Soybased Functional Foods with Reference to Probiotics and Isoflavones

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by
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Under the supervision of
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September 2010

DECLARATION

I hereby declare that, the thesis entitled **"Soybased Functional Foods with Reference to Probiotics and Isoflavones"** submitted to the University of Mysore, Mysore, for the award of the degree of Doctor of Philosophy in the Faculty of Microbiology is the result of work carried out by me under the guidance of **Dr. G. Vijayalakshmi,** Scientist, Department of Food Microbiology, Central Food Technological Research Institute, Mysore, during the period April 2005 – March 2008.

I further declare that, the result of this thesis has not been submitted by me for award of any other degree/diploma to this or any other University.

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I hereby certify that this thesis entitled **"Soybased Functional Foods with Reference to Probiotics and Isoflavones"** submitted by **Mrs. C.R. Rekha** for the award of **Doctor of Philosophy in Microbiology**, to the University of Mysore, is the result of research work carried out by her in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under my guidance during the period of April 2005 to March 2008.

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Abbreviations

AOAC	: Association of Official Analytical Chemists
β	: Beta
BMI	: Body mass index
BHA	: Brain heart infusion agar
BHI	: Brain heart infusion broth
cm	: Centimeter
CFU	: Colony forming units
CFU mL^{-1}	: Colony forming units /milliliter
°C	: Degree centigrade
MRS	: De Man Rogosa Sharpe agar
DPPH	: 1, 1-Diphenyl-2-picrylhydrazyl
DMRT	: Duncan's multiple range test
ESI	: Electron spray ionization
FAO	: Food and Agriculture Organization
FRSA	: Free radical scavenging activities
g	: Gram, gravity
GC	: Gas chromatography
GC-MS	: Gas chromatography-mass spectrometry
GRAS	: Generally recognized as safe
GDL	: Glucono-delta-lactone
HDL	: High-density lipoprotein
HPLC	: High Performance Liquid Chromatography
h	: Hour
kDa	: Kilodalton
Kg	: Kilogram
LAB	: Lactic acid bacteria
La	: *Lactobacillus acidophilus*
Lb	: *Lactobacillus bulgaricus*
Lc	: *Lactobacillus casei*
Lf	: *Lactobacillus fermentum*
Lh	: *Lactobacillus helviticus*
Lp	: *Lactobacillus plantarum*

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LC-MS	: Liquid chromatography – mass spectrometry
L	: Liter
LDL	: Low-density lipoprotein
LDPE	: Low-density polyethylene
MHz	: Mega hertz
mts	: Meters
µg	: Microgram
µg g^{-1}	: Microgram/gram
µL	: Microliter
µm	: Micrometre
µmol	: Micromole
µmol g^{-1}	: Micromole/gram
Mg	: Milligram
mL	: Milliliter
mm	: Millimeter
mmol l^{-1}	: Millimolar/ liter
mu	: Milli units
min	: Minute
M	: Molar
mol L^{-1}	: Mole/ liter
MWCO	: Molecular weight cut off
TEMED	: N,N,N',N' Tetramethylethylenediamine
nm	: Nanometre
N	: Newton, Normality
NA	: Nutrient agar
OD	: Optical Density
P-NPG	: P-Nitrophenyl-β-D-glucopyranoside
%	: Percentage
PUFA	: Poly unsaturated fatty acid
PDA	: Potato dextrose agar media
QDA	: Quantitative Descriptive Analysis
RSM	: Response Surface Methodology
rpm	: Revolutions per minute
Sb	: *Saccharomyces boulardii*
SEM	: Scanning Electron Microscope

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sec	: Second
SDE	: Simultaneous steam distillation and extraction
SDS	: Sodium dodecyl sulphate
SDS-PAGE	: Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TPCA	: Total plate count agar
uv	: Ultra violet
U	: Units (enzyme activity)
USDA	: United States Department of Agriculture
V	: Volts
v/v	: Volume/volume
w/v	: Weight/volume
WESK	: Water extract of soybean koji
wt	: Weight
YPD	: Yeast extract peptone dextrose agar medium

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Introduction

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PREFACE

Multifold benefits of soybean as healthy functional food for human reveals that, it contains more protein than beef, more calcium than milk, more lecithin than egg with more vitamins, minerals and many biologically active components. Soybean is cheap, conventional, convenient and richest source of protein for the fast expanding population. Efforts have been made to bring out different delicious novel foods from soybean to promote its wide consumption and acceptance.

Among the non fermented soyfood, tofu is popular for its protein content. It is usually coagulated with synthetic coagulants. It gets contaminated easily. Little information is available on the microbial quality of tofu. Likewise effect of various natural coagulants of plant origin on the amount of isoflavones and antioxidant activity of tofu is still unknown.

Soymilk fermented with lactic cultures and the bioconversion of isoflavones is extensively studied. However, there are only few studies with combination of yeast, stability of these cultures in fermented soymilk and survival of food borne pathogens like *Staphlococcus aureus* and *Listeria monocytogenes* in soymilk.

This thesis describes studies on soybean for use as food, microbiological quality of tofu and influence of coagulants of plant origin on the isoflavone content of tofu. It also explains the fermentation of soymilk with probiotic lactic acid bacteria with combination of probiotic yeast *Saccharomyces boulardii*. It elucidates the partial characterization of the antimicrobial compound and utilization of by-products of tofu preparation in Indian traditional foods, specifically taking into account its functional and nutritional values.

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1. Introduction

Functional foods are one of the most promising fields concerning nutritional sciences. The increasing interest of consumers in functional foods has brought about a rise in demand for functional ingredients obtained using ‘‘natural’’ processes. Therefore, there has been a growing interest in research, development and commercialization of functional food ingredients, around the globe (Shahidi, 2009). The goal of functional foods is to improve the quality of life, enhance health status and increase lifespan while maintaining health preventing diseases.

Potential of soybeans as a functional food is being currently explored by the food industry. Soybeans and soy foods like soymilk, miso and tofu, are widely promoted and eaten based on assumed relationships between its consumption and beneficial health effects in humans (Ikeda *et al*., 2006; Wu *et al*., 2008). During the last decade our knowledge about the dietary impact on health and well-being has increased substantially and often related to specific food components. Several classes of phytochemicals have been identified in soybeans, including protease inhibitors, phytosterols, saponins, phenolic acids, phytic acid and isoflavones (Romani *et al*., 2003). Of these, isoflavones are particularly noteworthy because soybeans are the only significant dietary source of these compounds which have received considerable attention due to their biological activity over the past 20 years. Isoflavones content in soybeans range from 0.4 to 9.5 mg per gram, which is influenced by genetics, crop year and growth location (Lee *et al*., 2003). More importantly, these compounds have shown several *in vitro* and *in vivo* beneficial properties consistent with the potential soybean effects on health.

Dairy foods containing probiotic bacteria are the main ‘‘bioactive ingredients” added to generate a health benefit which is gaining much importance. Soy is an excellent candidate for such products (Devaldez and Giori, 1993). A first benefit of soy beverage fermentation is the reduction of its ‘‘beany” flavour (Blagden and Gilliland, 2005). Soy is also considered a good substrate for functional foods since fermentation by probiotics has the potential to (1) reduce the levels of some carbohydrates which can be responsible for gas production in the intestinal system, (2) increase free isoflavone levels (Chien *et*

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al., 2006; Wei *et al*., 2007) and (3) favour desirable changes in bacterial populations in the gastrointestinal tract (Bouhnik *et al*., 2004).

Attention is focused on the use of natural antioxidants for inhibition or protection from oxidative damage (Fritz *et al*., 2003). Soybean and its products are important sources of antioxidant compounds. Consumption of foods containing significant amounts of antioxidants help the human body to reduce oxidative damage related to ageing and diseases, such as atherosclerosis, cancer and cirrhosis. The Food and Drugs Administration has approved a health claim for soy based on clinical trials and epidemiological data indicating that high soy consumption is associated with a lower risk of coronary artery disease (Rimbach *et al*., 2008). Hence Soy may be treated as a complete functional food because of its innumerable desirable characteristics (Tripathi and Misra, 2005).

The review of literature presented here focuses only of those aspects that are relevant to the present investigation.

1.1 Soybean

The soybean, *Glycine max* (L.) Merrill, belongs to the family *Leguminosae* and subfamily *Papilionoidae* originated in Eastern Asia. The plant is classed as an oilseed rather than a pulse. Hence Soybeans are primarily an industrial crop for oil and protein. It accounts for roughly 50% of the total oilseed production. Global annual consumption of soybeans has increased from 114 to 170 million tons during the past decade (Klejdus *et al*., 2004).

It is an annual plant that has been used in China for 5,000 years, primarily to add nitrogen to the soil as part of crop rotation. The plant is sometimes referred to as greater bean. The cultivated soybean first appeared in “Species Plantarum”, by Linnaeus, under the name *Phaseolus max* L. The combination *Glycine max* (L.) Merr., as proposed by Merrill in 1917, has become the valid name for this plant. As early as 5,000 years ago, records show that farmers in China grew soybeans as an important staple crop for their everyday diet. In fact, ancient Chinese scholars referred to soy as one of the "five sacred grains" (Tripathi and Misra, 2005). Although USA and Brazil account for most of the soybean production of the world, (**Table 1.1**) today the introduction of this crop to Western agriculture is quite recent.

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Table 1.1 Soybean producers
(Million metric tons)

United States	87.7
Brazil	52.4
Argentina	40.4
China	15.5
India	8.3
Paraguay	3.8
Canada	3.5
Bolivia	1.4
World Total	221.5

Source: UN Food & Agriculture Organisation (FAO)

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1.2 Physical characteristics

Soy varies in growth, habit and height. It grows to a height of 20 cm to 2 mts. The leaves are trifoliate, having 3-4 leaflets per leaf and the leaflets are 2-6 inches long and 1-3 inches broad. The leaves fall before the seed are mature. The flowers are borne in the axil of the leaf and are white, pink or purple.

The fruit is a hairy pod that grows in clusters of 3-5, each pod is 1-3 inches and usually contains 2-4 (rarely more) seeds which are 5-11 mm in diameter **(Fig. 1.1)**. Soybeans occur in various sizes and hull or seed coat varies in colour including black, brown, blue, yellow, green and mottled. The hull of the bean is hard, water resistant and protects the cotyledon and hypocotyls from damage. The scar visible on the seed coat is called the hilum. At one end of the hilum is the micropyle or small opening in the seed coat, which can allow absorption of water for sprouting.

