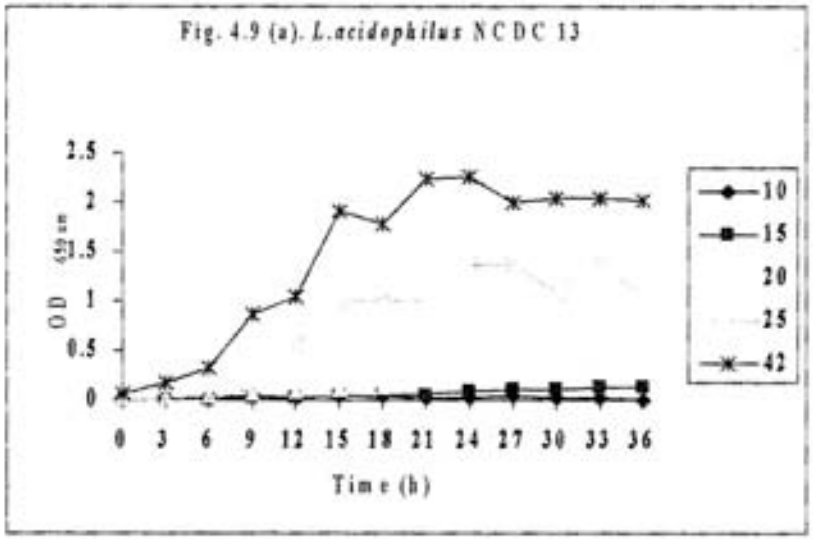


Fig 4.8 - 4.14: Growth curves of *L. acidophilus* strains at different temp (^{0}C) (a : $OD_{650\ nm}$; b: Log cfu/ml)







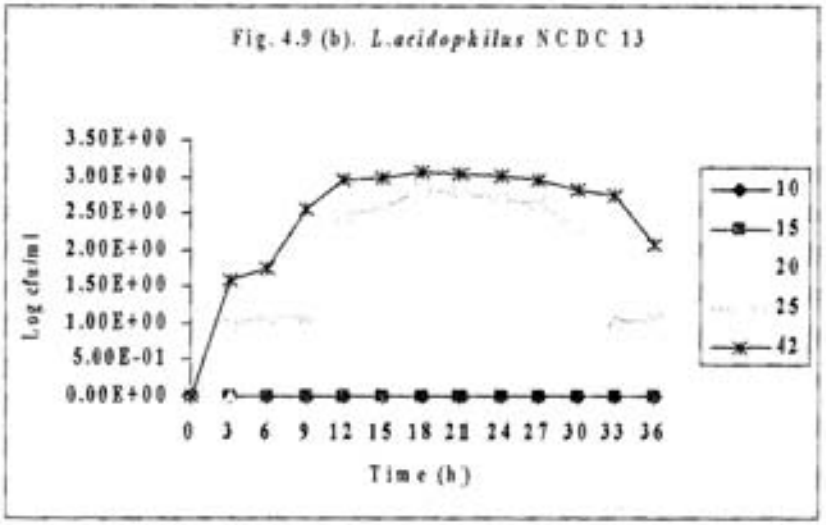


Fig. 4.10 (a). *L. acidophilus* NCDC 14

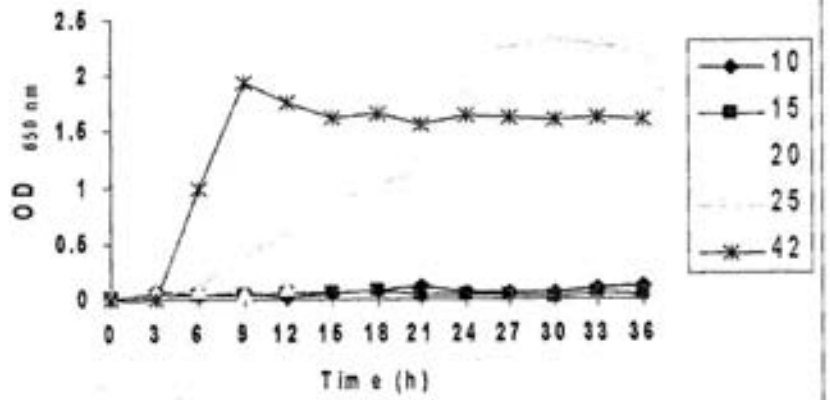


Fig. 4.10 (b). *L. acidophilus* NCDC 14

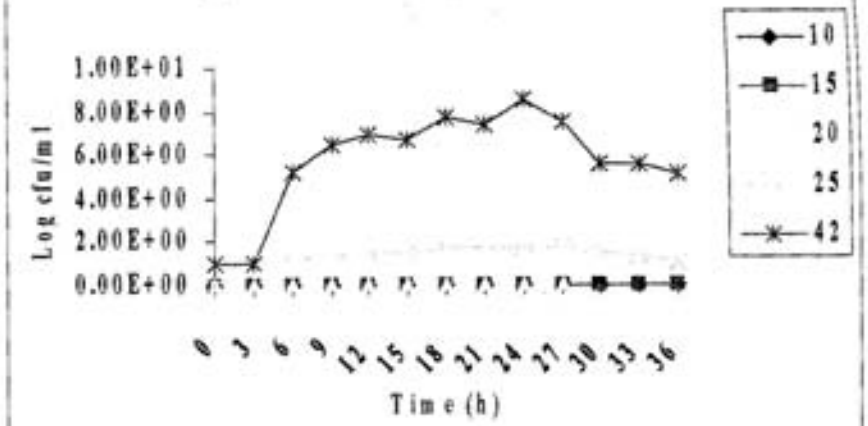


Fig. 4.11 (a). *L.acidophilus* NCDC 15

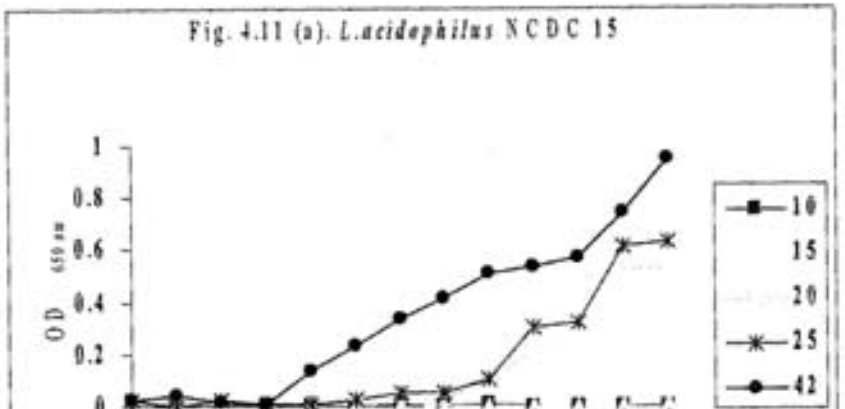
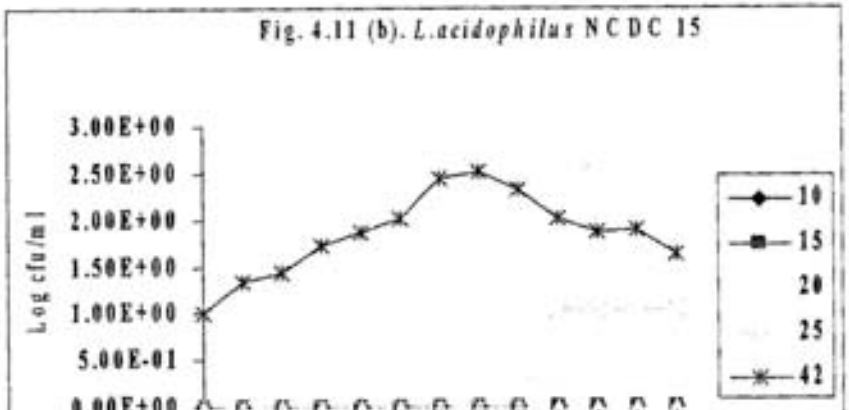
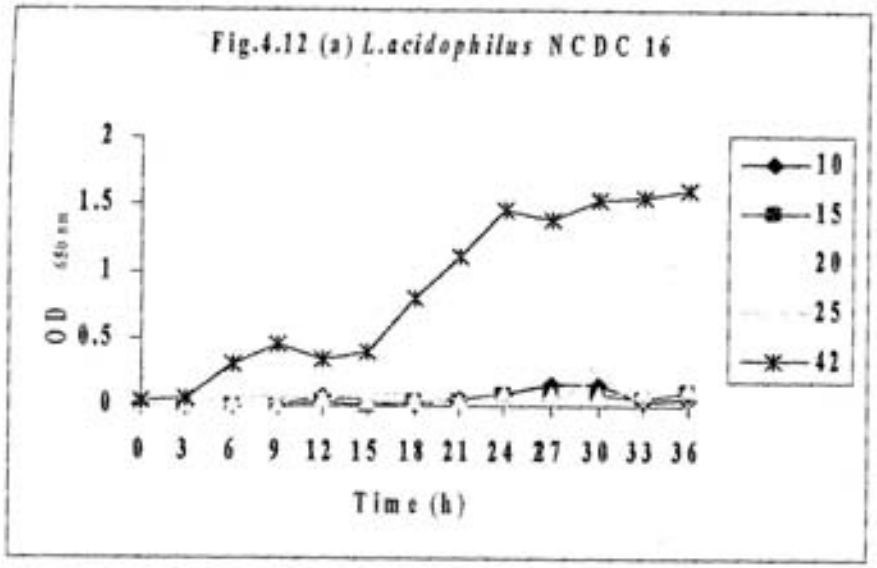
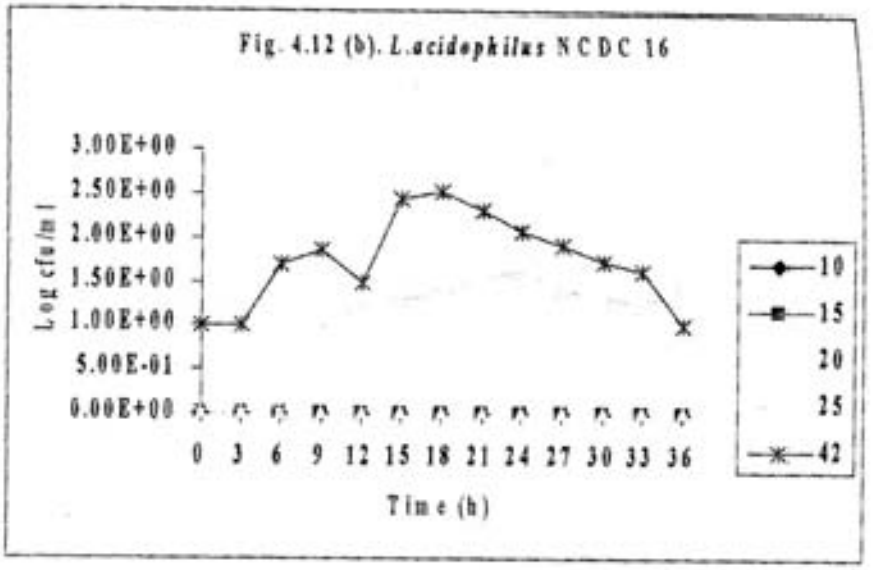
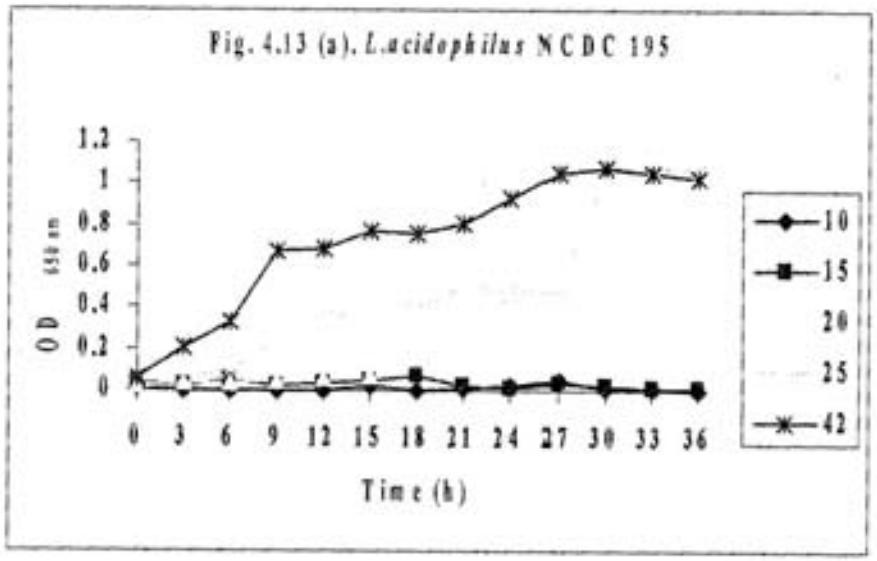


Fig. 4.11 (b). *L.acidophilus* NCDC 15




Fig.4.12 (a) L.acidophilus NCDC 16
OD 650 nm
2
1.5
1
0.5
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42


Fig. 4.12 (b). L.acidophilus NCDC 16
Log cfu/ml
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42


Fig. 4.13 (a). L.acidophilus NCDC 195
OD 650 nm
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42

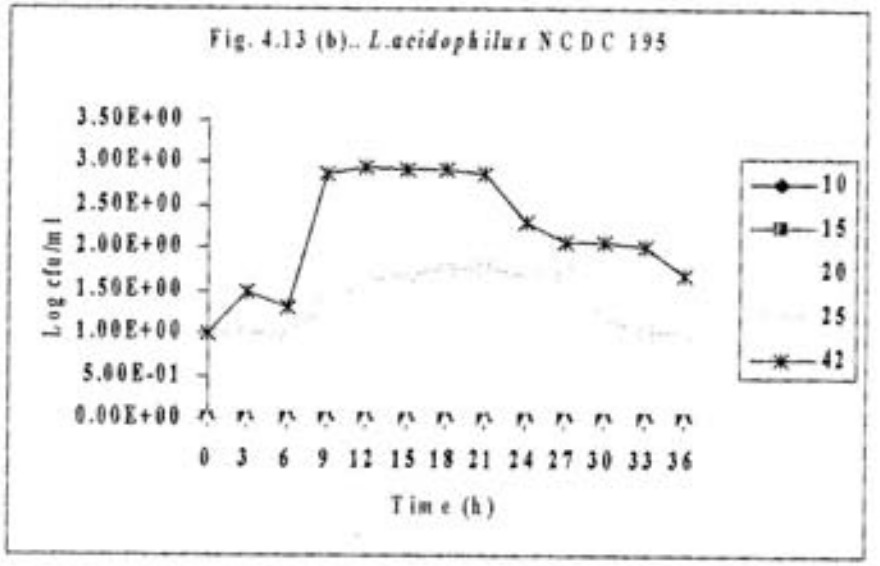

Fig. 4.13 (b).. L.acidophilus NCDC 195
Log cfu/ml
3.50E+00
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42

Fig. 4.14 (a). *L. acidophilus* NCDC 291

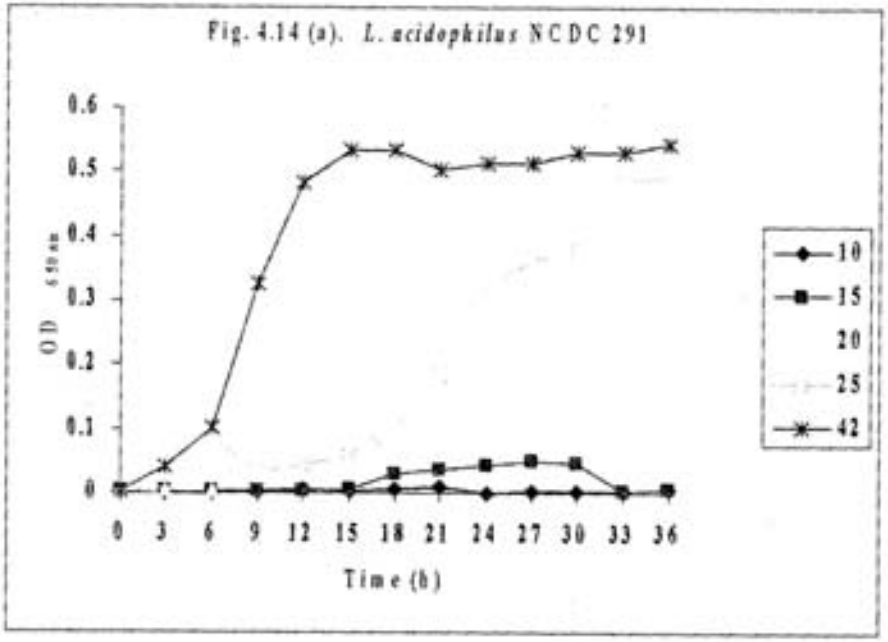


Fig. 4.14 (b). *L.acidophilus* NCDC 291

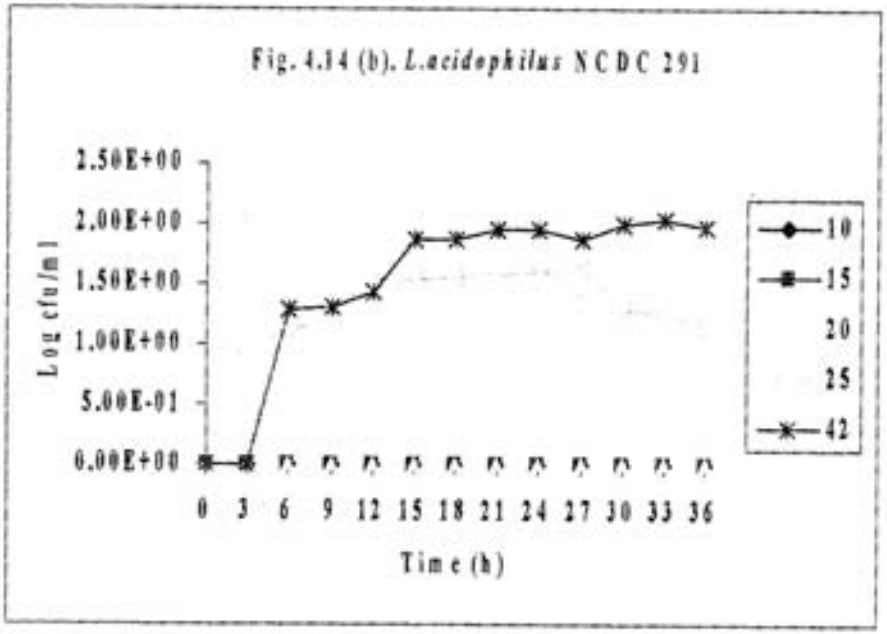


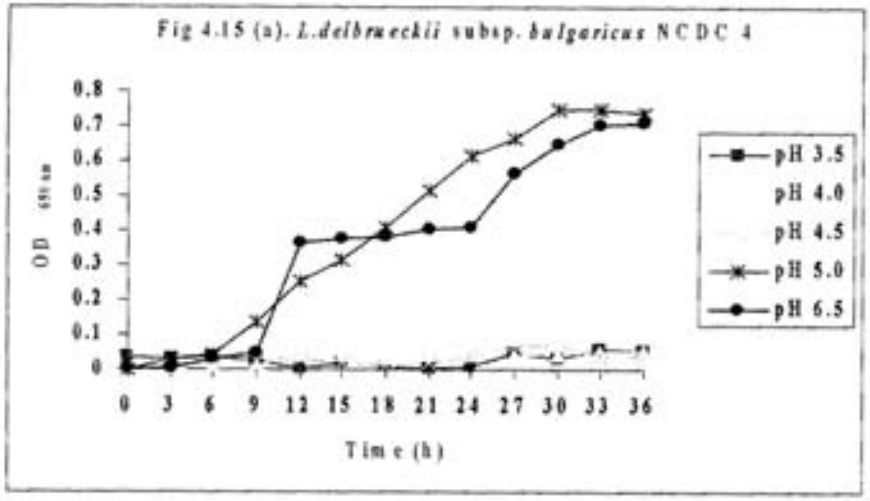
Table 4.3: Correlation coefficient (r^2) of *L. delbrueckii* subsp. *bulgaricus* strains ($OD_{650\ nm}$ versus Log cfu) at different pH and temp (^0C)

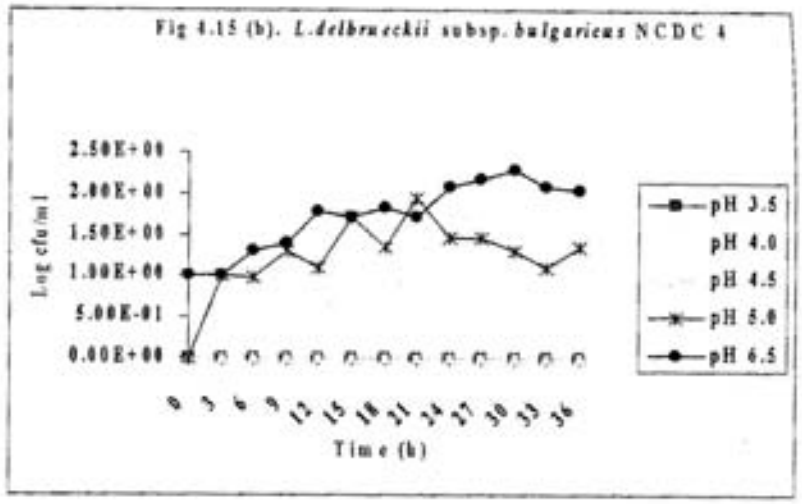
L. delbrueckii subsp. bulgaricus	pH			Temp. (^0C)		
	4.5	5.0	6.5	20	25	42
NCDC 04	0.00	0.5202	0.9425	0.00	0.9015	0.9285
NCDC 08	0.00	0.3359	0.3450	0.00	0.3164	0.3359
NCDC 09	0.00	0.5290	0.5756	0.00	0.6728	0.5533
NCDC 26	0.00	0.6733	0.8110	0.00	0.3939	0.8733
NCDC 27	0.00	0.2933	0.4206	0.00	0.3540	0.4208
NCDC 184	0.00	0.5368	0.8237	0.00	0.8027	0.5368
NCDC 253	0.00	0.034	0.353	0.00	0.3243	0.3442
NCDC 277	0.00	0.968	0.6202	0.00	0.00	0.8310
NCDC 281	0.00	0.0837	0.4309	0.00	0.5200	0.4309
NCDC 285	0.00	0.9490	0.7626	0.00	0.8152	0.7628
NCDC 293	0.00	0.3019	0.9249	0.00	0.6608	0.9249
NCDC 304	0.00	0.4124	0.6940	0.00	0.4124	0.6940
NCDC 305	0.00	0.3754	0.8101	0.00	0.3044	0.8101
NCDC 307	0.899	0.968	0.844	0.8674	0.6412	0.8442
NCDC 308	0.8314	0.9267	0.8396	0.00	0.9267	0.8396

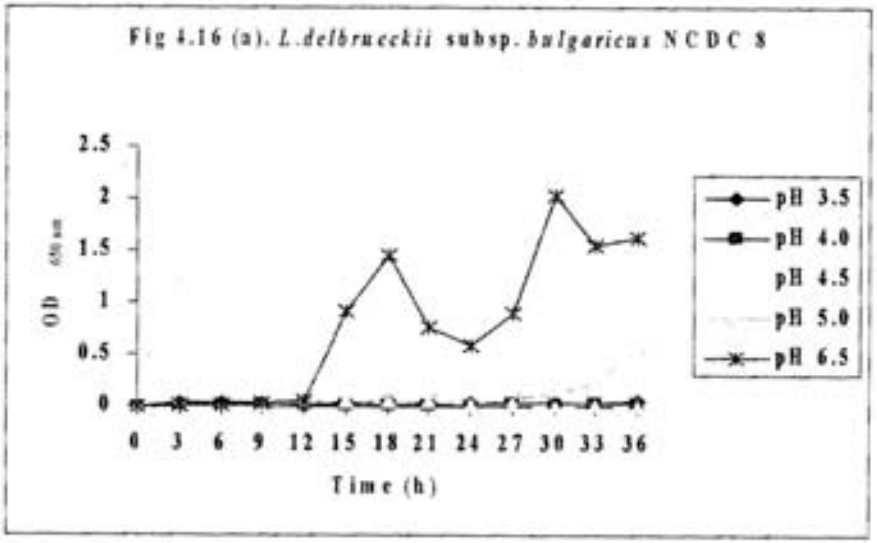
Table 4.4: Growth rate (μ_{max} / h) of *L. delbrueckii* subsp. *bulgaricus* strains at different pH and temp (^{0}C)

L. delbrueckii subsp. *bulgaricus*	pH			Temp. (^{0}C)		
	4.5	5.0	6.5	20	25	42
NCDC 04	0.00	0.007	0.047	0.00	0.004	0.047
NCDC 08	0.00	0.009	0.078	0.00	0.006	0.078
NCDC 09	0.00	0.002	0.013	0.00	0.009	0.013
NCDC 26	0.01	0.10	0.11	0.00	0.02	0.11
NCDC 27	0.00	0.001	0.076	0.00	0,05	0.076
NCDC 184	0.00	0.003	0.15	0.00	0.006	0.15
NCDC 253	0.01	0.004	0.11	0.00	0.002	0.11
NCDC 277	0.00	0.00	0.008	0.00	0.00	0.008
NCDC 281	0.00	0.003	0.16	0.00	0.011	0.16
NCDC 285	0.062	0.31	0.256	0.002	0.007	0.256
NCDC 293	0.00	0.09	0.054	0.001	0.003	0.054
NCDC 304	0.00	0.002	0.01	0.00	0.002	0.01
NCDC 305	0.00	0.11	0.10	0.00	0.001	0.10
NCDC 307	0.05	0.80	0.63	0.00	0.009	0.63
NCDC 308	0.00	0.01	0.001	0.00	0.010	0.001

Fig 4.15 - 4.29: Growth curves of *L. delbrueckii* subsp. *bulgaricus* strains at different pH (a: $OD_{650\,nm}$; b: Log cfu/ml)







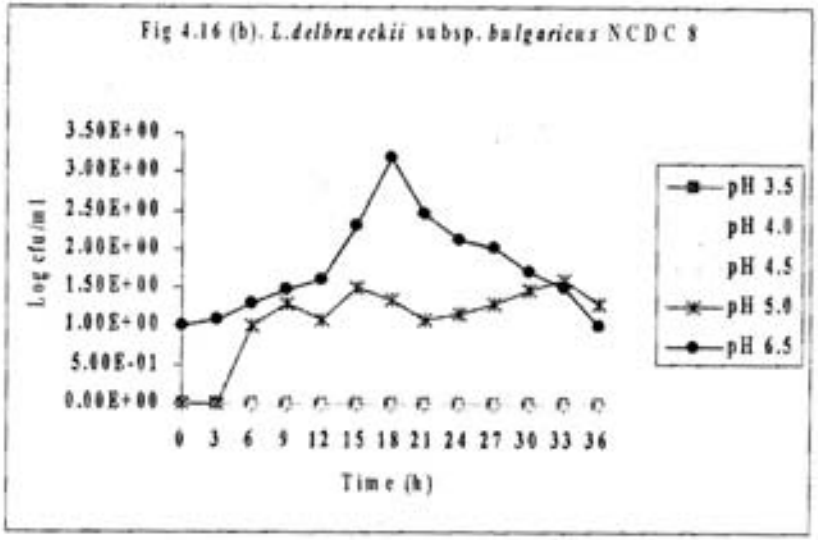


Fig. 4.17 (a). *L.delbrueckii subsp. bulgaricus* NCDC 09

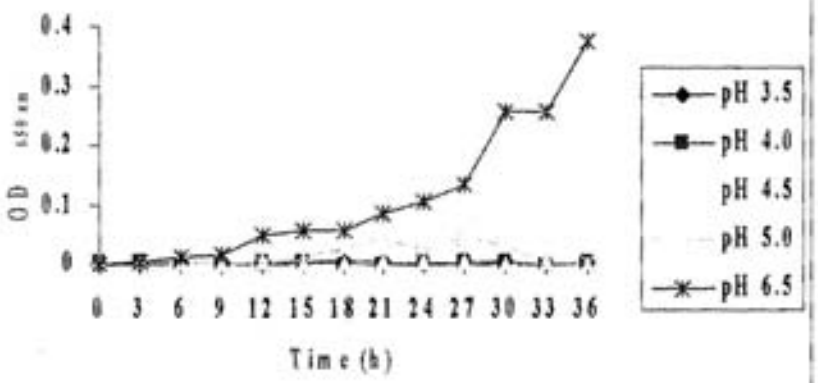


Fig.4.17 (b). *L.delbrueckii subsp. bulgaricus* NCDC 09

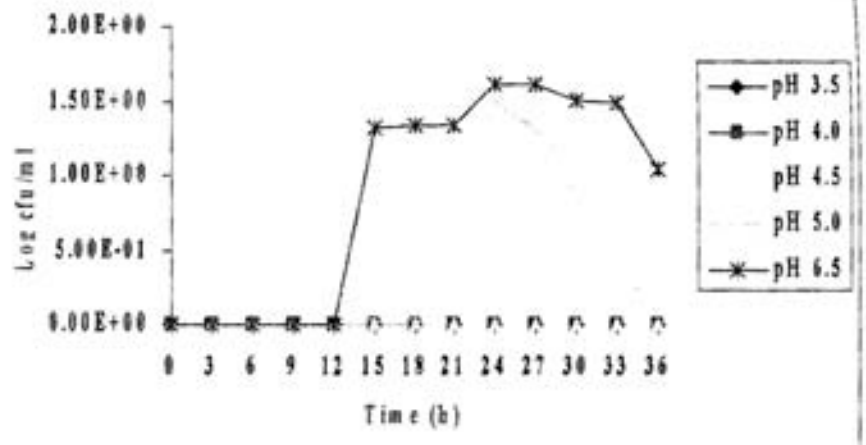


Fig. 4.18 (a). *L.delbrueckii subsp. bulgaricus* NCDC 26

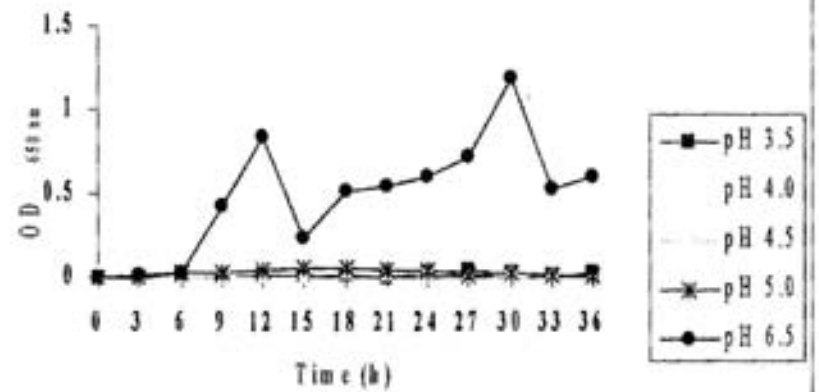


Fig. 4.18 (b). *L. delbrueckii subsp. bulgaricus* NCDC 26

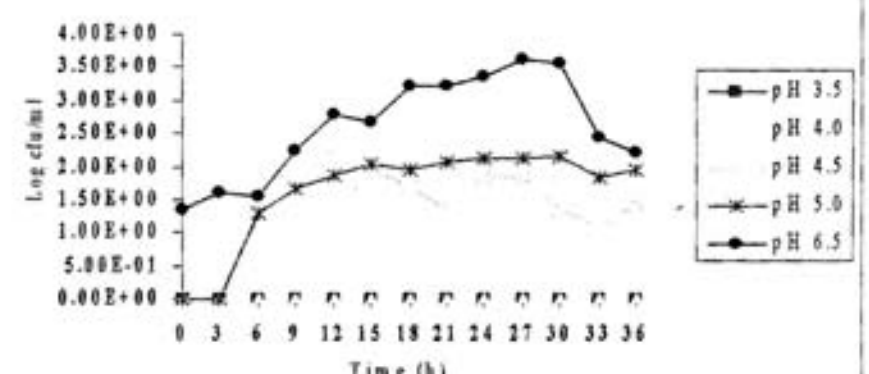


Fig. 4.19 (a). *L.delbrueckii subsp. bulgaricus* NCDC 27

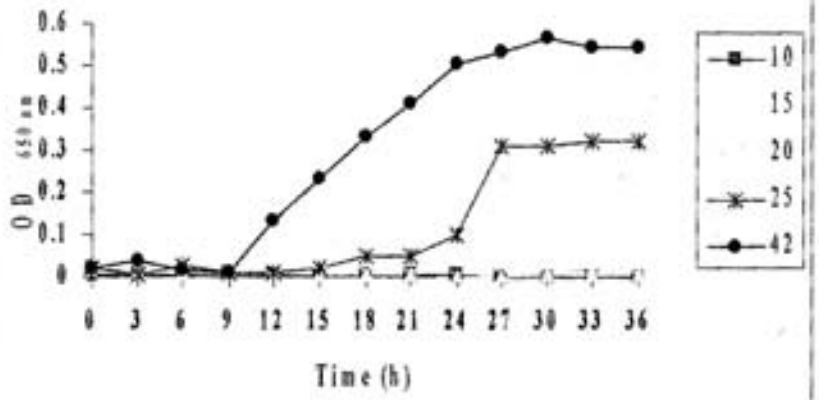


Fig. 4.19 (b). *L.delbrueckii subsp. bulgaricus* NCDC 27

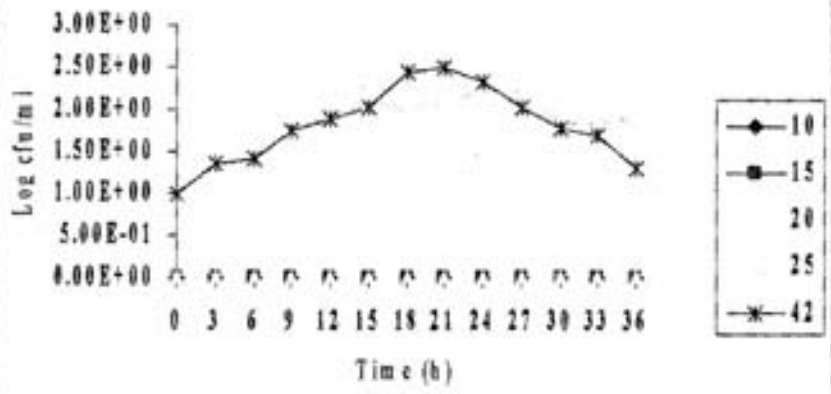


Fig. 4.20 (a). *L.delbrueckii subsp. bulgaricus* NCDC 184

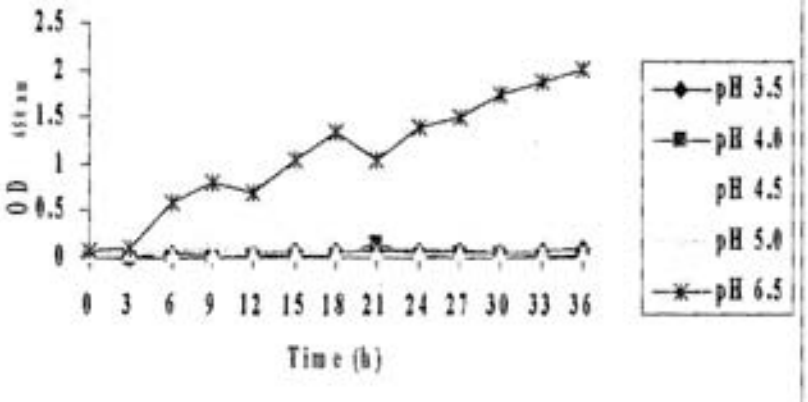


Fig. 4.20 (b). *L.delbrueckii subsp. bulgaricus* NCDC 184

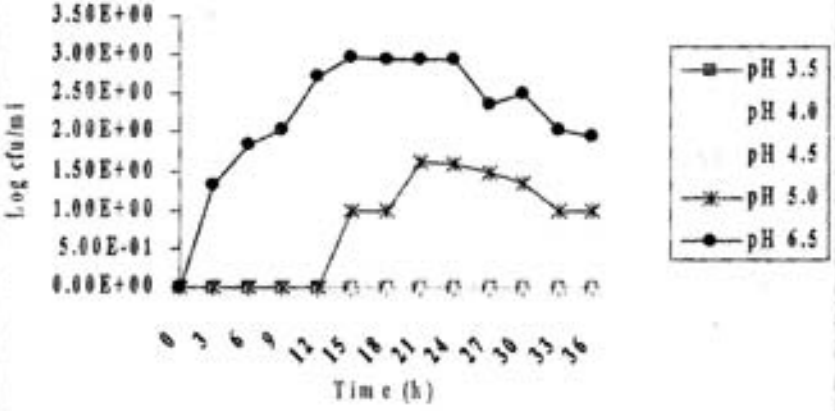


Fig. 4.21 (a) *L.delbrueckii* subsp. *bulgaricus* NCDC 253

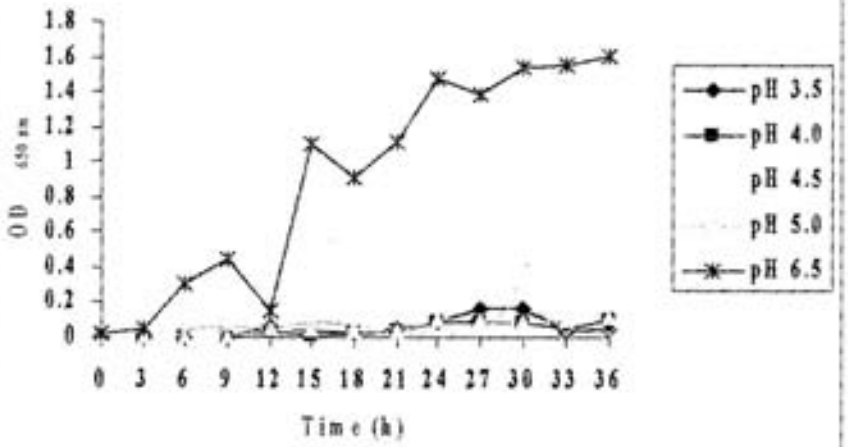


Fig.4.21 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 253

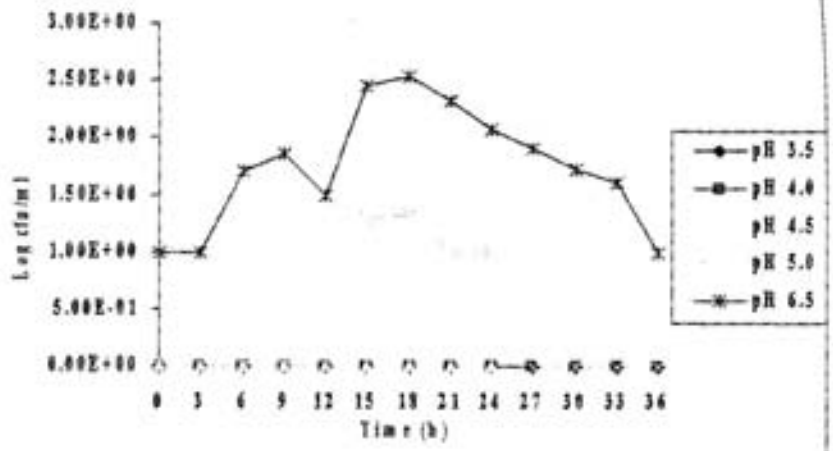


Fig. 4.22 (a). *L.delbrueckii* subsp. *bulgaricus* NCDC 277

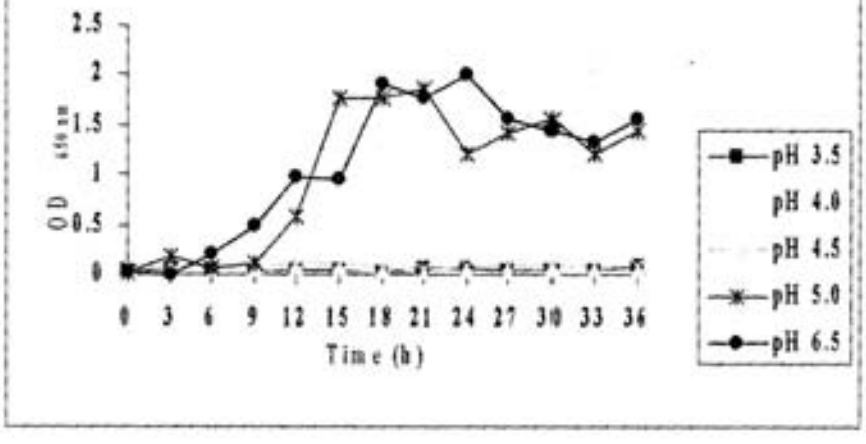


Fig. 4.22 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 277

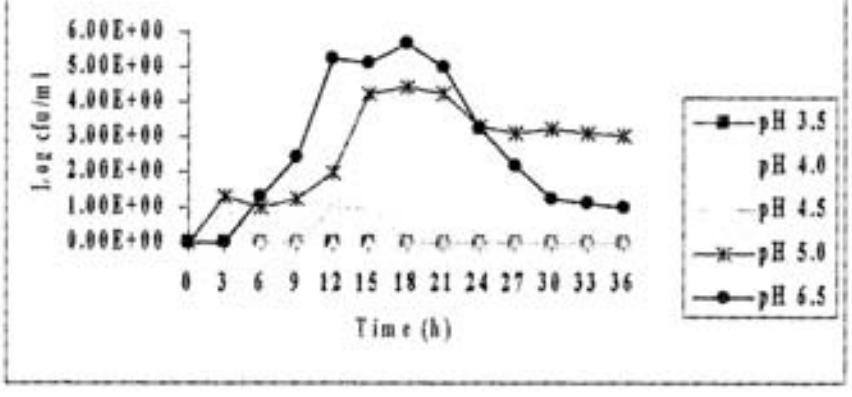


Fig. 4.23 (a). *L.delbrueckii* subsp. *bulgaricus* NCDC 281

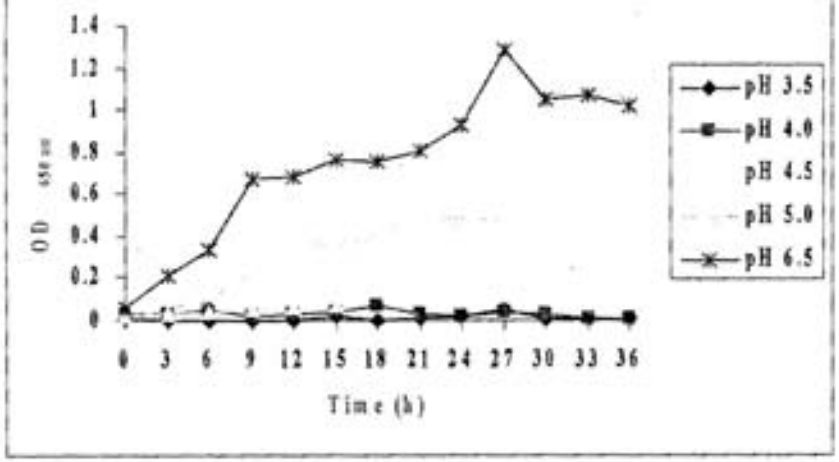


Fig. 4.23 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 281

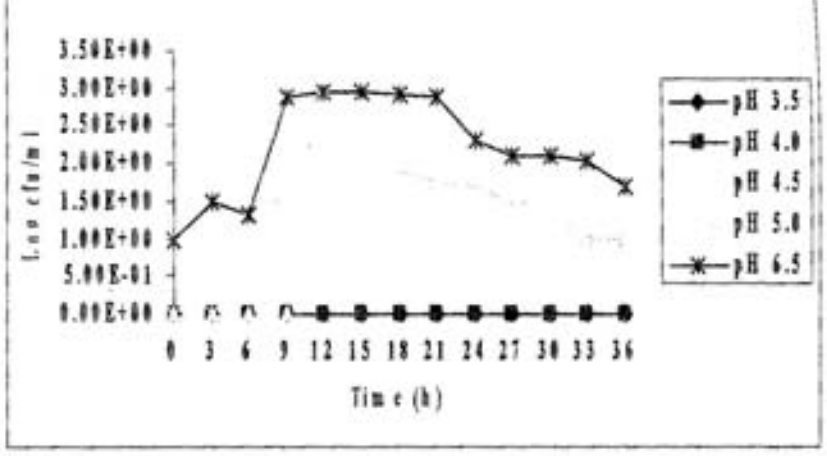


Fig 4.24 (a). *L.delbrueckii* subsp. *bulgaricus* NCDC 285

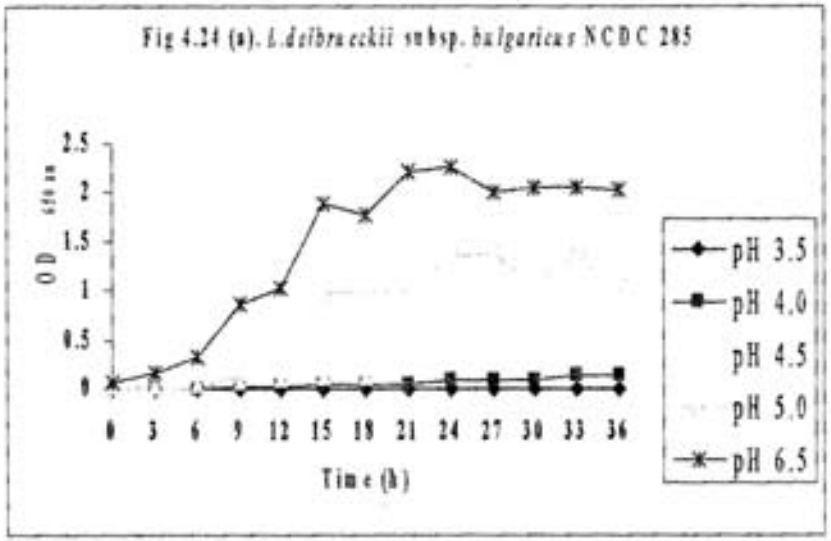
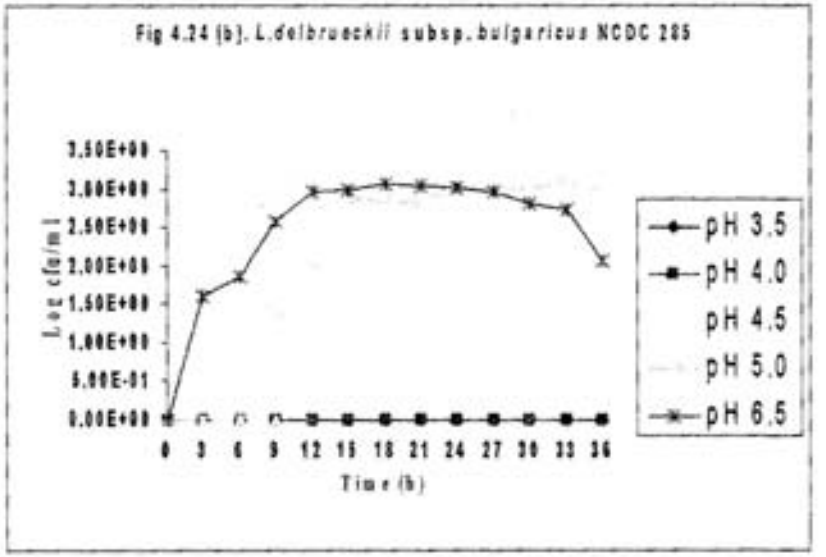
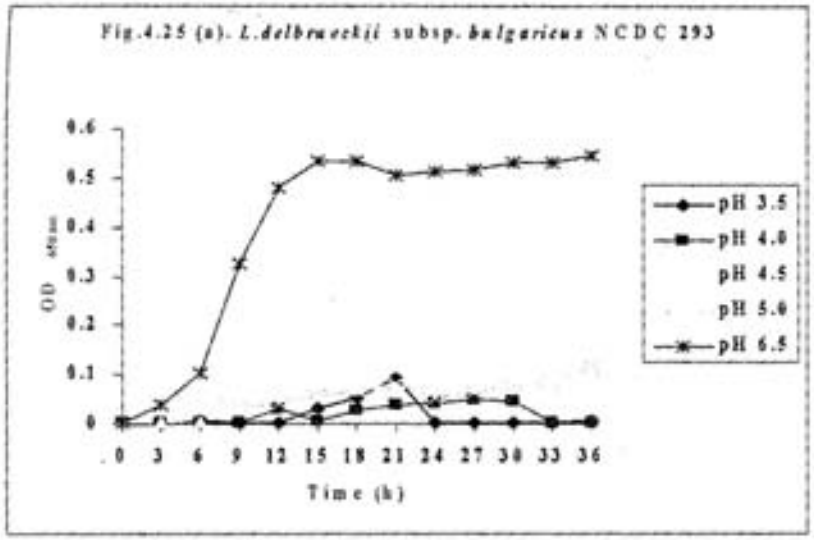
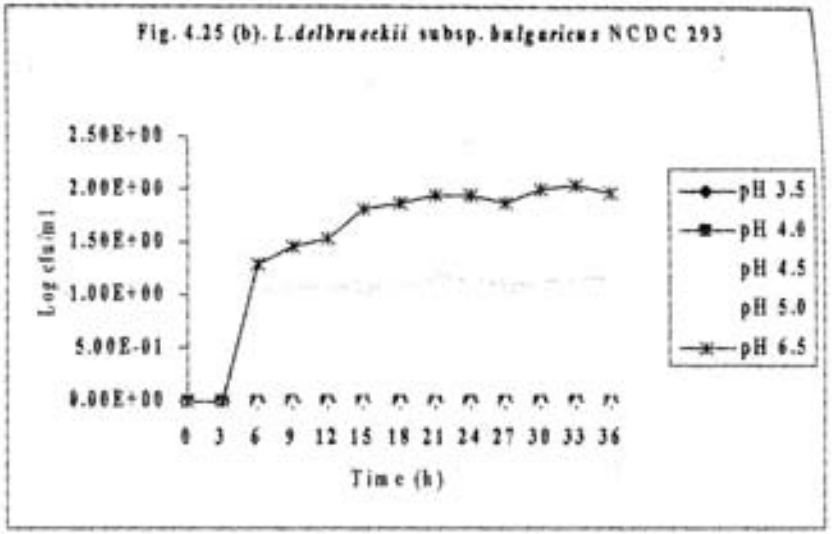
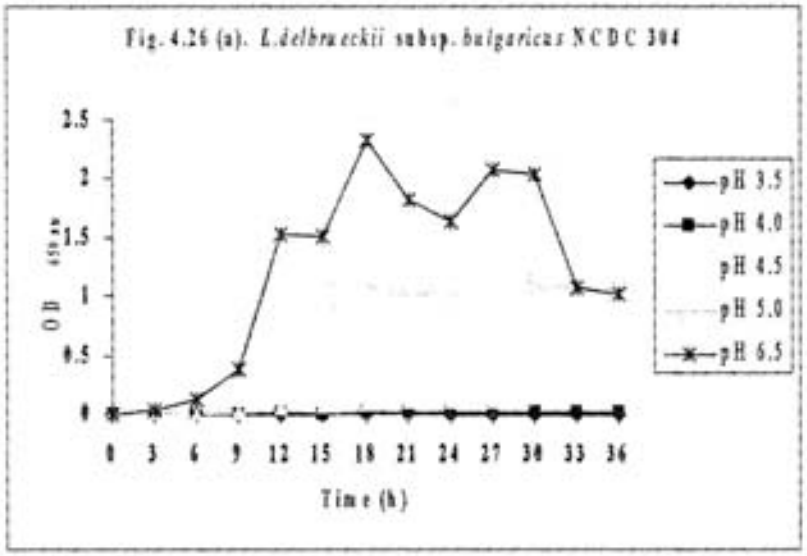


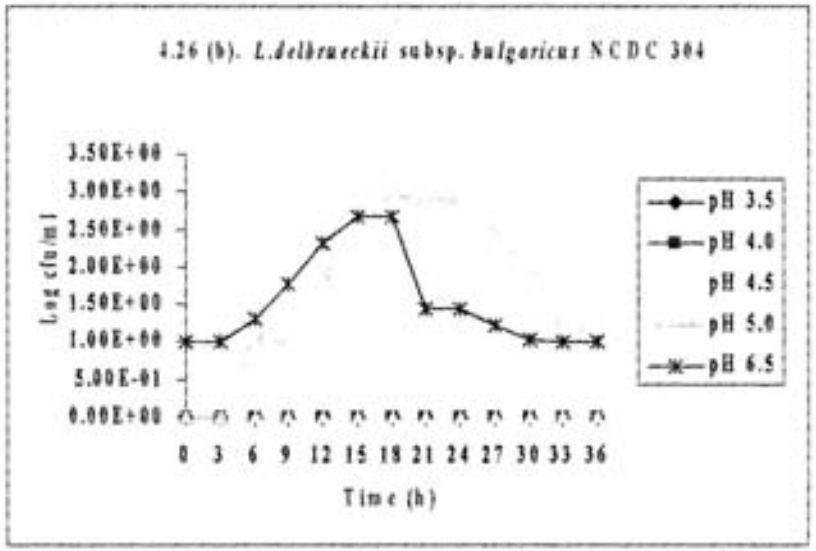
Fig 4.24 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 285

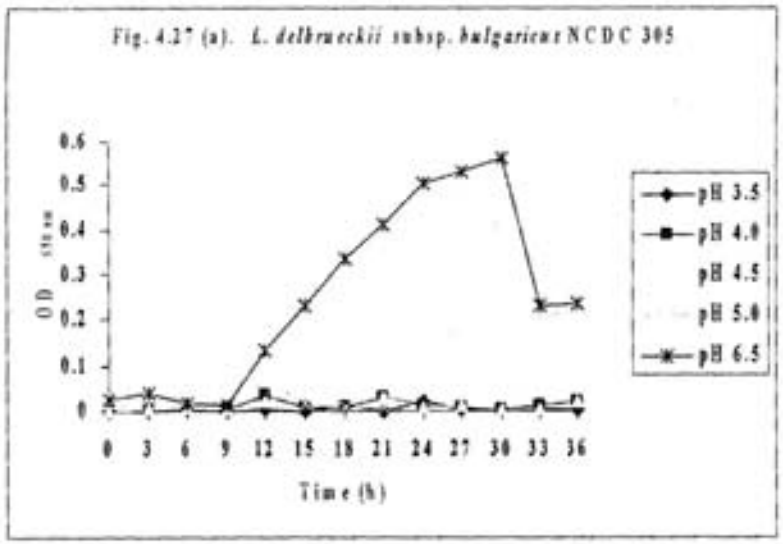


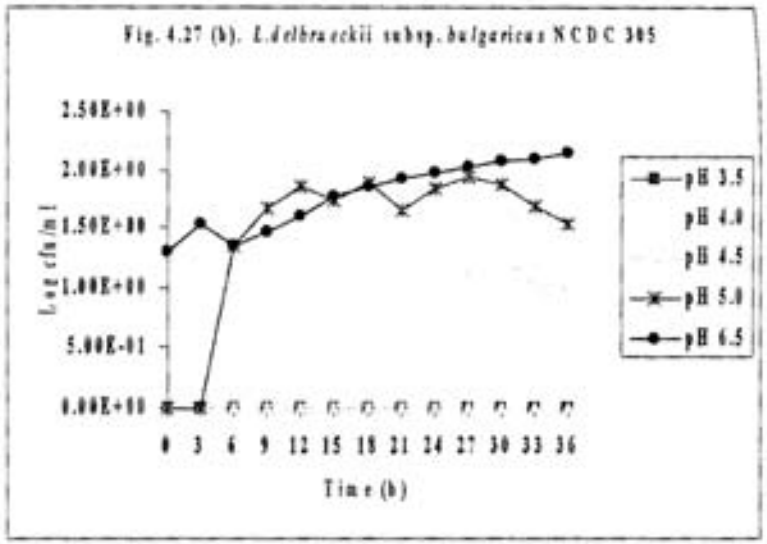

Fig.4.25 (a). L.delbrueckii subsp. bulgaricus NCDC 293
OD
0.6
0.5
0.4
0.3
0.2
0.1
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5

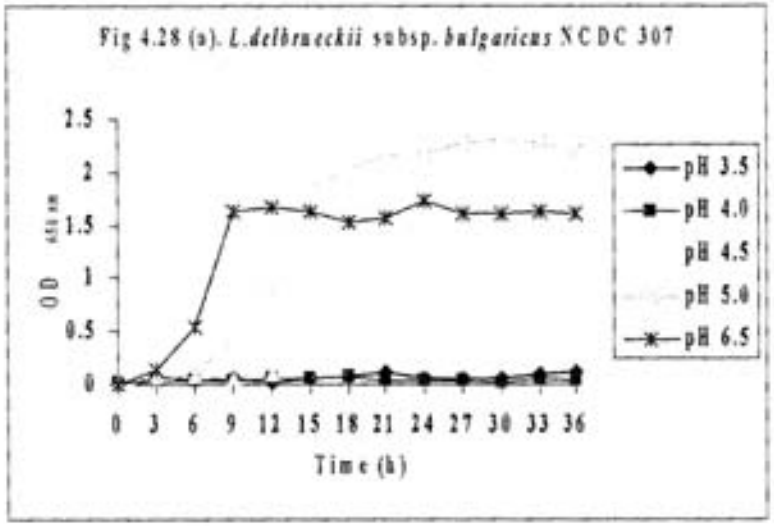

Fig. 4.25 (b). L.delbrueckii subsp. bulgaricus NCDC 293
Log cfu/ml
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig. 4.26 (a). L.delbrueckii subsp. bulgaricus NCDC 304
OD
2.5
2
1.5
1
0.5
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


4.26 (b). L.delbrueckii subsp. bulgaricus NCDC 304
Log cfu/ml
3.50E+00
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig. 4.27 (a). L. delbrueckii subsp. bulgaricus NCDC 305
OD
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig. 4.27 (b). L.delbrueckii subsp. bulgaricus NCDC 305
Log cfu/ml
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5


Fig 4.28 (a). L.delbrueckii subsp. bulgaricus NCDC 307
OD
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5

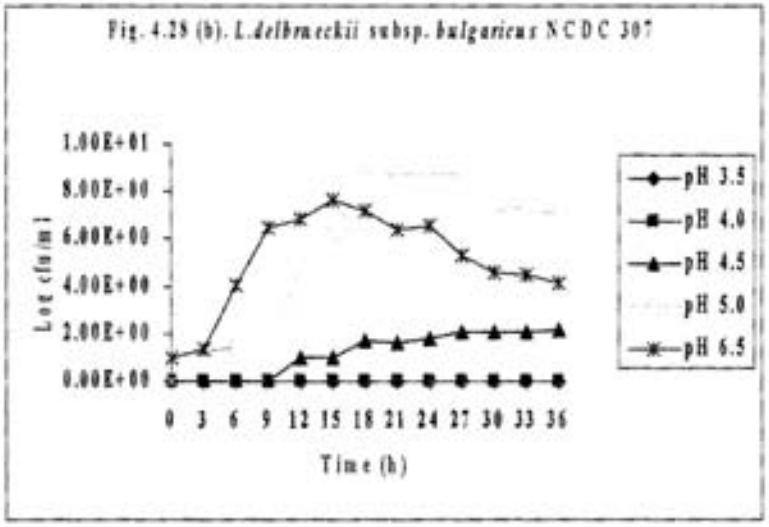

Fig. 4.28 (b). L.delbrueckii subsp. bulgaricus NCDC 307
Log cfu/ml
Time (h)
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 6.5