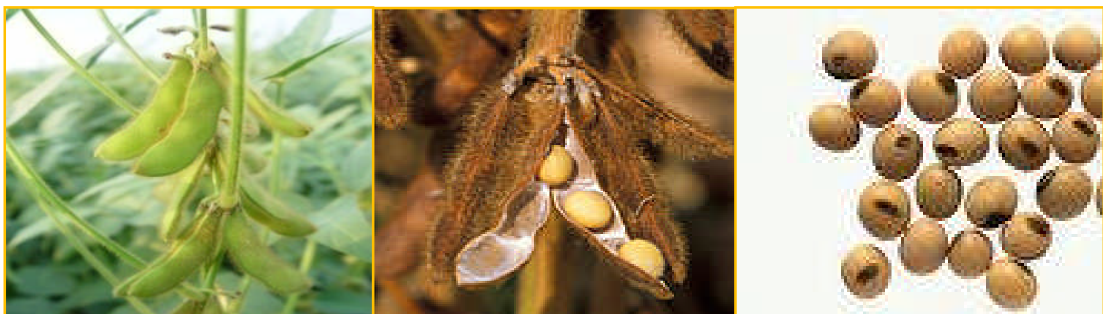

Fig. 1.1 Soybean pods and seeds

1.3 Chemical composition

Soybean is composed of macronutrients such as lipids, carbohydrates and proteins **(Table 1.2).** Soybean lipids, which are deprived of cholesterol, contain about 15% of saturated fat, 61% of polyunsaturated fat, and 24% of monounsaturated fat (USDA, 1979). Carbohydrates make up about 30% of the seed, with 15% being soluble carbohydrates (sucrose, raffinose, stachyose) and 15% insoluble carbohydrates (dietary fiber). Protein content of soybean varies from 36 to 46% depending on the variety (Garcia *et al*., 1997; Grieshop *et al*., 2003). Storage proteins are predominant, such as the 7S globulin (conglycinin) and 11S globulin (glycinin), which represent about 80% of total protein content, as well as less abundant storage proteins such as 2S, 9S, and 15S globulins (Garcia *et al*., 1997). Interestingly, conglycinin but not glycinin is capable of improving serum lipid profiles in mice and humans, in the absence of phytoestrogens (Kohno *et al*., 2006).

Soybeans also contain micronutrients, which include isoflavones, phytate, soyaponins, phytosterol, vitamins and minerals. Although beneficial effects of micronutrients such as saponins and phytosterols on cholesterol levels and absorption have been reported (Lukaczer *et al*., 2006), there is enormous literature suggesting that isoflavones may additionally have a beneficial role in lipid and glucose metabolism. Soybeans are the most abundant source of isoflavones in food. Studies have shown that there is a large variability in isoflavone content and composition in soybeans. It depends on the variety of soy grown, as well as environmental conditions (Wang and Murphy, 1994; Caldwell *et al*., 2005). Abiotic and biotic stresses such as variation in temperature, drought or nutritional status, pest attack or light conditions may modify isoflavone content and composition. As a consequence, total isoflavone content may vary up to 3-fold with growth of the same soy cultivar in different geographical areas and years (Wang and Murphy, 1994).

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Table 1.2 Approximate food value of 100 g edible soybean

Constituents	Value (g)	Remarks
Protein	43	Soybean is a rich source of best quality plant protein, PUFA rich oil, fibre and Soybean oil also contains omega-3 fatty acid minerals
Carbohydrates	21	
Fat	19	
Moisture	8	
Minerals	5	
Fibre	4	
Energy	430 kcal	It is a good source of dietary energy
Phosphorus	690 mg	Soybean is rich in phosphorus, calcium and magnesium
Calcium	240 mg	
Magnesium	175 mg	
Iron	10 mg	
Zinc	3 mg	
Manganese	2 mg	
Copper	1 mg	
Carotene	426 mg	It is reasonably a good source of carotene
Niacin	3 mg	
Thiamine	1 mg	
Riboflavin	1 mg	
Phytochemicals	Reasonable amount	It has phytochemicals like isoflavones, phytic acids, phytosterols, trypsin inhibitor, etc.

Source: Swaminathan and Chadha (2006)

1.4 Soy foods

Soy foods made from whole soybean can be classified into two categories:

1) Non fermented soyfood products like soymilk, tofu, fresh green soybeans, whole dry soybeans, soy nuts, soy sprouts and soyflour.

2) Fermented soy foods include Natto, Miso, Tempeh, Soy sauce, Taoco, sufu, oncom and fermented soymilk.

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New generation soy products are also being marketed to suit the western style. They are soy pudding, soy butter, soy candy bars, soy ice-cream, soy yoghurt, soy cheese, soy burger, soy bread, soy pastes, and soy snacks (Tripathi and Misra, 2005).

1.5 Tofu

Tofu is usually considered as salt- or acid-coagulated water based gel, with soy lipids and proteins trapped in the gel network (Kohyama *et al*., 1995). It is an inexpensive, nutritious and versatile meat or cheese substitute with bland taste and porous texture and hence called "Tofu is meat without bone". On the moisture free basis, tofu contains about 50% protein and 27% oil, and the remaining constituents are carbohydrates and minerals (Tripathi and Misra, 2005).

Its preparation generally includes soaking and grinding of soybeans in water, filtering, boiling and coagulation of soymilk, molding and pressing. The taste of tofu is significantly affected by its final texture (Kohyama and Nishinari, 1993; Jackson *et al*., 2002). The textural property is governed by soymilk concentration, coagulant type and concentration, gelation pressure and temperature and gelation time (Hou *et al*., 1997; Cai and Chang, 1998). The properties of gel can be properly unveiled with microscopy for the microstructure and with textural analysis or rheological study for the mechanical properties (Arltoft *et al*., 2007; Saowapark *et al*., 2008).

It is consumed in significant amounts in Asian countries because of their inexpensive high quality protein and isoflavones. Coward *et al*., (1993) analyzed tofu products of two different brands and reported that they contained 0.031 and 0.015 mg g^{-1} genistein and 0.249 and 0.269 mg g^{-1} genistin respectively. According to Wang and Murphy (1994), each gram of tofu contains 0.532 mg of isoflavones. In another study, total isoflavone was estimated to be 0.297 mg g^{-1} in raw tofu and 0.258 mg g^{-1} in cooked tofu (Franke *et al*., 1999). Variation in isoflavone contents of tofu products is governed by the native soybeans and loss occurs in whey during recovery of soybean curd. Most studies have addressed optimization of yield, physicochemical properties, and sensory qualities of tofu products as affected by variety, coagulant, and processing parameters (Wu *et al*., 2004). Transformation and recovery of isoflavones during tofu preparation,

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as affected by enzyme pretreatment, from soy milk for the purpose of enhancing the functional quality of tofu has not been given attention.

1.5.1 Factors affecting tofu quality and texture

Tofu making involves complex interactions of many factors that include intrinsic characteristics, such as soybean total protein of two major storage protein components, glycinin (11S) and β-conglycinin (7S), and their ratio (Saio *et al*., 1969). Shen et al., (1991) showed that yield of pressed glucono δ-lactone (GDL).tofu increased with protein content of soybeans and decreased with levels of phosphorus. Rheological studies on the gelation process of soybean 7S and 11S proteins in the presence of glucono-delta-lactone were studied by Kohyama and Nishinari (1993). The ratio of gelation for 7S was much lower and the gelatin time was longer for 11S (GDL). The gelation rate increased and the gelation time decreased with increasing GDL concentration at a constant 7S concentration. The minimum concentration of 7S protein for the gelation in the presence of GDL was lower than that of 11S-GDL system.

Processing method affected 7S and 11S protein contents of tofu and their contribution to tofu hardness yield, and sensory quality was shown by Cai and Chang (1999). Yagasaki *et al*., (2000) and Tezuka *et al*., (2000) reported the hardness of gels from glycinin decreased in the order group IIa, IIb, and I. The relative order of hardness for gels made from the β-conglycinin subunits was α ά and β (Mohamad Ramlan *et al*., 2004). Tezuka *et al*., (2000) evaluated the textural properties of tofu made from soy lines lacking different glycinin subunits, while Yagasaki *et al*., (2000) combined soymilk from low glycinin and low β-conglycinin soybeans for a series of 11S:7S ratios to evaluate the effects of different subunits on tofu gels. Mujoo *et al.,* (2003) reported that 11S fraction protein affected tofu yield though the firmness depended on 11S and the ratio with 7S fraction. The specific subunits within glycinin and β-conglycinin (Mohamad Ramlan *et al*., 2004) contribute differentially to protein gelling properties. The soy globulins differ in their functional properties, especially in gelation, with gels made from glycinin being harder than gels from β-conglycinin (Rickert *et al*., 2004). Both Poysa *et al*., (2006) reported that the group IIb (A3) glycinin subunit played a major role in contributing to

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tofu firmness, regardless of coagulant, while the group IIa (A4) subunit had a negative effect on tofu quality. Soybeans with the group I (A1, A2) subunit resulted in tofu with textural properties about one-third higher, expressed as a percent of Harovintons values, than tofu prepared from soybeans without the group I subunit. The individual components of group I had contradictory effects on GDL tofu quality with the A1 subunit having a negative effect and A2 having a major positive effect. Lack of the ά subunit of β-conglycinin increased gel hardness relative to the complete 7S protein.

Cai and Chang (1997) showed that solid content of 9.2° brix produced the lowest yield and coagulation time for 5 min resulted in a significantly lower tofu yield. In addition yield, quality and texture of tofu are influenced by several factors such as variety of soybeans and storage conditions, time and temperature of soaking soybeans, extent of heat treatment of soymilk, type and concentration of coagulant and rate of stirring and coagulation temperature (Sun and Breene, 1991). Soft tofu made by the highest stirring speed (285 rpm) had a lower yield, but tofu made from calcium sulphate and modified nigari stirred at 207 or 285 rpm decreased as stirring time increased to 30 sec. Textural properties were related to stirring time. Stirring time less than 25 sec was appropriate for soft tofu making (Hou *et al.,* 1997).

By Response surface methodology (RSM) Shih *et al.,* (1997) determined the optimum combination of four factors, solid content of soy milk (10-14° brix), concentration of coagulant (0.25-0.41% w/v), mixing temperature (75-91°C) and stirring time (5-25 sec) for producing soft tofu. Yield was affected by soymilk solid content and coagulant concentration. Solids and protein content of tofu were affected by 3 factors except mixing temperature. Optimum combinations were, soymilk solids 11.8 to 12.3° brix; coagulant 0.27 to 0.32%; mixing temperatures 85 to 91°C; and stirring time 5 to 11.3 sec.

Camparison of tofu was made by Cai and Chang (1997) using a bench scale (139 g bean, manual method) and a production scale (6500 g bean, automated machine method) showed that the bench scale resulted in greater varietal effects on tofu yield,

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moisture content, hardness and elasticity than the production scale. Soybean varieties high in protein, fat and phosphorus contents produced tofu with higher protein, fat and phosphorous contents (Lim *et al*., 1990). In another study the effect of soymilk coagulation on tofu yield and quality in two Proto and Vinton soybean varieties were studied (Cai and Chang 1998). They reported that stirring for 10 sec with the large propeller produced Proto tofu with a high yield and sensory quality but Vinton required a different coagulation condition for a high yield and quality. The soymilk from glycinin rich soybeans had a high protein particle content and formed harder tofu curd (Poysa *et al*., 2002; Guo and Ono, 2005).