Fig.4.29 (a). *L.delbrueckii* subsp. *bulgaricus* NCDC 308

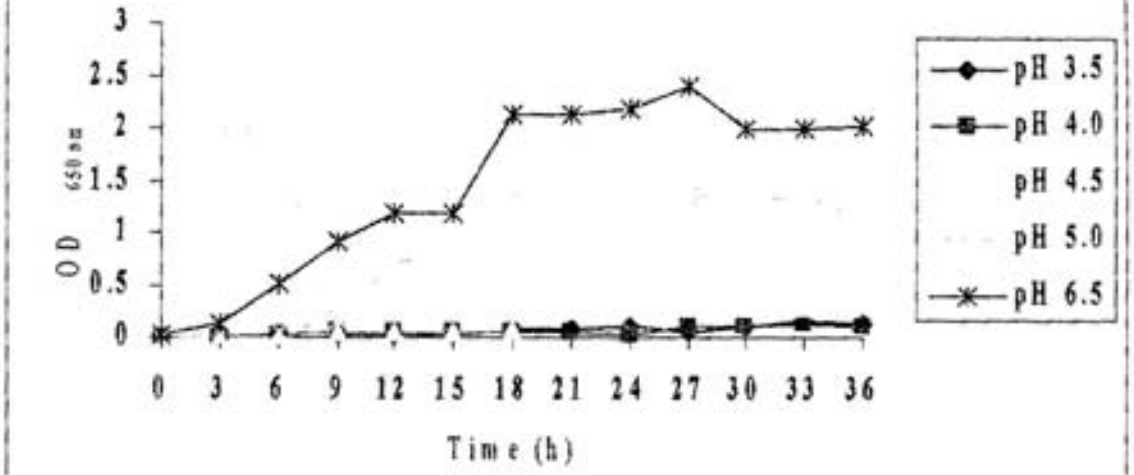


Fig.4.29 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 308

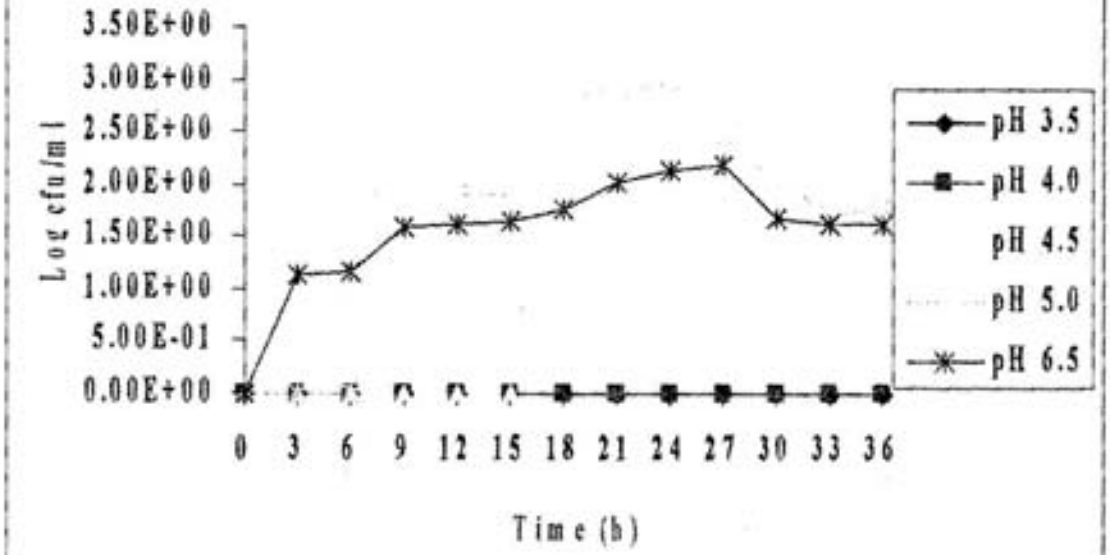
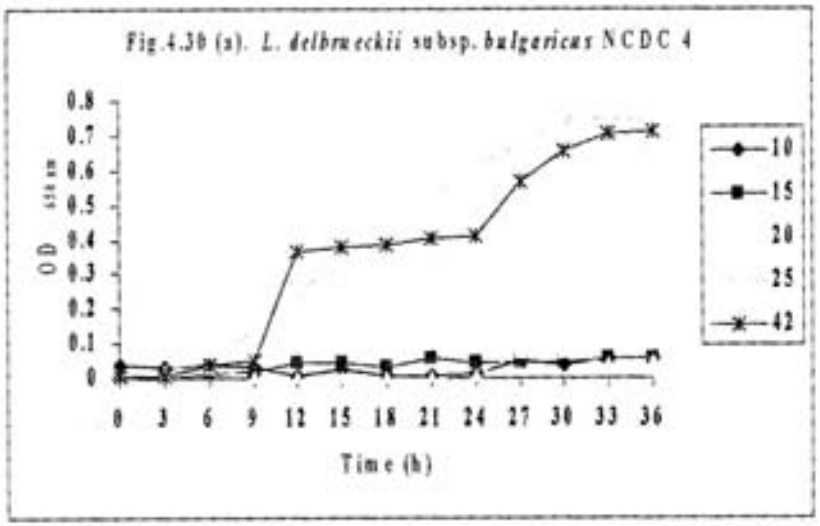
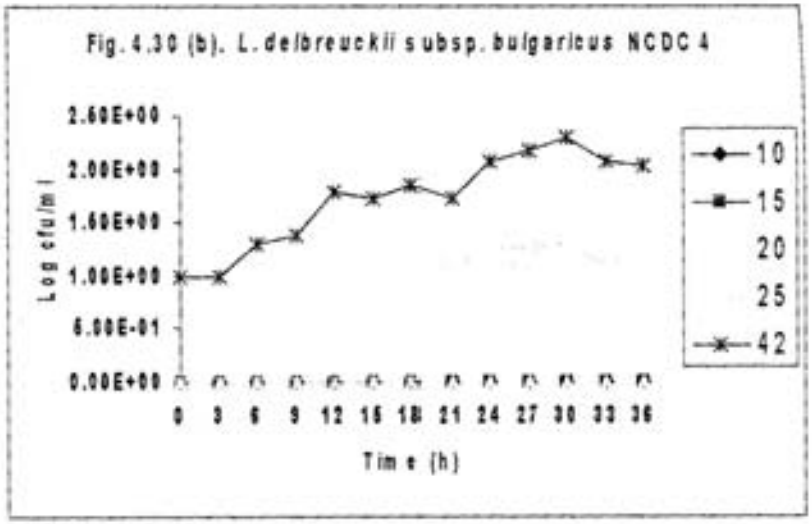
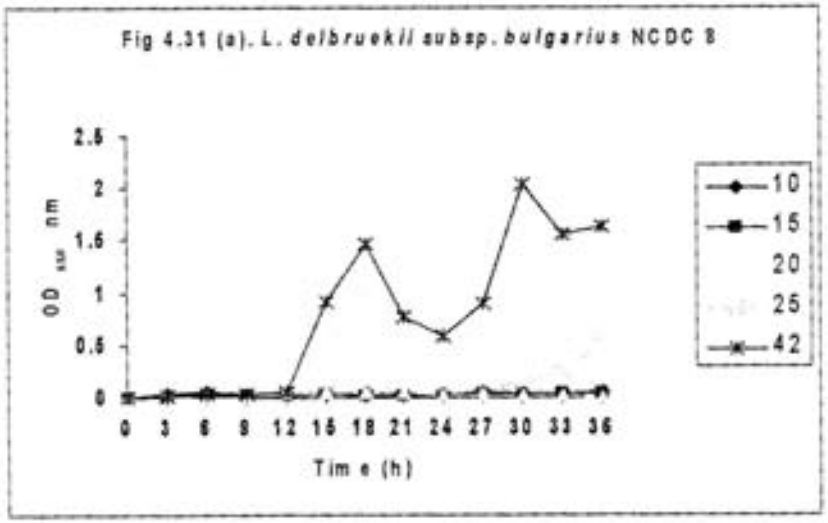
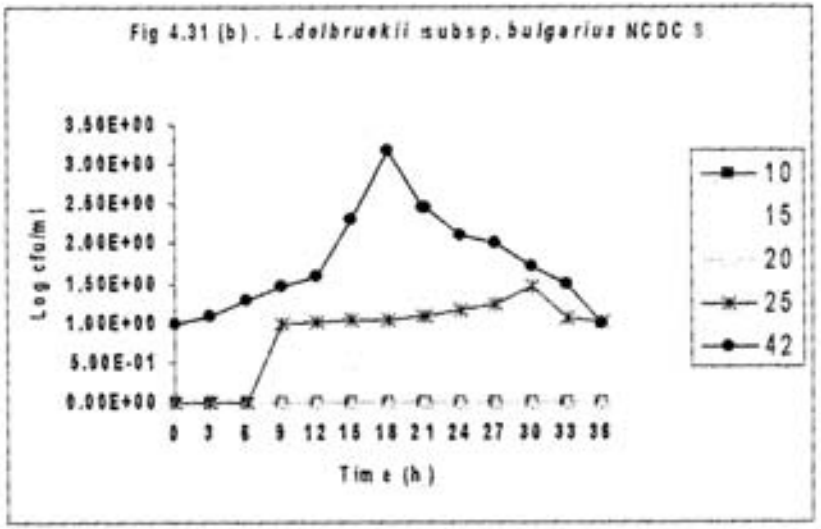


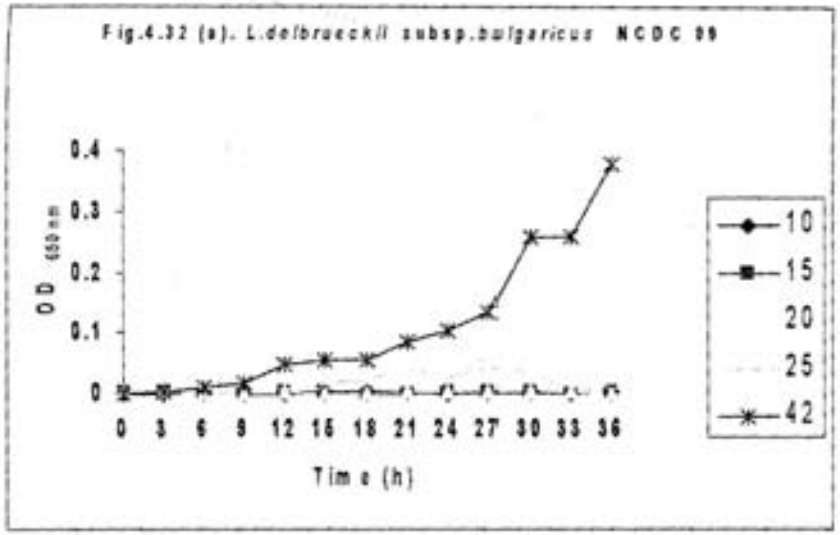
Fig 4.30 - 4.44: Growth curves of *L. delbrueckii* subsp. *bulgaricus* strains at different temp (^{0}C) (a: $OD_{650\ nm}$; b: Log cfu/ml)

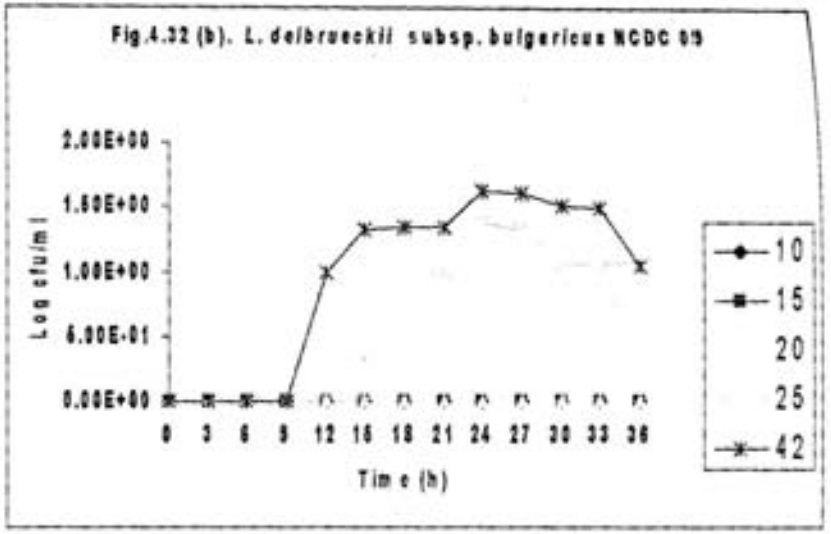


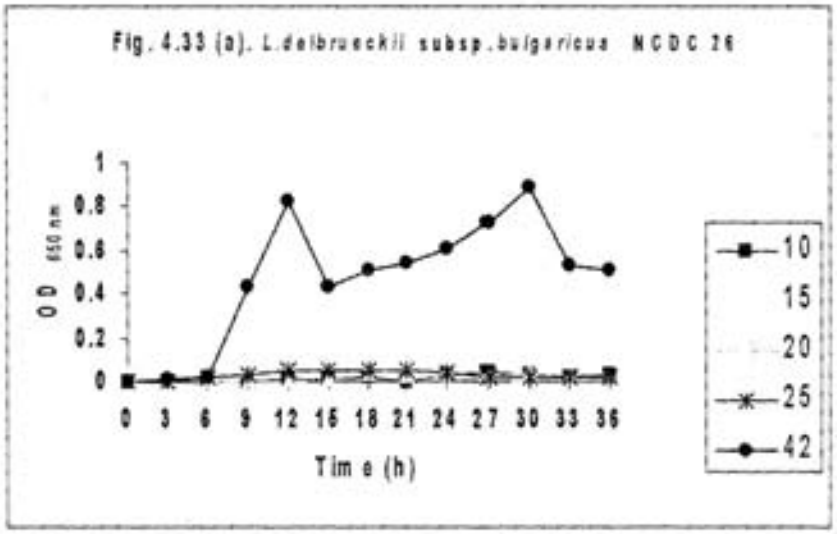


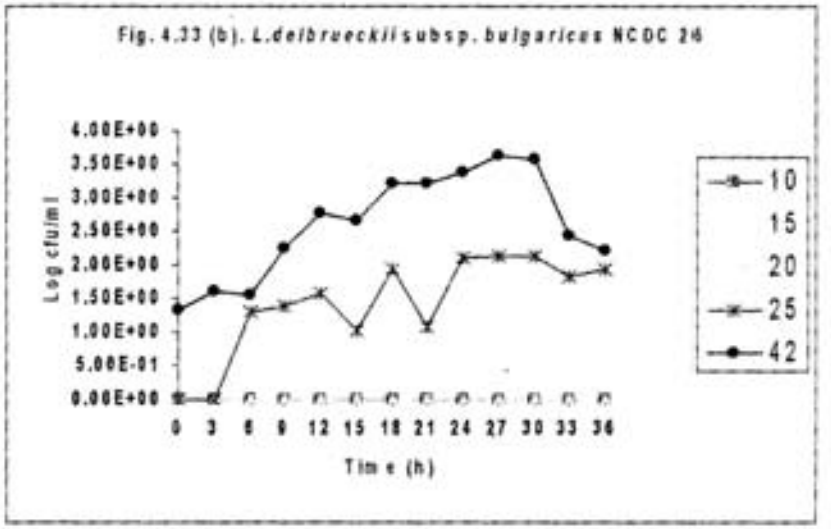


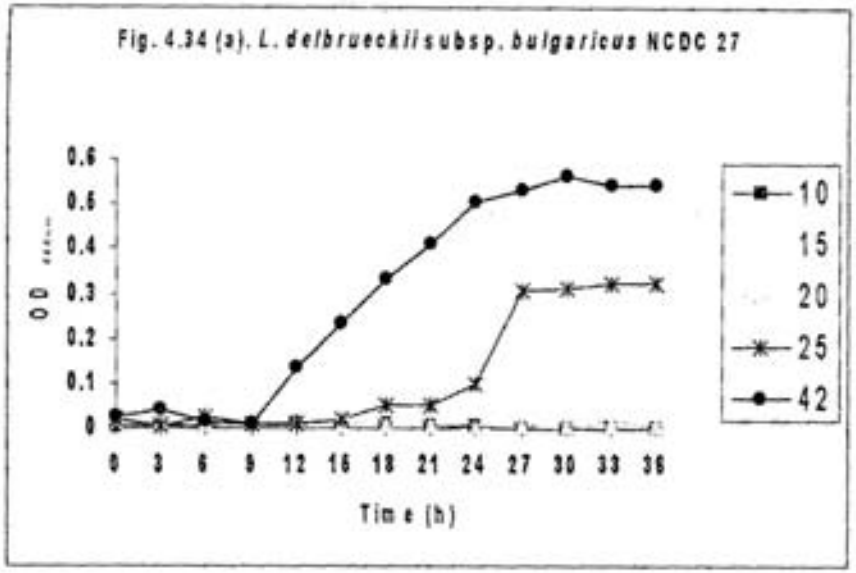


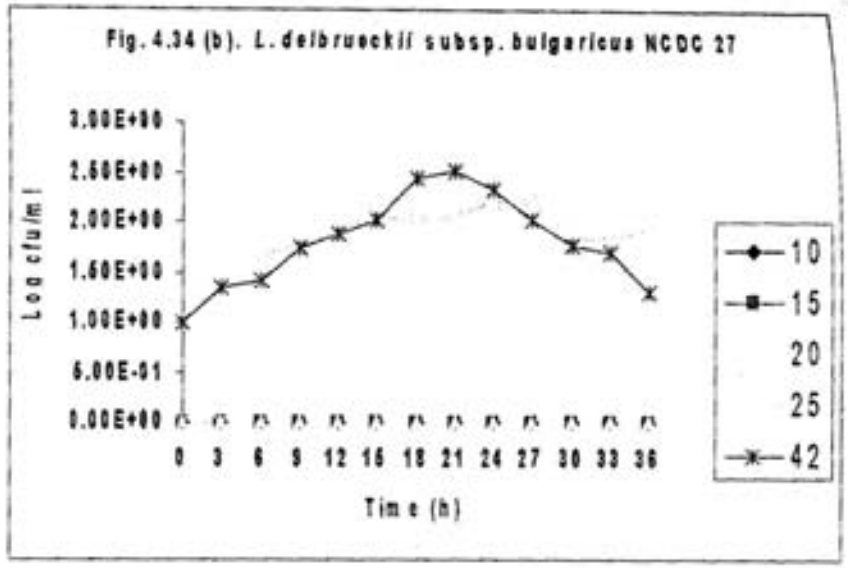

Fig.4.32 (a). L.delbrueckii subsp.bulgaricus NCDC 99
OD 650 nm
0.4
0.3
0.2
0.1
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42

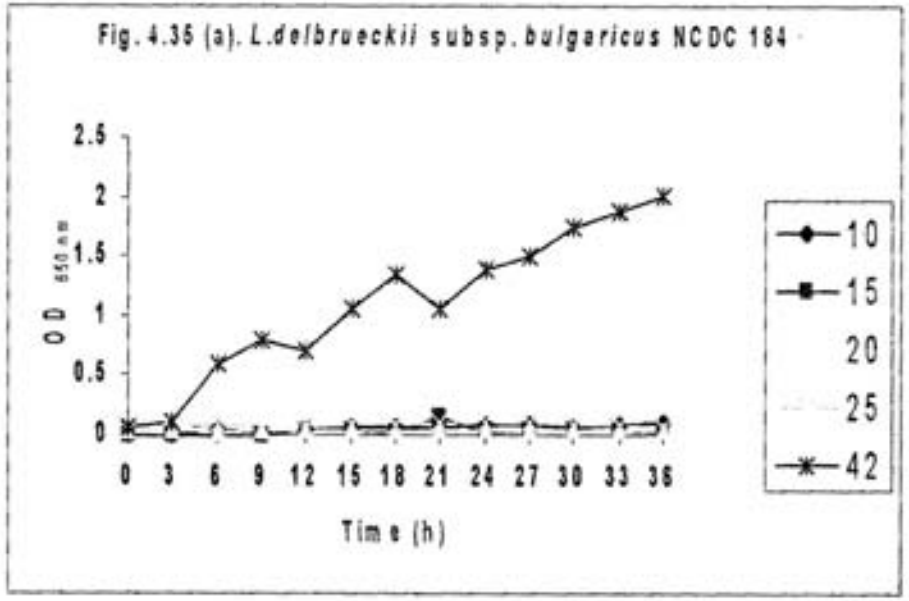

Fig.4.32 (b). L. delbrueckii subsp. bulgaricus NCDC 99
Log cfu/ml
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42


Fig. 4.33 (a). L.delbrueckii subsp.bulgaricus NCDC 26
OD 650 nm
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42


Fig. 4.33 (b). L.delbrueckii subsp. bulgaricus NCDC 26
Log cfu/ml
4.00E+00
3.50E+00
3.00E+00
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42


Fig. 4.34 (a). L. delbrueckii subsp. bulgaricus NCDC 27
OD
Time (h)
10
15
20
25
42


Fig. 4.34 (b). L. delbrueckii subsp. bulgaricus NCDC 27
Log cfu/ml
Time (h)
10
15
20
25
42


Fig. 4.35 (a). L. delbrueckii subsp. bulgaricus NCDC 184
OD 650nm
Time (h)
10
15
20
25
42

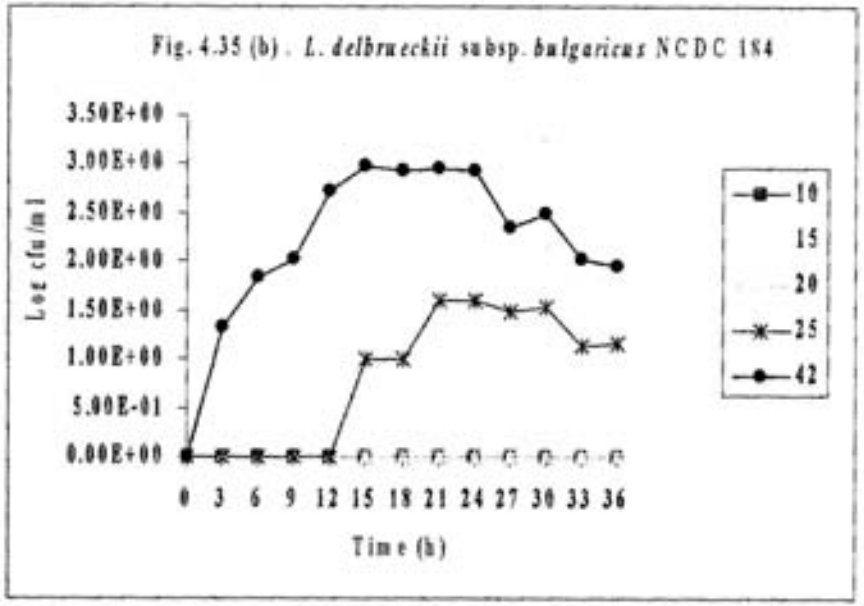

Fig. 4.35 (b). L. delbrueckii subsp. bulgaricus NCDC 184
Log cfu/ml
Time (h)
10
15
20
25
42

Fig.4.36 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 263

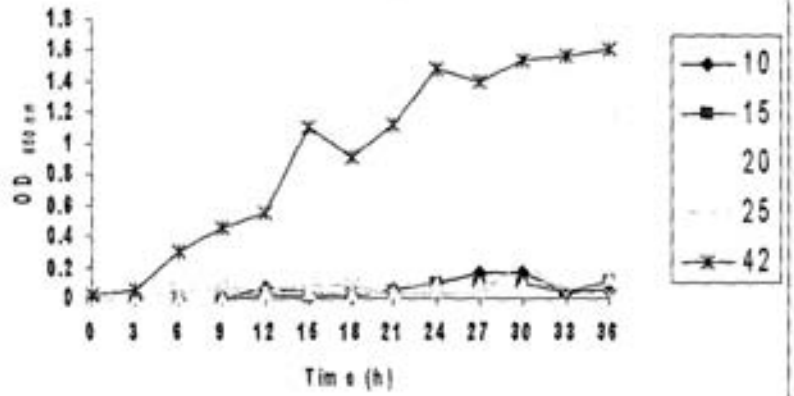


Fig.4.36 (b). *L. delbrueckii* subsp. *bulgaricus* NCDC 263

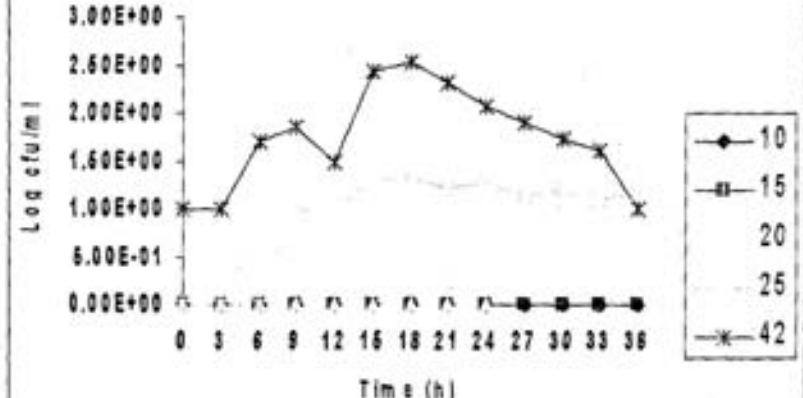


Fig. 4.37 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 277

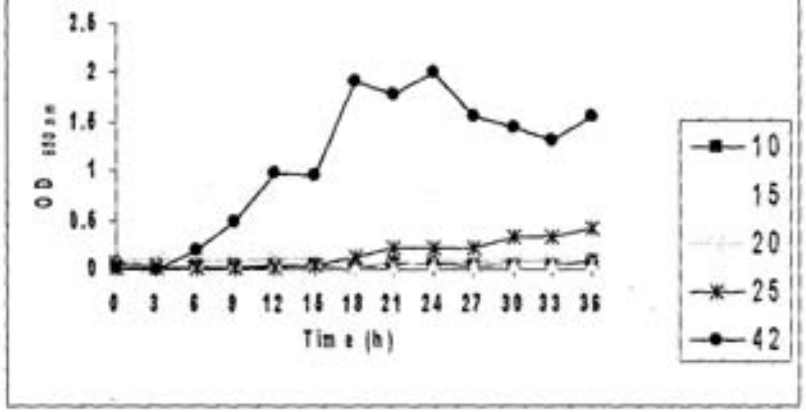


Fig. 4.37 (b). *L. delbrueckii* subsp. *bulgaricus* NCDC 277

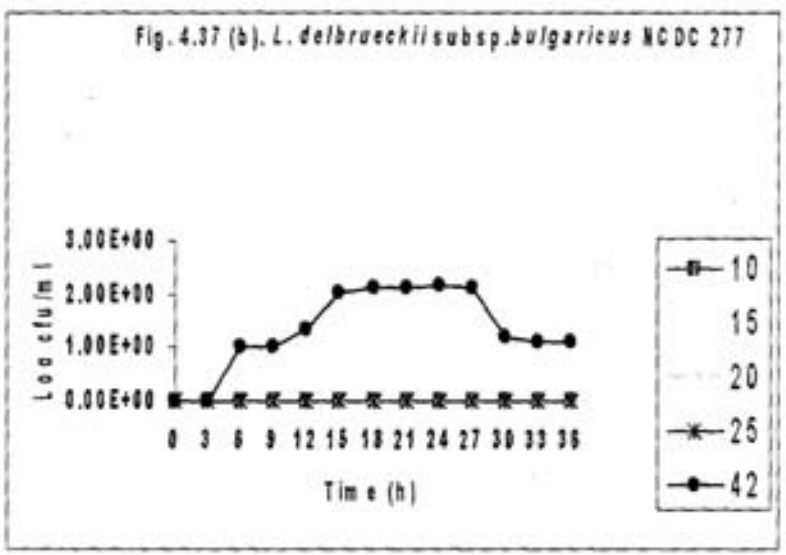


Fig. 4.38 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 281

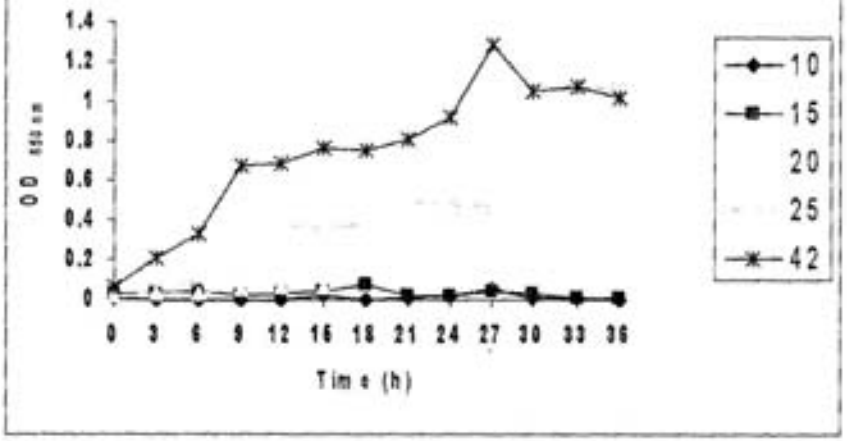


Fig. 4.38 (b). *L. delbrueckii* subsp. *bulgaricus* NCDC 281

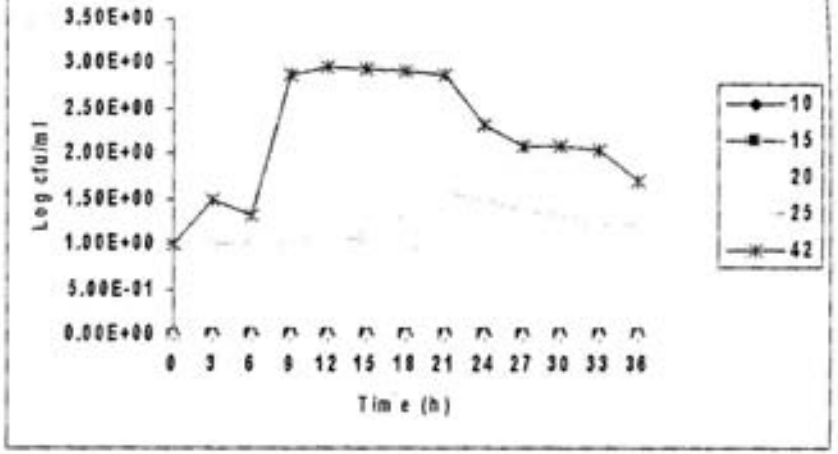


Fig 4.39 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 286

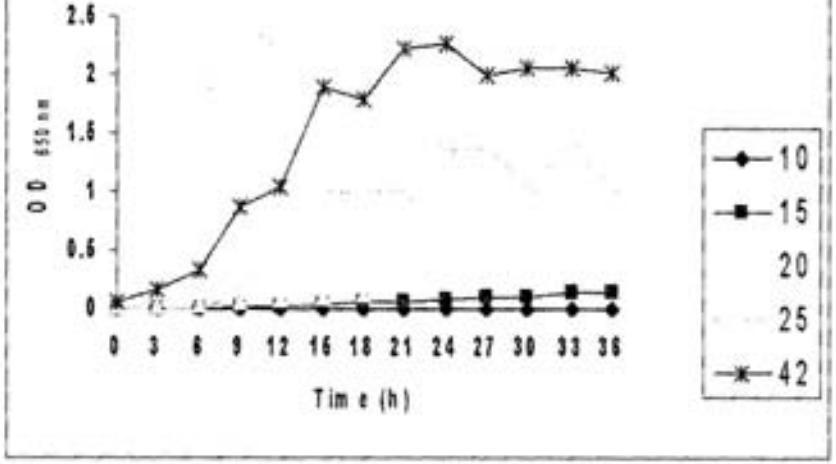


Fig 4.39 (b). *L. delbrueckii* subsp. *bulgaricus* NCDC 286

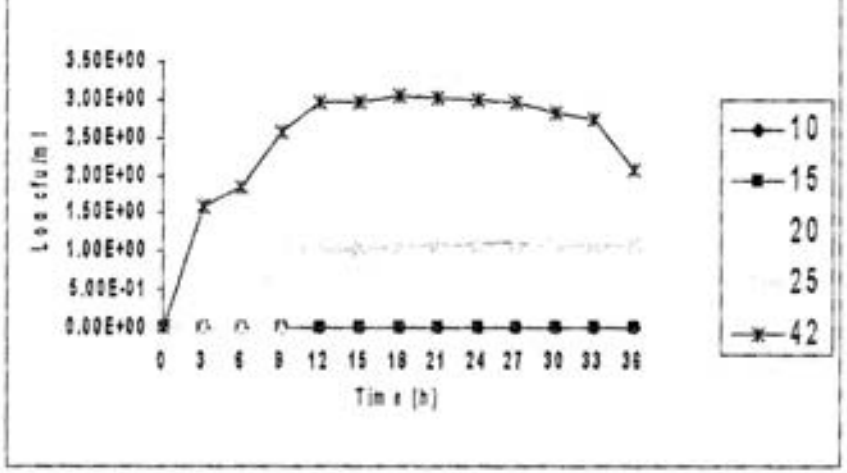


Fig. 4.40 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 293

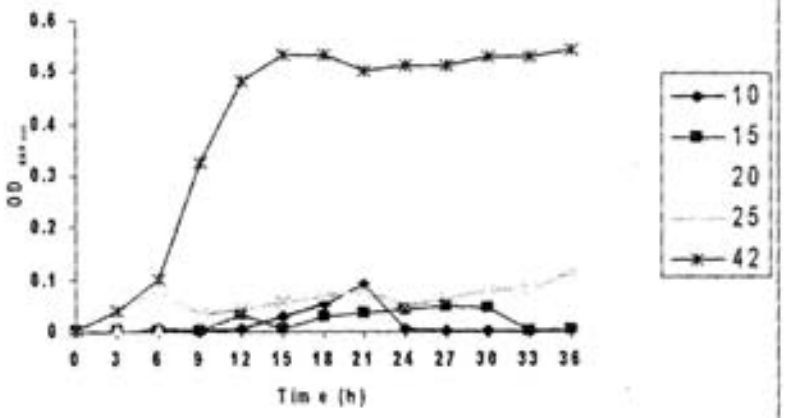


Fig. 4.40 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 293

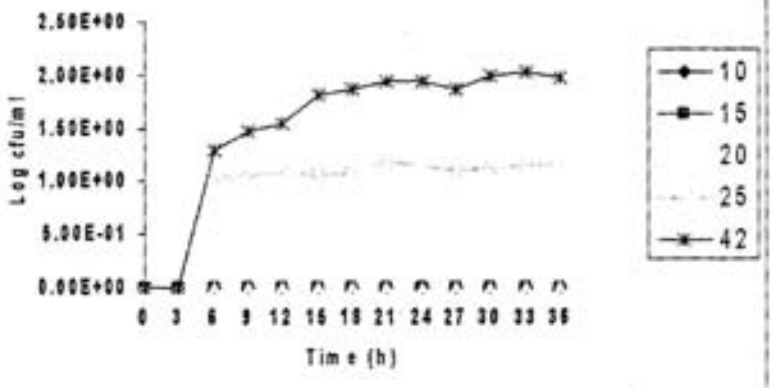
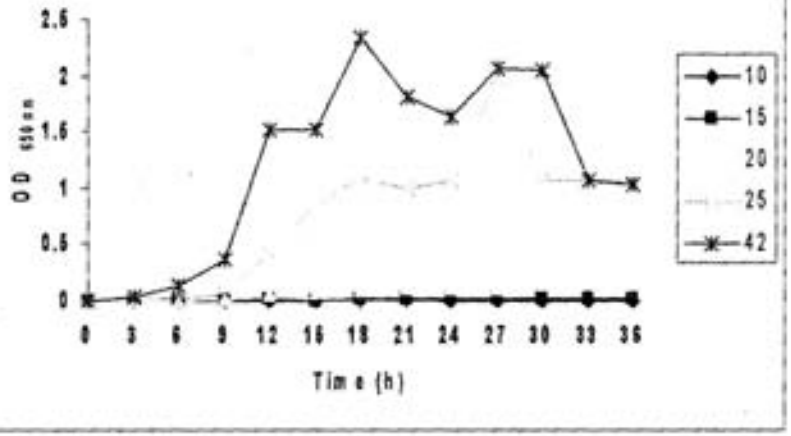
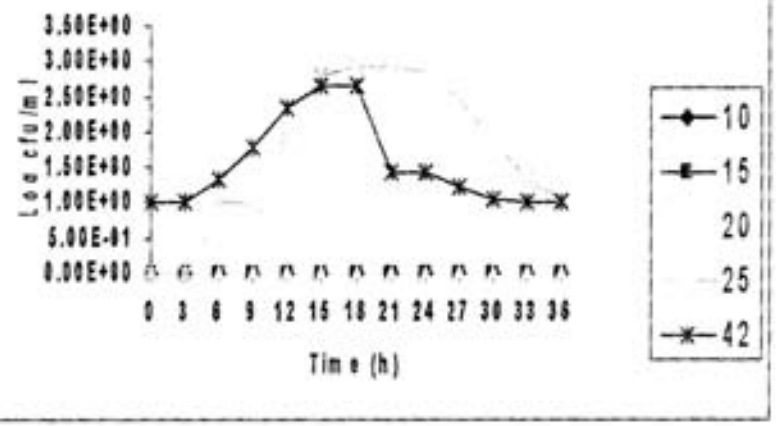
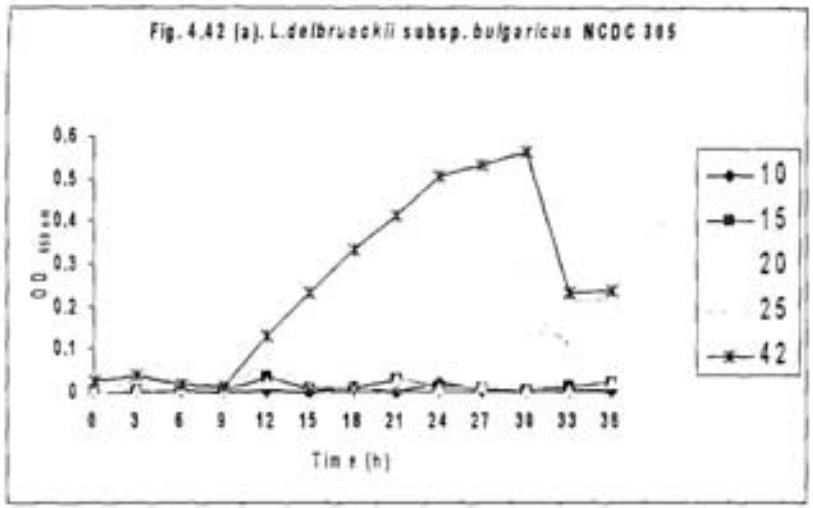


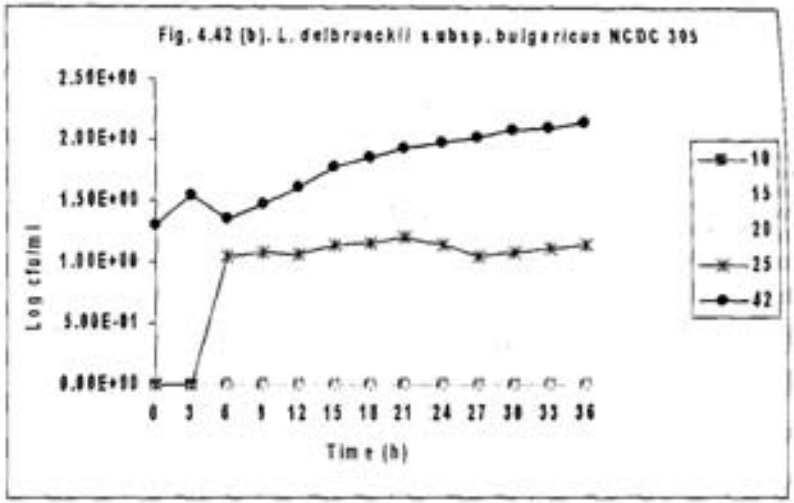
Fig. 4.41 (a). *L. delbrueckii* subsp. *bulgaricus* NCDC 304

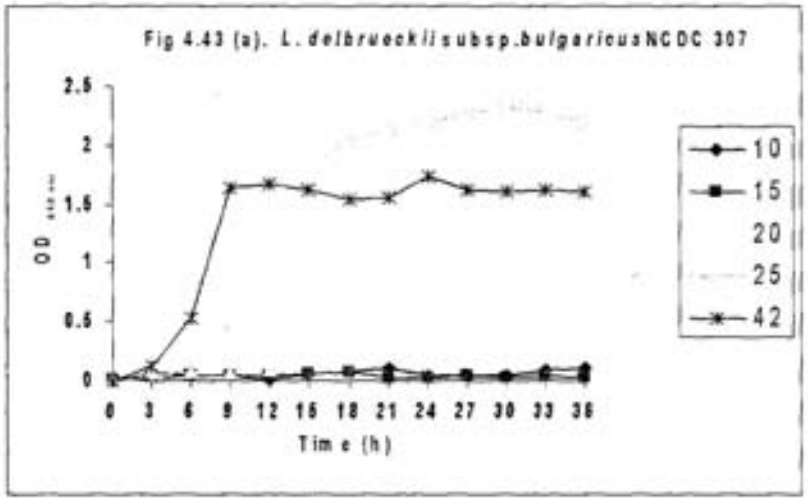


4.41 (b). *L. delbrueckii* subsp. *bulgaricus* NCDC 304




Fig. 4.42 (a). L.delbrueckii subsp. bulgaricus NCDC 305
OD 655 nm
Time (h)
10
15
20
25
42


Fig. 4.42 (b). L. delbrueckii subsp. bulgaricus NCDC 305
Log cfu/ml
Time (h)
10
15
20
25
42


Fig 4.43 (a). L. delbrueckii subsp.bulgaricus NCDC 307
OD 655 nm
Time (h)
10
15
20
25
42

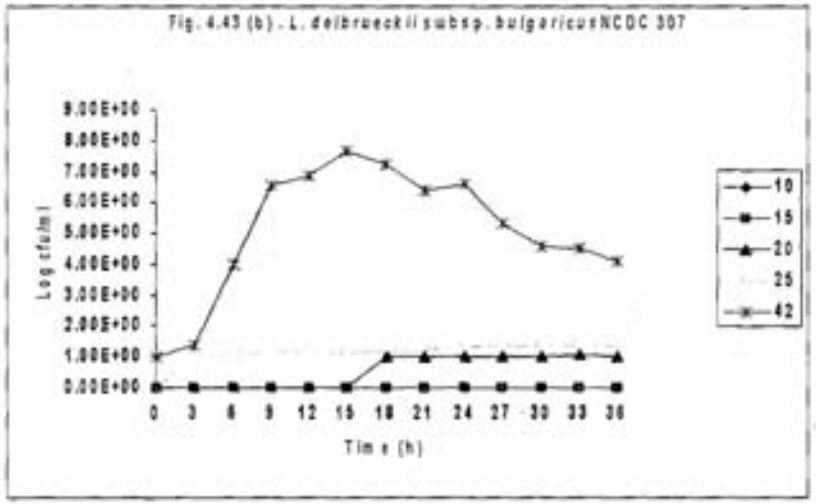

Fig. 4.43 (b). L. delbrueckii subsp. bulgaricus NCDC 307
Log cfu/ml
Time (h)
10
15
20
25
42

Fig. 4.44 (a). *L.delbrueckii* subsp. *bulgaricus* NCDC 308

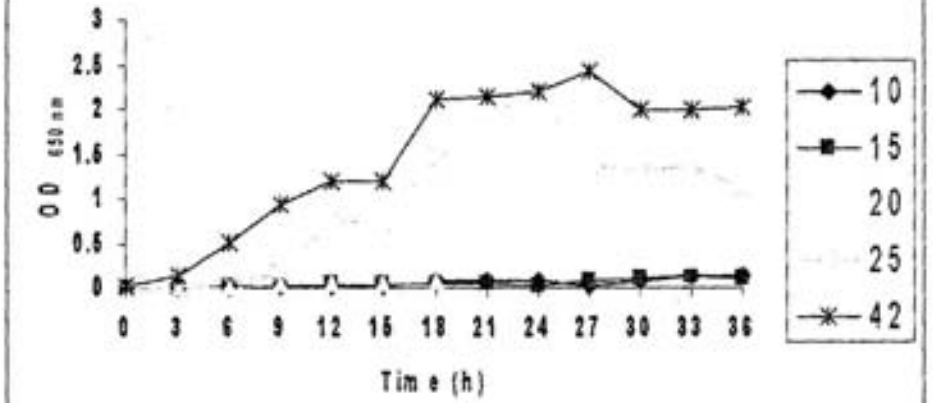


Fig.4.44 (b). *L.delbrueckii* subsp. *bulgaricus* NCDC 308



Table 4.5: Correlation coefficient (r^2) of *S. thermophilus* strains ($OD_{650\ nm}$ versus Log cfu value) at different pH and temp (^0C)

S.thermophilus strains	pH			Temp. (^0C)		
	4.5	5.0	6.5	20	25	42
NCDC 74	0.00	0.6689	0.8817	0.7265	0.8778	0.6689
NCDC 75	0.00	0.1123	0.7446	0.5698	0.6733	0.8111
NCDC 76	0.00	0.9037	0.8939	0.7780	0.7812	0.8939
NCDC 80	0.00	0.9208	0.8977	0.7152	0.8263	0.8977
NCDC 158	0.00	0.7443	0.7244	0.00	0.8027	0.7244
NCDC 175	0.00	0.8214	0.7501	0.00	0.9426	0.7501
NCDC 177	0.00	0.7973	0.8621	0.00	0.5492	0.8621
NCDC 199	0.00	0.5867	0.7825	0.00	0.8389	0.7825
NCDC 217	0.00	0.6584	0.7800	0.00	0.6733	0.7800
NCDC 218	0.00	0.8681	0.6860	0.00	0.3076	0.6859
NCDC 295	0.00	0.2373	0.9111	0.00	0.2373	0.9112
NCDC 303	0.00	0.8235	0.9249	0.00	0.5397	0.9249
NCDC 311	0.00	0.7478	0.8067	0.00	0.2499	0.6238
NCDC 312	0.00	0.6772	0.6738	0.00	0.2958	0.7612

Table 4.6: Growth rate (μ_{max} / h) of *S. thermophilus* strains at different pH and temp (^{0}C)

S.thermophilus strains	pH			Temp (^{0}C)		
	4.5	5.0	6.5	20	25	42
NCDC 74	0.00	0.12	0.53	0.008	0.26	0.53
NCDC 75	0.00	0.03	0.11	0.005	0.10	0.11
NCDC 76	0.00	0.035	0.17	0.00	0.02	0.17
NCDC 80	0.00	0.037	0.15	0.00	0.053	0.15
NCDC 158	0.00	0.00	0.15	0.00	0.030	0.15
NCDC 175	0.00	0.018	0.054	0.001	0.039	0.054
NCDC 177	0.00	0.025	0.25	0.00	0.050	0.25
NCDC 199	0.00	0.00	0.003	0.00	0.00	0.003
NCDC 217	0.00	0.006	0.11	0.001	0.016	0.14
NCDC 218	0.00	0.007	0.07	0.00	0.005	0.07
NCDC 295	0.00	0.003	0.054	0.00	0.00	0.054
NCDC 303	0.00	0.001	0.054	0.00	0.001	0.054
NCDC 311	0.00	0.001	0.006	0.00	0.007	0.060
NCDC 312	0.00	0.0008	0.14	0.002	0.006	0.14

The growth curves of 14 *S. thermophilus* strains at different pH have been drawn in Fig 4.45 to 4.58. All the strains demonstrated good growth at pH 5.0 and pH 6.5, but none survived at pH 4.5, 4.0 and 3.5. However, their growth rate was less as compared to that of the lactobacilli (Table 4.6). Thus amongst all the 36 cultures scanned at different pH only two cultures that too from genus *Lactobacillus* i.e. *L. acidophilus* NCDC 14 and *L. delbrueckii* subsp. *bulgaricus* NCDC 307 which showed better growth than others at low pH were selected for further acid adaptation studies.

Similarly all the 36 cultures were scanned at different temp ranges, for selection of a suitable culture for cold-stress studies. Fig 4.8 to 4.14 demonstrated growth curve of *L. acidophilus* strains at different temp and Fig 4.30 to 4.44 that of *L. delbrueckii* subsp. *bulgaricus* strains. All lactobacilli more or less grew at as low as 25 ^{0}C, however, some strains grew with a longer lag time at 20 ^{0}C. Fig 4.59 - 4.71 depicted the growth curves of *S. thermophilus* strains at different temp. All the strains showed a good growth at 42 ^{0}C and 25 ^{0}C, whereas some grew at 20 ^{0}C with the highest growth rate (μ_{max}/h) i.e. 0.008 attained by *S. thermophilus* NCDC 74 amongst all the TLAB (Table 4.6). Therefore *S. thermophilus* NCDC 74 was selected for further cold adaptation studies.

Several models like Gompertz (1825), Richards (1959), Stannard *et al.* (1985), Schnute (1981) etc. have been developed for treatment of growth related data of microorganisms. These models can be applied to determine the growth rate or to estimate the relative impact of the environmental factors on the microbial growth and thus to predict the behaviour of microorganisms in different stress conditions. The main objective of the use of the mathematical models is to describe the evolution of food-based microorganisms under the influence of intrinsic environmental factors (like pH, a_w) and extrinsic factors (like temp, gases etc.). In the present study, DMFit version 2.0 (Baranyi and Roberts, 1994) was used to analyze the growth data of the TLAB. The main difference between this model and other sigmoid curves like Gompertz, Logistic, etc. is that the mid-phase is really very close to linear, unlike those classical sigmoid curves which have a pronounced curvature at that position. In a study Zuera-Cosano *et al.* (2002) applied DMFit to evaluate the growth data of *Leuconostoc mesenteroides* for developing a response surface model. They observed that temp was the most decisive factor for growth rate (Gr) and there was a corresponding increase in the maximum specific growth rate with the increase in temp and pH. In a similar study Pin and Baranyi (1998) and Schepers, Thaibault and Lacroix (2002) estimated that pH was the most decisive factor in the growth of lactic acid bacteria. At the same time Garcia-Gimeno *et al.* (2002) noted that the conc of

NaCl, pH and temp had the most significant effect on the growth rate of *Lactobacillus plantarum*. An increase in lag time was observed when the pH and temp were decreased from optimal and sub optimal range during growth of lactic acid bacteria. In the present study an increase in lag time and decrease in growth rate have been observed in all the TLAB when subjected to growth conditions below the suboptimal range of temp and pH. The growth rate (μ_{max}/h) at various temp and pH levels was found to be strain and species specific. The same was demonstrated by Jan *et al.* (2000) in *Propionibacterium* while analysing the viability of different species and strains at various pH levels.

From the above analyses, three cultures viz. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307, *Lactobacillus acidophilus* NCDC 14, *Streptococcus thermophilus* NCDC 74 were selected for further studies and were subjected to acid and cold stress for their adaptation. Their DMFit curves have been presented in Fig 4.72 to 4.80.

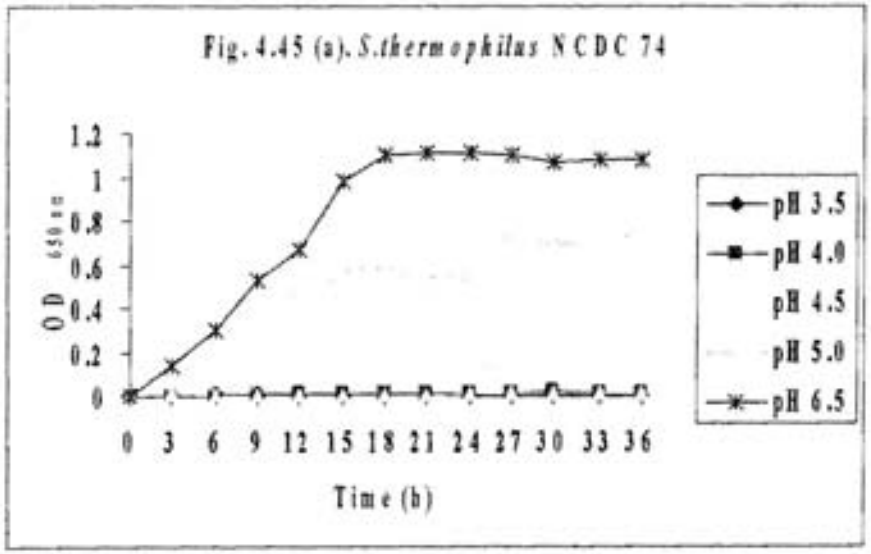
4.2 STRESS TOLERANCE RESPONSE OF THE TLAB

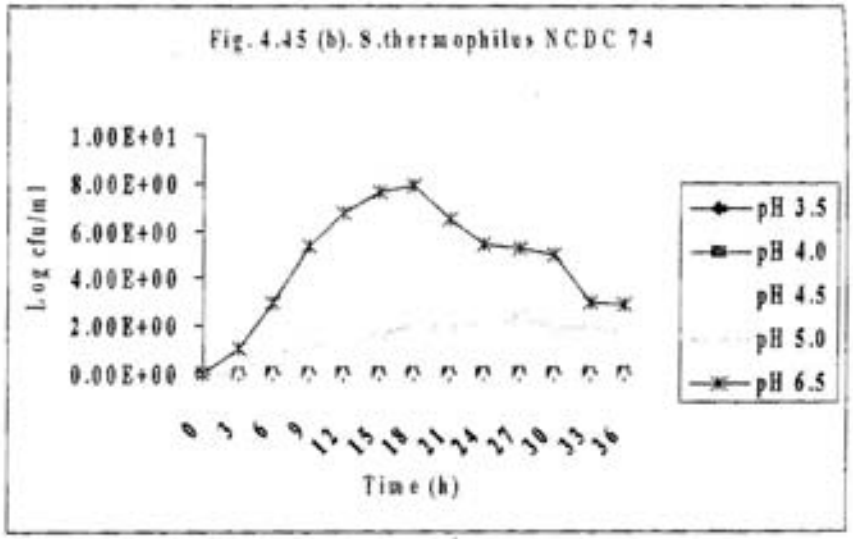
Before attempting to improve the selected TLAB with respect to their behaviour, acid tolerance response (ATR) in lactobacilli and cold shock response in *S. thermophilus* NCDC 74 were determined. The results of these experiments have been recorded in Fig 4.81 to 4.89.

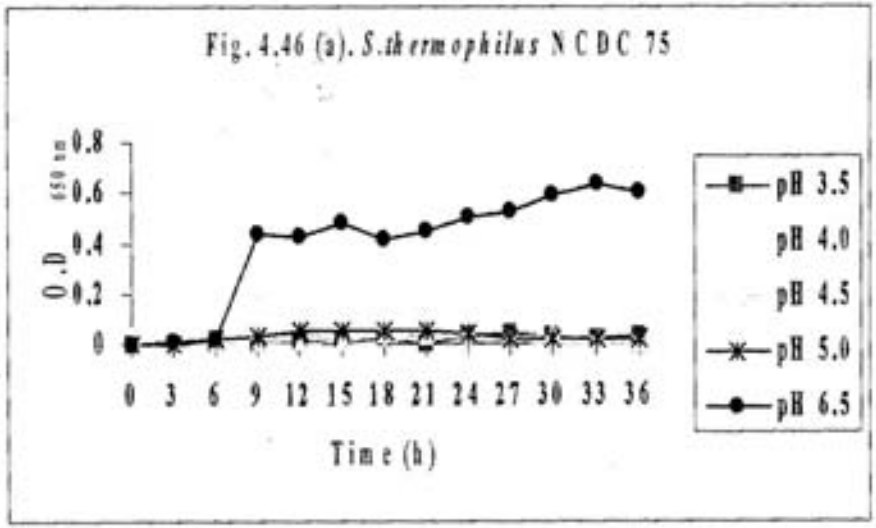
4.2.1 Acid tolerance Response (ATR) in *L. acidophilus* NCDC 14

Acid stress is considered as the exposure to pH values below the growth range (Jan *et al.*, 2000). Table 4.2 showed growth rate of this strain at different pH. No growth was observed at pH 4.0 and pH 3.5. At pH 4.5, growth rate was 0.047, with a longer lag time. ATR was characterised following acid exposure on log phase cells during 30 min at various pH values between 4 to 6 and subjected to acid challenge at pH 3.5. Cells exposed to pH 4.0 for 30 min showed the highest degree of resistance to a subsequent strong lactic acid challenge (pH 3.5) (Fig 4.81). Fig 4.82 showed the kinetics of acquisition of resistance of pH 4.0 adapted cells towards lactic acid challenge at pH 3.5. In the first 20 min, resistance increased exponentially

Fig 4.45 - 4.58: Growth curves of *S. thermophilus* strains at different pH (a: $OD_{650\ nm}$; b: Log cfu/ml)







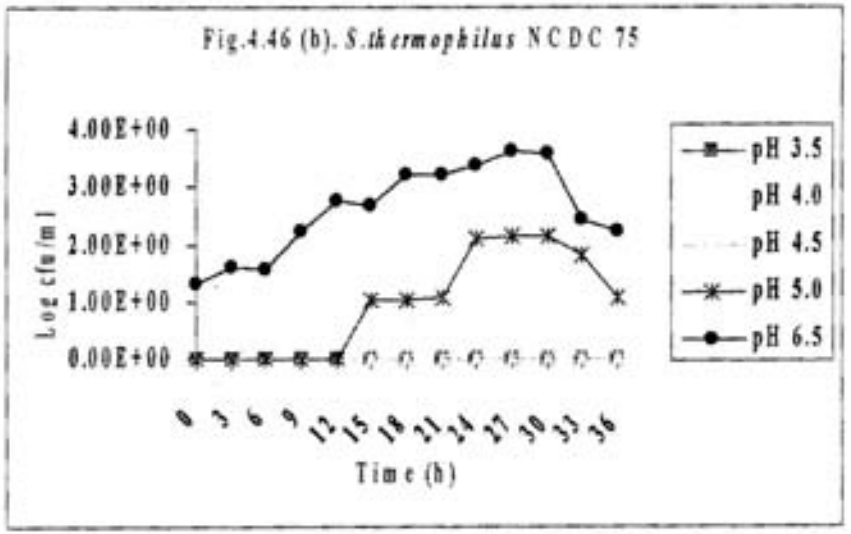


Fig. 4.47 (a). *S.thermophilus* NCDC 76

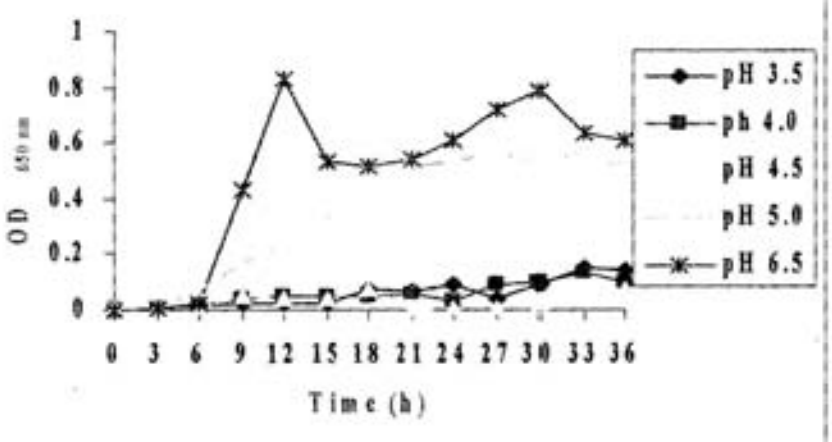


Fig. 4.47 (b). *S.thermophilus* NCDC 76

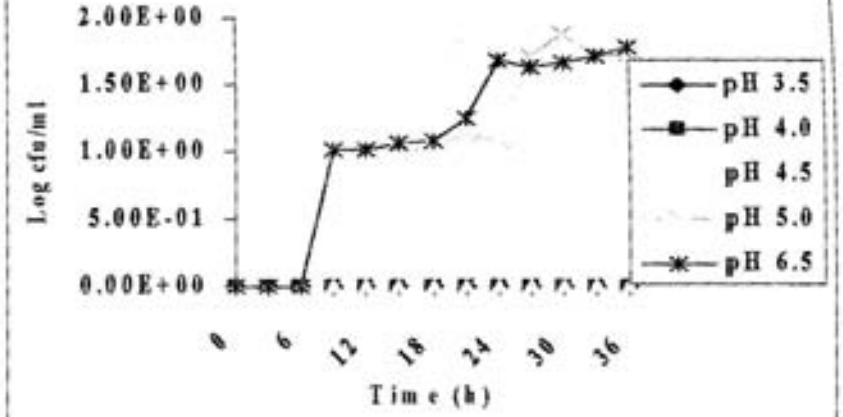


Fig. 4.48 (a). *S.thermophilus* NCDC 80

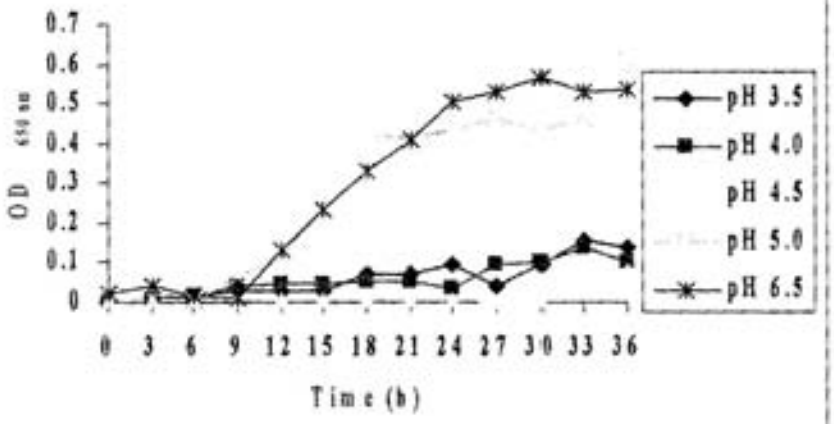


Fig. 4.48 (b). *S.thermophilus* NCDC 80

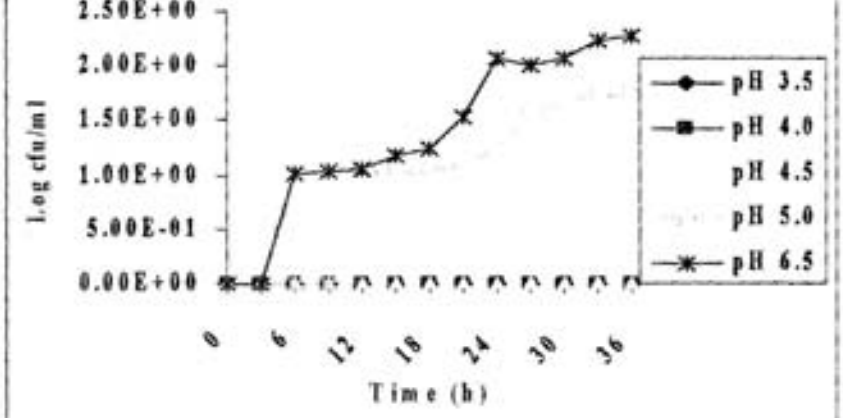


Fig 4.49 (a). *S.thermophilus* NCDC 158

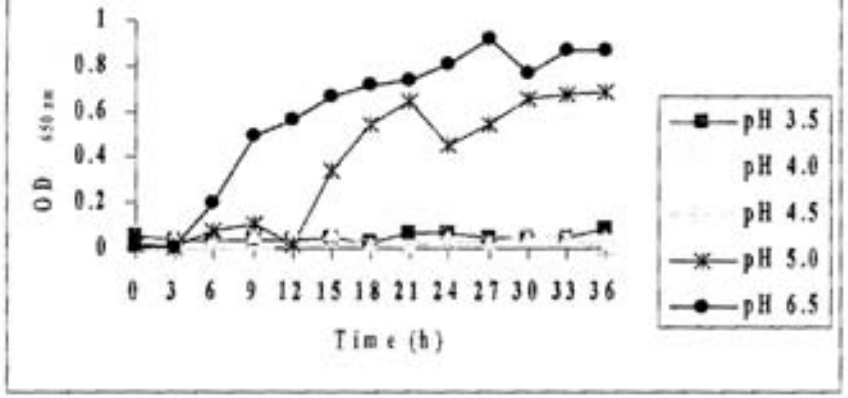


Fig 4.49 (b). *S.thermophilus* NCDC 158

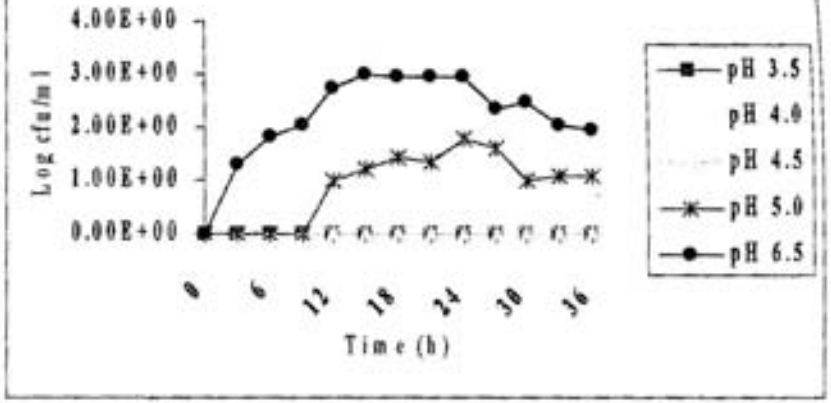


Fig. 4.50 (a). *S. thermophilus* NCDC 175

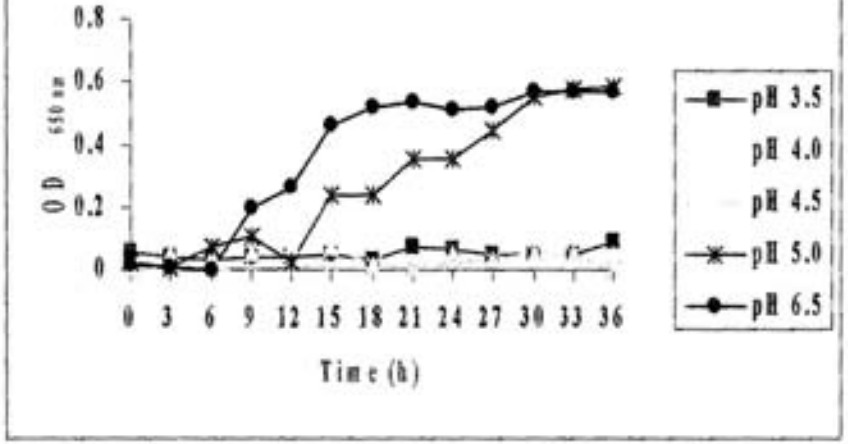


Fig. 4.50 (b). *S. thermophilus* NCDC 175

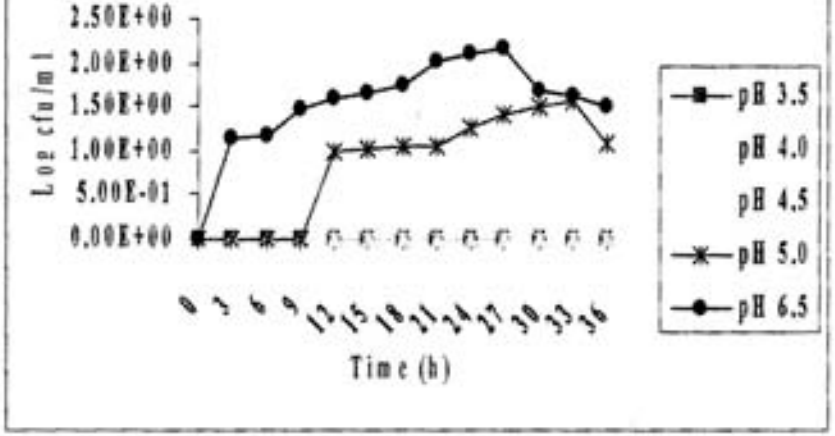


Fig. 4.51 (a). *S.thermophilus* NCDC177

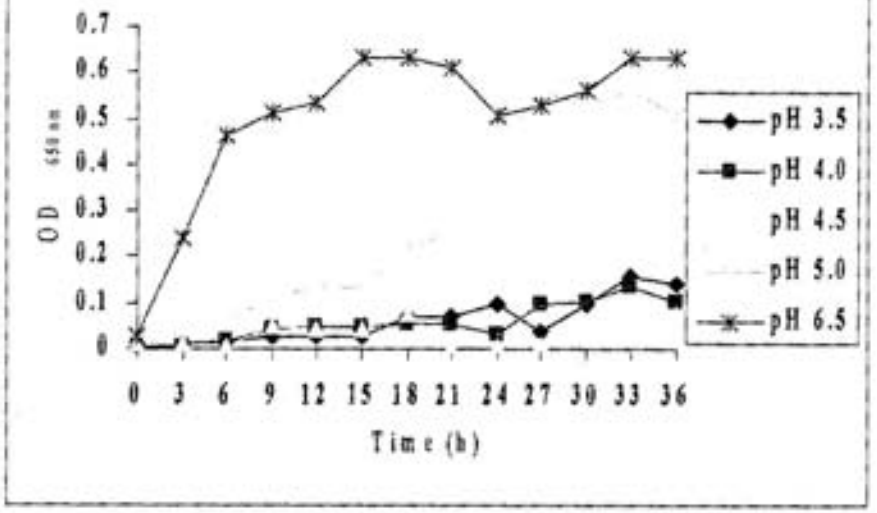


Fig. 4.51 (b). *S.thermophilus* NCDC177

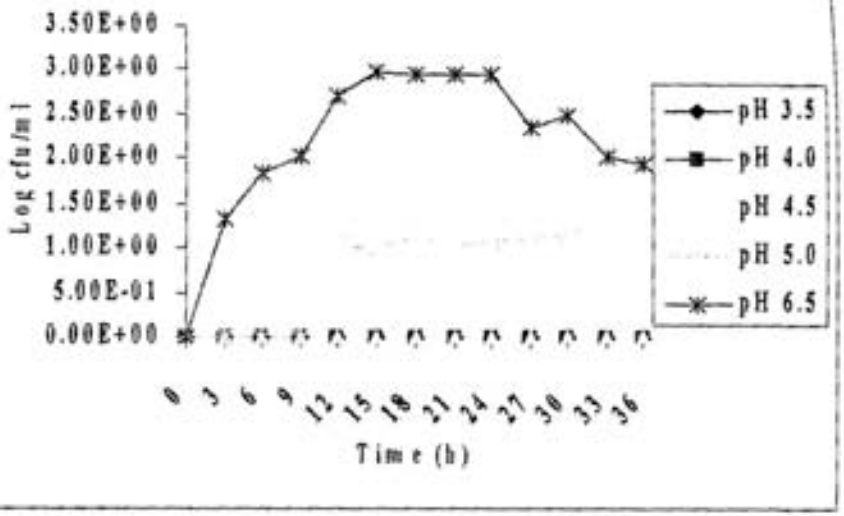


Fig. 4.52 (a). *S.thermophilus* NCDC 199

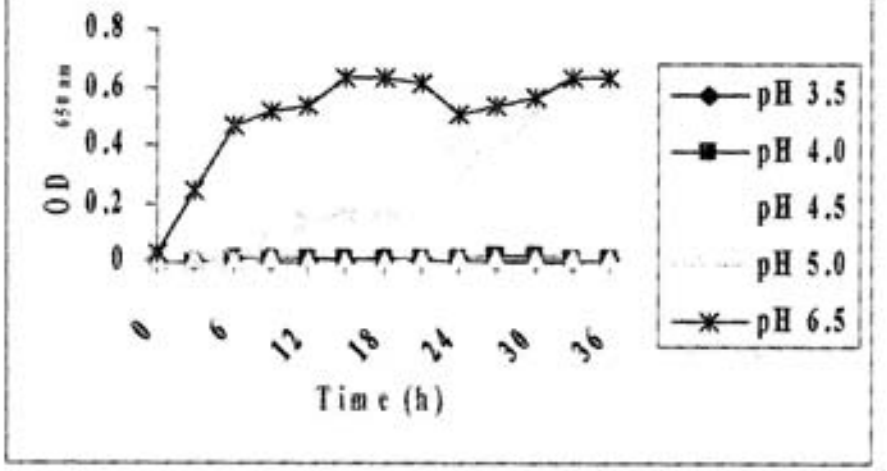


Fig. 4.52 (b). *S.thermophilus* NCDC 199

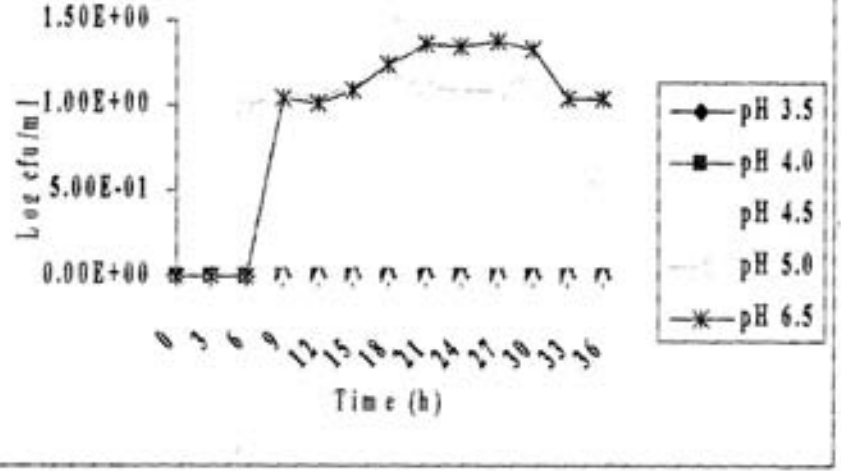


Fig. 4.53 (a). *S.thermophilus* NCDC 217

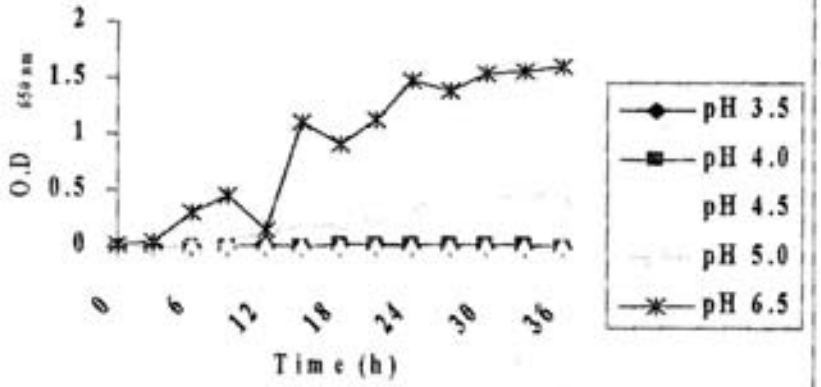


Fig. 4.53 (b). *S.thermophilus* NCDC 217

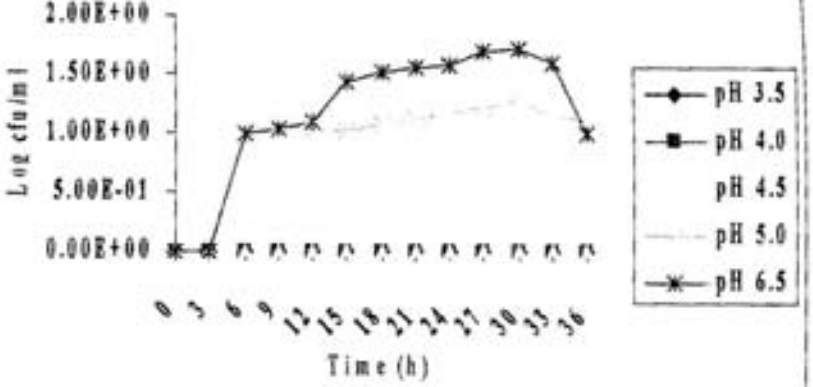


Fig. 4.54 (a). *S.thermophilus* NCDC 218



Fig. 4.54 (b). *S.thermophilus* NCDC 218



Fig. 4.55. (a). *S.thermophilus* NCDC 295

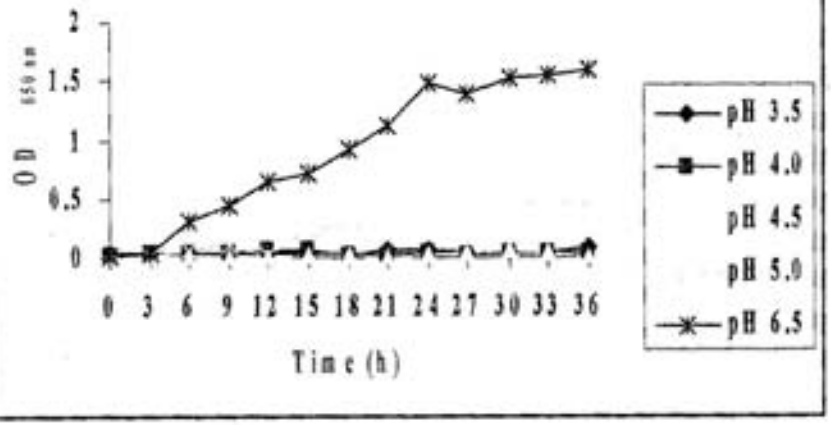


Fig. 4.55. (b). *S.thermophilus* NCDC 295

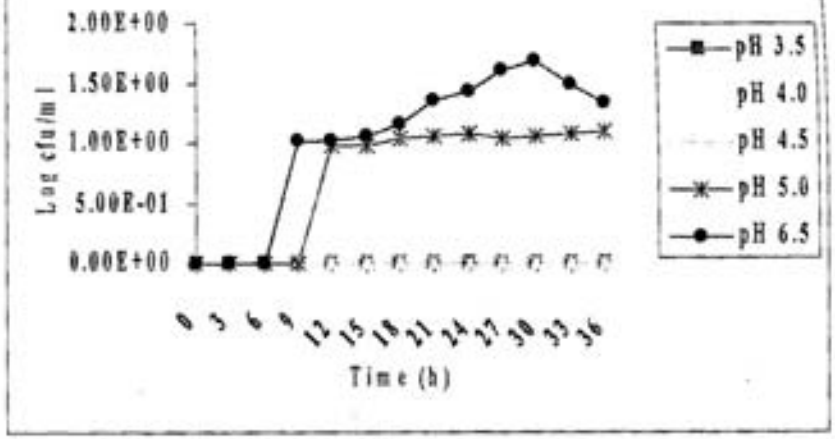


Fig. 4.56 (a). *S.thermophilus* NCDC 303

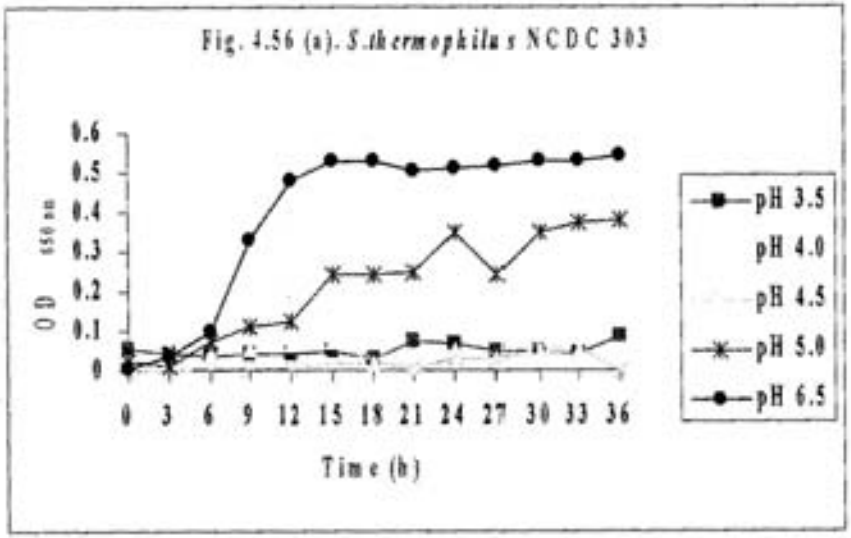


Fig. 4.56 (b). *S.thermophilus* NCDC 303

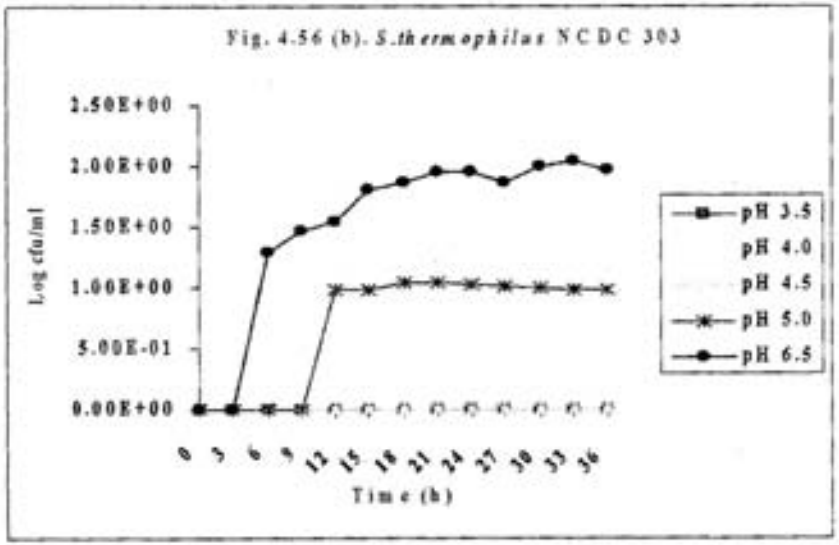


Fig. 4.57 (a). *S.thermophilus* NCDC 311

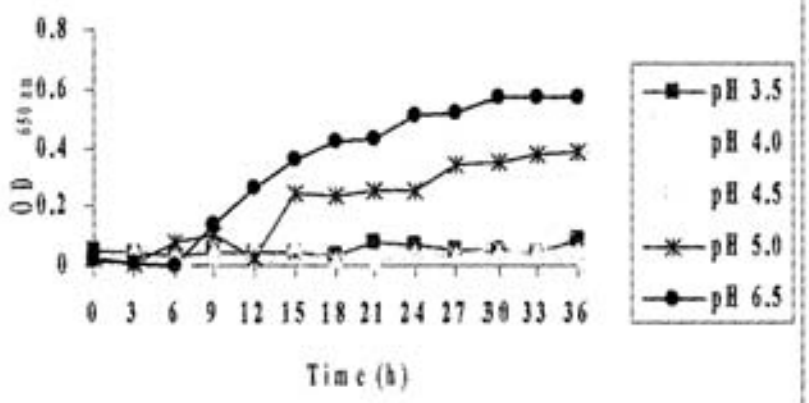


Fig. 4.57 (b). *S.thermophilus* NCDC 311

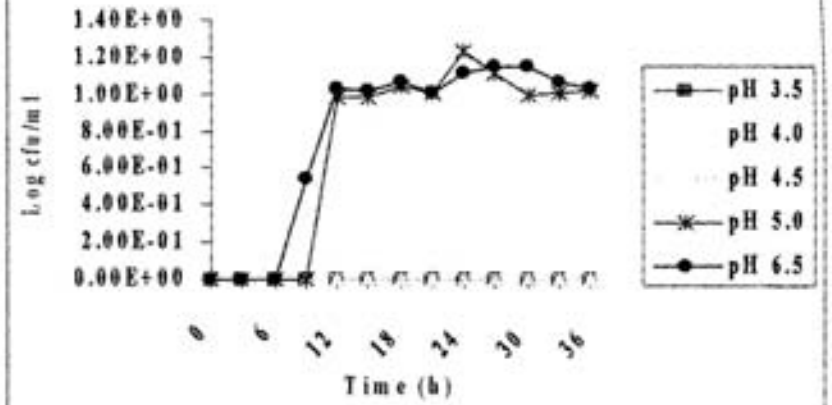


Fig. 4.58 (a). *S.thermophilus* NCDC 312

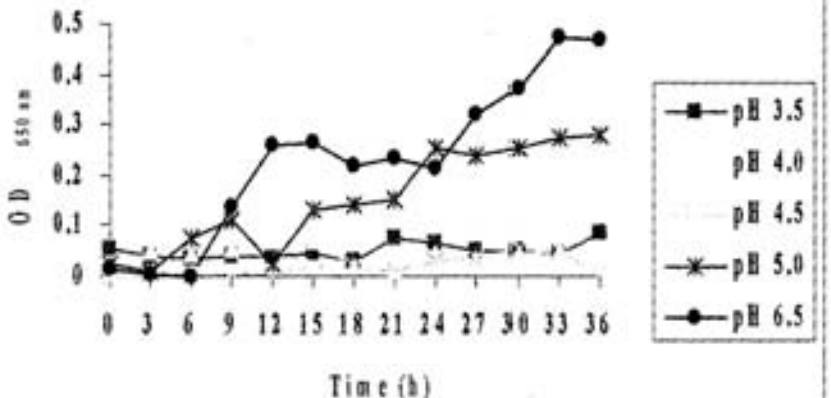


Fig. 4.58 (b). *S.thermophilus* NCDC 312

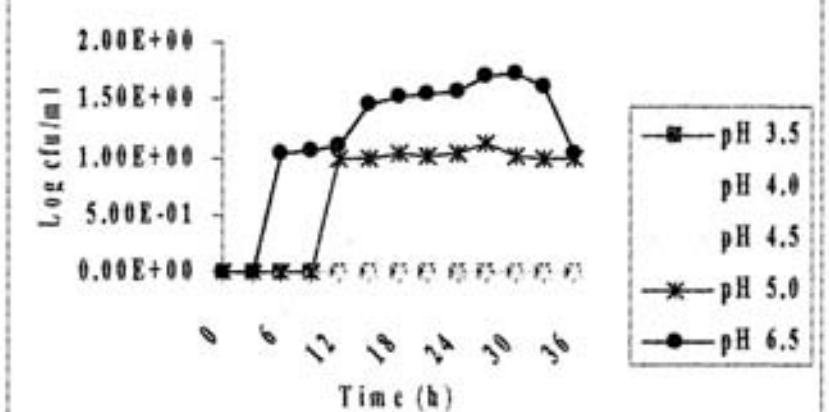
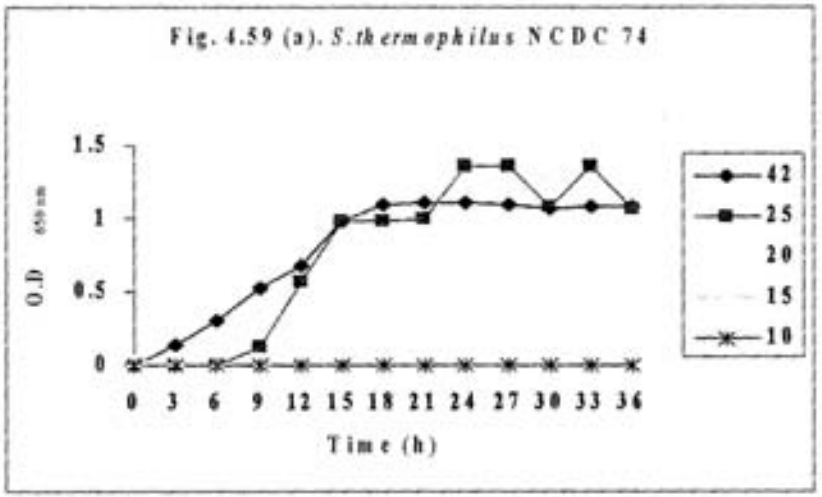
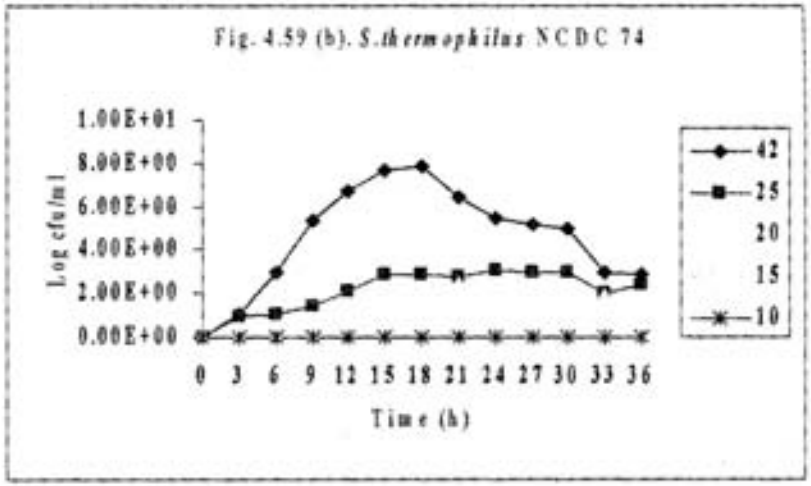
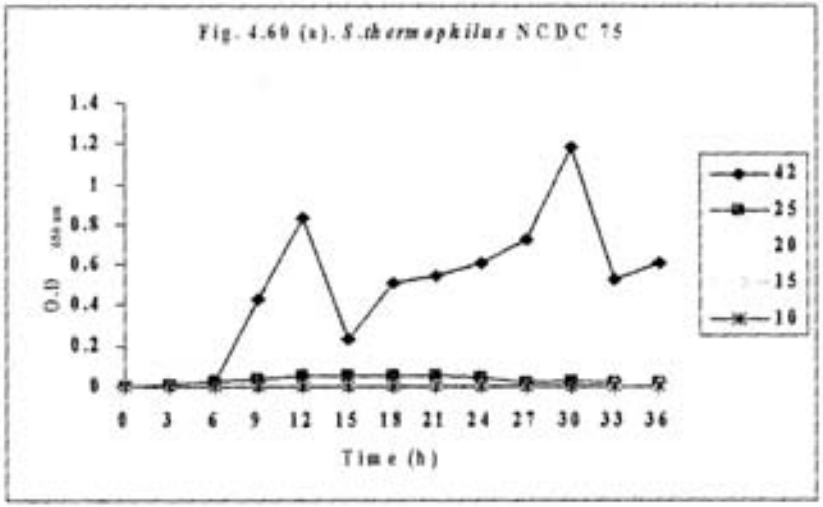


Fig 4.59 - 4.71: Growth curves of different *S. thermophilus* strains at various levels of temp (^{0}C) (a: $OD_{650\,nm}$; b: Log cfu/ml)







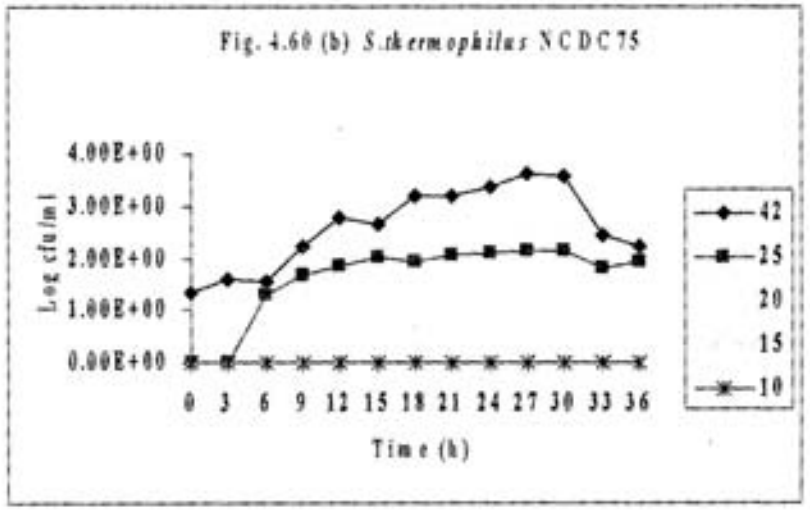


Fig. 4.61 (a). *S.thermophilus* NCDC 76

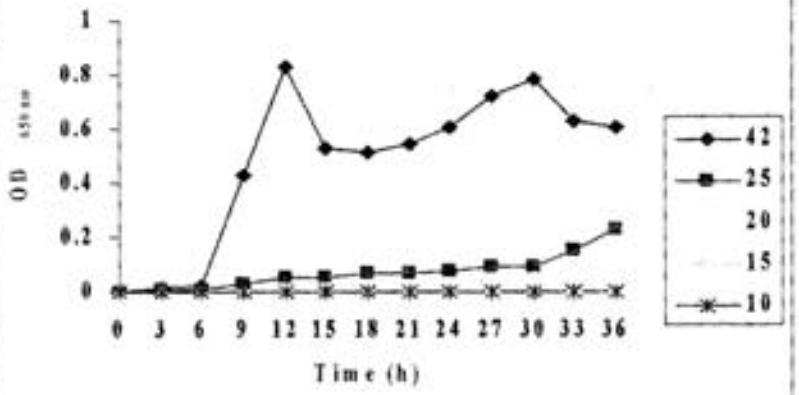


Fig. 4.61 (b). *S.thermophilus* NCDC 76

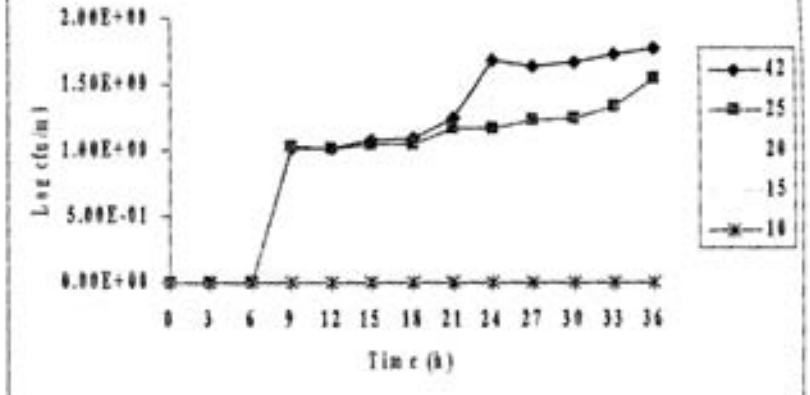


Fig. 4.62 (a). *S. thermophilus* NCDC 80

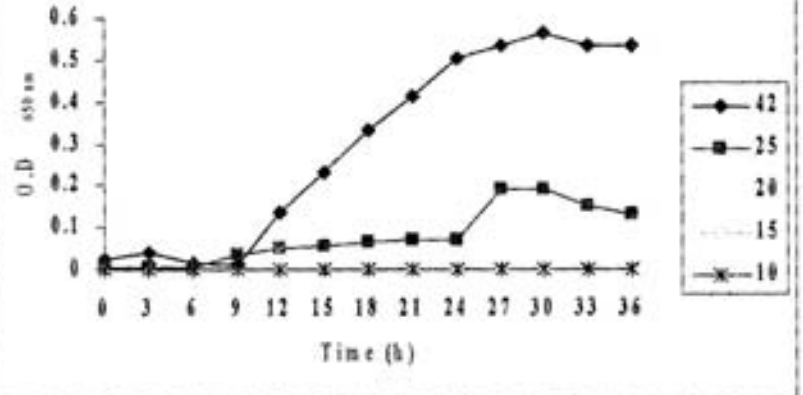


Fig. 4.62 (b). *S. thermophilus* NCDC 80

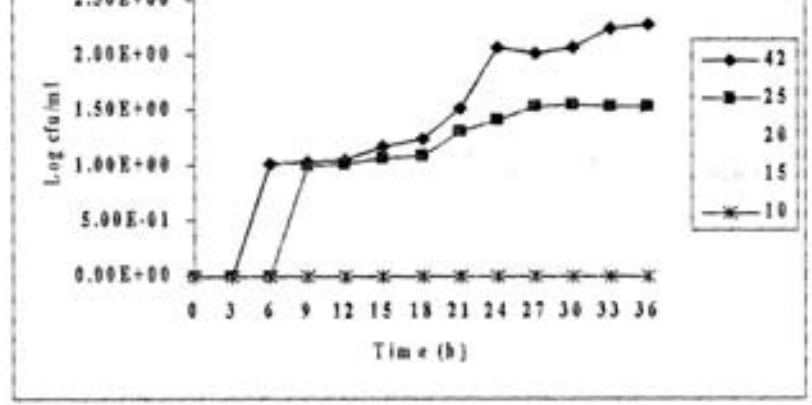


Fig. 4.63 (a). *S.thermophilus* NCDC 158

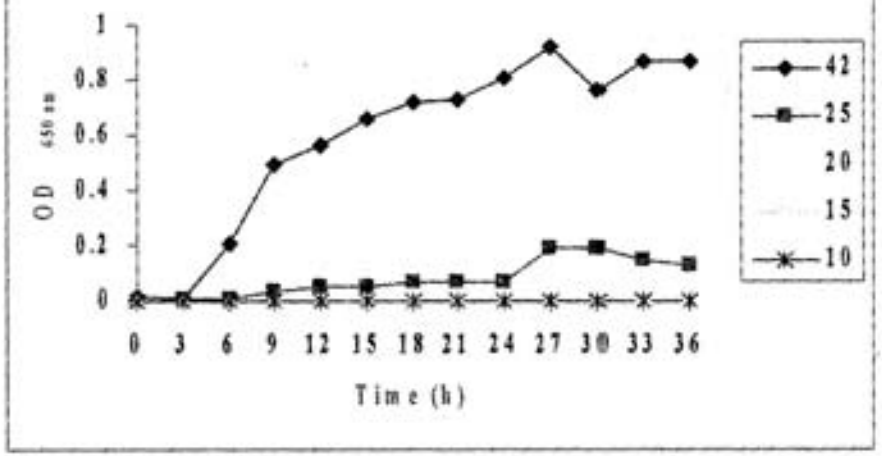


Fig. 4.63 (b). *S.thermophilus* NCDC 158

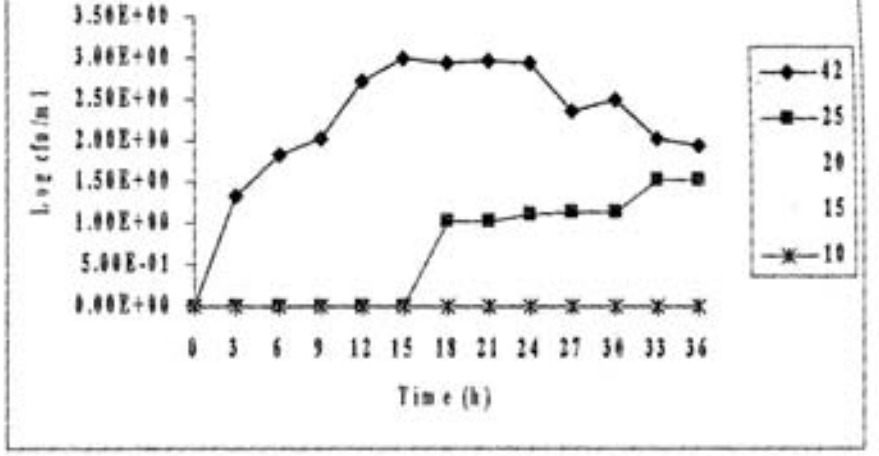


Fig. 4.64 (a). *S.thermophilus* NCDC 175

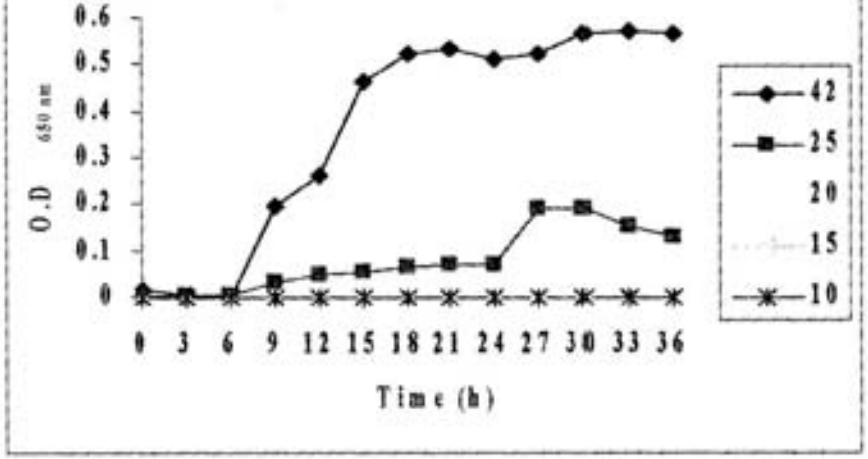


Fig. 4.64 (b). *S.thermophilus* NCDC 175

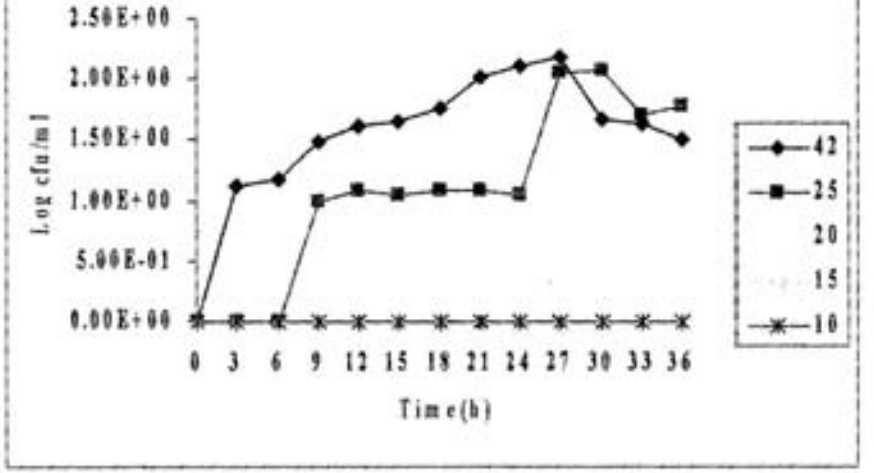


Fig. 4.65 (a). *S.thermophilus* NCDC199

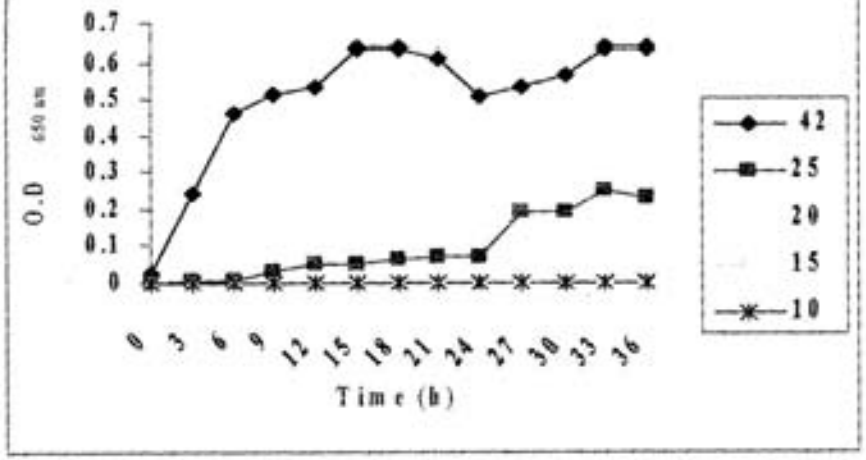


Fig. 4.65 (b). *S.thermophilus* NCDC199

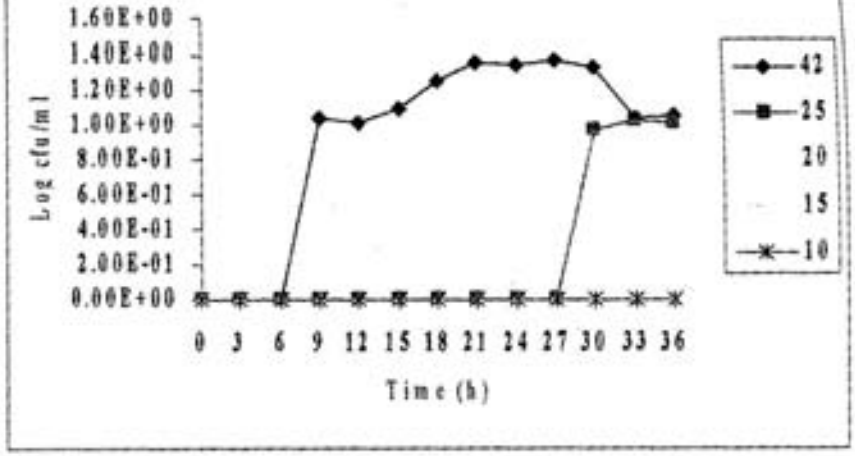


Fig. 4.66 (a). *S.thermophilus* NCDC 217

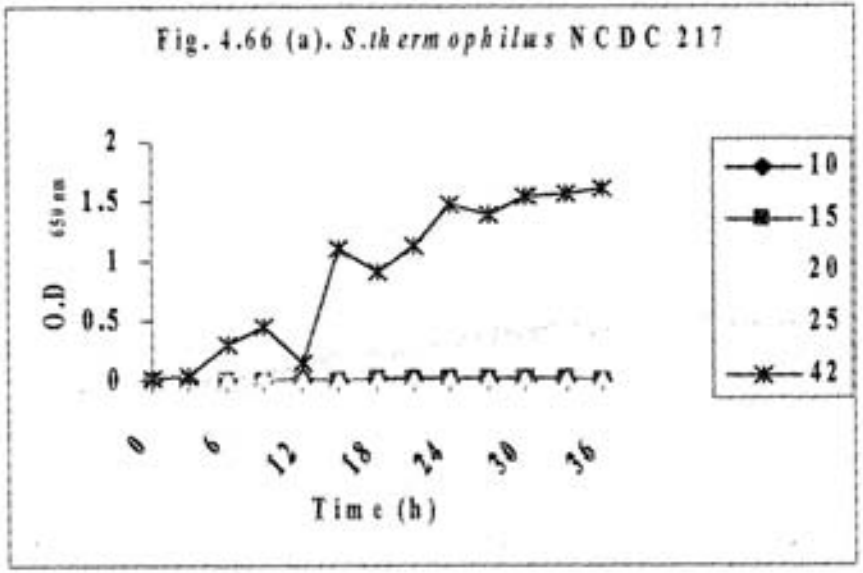


Fig. 4.66 (b). *S.thermophilus* NCDC 217

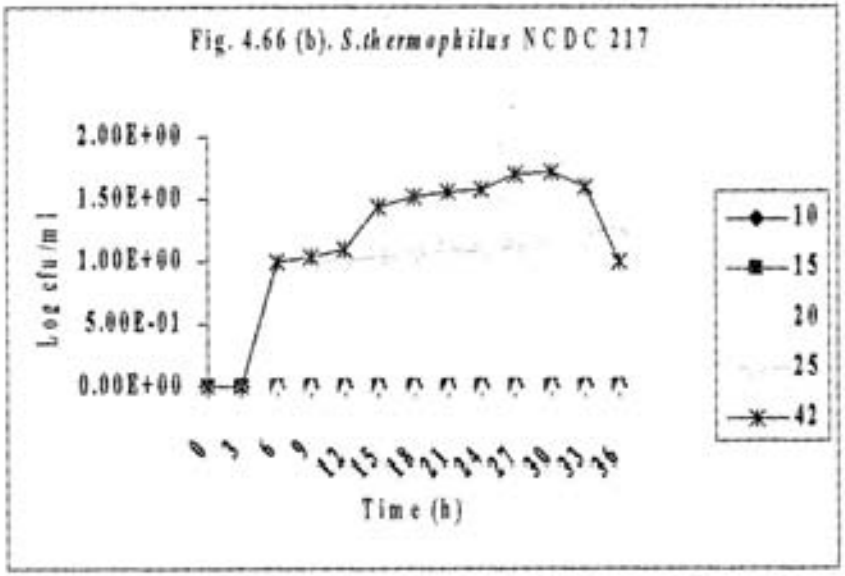


Fig. 4.67 (a). *S.thermophilus* NCDC 218

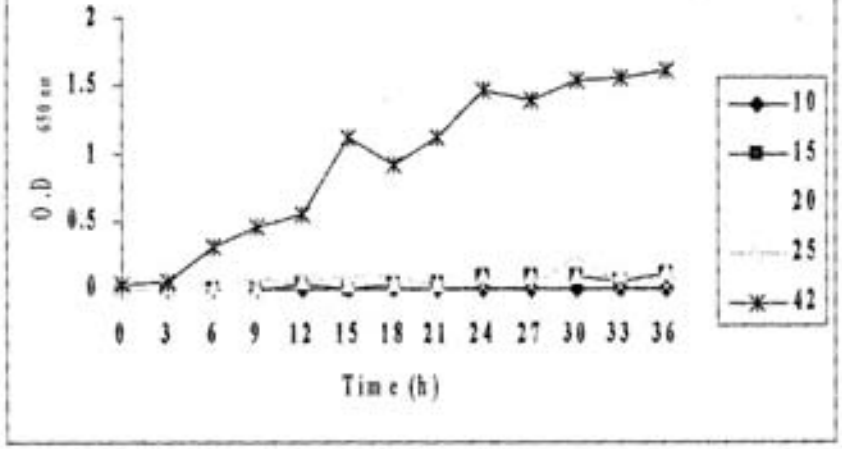


Fig. 4.67 (b). *S.thermophilus* NCDC 218

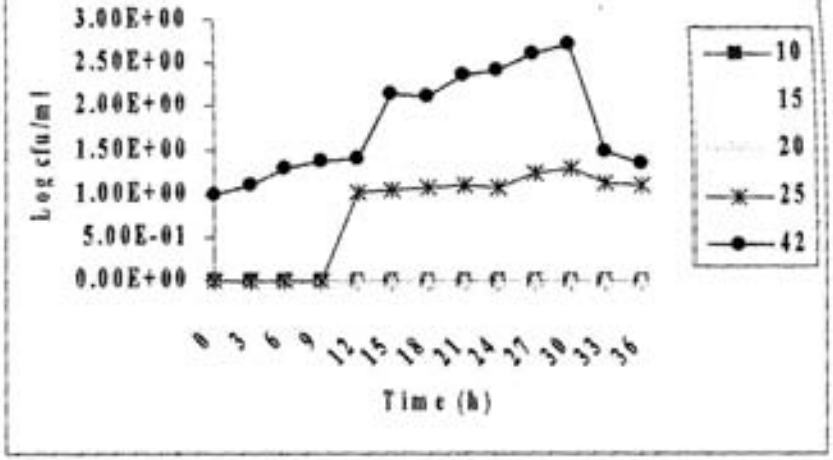


Fig. 4.68 (a). *S.thermophilus* NCDC 295

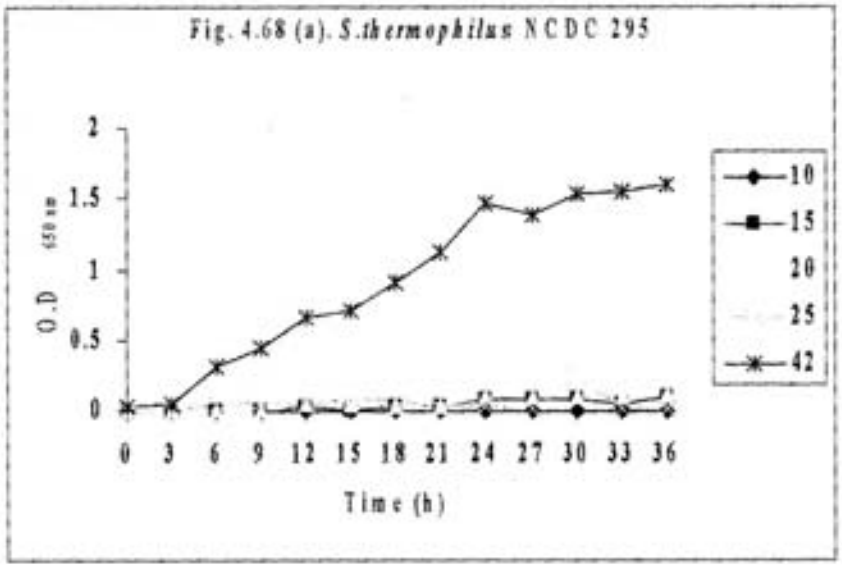


Fig. 4.68 (b). *S.thermophilus* NCDC 295

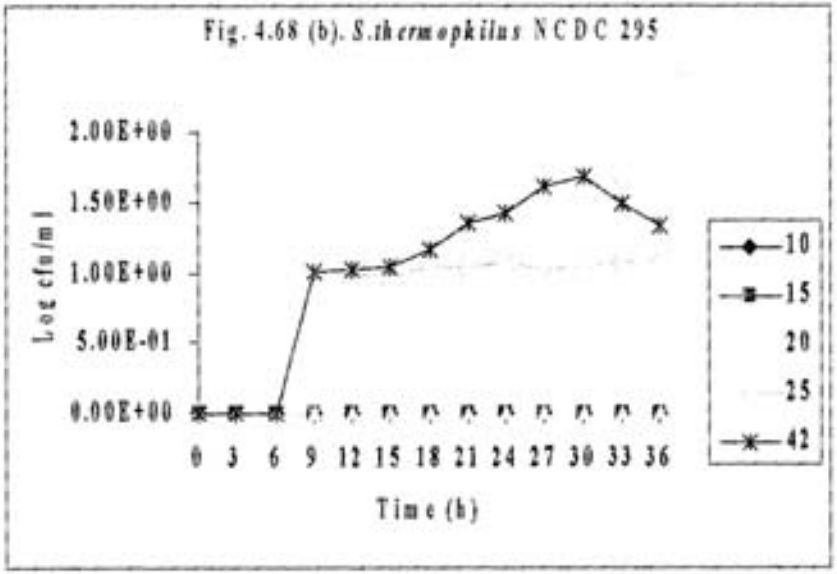


Fig. 4.69 (a). *S.thermophilus* NCDC 303

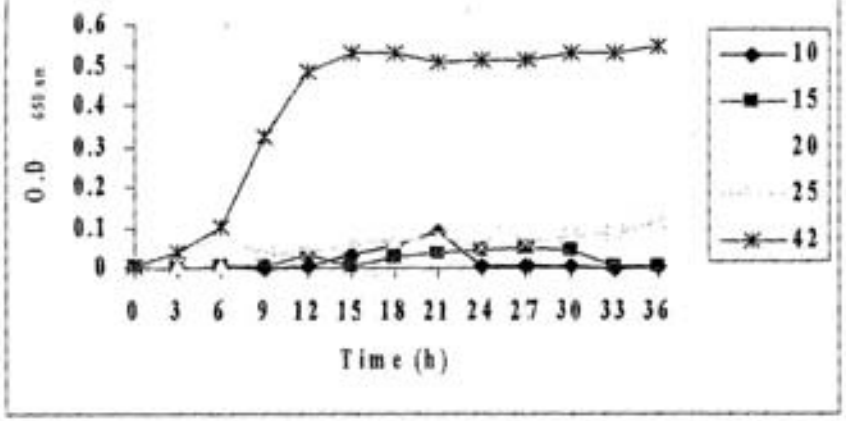


Fig. 4.69 (b). *S.thermophilus* NCDC 303

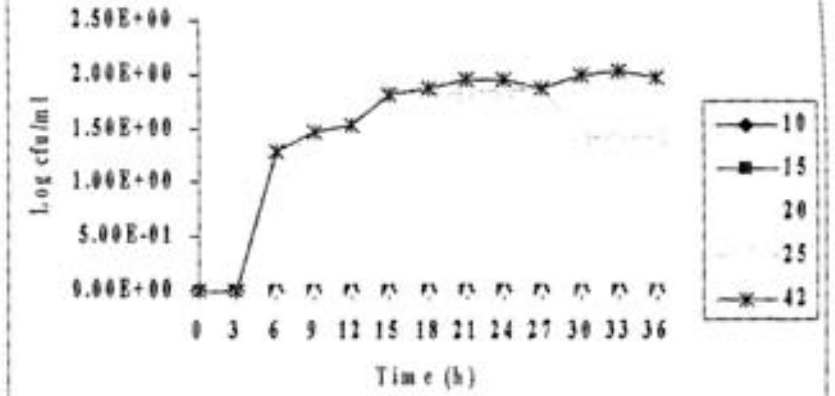


Fig. 4.70 (a). *S.thermophilus* NCDC 311

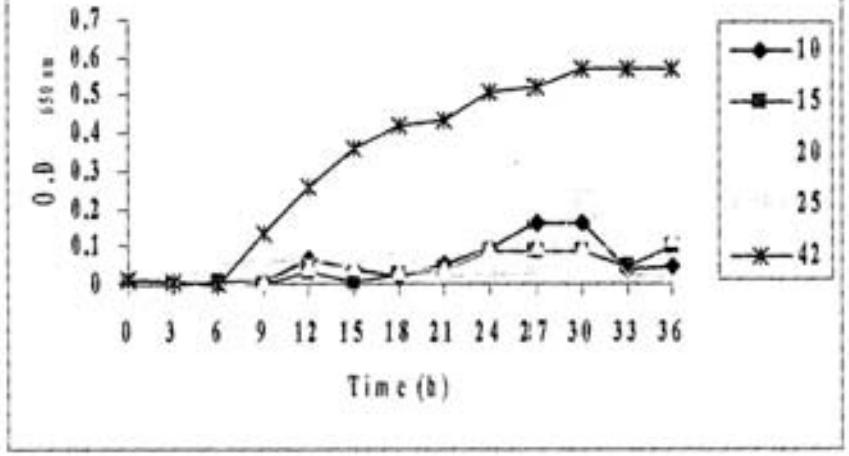
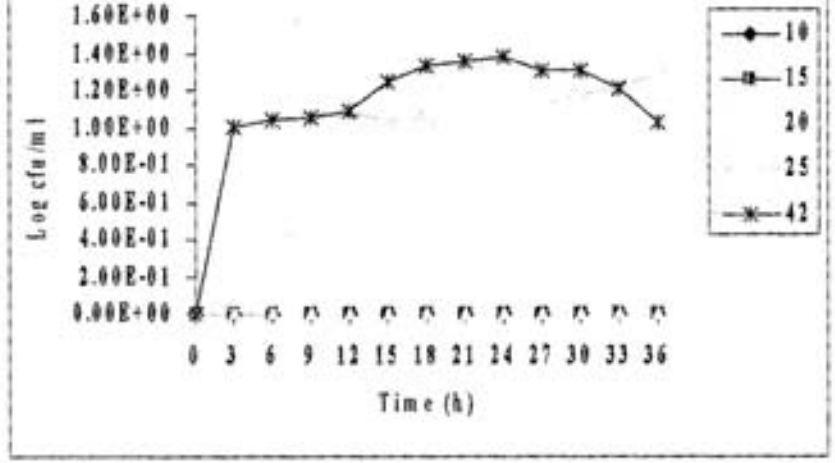
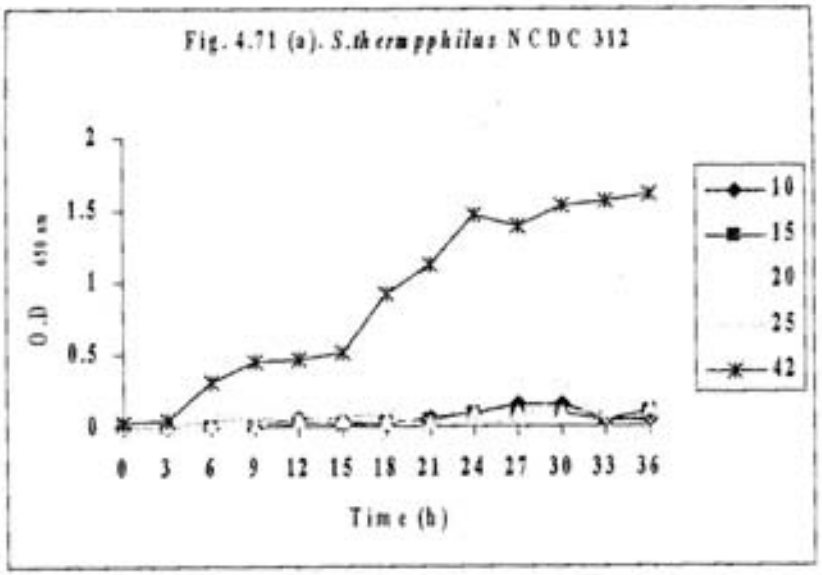