Tofu yield increased and its texture became soft when phytate was added to soymilk during the tofu-making process (Saio *et al*., 1969a). The textural properties of tofu such as hardness, cohesivenesss, gumminess and chewiness significantly changed by the addition of Chitosan to tofu (Kim and Han, 2002). Phytate is also considered to affect tofu texture by reacting with protein and coagulants such as calcium and magnesium salts (Toda *et al*., 2003). Hou and Chang (2003) investigating the effect of the phytate concentration in soymilk on the yield and texture of pressed tofu stated that the pressed tofu yield increased and that the texture was softer when phytate was reduced with phytase. Takahiro *et al.,* (2006) concluded that soybeans having more phytate results in soft textured tofu.

Mechanical evolution of tofu curd in gelation was investigated by Ting *et al*., (2009) using low-power ultrasound and textural analysis. Two independent ultrasonic parameters, velocity and attenuation, were measured at 1MHz frequency as a function of time after addition of the calcium sulphate coagulant to heated soy milk. The responsive ultrasonic velocity had a plateau in the beginning of gelation and reached a lower steady state after formation of tofu gels. Ultrasonic attenuation exhibited first-order kinetics that matches the development of firmness revealed by textural analysis.

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1.5.2 Effect of coagulants

Various coagulants have been used in the preparation of tofu. It has been reported that the variety and amount of coagulants used, affect the yield and quality of tofu (Sun and Breene, 1991; Jackson *et al*., 2002) varying from soft to firm tofu and with moisture content ranging from 70 to 90% (deMan *et al*., 1986). Calcium sulphate (gypsum) and nigari were suitable coagulants for making food quality Chinese style tofu, but not glucono-δ-lactone (Tsai *et al.,* 1981). Kohyama *et al.,* (1995) analyzed the gelation process of tofu by adding glucono-δ-lactone (GDL) or calcium sulfate and found that the structure of calcium gels was quite similar to that of glucono-δ-lactone (GDL) gels. Tofu made from modified nigari had lower textural parameter values than those made using calcium sulphate (Hou *et al.,* 1997).

Wang and Hesseltine (1982) found that the 0.02 M calcium sulphate suspension added to soybean milk at 70°C was the most suitable coagulant for making tofu of high bulk weight, high nitrogen recovery and smooth textured tofu of all the 5 coagulants used. Karim *et al.,* (1999) reported that addition of Carrageenan increased the yield of calcium sulphate and calcium acetate tofu by 33 and 46.7% respectively but no increase in yield of glucono δ lactone tofu was seen but texture of calcium sulphate tofu was harder than calcium acetate and glucono δ lactone tofu. Kao *et al*., (2003) studied the effects of different concentration of calcium sulphate from 0.2 to 0.5%, on the microstructure and physical properties and found that 0.4% $CaSO_4$ in soymilk produced tofu with a maximum tofu yield, maximum protein recovery, maximum solid recovery and a maximum water retention ability. All four parameters increased significantly as the $CaSO_4$ concentration increased from 0.2 to 0.4% but decreased as the $CaSO_4$ concentration increased from 0.4 to 0.5% **(Fig. 1.2 &1.3)**. The contribution of soybean protein to the physical properties of tofu, with coagulants such as calcium or magnesium chloride, was studied by comparing the properties of soy milk prepared from soybeans with different subunits of glycinin with amino acid residues deleted. The breaking stress value of the tofu curd is dependent upon the number of protein particles in the soy milk and determined by the proportion and structure of glycinin in the soybean (Tezuka *et al*., 2000).

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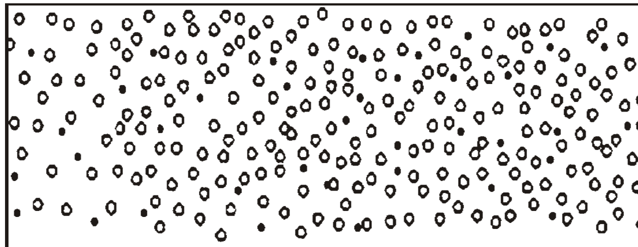

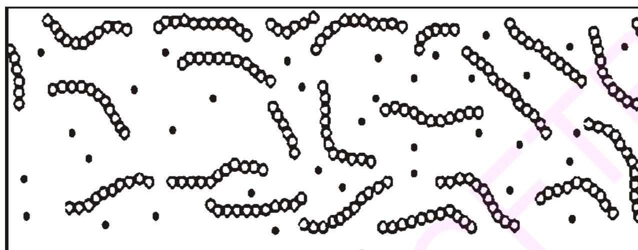

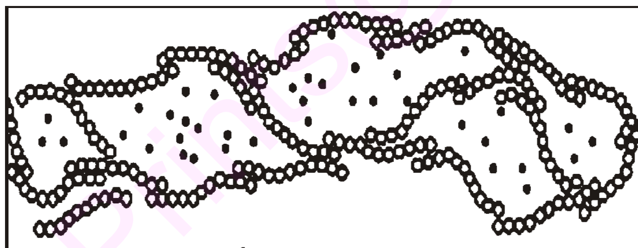

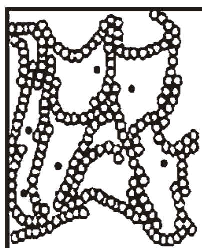

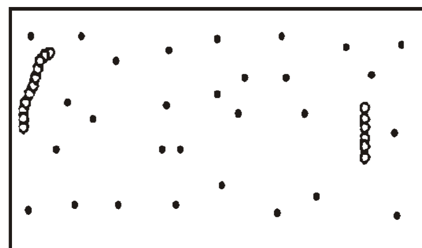

Fig. 1.2 Gelation mechanism of firm tofu in the presence of $CaSO_4$ (O) representing high molecular weight protein (•) representing low molecular weight protein

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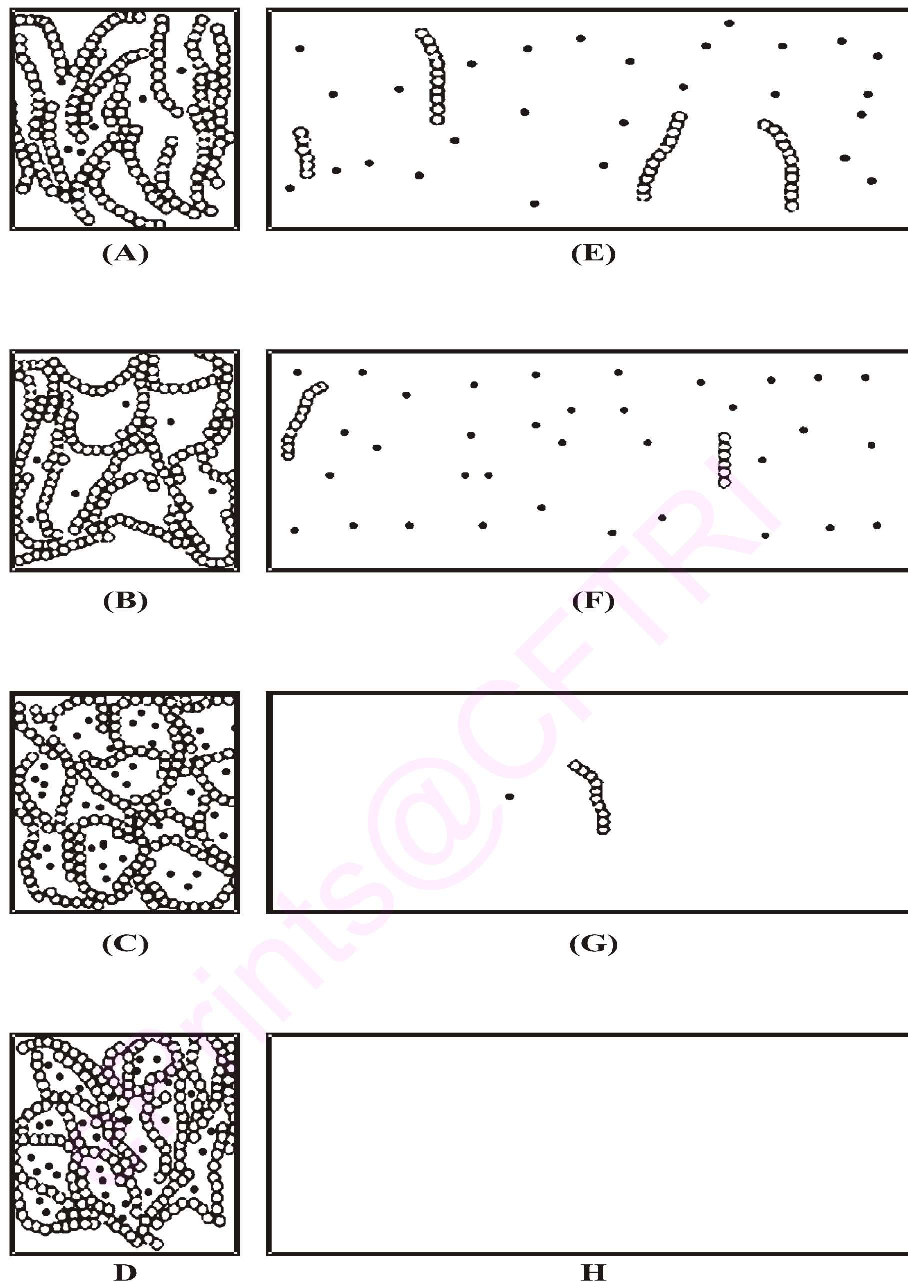

Fig. 1.3 Schematic representation of protein filaments and Low molecular weight proteins in tofu network and whey with different concentrations of $CaSO_4$ in soymilk; (O) high molecular weight protein; (•) low molecular weight protein. **(A)-(D)** 0.2,0.3.0.4 and 0.5% (tofu network) **(E)-(H)** 0.2, 0.3. 0.4 and 0.5% (tofu whey) The left-hand square areas represent the relative volumes of tofu, and the right-hand square areas represents the relative volumes of tofu whey.

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1.6 Isoflavones

Isoflavones are secondary plant metabolites, which constitute a group of natural bioflavonoids synthesized almost exclusively by plants of *Leguminasae* family (Fritsche and Steinhart, 1999). They occur in large amounts in soybeans and soy products, chickpeas, beans and other legumes, as well as clover, toothed medick and bluegrass (Reinli and Block, 1996). Isoflavones have a structure similar to estrogen and can exhibit weak estrogenic effects and therefore belong to phytoestrogens.