Fig. 4.70 (b). *S.thermophilus* NCDC 311




Fig. 4.71 (a). S.thermpphilus NCDC 312
O.D 650 nm
2
1.5
1
0.5
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42

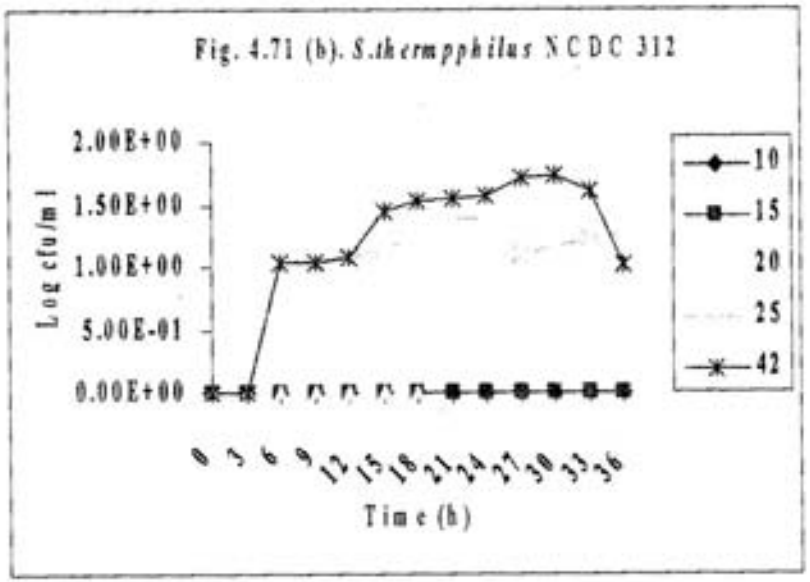
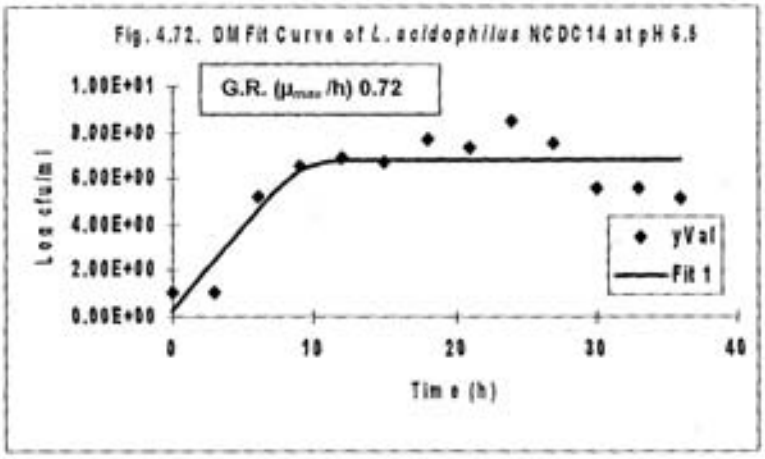
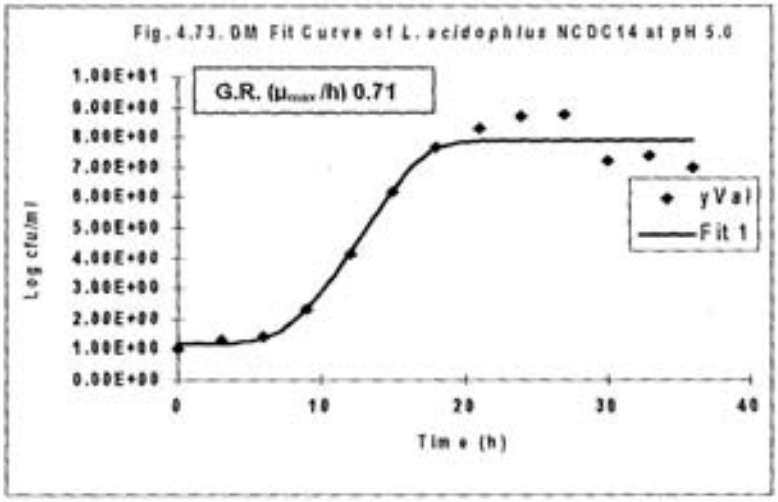

Fig. 4.71 (b). S.thermpphilus NCDC 312
Log cfu/ml
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
0 3 6 9 12 15 18 21 24 27 30 33 36
Time (h)
10
15
20
25
42

Fig 4.72 – 4.74: DMFit curve of *L. acidophilus* NCDC 14 at different pH





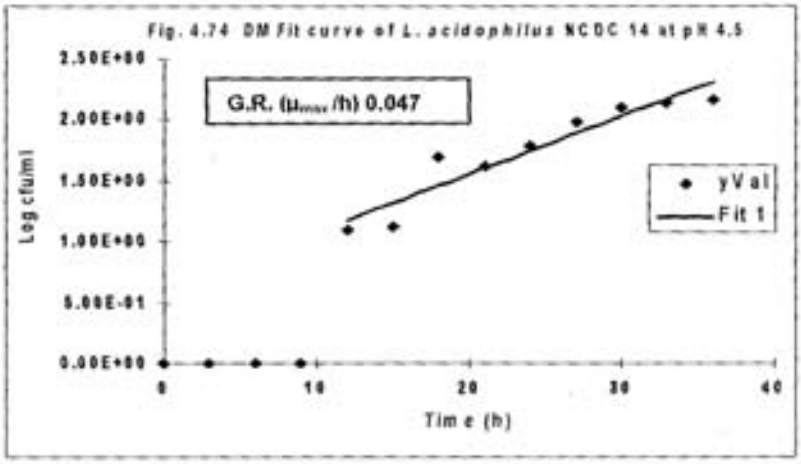
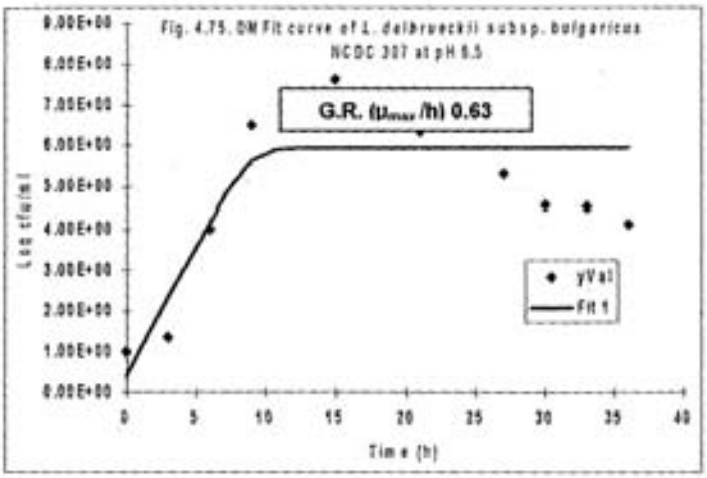
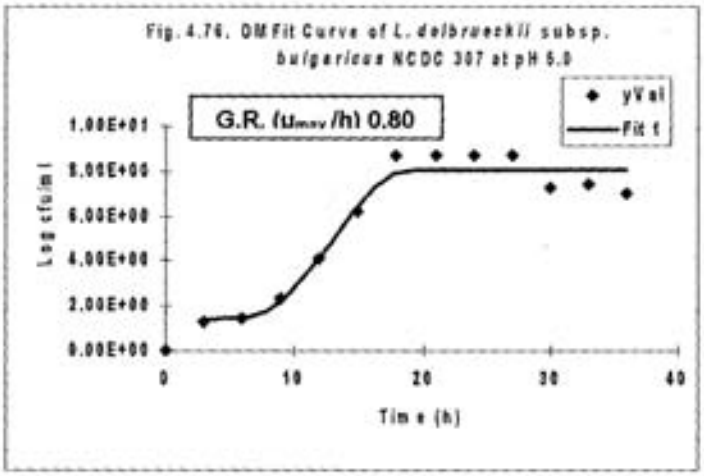


Fig 4.75 – 4.77: DMFit curve of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 at different pH





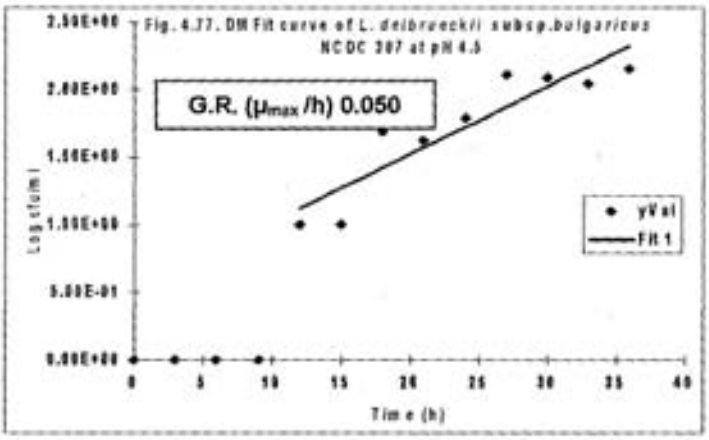
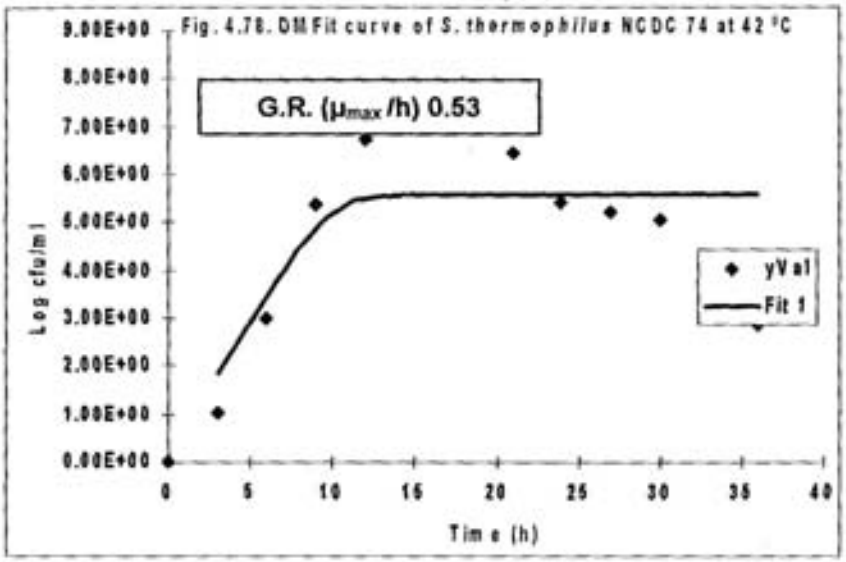
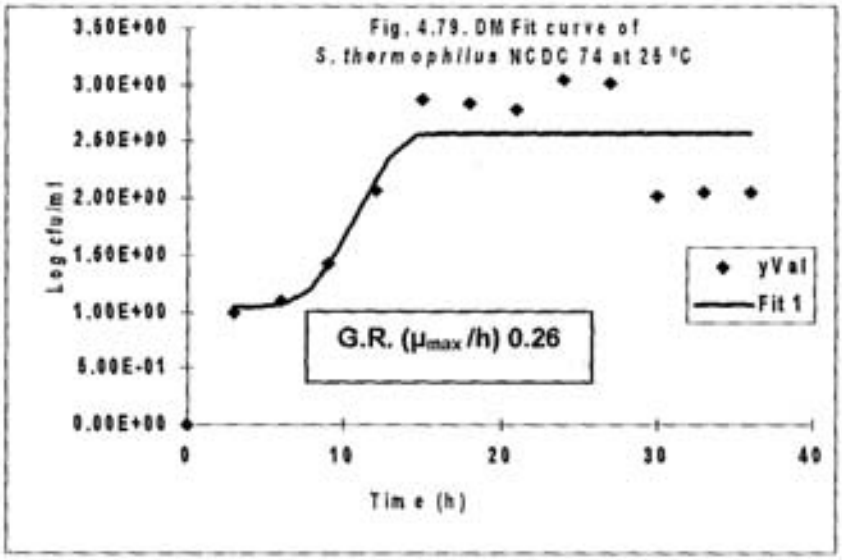
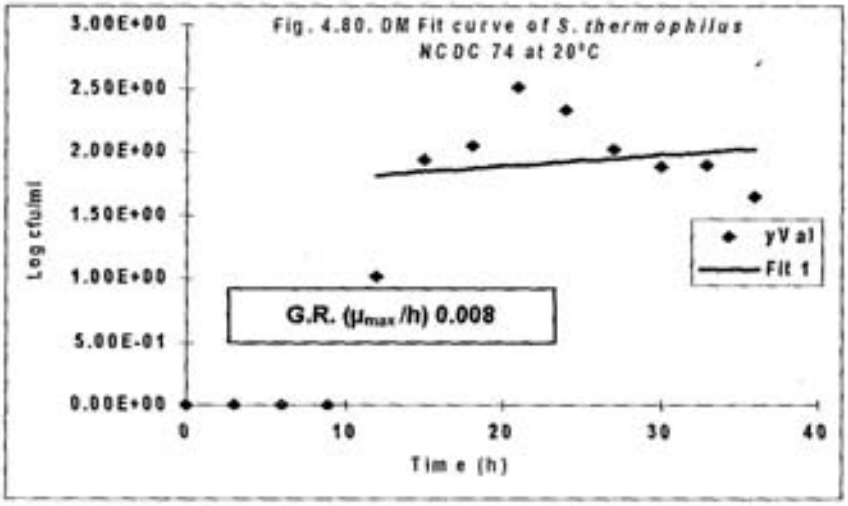


Fig 4.78 - 4.80: DMFit curve of *S. thermophilus* NCDC 74 at different temp (^{0}C)







and levelled off in the following 40 min. Log phase ATR was observed after a 30 min exposure to pH 4.0, ≈ 200 fold cells survived after exposure to lethal pH for 30 min. Chloramphenicol added cells showed a low survival as compared to adapted cells suggesting the role of *de novo* protein synthesis during acid stress (Fig 4.83).

4.2.2 Acid tolerance Response (ATR) in *L. delbrueckii* subsp. *bulgaricus* NCDC 307

Acid stress is considered as the exposure to pH values below the growth range (Jan *et al.*, 2000). Table 4.4 showed growth rate of this strain at different pH. No growth was observed at pH 4.0 and pH 3.5. At pH 4.5, growth rate was 0.05, with a lag time of ~ 9h. Thus pH 4.5 was considered as moderate acidic conditions. ATR was characterised following acid adaptation on log phase cells during 30 min at various pH values between 4 to 6 and subjected to acid challenge at pH 3.5. Cells exposed to pH 4.5 for 30 min showed the highest degree of resistance to a subsequent strong lactic acid challenge (pH 3.5) (Fig 4.84). Fig 4.85 showed the kinetics of acquisition of resistance of pH 4.5 exposed cells towards lactic acid challenge at pH 3.5. In the first 20 min, resistance increased exponentially and levelled off in the following 40 min. Log phase ATR was observed after a 30 min exposure to moderate stress (pH 4.5), ≈ 200 fold cells survived after exposure to lethal pH for 30 min. Chloramphenicol added cells showed a low survival as compared to adapted cells suggesting the role of *de novo* protein synthesis during acid stress (Fig 4.86).

Determination and characterization of ATR is important from practical point of view. It leads to selection of conditions for development of robust strains. In the present study a log phase ATR was demonstrated in both the lactobacilli. In LAB, acid tolerance (AT) increased in at least two distinct physiological states. (i) during logarithmic growth an adaptive response referred to as L-ATR, can be induced by incubation at a non-lethal acidic pH; (ii) after entry in the stationary phase, ATR increases as a result of the induction of a general stress response (Hartke *et al.*, 1996). The latter

response is usually independent of the external pH (pH_e), although in *L. acidophilus* CRL639 a low pH_e was needed (Lorca *et al.*, 2001). Log phase ATR had also been demonstrated in acid adapted cells (30 min incubation at PH 4.75 (pH adjusted with HCl)) of *L. delbrueckii* subsp. *bulgaricus* VI-1010 by Lim *et al.*, 2001. In the present study, log phase acid tolerance response has been observed in both *L. acidophilus* NCDC 14 and *L. delbrueckii* subsp *bulgaricus* NCDC 307 at pH 4.0 and 4.5 for exposure period of 30 min respectively. This implies that these strains have the capacity to adapt at low pH. It may be noted that acid adaptation protects the organisms from lethal stress and *de novo* protein synthesis may be required during the process.

4.2.2.1 Cold shock response in *S. thermophilus* NCDC 74

4.2.2.1.1 Effect of temp down shock on growth of S. thermophilus NCDC 74

S. thermophilus NCDC 74 grows optimally at 42 ^{0}C and has a minimal growth temp just below 20 ^{0}C. Growth parameters were calculated from DMFit version 2.0 programme. The growth rate (μ_{max}/h) significantly decreased at low temp (from 0.5 to 0.008 at 20 ^{0}C, Table 4.6). This strain was able to adapt relatively quickly (within 2 to 4 h) when shifted from 42 to 20 ^{0}C (Fig 4.87). This indicates that this strain has the capacity to efficiently adapt to low temps. However, a cold shock from 42 to 10 ^{0}C resulted in a growth block. This observation was in line with Wouters *et al.* (1999).

4.2.2.1.2 Increased survival after freezing following low temp treatment

The survival after freezing was determined for cells grown to mid-exponential phase at 42 ^{0}C and for cultures which were exposed to low temps (10, 15 or 20 ^{0}C) for 2, 4 and 6 h. After four consecutive freeze-thaw cycles, approximately 0.01 % of cells cultured at 42 ^{0}C survived. The exposure of cells to 20 ^{0}C for 2, 4 and 6 h results in an increased survival of approximately a factor of 100, 1000 and 500 fold respectively, compared to the survival of mid-exponential phase cells cultured at 42 ^{0}C (Fig 4.88). However, exposure to 10 and 15 ^{0}C for 2, 4 and 6 h results in only a small increase (10 to15 fold) in freeze survival (Fig 4.89).

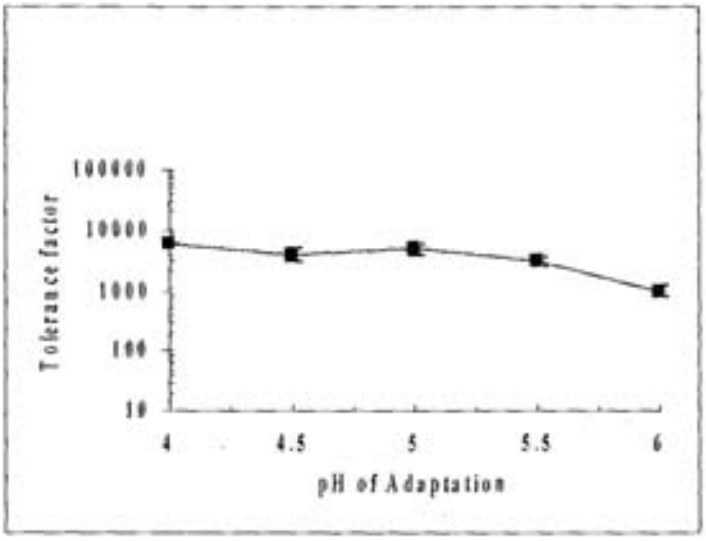


Fig 4.81: Determination of ATR in *L. acidophilus* NCDC 14 (Bars indicate standard error)

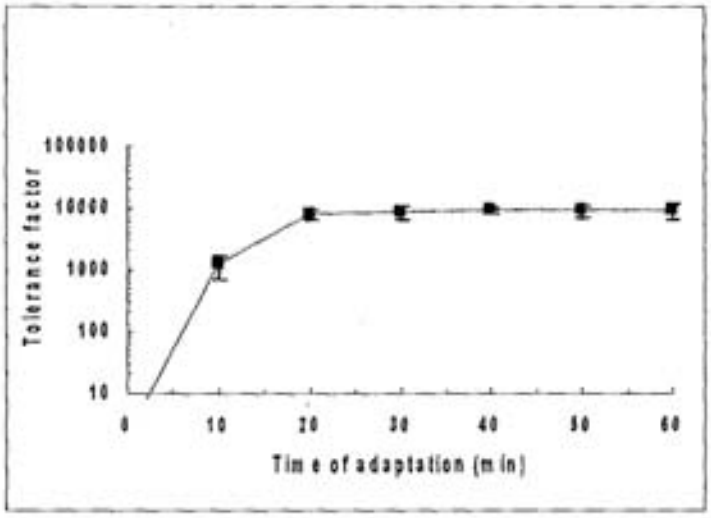


Fig 4.82: Kinetics of acquisition of acid resistance in *L. acidophilus* NCDC14 at pH 4.0 (Bars indicate standard error)

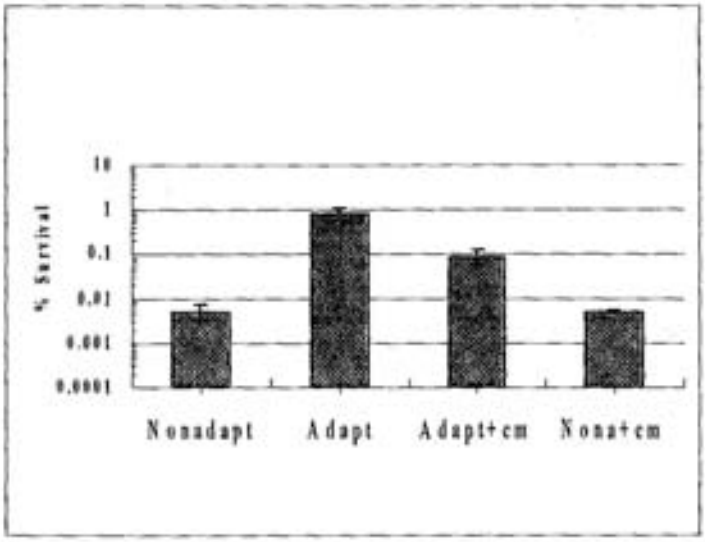


Fig 4.83: Acid stress response in *L. acidophilus* NCDC 14 at pH 4.0/30 min in the presence of 100 µg of Cm (chloramphenicol)/ml. (Bars indicate standard error)

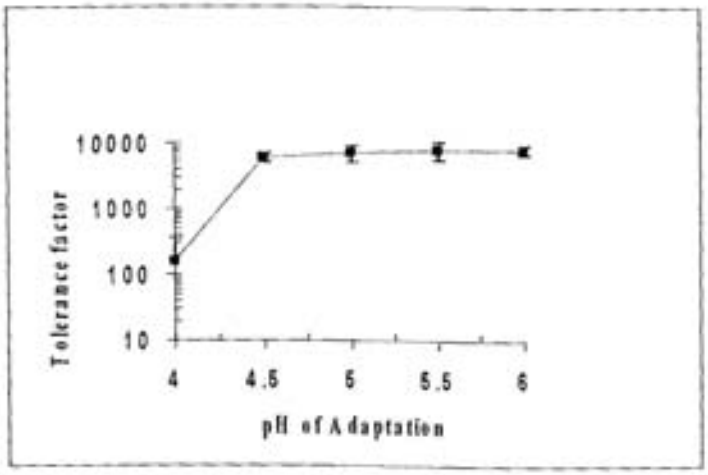


Fig 4.84: Determination of ATR in *L. delbrueckii* subsp. *bulgaricus* NCDC307. (Bars indicate standard error).

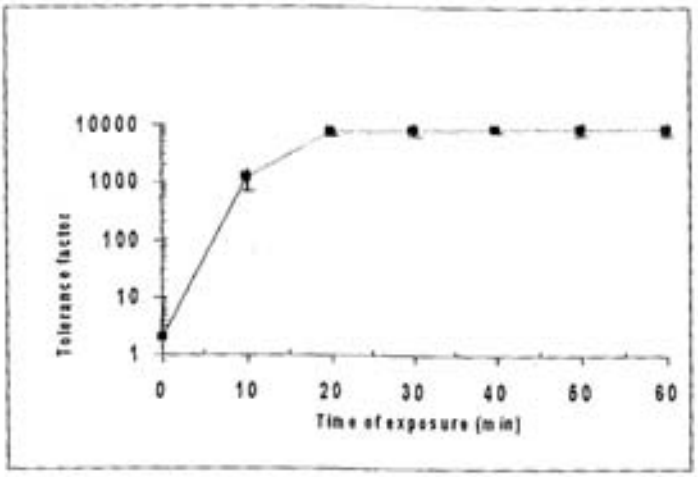


Fig 4.85: Kinetics of acquisition of acid resistance in *L. delbrueckii* subsp. *bulgaricus* NCDC307 at pH 4.5 (Bars indicate standard error)

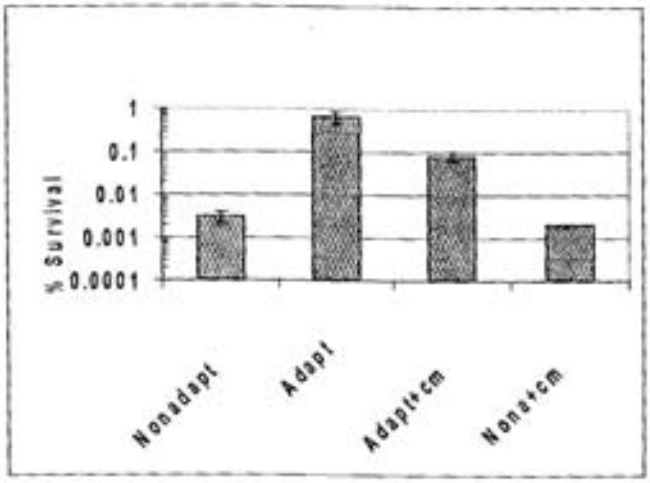


Fig 4.86: Acid stress response in *L. delbrueckii* subsp. *bulgaricus* NCDC307 at pH 4.5/30 min in the presence of 100 µg of Cm (chloramphenicol)/ml. (Bars indicate standard error)

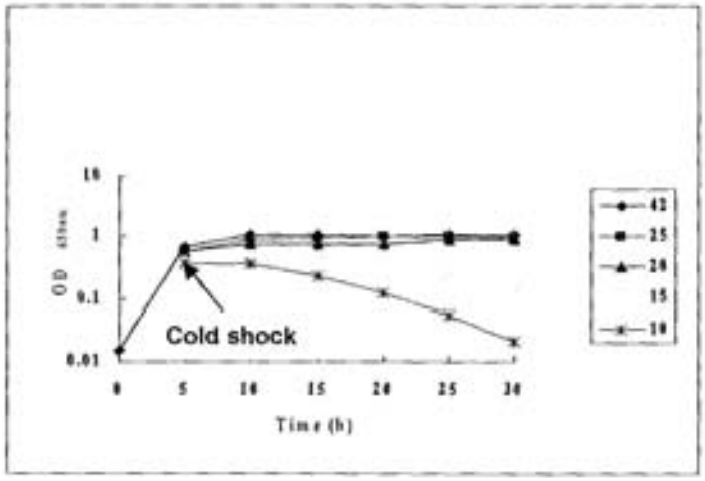


Fig 4.87: Growth of *S. thermophilus* NCDC 74 following a cold shock to different temp (^{0}C)

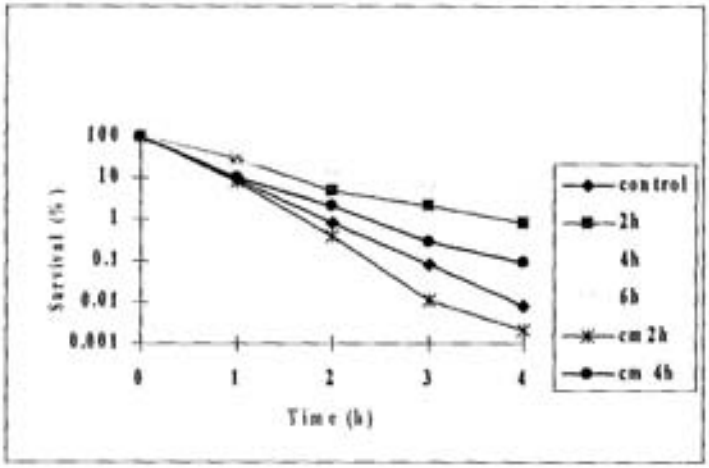


Fig 4.88: Freeze thaw survival of *S. thermophilus* NCDC 74 cold shocked from 42 to 20 ^{0}C

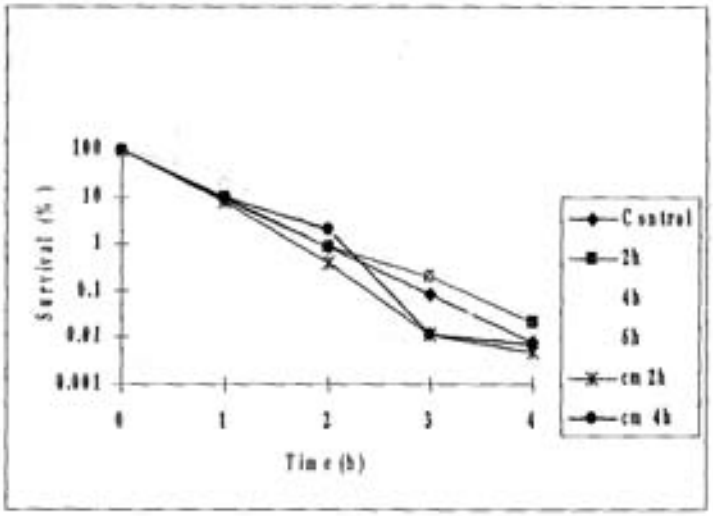


Fig 4.89 Freeze thaw survival of *S. thermophilus* NCDC 74 cold shocked from 42 to 10 ^{0}C

Upon addition of chloramphenical (100 µg/ml) during the cold shock treatment the adaptive response was completely blocked, indicating that protein synthesis was required in the adaptation process (Fig 4.88 and Fig 4.89).

4.3 DEVELOPMENT OF TLAB CAPABLE OF GROWING UNDER ACID AND COLD STRESS

Two methods - adaptation and mutation were tried to obtain stress tolerant cells. The cultures selected for pH/ acid-stress studies were *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307 and *Lactobacillus acidophilus* NCDC 14, whereas for low temp/ cold stress studies *S. thermophilus* NCDC 74 was taken.

4.3.1 Mutation

Mutation in the selected cultures was tried by both chemical treatment using NTG and physically by UV-irradiation.

4.3.1.1 N-methyl-N-nitro-N'-nitrosoguanidine (NTG)

The percent recovery of the cells of three LAB after treatment with NTG at different time intervals has been recorded in Tables 4.7. Lactobacillus counts abruptly decreased to around 1 % in a little less than 90 min while streptococcal counts decreased to a similar level in merely a little more than 60 min. The counts of lactobacilli still continued to decrease rapidly on further exposure to NTG beyond 90 min, the streptococci tended to resist the chemical up to 90 min but later got killed more rapidly than lactobacilli.

The survivors did not show demonstrable growth when inoculated into their respective broth media at different pH and temp levels. The results indicated that the NTG treatment might have killed the cells or injured them irreparably within 24 - 48 h as there was no growth below normal pH and temp levels. The total counts of the TLAB at different intervals during their exposure to NTG indicated that the rate of kill varied among the strains as well as among the exposure time intervals in any given strain. It increased sigmoidly with increasing time concomitantly with a decrease in their percent

survival. This might have been due to the decreasing resistance of the organisms and also due to squandering of the protective cell sap effects typical of the living cells. Overall, the results indicated that the streptococci were more sensitive compared to the lactobacilli to the killing effect of NTG (Table 4.7). As there were no parallel reports available the present findings could not be further evaluated.

Table 4.7: Survival of TLAB exposed to 400 µg NTG/ml

Time of exposure, min	*L. delbrueckii* subsp. *bulgaricus* NCDC 307		*L. acidophilus* NCDC 14		*S. thermophilus* NCDC 74	
	(Cfu x 10^6/ml)	Survival (%)	(Cfu x 10^6/ml)	Survival (%)	(Cfu x 10^6/ml)	Survival (%)
0	180	100	180	100	170	100
30	110	61	120	67	100	59
60	18.40	10	28	16	1.80	1.05
90	1.60	0.88	1.3	0.73	1.60	0.94
120	0.11	0.06	0.21	0.11	0.09	0.05
150	0.09	0.05	0.08	0.05	0.002	0.001

4.3.1.2 UV-irradiation

The percent recovery of the cells of three TLAB after exposure to UV-irradiation at different time intervals has been recorded in Tables 4.8. Both lactobacilli and streptococci counts abruptly came down to less than 1 % within 20 to 25 min of UV irradiation. There was no demonstrable difference in sensitivity of the rods vis-à-vis that of spheres to UV-rays. Also these seemed to be not much variation among the strains as well as among the exposure time intervals in any given strain. The sigmoidal increase in reduction of counts was more acute with increasing time and decreasing number of the survivors. The survivors when inoculated into their respective broth media at

different pH and temp levels did not show demonstrable growth and none survived below normal pH and temp.

Table 4.8: Survival of TLAB exposed to UV irradiation

Time of exposure, min	*L. delbrueckii* subsp. *bulgaricus* NCDC 307		*L. acidophilus* NCDC 14		*S. thermophilus* NCDC 74	
	(Cfu x 10^6/ml)	Survival (%)	(Cfu x 10^6/ml)	Survival (%)	(Cfu x 10^6/ml)	Survival (%)
0	153	100	165	100	142	100
10	88	58	97	59	78	55
15	47	30	67	41	37	26
20	4	3	9	6	4	3
25	1	0.65	1	0.60	1	0.70
30	0.002	0.0013	0.003	0.0018	0.002	0.0014

4.3.2 Adaptation

4.3.2.1 Adaptation at low pH and temperature

The lactobacilli were adapted at low pH and *Streptococcus* was adapted at low temp.

Acid-tolerant adapts were isolated by gradual decrease of medium pH from pH 5.0 to pH 4.2 for *L. delbrueckii* subsp. *bulgaricus* NCDC 307 and pH 4.5 to pH 3.9 in *L. acidophilus* NCDC 14. The survivors of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 were not found to be stable at 4.2, but stable at 4.3 i.e. the growth was repeatable. The survivors of *L. acidophilus* NCDC 14 were not found to be stable at pH 3.9, but were stable at pH 4.0. The growth rates pertaining to the above cultures at different pH are represented in the Table 4.9.

Till yet only countable reports are available among the adaptation of microorganisms. O'Driscoll *et al.* (1998) isolated mutants of *L.*

monocytogenes following prolonged exposure to pH 3.5 which constitutively demonstrated increased acid tolerance at all stages of the growth cycle. In a study two constitutively acid-tolerant mutants of *L. sanfranciscencis* CB1 were isolated using natural selection techniques after sequential exposure to lactic acid, pH 3.2 (De Angelis *et al.*, 2001). Chou and Weimer (1998) isolated acid- and bile-tolerant strains of *L. acidophilus* by using natural selection technique after sequential exposure to hydrochloric acid. In the present study a gradual adaptation method was used along with natural selection technique for isolating the acid-tolerant cells. All the adapts possessed higher growth rates over that of the parents at low pH, demonstrating the efficiency of adapts which has also been observed in *L. monocytogens*, *L. acidophilus* and *L. sanfranciscensis*. However there was no report regarding temp gradient method. In the present study, we could successfully isolate adapts of *S. thermophilus* that were capable to grow and survive at low temp.

Table 4.9: Growth rate (μ_{max}/h) of parent and adapts of lactobacilli

Culture	pH		
	4.5	5.0	6.5
L. delbrueckii subsp. *bulgaricus* NCDC 307 (Parent)	0.05	0.80	0.63
Adapts of NCDC 307	0.08	0.88	0.64
L. acidophilus NCDC 14 (Parent)	0.047	0.71	0.72
Adapts of NCDC 14	0.15	0.82	0.71

Specific growth rate (μ_{max}/h) of both control and adapts were identical at neutral pH, but the growth rate of the adapts was elevated at pH 5.0 and pH 4.5 relative to the rate of the wild type. For further studies, the stable adapts/ mutants were maintained at freezing temp in MRS broth adjusted at the pH giving stable adapts.

S. thermophilus NCDC 74 was adapted at low temp by gradually decreasing the temp from 20 ^{0}C to 10 ^{0}C. The culture was not found to be stable at 10 ^{0}C, but was stable at 12 ^{0}C. The later was maintained at freezing

temp for further studies. The growth rate of the adapts was higher at 25 ^{0}C and 20 ^{0}C relative to that of the parents (Table 4.10). However the adapts took longer time to grow as compared to parents.

Table 4.10: Growth rate (μ_{max}/h) of parent and adapts of *S. thermophilus* NCDC 74

Culture	42 ^{0}C	25 ^{0}C	20 ^{0}C
S. thermophilus NCDC 74 parent	0.53	0.26	0.008
S. thermophilus NCDC 74 adapts	0.54	0.35	0.11

4.4 CHARACTERISTICS OF ADAPTED CELLS

4.4.1 Gross Cell Morphology

The results of gross morphology as seen under light and electron microscopes have been presented in Plates 4.1 to 4.6.

4.4.1.1 Light microscopy

The adapts of all the three organisms viz., *L. delbrueckii* subsp. *bulgaricus* NCDC 307 ($^{4.3}pH_{307}$), *L. acidophilus* NCDC 14 ($^{4.0}pH_{14}$) and *S. thermophilus* NCDC 74 ($^{12}T_{74}$) showed apparent variations in their cell size, including length and diameter, cell shape of rods and cell arrangement regarding their aggregation and continuity.

4.4.1.1.1 Acid stress adapts of lactobacilli

The cylinders became shorter, thinner, usually curved. The chain length also decreased (Plates 4.1 to 4.4).

4.4.1.1.2 Cold stress adapts of streptococci

In parent cells, uniformity of cell size was disturbed. In general the cells became larger with some of those definitely attaining very large size. Some exceptions were there with slightly smaller size. The chains usually containing 2-6 cells, became shorter and chains involving more than 2 cells were much less. The cluster showing the components clearly in the parent culture turned into smaller bundles with non-discernible components as if they were very closely apposed (Plate 4.5 and 4.6).

4.4.1.2 Electron microscopy

The electron micrographs of the adapts of the test organisms confirmed the observations of light microscopy.

4.4.1.2.1 *Lactobacillus acidophilus*

The control cells of *L. acidophilus* NCDC 14 had the length of 2.30 (± 0.12) μm (Plate 4.7). Notable changes were observed in the adapt ($^{4.0}pH_{14}$) cells. Cell length was reduced to ~ 0.45 μm (Plate 4.8). However, the adapts were physiologically active. As was the case with *L. delbrueckii* subsp. *bulgaricus* NCDC 307, the abrupt exposure of parent cells to pH 3.5 caused notable change in morphological characteristics.

4.4.1.2.2 Lactobacillus delbrueckii subsp. *bulgaricus*

Parent cells when grown under standard conditions exhibited the characteristic rod shaped feature of dairy starters *Lactobacillus delbreueckii* subsp. *bulgaricus* NCDC 307. Their average length was 2.45 (±0.01) μm, (Plate 4.9) but the cell length of the adapts decreased to 1.06 (±0.01) μm, (Plate 4.10). However, when the parent cells were exposed for one h abruptly to pH 4.4 and pH 3.5 from normal medium pH, it caused drastic change in cell morphology. The cells became blurred as if integrity were lost. Clumping of the cells was observed, which might have been due to large scale destruction of cells.

These results illustrated the protective effect of gradual acid-adaptation at morphological level, which prevents the disruption of cell integrity by high lactic acid conc. Similar stress induced changes were reported previously for *Propionibacterium freudenreichii* (Jan *et al.*, 2001) and for other bacteria (Harley, 1997). There are no reports about such studies on the present test organisms. Therefore, the study is the first of its kind in reporting acid stress effects on *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*

4.4.2 Plasmid Analysis

The results regarding plasmid analysis of the TLAB under study and their adapts have been presented below as Plates 4.11 to 4.13.

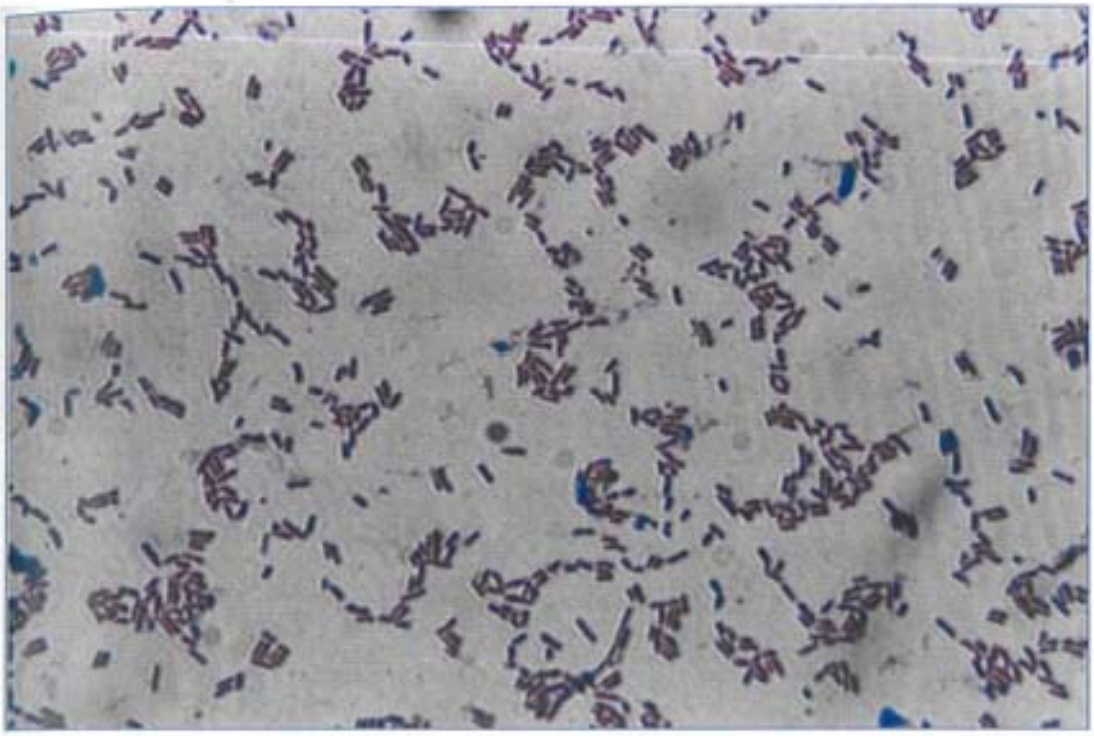


Plate 4.1: Parent cells (x1000) of *L. acidophilus* NCDC 14 (C_{14})

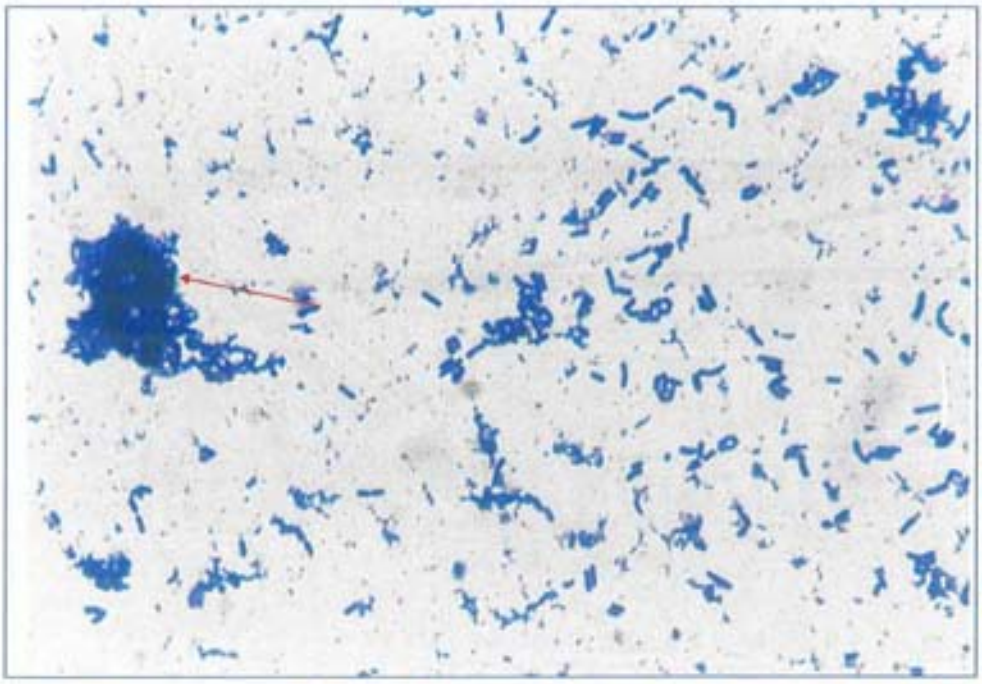


Plate 4.2: Adapts (x1000) of *L. acidophilus* NCDC 14 ($^{4.0}$ pH_{14})

→ Arrow indicates clumping of cells

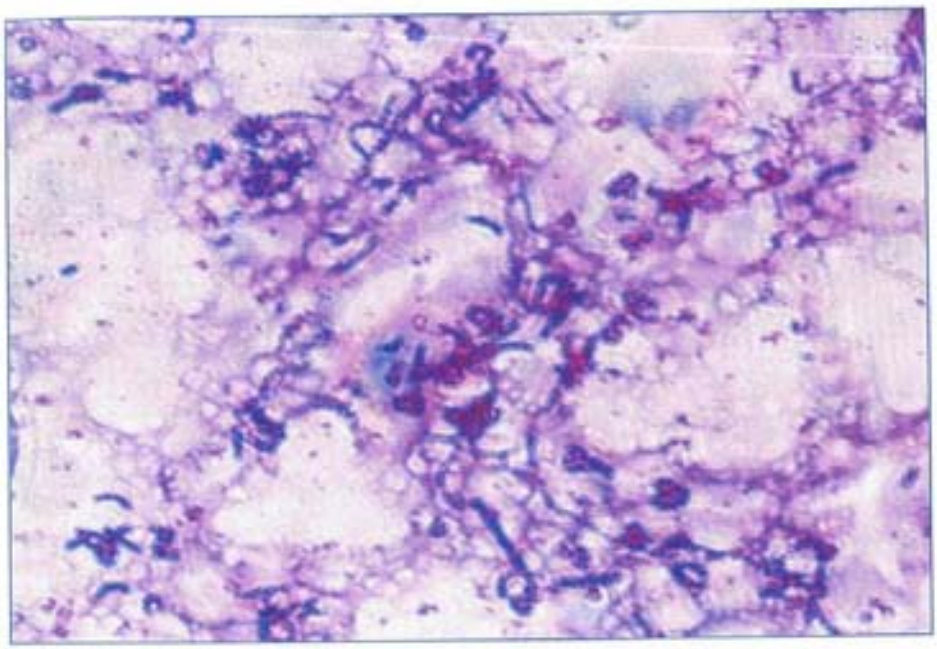


Plate 4.3: Parent cells (x1000) of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 (C_{307})

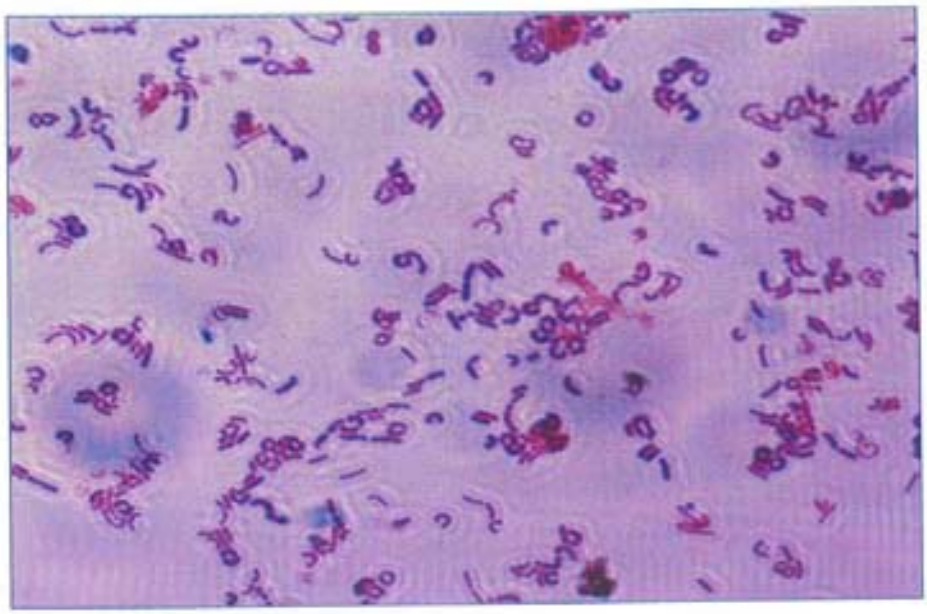


Plate 4.4: Adapts (x1000) of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 ($^{4.3}$ pH_{307})



Plate 4.5: Parent cells (x1000) of *S. thermophilus* NCDC 74 (C_{74})

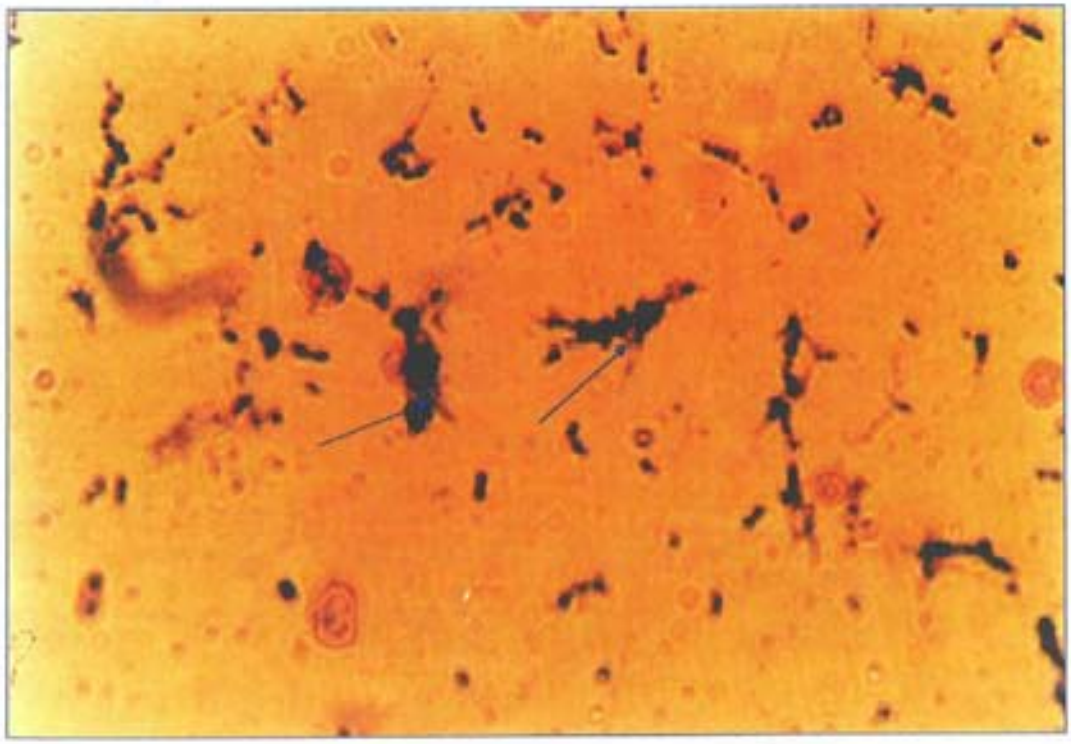


Plate4.6: Adapts (x1000) of *S. thermophilus* NCDC 74 ($^{12}T_{74}$)

→ Arrows indicate clumping of cells



Plate 4.7: SEM of Parent cells (x5000) of *L. acidophilus* NCDC 14 (C_{14})
(Avg. cell length 2.30 µm)



Plate 4.8: SEM of Adapts (x2000) of *L. acidophilus* NCDC 14 ($^{4.0}$ pH_{14})
(Avg. cell length 0.45 µm)

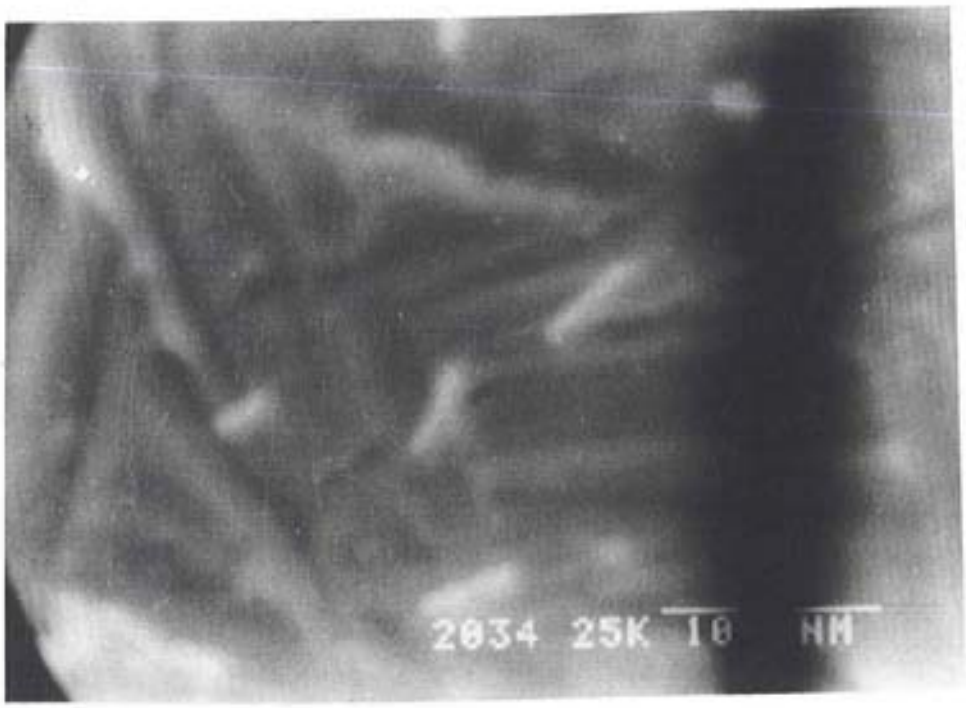


Plate 4.9: SEM of Parent cells (x3000) of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 (C_{307}) (Avg cell length 2.45 µm)



Plate 4.10: SEM of Adapts (x3000) of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 ($^{4.3}pH_{307}$) Avg cell length 1.06 µm

4.4.2.1 *Lactobacillus acidophilus* NCDC 14

The acid stress adapts ($^{4.0}pH_{14}$) and the parent cells depicted two bands of fluorescence (one chromosomal and one plasmid) those corresponded to the 10 kDa marker. The electrophoretic pattern indicated the presence of the same plasmid in the adapt which was already present in the parent also before adaptation. Hence the plasmid was unrelated to the stress factor of the cells.

4.4.2.2 *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307

The acid stress adapts ($^{4.3}pH_{307}$) and the parent cells depicted only one band of fluorescence that corresponded to the chromosomal DNA and to 10 kDa marker (Plate 4.12). This indicated that the parent cells were devoid of plasmids, which was thus absent in adapts, too.

4.4.2.3 *Streptococcus thermophilus* NCDC 74

The cold stress adapts ($^{12}T_{74}$) and the parent cells depicted chromosomal DNA band of fluorescence that corresponded to the 10 kDa marker (Plate 4.13). This indicated that the parent cells lacked plasmids consequently the adapts also were devoid of any plasmids.

4.4.3 Protein Analysis

4.4.3.1 Protein analysis of *Lactobacillus acidophilus* NCDC 14 adapts

Laemelli (1970) method was initially used as such to differentiate between protein patterns in the parent *L. acidophlilus* NCDC 14 and its adapts. However, the resolution obtained was poor at 12 and 15 % conc of the separating gel using 4 % stacking gel conc. The method was then modified with respect to certain of its components. A higher conc of tris (3M) was used in the resolving gel. Similarly, glycine at a higher conc (0.38M) was used in the electrode buffer which was 0.19 M in the original method. The conc of the stacking gel was also increased to 7%. Resolution of the protein bands in 12 % separating gel thus obtained was not good enough but it was better resolved in 15 % separating gel with stacking gel conc of 7 % (Plate 4.14). From densitometric analysis it was indicated that in adapts ($^{4.0}pH_{14}$) the protein bands of from ~ 10 kDa to ~ 85 kDa were up regulated (i.e. more

production of proteins under stress conditions) whereas beyond this limit these were down regulated (Fig 4.90). The arrow in (Plate 4.14) indicated the protein of MW ~205 kDa was absent in adapts. The extra production of such proteins by cells under stress suggested the protective role of these proteins during acid stress or acid adaptation. Earlier workers could characterize only few of such putative acid-resistance proteins (Hartke *et al.*, 1996; Guzzo *et al.*, 1997; Lim *et al.*, 2000; De Angelis *et al.*, 2001; Giard *et al.*, 2001; Williams *et al.*, 2001).

4.4.3.2 Protein analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307 adapts

As for *L. acidophilus*, the method of Laemelli (1970) as modified was also followed for resolving the proteins of the acid adapts of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Optimum resolution of protein bands was observed in 15 % separating gel with 7 % stacking gel conc. The dye intensity of the band varied in this case. Some bands showed increased intensity whereas some showed decreased intensity (Plate 4.15). The intensity indicated the protein conc. Thus, these increased proteins might have a role played during acid adaptation of the organism. Lim *et al.* (2001) compared the protein patterns after 2D gel electrophoresis of acid stressed *L. delbrueckii* subsp. *bulgaricus* to an acid stress. After 2D electrophoretic separation, the protein patterns were compared. At least 30 protein spots were induced under acidic conditions. Three highly induced proteins, GroES, GroEL and DnaK were identified as heat shock proteins. No such efforts could be made to identify the acid shock proteins in the present study. However, from protein profile of 1D gel, increased intensity of bands were observed. It was apparent that the proteins might have clubbed together, which however could be separated by high resolution 2 D gel electrophoresis technique.

4.4.3.3 Protein analysis of *Streptococcus thermophilus* NCDC 74 adapts

Laemelli (1970) method was initially used to differentiate between protein patterns in the parent *S. thermophilus* NCDC 74 and its adapts ($^{12}T_{74}$). Here also optimum resolution was obtained in 15 % separating gel with 7% stacking gel conc.

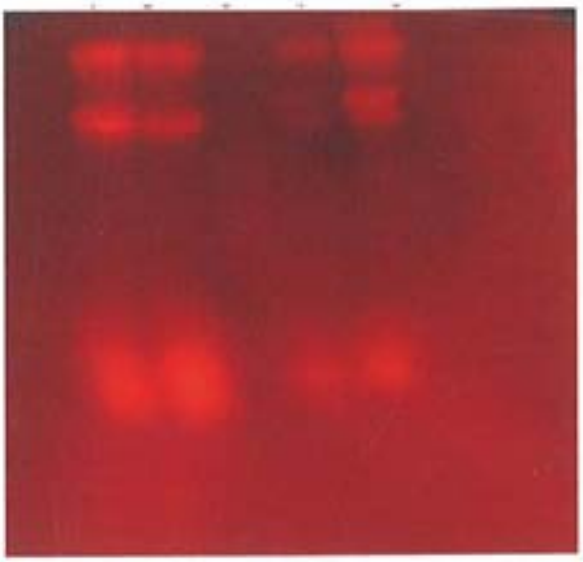


Plate 4.11: Plasmid profile of parent *L. acidophilus* NCDC 14 (C_{14}) and adapts ($^{4.0}pH_{14}$)

Lane 1&2: Parent (C_{14}) Lane 3&4: Adapts ($^{4.0}pH_{14}$)

(Mw: Molecular weight markers (Da))

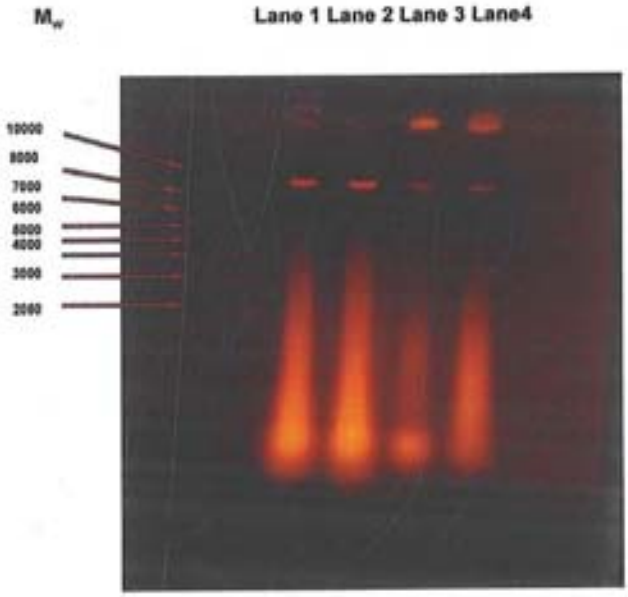


Plate 4.12: Plasmid profile of parent *L. delbrueckii* subsp. *bulgaricus* NCDC 307 (C_{307}) and adapts ($^{4.3}pH_{307}$)

Lane 1&2: Parent (C_{307}); Lane 3&4: Adapts ($^{4.3}pH_{307}$)

(Mw: Molecular weight markers (Da))

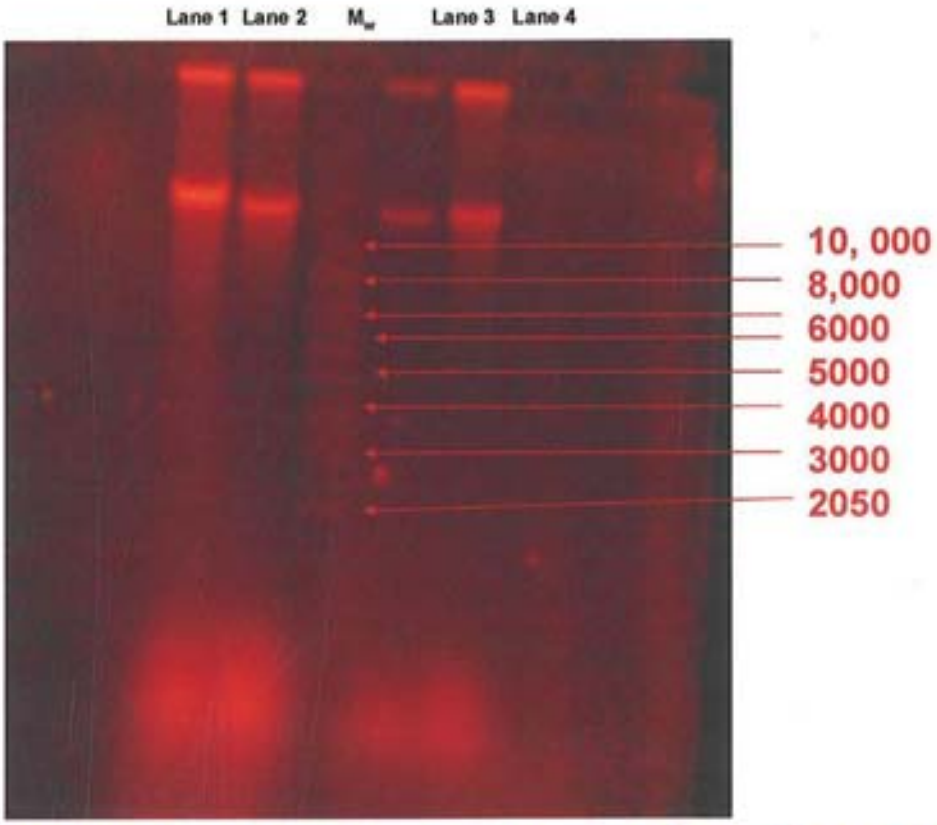


Plate 4.13: Plasmid profile of parent *S. thermophilus* NCDC 74 (C_{74}) and adapts ($^{12}T_{74}$)

(Lane 1&2: Parent (C_{74}) ; Lane 3&4: Adapts ($^{12}T_{74}$)

(Mw: Molecular weight markers (Da))

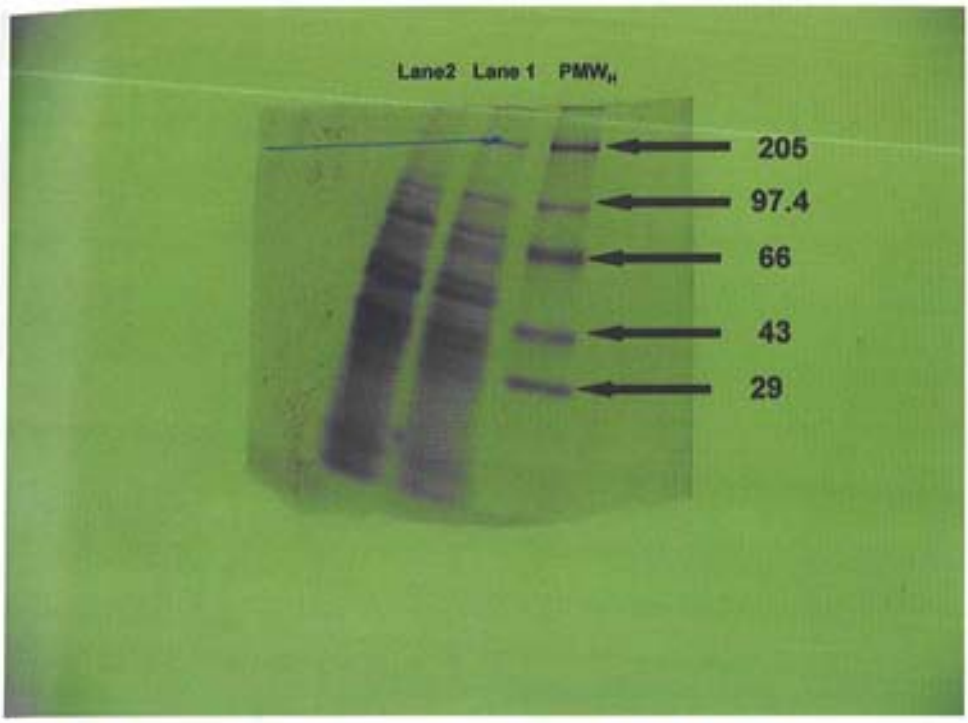


Plate 4.14: Protein profile of parent *L. acidophilus* NCDC 14 (C_{14}) and adapts ($^{4.0}pH_{14}$)

Lane 1: Parent (C_{14}); Lane 2: Adapts ($^{4.0}pH_{14}$); PMW_H (kDa) : High molecualr weight marker
(The arrow indicates the protein of Mw ~205 kDa was absent in adapts (repressed protein)

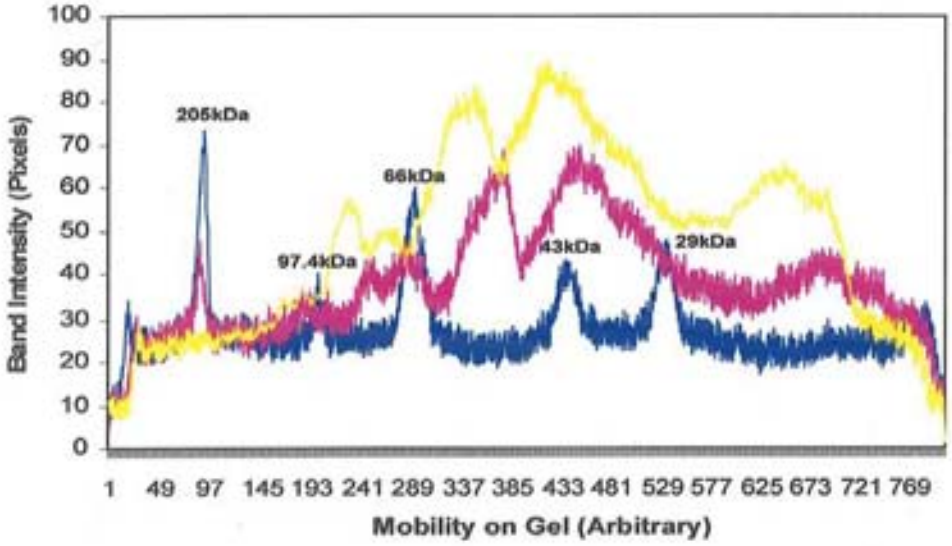


Fig 4.90: Densitometric analysis of protein profile of *L. acidophilus* NCDC 14 adapted at low pH.

No distinguished and separate bands were observed between the parent and adapted cells of *S. thermophilus* (Plate 4.16). The number of bands from the adapts was apparently the same as that from the parent cells of *S. thermophilus* NCDC 74. However, the intensity of the colour of protein bands and their thickness were more from the charge prepared from adapted cells. This might have been due to enhanced production of the proteins that might have helped them to adapt at low temp.

Similar studies conducted in *S. thermophilus* by Wouters *et al.* (1999a), revealed that the CSP levels, did not increase compared to cold-induced levels of log phase cells, but they still exhibited 20 fold higher survival rates after freezing than exponentially growing cells. The authors did not afford any explanation for the phenomenon. In a later study, six putative CSPs (CSPA to CSPF) have been detected in *S. thermophilus*. Five of these appeared to be induced after cold stock, whereas the last one, CSPF was expressed at low levels both at 42 and 20 ^{0}C (Wouters *et al.*, 1999b).

CSPs appear to be involved in cold adaptation by an overlapping regulatory mechanism role due to expression of both CSPs and several CIPs. They probably also play an important role during growth at optimal temp as suggested by the requirement of a minimal CSP level in the cell (Guchette *et al.*, 2002). The present study correlated well with that of the above authors in that, here the protein pattern did not change but only the intensity of existing bands increased. It is quite probable that the CSP numbers might have not changed in the adapts but the existing low profile CSPs might have been induced in the instant study.

4.4.3.4 Identification of cold shock proteins in *S. thermophilus* NCDC 74 by proteomic analysis

4.4.3.4.1 Changes in protein synthesis in S. thermophilus NCDC 74 during cold adaptation

Stress adaptation often involves modifications of gene expression. Protein synthesis in both non adapted cells (control, 42 ^{0}C) and cells cold shocked for 2, 4 and 6 h at 20 ^{0}C of *S. thermophilus* NCDC 74 respectively

was demonstrated initially by Tricine SDS PAGE followed by 2 D-PAGE as shown in Plate 4.17 to Plate 4.21.

4.4.3.4.1.1 Tricine SDS PAGE of S. thermophilus NCDC 74 during cold shock treatment

As can be evidenced from Plate 4.17, no discrete bands were observed between control, 2 h, 4 h and 6 h cells on SDS PAGE. Densitometric analysis depicted that with the increased period of cold stress, more proteins of molecular weight range ~10 kDa to ~150 kDa were produced indicating their essentiality (Fig 4.91 to Fig 4.93). The low molecular weight range of proteins were expressed at 6 h. The densitometric analysis result indicated that some protein bands may be clubbed which, however, could be separated by high resolution 2D-PAGE. The detailed analysis of the results regarding the study are given below.

4.4.3.4.1.2 2D Gel Electrophoresis (2D-PAGE) of S. thermophilus NCDC 74 during cold shock treatment

By using the described separation and staining conditions, a total 36 spots could be identified. Major cold induction was observed for cells exposed to 20 ^{0}C for 6 h.

A total of 14 spots could be differentiated among control and 6 h gels. A total of six new spots (Spot No. 1, 1^{1}, 8, 9, 11 and 13) were seen (Table 4.11) (Plate 4.18 and Plate 4.21). All these are of low molecular mass (<14 kDa region) and PI ranged between 4.0 to 6.0 region. The most prominent spot was spot no. 1 which was further characterised by peptide mass finger printing analysis (Plate 4.21).

Among the induced spots, 6 were characterised (spot no. 2, 3, 7, 10, 12 and 14). Spot no. 3 was highly induced followed by spot no. 2 and 7. These were again of low molecular region i.e. 7 to 10 kDa (Plate 4.18 to Plate 4.21). These proteins seem to play a significant role in cold-adaptation.

Furthermore, repressed proteins were also observed in this group, spot 5 was totally repressed (i.e. absent) in 6 h, while spot 4 and 6 were highly repressed (Present in relative low amounts than control) (Plate 4.21).

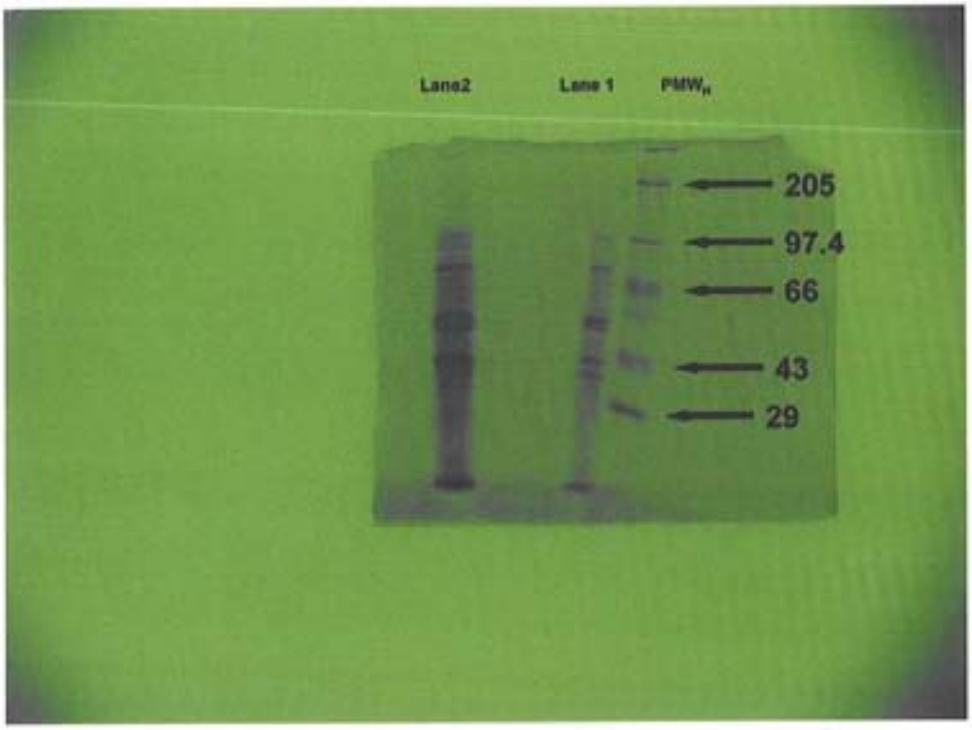


Plate 4.15: Protein profile of parent *L. delbrueckii* subsp. *bulgaricus* NCDC 307(C_{307}) and adapts ($^{4.3}pH_{307}$)

Lane 1: Parent (C_{307}); Lane 2: Adapts ($^{4.3}pH_{307}$); PMW_H (kDa) : High molecualr weight marker

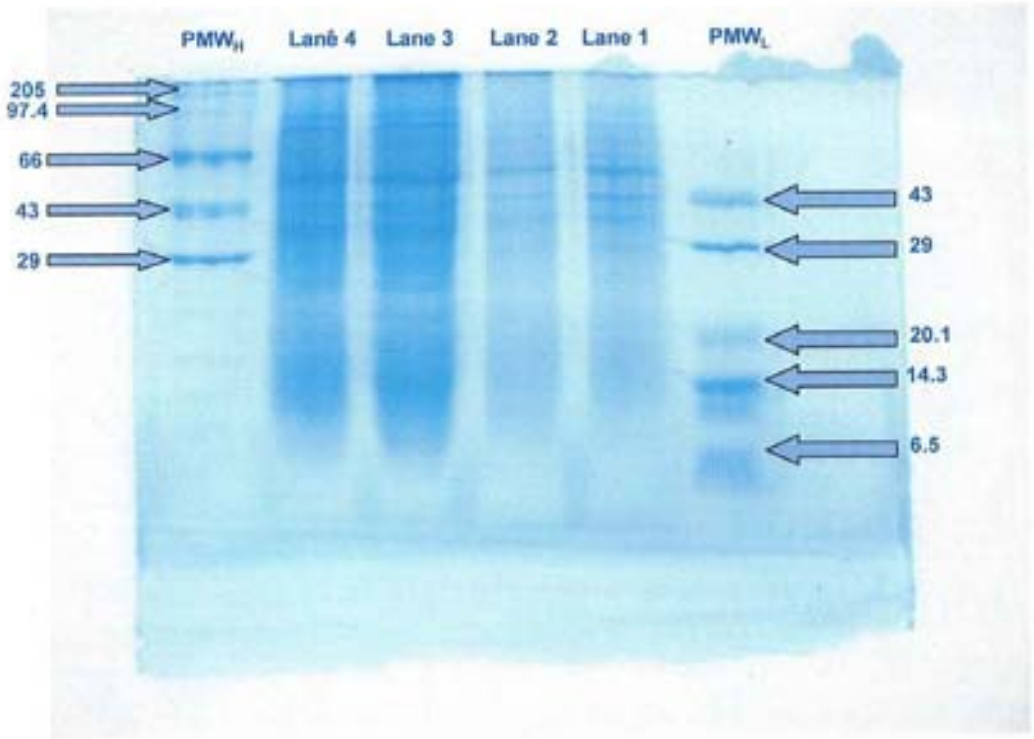


Plate 4.16: Protein profile of parent *S. thermophilus* NCDC 74 (C_{74}) and adapts ($^{12}T_{74}$)

Lane 1 & 2: Parent (C_{74}); Lane 3 & 4: Adapts ($^{12}T_{74}$); PMW_H (kDa): High molecular weight marker; PMW_L (kDa): Low molecular weight. marker

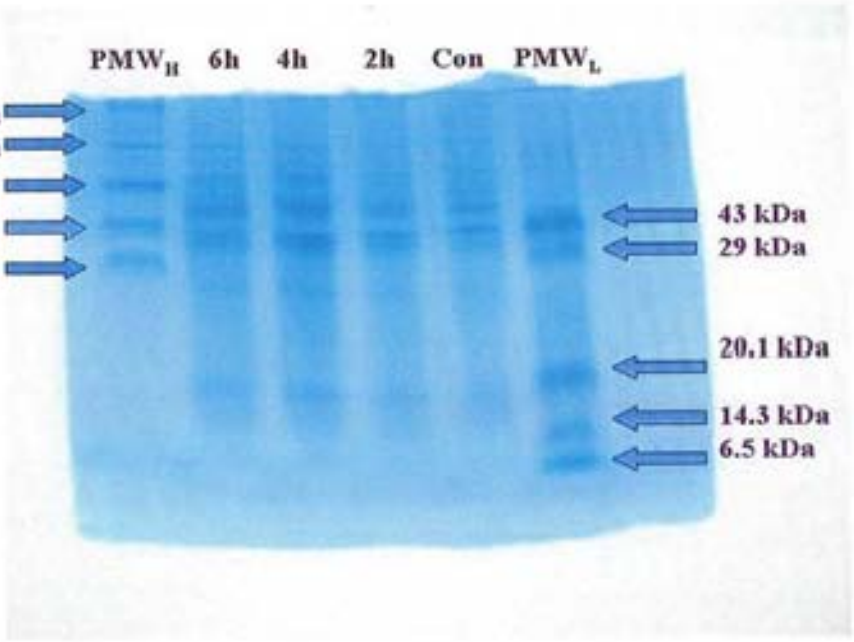


Plate 4.17: Protein profiles of *S. thermophilus* NCDC 74 upon exposure to 20 °C on Tricine SDSPAGE

PMW$_H$ (kDa) : High molecular weight marker
PMW$_L$ (kDa) : Low molecular weight marker
Con : Cells grown at 42 °C,
2 h, 4 h and 6 h : Cells cold shocked at 20 °C for 2, 4 and 6 h

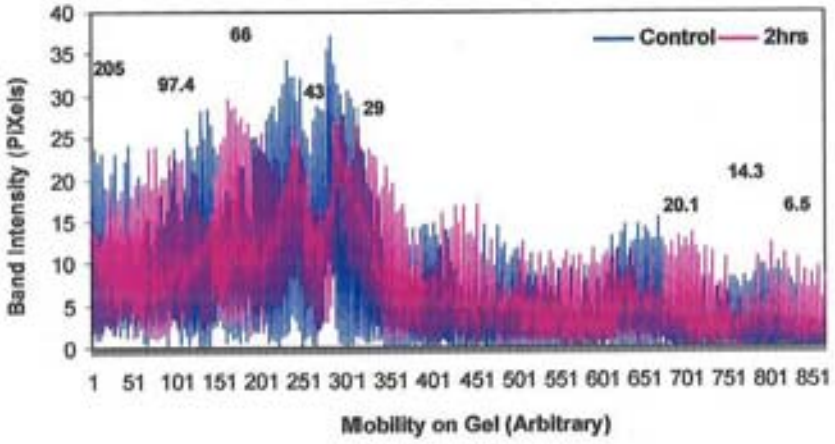


Fig 4.91: Densitometric analysis of protein profile of *S. thermophilus* NCDC 74 upon exposure to low temp (20 °C/ 2 h)

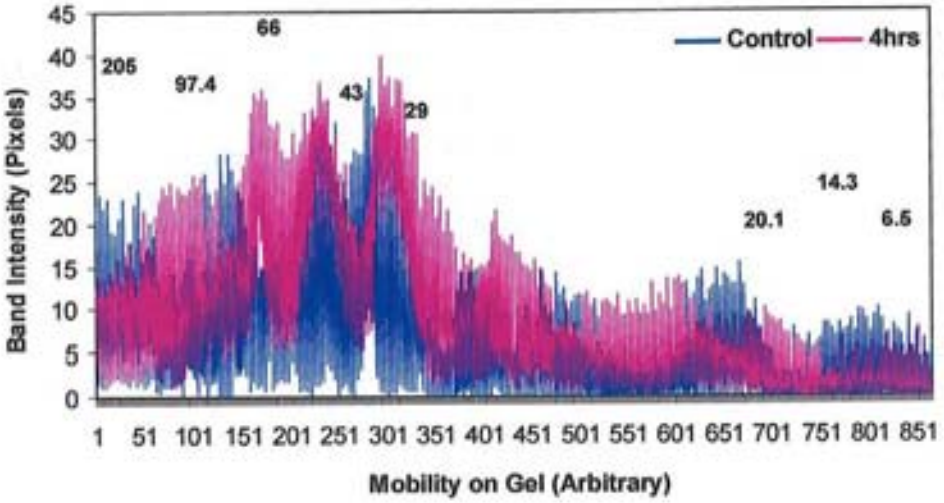


Fig 4.92: Densitometric analysis of protein profile of *S. thermophilus* NCDC 74 upon exposure to low temp (20 ^{0}C/ 4 h)

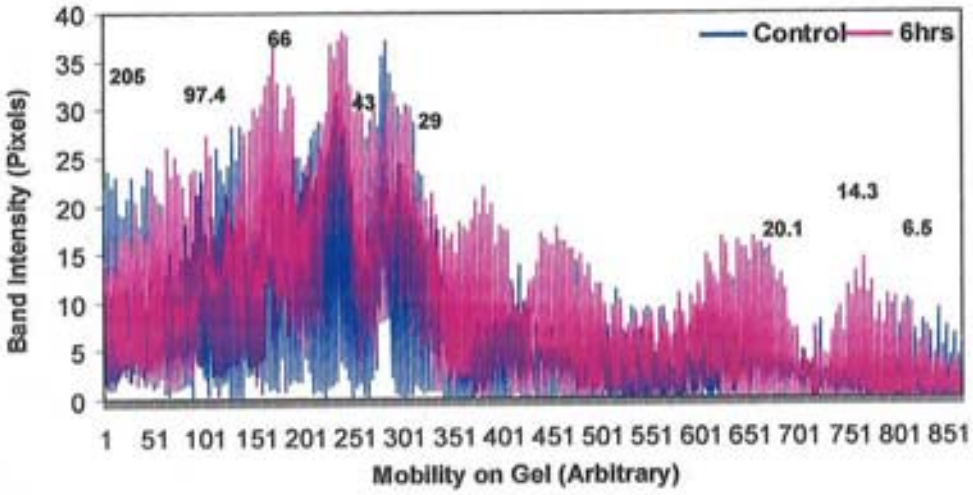


Fig 4.93: Densitometric analysis of protein profile of *S. thermophilus* NCDC 74 upon exposure to low temp (20 ^{0}C/ 6 h)

Table 4.11: Spot differences in Control and 6 h 2D-PAGE

Spot no / New Spots	Control	6h
1	Absent	Present
1'	Absent	Present
8	Absent	Present
9	Absent	Present
11	Absent	Present
13	Absent	Present
Induced spots	**Control**	**6h**
2	Light	Prominent
3	Light	Prominent
7	Light	Prominent
10	Light	Prominent
12	Light	Prominent
14	Light	Prominent
Repressed spots	**Control**	**6h**
4	Prominent	Light
5	Present and prominent	Absent
6	Dark	Light

Comparison of four 2D PAGE gels revealed 22 different spots both including, induced, repressed and new proteins (Table 4.12, Plates 4.18 to Plates 4.21). Spot A and spot V appeared to be new proteins. 11 spots were found to be induced whereas six spots were found to be repressed (Table 4.16). Spot C and Spot J were highly induced at 4 h whereas the intensity of spot V was highest in 2 h. Among others, spot D, spot K, spot M, spot Q, spot R were highly induced after 6 h of cold shock treatment. Among the repressed

proteins spot P was found to be important being present only in control gels. Spot I was present in all gels except in 6 h treated sample (Table 4.12).

Table 4.12: Spot differences in Control, 2 h, 4 h and 6 h 2D PAGE

Spot no / New Spots	Control	2 h	4 h	6 h
A	Absent	Light	Light	**Prominent**
V	Absent	Absent	Absent	**Prominent**
Induced spots	**Control**	**2h**	**4h**	**6h**
B	Light	Light	Light	Prominent
C	Dark	Absent	**Most Prominent**	Prominent
D	Absent	Absent	Light	**Prominent**
G	Light	Light	Prominent	Prominent
J	Light	Dark	**Most Prominent**	Dark
K	Light	Light	Prominent	Prominent
L	Light	Light	Prominent	Visible
M	Light	Light	Visible	Prominent
N	Absent	Light	Prominent	Absent
R	Light	Light	Light	**Prominent**
U	Visible	**Most Prominent**	Visible	Light
Repressed spots	**Control**	**2h**	**4h**	**6h**
P	**Prominent**	Absent	Absent	Absent
I	**Prominent**	**Prominent**	**Prominent**	Absent
E	**Prominent**	Absent	Light	Light
F	**Prominent**	Light	Light	Light
H (Cluster of four spots)	All prominent	Three prominent	Three prominent	All prominent
O	Prominent	Prominent	Prominent	Absent

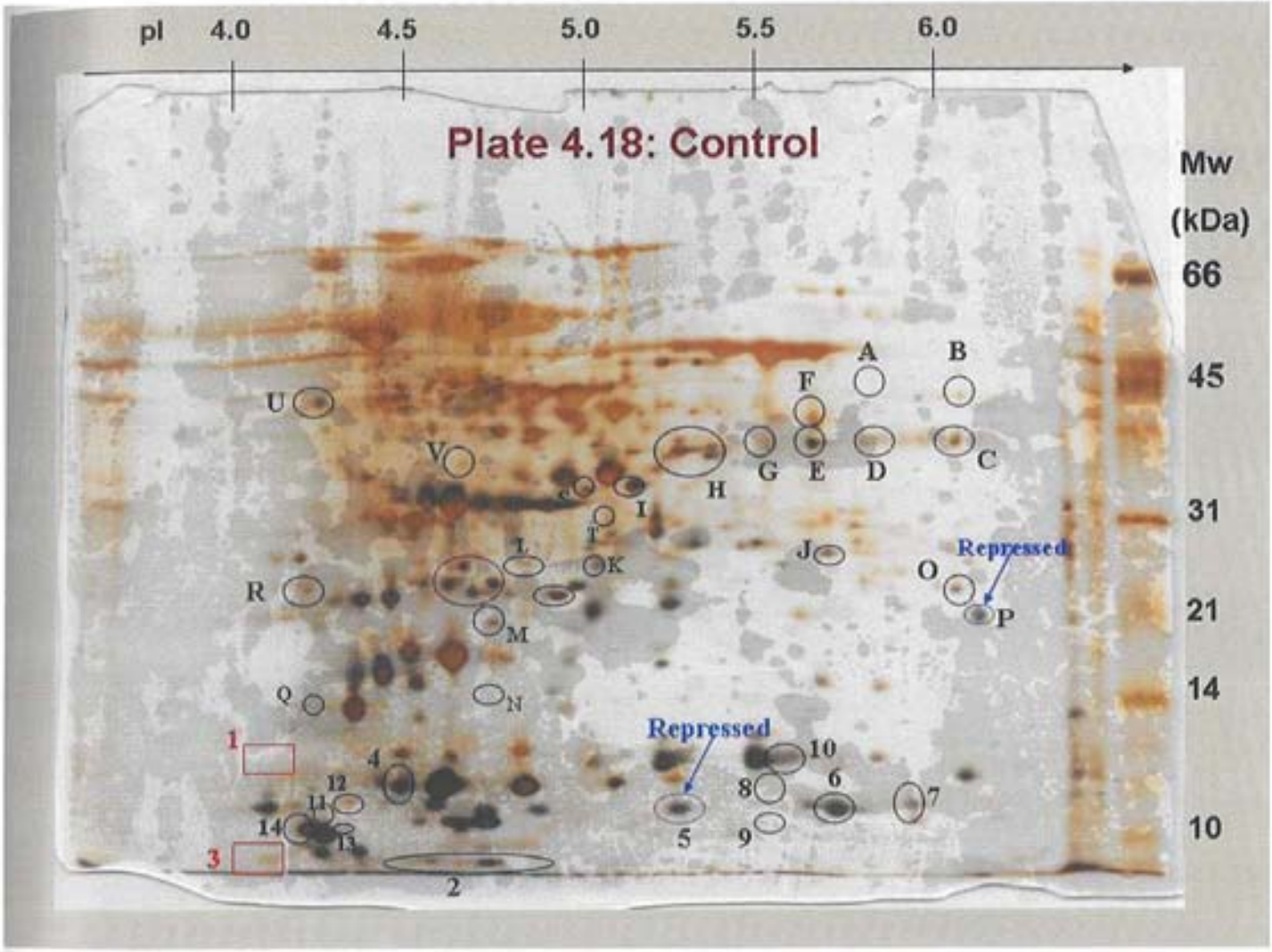


Plate 4.18: 2D-EF of total protein extracts from log phase cells of *S. thermophilus* NCDC 74 grown at 42 ^{0}C (Control)

Mw : Molecular weight marker

pI : Isoelectric point range from 4.0 to 6.0 (IPG strips, Biorad), shown on top

(New proteins are marked as red, Repressed proteins are marked as blue, Induced proteins are marked as merune)

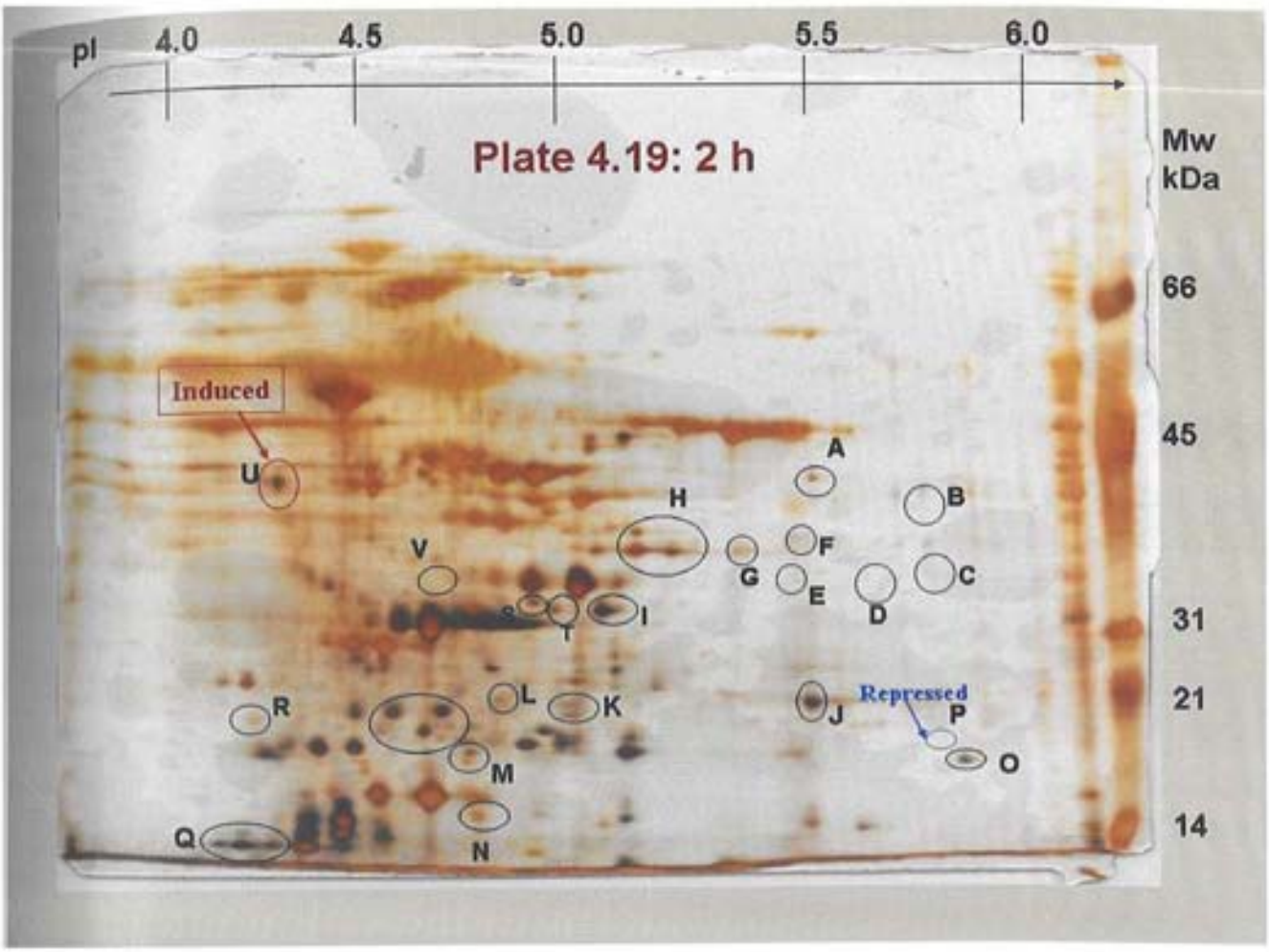


Plate 4.19: 2D-EF of total protein extracts from log phase cells of *S. thermophilus* NCDC 74 exposed to cold shock from 42 to 20 °C for 2 h

Mw: Molecular weight marker
pI: Isoelectric point range from 4. 0 to 6. 0 (IPG strips, Biorad), shown on top
(New proteins are marked as red, Repressed proteins are marked as blue, Induced proteins are marked as merune)

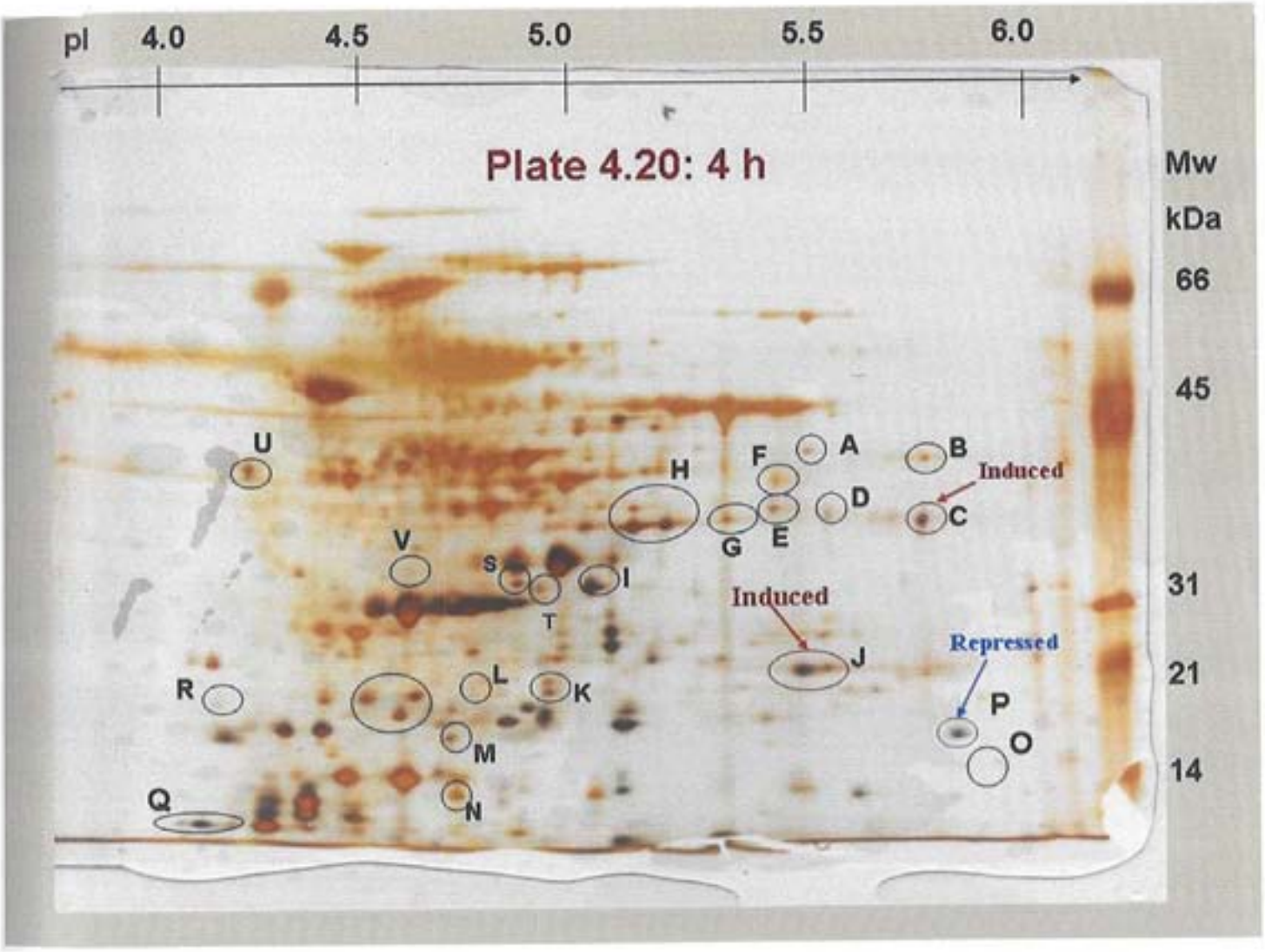


Plate 4.20: 2D-EF of total protein extracts from log phase cells of *S. thermophilus* NCDC 74 exposed to cold shock from 42 to 20 ^{0}C for 4 h

(Mw: Molecular weight marker
pI: Isoelectric point range from 4. 0 to 6. 0 (IPG strips, Biorad), shown on top
(New proteins are marked as red, Repressed proteins are marked as blue, Induced proteins are marked as merune)

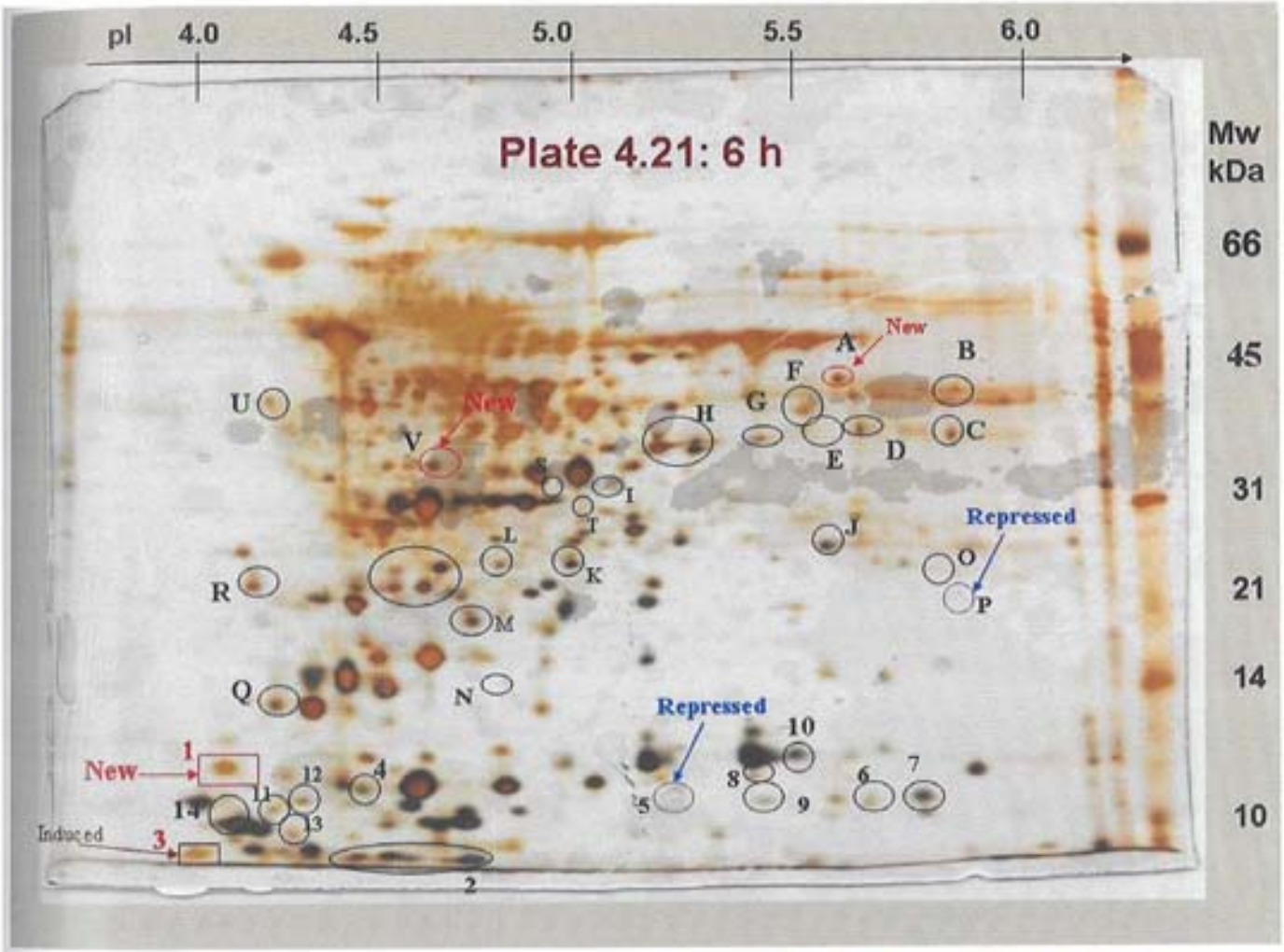


Plate 4.21: 2D-EF of total protein extracts from log phase cells of *S. thermophilus* NCDC 74 exposed to cold shock from 42 to 20 ^{0}C for 6 h

Mw : Molecular weight marker
pI : Isoelectric point range from 4. 0 to 6.0 (IPG strips, Biorad), shown on top

Spot 1 (red box) indicated the expression of a new cold shock protein

Spot 3 (red box): Induced cold shock protein

(New proteins are marked as red, Repressed proteins are marked as blue, Induced proteins are marked as merune)

4.4.3.4.1.3 Identification of cold shock proteins in S. thermophilus NCDC 74

Out of 36 spots (including repressed, induced and new), 18 spots that appeared to be the most important were identified by peptide mass finger printing (PMF) using 2D-nano LC-MS (LC-MSD Trap XCT, Agilent 1100 series) and MALDI TOF/TOF (Bruker Ultraflex, MALDI TOF/TOF).

MS/MS provides information that is highly complementary to that provided by Edman degradation. In addition, MS/MS has several advantages relative to Edman degradation for determining peptide sequence. First MS/MS can produce sequence information for blocked or otherwise modified peptides that are either difficult or impossible to sequence by Edman degradation. Secondly, the ability to individually select and fragment peptides in mixtures eliminates the need for extensive purification of peptides prior to sequence analysis. Third, MS-based peptide sequencing is faster than Edman sequencing because it is not a stepwise process. All of the structural information, in the form of fragment ions, is presented in single mass spectrum acquired in a single experiment. Finally MS-based approaches are now at least as sensitive as Edman degradation (e.g. 1 to 5 pmol of peptide or protein is required) and state of the art MS-based sequencing by either ESI-MS or MALDI-MS is capable of providing sequence information at the 50-to-250-fmol level. Finally, a variety of computer based searching strategies have been developed whereby known proteins can be identified from protein and DNA databases by searching MS-derived molecular weights with or without partial sequence information (Pappin *et al.*, 1993; Eng *et al.*, 1994; Hall *et al.*, 1996).

4.4.3.4.1.4 Analysis of control and 6 h gel

Among all the gels, control and 6 h gels were resolved up to low molecular mass region (<10 kDa) whereas 2 and 4 h gels could not. A total of 14 spots were differentiated among control and 6 h gels. Out of 14, six important spots were characterised. **Spot 1** in 6 h gel (red box) (Plate 4.18 and Plate 4.13) appeared as new protein (MW ~12kDa) whereas **spot 3** appeared to be highly induced cold shock protein of molecular weight ~ 8

kDa. PMF (peptide mass finger printing) analysis revealed that spot 1 matched with cold shock protein A (CSP A) fragment of *Lactobacillus casei* with a highest probability based Mowse score of 164 ($P<0.05$) (Table 4.13) (Annexure - IV) . Two other important peptide fragments of this protein matched with Holo (acyl-carrier-protein) synthases (EC 2.7.8.7) from *Mycoplasma mycoides* (Mowse score, 54) and zinc transport transcriptional repressor from *S. thermophilus* CNRZ1066 (Mowse score, 44) respectively.

The aforesaid result suggested that these proteins might play a role in cold adaptation of *S. thermophilus* NCDC 74. As per earlier reports, only two findings are available regarding cold shock responses of *S. thermophilus*. Among other proteins, 7 kDa cold shock proteins were identified in *S. thermophilus* CNRZ 302 by Wouters *et al.*, 1999; whereas Perrin *et al.*, 1999 reported the presence of a new cold shock protein of 21.5 kDa in *S. thermophilus* PB18. The present result thus suggested Spot 1 (red box) in 6 h gel is a new cold shock protein of pI ~ 4.1 and MW of ~ 12 kDa.

Spot 3 appeared to be a highly induced protein present in 6 h gel, having molecular weight of ~ 8 kDa region. This protein was matched with major cold shock protein fragment of *Listeria grayii* (Mowse score, 48) (Annexure – IV). This protein was highly induced at cold shock from 42 to 20 ^{0}C, and therefore, seemed to be the presence of family of CSP proteins in *S. thermophilus*. This result was in agreement with the earlier reports (Wouters *et al.*, 1999; Perrin *et al.*, 1999) in the sense that both of them identified cold shock proteins in *S. thermophilus* of molecular weight about 7 to 7.5 kDa by 2D-Gel electrophoresis . Other proteins (spot 11, 13, and 2) along with spot 3 were also induced upon cold shock to 20 ^{0}C for 6 h, and these might also play a role in cryoprotection.

4.4.3.4.1.4.1 Cold shock induce general stress protein synthesis

Cold shock also induced the expression of some general stress proteins in *S. thermophilus* NCDC 74. The peptide fragments characterised from spot 2 were hypothetical protein lp_0704 (M_w~8 kDa) from *L. plantarum*, hypothetical protein from *E. faecalis*, chromosomal replication initiation protein

dna A from *L. monocytogenes*. Spot No.7 matched with 6 proteins, including HSP60 fragment from *Pediococcus pentosaceous*, putative extra cellular matrix binding protein from *S. pyogenes* as given in the NCBInr protein database (Table 4.13). These proteins were also reported to be produced against other stresses (oxidative, acid and heat shock) as revealed by Leverrier *et al.* (2003).

4.4.3.4.1.4.2 Repressed proteins

In the present study an attempt was also made to identify the repressed protein expression on cold shock. Two repressed proteins were characterised by PMF. Spot no 5 was completely absent whereas spot 6 was repressed several fold as the intensity was decreased. The spot 5 revealed, Hsds, AE007317, AE0016747 NID, from *M. gallisepticum*, *S. pneumoniae*, *S. epidermidis* etc. (Table 4.13). Spot 6 demonstrated methyl-accepting chemotaxis protein t1pA from *C. tetani*, probable ABC transporter from *C. perfringenes* among others (Table 4.13). Some repressed spots were observed in cold shocked cells of *S. thermophilus* exposed to 20 ^{0}C for 6 h and 4 h (Wouters *et al.*, 1999). However, they were not characterised further. The present study suggested the role of down regulation of these proteins during cold shock.

4.4.3.4.1.5 Analysis of control, 2, 4 and 6 h gels

Comparison of control 2, 4 and 6 h gels revealed 22 spots (including induced, repressed and new proteins). 12 spots (important ones) were characterised including repressed spots (Table 4.12). Spot V appeared to be new, whereas spot A appeared to be highly induced at 6 h. Spot V was matched with cell surface protein of *B. cereus* strain ATCC 14579 (Mowse score, 74), along with other protein. Similarly spot A matched with glyceraldehyde-3-phosphate-dehydrogenase (phosphorylating) (EC1.2.1.12) of *Lactobacilli* (Mowse score, 44) along with other proteins. Spot K on the other hand matched with Cu/Zn containing superoxide dismutase of *B. cereus* along with others. Spot M matched with L-serine-dehydratase alpha subunit of

S. thermophilus CNRZ1066. Among other matched protein, one is two component sensor histidine kinase BH0397 from *B. halodurans*. Spot C and Spot J are highly induced at 4 h. Spot C matched with, oxidoreductase (EC 1.-.-.-) of *L. plantarum*, probable reductase of *L. johnsonii* along with other proteins. Spot J showed similarity with 3-hydroxyacyl – CoA dehydrogenase and enoyl-CoA hydratase of *Bacillus cereus*. The intensity of spot U was highly induced at 2 h. The important matches were cell division initiation protein DivIVA of *L. plantarum*, Tuf (fragment) of *L. casei*, L-lactate dehydrogenase of *L. casei*.

From the results, it was inferred that there were several stress protein produced in higher intensity during cold shock indicating a general stress system. This finding is consistent with previous observations, indicating that, in other bacteria, many stresses induced a set of general stress proteins via a general sigma factor. These include oxiclative stress proteins, glyceraldehyde 3-phosphate dehydrogenase, two component sensor histidine kinase, oxidoreductase etc. (Graumann *et al.*, 1996). Superoxide dismutase have been shown to be a σ^B-dependent general stress proteins in *B. subtilis* (Peterson *et al.*, 2001).

Spot M was matched with two component sensory histidine kinase from *B. halodurans* (Table 4.14). This protein was clearly characterised as the response regulator of a two-component signal transduction. When phosphorylated, it binds DNA and cause, in *B. subtilis*, specific alterations of gene expression, allowing sensing of high salt conc, phosphate starvation or attractants and repellants in the chemotaxis system (Msadek, 1999). Such a chromosomal colocalization is typical of genes encoding partners of two components systems and suggests an operon like structure. It is thus tempting to speculate that the cold shock response in *S. thermophilus*, involves extra cellular stimulus detection and signal transduction. By using PMF technique, similar protein was characterised from bile salt stressed cells of *P. freudenreichii* (Leverrier *et al.*, 2003).

Two important repressed (spot P and spot I) proteins were also characterised in the current study. Spot P was identified as ATP-dependent protease, ATP finding chain (C1pB). These proteins played a central role in the *Bacillus subtilis* signal transduction network (Msadek, 1999). This protein seemed to play an important role in bile salt adaptation process, identified both in *E. coli* (Rajgopal *et al.*, 2002) and *P. freudenreichii* (Leverrier *et al.*, 2003). However, this is induced in bile stressed cells of *E. coli* and *P freudenreichii*, whereas the present study demonstrated that it was repressed in *S. thermophilus* NCDC 74 after cold stress. Thus, it may be note worthy that, down regulation of these proteins were involved during cold shock. Spot I appears to resemble as pyruvate kinase of *L. casei*.

The proteomic approach in the present study allowed identification of cold-shock proteins in *S. thermophilus* NCDC 74. PMF analysis using 2D-nano-LCMS conferred characterisation of 18 spots that include induced, repressed and new proteins. Present findings in this regard demonstrated the induction and repression of several general stress proteins during cold shock treatment in this firmicute. This suggests a role of general stress system in *S. thermophilus* along with the specific system. On detailed analysis two cold shock proteins were identified with a molecular weight of ~12 kDa and ~7 kDa respectively. The elucidation of the mechanisms involved in tolerance acquisition by a proteomic approach is however, limited mainly by the lack of an available completed *S. thermophilus* genome. Ongoing studies should allow identification of the genes differentially expressed under stress conditions. Once this obstacle is overcome, these investigations will lead to crucial information on stress protection conferred by either homologous or heterologous stimuli and on the cellular and molecular mechanisms triggered in *S. thermophilus*. The study will also provide molecular markers corresponding to these tolerance states as well as efficient tools for screening of collections of *S. thermophilus* for tolerant strains. The presence of CSPs and CSP like proteins in this organism might play a crucial role in cryoprotection.

Table 4.13: Peptide mass fingerprinting (PMF) analysis of *S. thermophilus* NCDC 74 during cold stress (Control and 6h gel)

Spot no.	Homologous protein	Microorganisms	NCBInr accession no[a]	No. of peptide mass values matched	Mowse score[b]	pI	Molecular mass (kDa)
New							
1	Cold shock protein A	*Lactobacillus casei*	Q70LR3_LACCA	6	164	4.1	~1
Induced							
2	Hypothetical protein lp_0704	*Lactobacillus plantarum*	Q88YP5_LACPL	1	48	4.5	~8
3	Major cold-shock protein (fragment)	*Listeria grayi*	Q48555_LISGR	1	48	4.1	~ 8
7	HSP 60 (Fragment)	*Pediococcus pentosaceus*	Q8KWJ2_PEDPE	2	45	6	~11
Repressed							
5	HsdS	*Mycoplasma gallisepticum*	Q7NAH4_MYCGA	2	47	5.2	~ 11
6	Methyl accepting chemotaxis protein t1pA	*Clostridium tetani*	Q895R0_CLOTE	2	47	5.6	~11

[a] The NCBInr library was searched by using the Mascot programme (http://www.matrixscience.com)

[b] Probability-based Mowse score: (-10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

Table 4.14: Peptide mass fingerprinting (PMF) analysis of *S. thermophilus* NCDC 74 during cold stress (Control, 2 h, 4 h and 6 h gel)

Spot no.	Homologous protein	Microorganisms	NCBInr accession no[a]	No. of peptide mass values matched	Mowse score[b]	pI	Molecular mass (kDa)
New							
A	Glyceraldehyde-3-phosphate dehydrogenase	*Lactobacillus delbrueckii*	Q6F1H0_MESFL	2	44	5.5	45
V	Cell surface protein	*Bacillus cereus*	Q816D8_BACCR	2	48	4.5	33
Induced							
C	Oxidoreductase (EC 1.-.-.-)	*Lactobacillus plantarum*	Q88SL1_LACPL	7	140	6	40
D	Phage terminase, large subunit	*Bacillus licheniformis*	Q62W49_BACLD	1	47	5.7	40
J	3-hydroxyacyi-CoA dehydrogenase/ carnithine racemase fusion (EC 4.2.1.17).-	*Bacillus cereus*	Q631W2_BACCZ	2	44	5.6	30
K	Cu/Zn-containing superoxide dismutase	*Bacillus cereus*	Q6RT16_BACCE	1	49	4.9	30
M	L-serine dehydratase alpha subunit	*Streptococcus thermophilus* CNRZ1066	Q5LZ51_STRTR	1	59	4.6	21
Q	AE001437 NID	*Clostridium acetobytylicum*	AAK78123	1	41	4.1	15
U	Cell division initiation protein DivIVA	*Lactobacillus plantarum*	Q88V88_LACPL	2	72	4.2	43

Spot no.	Homologous protein	Microorganisms	NCBInr accession no[a]	No. of peptide mass values matched	Mowse score[b]	pI	Molecular mass (kDa)
Repressed							
P	ATP-dependent C1p protease, ATP-binding subunit C1pB	*B.thuringiensis*	Q6HM12_BACHK	3	50	6.1	21
I	Pyruvate kinase	*Lactobacillus casei*	Q6XBK1_LACCA	2	106	5	33
E	UTP—glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	*Lactobacillus plantarum*	Q88YK0_LACPL	5	140	5.6	41

[a] The NCBInr library was searched by using the Mascot programme (http://www.matrixscience.com)

[b] Probability-based Mowse score: (-10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hit

4.4.4 Biochemical Characteristics

The results obtained from the biochemical evaluation of the parents and adapts in terms of titratable acidity produced (as % lactic acid) and proteolytic activity (as mg tyrosine released/ ml) have been presented in the Table 4.15.