The basic isoflavone structure is a flavone nucleus, with two benzene rings linked to a heterocyclic ring. The position in the benzene ring is the basis for the categorization of the flavanoid class (position 2) and the isoflavonoid class (position 3). Twelve isoflavones have been isolated from soybeans. Three free isoflavones (genistein, daidzein and glycitein) and their respective nine glucosidic conjugates are shown in **Fig 1.4** (Jackson *et al.,* 2002). The glucosides include three β-glucosides forms (genistin, daidzin and glycitin), three malonylglucosides (6''-*O*-malonylgenistin, 6''-*O*-malonyldaidzin and 6''-*O*-malonylglycitin) and three acetylglucosides (6''-*O*-acetylgenistin, 6''-*O*-acetyldaidzin and 6''-*O*-acetylglycitin).

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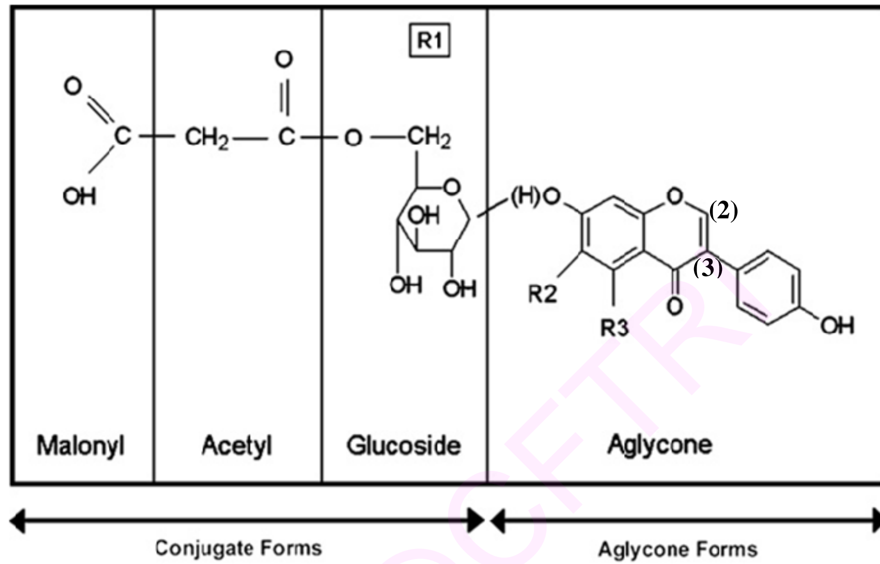
1.6.1 Isoflavone content in soyfoods

Concentrations of total isoflavones in different types of soy flour range from 60 to 265 mg 100 g^{-1}. Tofu contain a total isoflavones of 5.1 to 64 mg 100 g^{-1}, soy milk between 1.3 and 21 mg 100 g^{-1}, miso between 23 and 126 mg 100 g^{-1}, natto between 20 and 124 mg 100 g^{-1} and tempeh between 6.9 and 63 mg 100 g^{-1}. Soy yoghurts (1.6–11.8 mg 100 g^{-1}) soy milk drinks (1.0–11 mg 100 g^{-1}) and soy cheeses (2.3–33 mg 100 g^{-1}) also contain isoflavones (Mortensen, 2009).

Concentration and distribution of isoflavone forms in soybeans is influenced by the genotype, location and crop year (Wang and Murphy, 1994), whereas in processed soy products it depends on the sort of soybean used as well as on the type of processing (Murphy *et al*., 2002).

Isoflavone profile of raw soybeans alters due to processing. Processing of soybeans affects the nutritional content of products significantly (Wang and Murphy 1994 and 1996). Seventeen Ohio soybeans were screened for isoflavone content and

antioxidant activity by Lee *et al*., (2004). The highest and lowest total isoflovone contents were 11.75 and 4.20 µmol g^{-1}, while the average was 7.12 µmol g^{-1}. Antioxidant activities of soybean extracts ranged from 7.51 to 12.18 µmol g^{-1} butylated hydroxyl toluene (BHT) equivalent.



Isoflavone	**Symbol**	**R1**	**R2**	**R3**
Genistein	Ge	H	H	OH
Daidzein	De	H	H	H
Glycitein	Gle	H	OCH_3	H
Genistin	Gi	$C_6O_5H_{11}$	H	OH
Daidzin	Di	$C_6O_5H_{11}$	H	H
Glycitin	Gly	$C_6O_5H_{11}$	OCH_3	H
Acetyl-genistin	AGi	$C_6O_5H_{11}$+$COCH_3$	H	OH
Acetyl-daidzin	ADi	$C_6O_5H_{11}$+$COCH_3$	H	H
Acetyl-glycitin	AGly	$C_6O_5H_{11}$+$COCH_3$	OCH_3	H
Malonyl-genistin	MGi	$C_6O_5H_{11}$+$COCH_2COOH$	H	OH
Malonyl-daidzin	MDi	$C_6O_5H_{11}$+$COCH_2COOH$	H	H
Malonyl-glycitin	MGly	$C_6O_5H_{11}$+$COCH_2COOH$	OCH_3	H

Fig. 1.4 Chemical structures of soybean isoflavones and abbreviations

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Ishihara *et al.,* (2007) studied the total isoflavone content and their antioxidant capacity in homemade and commercial tofu and soymilk. The total isoflavone, aglycone and antioxidant levels were significantly higher in homemade soymilk and tofu (1571 µg) than in commercial samples. A strong positive correlation was observed between the total isoflavone, aglycone conjugates and genistein series concentration and antioxidant capacity of soymilk.

There are various reports on the isoflavone content of some soybean varieties and soy foods as well as the effects of processing on these compounds (Jackson *et al*., 1999). Distribution of isoflavones in commercial soy food products is determined by the variety of soybeans, the processing conditions and by dilution with non-soy ingredient (Wang and Murphy, 1994). Heat processing, enzymic hydrolysis and fermentation significantly alter the distribution of the isoflavone components in soy foods. Hui *et al*., (2001) found a 2-3-fold difference in isoflavone content between different brands or varieties of tofu. These variations in isoflavone content may result from different processing techniques and soybean varieties.

Certain processing methods, such as boiling, milling and protein coagulation in tofu production do not destroy daidzein or genistein significantly, while other methods such as roasting (high heat treatment) result in a 15– 21% loss of daidzein and genistein, respectively (Franke *et al.,* 1995). Defoaming during the heating process of soy beverage production may also remove isoflavones (Okubo *et al.,* 1983). Kao *et al*., (2004) reported that tofu made with 0.3% calcium sulphate (protein coagulant) was found to contain the highest yield (2272.3 $\mu g\ g^{-1}$) of total isoflavones whereas, a higher level (0.7%) of calcium sulphate resulted in a lower yield (1956.6 $\mu g\ g^{-1}$) of total isoflavones in tofu.

Jackson *et al*., (2002) investigated the effect of the processing of soybean on the total content of isoflavones (including aglycones and glucosides) and the relative concentrations of 12 isoflavone compounds during the preparation of soy beverage and tofu. The mean recoveries of isoflavones in soy beverage and tofu in relation to their initial concentration in the raw soybeans were 54 and 36%, respectively. The estimated percentage of total isoflavones lost in the water used to soak raw soybeans, the okara (waste from heat-treated slurry) and whey were 4, 31and 18%, respectively. Thermal

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processing of tofu mainly decreases daidzein than genistein (Grun *et al.,* 2001). In control and enzyme-treated tofu products, the total recoveries of daidzin, genistin, daidzein, and genistein in the tofu products increased from 54.9% to 64.2% (Wu *et al.*, 2004). Tofu coagulated with different coagulants was found to contain varied amounts of isoflavones and calcium sulphate was found to be the most suitable coagulant for tofu making in terms of its high yield, retention of maximum amount of isoflavones and in obtaining a firm, but not hard texture of tofu (Prabhakaran *et al*., 2006).

1.6.2 Absorption and metabolism of isoflavones

The metabolism of isoflavones is rather complex. The two major isoflavones, genistein and daidzein, are present in soy as glycosides, namely genistin and daidzin which are biologically inactive (Setchell, 1998). Intestinal bacteria play an essential role in isoflavone metabolism.

Once ingested, isoflavone glycosides are hydrolyzed to their corresponding bioactive aglycones (genistein and daidzein) by β-glucosidase of intestinal bacteria, whereas, 6"-*O*-malonyl glucosides and 6"-*O*-acetyl glucosides are not hydrolyzed. Only the aglycone forms are absorbed by the intestinal tract and are therefore biologically active. Daidzein are further metabolized to equol and *O*-demethyangolensin and genistein to p-ethyl phenol before absorption by bacteria. Thus daidzein, genistein, equol and *O*-demethyangolensin are the major isoflavones detected in the blood and urine of humans and animals (Setchell, 1998).

1.6.3 Importance of isoflavones

Epidemiological studies have shown that type-2 diabetes is four times less prevalent in Japanese people in Tokyo than in Japanese- Americans in Seattle (Fujimoto *et al*., 1991). Consumption of more than 12.6 g of soy protein per day is associated with a lower risk of glycosuria, a strong predictor of diabetes (Yang *et al*., 2004). Additionally, a 6-month clinical trial was conducted to compare the effects of isoflavones with that of conjugated estrogens on blood glucose, insulin, and lipid profiles in postmenopausal Taiwanese women. The study (Cheng *et al*., 2004) revealed that during fasting both glucose and insulin levels were significantly reduced by soy isoflavones (100 mg/day) and conjugated estrogens (0.625 mg/day).

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Several studies have reported that isoflavone consumption by postmenopausal women correlated with lower body mass index (BMI) and higher HDL levels (Goodman and Kritz, 2003). Clinical studies also suggest that soy protein or isoflavones may improve metabolic parameters. Reports (Jayagopal *et al*., 2002; Greany *et al*., 2004) demonstrated a significant reduction in plasma concentrations of total and LDL cholesterol in humans exposed to soy proteins. In addition, postmenopausal Japanese women treated for 24 weeks with isoflavones exhibited a lower fat mass (Wu *et al*., 2006). Obese patients treated with soy protein isolates for 12 weeks had lower body weight and BMI, with decreased cholesterol and LDL levels in the blood (Allison *et al*., 2003).

In contrast to the above mentioned trials, a significant number of studies reported an absence of beneficial effects of soy on classical metabolic parameters such as bodyweight, serum lipid profiles, fat mass, blood glucose and insulin profiles (Li *et al*., 2005; Hall *et al*., 2006; Ikeda *et al*., 2006; Anderson *et al*., 2007). These discrepancies make it difficult to draw firm conclusions regarding the beneficial effect of soy on glucose and lipid metabolism.

Studies indicate that consumption of isoflavones can have bone-sparing effects over the long term (Setchell and Lydeking, 2003) not only by attenuating bone loss (Atkinson *et al*., 2005) but also by enhancing calcium absorption (Zafar *et al*., 2004).