Table 4.15: Biochemical characteristics of Adapts

Strain No.	Titratable acidity (% Lactic acid)	Proteolytic activity (mg tyrosine released/ ml)
Parent *L. delbrueckii* subsp. *bulgaricus* NCDC 307 $\mathbf{C_{307}}$	0.92	0.16
Adapt of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 $^{4.3}\mathbf{pH_{307}}$	1.02	0.19
Parent *L. acidophilus* NCDC 14 $\mathbf{C_{14}}$	1.00	0.17
Adapt of *L. acidophilus* NCDC 14 $^{4.0}\mathbf{pH_{14}}$	1.10	0.21
Parent *S. thermophilus* NCDC 74 $\mathbf{C_{74}}$	0.825	0.225
Adapt of *S. thermophilus* NCDC 74 $^{12}\mathbf{T_{74}}$	0.840	0.28

4.4.4.1 Lactobacilli

The adapts of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307 and *Lactobacillus acidophilus* NCDC 14 produced 1.02 and 1.10 percent titratable acidity as lactic acid in milk. The adapts exhibited 0.1 % increase in % LA over that produced by the parents. A slight increase in total proteolytic activity was also observed in case of the adapts (15 % in $^{4.3}pH_{307}$; 23% in $^{4.0}pH_{14}$) (Table 4.15).

In a related study on *Lactobacillus* species, Prasad (1991) noted that improved variants with respect to antibiotic resistance exhibited almost similar lactic acid production and moderate proteolytic activity not much different from that of the parent cultures. However, Prasad did not report on tolerance of the variants for high acidity. At the same time the variants did not show improved

proteolytic activity. Chou and Weimer (1998) have indicated that the protease activity of acid and bile tolerant isolates of *L. acidophilus* ATCC 33200 series was similar to that of the parents. De Angelis *et al.* (2001) demonstrated higher aminopeptidase and iminopeptidase activities in acid tolerant mutants (CBI-7R) of *L. sanfranciscensis* CBI. To date no published reports are available regarding the gradual adaptation of *L. delbrueckii* subsp. *bulgaricus*. Only one report is available regarding the acid-stress response by Lim *et al.* (2000) but the authors not mentioned about the production of lactic acid or proteolytic activity. The present study has amply shown that some improvements were achieved with the gradual adaptation methods, without manipulating the organism genetically. However, there is plenty of scope to further exploit this technique on industrial scale.

4.4.4.2 *Streptococcus thermophilus*

As is evident from the Table 4.15, the developed acidity was 0.84% in adapt ($^{12}T_{74}$) whereas parent culture succeeded in developing an acidity of 0.825%. The result indicated a non significant about 1.5% increase in LA by adapts over that of the parents. The proteolytic activity in adapts (0.28 mg) increased notably by 20 % over that by the parent (0.22 mg) in the present study. The results indicated that the parent culture may not thus be rated as an ideal starter culture. Higher levels of lactic acid and proteolytic activity in the adapts indicated that the adaptation method could be suitable for improving the metabolic activity of the original culture. In a related study Gupta (1991), reported an increased production of LA and proteolytic activity by the recombinants of *L. lactis* subsp. *lactis* over that of the parents. All these results indicate that the possibilities now exist for developing/improving streptococcal strains with enhanced metabolic capabilities by environmental and genetic endeavours.

4.4.5 Freeze Thaw Survival Capacity

4.4.5.1 *Lactobacillus acidophilus*

About 0.05% of the parent cells survived after the fourth cycles, whereas in the adapts, 1% survivability was observed. A continuous increase of 4, 8, 14 and 20 fold survivability was seen in adapts over the parent cells.

In chloramphenicol treated cells the survivability was drastically and comparably reduced.

Cross protection was observed in both lactobacilli as they are adapted to low pH. Chloramphenicol treatment showed protein synthesis may require during adaptation process. In a related study, freeze thaw survivabilty in acid and bile tolerant mutant of *L. acidophilus* was observed (Chou and Weimer, 1999).

4.4.5.2 *Lactobacillus delbreueckii* subsp. *bulgaricus*

The freeze thaw survivability of both the parent and adapt was determined during four consecutive freeze thaw cycles. Control and adapts observed a downward trend, and the survivability was reduced (Fig 4.95). Approximately 0.05% of the parent cells grown in MRS broth survived after the fourth cycle, compared to 1.02 % of the adapts. The adapts demonstrated a continuously higher survival rates, i.e. 5, 9, 15 and 20 fold over that of parent after first to fourth cycles respectively. In the presence of chloramphenicol, the survivability of both the parent and adapt was highly reduced and comparable to each other. This indicated the inability of the organisms to produce new/more protein in the presence of chloramphenicol.

4.4.5.3 *Streptococcus thermophilus*

The survival of both the parents and the adapts was determined during four consecutive freeze-thaw cycles. Approximately, 0.05% of the parent cells grown in normal LM17 broth survived after the fourth cycle. The adapts showed a 65 fold more survival over that of the parents (Fig 4.96). Overall, the adapts showed a continuous and almost constant increase in freeze survival rates i.e. 8, 24, 43, and 65 fold over that of the parents after 1st, 2nd, 3rd, and 4th cycle, respectively. At the same time the adapts showed a similar downward trend but with a steeper slope upon addition of chloramphenicol (100 μg/ ml) to the LM17 broth, during the adaptation process.

In the dual treatment comprising chloramphenicol and temp, the survivability was drastically reduced (Fig 4.96) after the four freeze thaw cycles where only 0.042, 0.010, 0.001 0.003 fold survivability after 1st, 2nd,

3rd, and 4th cycles respectively was observed in comparison to the adapts in the treatment only with low temp without chloramphenicol.

The downward trend was also observed in chloramphenicol treated parent cells, with the survivability of 0.86, 0.85, 0.45 and 0.2 fold in the four consecutive cycles, respectively.

Pre-submission of the parent cells to milder stress (i.e. at suboptimal temp) induced higher resistance to a lethal stress. The phenomenon is known as tolerance, phenotypic adaptation or habituation (Thammavongs *et al.*, 2000). Adaptation of bacterial cells to suboptimal temp results in an increased survivability during freezing. This phenomenon is known as cryotolerance (Panoff *et al.*, 2000).

In the present investigation, cryotolerance was demonstrated in *S. thermophilus* NCDC 74 and when the cells were subjected to gradually decreasing temp. The adapted cells showed a higher survival (~ 65 fold) over the parent cells after the four freeze thaw cycles. Cryotolerance has been generated in *L. lactis* (Kim and Dunn, 1997); *L. acidophilus* (Lorca and Valdez, 1998); *L. bulgaricus* (Urazza and Antoni, 1997) and *S. thermophilus* (Wouters *et al.*, 1999) by incubation of sub-optimal temps. The present findings are in agreement with those of Kim and Dunn (1997). They observed that the survival of *S. thermophilus* TS2 after freezing was increased when the cells were adapted at 10 ^{0}C. Effective protection of *S. thermophilus* CNRZ302 can be achieved by pre-treatment at 20 ^{0}C as demonstrated by Wouters *et al.* (1999). However, they observed that exposure to 10 ^{0}C for 2 and 4 h results in only a small increase (5 and 10 fold respectively) in freeze survival compared to 100 and 1,000 fold survivability on exposure at 20 ^{0}C. As such theirs was a cold shock treatment method, whereas in the present study it was a gradual adaptation approach. The higher survivability in *S. thermophilus* NCDC 74 (~ 65 fold) adapted at 12 ^{0}C in the present study may be due to the fact that, the cells here adapted to a continuous and gradual temp, gradually. This is in accordance with the similar study, by Baati *et al.*, 2000, where *L. acidophilus* resulted in a survival rate of 74 %, when it was subjected to a slow, step-wise decrease in the incubation temp.

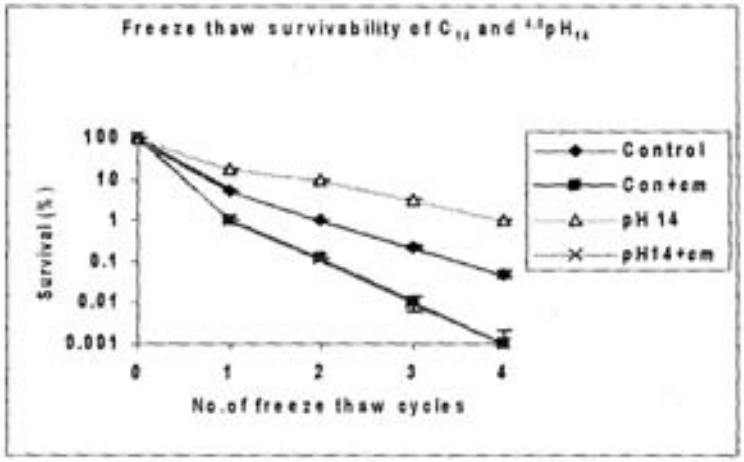


Fig 4.94: Freeze thaw survivability of C_{14} and $^{4.0}pH_{14}$. cm: chloramphenicol (Bars indicate standard errors).

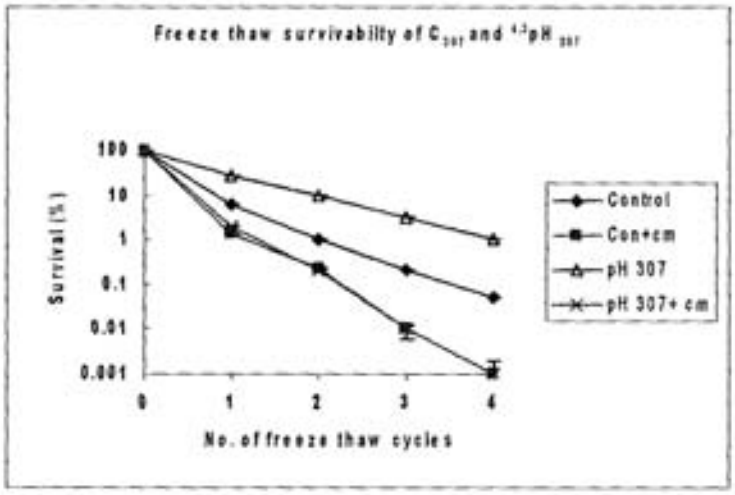


Fig 4.95: Freeze thaw survivability of C_{307} and $^{4.3}pH_{307}$. cm: chloramphenicol. (Bars indicate standard errors).

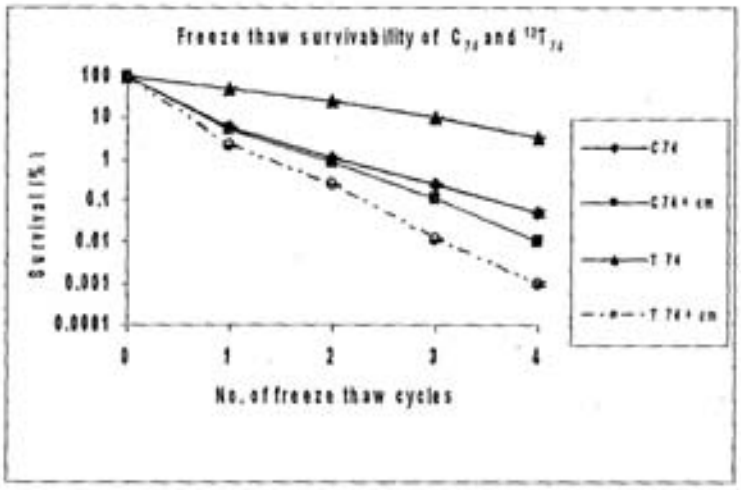


Fig 4.96: Freeze thaw survivability of C_{74} and $^{12}T_{74}$. cm: chloramphenicol. Results are the means of three independent experiments (Bars indicate standard errors).

4.4.6 Probiotic Properties

4.4.6.1 Tolerance to low pH/ acid stability

Different regions of the gastrointestinal tract have varying levels of acidity. The pH of the stomach and duodenum may fall to as low as 1.5 due to the secretion of hydrochloric acid by the gastric parietal (oxynytic) cells (Mitsuoka, 1990). However, consumption of milk and milk products decreases gastric pH to a greater than 2.7 for 3 h following ingestion (Martini *et al.*, 1987). The rate of food passage through the stomach depends upon composition of food, primarily fat content. Berrada *et al.* (1991) reported 80 % gastric emptying within 90 min for two fermented milk products containing bifidobacteria. If a fermented dairy product is consumed along with other food items or as part of a meal, the length of time spent in the stomach might be increased (Pochart *et al.*, 1992). This could affect the survival of probiotic bacteria present in the fermented milk product during its passage through the stomach. Therefore, the microorganisms (parents as well as the adapts) taken up for the present study were screened for their in vitro tolerance to varying levels of pH (1.0, 2.0 and 3.0) during 1, 2, and 3 h exposure at 37 ^{0}C. The results of the experiment have been recorded in Fig 4.97 to 4.102.

The parent *L. acidophilus* NCDC 14 showed the same initial survivability at pH 3 as that of pH 6.5. It decreased to some extent at pH 2.0 but it was drastically reduced at pH 1.0. But the adapt was such less adversely affected at pH 1.0. After 1 h the growth of parents at pH 2 and 3 was same but that at pH 1.0, it was reduced still further. The increasing incubation at 2 h affected the growth adversely but at 3 h, the proportion appeared to be similar as at 2 h. The adapts ($^{4.0}pH_{14}$) followed the same pattern at pH 6.5 but at other level of pH they resisted the deleterious effect of acid. The survivability of pH 1 was much more than that of the parents during whole of the metabolism.

In general, the result of *L. delbrueckii* subsp. *bulgaricus* followed almost the same trend as that of *L. acidophilus*. However, a lower survivability was observed in most of the cases. A 3 log reduction in viable counts was

observed at pH 1 in 3 h as compared to 0 h. At pH 2, the reduction is nominal after 1 h, but ~ 3 log reduction occurred after 3 h of incubation. However, 70% cells were survived compared to pH 1. pH 3 cells depicted 2 log reduction as compared to 0h cells.

Adapts ($^{4.3}pH_{307}$) followed the same pattern with control at pH 6.5, but at other levels, they resisted deleterious effects of acid, ~ 25% cells were survived at pH 1 after 3 h whereas, in adapts it was 3 fold (~75%) more than parents. At pH 2, reduction of ~ 50% cells was observed as in parents, whereas the percent loss in adapts is only 15% (Fig 4.97 to Fig 4.102). The Fig 4.101 and Fig 4.102 represented the tolerance of low pH in *S. thermophilus*. The results indicated that the *S. thermophilus* is less resistant as compared to lactobacilli. In parents, 1-, 3-, and 3-log reduction in viable counts were detected (Fig 4.101), over the period of 3 h at pH 1, 2 and 3 respectively, whereas control showed consistent result. In adapts, ($^{12}T_{74}$), 1-, 2- and 3- log reduction was observed at pH 1, 2, 3 respectively after 3 h (Fig 4.102).

Results obtained in the present study are in agreement with the earlier observations of tolerance to low pH in screening of *Lactobacilli* strains for probiotics attributes (Salomi, 1997). Two log reductions in cell counts were observed in pH 2 after 2 and 3 h of exposure. At pH 1, the number of survivors dropped from 10^4 to 10^2 cfu/ml after 36 h, in *Lactobacillus acidophilus* NCDC 14. They confirmed the fact that the tolerance to pH of human stomach is species and strain dependent in lactobacilli and bifidobacterial species. The better survivability of the adapts at low pH in both the lactobacilli was in agreement with the findings of Chow and Weimer (1998). In their study, they observed the acid-tolerant mutants of *L. acidophilus* (LSC13) isolated by them tolerate lethal pH (pH 3.5). In another report, Jan *et al.* (2001), revealed the acid tolerance response (ATR) in probiotics *Propionibacterium freudenreichii* subsp. *shermanii* SI 41, where they reported 100 % survival of acid adapted cells at pH 2.0.

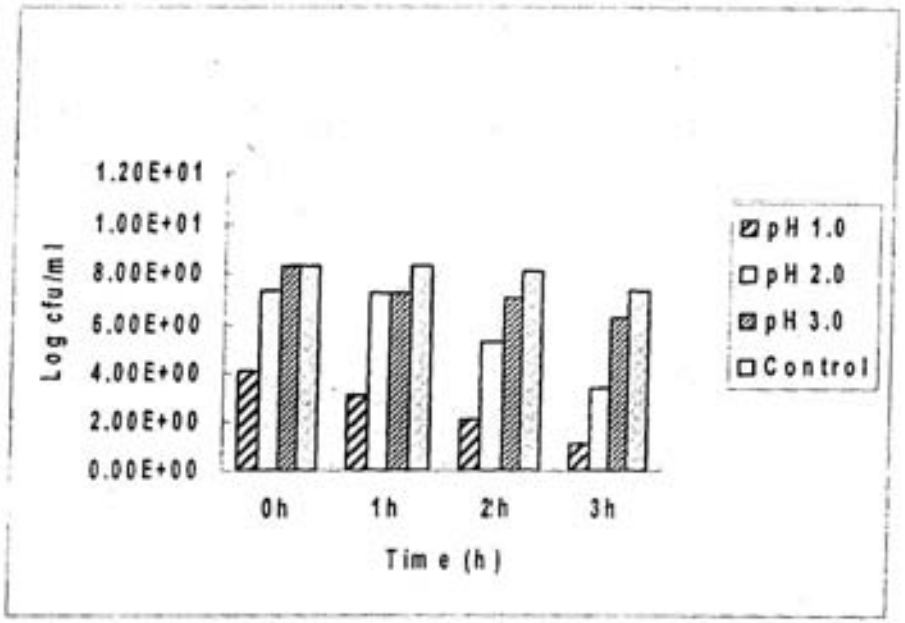


Fig. 4.97: Survival of *L. acidophilus* NCDC 14 (C_{14}) during 3 h in HCl

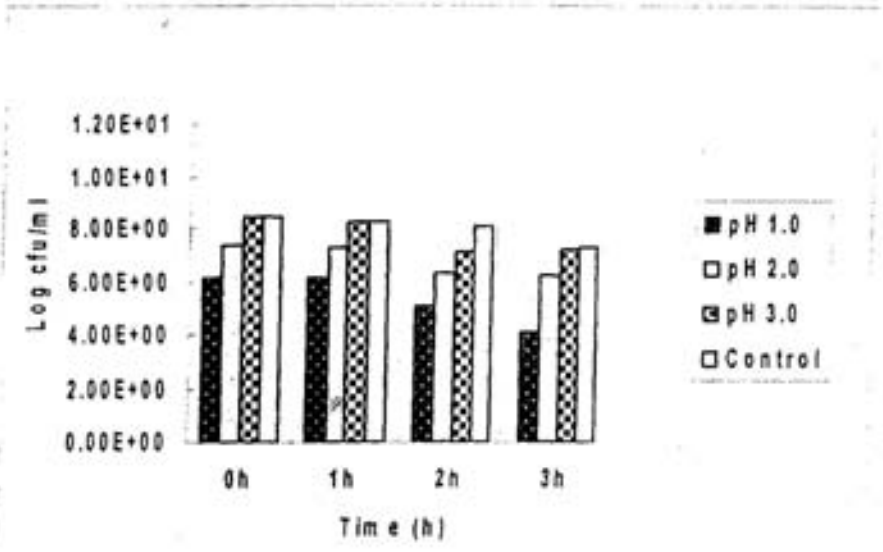


Fig 4.98: Survival of *L. acidophilus* NCDC 14 adapts ($^{4.0}$ pH_{14}) during 3 h in HCl

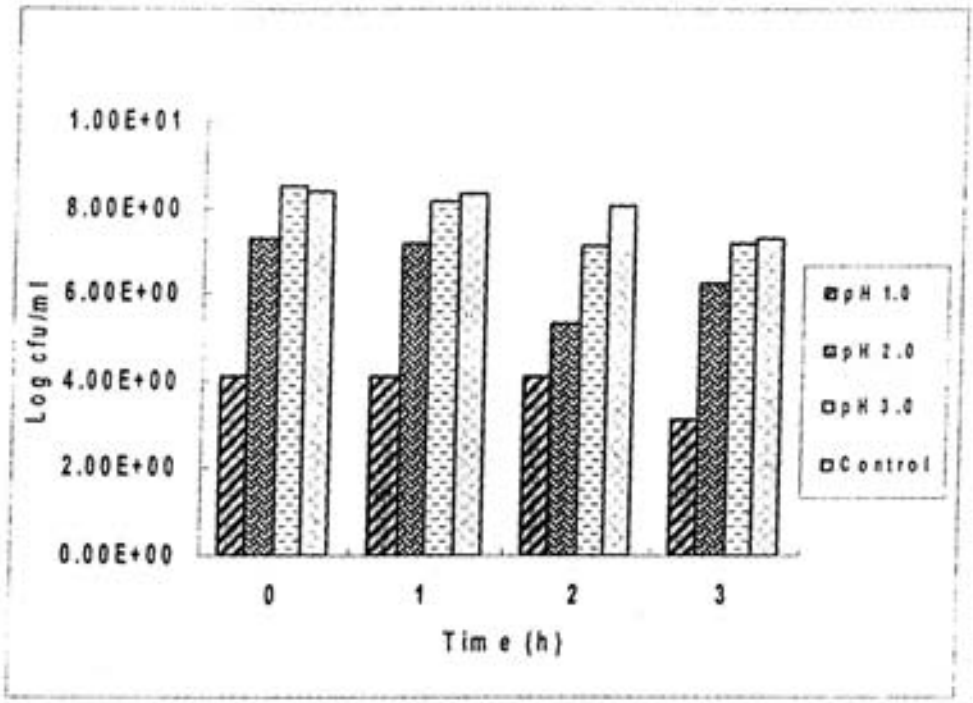
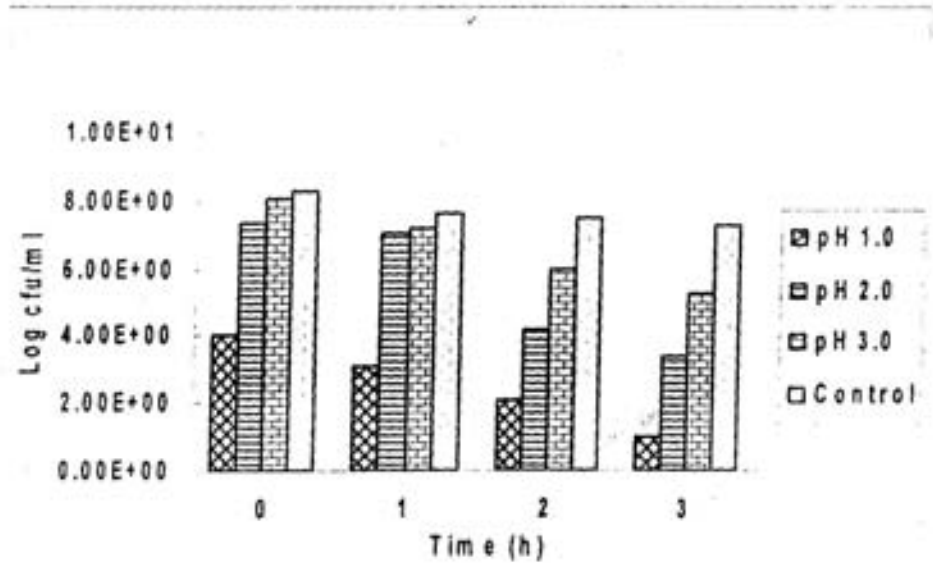


Fig 4.99: Survival of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 (C_{307}) during 3 h in HCl



4.100: Survival of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 adapts ($^{4.3}pH_{307}$) during 3 h in HCl

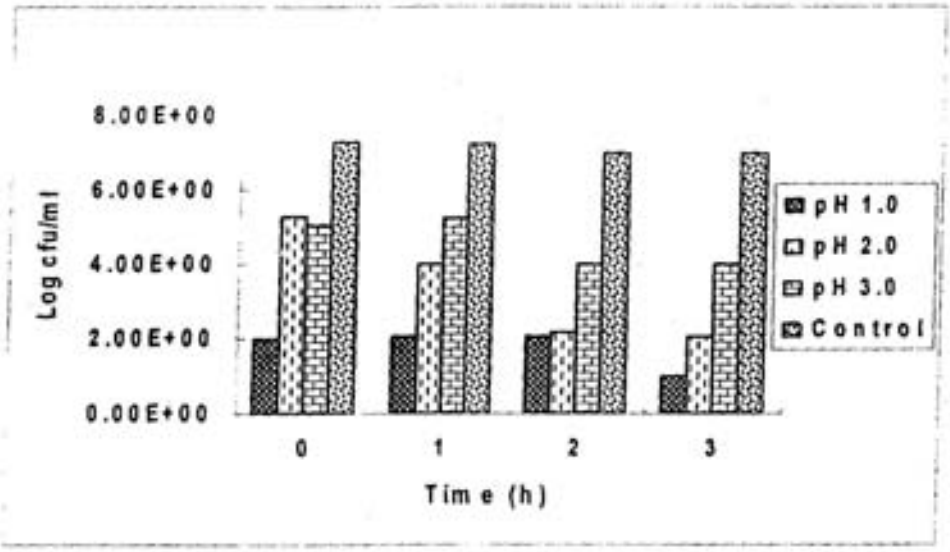


Fig 4.101 : Survival of *S. thermophilus* NCDC 74 (C_{74}) during 3 h in HCl

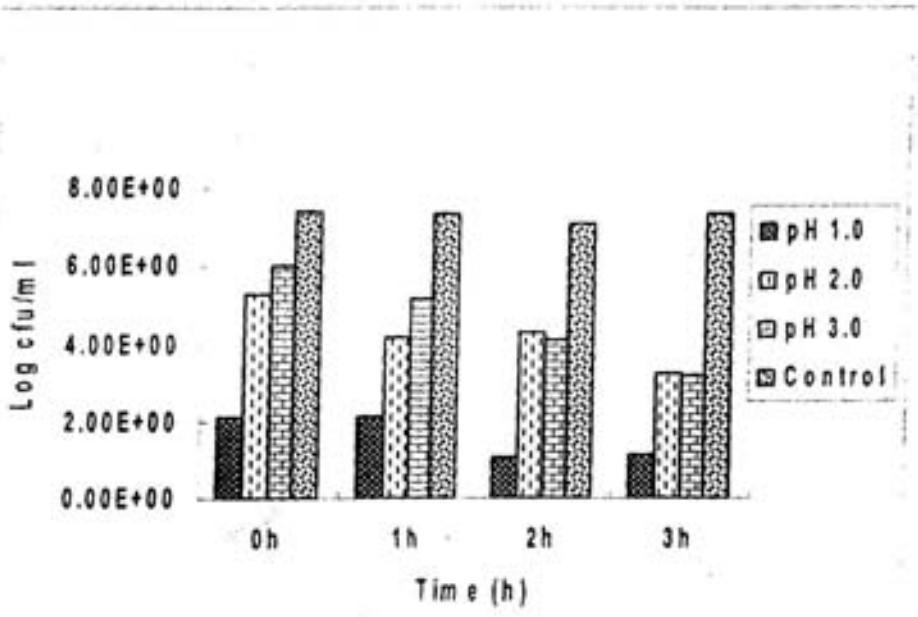


Fig 4.102: Survival of *S. thermophilus* NCDC 74 adapts ($^{12}T_{74}$) during 3 h in HCl

The present study thus depicts the protective effect of acid pretreatment/ acid adaptation in both *L. bulgaricus* and *L. acidophilus* adapts.

In *S. thermophilus*, a significant reduction of cells was observed as compared to lactobacilli. However, in adapts ($^{12}T_{74}$), at pH 2, two log reduction of cells were occurred, whereas in parents only 3 log reduction was observed. This result indicates a cross-protection mechanism in case of *S. thermophilus* NCDC 74 because *S. thermophilus* NCDC 74 was adapted at low temp.

Similar observation were reported by Baati *et al.* (2000) in *L. acidophilus*, where they reported no significant loss of viability occurred during freezing, after exposure of cells to low pH.

4.4.6.2 Tolerance to bile salt

The parent cells of *L. acidophilus* NCDC 14 demonstrated a decreased tolerance to bile salts (1%) as compared to control cells (Fig 4.103). In 2 % no organisms remain to be viable. Similar observations were reported by Salomi in *L. acidophilus* stains. At 1 % conc, only 32 % cells were survived at the end of 3 h whereas at the end of 12 h only 7.5 % cells were survived. In adapts, at the end of 3 h in 1 % conc 50 % cells were survived (Fig 4.104). At 2 % some of the cells grow as opposed to parents. This might be due to pre-adaptation of the microorganisms to an acid stress.

In *L. bulgaricus*, both in parent and adapts, at 1 % bile conc no viable cells were seen at the end of 12 h (Fig 4.105 and Fig 4.106). Adapts ($^{4.3}pH_{307}$), tolerated 2 % initial bile salt conc whereas parents could not. However, the adapts ($^{4.3}pH_{307}$) showed a decreasing number of cells in 1 % bile conc at 0 h (Fig 4.106).

In, *S. thermophilus*, the viable number was decreased as compared to lactobacilli in control bile conc. The deleterious effects of bile salts were observed in all other conditions in both the cases (Fig 4.107 and Fig 4.108).

In the present study, adapted cells of lactobacilli showed a better resistance to bile salt as compared to parents. During a study of susceptibility and adaptive response to bile salts in *P. freudenreichii* SI41, Leverrier *et al.* (2003),

observed a cross protection mechanism against bile salt, when cells are exposed to 42 ^{0}C. However, they reported acid pre-treatment against bile salts rather acid-adapted cells were less tolerant to bile salts than non-adapted ones. In an earlier report, Flahaut *et al.* (1996) observed, acid pre-treatment does not induce protection against bile salts, but also does not induce sensitivity. In LAB, the strong cross-protection between bile and heat-shock which was observed in some species, suggests overlapping regulatory pathways (Guchte *et al.*, 2002). It has been already proved by various proteomics studies that, some heat shock protein (Hsps) were highly induced under acid stress conditions. For example, GroES, GroEL and DnaK were highly induced under acidic conditions in *L. bulgaricus* (Lim *et al.*,2001); DnaK and GroEL in *L. lactis* under acid stress conditions (Hartke *et al.*,1996). In *E. faecalis*, the conserved chaperones DnaK and GroEL were identified in bile stressed cells (Flahaut *et al.*, 1996). From these findings it can be suggested that there is a regulatory network exists between acid, bile and heat shock response. However, much remains to be elucidated on this line. The present study only confirmed a cross protection between bile and acid in physiological level in lactobacilli. In *S. thermophilus* no cross protection observed during bile tolerance studies of cold-adapted cells.

4.4.6.3 Cell-surface hydrophobidity

Cell surface hydrophobicity was determined by percent hydrophobicity and salting out aggregation (SAT) values, the results of which are shown in Tables 4.16.

In the present study, the cell surface hydrophobicity and SAT values is slightly increase in adapts (Table 4.16). This suggests their better performance in relation to the above activity than parents. This is in consistent with their superior tolerance to low pH and bile salts. However, the study by Prasad (1991) in different lactobacilli did not show superior tolerance to low pH, bile salt, that may showed adhesion properties. It correlates with the similar observations in parent culture in the present study but not in adapts.

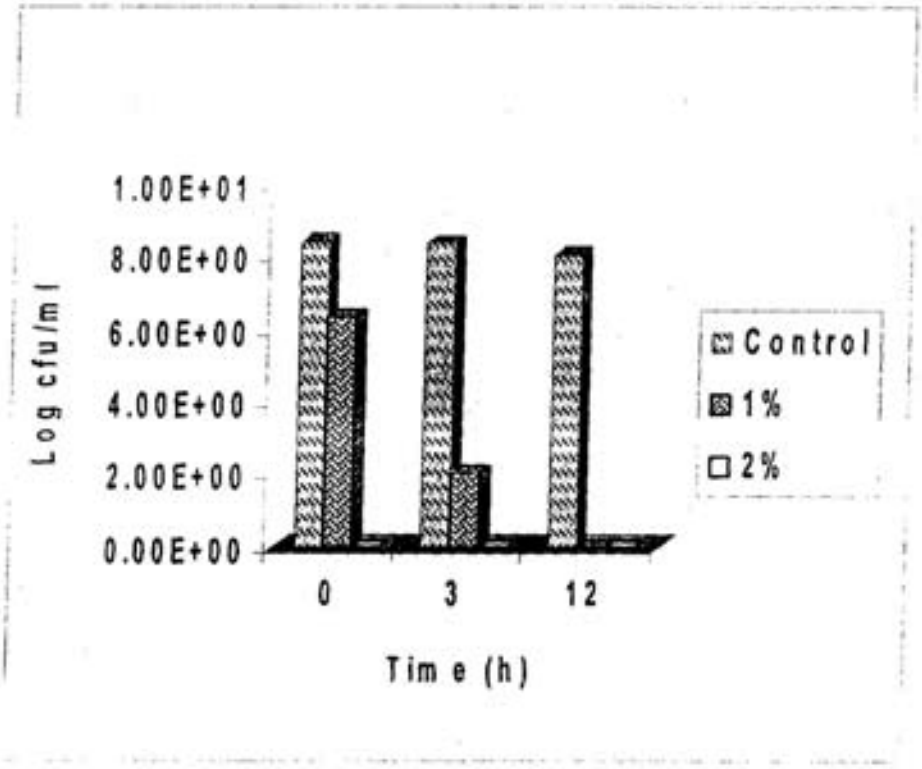


Fig 4.103: Bile tolerance of C_{14}

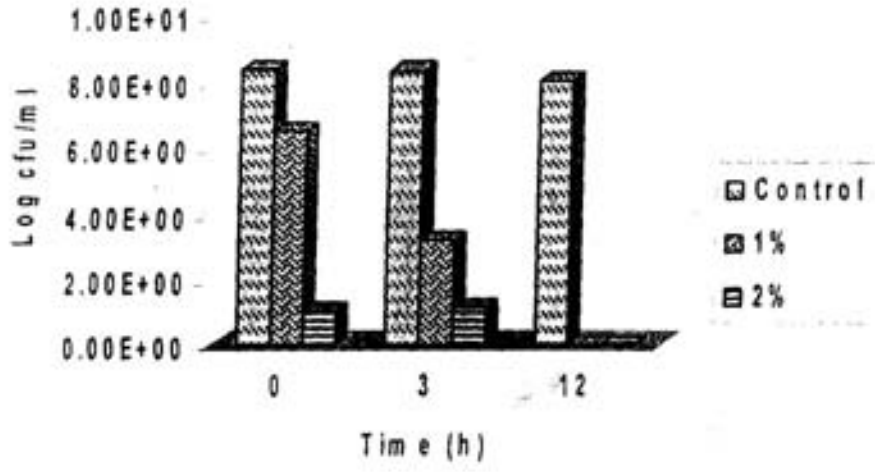


Fig 4.104: Bile tolerance of $^{4.0}$ pH_{14}

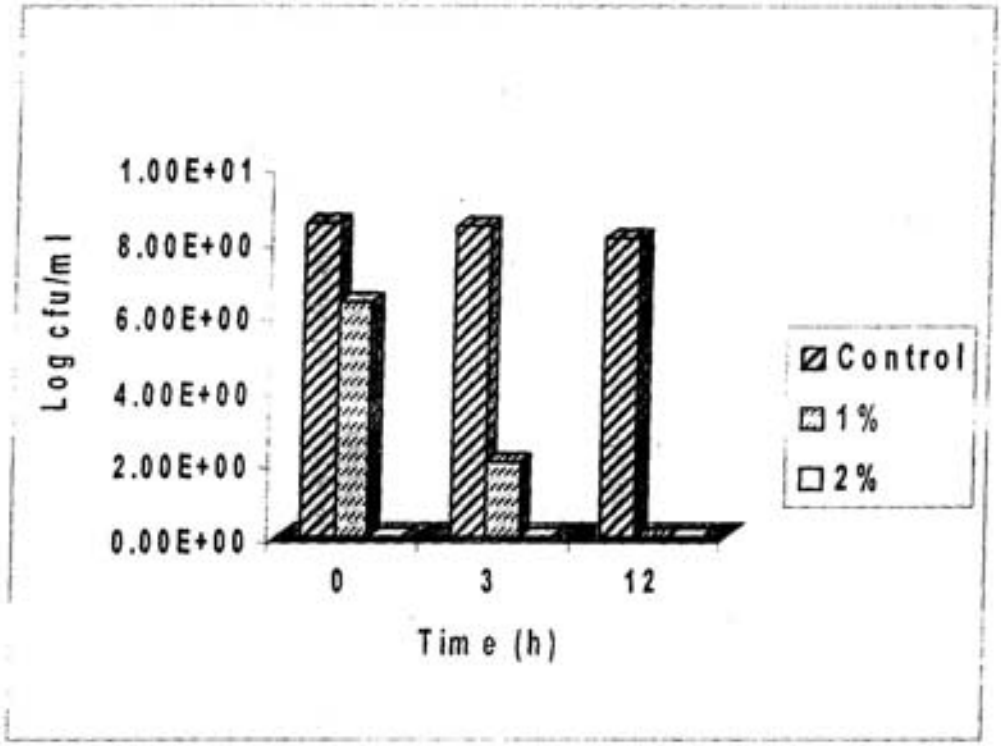


Fig 4.105: Bile tolerance of C_{307}

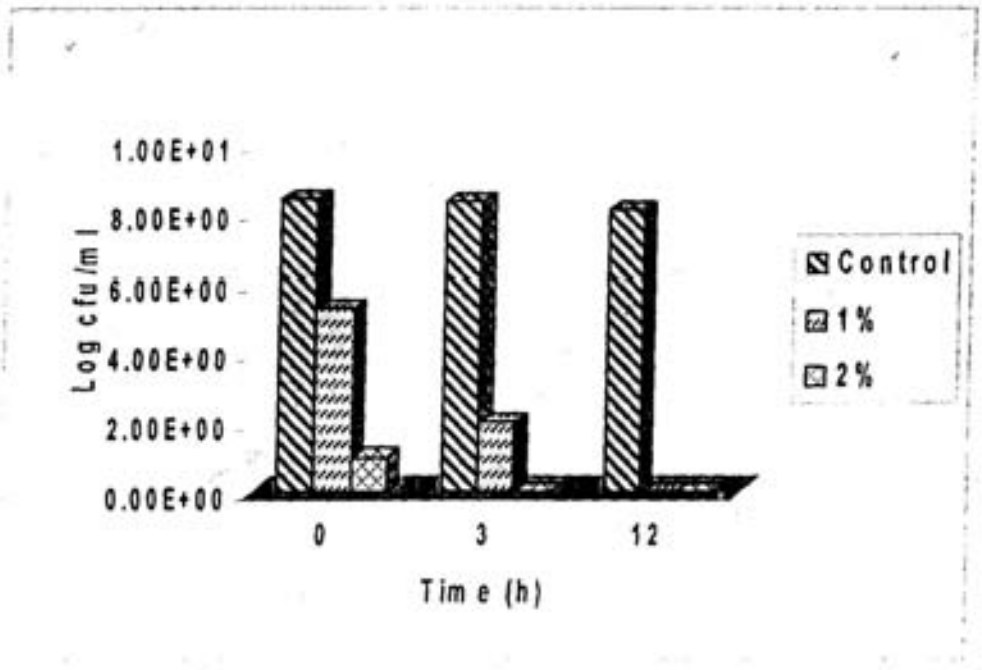


Fig 4.106: Bile tolerance of $^{4.3}$ pH_{307}

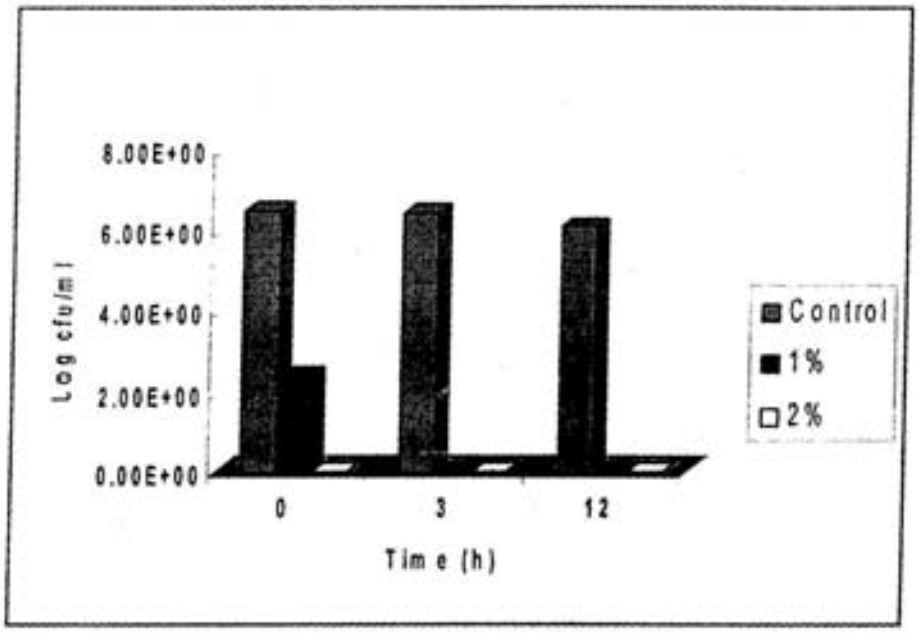


Fig 4.107: Bile tolerance of C_{74}

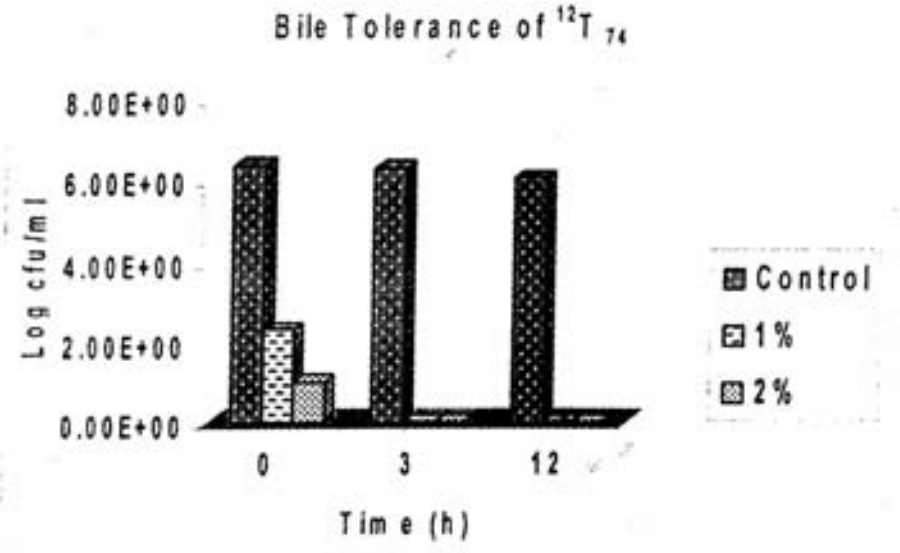


Fig 4.108: Bile tolerance of $^{12}T_{74}$

Therefore, it may be suggested that the adaptation procedure followed here may serve as model for other probiotic organisms of industrial significance.

Tables 4.16: Cell surface hydrophobicity of Adapts

Culture	Cell surface hydropobicity (% hydrophobicity)	SAT values
Lactobacillus acidophilus NCDC 14 (C_{14})	11.50	1.50
Adapt of *Lactobacillus acidophilus* NCDC 14 ($^{4.0}pH_{14}$)	14.31	1.50
L. delbrueckii subsp. *Bulgaricus* NCDC 307 (C_{307})	12.6	1
Adapt of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 ($^{4.3}pH_{307}$)	12.82	1.1
S. thermophilus NCDC 74 (C_{74})	4.56	0.7
Adapt of *S. thermophilus* NCDC 74 ($^{2}T_{74}$)	4.58	0.75

4.4.6.4 Antimicrobiocity

No demonstrable difference with regard to antimicrobiocity has been observed between parents and adapts in the present study.

4.5 GENOME SHUFFLING / GENETIC IMPROVEMENT OF SELECTED CULTURES USING PROTOPLAST FUSION

4.5.1 Protoplast Formation

The protoplast formation of *S. thermophilus* NCDC 74 and adapts ($^{12}T_{74}$), lactobacilli (C_{14}, C_{307}) and their adapts ($^{4.0}pH_{14}$, $^{4.3}pH_{307}$) was optimized at 10 mg/ml lysozyme and 50 μg/ml mutanolysin conc. Fig 4.109 shows the rate of production of osmotically fragile cells (protoplasts) during incubation with enzyme mixture. The number of osmotically resistant, i.e. normal cells (count on LM17 and MRS Agar respectively) per ml of the suspension decreased during incubation irrespective of the type of organisms. Lactobacilli were more sensitive as compared to *S. thermophilus* NCDC 74, After 30 min, the osmotically stable cell count of lactobacilli, reduced by 5 to 6 log units, whereas in *S. thermophilus* and its adapts it was reduced to four log units.

Moreover, after 120 min incubation, lactobacilli did not show any osmotically stable cells, whereas *S. thermophilus* and $^{12}T_{74}$ showed residual osmotically stable cells to the extent of 2.23 and 1.04 log units respectively. In *S. thermophilus* the formation of protoplast was recorded 99.99 % after 120 min incubation. Similar condition reached in 90 min in case of lactobacilli (Fig 4.109).

As illustrated in Fig 4.109, contrary to osmotically stable cell count, the log number of regenerated cells increased. For instance, in case of *L. delbrueckii* subsp. *bulgaricus* (C_{307}), the log counts of regenerants per ml after 30 min was 9.75, but after 90 min, the log count increased to 9.91. The log count of all the three cultures and their adapts tested were decreased after 90 min (Fig 4.109).

4.5.2 Microscopic Examination

The formation of protoplasts also judged by light and electron microscopy. The appearance of spherical cells as judged by light microscopy was used as an indicator of protoplast formation (Plate 4.22).

4.5.3 Genome Shuffling/ Protoplast Fusion

Genome shuffling amplifies the genetic diversity within a selected population through extensive recombination between the individual members. Thus it accelerates directed evolution (Zhang *et al.*, 2002). Genome shuffling by protoplast fusion has recently been reported in a lactobacillus strain, where the strain was the starting point of the evolution program (Patnaik *et al.*, 2002).

In the present study, the same approach was used as the starting materials were protoplasts of a single TLAB strain and its adapt. No antibiotic resistance marker was used for fusion studies and selection of expected fusants. The protoplasts obtained from the parent as well as adapts were fused and fusants were grown in regeneration medium. The fusants grown in regeneration medium were scraped, passaged in LM17 and MRS media at least for five times and kept for further analysis (Table 4.17 to Table 4.20).

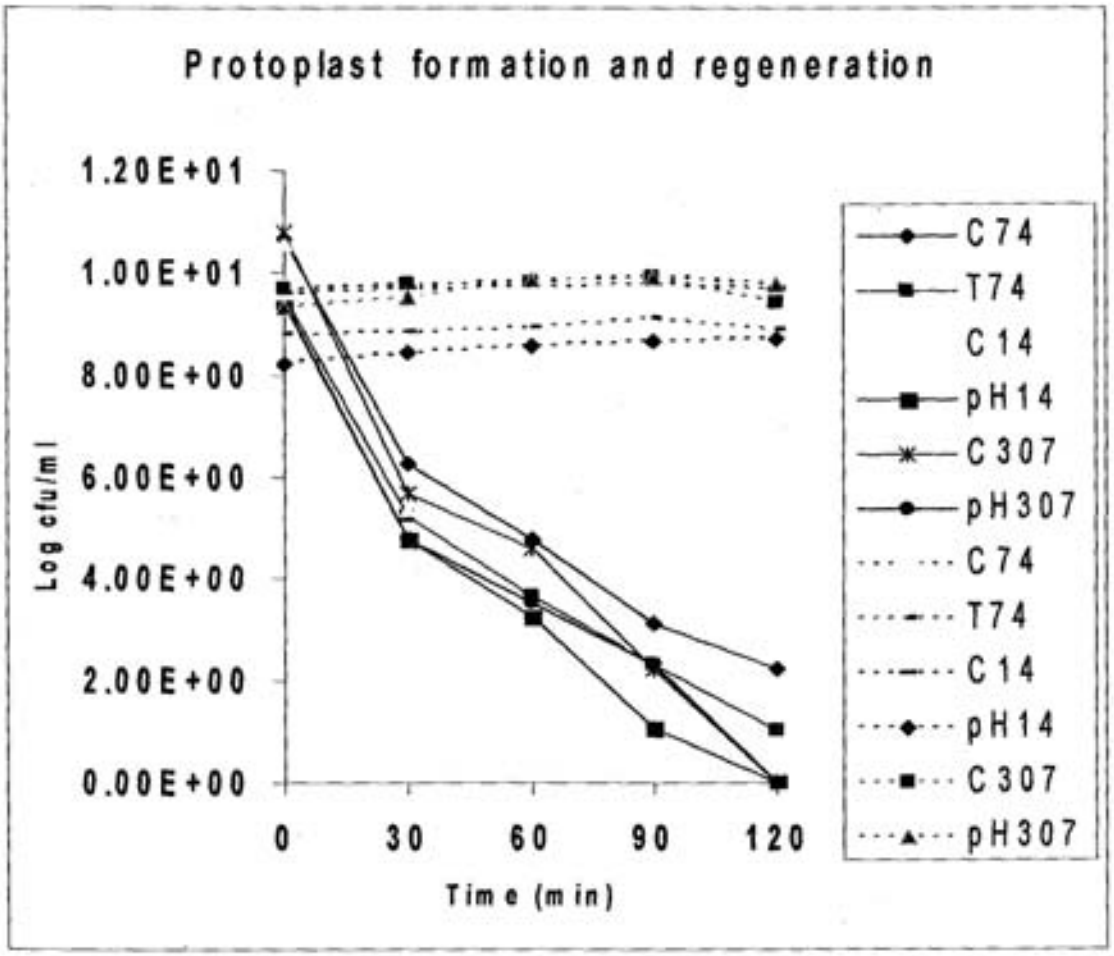


Fig 4.109 : Protoplast formation and regeneration in parents and adapts of TLAB

The isolation history of the parents and adapts are presented in Table 4.17 to Table 4.19.

4.5.3.1 Screening and characterization of individual isolates/ strains

4.5.3.1.1 Screening of individual population

To achieve better quantification of the improved tolerance to stress conditions of the shuffled strains, various temp and pH levels were used for streptococci and lactobacilli respectively (Table 4.20).

Table 4.17: Isolation history of fuasnts of *L. acidophilus* NCDC 14

Strain designation	Isolation history
C_{14}	Parent strain of *L. acidophilus* NCDC 14
$^{4.0}pH_{14}$	Low pH adapt of C_{14}
F1 $^{4.0}pH_{14}$	Population generated after fusion between protoplasts of C_{14} and $^{4.0}pH_{14}$
F2 $^{4.0}pH_{14}$	Population generated after fusion between protoplasts of C_{14}, $^{4.0}pH_{14}$ and F1 $^{4.0}pH_{14}$
F3 $^{4.0}pH_{14}$	Population generated after fusion between protoplasts of F1 $^{4.0}pH_{14}$ and F2 $^{4.0}pH_{14}$
F3 $^{4.0}pH_{14}$ – 6 & F3 $^{4.0}pH_{14}$ – 14	Colonies selected from the F3 $^{4.0}pH_{14}$ library for further analysis

Table 4.18: Isolation history of fuasnts of *L. delbrueckii* subsp. *bulgaricus* NCDC 307

Strain designation	Isolation history
C_{307}	Parent strain of *L. delbrueckii* subsp. *bulgaricus* NCDC 307
$^{4.3}pH_{307}$	Low pH adapt of C_{307}
F1 $^{4.3}pH_{307}$	Population generated after fusion between protoplasts of C_{307} and $^{4.3}pH_{307}$
F1 $^{4.3}pH_{307}$ -1	Colony selected from the F1 $^{4.3}pH_{307}$ library for further study

Table 4.19: Isolation history of fuasnts of *S. thermophilus* NCDC 74

Strain designation	Isolation history
C_{74}	Parent strain of *S. thermophilus* NCDC 74
$^{12}T_{74}$	Low temp adapt of C_{74}
$F1^{12}T_{74}$	Population generated after fusion between protoplasts of C_{74} and $^{12}T_{74}$
$F1^{12}T_{74}$-2 & $F1^{2}T_{74}$-13	Colonies selected from the $F1^{12}T_{74}$ library for further analysis

Table 4.20: Temp and pH selected for screening of fusants from lactobacilli and streptococci

Organisms	Screening temp/ pH (^{o}C)
S. thermophilus NCDC 74	42, 25, 20, 15, 10
L. bulgaricus NCDC 307 and *L. acidophilus* NCDC 14	6.5, 5.0, 4.5, 4.0, 3.8, 3.5

For streptococci, the serial dilutions of C_{74}, $^{12}T_{74}$ and $F1^{12}T_{74}$ each were plated in LM17 agar and incubated at different temp for 48 h as mentioned in Table 4.20. MRS agar at various pH (Table 4.20) adjusted with lactic acid was used for plating of lactobacilli. The total colony forming units/ml of each population have been presented in Fig 4.110 to Fig 4.112.

It is apparent from the Fig 4.110 that all the parents could not survive at pH 4.0 to pH 3.5, whereas the F3 $^{4.0}pH_{14}$ population grew at pH 3.5 and the colonies were very small. At pH 3.8 the colonies of F1 $^{4.0}pH_{14}$ and F2 $^{4.0}pH_{14}$ populations were smaller as compared to F3 $^{4.0}pH_{14}$ populations. They also took longer time to grow. Therefore, the F3 $^{4.0}pH_{14}$ population grew at pH 3.8 were taken for further study.

It is clear from the Fig 4.111 that at pH 3.8 only the population of F1 $^{4.3}pH_{307}$ survived. However, higher counts of $^{4.3}pH_{307}$ population were observed in all other pH from that of F1 $^{4.3}pH_{307}$. As in other cases, here also F1 $^{4.3}pH_{307}$ were taken for further screening as no colonies were observed at pH 3.8 with regard to rest of the population.

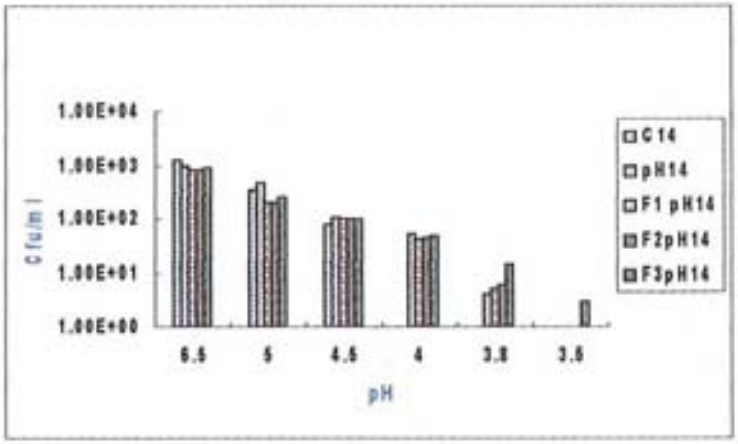


Fig 4.110 : Screening of adapts and fusants of *L. acidophilus* NCDC 14 at different pH
(C_{14} : Parent *L.acidophilus* NCDC 14; $^{4.0}pH_{14}$: Adapts of *L. acidophilus* NCDC 14; F1 $^{4.0}pH_{14}$: F1 population, F2 $^{4.0}pH_{14}$: F2 population, F3 $^{4.0}pH_{14}$: F3 population)

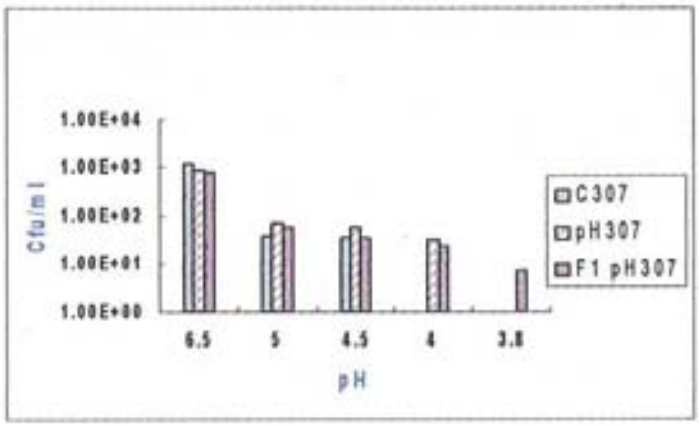


Fig 4.111 : Screening of adapts and fusants of *L. delbrueckii* subsp. bulgaricus NCDC 307 at different pH
(C_{307} : Parent *L. delbrueckii* subsp. bulgaricus NCDC 307; $^{4.3}pH_{307}$: Adapts of *L. delbrueckii* subsp. bulgaricus NCDC 307; F1 $^{4.3}pH_{307}$: F1 population)

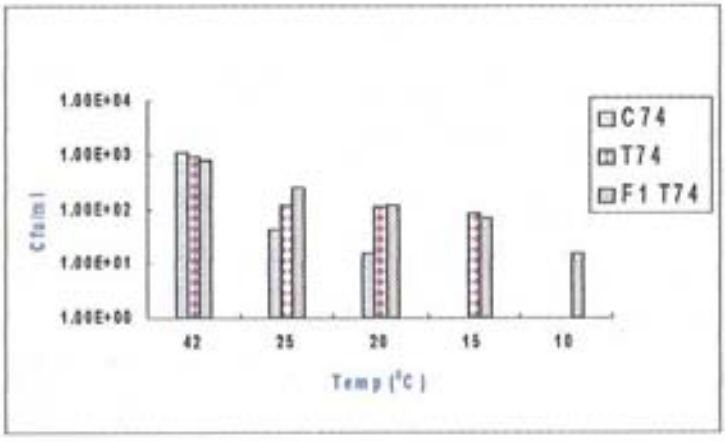


Fig 4.112 : Screening of adapts and fusants of *S.thermophilus* NCDC 74 at different temp (^{0}C)
(C_{74} : Parent *S. thermophilus* NCDC 74; $^{12}T_{74}$: Adapts of *S. thermophilus* NCDC 74; F1 $^{12}T_{74}$: F1 population)

Fig 4.112 indicated the populations of *S. thermophilus* survived at different temp. All populations survived equally well at 42 ^{0}C. At 25 ^{0}C, the cfu was less as compared to that of T_{74} and $F1^{12}T_{74}$. The control cells did not survive below 25 ^{0}C. At 10 ^{0}C, only members of the $F1^{12}T_{74}$ populations were survived. Thus, we expected, the shuffled population may contain new strains that have the capability to grow at 10 ^{0}C, below the temp level inhibitory to C_{74} and $^{12}T_{74}$.

4.5.3.1.2 Screening of individual isolates

Screening of individual isolates was performed on the basis of cell density measured at 650 nm.