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1.7 Fermented soymilk

Fermented foods that have potential probiotic properties are produced worldwide from a variety of food substrates (Farnworth, 2005). Yoghurt produced from cows' milk is consumed in both developing and industrialized countries. However, the demand for alternatives to cow’s milk is growing due to problems with allergenicity and desire for vegetarian alternatives. Probiotic milk-based yoghurts are now being marketed and consequently it would be desirable to know if probiotic bacteria can also be incorporated into soy-based yoghurt-type fermentations (Farnworth *et al.,* 2007).

Fermentation by lactic acid bacteria - Probiotic bacteria generally do not grow rapidly in cow’s milk. Thus, in yoghurt manufacture, they do not attain as high numbers

as the starter cultures (Champagne and Gardner, 2005). However, many studies indicate that soy is a good substrate for probiotic bacteria (Scalabrini *et al*., 1998), but not for the traditional yoghurt starter *L. delbrueckii subsp. bulgaricus* (Wang *et al*., 2002a). These findings suggest that some probiotic bacteria could better compete with yoghurt cultures in a soy-based substrate. Results of Murti *et al*., (1993) suggest that *Bifidobacterium* can indeed grow more extensively in soy than in cow's milk under comparable conditions. However, very wide variations have been noted in the growth abilities of strains within a given species (Murti *et al*., 1993a; Scalabrini *et al*., 1998), and more data are needed to characterize the potential of soy as a substrate to support good growth of bifidobacteria in combination with yoghurt strains.

Lactobacilli are also extensively used as probiotics. Soy has been examined as a substrate for the Lactobacillus species, *L. casei* (Murti *et al*., 1993; Garro *et al*., 1999), *L. helveticus* (Murti *et al*., 1993), *L. fermenti* Chumchuere and Robinson, 1999), *L. fermentum* (Garro *et al*., 2001, 2004), *L. reuteri* (Tzortzis *et al*., 2004) and *L. acidophilus* (Wang *et al*., 2002, 2003). Farnwarth *et al*., (2007) studied the ability of *L. rhamnosus, L. johnsonii* and various bifidobacteria to grow in mixed cultures with yoghurt strains in a soy beverage and cows' milk and found that the *Lactobacilli* can compete better with the yoghurt strains in a soy beverage than in cows' milk.

Fermentation by lactic acid bacteria and yeasts - According to Narvhus and Gadaga, (2003) presence of large number of yeasts, with lactic acid bacteria in fermented milk suggested that yeasts contribute to important product characteristics. The changes in growth and metabolite profiles in yeast–LAB co-cultures are evidence of synergistic growth. Naturally fermented milks are thus a result of mixture of many organisms.

It is generally believed that the yeasts excrete nutrients that benefit the LAB. This is indeed the case with some LAB and yeasts (Liu and Tsao, 2009). Not all LAB responds to the presence of yeasts with respect to their stability suggesting that there may be different mechanisms for different yeast–LAB combinations. Liu *et al*., (2009) reported that 5 different yeasts examined significantly improved the survival of *L. rhamnosus* DR20 by 10^3 to 10^6-fold compared with the control (no added yeast). Thus yeasts possess stability-enhancing effects on LAB and that the specific effects of yeasts on LAB stability vary with yeasts as well as with LAB.

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Martin *et al*., (2008) studied the role of yeast and lactic acid bacteria in ultra high treated skimmed milk separately and in co-culture. The yeasts and LAB species studied showed both stimulatory and inhibitory effects on one another, depending on the combination. The production of volatile components varied widely between single and co-cultures, with no clear tendency observed. Characteristically, diacetyl was mostly produced by LAB strains, while yeasts were responsible for producing a majority of maltic compounds.

1.7.1 Advantages of soymilk fermentation

Soy is considered as a good substrate for functional foods since fermentation by probiotics has the potential to reduce beany flavor, reduction of oligosaccharides, release of free isoflavones, improves antioxidant activity and has the beneficial effect of using probiotics.

1.7.1.1 Reduction in beany flavor

A first benefit of soy beverage fermentation is the reduction of ‘‘beany” flavour (Blagden and Gilliland, 2005). Fermentation of soymilk with various microorganisms, especially lactic acid bacteria, has been attempted to overcome the problem of beany flavour and increase acceptability (Denkova and Murgov, 2005). Some studies (Donkor *et al*., 2007) report that lactic acid bacteria fermentation provide an improved volatile profile to soymilk. Fermentation processes may improve the sensory attributes and also decrease or mask the properties of undesirable compounds. Several studies have reported the use of soymilk for the production of yoghurt-like products (Kovalenko and Briggs, 2002; Denkova and Murgov, 2005). Blagden and Gilliland (2005) reported that methanol, acetaldehyde, ethanol and hexanal were the 4 major volatiles detected in unfermented soymilk. Eight cultures of lactobacilli or streptococci during soymilk fermentation significantly reduced the levels of methanol and hexanol, *Streptococcus thermophilus* OSU-2 lowered ethanol, *L. acidophilus* C19 and *L. casei* E5 lowered acetaldehyde.

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1.7.1.2 Reduction of oligosaccharides

Soymilk contains oligosaccharides, principally sucrose, raffinose and stachyose, which are recognized as the flatulence factors. This can be reduced by lactic fermentation as shown by Wang *et al*., (2003). This study showed a reduction in stachyose and raffinose and increase in sucrose, fructose, galactose in soymilk fermented with mixed cultures of *Bifidobacteria* and lactic acid bacteria than that of single culture. The reduction of oligosaccharides by *Bifidobacterium longum* CRL 849 was due to maximum hydrolysis of stachyose (49.3%) during the first 7 h of incubation and 79.3% decrease in sucrose concentration after 9 h (Garro *et al*., 1999).

1.7.1.3 Biotransformation of isoflavones

In nonfermented soy food products, isoflavones predominantly exist as biologically inactive glycoside conjugates ranging from 83.90% to 98.37% (King and Bignell, 2000). In humans, the isoflavone aglycones are absorbed faster and in greater amounts than their glycosides counterpart. Aglycones are absorbed directly through the gut wall, while isoflavone glycosides are very poorly absorbed from the gut due to their higher hydrophilicity and larger molecular weight (Izumi *et al*., 2000).

It is generally thought that isoflavone glycosides are converted to their corresponding aglycones by gut microflora or gut glucosidases and then absorbed from the small intestine (Izumi *et al*., 2000). Several groups of gut bacteria such as *Bifidobacterium*, due to *β*-glucosidase activity, are able to hydrolyze isoflavone glycosides to aglycones (Tsangalis *et al*., 2002; Hughes *et al*., 2003). Probiotic micro-organisms including *Lactobacillus* and *Bifidobacterium* possess endogenous β-glucosidases which can play an important role in altering the profile of isoflavones during fermentation (Otieno *et al*., 2005). Although each group of probiotics has vary in their potential, in the hydrolysis of isoflavones during fermentation (Otieno *et al*., 2006a), the hydrolytic action has been found to cause major increase in the concentration of bioactive isoflavone aglycones and concomitant decrease in the concentration of isoflavone glycosides (Otieno *et al*., 2006a). Microorganisms in soymilk may lead to a combination of benefits as probiotics as well as the transformation of isoflavone glycosides to bioactive isoflavone aglycones. In addition, aglycones have been reported to be more

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stable than isoflavone glycosides during the storage at different temperatures (Otieno *et al*., 2006). Consequently, providing food products with aglycones would be considered as a novel trend for the food industry.

Tsangalis *et al*., (2002) studied the enzymic transformation of isoflavone phytoestrogens in soymilk by *β*-glucosidase-producing bifidobacteria. Otieno *et al*., (2005) reported the evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus,* and *Lactobacillus casei*. Similarly, Chien *et al*., (2006) studied the transformation of isoflavones during the fermentation of soymilk with lactic acid bacteria and *bifidobacteria*. However, in these studies, the rate of transformation of isoflavone glycosides to aglycones was low (6.42% of the total isoflavone glycosides in soymilk were fermented by *B. longum* after 32 h fermentation at 37°C). To enhance the rate of biotransformation of isoflavone glycosides to aglycones as well as to improve the quality of fermented soymilk, skim milk powder was supplemented (Shah, 2006). Lactose present in skim milk powder reported by enhanced the growth and metabolism of beneficial bacteria such as *lactobacilli* and *bifidobacteria* (Kontula *et al*., 1999). Two probiotic strains, *Bifidobacterium animalis* A and B, biotransformed glycosides to aglycones significantly in soymilk supplemented with skim milk powder (Pham and Shah, 2007)**.**

Tang *et al*., (2007) reported that fermenting calcium-fortified soymilk with the selected probiotics can potentially enhance calcium bioavailability of calcium-fortified soymilk due to increased calcium solubility and bioactive isoflavone aglycone enrichment. Pham and Shah (2008) studied the effect of lactulose, which is produced during the heat treatment of lactose on the biotransformation of glycosides to aglycones and reported that biotransformation occurred rapidly during the initial 12 h incubation, in both soymilk supplemented with lactulose and Soymilk. Among the 4 *Lactobacillus* strains, *L. acidophilus* 4461 biotransformed the highest level (88.8%) of glycosides to aglycones in soymilk lactulose compared to 68.2% in soymilk after 24 h of incubation. Chun *et al*., (2007) while studying the biotranformation of glucosides to aglycones in soymilk fermented with lactic acid bacteria, found that the rates of hydrolysis of glucosides varied depending on the species of LAB.

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1.7.1.4 Improved antioxidant activity

Antioxidant activity of fermented soy foods increases due to isoflavone bioconversion during fermentation. Fermentation of soybean with 4 bacterial strains *Lactobacillus plantarum* KFRI 00144, *Lactobacilllus delbrueckii subsp.lactis* KFRI 01181, *Bifidobacteria themophilum* KFRI 00748 and *Bifidobacteria breve* K-10, resulted in a significant increase in the antioxidant capacity campared to control which was fermented without the above bacteria (Pyo *et al*., 2005). Thus antioxidant activity of fermented soymilk varied with the starter cultures used (Wang *et al*., 2006). However the antioxidative activity in soymilk fermented with lactic acid bacteria and *bifidobacteria* simultaneously significantly increased than that fermented either individually.

Zhu *et al*., (2008) evaluated the antioxidant activity of Chinese traditional fermened okara, Meitauza. The water extract of soybean koji (WESK), water extract of okara koji (WEOK) showed higher 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and reducing power. Wang *et al.,* (2008) showed that the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food) had excellent antioxidant activities. The antioxidant properties and total phenolic compounds of soybean extract fermented with *Saccharomyces cerevisiae* during 24 h have also been evaluated by (Ana *et al*., 2004). The extract exhibited excellent DPPH scavenging and reducing power, with superoxide radical scavenging activities.