4.5.3.1.2.1 Lactobacilli

Fig 4.113 depicted the OD_{650} measurement of all the *L. delbrueckii* subsp. *bulgaricus* NCDC 307, adapts and fusants, individual colonies from pH 3.8 (~15 colonies) plates were selected. The parent culture (C_{307}), adapts ($^{4.3}pH_{307}$) along with $F1^{4.3}pH_{307}$ derivatives were grown in 10 ml MRS broth adjusted at different pH and OD_{650} value was recorded. Out of all the strains screened only one hits demonstrated better growth than that of parents and adapts at all pH ranges. At pH 3.5 none depicted growth. At pH 3.8 all the isolates of $F1^{4.3}pH_{307}$ library depicted better cell density than that of parents and adapts, highest recorded in $F1^{4.3}pH_{307}$ -1. Thus, only $F1^{4.3}pH_{307}$ -1 was taken for further study.

As per Fig 4.114, individual colonies from F3 $^{4.0}pH_{14}$ grown at pH 3.8 were selected. Parent (C_{14}) and adapts ($^{4.0}pH_{14}$) grown in liquid media were taken for comparison purpose. All the strains were cultured in MRS media adjusted to different pH with lactic acid and cell density were recorded after 30 h. Two strains selected for further analysis based on growth studies were F3 $^{4.0}pH_{14}$- 6 and F3 $^{4.0}pH_{14}$- 14 (Fig 4.114).

4.5.3.1.2.2 Streptococcus thermophilus

Individual colonies (~15) from $F1^{12}T_{74}$ population growing on LM17 agar at 10 ^{0}C were selected. For comparison C_{74} and $^{12}T_{74}$ were taken and

grown in LM17 broth. All the selected colonies were grown in 10 ml LM 17 broth. Cell density was recorded after 30 h at 42 ^{0}C, 15 ^{0}C and 10 ^{0}C in all the cases (Fig 4.115). At 42 ^{0}C, 13 isolates showed a cell density less than C_{74} and $^{12}T_{74}$. But at 15 and 10 ^{0}C, the recorded OD_{650} was higher in all the isolates than control and adapted ones. The best performing hits numbered as F1$^{12}T_{74}$-1 and F1$^{12}T_{74}$ – 13 were presented for further analysis.

Table 4.21: Fusants selected for further analysis

Strain designation	Parent strains
F1 $^{12}T_{74}$ -2	*S. thermophilus* NCDC 74
F1 $^{12}T_{74}$ - 13	*S. thermophilus* NCDC 74
F1 $^{4.3}pH_{307}$-1	*L. delbrueckii* subsp. *bulgaricus* NCDC 307
F3 $^{4.0}pH_{14}$- 6	*L. acidophilus* NCDC 14
F3 $^{4.0}pH_{14}$- 14	*L. acidophilus* NCDC 14

4.5.3.1.3 Analysis of fusants

4.5.3.1.3.1 Probiotic properties

4.5.3.1.3.1.1 Acid tolerance

Fig 4.116 and Fig 4.117 indicated the acid tolerance of fusants of *L. acidophilus* NCDC 14. In comparison to adapts ($^{4.0}pH_{14}$), F3 $^{4.0}pH_{14}$-6 and F3 $^{4.0}pH_{14}$-14 demonstrated increased tolerance to pH 1.0 up to 3 h (~ 2 and ~3 log cfu respectively). From Fig 4.118 it was illustrated that, the acid tolerance of fusant F1 $^{4.3}pH_{307}$-1 was increased in fusant than that of parents and adapts.

Acid tolerance in *S. thermophilus* NCDC 74 fusants were also increased from that of parents and adapts (Fig 4.119 and Fig 4.120).

4.5.3.1.3.1.2 Bile tolerance

Fig 4.121 and Fig 4.122 indicated the bile tolerance of two promising fusants of *L. acidophilus* NCDC 14 i.e. F3 $^{4.0}pH_{14}$- 6 and F3 $^{4.0}pH_{14}$- 14. Adapts ($^{4.0}pH_{14}$) tolerated 2% bile salt up to 3 h, but after 12 h none survived.

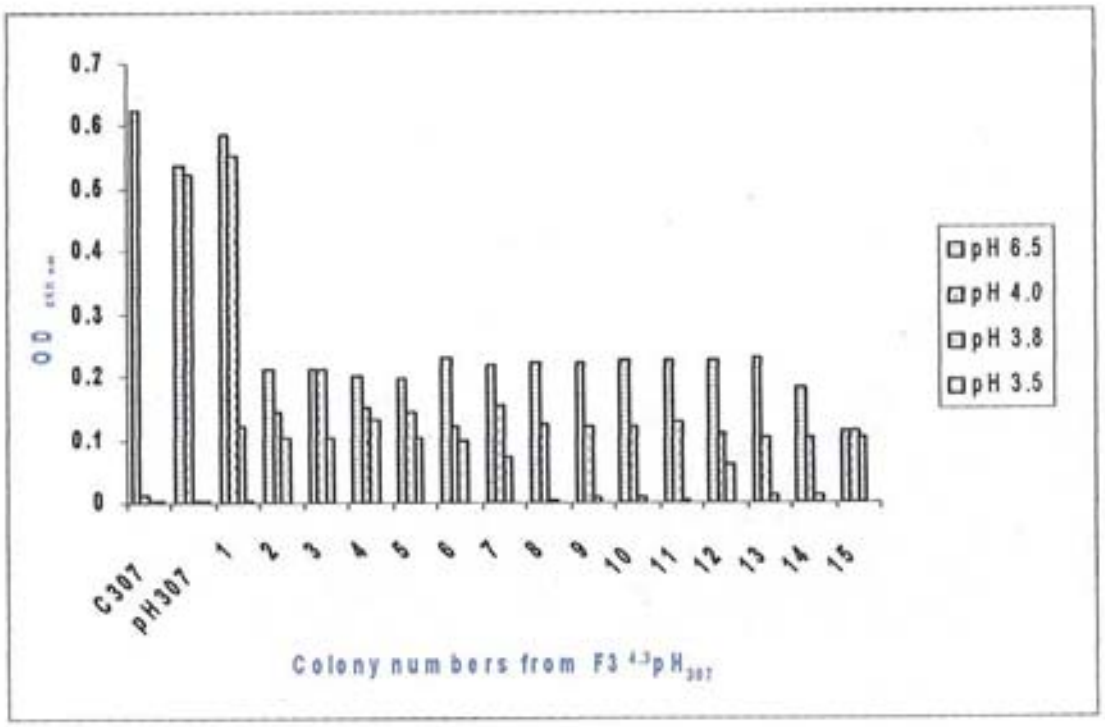


Fig 4.113: Screening of individual isolates from F3 $^{4.3}pH_{307}$ population grown at pH 3.8

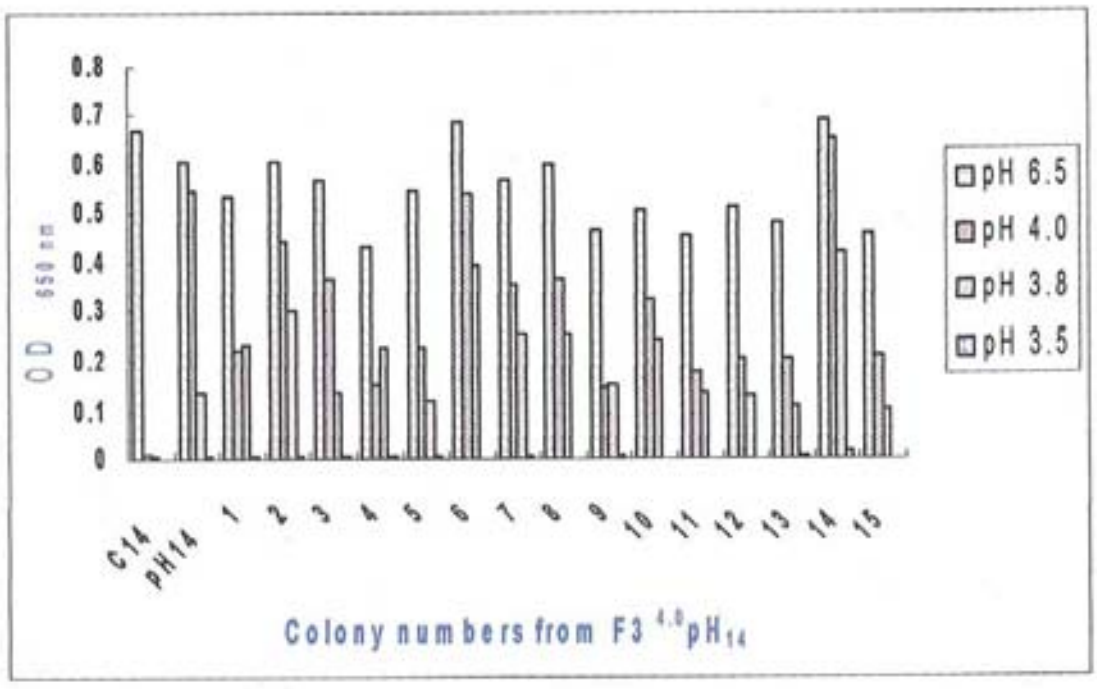


Fig 4.114: Screening of individual isolates from F3 $^{4.0}pH_{14}$ population grown at pH 3.8

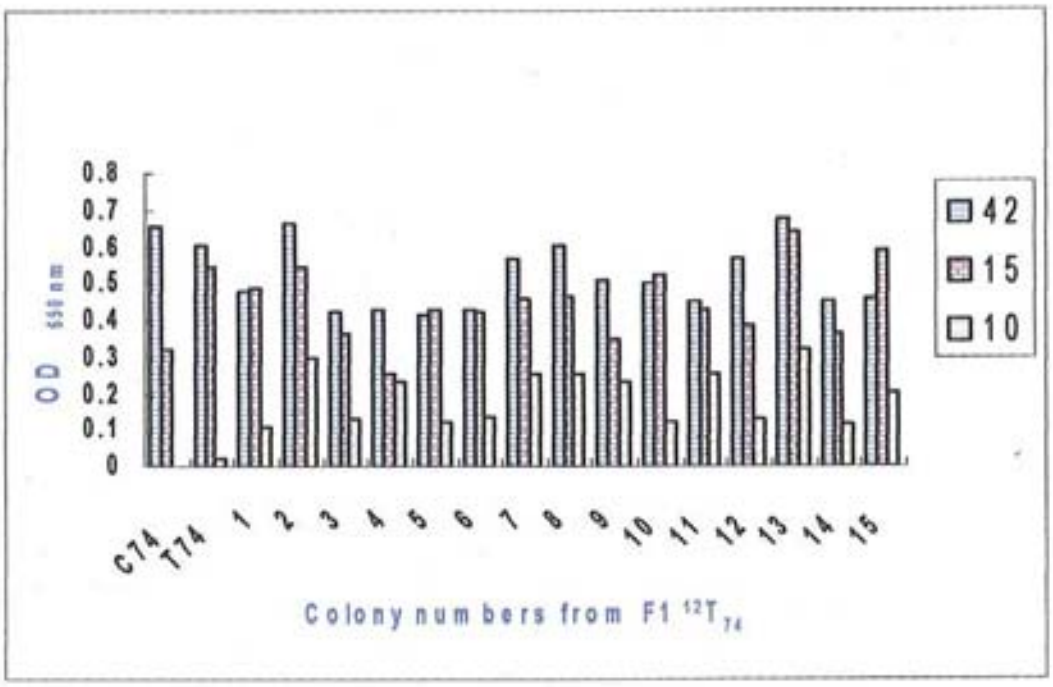


Fig 4.115: Screening of individual isolates from F1 $^{12}T_{74}$ population grown at 10 ^{0}C

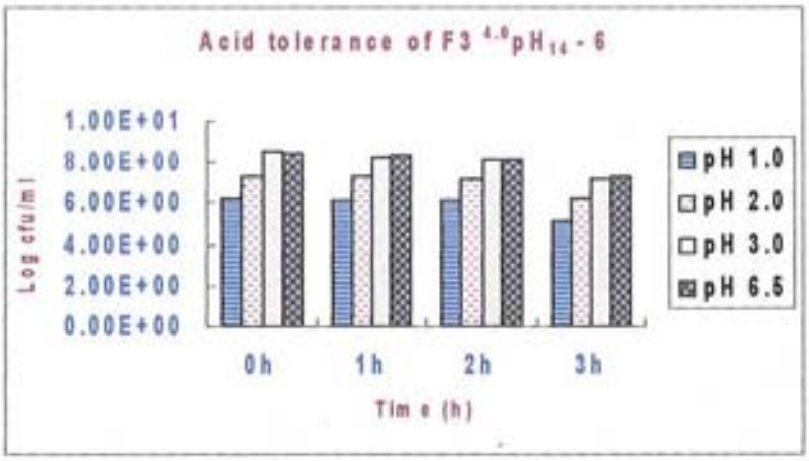


Fig 4.116: Acid tolerance of F3 $^{4.0}pH_{14}$ - 6

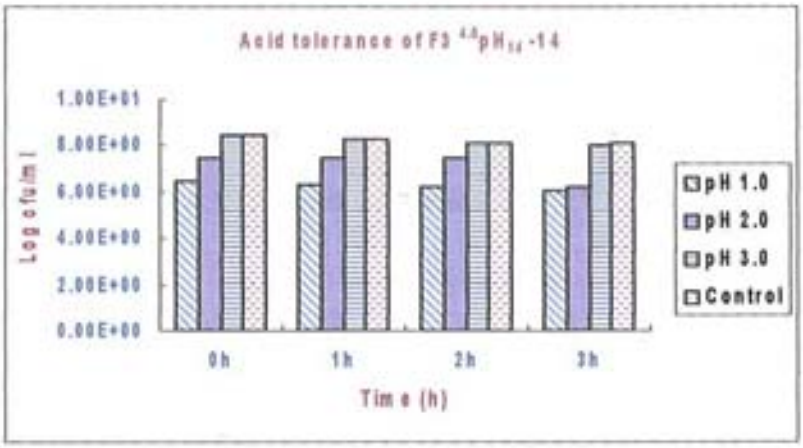


Fig 4.117: Acid tolerance of F3 $^{4.0}pH_{14}$ - 14

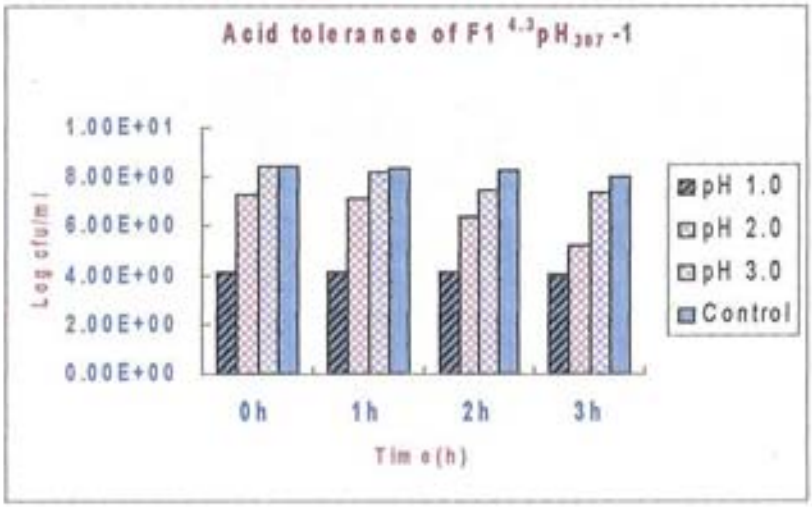


Fig 4.118: Acid tolerance of F1 $^{4.3}pH_{307}$ -1

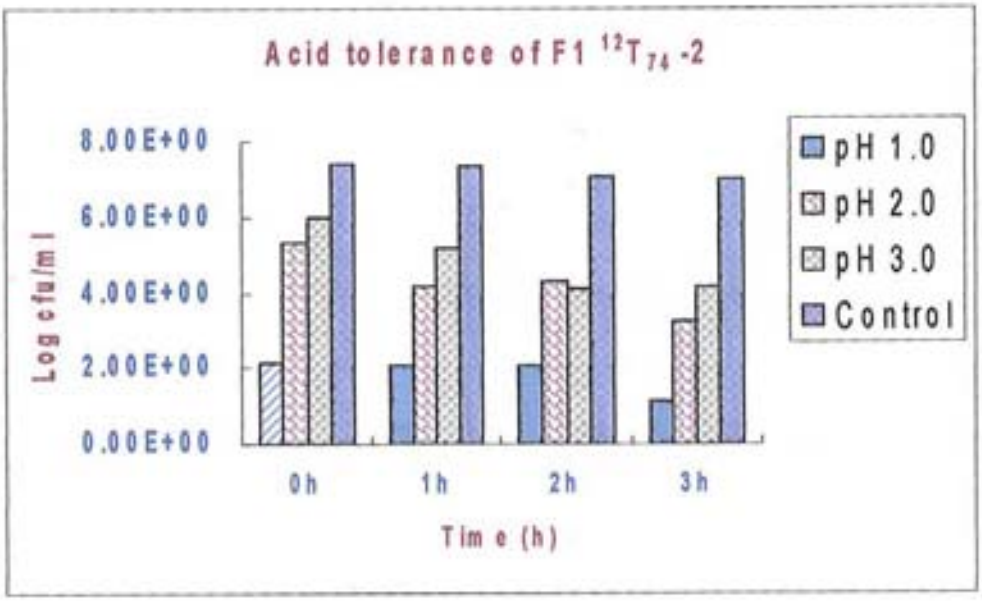


Fig 4.119: Acid tolerance of F1 $^{12}T_{74}$ -2

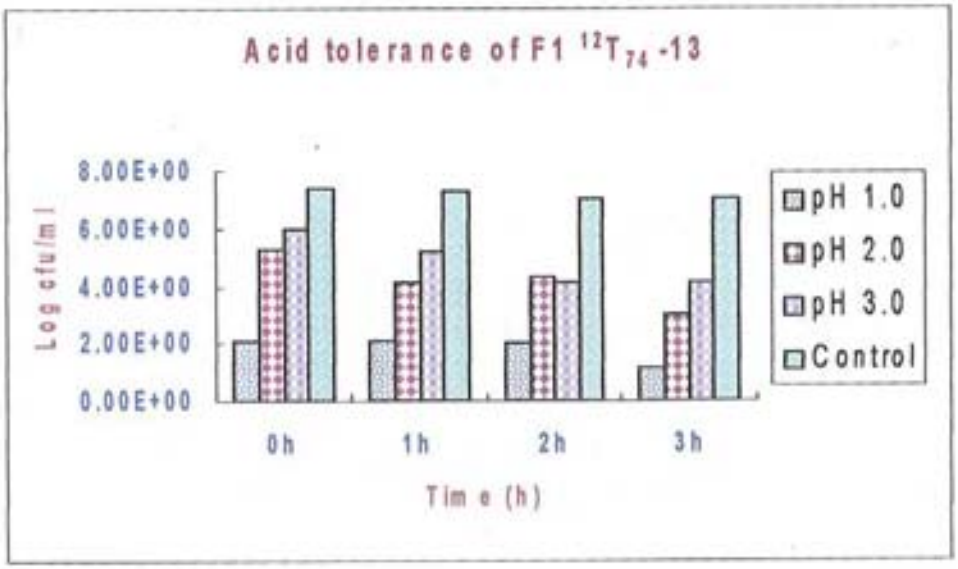


Fig 4.120: Acid tolerance of F1 $^{12}T_{74}$ -13

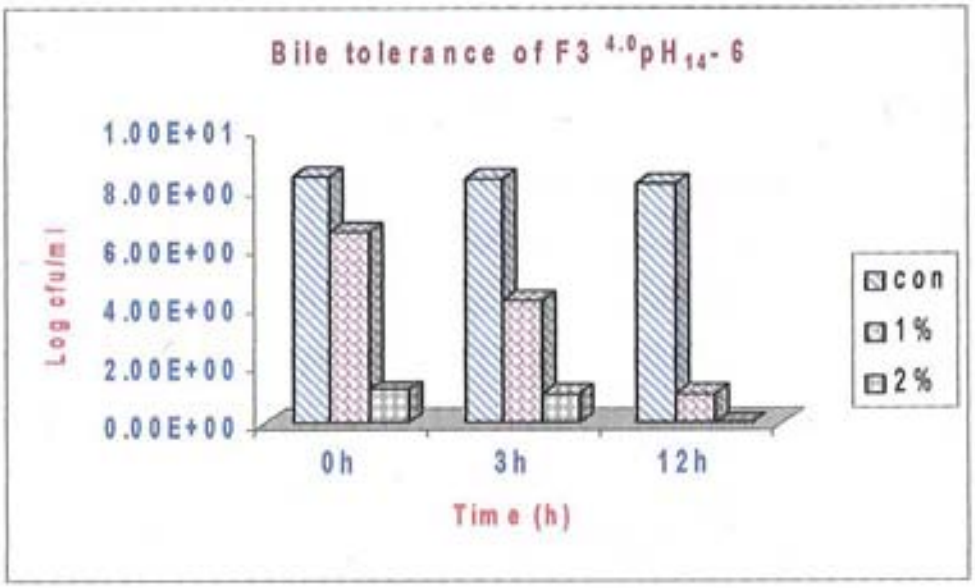


Fig 4.121: Bile tolerance of F3 $^{4.0}pH_{14}$ -6

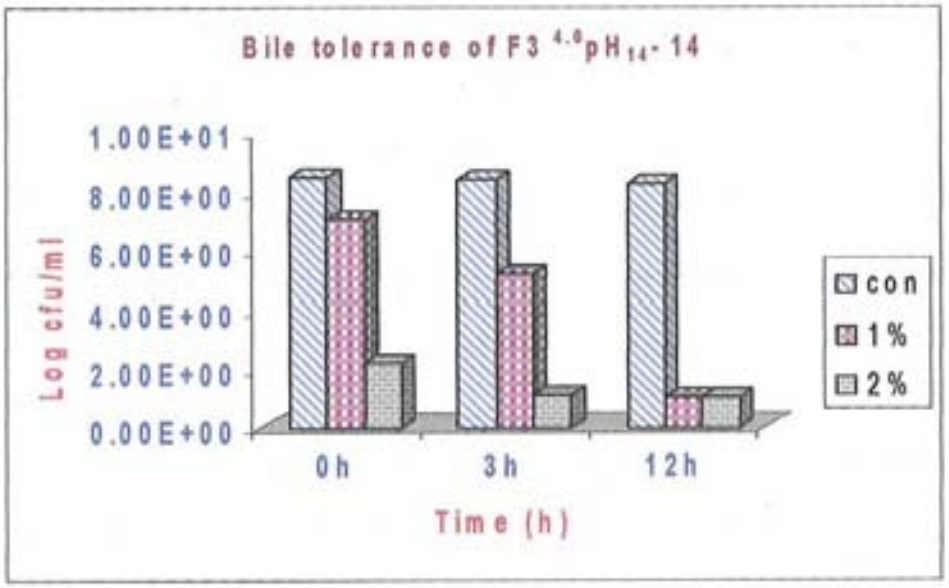


Fig 4.122: Bile tolerance of F3 $^{4.0}pH_{14}$ -14

But fusant F3 $^{4.0}pH_{14}$-6 tolerated 1 % bile salt up to 12 h whereas F3 $^{4.0}pH_{14}$-14 tolerated 1 and 2 % bile salt up to 12 h. Fig 4.123 indicated bile tolerance of F3 $^{4.3}pH_{307}$- 1. The adapts $^{4.3}pH_{307}$ tolerated 1 % bile salt up to 3h with log cfu of 2.01 whereas fuasnts tolerated 1 % salt up to 12 h. The adapts of *S. thermophilus* NCDC 74 didn't tolerate 1 % bile salt up to 1h whereas both the fuasnts able to tolerate 1 % up to 3 h (Fig 4.124 and Fig 4.125).

4.5.3.1.3.2 Cell surface hydrophobicity

Table 4.21 indicates cell surface hydrophobicity in adapts and fusants. 1% increase in cell surface hydrophobicity was occurred in fusants of *S. thermophilus* and *L. delbreueckii* subsp. *bulgaricus,* whereas ~ 9% increase was experientialed in F3 $^{4.0}pH_{14}$- 14.

Table 4.22: Cell surface hydrophobicity of fusants

Culture	Cell surface hydropobicity (% hydrophobicity)	SAT values
Lactobacillus acidophilus NCDC 14	11.50	1.50
$^{4.0}pH_{14}$ (Adapt)	14.31	1.50
F3 $^{4.0}pH_{14}$- 6 (Fusant)	14.4	1.2
F3 $^{4.0}pH_{14}$- 14 (Fusant)	15.82	1.70
L. delbrueckii subsp. *bulgaricus* NCDC 307	12.6	1
$^{4.3}pH_{307}$ (Adapt)	12.82	1.1
F1 $^{4.3}pH_{307}$-1 (Fusant)	12.95	1.2
S. thermophilus NCDC 74	4.56	0.7
$^{12}T_{74}$ (Adapt)	4.56	0.7
F1 $^{12}T_{74}$ -2 (Fusant)	4.61	0.78
F1 $^{12}T_{74}$ – 13 (Fusant)	4.6	0.76

4.5.3.1.3.3 Freeze thaw survivability

Fig 4.126 to Fig 4.128 represented freeze thaw survivabilty of fusants (F3 $^{4.0}pH_{14}$- 6, F3 $^{4.0}pH_{14}$- 14, F1 $^{4.3}pH_{307}$-1, F1 $^{12}T_{74}$ -2 and F1 $^{12}T_{74}$ – 13). About 40 fold F3 $^{4.0}pH_{14}$- 14 cells survived after fourth freeze thaw cycle, whereas ~20 fold survival was occurred after fourth cycle in F1$^{4.3}pH_{307}$-1.

About 200 fold survival was demonstrated in fusants of *S. thermophilus* NCDC 74 after fourth cycle.

Genome shuffling accelerates directed evolution by facilitating recombination between members of a diverse selected population (Zhang et al, 2002). Because a single strain was the starting point of the evolution program, an improved population for breeding was required. In the present study populations were generated by a straightforward stepwise adaptation method in both streptococci and lactobacilli. The parents and adapts were shuffled by means of pool wise recursive protoplast fusion as per the protocol of Patnaik *et al.*, 2002. The resultant fusants of *S. thermophilus* were able to grow at 10 ^{0}C whereas the fusants of lactobacilli were competent to grow and survive at pH as low as 3.5. From the abovementioned result all the fusants illustrated better growth, biochemical activity, probiotic attributes and freeze thaw survivabilty over their respective parents and adapts. The resultant fusants thus have the potentiality to survive extreme pH and temp, and thus prove to be promising commercially. However the commercial utility of the evolved strains will require further characterization under true process conditions. All the cultures were deposited at the National Collection of Dairy Cultures, NDRI, Karnal. Thus further shuffling and evolution of this population can be pursued to meet a commercial goal. The present investigation is in line with the Patnaik *et al.*, 2002, where they developed lactobacilli that grew at pH 3.8 and produced threefold more lactic acid than wild type cells at pH 4.0. Genome shuffling thus seems broadly useful for the rapid evolution of tolerance and other complex phenotypes in industrial microorganisms. The fusant F3 $^{4.0}pH_{14}$-14 that demonstrated best performance with regard to probiotic and biochemical activities was taken for application in a fermented product.

4.6 APPLICATION OF THE DEVELOPED STRAIN IN THE PRODUCT

4.6.1 Sensory Evaluation

Unless and until the improved/newly developed organisms is put to test for a product formation and industrial utilisation, the acceptability of the

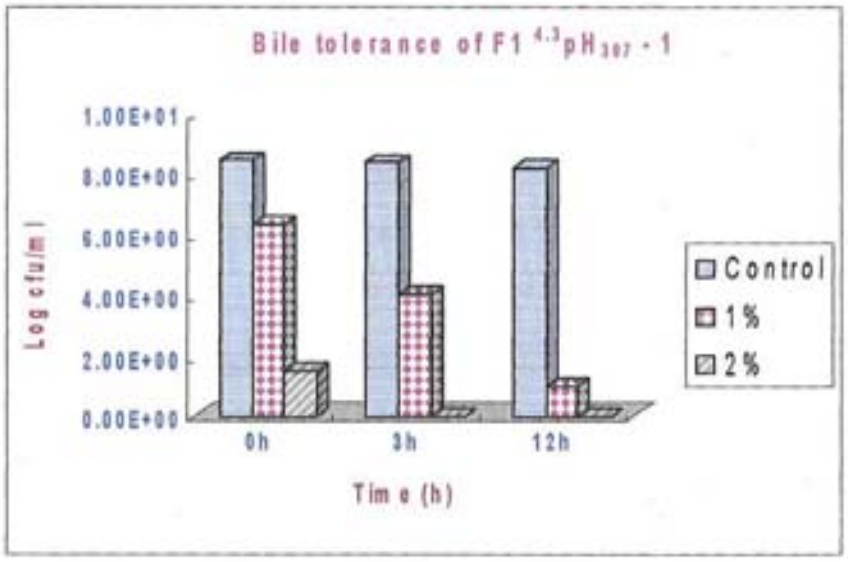


Fig 4.123: Bile tolerance of F1 $^{4.3}pH_{307}$ -1

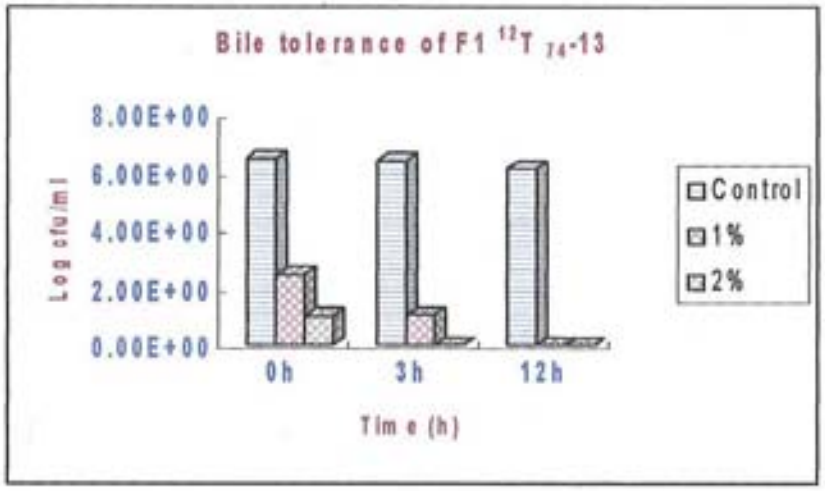


Fig 4.124: Bile tolerance of F1 $^{12}T_{74}$ -2

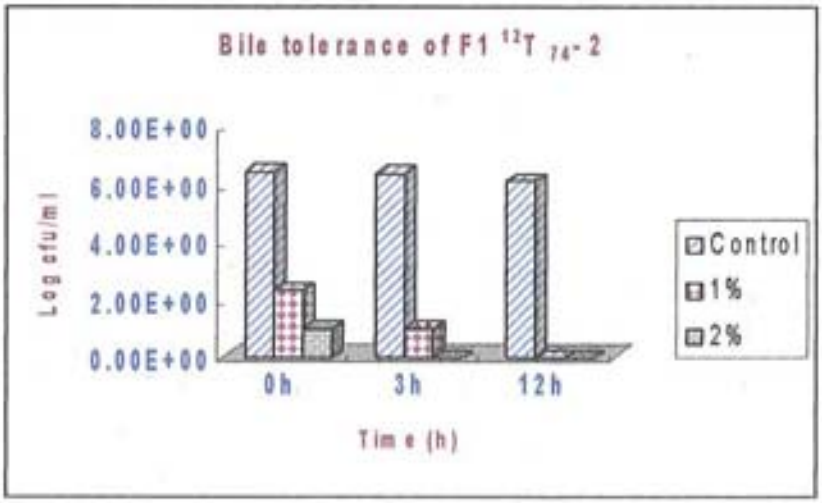


Fig 4.125: Bile tolerance of F1 $^{12}T_{74}$ -13

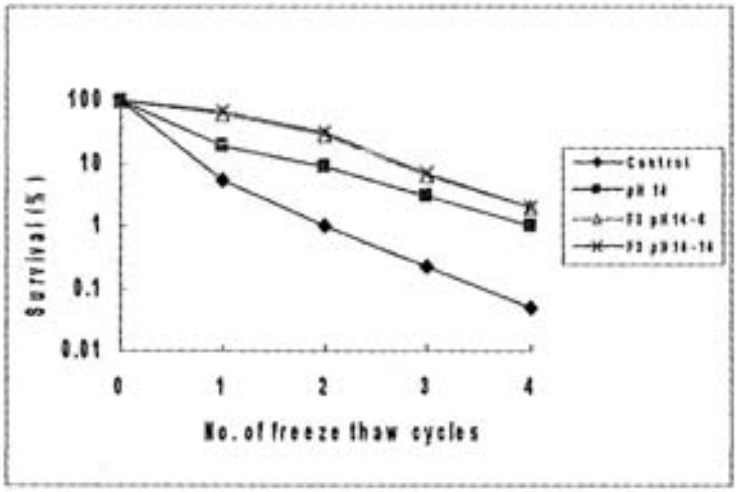


Fig 4.126: Freeze thaw survivability of C_{14}; $^{4.0}pH_{14}$; F3 $^{4.0}pH_{14}$-6 and F3 $^{4.0}pH_{14}$-14

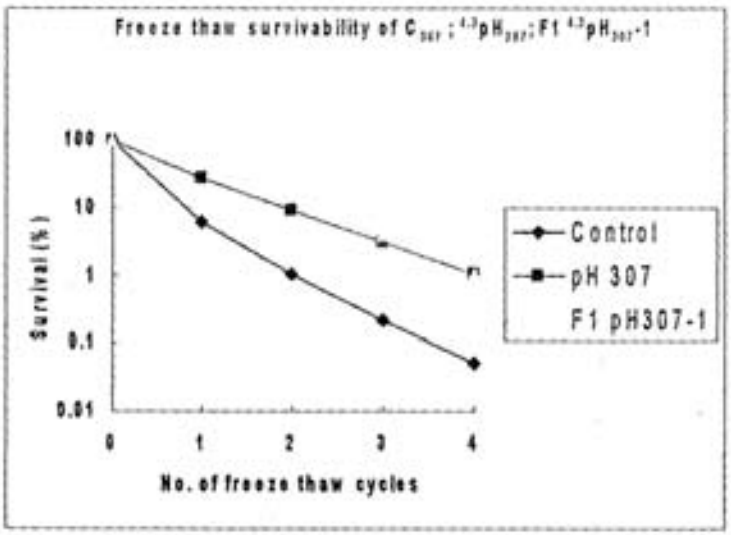


Fig 4.127: Freeze thaw survivability of C_{307}; $^{4.3}pH_{307}$; F1 $^{4.3}pH_{307}$-1

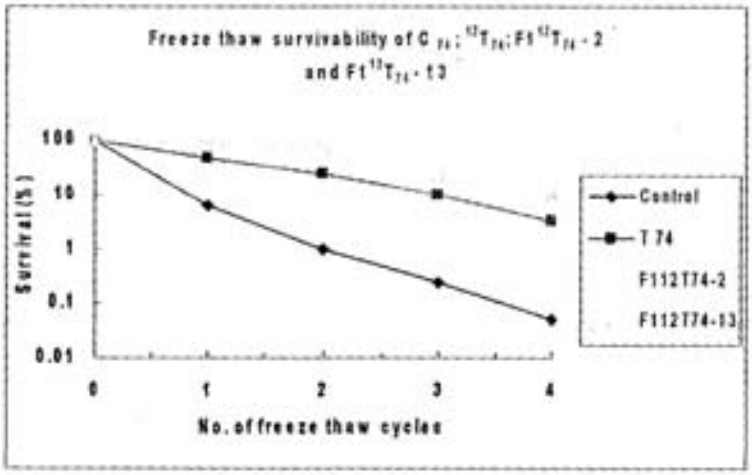


Fig 4.128: Freeze thaw survivability of C_{74}; $^{12}T_{74}$; F1 $^{12}T_{74}$-2 and F1 $^{12}T_{74}$-13

organism does not sustain and the effort in developing it becomes a wasteful exercise. It may then turn out to only be of academic interest. Among all the parents, adapts and their fusants belonging to *L. acidophilus* NCDC 14 proved to be the most promising candidates because of their biochemical and probiotic attributes. Yoghurt, an industrial milk product has the other two TLAB as authentic cultures for its preparation. It was therefore selected for fortification with LAB fusants to study any improvement in its characteristics and its subsequent industrial application. The fortified yoghurt product prepared as detailed in section 3.9 revealed encouraging responses by the evaluators. However, scores of sensory evaluation were significantly different between yoghurt prepared by Group III and Group IV and control ($P<0.05$) whereas no significant difference was obtained between Group I and Group II.

4.6.2 Storage Studies

One important property of probiotic cultures to be used as dietary adjuncts is that they must remain viable and active in the carrier food during storage until consumption (Gilliland, 1989; Hull *et al.*, 1992).

Consumption of *L. acidophilus* or *Bifidobacteria* at viable populations of $10^5 - 10^7$ cfu/ml is considered necessary to derive the probiotic benefit, and in some countries standards have been established to govern this minimum requirement (Ishibashi and Shimamura, 1993; Lee and Salminen, 1995). However, there are reports that the incorporated probiotic species do not survive well in yoghurts and other commercial preparations during retail storage (Hull *et al.*, 1984; Modler and Villa-Garcia, 1993; Shah *et al.*, 1995 Dave and Shah, 1997; Rybka and Fleet, 1997). At the time of sale their viable populations might not be sufficient to meet the criteria required to provide the reported health benefits. High levels of developed acidity, low pH values ($\leq$ 4.0), fluctuating storage temp, leakage of air through packaging material of the container and cultural incompatibility have been cited as some reasons, for the loss in viability of incorporated probiotic bacteria in yoghurts.

In the present study, changes in pH, titratable acidity, viable count, were observed during a 15 d period of refrigerated storage (4 - 7 ^{0}C).

4.6.2.1 Changes in the pH and titratable acidity of yoghurt and probiotic yoghurt during refrigerated storage

The values of pH and titratable acidity (% LA) during the storage period were given in the Table 4.23. During the period of storage the pH finally decreased below 4.0 in control yoghurt (Group I) whereas it was maintained much above pH 4.0 in all the other products. However, the pH remained above 4.6 throughout with adapts (Group III) and fusants (Group IV). The titratable acidity (% lactic acid) was increased from 0.69 to 0.91 per cent in control yoghurt whereas in Group III and Group IV. The titratable acidity was increased from 0.69 to 0.91 % LA in control yoghurt whereas in Group III and Group IV it was increased initially up to 10 d and tended to stabilse thereafter particularly in group III.

In one previous study, titratable acidity and pH of milk fermented with *L. acidophilus* and *Bifidobacterium* spp. showed little change during storage at 4 ^{0}C for 15 d (Hughes and Hoover, 1995). Viability of probiotic bacteria in yoghurt has been reported to decrease rapidly at pH values less than 4.6 units (Martin and Chou, 1992; Rybka and Fleet, 1997) during refrigerated storage. The present result were encouraging as during refrigerated storage for 15 d the Group III and Group IV samples pH remained at more than 4.6 units till the end.

Table 4.23: Effect of refrigerated storage on pH and acid development (in terms of %LA) in yoghurt fortified with *L. acidophilus* NCDC 14, its adapts and Fusants.

Storage period (d)	Group I		Group II		Group III		Group IV	
	pH	% LA	pH	% LA	pH	% LA	pH	% LA
0	4.59	0.69	4.68	0.68	5.33	0.65	5.31	0.62
5	4.38	0.76	4.65	0.75	4.93	0.71	4.92	0.67
10	4.12	0.83	4.53	o.76	4.83	0.79	4.87	0.69
15	3.97	0.91	4.11	0.83	4.81	0.79	4.82	0.71

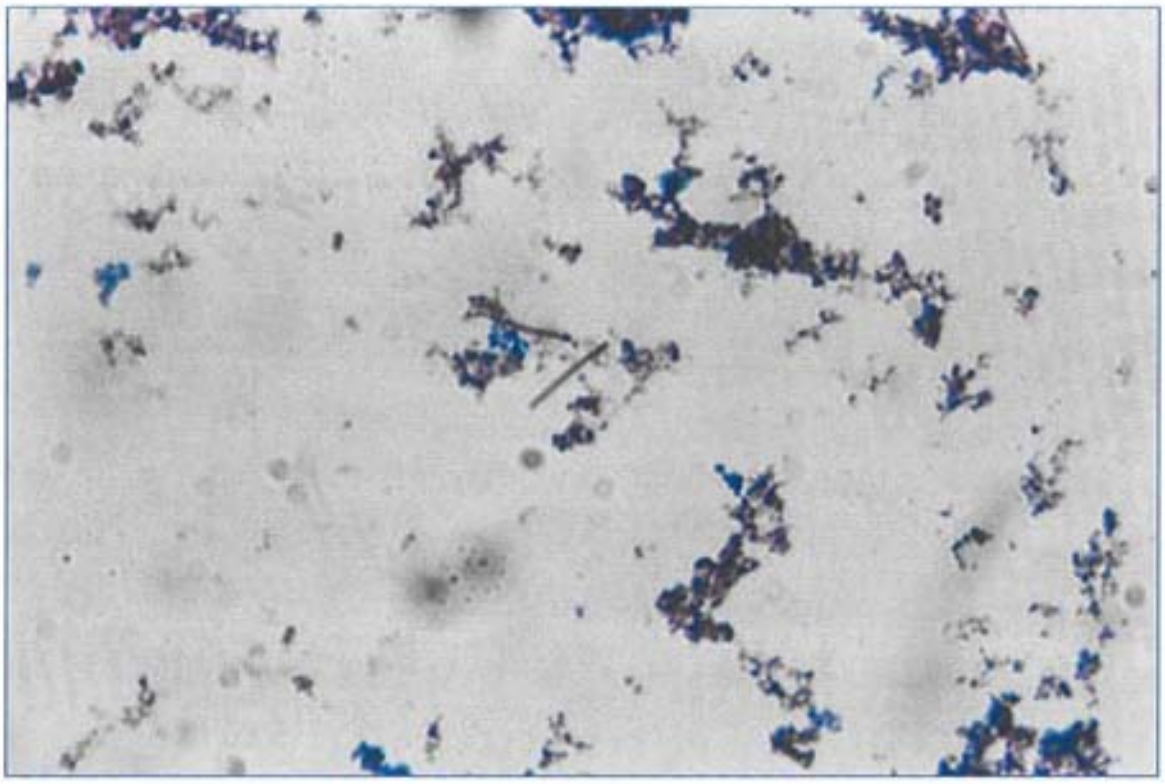


Plate 4.23: Yoghurt containing lactobacilli and streptococci (x1000)

4.6.2.2 Viability of the yoghurt bacteria during storage

4.6.2.2.1 Normal yoghurt organisms

Streptococcus thermophilus and *L. delbrueckii* subsp. *bulgaricus* cell counts increased slightly from initial 8.23 to 8.78 and 9.02 to 9.58 log cfu/ml respectively during the first 5 d of storage (Fig 4.129), in GP II. The number of viable cells fell marginally to 7.28 log cfu/g in case of *L. bulgaricus* and 7.30 in case of *S. thermophilus*.

A similar trend in viable numbers was seen in case of control yoghurt. However, a slight increase in viable count was observed in Group III and Group IV. This might have been due to the occurrence of prevalent levels of titratable acidity and pH at the end of 15 d in these groups of product (Fig 4.129 and Fig 4.130).

4.6.2.2.2 *L. acidophilus organism supplemented as probiotic adjunct*

Freshly prepared yoghurt contained 7.35 – 7.4 log cfu/g of *L. acidophilus*. The viable counts of C_{14} decreased from 7.38 to 6.92 log cfu/ g during 5 d of storage. Viable number of this organism fell further by two log cycles to 4.78 log cfu/ g by the 10 d of refrigerated storage, after which the cfu count/g decreased slowly to 4.51 during the last phase of storage. However, the viable count of acid adapted strain $^{4.0}pH_{14}$ only marginally decreased from 7.35 log cfu/ g to 7.1 log cfu/ g during first five d of storage and then reduced to one log cycle after 10 d (6.58) and remained at 5.9 after 15 d of storage. Peculiarly enough the rate of fall in counts of adapts after 10 d was faster than the other two groups but having better overall survivabilty than the parent. The viable populations of the shuffled strain (the Fusants, Group IV) remained at the level of 6.91 log cfu/g from 7.4 log cfu/ g indicating the highest survivability (Table 4.24). From the result it was inferred that the survivability of the adapted strains during long term storage period were superior as compared with the control strain. The result was in consistent with Shah *et al.* (1995) where they observed the survivabilty of acid adapted cells of *Lactobacillus acidophilus* in yoghurt for 5 weeks. In a study De Angelis *et al.* (2001)

reported higher acidification rate and survivabilty of acid-tolerant mutant and adapted strains of *L. sanfranciscensis* CB1 during sour dough fermentation.

Table 4.24: Viable counts of *L. acidophilus* (C_{14}), adapts ($^{4.0}pH_{14}$) and fusants (F3 $^{4.0}pH_{14}$ -14) as adjunct in yoghurt during refrigerated storage

	Log viable cell counts of *L. acidophilus*		
Storage, d	C_{14}	$^{4.0}pH_{14}$	F3 $^{4.0}pH_{14}$ -14
0 d	7.38	7.35	7.4
5 d	6.92	7.1	7.21
10 d	4.78	6.58	6.95
15 d	4.51	5.9	6.91

In the present study shuffled strain of *L. acidophilus* NCDC 14 was used for application purpose along with the adapts and parent cells. Genome shuffling proved to be straightforward to implement and well suited for industrial applications, where time is of essence. For one, it now seems possible to obtain strains that would be able to function satisfactorily under unconventional environmental conditions, such as high/low temp., low pH, or high osmotic pressures, thus freeing fermentation conditions from some rather onerous constraints. The shuffled strains developed in the present work was found to be promising to meet industrial challenges. These strains have the capability to remain viable up to desired level without affecting the post-processing acidification and the pH of the product. Thus, the products retain their ability to deliver sufficient viable numbers of probiotic bacteria up to 15 d of refrigerated storage without affecting post processing acidification. This study also confirmed that the methods used here for the strain development could be serve as a model for developing other industrial probiotic strains so as the final product reaching to the consumer contained sufficient number of viable probiotic organisms.

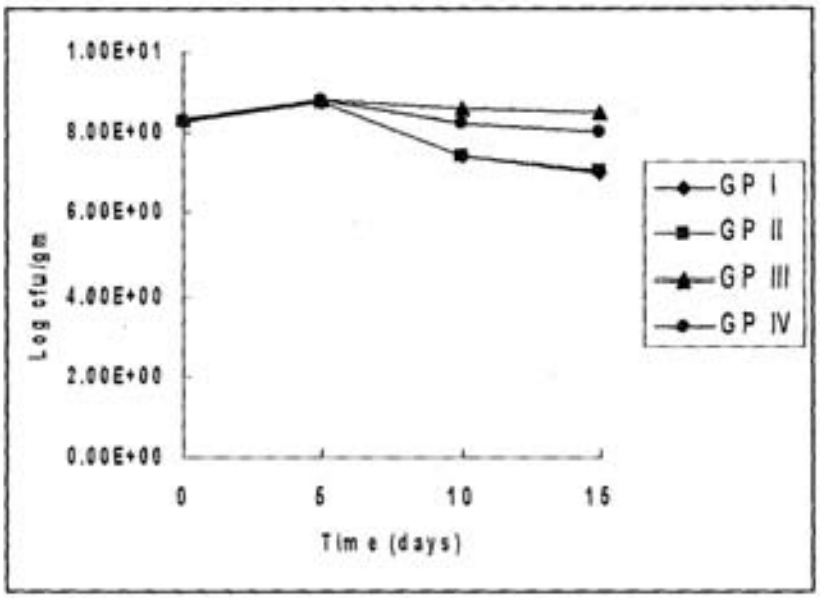


Fig 4.129: Viability of the lactobacilli in all groups of yoghurt during refrigerated storage

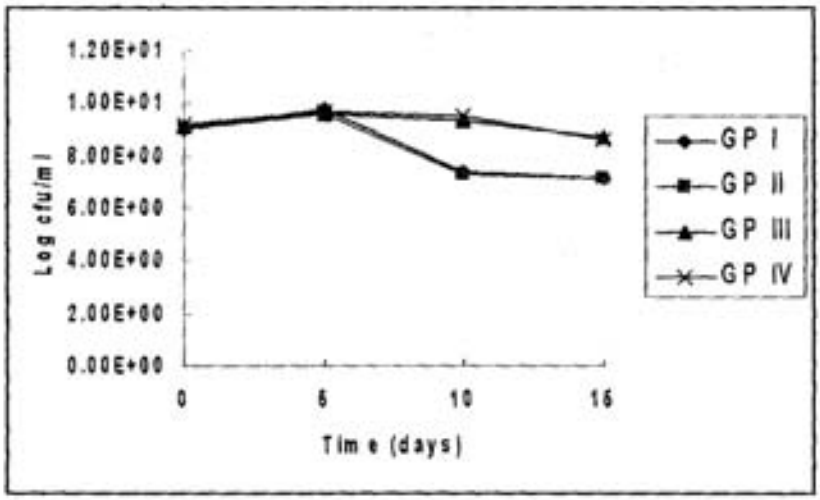


Fig 4.130: Viability of the *S. thermophilus* in all groups of yoghurt during refrigerated storage

5.0 SUMMARY AND CONCLUSIONS

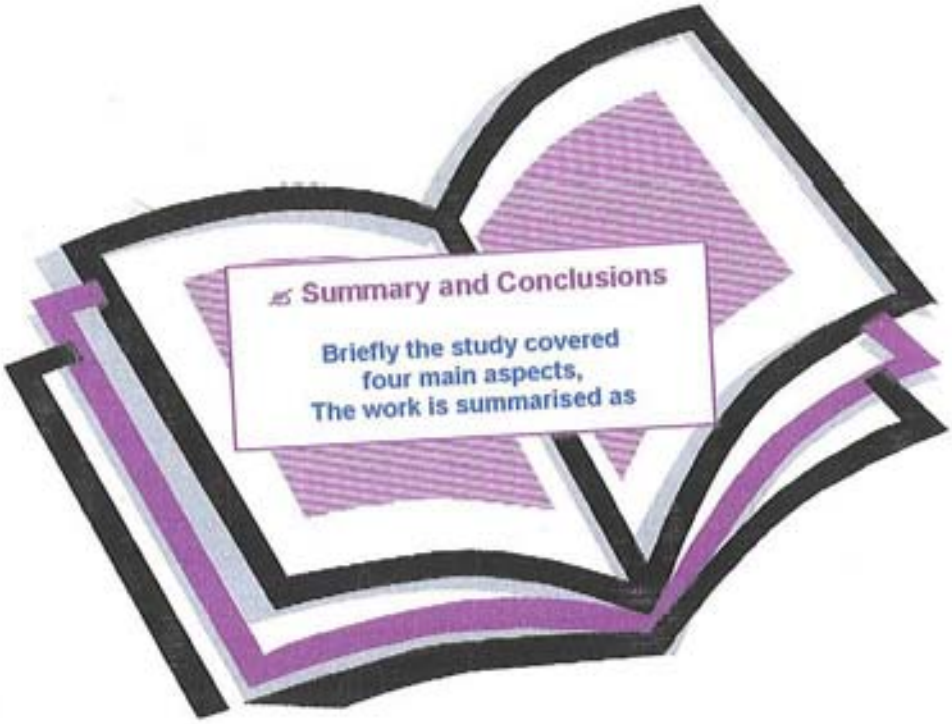
Lactic acid bacteria experience various environmental stresses during industrial fermentations for milk products formation. Mesophilic lactic acid bacteria have been extensively studied for such stress responses but TLAB lack much behind. To make up the gap, develop stronger biotechnological means and their evaluation the present study was undertaken. The TLAB available with NCDC, DM division, NDRI, Karnal were extensively studied for this purpose. The study was spread over four aspects: growth analysis for selecting the promising strains; strain improvement by adaptation and protoplast fusion; biochemical activities, probiotic attributes, freeze thaw survivability, plasmid and proteomic analysis of the improved strains and finally application of one of the most potential strains as adjunct in yoghurt. The findings of the present study are summarised as follows:

1. A total of 36 thermophilic lactic cultures comprising 14 strains of *Streptococcus thermophilus,* 15 of *L. delbrueckii* subsp. *bulgaricus*, and 7 of *L. acidophilus* were screened for growth at different pH (3.5, 4.0, 4.5, 5.0 and 6.5) at 42 ^{0}C and temp (10, 15, 20, 25 and 42 ^{0}C) at pH 6.5.

2. For all the ten atmospheric conditions studied and the 36 cultures under test, a total of 360 growth curves were obtained from the resultant OD (optical density) and total viable cell counts (Log cfu/ml).

3. All the strains of *L. acidophilus* grew well at pH 6.5 and pH 5.0, showed a longer lag time at pH 4.5, but no apparent growth occurred at pH 4.0 and pH 3.5. *L. acidophilus* NCDC 14 showed higher growth rate (μ_{max}/h) at pH 4.5, 5.0 and 6.5 with values 0.047, 0.71 and 0.72, respectively.

4. All the strains of *L. delbrueckii* subsp. *bulgaricus* grew well at pH 5.0 and 6.5. Strains NCDC 307 and NCDC 308 grew even at pH 4.5 but with a longer lag time ~ 9h in the former and ~ 15 h in the latter. *L.*



Summary and Conclusions

delbrueckii subsp. *bulgaricus* NCDC 307 showed a better overall growth rate (μ_{max}/h) of 0.05, 0.80, and 0.63 at pH 4.5, 5.0 and 6.5 respectively.

5. All the strains of *S. thermophilus* demonstrated good growth at pH 5.0 and pH 6.5. However, their growth rate was less as compared to that of the lactobacilli. None survived at pH 4.5, 4.0 and 3.5.

6. In an attempt for selection of a suitable culture for cold stress studies *S. thermophilus* NCDC 74 was found to show the highest growth rate (μ_{max}/h) i.e. 0.008, 0.26, 0.53 at 20 ^{0}C, 25 ^{0}C and 42 ^{0}C, respectively.

7. Thus based upon growth analysis three cultures i.e. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 307, *Lactobacillus acidophilus* NCDC 14, for pH/acid stress studies and *S. thermophilus* NCDC 74 for low-temp/cold stress studies were selected.

8. After exposure for 30 min to medium at initial pH 4.0 and 4.5 the log phase cells of *L. acidophilus* NCDC 14 and *L. delbrueckii* subsp. *bulgaricus* NCDC 307, respectively showed their potential acid tolerance response (ATR). This implies that these strains have the capacity to adapt at low pH. Chloramphenicol treatment adversely affected the cells grown in high pH more than those grown in low pH.

9. Growth rate (μ_{max}/h) of *S. thermophilus* NCDC 74 significantly decreased at low temps (from 0.5 to 0.008 at 20 ^{0}C). This strain was able to adapt relatively quickly (within 2 to 4 h) when shifted from 42 to 20 ^{0}C. This indicates that this strain has the capacity to efficiently adapt to low temp.

10. Mutation and adaptation were tried for development of potential stress tolerant cells, Mutation attempts by both UV-irradiation and NTG were not successful whereas gradual/stepwise adaptation yielded the desired improved adapts/strains.

11. Acid-tolerant adapts were developed by gradual decrease of initial medium reaction from pH 5.0 to pH 4.2 for *L. delbrueckii* subsp. *bulgaricus* NCDC 307 and from pH 4.5 to pH 3.9 in *L. acidophilus*

NCDC 14. The survivors of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 were not stable (repeatable growth) at pH 4.2, but stable at pH 4.3. The survivors of *L. acidophilus* NCDC 14 were not stable at pH 3.9, but were stable at pH 4.0.

12. *S. thermophilus* NCDC 74 was adapted at low temp by gradually decreasing the temp from 20 ^{0}C to 10 ^{0}C. The culture was not found to be stable at 10 ^{0}C, but was stable at 12 ^{0}C. These adapts of all the three TLAB were maintained at freezing temp for further studies.

13. The adapts were christened as $^{4.0}\mathbf{pH_{14}}$, $^{4.3}\mathbf{pH_{307}}$ and $^{12}\mathbf{T_{74}}$ for further studies.

14. Gross cell morphology by light and scanning electron microscopy confirmed the reduction in rod size and clumping of cocci in the adapts.

15. No change in plasmid profiles of the parents was observed on adaptation.

16. Glycine SDS-PAGE protein analysis showed increased or decreased band intensity in *L. acidophilus* NCDC 14, *L. delbrueckii* subsp. *bulgaricus* NCDC 307 and their adapts ($^{4.0}pH_{14}$ and $^{4.3}pH_{307}$). Densitometric analysis indicated that there was more production of proteins in $^{4.0}pH_{14}$ from 10 kDa to 85 kDa under acid stress conditions whereas beyond this limit these were down regulated.

17. Similarly the adapt ($^{12}T_{74}$) of *S. thermophilus* gave more intensed but same bands with glycine SDS-PAGE protein profile.

18. Densitometric analysis of Tricine SDS-PAGE demonstrated more protein production in molecular weight range of 10 kDa to 150 kDa in *S. thermophilus* under cold stress indicating their essentiality. The low MW proteins were expressed during 6 h at 20 ^{0}C. Some protein bands might have clubbed which however could be separated by high resolution 2D-PAGE.

19. By 2D gel electrophoresis a total of 36 spots of repressed, induced and new proteins were differentiated. Among these 18 spots appeared to

be most important and were subjected to identification by peptide mass finger printing (PMF).

20. A total of 14 spots could be differentiated between control and cells exposed to 20 ^{0}C for 6 h. **Spot 1** in **6 h** gel appeared as a **new** protein (~12 kDa, pI ~ 4.1) whereas spot 3 appeared to be highly induced cold shock protein of molecular weight ~ 8 kDa, pI 4.1. PMF analysis revealed that **spot 1** matched with cold shock protein A **(CSP A)** of *Lactobacillus casei* with a highest probability based Mowse score of 164 ($P<0.05$).

21. Among the induced spots, 6 were characterized to be small (7 to 10 kDa). Spot No. 3 was highly induced followed by spot no 2 and 7. PMF analysis revealed that **spot 3** protein matched with major cold shock protein **(CSP** fragment of *Listeria grayii* (Mowse score: 48).

22. PMF analysis of control, 2 h, 4 h and 6 h 2D gels confirmed the presence of induced, repressed and new proteins in *S. thermophilus* NCDC 74.

23. General stress proteins e.g. HSP60, oxidative stress proteins like Cu/Zn containing superoxide dismutase, dna A etc were induced. But proteins like ATP-dependent protease, ATP binding chain (C1pB), Hsds), methyl-accepting chemo taxis protein t1pA were repressed.

24. All the adapts exhibited 0.1 % (lactobacilli) to 1.5 % (streptococci) increase in lactic acid production. An increase in total proteolytic activity was also observed in case of the adapts (15 % in $^{4.3}pH_{307}$; 23 % in $^{4.0}pH_{14}$ and 20 % in $^{12}T_{74}$).

25. The adapted cells ($^{12}T_{74}$) of *S. thermophilus* NCDC 74 showed a higher survival (65 fold) over the parent cells after four freeze thaw cycles. The adapts of *L. delbrueckii* subsp. *bulgaricus* NCDC 307 ($^{4.3}pH_{307}$) and adapts of *L. acidophilus* NCDC 14 ($^{4.0}pH_{14}$) showed 20 fold survivability over their parent cells.