After consumption the glycosides are hydrolysed in the human gut to their aglycones, which are further metabolised, and excreted (Kulling *et al*., 2002). For this reason, several authors preferred to determine the aglycones formed after acid (Wang *et al*., 1990; Mullner and Sontag, 2000) and enzymatic (Franke *et al*., 1994) hydrolysis in order to study the hydrolysis.

1.8 Probiotics as functional food

One of the biggest problems for humans in 21st century is to control infectious diseases. These diseases are caused by pathogenic microorganisms like pathogenic *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Campylobacter* and rotavirus which are the main causes of death in developing countries. Even in a developed country, like USA, 21–37 million cases of diarrhea occur annually in a population of 16.5 million children

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(Glass *et al*., 1991). Excess use of antibiotics has led to antibiotic resistant bacteria, reduction in their potency and efficiency. As such, attention has turned towards food materials that offer improved health benefits. Under these circumstances, some useful bacteria contained in yoghurt, beverages, and other fermented foods have been medically recognized as probiotics (Fuller, 1989; Reid *et al*., 2005).

There is a great demand for functional foods containing specific ingredients. Dairy foods containing probiotic bacteria are the main ''bioactive ingredients" added in order to generate this health benefit and gaining much importance, accounting for nearly 33% of the market worldwide (Leatherhead Food International, 2006). Probiotics are defined as ''live microorganisms which, when administered in adequate amounts, provide a health benefit the host" (Araya *et al*., 2002). Probiotic containing drinks are the fastest-growing dairy product in Europe and data show that the global market for probiotic functional foods has grown by 19% in recent years and is expected to grow by 5% annually between 2006 and 2011 (Nutra ingredients-USA, 2007). New probiotic-containing products have been launched, particularly in fruit-based drinks and cereals. Soy is an excellent candidate for such products (DeValdez and Giori, 1993).

A feature of soy fermentation by probiotics is the bacterial strain-linked variability of the acidification rate. The study by Stern *et al*., (1977), which included eight *Lactobacillus acidophilus* cultures, and similar studies using bifidobacteria (Scalabrini *et al*., 1998; Tsangalis *et al*., 2002), revealed sharp differences between strains in the rate of acid production. Furthermore, probiotic cultures alone can generate products with unpleasant flavours (Macedo *et al*., 1998). A potential solution to these two problems is the use of mixed cultures with a yoghurt bacterial strain. However, little is known about the behavior of probiotics when used in soy beverages along with typical yoghurt starter culture. Pairing of probiotic cultures can be very disadvantageous to some strains (Macedo *et al*., 1998) and data show that combinations with *Streptococcus thermophilus* can be detrimental to *L. acidophilus* (De Valdez and Giori, 1993) and bifidobacteria (Murti *et al*., 1993). Thus, the development of a fermented soy product containing probiotics will require strain selection for ability to grow in the substrate as well as ability to compete or even establish a synergy between strains.

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The main probiotic bacteria studied in the past for growth in soy beverages are *L. acidophilus*, *L. fermentum* and *bifidobacteria*. Little is known of probiotic *L. rhamnosus*, *L. helveticus*, *L. delbrueckii ssp. lactis*. Champagne *et al*., (2009) studied the growth of various bacteria like *Lactobacillus delbrueckii* subsp. *lactis* R0187, *L. helveticus* R0052, *L. rhamnosus* R0011 and *B. longum* R0175 were examined for their ability to grow in combination with *Streptococcus thermophilus* cultures in milk and a laboratory soy beverage. They found that strains R0011 and R0187 did not rapidly acidify the soy beverage despite good growth rates on soy carbohydrates. The populations of *L. helveticus* in the fermented products were similar in pure cultures or in the presence of the Streptococci. The probiotic populations in the mixed culture were influenced by the *S. thermophilus* strain and time of fermentation. The influence of temperature on the growth and biological activity of two probiotic strains (*Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251) as pure and mixed cultures in soymilk (Garro, *et al*., 2004) showed maximum growth at 37°C in both mixed and pure cultures.

Gibson (2004) studied the effect of bifidobacteria and lactobacilli on gut disorder. Dietary carbohydrates had a selective metabolism within the gut flora thereby shifting the community towards a more advantageous structure. Conventional fibres like pectins, cellulose, etc. are not selectively metabolised by gut bacteria. However, certain oligosaccharides do have this capability. Most research has been conducted with fructooligosaccharides, like inulin, which have a powerful bifidogenic effect. Trials are ongoing to determine the clinical benefits of prebiotic use. Intestinal disorders like ulcerative colitis, gastroenteritis and irritable bowel syndrome are particular targets. Matto *et al*., (2005) reported the intestinal survival and persistence of probiotic strains *Lactobacillus* F19, *Lactobacillus acidophilus* NCFB 1748, and *Bifidobacterium animalisl subsp.lactis* Bb-12 in the human gastrointestinal tract when yoghurt is consumed.

Wang *et al*., (2004) studied the survival of lactic acid bacteria and bifidobacteria during drying process, subsequent rehydration at different temperatures, and during a 4-month period of storage under different storage conditions. The survival of Lactic acid bacteria and *bifidobacteria* were higher, in freeze dried samples than spray dried. A higher survival rate was noted for both the starter organisms when the dried fermented soymilk was stored at 4°C than at 25°C. Among all the packaging materials and storage

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temperatures tested, starter organisms were most stable in the dried fermented soymilk stored at 4°C in laminated pouch.

The effects of soybean isoflavones with or without probiotics on tissue fat deposition, plasma cholesterol, and steroid and thyroid hormones were studied in SHR/N-cp rats, an animal model of obesity, and were compared to lean phenotype. In both phenotypes, isoflavones lowered fat deposition. Probiotics alone had no significant effect on fat deposits. Isoflavones lowered total, LDL, and HDL cholesterol in lean rats, but in obese rats isoflavones lowered only total and LDL cholesterol. Probiotics had no significant effect on cholesterol or hormones. Thus, it showed that soy isoflavones also lower plasma cholesterol and that the hypocholesterolemic effect appears to be due in part to the modulation of steroid hormones. Probiotics did not seem to enhance the effect of isoflavones (Ali *et al*., 2004). Isoflavones also lowered many of the steroid hormones involved in lipid metabolism but had no significant effect on thyroid hormones.

Soybean and its isoflavones have been shown to have beneficial effects on carbohydrate and lipid metabolism and on renal function. Probiotics may potentiate the beneficial effects of isoflavones by converting the inactive isoflavone glycoside to aglycones, which are biologically active, thereby producing a synergistic effect. Isoflavones given alone lowered plasma glucose in both phenotypes while triglyceride decreased only in lean animals. Isoflavones also lowered aspartate amino transferase and alanine amino transferase in both phenotypes. Isoflavones had significant effect on plasma insulin, leptin and glucagon in lean rats but not in obese rats. Thus, it showed that in lean animals, isoflavones have hypoglycemic and hypolipidemic effect and the effect is mediated by changes in peptide hormones. When lipid levels are high, as in obese rats, isoflavones fail to lower plasma triglyceride levels. Probiotics do not appear to enhance the effect of isoflavones (Ali *et al*., 2005).

1.8.1 Probiotic microorganisms

The most commonly used microorganisms are lactic-acid producing bacteria, *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Bifidobacteria* (Gibson, 2004). The non-lactic acid bacteria include *Bacillus* and the yeast, *Saccharomyces*. Both groups have different mechanisms of action, metabolism

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and sensitivity to antibiotics. Since *Saccharomyces* is not affected by antibiotics, it has an advantage in probiotic preparations used for preventing disruptions in the normal microflora by antimicrobial agents.

Lactic acid bacteria have the ability to digest lactose, converting it into lactic acid and therefore lowers pH. They are facultative anaerobes, Gram-positive and mostly of human origin. *Lactobacilli* strains vary in their fermentation process, hydrogen peroxide and bacteriocin production. These features make them a versatile group suitable for different conditions. Recently, they have been reported to colonise *in vivo* (Alander *et al*., 1999) and promote vitamin production and food digestion (Denter and Bisping, 1994). They are available commercially as live in food preparations or as live, or in a heat-inactivated form. The yeast *Saccharomyces boulardii* has been used in gut disorders. Several trials confirmed its efficacy in diarrhea (Surawicz *et al*., 1989) and they have the advantage of being resistant to antibacterial agents. They are available in a lyophilised formulation.

1.8.2 Characteristics of probiotics

A number of lactic acid bacteria have been classified as probiotics and are either incorporated into functional foods or marketed as lyophilized cells in capsules (Fuller, 1989; Svensson, 1999). Health benefits described for probiotics include prevention or treatment of infectious diseases, irritable bowel syndrome, allergies, lactose intolerance, colon cancer and reduction of serum cholesterol levels (Andersson *et al*., 2001). Criteria for selection of a probiotic vary, but usually includes the ability to adhere to mucus and epithelial cells (Gorbach, 2002), survival at low pH (1.0 to 3.0) and bile salts of approximately 0.3% (Mainville *et al*., 2005).

Mishra and Prasad (2005) examined different *in vitro* characteristics of seven *Lactobacillus casei* strains. All those strains able to resist pH 3 for 3 h, were antagonistic to common pathogens with cholesterol reducing ability. Adhesion studies on stainless steel chips did not reveal any specific attachment to surfaces by any of strains. Shimakawa *et al*., (2003) evaluated the effects of *B. breve* strain Yakult fermented soymilk could be a novel type of probiotic food.

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Table 1.3 Probiotics used in experimental studies

Lactobacilli	*Bifidobacteria*	**Others**	**Non-lactic acid producing bacteria**
L. acidophilus	*B. adolescentis*	*E. faecalis*	*B.cereus ('toyoi)*
L. casei	*B. animalis*	*E. faecium*	*B. subtilis*
L .crispatus	*B. bifidum*	*L. lactis*	*S. boulardii*
L. gasseri	*B. infantis*	*P. acidilactici*	*S. cerevisiae*
L. reuteri	*B. lactis*	*S. thermophilus*	
L. johnsonii (L. paracasei)	*B. breve*		
L. rhamnosus	*B. cereus*		
L. delbrueckii subsp bulgaricus	*B. longum*		
L. brevis	*B. thermophilus*		
L. cellobiosus			
L. fermentum			
L. curvatus			
L. rhamnosus (GG)			
L. plantarum			
L. salivarius			
L. helviticus			
L. farciminis			

Source: Penner *et al.,* (2005).

1.8.3 Beneficial effects and therapeutic application of probiotic bacteria in humans (Fuller, 1989)

Beneficial effects

- Maintenance of normal intestinal microflora
- Enhancement of the immune system
- Resuction of lactose intolerance
- Reduction of serum cholesterol levels
- Anticarcinogenic activity
- Improved nutritional value of foods

Therapeutic applications

- Prevention of urogenital infection
- Protection against traveller's diarrhea
- Reduction of antibiotic induced diarrhea
- Alleviation of constipation
- Prevention of infantile diarrhea
- Prevention of hypercholesterolaemia

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- Protection against colon /bladder cancer
- Prevention of osteoporosis

The effect of administration of *S. boulardii* in hospitalized patients, receiving antibiotic therapy showed that 9.5% of patients treated with *S. boulardii* developed antibiotic associated diarrhea, while in the control group the percentage was 22% (Surawicz *et al*., 1989). The effect of *S. boulardii* to control travellers diarrhea in Austrian tourists found the yeast to significantly reduce the frequency of diarrhea in a dose dependent manner (Kollaritsch and Wiedermann, 1990).

1.9 Bacteriocins

Natural, traditional and processed foods without chemical preservatives are gaining much attention due to their health benefits. Lactic acid bacteria (LAB) are widely used for the fermentation and preservation of a wide range of milk, meat and vegetable foods (Zhu *et al*., 2000). In fermented foods, Lactic acid bacteria (LAB) have long been used as starter cultures because they significantly contribute to flavour, texture, and nutritional value of the food products (McKay and Baldwin, 1990). LAB's play an important role in preservation and microbial safety of fermented foods (Caplice and Fitzgerald, 1999), thus promoting the microbial stability of the final products of fermentation (Mensah *et al*., 1991). The preservative effect is not only due to acidic conditions but also due to inhibitory substances such as carbon dioxide, ethanol, hydrogen peroxide and diacetyl (Atrih *et al*., 2001) or bacteriocins (DeVuyst and Vandamme, 1994).

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1.9.1 Bacteriocins of lactic acid bacteria

The term "bacteriocin" was coined in 1953 to define colicin produced by *Escherichia coli*. After the first discovery of bacteriocin by Gratia in 1925 (Garneau *et al*., 2002), bacteriocin production has been found in numerous species of bacteria. LAB have attracted great interest in terms of food safety because they are "Generally Recognised As Safe" GRAS). As LAB bacteriocins are food-grade, they can be used in both fermented and non-fermented foods to prevent unwanted organisms. Bacteriocins produced by lactic acid bacteria (Cotter *et al*., 2005) are small, ribosomally synthesised, antibacterial, extracellularly released lowmolecular mass peptides or proteins (usually

30–60 amino acids) which have a bactericidal or bacteriostatic effect either in the same species (narrow spectrum) or across genera (broad spectrum). Some of them are inhibitory towards food spoilage and foodborne pathogenic bacteria (Zamfir *et al*., 1999). Numerous reviews have suggested that some LAB were able to control the growth of pathogens such as *Listeria monocytogenes* in food products (Callewaert *et al*., 2000; Mataragas *et al*., 2003). However, the only bacteriocin approved for utilization as a preservative in many foods by the US Food and Drug Administration is nisin (Federal Register, 1988), commercially available as Nisaplin™ (Danisco, Copenhagen, Denmark).

1.9.2 Classification of LAB bacteriocin

The bacteriocins of LAB are commonly categorized into three groups: class I—the lantibiotics, class II—the heat stable unmodified bacteriocins, class III—the larger heat labile bacteriocins. Class II can also be subdivided into class IIa, class IIb and class IIc (Nes and Holo, 2000). Most of the bacteriocins of *Lactobacillus* species belong to the class II bacteriocins, which are small (<10 kDa), heat-stable, unmodified peptides of 37–48 amino acids and having high antilisterial activity (Cleveland *et al*., 2001; Deegan *et al*., 2006). Additionally, class IIa bacteriocins can also inhibit growth of some food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium perfringens, Staphylococcus aureus*, and *Escherichia coli* (Barefoot and Klaenhammer, 1983; Rodriguez *et al*., 2005). Class IIa bacteriocins are by far the most investigated with respect to production, structure–function relationship, and have been considered as one of the most interesting and potential groups of antimicrobial peptides for use in food preservation (Eijsink *et al*., 2002; Fimland *et al*., 2005 Calo *et al*., 2008).

1.9.3 Desirable characters of Bacteriocins in food preservation

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation. They are (i) generally recognised as safe (ii) not active and nontoxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) usually pH and heat-tolerant, (v) have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria (vi) show a bactericidal mode of action, usually acting on the bacterial

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cytoplasmic membrane without cross resistance to antibiotics, and (vii) genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Galvez *et al.*, 2007).

1.9.4 Application of Bacteriocins

Bacteriocin-producing LAB have been suggested for use as natural or biopreservative agents to improve safety and storage-life of foods. Bacteriocinogenic strains can be used either directly as starter cultures, as adjuncts or co-cultures in combination with a starter culture, or as protective cultures (Galvez *et al.*, 2007). Ponce *et al.*, (2008) found four LAB strains for application as biopreservatives in minimally processed vegetables. Forty-five strains of LAB isolated from vegetables, were investigated for their antimicrobial activity against taxonomically related microorganisms. It was found to produce bacteriocin-like substances which were active against Gram-positive bacteria and Gram-negative foodborne pathogens like *L. monocytogenes* and *E. coli*. The proteinaceous nature of inhibition was confirmed by inactivating them with proteases. It was stable after extended refrigerated storage and freezing–thawing cycles. Leroy *et al.*, (2003) found that the *Enterococcus faecium* RZS C5, a natural cheese isolate, had a strong activity towards *Listeria monocytogenes*. Hence, the strain may be applicable as a bacteriocin-producing co-culture in food fermentation in order to reduce the risk on *Listeria* outgrowth. Aslim *et al.*, (2005) determined bacteriocin producing ability of 19 LAB's isolated from Turkish fermented dairy products, and their effect against food borne pathogenic bacteria. Only three strains produced inhibitory activities due to bacteriocin-like substances which were resistant to heat. Thus the bacteriocin producing strains or heat resistant bacteriocin may be used as biopreservative in food products. Salim *et al.*, (2006) studied the antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from meat in biofilms, as a real mode of bacterial attachment. It was found that certain bacteriocin-like produces repressed the growth of some undesirable microorganisms in dual species biofilms and thus suggested their used as barrier flora against the settlement of these undesirable microorganisms on the processing surfaces equipment in meat small-scale facilities.

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1.9.5 Benefits of bacteriocins in food preservation

The accumulation of studies carried out in recent years indicates that the application of bacteriocins in food preservation can offer several benefits. (i) An extended shelf life of foods, (ii) Provide extra protection during temperature abuse conditions, (iii) Decrease risks of food borne pathogen transmission through the food chain, (iv) Reduce economic losses due to food spoilage, (v) Reduce application of chemical preservatives, (vi) Permit application of less severe heat treatments without compromising food safety; better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (vii) Permit marketing of "novel" foods (less acidic, with a lower salt content, and with a higher water content), and (viii) Satisfy industrial and consumers demands (Thomas *et al*., 2000).

1.10 Functional foods

It is well known that consumption of plant-based foods, which includes fruits, vegetables, whole grains, cereals and nuts have health benefits and reduce diseases. A functional food can be defined as a food that produces a beneficial effect in one or more physiological functions, increases the welfare or decreases the risk of suffering from a particular disease. Functional foods are obtained from traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health (Miguel *et al*., 2006). These food-stuffs are important for maintaining health, preventing diseases and for added value of products.

There are several raw materials that can be used for healthy purposes and soybeans are one among them. Soybeans are one of most produced and commercialized commodities worldwide. Foods derived from soybeans such as soy milk, tofu and tempeh, and the consumption and use of soybeans by the food industry is increasing every year (Rostagnoa, 2009).

Although soymilk has large sales in the United States, a by-product from manufacturing tofu, okara, has not been utilized into soy foods. Okara is mainly treated as industrial waste or used in animal feeds (Ohno *et al*., 1996; Chan and Ma, 1999). Okara is fibrous residue after soymilk production, with good amino acid profiles. It contains 20 to 27% protein (dry weight basis) and 52% to 58% dietary fiber (Chan and

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Ma, 1999). Genta *et al.,* (2002) used okara to make a soy candy to increase the availability of soy proteins for human consumption and production of soybean products. Thus okara can be utilized to develop and commercialize soy-based snack foods, to improve human health, and reduce environmental wastes (Katayama *et al*., 2008).

Another by-product, soybean whey, has been considered as a waste product by the food industry and its disposal actually constitutes an environmental and the industrial problem. However, soy-whey is a good source of proteins, polyunsaturated fat and bioactive substances, such as isoflavones and oligosaccharides, and thus should not be considered a waste.

Soybean and soybean foods are known to have good nutritional and functional qualities, not only for their high protein and oil content, but also because they are a source of phytochemicals called isoflavones (Dyah *et al*., 2010). Consumption of 25 g soybean protein per day can contribute to the lowering of serum cholesterol levels and the prevention of heart disease (FDA). This health claim places soy foods among a selected category of '**functional foods'** possessing unique medicinal, as well as nutritional value.

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THE PROBLEM

Soy protein is a popular food ingredient for its nutritional and functional properties which is transformed into various forms, among which tofu is a widely accepted soy food. Isoflavones in tofu have been credited with performing several health-promoting functions, like lowering the incidence of breast cancer, prostate cancer, colon cancer and reducing the risk of cardiovascular problem, osteoporosis, menopausal symptoms etc.

But, little information is available on the effect of various coagulants on the level of isoflavones in tofu. Calcium sulphate, calcium chloride, magnesium sulphate and magnesium chloride are different types of coagulants used on an industrial scale for the preparation of tofu. The physical properties such as the hardness, texture, moisture and colour of tofu determine the quality of tofu, because of its bland nature. Hence, evaluation of these quality parameters along with the isoflavone levels in tofu prepared from different coagulants, were carried out in this study.

Soymilk provides high quality proteins containing no cholesterol, gluten or lactose. However, soymilk has often limited human use in Western diets due to undesirable flavour and flatulence caused by high levels of oligosaccharides. Fermentation of soymilk with some organisms, mainly Lactic Acid bacteria (LAB), has been attempted to overcome these limitations. *Lactobacillus, Streptococcus,* and *Bifidobacterium* are the most commonly used organisms for fermentation of dairy products. There is a continuing need to improve existing cultures or to screen new organisms for development of new products. There are many studies on fermentation of soymilk with different species of LAB, but no information about probiotic yeast *Sacharomyces boulardii* related with bioconversion of isoflavones glucosides and growth characteristics in soymilk is available.

Therefore, the objective of this study was to investigate changes in the contents of isoflavone glycosides (daidzin and genistin) and aglycones (daidzein and genistein) and other characteristics (viable cell growth, acid production, and β-glucosidase activity) in soymilk fermented with LAB and *S. boulardii.*

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Lactic acid bacteria are traditionally used as starter cultures for the fermentation of foods and beverages, because of their contribution to flavour, aroma development and to spoilage retardation. The preservative effect is mainly due to acidic conditions that these bacteria create in food during their development, but they are capable of producing and excreting a variety of antimicrobial compounds, including bacteriocins.