26. With respect to their probiotic attributes the adapts of *L. acidophilus* NCDC 14 ($^{4.0}pH_{14}$) followed the same pattern as that of their parents at

pH 6.5 but at lower pH they resisted the deleterious effect of acid. The survivability of adapts at pH 1 was much more than that of the parents.

27. Acid tolerance results indicated that the *S. thermophilus* adapts were less resistant as compared to lactobacilli. In adapts ($^{12}T_{74}$), at pH 2, two log reduction of cells were occurred, whereas in parents only 3 log reduction was observed.

28. Adapts of lactobacilli showed a better resistance to bile salt compared to their parents. However, cold adapts of *S. thermophilus* did not show any demonstrable difference with regard to bile tolerance.

29. No demonstrable difference between all the parents and their adapts in cell surface hydrphobicity and antimicrobiocity was observed.

30. Recursive protoplast fusion technique for genome shuffling was used to develop improved breeds of the three TLAB. Among the F1 fusants (parents x adapts) of the three TLAB, the F1 $^{4.0}pH_{14}$ (i.e. C_{14} x $^{4.0}pH_{14}$) showed maximum growth, probiotic and biochemical attributes and was used for further strain improvement. Fusion between F1, C_{14} and $^{4.0}pH_{14}$ gave the second generation fusants F2 $^{4.0}pH_{14}$ and further **F3 $^{4.0}pH_{14}$** were obtained by fusion of F1 and F2 protoplasts.

31. Fusants from colony number **F3 $^{4.0}pH_{14}$ – 14** exhibited the best probiotic and biochemical characteristics among all the TLAB, their adapts and fusants.

32. Based on similar parameters of performance C_{14}, $^{4.0}pH_{14}$ and F3 $^{4.0}pH_{14}$ – 14 were used as adjuncts in yoghurt otherwise prepared normally. Its sensory evaluation scores were significantly ($p<0.05$) more than that from normal/control yoghurt (without adjunct). Their scoring rate was compared as follows: control yoghurt< adjunct = C_{14}< $<^{4.0}pH_{14}$ < F3 $^{4.0}pH_{14}$ – 14 ($p<0.05$).

33. During 15 d period of refrigerated storage the control yoghurt pH decreased to below pH 4.0 but it remained above pH 4.0 in all other formulations. However, the reaction remained above pH 4.6 in products co-inoculated with $^{4.0}pH_{14}$ adapt and fusant F3 $^{4.0}pH_{14}$ – 14.

34. Viable numbers of C_{14} decreased from 7.38 log cfu/g to 4.78 log cfu/ g by the 10 d of storage, after which these stabilized at around 4.51 log cfu/ g during the last phase of storage. In comparison the log viable count/g of acid adapted strain $^{4.0}pH_{14}$ decreased from 7.35 to 6.8 only during first five d to 6.58 in 10 d and remained at 5.9 after 15 d of storage. The viable populations of the genomically shuffled strain F3 $^{4.0}pH_{14}$ – 14 decreased insignificantly to 6.91 log cfu/g from 7.4 log cfu/ g after 15 d of storage.

CONCLUSIONS

Ever since the advent of knowledge about preservation, conservation and value addition of food by microbial fermentation, man has endeavoured to enhance the efficiency of the useful organisms. Among various techniques, their genetic improvement has now become most fascinating. Milk being the most basic and nutritious food for any mammal has attracted the maximum attention. The fermenting organisms in general and the lactic acid bacteria (LAB) in particular experience lot of stresses during their growth and activity in various food fermentations which may even terminate their activity. With respect to their stress response the mesophilic LAB have very widely been studied but the thermophiles (TLAB) somehow have remained in the background.

The present study has been a moderate attempt to characterise the stress response of TLAB with respect to high acid i.e. low pH and low temperature, their phylogenetic improvement by adaptation and genome shuffling technique and their applicability in biotechnologically better fermented product formation. From among 36 TLAB strains procured from NCDC unit of DM division, NDRI, Karnal three strains were selected based on their growth at different temperature and pH. While lactobacilli endured higher acidity and streptococci endured lower temperature than their established growth sustaining limits *L. delbrueckii* subsp. *bulgaricus* NCDC 307, *L. acidophilus* NCDC 14 and *S. thermophilus* NCDC 74 proved promising. Their good acid tolerance and cold shock tolerance responses implied that these strains had capacity to adapt to high acid and low temperature. This could also be concluded from the fact that chloramphenicol adversely affected their *de novo* protein synthesis but the adapts would maintain themselves in respective stress conditions. Failure to get mutants by NTG and UV treatment indicated the ineffectiveness of the particular doses used, or, may be, even

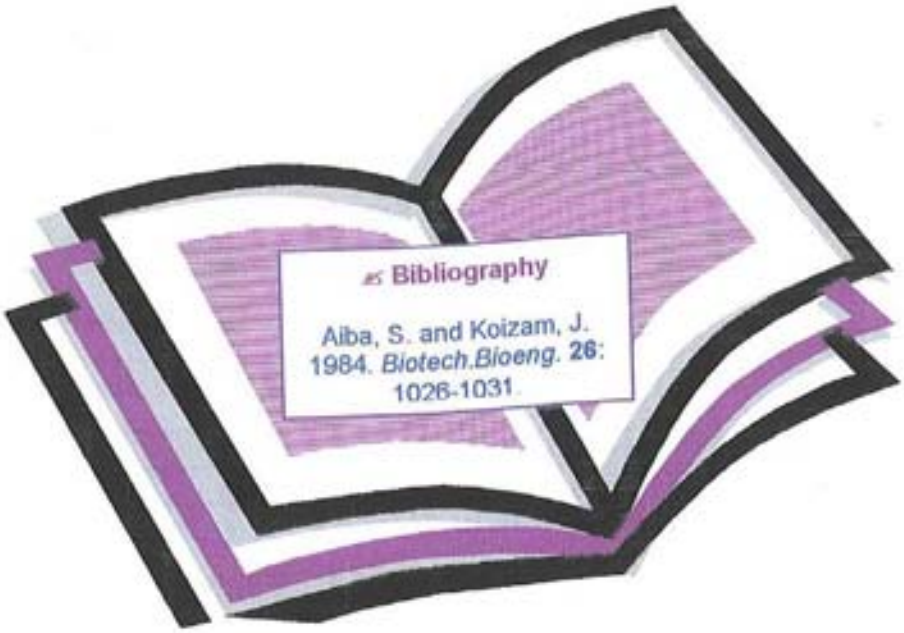
this approach in the present case. Another conventional approach – adaptation - both a bit abruptly or much slowly and gradually was successful, particularly the latter one and stable high acid and low temp adapts could be obtained those could also easily survive bile and a pH of 1.0. This finding also gets strength from the fact that microorganisms can adapt to any conditions on long and sustained exposures to such conditions. As may be normally expected the cells made to grow under such extreme conditions got some alterations in their gross morphology and, of course, in their physiology. However, this change was not detrimental to their life. Hence, such studies as to improve overall capability of useful organisms should be conducted in the interest of man kind. The general lack of plasmids in these TLAB indicated that the sought for fermentative activity was chromatid controlled and as such the transfer of these traits through plasmids was not possible. However, in such cases cloning of the controlling genes is a suggested method for transformation of other target organisms lacking the traits. The protein profiles amply illustrated that the adaptation protects the organisms from lethal stress and that *de novo* synthesis/activation of certain stress related proteins may also be required and occur during the slow adaptation processes as they have protective role for the producing organisms. The intra strain genome shuffling increased the survivability and fermentative activity of the fusants under stress conditions indicates that the transfer of such genes as express these protective proteins was conveniently possible.

The findings of the present study provide thus comprehensive information regarding the stress response studies in thermophilic lactic acid bacteria. The improved acid adapted strains can be used as probiotic adjuncts. The method developed can serve as a reference model for strain improvement of other potential microorganisms. Stress response studies also play a significant role in quality assurance, as these developed strains survive and remain metabolically active under harsh conditions, implying that they can inhibit the growth of pathogenic organisms. The fusants developed in this study have the potentiality to survive at extreme pH and temp and thus show

commercial promise. However the commercial utility of the evolved strains will require further characterization under true process conditions.

The specific study on cold shock response in *S. thermophilus* provides evidence for its active low temp adaptation. The presence of various CSP and induction of general stress proteins was also confirmed in this organism. Further identification of CIPs and their connection with CSPs is still required for a better understanding of the low temp adaptation in thermophilic lactic acid bacteria.

The present investigation thus convincingly shows that the research regarding stress responses in thermophilic starters is attaining new and excting dimensions. The wealth of genomic and proteomic information gathered in the study will not only aid our understanding of the molecular biology and physiology of TLAB but also facilitate selection of such appropriate starters by the food and fermentation industry. Future research needs to focus on proteomic, genomic and transcriptomic linkages in these organisms to improve the knowledge regarding the proper metabolic engineering of such organisms. Mutagenesis, recombination and selection efforts might further contribute to a next generation of thermophilic lactic acid bacterial starters possessing enhanced fermentation capability.



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Appendices

A. MEDIA AND REAGENTS

1) MRS Broth/Agar (deMan, Rogosa and Sharp, 1960)

Ingredients		*g/ l*
Peptone	:	10.00
Beef extract	:	10.00
Yeast extract	:	5.00
D-glucose	:	20.00
Sodium acetate	:	5.00
Tri-ammonium Citrate:		2.00
K_2HPO_4	:	1.00
$MgSO_4.7H_2O$	:	0.20
Tween 80	:	1 ml

All the above ingredients except D-glucose were dissolved in 800 ml dist. water, final pH was adjusted to 6.5 to 6.6. The broth was filtered through a muslin cloth followed by sterilization in an autoclave at 121 ^{0}C (1 atm or 1.1 kg per sq cm) for 20 min. D-glucose was either autoclaved in 200 ml dist water or filter sterilized (0.45 μm membrane filter) separately and added aseptically. Whenever agar was required 20 g agar powder was added to the broth and autoclaved.

2) M17 Broth/ Agar (Terzaghi and Sandine, 1975)

Ingredients		*g/ l*
Disodium β-Glycerophosphate	:	19.00
Polypeptone	:	5.00
Beef extract	:	5.00
Soya peptone	:	0.5
Yeast extract	:	2.5
L.-ascorbic acid	:	0.5
$MgSO_4$	:	0.25
pH of the media was adjusted to	:	6.1 ± 0.2

Lactose was added separately after sterilization. The procedure was same as mentioned above for D-glucose in MRS broth. Unless otherwise

stated, the lactose conc was 5 g/l. Whenever agar was required 20 g agar powder was added to the broth and autoclaved

3) Sterilized Skim Milk

Commercial skim milk powder obtained from the Experimental Dairy Unit, NDRI was reconstituted at the rate of 10 per cent (w/v) in lukewarm dist water. This was distributed in 10 ml aliquots in 20 ml test tubes and sterilized by autoclaving at 121 ^{0}C (1 atm or 1.1 kg per sq cm) for 20 min.

Azolitmin (0.75 g) was dissolved in 10 ml dist water. The pH was adjusted to 7.0 by adding 10 per cent Na_2CO_3 sol. The resulting sol was added to skim milk or RSM. Added a pinch of chalk powder ($CaCO_3$) into each tube and distributed the above treated skim milk into them. Sterilization was done at 121 ^{0}C (1 atm. or 1.1 kg per sq cm) for 20 min. After sterilization the colour of the tube became colorless. Then all the tubes were put in 37 ^{0}C for 24 h. After 24 h the tubes regained the colour of the indicator and thus were ready for use.

B. PREPARATION OF REAGENTS FOR SEM

1. Cacodylate buffer, 0.1 M

To prepare 0.1M cacodylate buffer, 1.6 g of sodium cacodylate (Fluka Chemical Co.) was dissolved in 100 ml of glass dist. water. The pH was adjusted to 7.2 with 1N NaOH using a pH meter (water proof pH scan BNC microprocessor based pocket pH meter; Eutech Instruments, Singapore).

2. Buffered Osmic acid Solution, 2 %

The surface of the 1 g (Commercial Osmium tetroxide, Sigma Chemical Co., USA) vial was thoroughly cleaned with soap and dist water to remove any organic matter present on glass surface. The glass vial was cracked in the middle into two halves using iron file under a hood. The content was transferred with no loss of time to a clean amber coloured glass stoppered bottle containing 25 ml of deionised water. The stopper of bottle was well tightened and stored at low temp in dark place. All the precautions were taken not to come in direct contact with the sol or the fumes. The final conc of 2 %

OsO_4 was prepared by mixing equal vol of stock sol and 0.1M cacodylate buffer adjusted to pH 7.2.

3. Buffered Glutaraldehyde fixative solution, 2.5%

(Sabitini *et al.*, 1963)

25% Glutaraldehyde, EM grade (Polysciences)	: 2.5 ml
0.1M Cacodylate Buffer (pH 7.2)	: 12.5 ml
Dist water	: 10.00 ml
Total vol	: 25.00 ml

4 . Dehydration agents

For the purpose of dehydration, a graded series of ethanol-water mixture of 30, 50, 70, 90 percent alcohol was prepared. Acetone or absolute alcohol was used for further dehydration.

C. REAGENTS FOR PLASMID ISOLATION

1. Solution I (pH 8.0)

Ingredients	***g/100 ml***
Sucrose	25
25 mM Tris-HCl	0.301
10 mM EDTA	0.372

2. Solution II (Freshly prepared)

Ingredients	***g/100 ml***
0.2 N NaOH	0.8
3 % SDS	3.0

Working sol of SDS (3 %) was prepared fresh from a stock sol of 10 % SDS.

3. Solution III (pH 4.8)

Ingredients	***g/100 ml***
3M sodium acetate	20.4

Dissolved chemicals in 50 ml milli Q water, pH was adjusted with glacial acetic acid, vol was adjusted to 100 ml in volumetric flask.

4. Solution IV

Ingredients

7.5 M ammonium acetate	57.75
Ethidium bromide	50 mg
10 mM EDTA	0.372

5. **Chloroform: Iso-amyl alcohol mixture (24:1)**

6. **Tris-EDTA Buffer (pH 8.0):**

Ingredients	*g/100 ml*
100 mM Tris	0.1211
1M EDTA	0.0372

All sol were made with double dist water and filtered. All the above reagents except sol II, sol IV and chloroform: isoamyl alcohol were autoclaved at 121 ^{0}C, 1 atm or 1.1 kg per sq cm for 20 min.

D. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

1. Tris acetate-EDTA (TAE) buffer (pH 8.0) (50X)

Ingredients	*g/100 ml*
Tris base	24.2
Glacial acetic acid	5.71 ml
0.5 M EDTA (pH 8.0)	10.00 ml

Working sol (1X): It was prepared by diluting 10 ml of 50X TAE to 500 ml with double dist water.

2. Tracking Dye (6X)

0.05% bromophenol Blue	0.05 ml
66% glycerol (w/v)	47.60 ml
0.5 M EDTA (w/v)	10.00 ml

Staining solution: working sol of EtBr was prepared by adding one ml of stock sol of EtBr (0.5 mg/ml) to one l of double dist water to give a final conc of 0.5 µg/ml.

Agarose: Agarose (sigma) gel of 0.7 percent conc was prepared in appropriate vol of 1X TAE buffer (pH 8.0) in microwave oven or by dissolving the agarose in boiling water bath.

E. REAGENTS FOR PROTEIN ANALYSIS

1. 50 mM Phosphate Buffer

Ingredients	*g/100 ml*
Monosodium hydrogen orthophosphate ($NaHPO_4$)	0.69
Disodium hydrogen orthophosphate (Na_2HPO_4)	0.71

To disodium hydrogen orthophosphate sol, monosodium hydrogen orthophosphate was added till the pH of the sol was 7.0.

2. **Sonication Buffer**

Ingredients	*g/100 ml*
β – Mercaptoethanol (3 mM)	0.2
EDTA (0.3 mM)	0.0112
Tris-HCl (150mM)	2.364

Equal amounts of all the three ingredients were mixed. The pH was adjusted to 7.0 by adding 0.1N NaOH.

3. **Reagents for Bradford (1976) method**

a. 50 mM Phosphate Buffer (pH 7.0) : as above

b. Bovine Serum Albumin (BSA) Stock Sol

1g of BSA was dissolved in 100 ml of 50mM phosohate buffer (pH 7.0)

c. Bradford Reagent (Dye Reagent)

100 mg of Coomassie Brilliant Blue (CBB) G-250 in 50 ml of 95 % ethanol was added. To it 100 ml of 85 % (w/v) phosphoric acid was added. The vol was made upto 1l with double dist water.

F. REAGENTS FOR TRICINE SDS PAGE (Schagger and Jaggow, 1987)

Stock Solutions

a. **Stock acrylamide (T)/bis-acrylamide (C) (33% T / 3% C)**

16.005 g of acrylamide and 0.495 g of bis-acrylamide were dissolved in triple dist water and total vol made up to 50 ml. The sol was filtered through Whatman No. 42 filter paper and stored at 4 ^{0}C in a dark amber coloured bottle.

b. **3M Tris HCl, pH 8.45**

36.3 g of tris-base was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tris- base were dissolved. The pH was adjusted to 8.45 with 1N HCl. The final vol was made up to 100 ml with triple dist water and stored at 4 ^{0}C.

c. **1.36 M Tris HCl, pH 6.8**

16.456 g of Tris-base was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of

tris- base were dissolved. The pH was adjusted to 6.8 with 1N HCl. The final vol was made up to 100 ml with triple dist water and stored at 4 ^{0}C.

d. 1.5 M Tris HCl, pH 8.8

36.342 g of Tris-base was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tris- base were dissolved. The pH was adjusted to 8.8 with 1N HCl. The final vol was made up to 200 ml with triple dist water and stored at 4 ^{0}C.

e. 1M Tricine

17.918 g of Tricine was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tricine were dissolved. The final vol was made up to 100 ml with triple dist water and stored at 4 ^{0}C.

f. 10% SDS

10 g of SDS was dissolved in 100 ml of triple dist water.

g. Upper Tank (Cathode) Buffer

0.1 M Tris (13.33 ml of 1.5M Tris-HCl, pH 8.8)
0.1 M Tricine (20 ml of 1M Tricine)
0.1% SDS (2ml of 10% SDS)

The above mentioned chemicals were mixed in desire proportion from stock sol. The final pH of the resulting sol was around pH 8.25 and final vol was made up to 250 ml with dist water.

h. Lower tank Buffer, 0.2M Tris, pH 8.9

33.4 mi of 1.5M Tris HCl was mixed with 200 ml double dist water. The pH was adjusted to pH 8.9 with 1N HCl and the vol was made up to 250 ml.

i Sample Buffer (3X)

Tris-HCl (0.1M)	551 µl of 1.36M Tris HCl, pH 6.8
β – mercaptoethanol (2% v/v)	300 µl
Glycerol (20% w/v)	3 ml
Bromophenol Blue	0.0125 g
SDS (1%)	1.5 ml 10 % SDS

j. Sample Buffer (6X)

Tris-HCl (0.1M)	551 µl of 1.36M Tris HCl, pH 6.8
β – mercaptoethanol (4% v/v)	300 µl
Glycerol (40% w/v)	3 ml
Bromophenol Blue	0.0125 g
SDS (1%)	1.5 ml 10% SDS

k. Separating Gel (16.5% T, 3% C)

3M Tris, pH 8.45	1.667 ml
2 × T+C (33%T, 3% C)	2.5 ml
Glycerol	650 µl
5% APS (prepared fresh daily)	50 µl
TEMED	2.5 µl
Double dist. water	1.31 µl

l. Separating Gel (12.5% T, 3% C)

3M Tris, pH 8.45	1.97 ml
2 × T+C (33%T, 3% C)	1.893 ml
Glycerol	650 µl
5% APS (prepared fresh daily)	50 µl
TEMED	2.5 µl
Double dist. water	450 µl

m. Spacer Gel (9%T, 3%C), 1 ml

1.36 M Tris, pH 6.8	735 µl
2 × T+C (33%T, 3% C)	273 µl
5% APS (prepared fresh daily)	10 µl
TEMED	0.5 µl

n. Spacer gel (10%T, 3%C), 1ml

1.36 M Tris, pH 6.8	735 µl
2 × T+C (33%T, 3% C)	300 µl
5% APS (prepared fresh daily)	10 µl
TEMED	0.5 µl

o. Stacking Gel (4%)

1.36 M Tris, pH 6.8	1.47 µl
2 × T+C (33%T, 3% C)	242 µl
5% APS (prepared fresh daily)	1 µl
TEMED	267 µl

p. Fixation

After electrophoresis the gel was dipped in fixing sol. and kept at 60 ^{0}C for 20 min.

Fixative Sol. (100 ml)
Methanol – 50 ml
Glacial Acetic Acid – 10 ml
Water – 40 ml

q. Staining

At the end of fixation step, the gel was kept in staining sol. for 40-45 min.

Staining Sol (0.25%)
Fixative Sol – 100 ml
Coomassie Brilliant Blue G-250 or R-250 – 0.25g

r. Destaining Solution

After staining for 40 to 45 min, the gel was transferred to the destaining sol (10% glacial acetic acid) at room temp for 2 h with renewal of destaining sol after every 30 min. During destaining the gel was never swirled to avoid diffusion of protein bands.

G. GLYCINE SDS-PAGE (LAEMELLI, 1970)

a. Stock acrylamide (T)/bis-acrylamide (30%T/2.67%C)

29.2 g of acrylamide and 0.8 g of bis-acrylamide were dissolved in triple dist water and total vol was made up to 100 ml. The sol was filtered through Whatman No 42 filter paper and stored at 4 ^{0}C in a dark amber coloured bottle for not more than 30 days.

b. 3M Tris-HCl, pH 8.85

36.3 g of Tris-base was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tris- base were dissolved. The pH was adjusted to 8.85 with 1N HCl. The final vol was made up to 100 ml with triple dist. water and stored at 4 ^{0}C.

c. 0.5M Tris-HCl, pH 6.8

6 g of Tris-base was dissolved in 50 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tris-base were dissolved. The pH was adjusted to 6.8 with 1N HCl. The final vol was made up to 100 ml with triple dist water and stored at 4^{0}C.

d. 1.5M Tris-HCl, pH 8.8

36.342 g of Tris-base was dissolved in 80 ml of triple dist water. The reagent was mixed properly in a magnetic stirrer till all the molecules of tris- base were dissolved. The pH was adjusted to 8.8 with 1N HCl. The final vol was made up to 200 ml with triple dist water and stored at 4 ^{0}C.

e. 10% SDS

10 g of SDS was dissolved in 100 ml of triple dist. water.

f. 5X Electrode (Running) Buffer, pH 8.3

Tris base	45g	(15 g/l)
Glycine	216g	(72 g/l)
SDS	15g	(5 g/l)
		to 3 l with dist. water

The sol. was stored at 4 ^{0}C. Warmed to 37 ^{0}C before use when precipitation occurred. Diluted to 300 ml 5X stock with one, 200 ml dist water for one electrophoresis run in large format gel and two electrophoresis run in minigel. The pH was not adjusted with acid or base.

g. Stacking Gel (4%)

Dist. water	3 ml
30% Acrylamide	0.65 ml
10% SDS	50 µl
0.5M Tris-HCl, pH 6.8	1.25 ml
10% APS (prepared fresh daily)	25 µl
TEMED	5 µl

h. Separating Gel (15%)

Dist. water	1.15 ml
30% Acrylamide	2.5 ml
(Degassed for ≥ 15 min. at room temp.)	
10% SDS	50 µl
1.5M Tris-HCl, pH 8.8	1.25 ml
10% APS (prepared fresh daily)	50 µl
TEMED	10 µl

H. REAGENTS FOR MODIFIED LAEMELLI METHOD MADE IN THIS STUDY

a. Stacking Gel (7%)

Dist. water	1.5 ml
30 % Acrylamide	0.7ml
10 % SDS	40 µl
0.5M Tris HCl, pH 6.8	1 ml
10% APS (prepared fresh daily)	40 µl
TEMED	6 µl

b. Separating Gel (15%)

Dist. water	1.342 ml
30% Acrylamide (Degassed for ≥ 15 min at room temp.)	3 ml
10% SDS (prepared fresh)	60 µl
3M Tris HCl, pH 8.85	1.5 ml
10 % APS (prepared fresh daily)	45 µl
TEMED	6 µl

c. 1 X Electrode (Running) Buffer, pH 8.3

Tris base	3.03 g
Glycine	14.415 g
SDS	0.5 g
	to 500ml dist. water

The electrode buffer was prepared fresh each time.

Electrophoresis was carried out at 50 V (13 mA) for stacking gel and 70 to 80 V (18-20 mA) for separating gel. Water circulation was performed to prevent heating of the chamber.

I. REAGENTS FOR PROTEOLYTIC ACTIVITY (Hull, 1947; Lowry *et al.*, 1951)

(a) 2 % Na_2CO_3 in 0.1N NaOH

(b) 1% solution of sodium potassium tartarate

(c) 0.5% copper sulphate

Alkaline reagents was prepared by mixing 48 parts of reagent (a) and one part each of reagents (b) and (c) just before use.

(d) 12.5% or 0.72N Trichloroacetic acid (TCA)

(e) Folin-Ciocaltau reagent (1N)

J. MEDIA AND REAGENTS FOR PROTOPLAST FORMATION, REGENERATION AND FUSION

(a) Tris HCl buffer (pH 8.0)

Ingredients	*g/100 ml*
Tris HCl (30 mM)	0.4728
$MgCl_2$ (3 mM)	0.061
Sucrose (20%)	20.00

(b) THMS buffer pH 8.0 (For *S. thermophilus*)

Ingredients	*g/100 ml*
Tris-HCl (30mM)	0.4728
$MgCl_2$ (3 mM)	0.061
Sucrose (20%)	20.0

The buffer was filter sterilized using 0.2 µm nitrocellulose membrane filter (Millipore).

(c) Lactobacillus protoplasting buffer (LBP), pH 6.3

Tris-HCl (10 mM)	0.1576
$CaCl_2$ (20 mM)	0.222
Sucrose (0.5 M)	17.115

In both the buffers lysozyme (Sigma Chemical Co., USA) at a conc of 10 mg/ml and mutanolysin (Sigma Chemical Co., USA) at a conc of 30 µg/ml was added.

d. Protoplast Regeneration

Regeneration medium used for the lactobacilli consisted of MRS agar without Tween 80 and fortification with sucrose, gelatin, $MgCl_2$ and bovine serum albumin as follows.

Ingredients	*g/100 ml*
Peptone	1
Beef Extract	0.8
Yeast Extract	0.4
Dextrose	2
K_2HPO_4	0.2
Sodium Acetate, trihydrate	0.5

Trammonium citrate	0.2
$MnSO_4$	0.05
$MgSO_4$	0.02
Gelatin	2.5
BSA	0.5
$MgCl_2$ (20mM)	0.396
Sucrose(0.5M)	17.115
Agar	2

e. Regeneration medium for *Streptococci*

M17 agar fortified with osmotic stabilizers namely sucrose (0.5 M), gelatin (2.5%), BSA (0.5%), $MgCl_2$ (20 mM) was used for regeneration of *S. thermophilus* protoplasts. The composition is given below.

Ingredients	***g/100 ml***
Disodium β-glycerophosphate.	1.9
Polypeptone	0.5
Soyapeptone	0.05
Beef Extract	0.5
Yeast Extract	0.25
Ascorbic acid	0.05
Lactose	0.5
$MgSO_4$	0.025
$MgCl_2$	0.396
BSA	0.5
Gelatin	2.5
Sodium Acetate, trihydrate	0.5
Sucrose	17.115
Agar	2

K. REAGENT FOR ADHESION TO HEXADECANE

a. Phosphate urea magnesium sulphate (PUM) buffer

Ingredients	*g/100ml*
$K_2HPO_4.3H_2O$	22.2
KH_2PO_4	7.26
Urea	1.8
Magnesium sulphate	0.2
pH	7.1

L. REAGENTS FOR 2D PAGE

1. Sonication buffer

Ingredients	*g/100 ml*
Tris HCl (150 mM)	2.364 g
EDTA (1 mM)	0.112g
β-mercaptoethanol (1 mM)	0.2 ml
Phenylmethane sulphonyl fluoride PMSF) (1 mM)	0.0175 g
pH 7.0 adjusted with 0.1 N NaOH	

2. Rehydration buffer

Ingredients	*g/10 ml*
8M Urea	4.8 g
2% CHAPS	200mg
50 mM dithiothreitol (DTT)	77mg
Bromophenol blue	Traces
(Few particles just to give bluish tinge)	
Bio-Lytes (3-10) (0.2%)	20 mg

3. SDS-PAGE Equilibration buffer I (with DTT) - (For 50 ml sol)

Urea (8 M)	-	18 g
DTT (2 % W/V)	-	1 g
SDS (2 %)	-	1 g
1.5 M Tris HCl (pH 8.8)	-	12.5 ml
Glycerol (20%)	-	10 ml

4. SDS-PAGE Equilibration buffer II (with Iodoacetamide)

Iodoacetamide (2.5 % w/v)	-	1.25 g
SDS (2 %)	-	1 g
Urea (6 M)	-	18 g
1.5 M Tris HCl (0.375M) (pH 8.8)	-	12.5 ml
Glycerol (20% w/v)	-	10 ml

Annexure - II

SCORE CARD FOR SENSORY EVALUATION OF YOGHURT

Date:

Attribute	Sample	1	2	3	4
Flavour (50)	***Score***				
	Astringent				
	Bitter				
	Cheesy				
	Fermented				
	Foreign				
	High Acid				
	Lacks Acid (flat)				
	Lacks freshness				
	Starchy/Doughy				
	Unclean				
	Yeasty				
Consistency (25)	***Score***				
	Curdy				
	Gassy				
	Lumpy				
	Too Firm (over stabilized)				
	Too Thin (weak)				
	Wheyed off (Synersis)				
Texture (15)	**Score**				
	Hard				
	Gritty				
	Coarse				
	Sandy				
Colour and Appearance (10)	***Score***				
	Dryness				
	Dull (lacks gassy)				
	Lacks uniformity				
	Unnatural colour				
Total Score (100)	Score Per Sample				

Comments: _____

_____

_____

Signature:

Name :

Suggested Score(s) for a defective product

Attribute	Defect	Degree of Defect		
		Slight	Moderate	Pronounced
Flavour	Bitter	40-42	35-37	32-34
	Cheesy	40-42	35-37	32-34
	Flat	42-44	37-39	34-36
	Low Acid	40-42	37-39	34-36
	High Acid	40-42	37-39	34-36
	Yeasty	38-40	35-37	32-34
	Foreign	38-40	35-37	32-34
	Unclean Flavor	38-40	35-37	32-34
	Lacks freshness	40-42	37-39	34-36
	Starchy	40-42	37-39	34-36
	Lacks Fine Flavor	40-42	37-39	34-36
Consistency	Curdy	20-22	18-20	15-17
	Gassy	20-22	18-20	15-17
	Lumpy	19-21	17-18	14-16
	Ropy	19-21	17-18	14-16
	Thin	19-21	17-18	14-16
	Wheyed Off	19-21	17-18	14-16
	Gel Like	19-21	17-18	14-16
Texture	Hard	11-13	8-10	6-7
	Gritty	10-12	7-9	5-8
	Coarse	11-13	8-10	6-7
	Sandy	10-12	7-9	5-8
Colour & Appearance	Chalky/Dull	9	8	7
	Atypical Color	9	8	7
	Color Leaching	9	8	7
	Lumpy	9	8	7
	Wheyed Off (Synersis)	9	8	7
Sediment		4	3	2

ANNEXURE III

SPECIFICATIONS OF HITACHI S-405 A SCANNING ELECTRON MICROSCOPE

Resolution	70 Å	
Magnification range	30 150,000x	
Electron optical system	Filament	Pre-centered hairpin type filament
	Accelerating voltage	5, 15, 25 KV
	Lens system	3-lens system
	Objective lens aperture	Movable aperture (0.1, 0.2 mm dia. Openings)
Specimen stage	X-traverse	0 20 mm (continuous)
	Y-traverse	0 10 mm (continuous)
	Tilting angle	-20 +70° (continuous)
	Rotation angle	360° (continuous)
	Z-traverse	5, 15 mm (semi-fixed); working distance (W.D.)
	Specimen size	60 mm dia.x1 mm H (max) 15 mm dia.x10 mm H (max)
Display unit	CRT	(afterglow type 150 x 135 mm) x 1
	Scanning speed (Raster scan)	
	For viewing	0.06 sec (rapid scan) 0.5 sec 10 (30) sec
	For photographing	50 (150) sec Parenthesized figures indicate the scan speed with SIGNAL SELECTOR (X-RAY) depressed.
Scanning Mode	Slow scan	
	Photo scan	
	Reduced area rapid scan	
Image Mode	Focus monitor	
	Stigmator monitor	
	Dynamic focus	
Evacuating system	Type	Automatic valve control
	Vacuum gauge	Pirani gauge
	Ultimate vacuum	5×10^{-6} Torr
	Vacuum pump	DP 400 1/sec x 1, RP 160 1/ min x 1
	Evacuating time	About 2 min.

ANNEXURE-III

NANOMETER/ MICRON MARKER SCALE FOR HITACHI S-405 A. SEM

Magnification	Marker Position	Microns (μ)
X 100	30 x 4 NM	300
X150	20 x 4 NM	200
X 200	15 x 4 NM	150
X 300	10 x 4 NM	100
X 400	75 x 3 NM	75
X 500	60 x 3 NM	60
X 1, 000	30 x 3 NM	30
X 1, 500	20 x 3 NM	20
X 2, 000	15 x 3 NM	15
X 3, 000	10 x 3 NM	10
X 4, 000	75 x 2 NM	7.5
X 5, 000	60 x 2 NM	6.0
X 6, 000	50 x 2 NM	5.0
X 7, 500	40 x 2 NM	4.0
X 10, 000	30 x 2 NM	3.0
X 15, 000	20 x 2 NM	2.0
X 20 000	15 x 2 NM	1.5
X 30, 000	10 x 2 NM	1.0
X 40, 000	75 x 1 NM	0.75
X 50, 000	60 x 1 NM	0.60

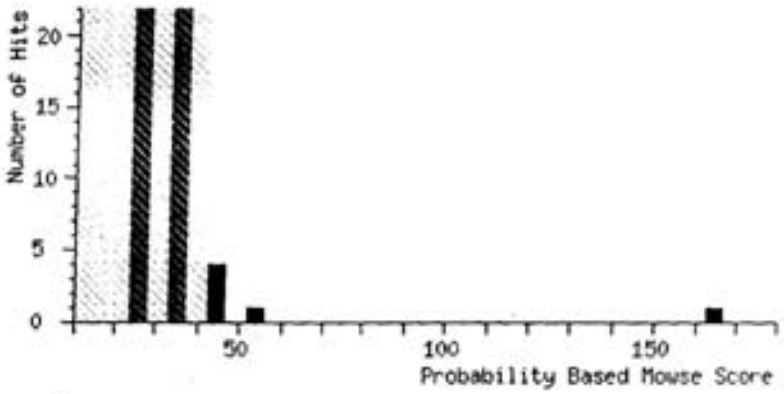
ANNEXURE-IV

{MATRIX} {SCIENCE} Mascot Search Results

User : reena
Email : arorareena12@yahoo.co.in
Search title :
MS data file : \\Chemstation01\Data (D)\Data\1'-6HRNDRIFINAL.mgf
Database : MSDB 20050227 (1942918 sequences; 629040812 residues
Taxonomy : Firmicutes (gram-positive bacteria) (170104 sequence
Timestamp : 21 Sep 2005 at 10:40:56 GMT
Significant hits: Q70LR3_LACCA Cold shock protein A.- Lactobacillus (
Q6MT35_MYCMS Holo-[acyl-carrier-protein] synthase (
Q5M1P4_STRTR Zinc transport transcriptional repress

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 42 indicate identity or extensive homology ($p<0.05$).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As | Peptide Summary

Significance threshold p< 0.05 Max. number of hits 20
Standard scoring ◉ MudPIT scoring ○ Ions score cut-off 0
Show pop-ups ◉ Suppress pop-ups ○ Sort unassigned Decreasi

Select All | Select None | Search Selected | ☐ Error tolerant

Archive Report

1. Q70LR3_LACCA Mass: 7198 Score: 164 Queries m
Cold shock protein A.- Lactobacillus casei.
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expec
116	390.19	778.37	779.36	-0.99	0	36	0.(
240	949.16	1896.31	1896.83	-0.53	0	(45)	0.01
179	633.11	1896.31	1896.83	-0.53	0	(38)	0.2
241	949.47	1896.93	1896.83	0.09	0	79	7.4e-00
227	921.92	2762.74	2763.29	-0.56	1	36	0.2
234	936.50	2806.48	2806.29	0.19	0	14	4

---

2. Q6MT35_MYCMS Mass: 12702 Score: 54 Queries m
Holo-[acyl-carrier-protein] synthase (EC 2.7.8.7).- M
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect I
210	738.10	1474.19	1473.75	0.44	0	54	0.0032

---

3. Q5M1P4_STRTR Mass: 17386 Score: 44 Queries m
Zinc transport transcriptional repressor.- Streptoco
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
116	390.19	778.37	778.35	0.01	0	44	0.099

---

4. Q8VT46_ENTFA Score: 41 Queries matched: 1
Putative ssb protein (Single-strand binding protein)
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
210	738.10	1474.19	1473.83	0.35	1	41	0.07

Proteins matching the same set of peptides:

Q9F1G6_ENTFA Score: 41 Queries matched: 1
Q82YK1_ENTFA Score: 41 Queries matched: 1

AAO80351 Score: 41 Queries matched: 1

---

5. E86802 Score: 41 Queries matched: 1
hypothetical protein pi348 [imported] - Lactococcus
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
210	738.10	1474.19	1473.83	0.35	1	41	0.07

---

6. Q72XQ2_BACC1 Mass: 26137 Score: 40 Queries m
Hypothetical protein.- Bacillus cereus (strain ATCC
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	971.58	0.57	1	40	0.12

---

7. AAK79568 Score: 39 Queries matched: 2
AE001437 NID: - Clostridium acetobutylicum ATCC 824
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	972.49	-0.34	0	31	0.98
219	769.11	1536.21	1534.79	1.42	1	12	90

Proteins matching the same set of peptides:
E97097 Score: 39 Queries matched: 2

---

8. Q6HRJ7_BACAN Mass: 225063 Score: 38 Queries m
Cell surface protein, gram positive anchor.- Bacillu
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
191	651.68	1301.35	1300.70	0.65	1	41	0.13

Proteins matching the same set of peptides:
Q816D8_BACCR Mass: 376330 Score: 38 Queries m
Cell surface protein.- Bacillus cereus (strain ATCC
AAP11800 Mass: 376530 Score: 38 Queries m

AE016877 NID: - Bacillus cereus ATCC 14579

Q6HBX5_BACHK Mass: 386099 Score: 37 Queries m
Hypothetical protein.- Bacillus thuringiensis (subsp.

Q632D5_BACCZ Mass: 386583 Score: 36 Queries m
Cell surface protein.- Bacillus cereus (strain ZK).

---

9. O69111_PAEMA Score: 37 Queries matched: 1
Uroporphyrinogen-III methyltransferase/uroporphyrinoc
☐ Check to include this hit in error tolerant search (

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	972.49	-0.34	0	37	0.23

---

10. Q9KW88_CLOBO Mass: 19110 Score: 37 Queries m
Botulinum neurotoxin type E (Fragment).- Clostridium
☐ Check to include this hit in error tolerant search (

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 217	752.68	1503.35	1502.80	0.55	0	37	0.16

---

11. Q6HMZ3_BACHK Score: 36 Queries matched: 1
Hypothetical protein.- Bacillus thuringiensis (subsp.
☐ Check to include this hit in error tolerant search (

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	972.52	-0.38	0	36	0.35

Proteins matching the same set of peptides:

Q63FI7_BACCZ	Score: 36	Queries matched: 1
Q81UN9_BACAN	Score: 36	Queries matched: 1
AAP24828	Score: 36	Queries matched: 1

---

12. Q8GKQ5_ENTFA Score: 36 Queries matched: 1
SfeI DNA methyltransferase.- Enterococcus faecalis (S
☐ Check to include this hit in error tolerant search (

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	971.57	0.58	0	36	0.35

Proteins matching the same set of peptides:
P71445_9LACT Score: 36 Queries matched: 1

---

13. Q8GQB7_LACSK Score: 36 Queries matched: 1
ORF-13.- Lactobacillus sakei.
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	971.58	0.57	1	36	0.35

---

14. Q62Y16_BACLD Score: 36 Queries matched: 1
Aldehyde dehydrogenase.- Bacillus licheniformis (str
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
151	487.08	972.15	971.54	0.61	0	36	0.35

---

15. S66016 Mass: 40272 Score: 35 Queries m
probable GTP-binding protein yyaF - Bacillus subtili
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
193	669.07	2004.19	2003.01	1.18	1	12	97
☑ 212	742.23	2223.67	2224.07	-0.40	1	24	6.2

---

16. Q6G6M2_STAAS Mass: 46204 Score: 35 Queries m
Putative glycine betaine/carnitine/choline transport
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 159	545.71	1089.41	1090.59	-1.19	1	35	0.32

Proteins matching the same set of peptides:
Q6GDY4_STAAR Mass: 46286 Score: 35 Queries m
Putative glycine betaine/carnitine/choline transport
Q8CN49_STAEP Mass: 47730 Score: 35 Queries m

Glycine betaine/carnitine/choline ABC transporter (A)
Q8CQB8_STAEP Mass: 44725 Score: 35 Queries m
Glycine betaine/carnitine/choline ABC transporter (A)
Q8NUY8_STAAW Mass: 46448 Score: 35 Queries m
Glycine betaine/carnitine/choline ABC transporter opu
AAO03819 Mass: 44925 Score: 35 Queries m
AE015929 NID: - Staphylococcus epidermidis ATCC 1222
AAO05659 Mass: 47930 Score: 35 Queries m
AE015929 NID: - Staphylococcus epidermidis ATCC 1222
A99947 Mass: 46462 Score: 35 Queries m
hypothetical protein opuCA [imported] - Staphylococc

---

17. Q8KST0_STRMU Score: 35 Queries matched: 1
Putative trehalose-6-phosphate hydrolase TreA.- Strep
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
159	545.71	1089.41	1089.58	-0.18	1	35	0.3

---

18. Q9A0K5_STRPY Score: 34 Queries matched: 2
Putative extracellular matrix binding protein.- Strep
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
186	636.84	1271.67	1270.75	0.92	1	14	6
191	651.68	1301.35	1301.65	-0.30	1	26	3.8

Proteins matching the same set of peptides:
AAK33683 Score: 34 Queries matched: 2

---

19. Q8KYN7_BACAN Score: 33 Queries matched: 1
Hypothetical protein BXA0091.- Bacillus anthracis st
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
116	390.19	778.37	779.35	-0.98	1	33	1

Proteins matching the same set of peptides:

Q74P48_BACC1 Score: 33 Queries matched: 1
A59099 Score: 33 Queries matched: 1

---

20. AAK80625 Mass: 78820 Score: 33 Queries m
AE001437 NID: - Clostridium acetobutylicum ATCC 824
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
107	340.40	678.79	679.37	-0.58	1	17	23
☑ 172	606.58	1816.72	1815.94	0.77	0	16	38

Proteins matching the same set of peptides:
F97229 Mass: 78620 Score: 33 Queries m
hypothetical protein CAC2678 [imported] - Clostridiur

Peptide matches not assigned to protein hits: (no details means no match)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Exp
☑ 103	655.17	654.16	652.35	1.81	0	32	0
☑ 247	737.39	2209.15	2208.10	1.05	0	32	0
☑ 149	483.33	1446.97	1446.66	0.31	0	32	
☑ 124	415.40	1243.18	1242.59	0.59	1	30	
☑ 221	800.66	1599.31	1597.80	1.50	1	30	0
☑ 141	435.72	869.43	870.53	-1.10	1	29	
☑ 129	421.56	841.11	841.54	-0.43	0	27	
☑ 166	583.17	1164.33	1163.62	0.71	1	26	
☑ 128	421.55	841.09	841.54	-0.45	0	25	
☑ 130	421.59	841.17	841.54	-0.37	0	25	
☑ 169	590.14	1178.27	1178.62	-0.36	1	24	
☑ 198	692.13	1382.25	1382.69	-0.44	0	24	
☑ 134	421.94	841.87	843.52	-1.65	1	24	
☑ 203	710.86	1419.71	1418.71	1.00	0	24	
☑ 114	375.19	748.37	746.45	1.91	0	24	
☑ 126	419.09	836.17	837.47	-1.31	0	24	
☑ 140	435.59	869.17	870.55	-1.39	1	23	
☑ 156	523.41	1044.81	1044.58	0.22	0	23	
☑ 138	434.84	867.67	867.55	0.11	0	23	
☑ 97	316.51	946.51	944.51	2.00	0	23	
☑ 154	517.25	1032.49	1032.59	-0.10	0	22	
☑ 214	744.22	1486.43	1487.70	-1.28	1	22	

☑	164	*579.40*	*1156.79*
☑	167	*583.58*	*1165.15*
☑	168	*583.79*	*1165.57*
☑	202	*703.47*	*1404.93*
☑	245	*989.05*	*1976.09*

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : Trypsin
Fixed modifications     : Carbamidomethyl (C)
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 2 Da
Fragment Mass Tolerance: ± 0.8 Da
Max Missed Cleavages    : 1
Instrument type         : ESI-TRAP
Number of queries       : 249
```

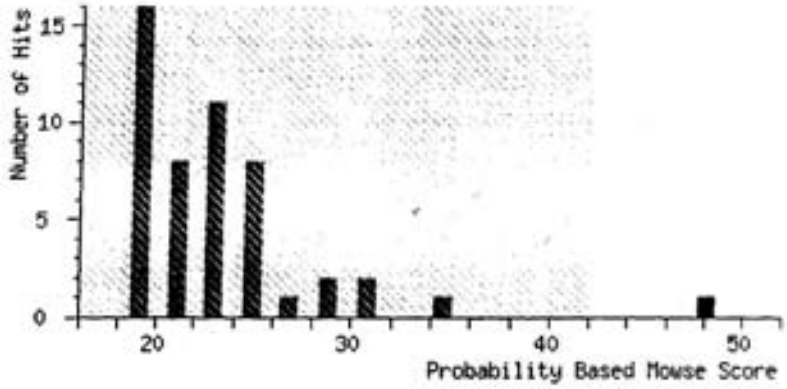
Mascot: http://www.matrixscience.com/

Mascot Search Results

User : reena
Email : arorareena12@yahoo.co.in
Search title :
MS data file : \\Chemstation01\Data (D)\Data\3-6HR-NDR-FINAL.mgf
Database : MSDB 20050227 (1942918 sequences; 629040812 residues)
Taxonomy : Firmicutes (gram-positive bacteria) (170104 sequences)
Timestamp : 22 Sep 2005 at 08:43:40 GMT
Significant hits: Q48555_LISGR Major cold-shock protein (Fragment).- Listeria grayi (Listeria mu

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
match is a random event.
Individual ions scores > 42 indicate identity or extensive homology (p<0.05).
(p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.
ranking protein hits.



Peptide Summary Report

Format As | Peptide Summary

Significance threshold p< 0.05 Max. number of hits 20

Standard scoring MudPIT scoring Ions score cut-off 0

Show pop-ups Suppress pop-ups Sort unassigned Decreasi

Select All | Select None | Search Selected | Error tolerant

Archive Report

Major cold-shock protein (Fragment).- Listeria grayi (Listeria murrayi).

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 130	962.84	1923.67	1923.87	-0.20	0	48	0.0099

---

2. Q8EMU2_OCEIH Mass: 48062 Score: 35 Queries matched: 1

Hypothetical protein OB2748.- Oceanobacillus iheyensis.

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 99	435.80	869.59	870.59	-1.00	1	35	0.33

---

3. Q66UK1_LEUME Mass: 7216 Score: 30 Queries matched: 1

Cold shock protein.- Leuconostoc mesenteroides.

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 77	407.27	812.53	812.42	0.11	0	30	1

---

4. F90549 Mass: 18502 Score: 30 Queries matched: 1

conserved hypothetical protein MYPU_3020 [imported] - Mycoplasma pulmonis

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 114	412.83	1235.47	1236.72	-1.25	0	30	0.89

---

5. Q5KW40_GEOKA Mass: 40457 Score: 30 Queries matched: 1

3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; chorismate mutase (

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 112	592.62	1183.23	1183.68	-0.46	0	30	1

---

6. Q62UI4_BACLD Score: 28 Queries matched: 2

Aconitate hydratase (Aconitase).- Bacillus licheniformis (strain DSM 13 /

☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	F
	73	364.94	727.87	728.44	-0.58	0	16	54	
☑	120	691.21	1380.41	1380.72	-0.32	0	14	32	

Proteins matching the same set of peptides:
Q65J27_BACLI Score: 28 Queries matched: 2

---

7. C98020 Mass: 15176 Score: 27 Queries matched: 1
cytidine deaminase (EC 3.5.4.5) [imported] - Streptococcus pneumoniae (str
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	F
☑	125	747.05	2238.13	2238.11	0.02	0	27	2.6	

---

8. Q8EV19_MYCPE Mass: 19501 Score: 25 Queries matched: 3
Hypothetical protein MYPE7480.- Mycoplasma penetrans.
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	F
	79	409.27	816.53	816.42	0.11	0	(19)	20	
☑	81	409.44	816.87	816.42	0.45	0	28	2.4	
☑	82	409.88	817.75	816.42	1.33	0	(18)	23	

---

9. T12891 Mass: 34183 Score: 25 Queries matched: 2
hypothetical protein yorF - Bacillus subtilis phage SPBc2
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
	82	409.88	817.75	818.45	-0.70	0	12	80
☑	90	419.43	836.85	835.41	1.44	0	13	56

---

10. BAC13507 Mass: 28956 Score: 25 Queries matched: 4
BA000028 NID: - Oceanobacillus iheyensis HTE831
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	F
☑	94	421.60	841.19	841.54	-0.35	0	25	3.7	

☑	95	421.68	841.35	841.54	-0.19	0	(19)	14
☑	96	421.73	841.45	841.54	-0.09	0	(23)	5.5
	97	421.77	841.53	841.54	-0.01	0	(17)	26

---

11. Q7NAR7_MYCGA Score: 24 Queries matched: 1

ValS.- Mycoplasma gallisepticum.

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
81	409.44	816.87	817.44	-0.58	1	24	5.7

---

12. Q8DY02_STRA5 Score: 24 Queries matched: 1

Sucrose operon repressor ScrR.- Streptococcus agalactiae (serotype V).

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
81	409.44	816.87	817.47	-0.60	1	24	5.7

Proteins matching the same set of peptides:

AAC31628 Score: 24 Queries matched: 1

AAN59466 Score: 24 Queries matched: 1

G98067 Score: 24 Queries matched: 1

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13. AAM23449 Score: 24 Queries matched: 1

AE008691 NID: - Thermoanaerobacter tengcongensis MB4

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
81	409.44	816.87	818.37	-1.50	0	24	5.7

---

14. B25039 Score: 24 Queries matched: 1

outer cell wall protein precursor - Bacillus brevis (strain 47)

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
81	409.44	816.87	817.42	-0.55	0	24	5.7

---

15. AAK78485 Score: 24 Queries matched: 1

AE001437 NID: - Clostridium acetobutylicum ATCC 824

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
81	409.44	816.87	817.39	-0.53	1	24	5.7

---

16. Q899K2_CLOTE Score: 23 Queries matched: 1

Glutamine synthetase (EC 6.3.1.2).- Clostridium tetani.

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
99	435.80	869.59	870.52	-0.93	1	26	2.7

Proteins matching the same set of peptides:

AAO34822 Score: 23 Queries matched: 1

---

17. Q739Z9_BACC1 Score: 23 Queries matched: 1

ABC transporter, ATP-binding protein.- Bacillus cereus (strain ATCC 10987

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
99	435.80	869.59	870.52	-0.93	0	23	5.1

Proteins matching the same set of peptides:

AAS40912 Score: 23 Queries matched: 1

---

18. H83899 Score: 23 Queries matched: 1

2-hydroxyhepta-2,4-diene-1,7-dioate isomerase BH2000 [imported] - Bacillu

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
99	435.80	869.59	870.48	-0.90	0	23	5.1

---

19. AJBSQS Score: 23 Queries matched: 1

glutamate-ammonia ligase (EC 6.3.1.2) [validated] - Bacillus subtilis

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
112	592.62	1183.23	1182.66	0.56	0	23	4.6

Proteins matching the same set of peptides:

GLNA_BACSU Score: 23 Queries matched: 1

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20. Q74LP1_LACJO Score: 23 Queries matched: 1

Tagatose 1,6-diphosphate aldolase.- Lactobacillus johnsonii NCC 533.

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
99	435.80	869.59	870.52	-0.93	1	23	5.7

Proteins matching the same set of peptides:

AAS08124 Score: 23 Queries matched: 1

Peptide matches not assigned to protein hits: (no details means no match)
no match)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect
☑ 79	409.27	816.53	816.47	0.05	0	25	5.1
☑ 103	525.05	1572.13	1572.69	-0.56	0	24	
☑ 117	639.25	1276.49	1277.58	-1.10	1	22	5.7
☑ 57	301.34	600.67	602.34	-1.67	1	22	9.9
☑ 98	428.63	855.25	855.48	-0.24	0	22	5.9
☑ 91	419.46	1255.36	1254.52	0.84	0	21	1
☑ 97	421.77	841.53	843.52	-1.99	1	20	12
☑ 51	531.35	530.34	530.28	0.06	0	19	9.3
☑ 60	309.36	616.71	618.29	-1.59	0	19	2
☑ 127	883.79	1765.57	1765.77	-0.20	1	18	1
☑ 86	413.12	1236.34	1236.65	-0.31	0	18	2
☑ 73	364.94	727.87	729.37	-1.50	0	18	36
☑ 87	419.17	1254.49	1255.66	-1.18	1	18	3
☑ 111	591.30	1180.59	1179.59	0.99	0	17	1
☑ 83	412.64	823.27	821.41	1.85	0	17	1
☑ 124	737.97	1473.93	1472.77	1.15	0	17	1
☑ 129	904.21	1806.41	1804.97	1.44	1	17	1
☑ 100	437.45	872.89	872.51	0.38	0	17	3
☑ 109	586.32	1170.63	1169.52	1.10	0	17	2
☑ 101	472.42	942.83	942.44	0.39	0	16	2
☑ 123	737.89	1473.77	1473.78	-0.02	1	16	4

☑	116	633.04	1264.07	1264.65	-0.58	1	16	2
☑	119	679.44	1356.87	1355.72	1.15	1	16	4
☑	84	412.73	823.45	822.47	0.97	0	15	2
☑	78	409.17	816.33	815.47	0.86	1	15	4
☑	89	419.35	1255.03	1253.59	1.44	1	15	5
☑	110	590.79	1769.35	1767.92	1.43	1	15	5
☑	58	301.36	600.71	602.32	-1.62	1	15	9
☑	113	617.20	1848.58	1848.07	0.51	0	15	5
☑	92	419.52	837.03	835.46	1.57	1	14	3
☑	93	419.53	837.05	837.39	-0.34	0	14	3
☑	105	569.24	1704.70	1703.76	0.94	0	14	6
☑	68	321.24	960.70	962.47	-1.77	0	14	7
☑	59	301.54	601.07	601.32	-0.25	0	14	1.2e+002
☑	80	409.28	816.55	815.47	1.08	1	14	6
☑	88	419.32	836.63	835.46	1.17	1	14	4
☑	126	751.34	1500.67	1498.84	1.83	0	13	4
☑	106	572.78	1143.55	1141.66	1.88	1	13	8
☑	121	695.60	1389.19	1388.77	0.42	1	13	4
☑	72	347.64	693.27	693.36	-0.09	0	13	6
☑	53	547.27	546.26	544.30	1.97	0	12	7
☑	104	1100.46	1099.45	1097.59	1.86	0	12	6
☑	128	895.14	1788.27	1787.93	0.34	0	12	4
☑	74	375.58	749.15	748.41	0.74	1	12	9
☑	85	412.82	823.63	824.39	-0.77	0	10	8
☑	67	320.48	638.95	637.29	1.66	0	10	8
☑	102	508.13	1014.25	1012.49	1.76	1	10	1.1e+002
☑	62	635.30	634.29	634.27	0.02	0	9	1.9e+002
☑	71	347.54	693.07	692.40	0.67	0	9	1.4e+002
☑	69	683.30	682.29	683.31	-1.02	0	9	1.2e+002
☑	122	695.90	1389.79	1391.73	-1.95	1	8	1.3e+002
☑	52	531.45	530.44	529.30	1.15	1	7	1.5e+002
☑	131	983.39	1964.77	1965.97	-1.20	0	7	1.4e+002
☑	1	*301.30*	*300.29*					
☑	2	*302.50*	*301.49*					
☑	3	*309.02*	*308.01*					
☑	4	*316.52*	*315.51*					
☑	5	*316.77*	*315.76*					
☑	6	*365.03*	*364.02*					
☑	7	*386.78*	*385.77*					
☑	8	*387.27*	*386.26*					
☑	9	*387.27*	*386.26*					
☑	10	*404.26*	*403.25*					

☑	11	409.23	408.22
☑	12	409.33	408.32
☑	13	409.41	408.40
☑	14	415.62	414.61
☑	15	418.46	417.45
☑	16	418.94	417.93
☑	17	419.02	418.01
☑	18	419.02	418.01
☑	19	419.05	418.04
☑	20	419.06	418.05
☑	21	419.07	418.06
☑	22	419.16	418.15
☑	23	419.16	418.15
☑	24	419.17	418.16
☑	25	419.18	418.17
☑	26	419.27	418.26
☑	27	419.29	418.28
☑	28	419.34	418.33
☑	29	419.35	418.34
☑	30	419.36	418.35
☑	31	419.37	418.36
☑	32	419.37	418.36
☑	33	419.39	418.38
☑	34	419.39	418.38
☑	35	419.39	418.38
☑	36	419.42	418.41
☑	37	419.44	418.43
☑	38	419.44	418.43
☑	39	419.44	418.43
☑	40	419.47	418.46
☑	41	419.49	418.48
☑	42	419.51	418.50
☑	43	419.56	418.55
☑	44	419.56	418.55
☑	45	421.70	420.69
☑	46	453.50	452.49
☑	47	459.02	458.01
☑	48	463.39	462.38
☑	49	481.36	480.35
☑	50	503.13	502.12
☑	54	579.06	578.05

☑	55	*579.21*	*578.20*
☑	56	*301.12*	*600.23*
☑	61	*625.06*	*624.05*
☑	63	*636.43*	*635.42*
☑	64	*636.94*	*635.93*
☑	65	*636.94*	*635.93*
☑	66	*637.11*	*636.10*
☑	70	*347.50*	*692.99*
☑	75	*790.27*	*789.26*
☑	76	*795.26*	*794.25*
☑	107	*579.35*	*1156.69*
☑	108	*583.67*	*1165.33*
☑	115	*625.28*	*1248.55*
☑	118	*1305.21*	*1304.20*

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 2 Da
Fragment Mass Tolerance: ± 0.8 Da
Max Missed Cleavages : 1
Instrument type : ESI-TRAP
Number of queries : 131

Mascot: http://www.matrixscience.com/

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