In recent years, many bacteriocins have been described to have anti-listeria activity and there is an increasing interest in using them as natural food preservatives in food systems. Bacteriocin producing LAB strains with strong anti-listeria activity have been isolated and characterized.

Processing parameters, coagulant type and concentration, affect the quality and texture of tofu. The different parameters like thermal treatment of soybeans with sodium bicarbonate, solid content of milk, stirring time after adding coagulant and molding of tofu on the texture and quality of tofu were optimized.

The quality of tofu in terms of yield, texture, protein, by coagulating with synthetic coagulants like magnesium chloride and calcium sulphate individually and in combination was studied. Tofu is usually prepared with synthetic coagulants. An attempt was made to prepare tofu with coagulants of plant origin and the antioxidant activity and isoflavone profile were compared to synthetic coagulant.

Based on the reported literature, tofu processors can minimize the contamination during various stages of tofu preparation by studying the microbiological quality of tofu. Hence studies on microbial count of fresh tofu and tofu stored in different containers at 4°C for 12 days were examined for aerobic mesophiles, yeasts and molds. The details of the study are described in the **First part** of the thesis.

Literature survey reveals that, Lactic acid bacteria are used as starter cultures in soymilk fermentation to obtain the bioactive isoflavones, genistein and daidzein. Studies using probiotics lactic cultures and probiotic yeast *Saccharomyces boulardii* showed correlations between the level of growth and β-glucosidase activity of each strain. Nutritional profile, bioavailability of minerals, vitamin B-complex and flavours in fermented soymilk were studied. These results are described in the **Second part** of the thesis

Probiotic Lactic acid bacteria were screened for their antimicrobial properties against pathogenic food borne bacteria and three isolates *L. acidophilus* B4496, *L. casei* B1922 and *P. acidilactici* K7 were selected. Partial characterization of antimicrobial compound determined the molecular size of the compound (4.8 KDa). The survivability of food borne pathogens in the co-cultivation of lactic acid bacterial isolates in soymilk found to be bacteriostatic and bactericidal. The details are described in the **Third part** of the thesis.

By-products of soymilk extraction and tofu coagulation suggested its application to Indian traditional foods like Idli and Vada. Tofu was also added to Tofu masala to improve the nutritional value. A method to use whey as a nutrient in the growth medium of *Monascus purpureus* for the production of natural food colourant. The **Fourth part** of the thesis describes these results.

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Materials and Methods

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2.1 Materials

Soybean and fruits like *Citrus limonum* (Lemon), *Garcinia indica* (Garcinia), *Tamarindus indica* (Tamarind), *Phyllanthus distichus* (Gooseberry) and *Passiflora edulis* (Passion fruit) were procured from local market.

Rice soji (*Oryza sativa*), black gram dhal (*Phaseolus mungo*), gram dhal (*Cicer arietinum*), green chillies (*Capsicum frutescens*), ginger (*Zingiber officinale*), onion (*Allium cepa*), coriander (*Coriandrum sativum*) and curry leaves (*Murraya koenigii*), salt, groundnut oil (*Arachis hypogaea*), chilli powder (*Capsicum frutescens*), garlic paste *(Allium sativum)*, turmeric powder (*Curcuma longa*), garam masala, tomato puree (*Lycopersicum esculentum*), and salt were purchased from a local market.

For storage studies of tofu in different containers, low density polyethylene pouches, earthern pots and steel containers were also purchased from a local market.

2.2 Chemicals

Calcium sulphate and Magnesium chloride used for tofu preparation were obtained from Rankem Fine Chemicals, USA. Ascorbic acid, Potassium Ferricyanide, Trichloro acetic acid and Ferrichloride were obtained from Sisco Research Laboratories, India. The substrate for enzyme assay P-Nitrophenyl-β-D-glucopyranoside (pNPG) was purchased from SRL Chemicals Co. Ltd, Mumbai, India.

The culture medium such as Potato dextrose agar (PDA), Total plate count agar (TPCA), de Man Rogosa Sharpe agar (MRS), Eosin methylene blue agar, Listeria oxford agar, Baird-Parker agar and Brain heart infusion broth (BHI), antifungal agent (Amphotericin B), ion partitioning reagents like pentane sulfonic acid and heptane sulfonic acid were all purchased from Hi-Media Laboratories Pvt Limited, Mumbai, India.

The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

- ❖ Isoflavones genistin, daidzin, genistein, and daidzein
- ❖ 1,1-diphenyl-2-picrylhydrazyl (DPPH) and BHA for antioxidant activity
- ❖ Taka diastase and Papain enzymes for vitamin quantification
- ❖ Pancreatin-bile and Pepsin for minerals estimation

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- Protease, Trypsin and Papain enzymes for protease activity of the lactic acid bacteria

Dialysis tubing of Molecular weight cutoff of 6-8K, was obtained from Spectrapor I. Spectrum Medical Industries, Inc., Queens. USA

The different organic solvents like methanol, ethanol, chloroform and acetone were obtained from Qualigenes Fine chemicals, Mumbai, India.

Reagents for electrophoresis like Tris base, Tricine, Sodium dodecyl sulphate (SDS), acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate, N,N,N',N'-Tetramethylethylenediamine (TEMED), 2-mercaptoethanol were from BDH Laboratories and Sigma Chemical Co., St. Louis, MO, USA. Low molecular weight protein marker was obtained from Bangalore Genei, India.

Molecular mass standard	**KDa**
Ovalbumin	43.0
Carbonic anhydrase	29.0
Soyabean trypsin inhibitor	20.0
Lysozyme	14.3
Aprotinin	6.5
Insulin (α and β chains)	3.0

All other chemicals used were of analytical grade.

2.3 Organisms

Lactic cultures namely *Lactobacillus acidophilus* B4496, *Lactobacillus bulgaricus* CFR 2028, *Lactobacillus casei* B1922, *Lactobacillus plantarum* B4495, *Lactobacillus fermentum* B4655, *Lactobacillus helviticus* B4526, *Lactobacillus amylophilus* B4437*, Streptococcus thermophilus* 074*, Pediococcus acidilactici* K7 *and Lactobacillus lactis* B3 used in this study were obtained from coworkers of Food Microbiology Department, Central Food Technological Research Institute (CFTRI), Mysore, India.

Saccharomyces boulardii was isolated from the dietary supplement sachet 'Darolac' obtained from local drug shop. Their morphological features were reconfirmed

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by staining and observing under phase contrast microscope and Scanning Electron Microscope. Probiotic properties were confirmed by conducting various biochemical tests (Bergey's manual of determinative bacteriology).

Food borne pathogenic bacteria such as *Listeria monocytogenes* Scott A*, Yersinia enterocolitica* MTCC 859*, Staphlococcus aureus* FRI 722*, Enterococcus feacalis* MTCC 5153, *Pseudomonas aeruginosa* CFR 1734 *Enterobacter aerogenes* MTCC 111, *Bacillus cereus* F4810*, Escherichia coli* MTCC 118 and *salmonella typhi* MTCC 733 used as indicator organisms were also obtained from Food Microbiology department.

2.4 Maintenance of cultures

Lactobacillus acidophilus B4496, *Lactobacillus bulgaricus* CFR 2028, *Lactobacillus casei* B1922, *Lactobacillus plantarum* B4495, *Lactobacillus fermentum* B4655 and *Lactobacillus helviticus* B4526 were maintained on MRS agar stabs. *S. boulardii* was maintained on potato dextrose agar slants and stored at 4°C.

Pathogenic food borne bacteria such as *Listeria monocytogenes* Scott A*, Yersinia enterocolitica* MTCC 859*, Staphlococcus aureus* FRI 722*, Enterococcus feacalis* MTCC 5153, *Pseudomonas aeruginosa* CFR 1734 *Enterobacter aerogenes* MTCC 111, *Bacillus cereus* F4810*, Escherichia coli* MTCC 118 and *salmonella typhi* MTCC 733 were cultured in Brain heart infusion agar slopes and stored at 4°C. These isolates were sub cultured twice (1% inoculums, 18 h, 30°C) in 10 mL MRS broth and kept frozen at -20°C in MRS supplemented with 10% glycerol.

2.5 Cultivation of cultures

For lactic acid bacterial cultures, after two successive transfers in MRS broth at 37°C for 12–16 h, the activated culture was again inoculated into MRS broth, incubated at 37°C for 16 h. This was diluted to obtain a population of 6-7 $\log_{10}$ CFU mL^{-1} and served as the initial inoculum.

Yeast *S. boulardii*, after two transfers in Yeast extract peptone dextrose agar medium (YDD) at 30°C, was reinoculated into and incubated at 30°C for 16 h, diluted to obtain a population of 7-8 $\log_{10}$ CFU mL^{-1} and served as the inoculum.

For conventional SEM, cultures grown on MRS stabs and YPD slants were used. Pathogenic food borne bacteria such as *Listeria monocytogenes* Scott A, *Yersinia enterocolitica* MTCC 859, *Staphlococcus aureus* FRI 722, *Enterococcus feacalis* MTCC 5153, *Pseudomonas aeruginosa* CFR 1734 *Enterobacter aerogenes* MTCC 111, *Bacillus cereus* F4810, *Escherichia coli* MTCC 118 and *salmonella typhi* MTCC 733 used as indicator organisms for antimicrobial activities were cultured in Brain heart infusion broth (BHI), at 37°C for 18-24 h before use.

For the extraction of antimicrobial compound, strains were grown in MRS broth for 24 h at 30°C. Cell-free supernatants were obtained by centrifuging the culture broth at 8000 rpm for 20 min at 20°C. To rule out the possibility that the inhibition of indicator organism may be due to the acidification of the media produced by LAB metabolism, pH of the supernatants was adjusted to 6.5.

2.6 Biochemical tests of LAB to characterize probiotic properties

2.6.1 Acid tolerance

The isolates were evaluated for acid tolerance according to the method described by Mishra and Prasad (2005). The solutions were prepared by adjusting double distilled water to pH 1, 2 and 3 with hydrochloric acid solution. Sterile double distilled water (pH 6.5) served as a control. Solutions were autoclaved and stored at room temperature. After thorough mixing 10 mL of each pH solution was taken in sterilized test tubes. A cell suspension containing about 10^8 cells mL^{-1} was added to each pH solution of 1, 2, 3 and control and mixed. One milliliter from each pH solutions was taken at 0, 1, 2 and 3 h and serial dilutions were prepared using 0.85% sterile saline. Appropriated dilutions were pour plated in MRS agar and incubated for at 37°C for 72 h.

2.6.2 Bile tolerance

The bile salt solution (1% and 2% ox gall) was prepared. Sterile double distilled water without ox gall was used as control. All solutions were autoclaved and stored at room temperature. Ten milliliters of each solution was transferred into sterile test tubes. Cell suspensions containing about 10^8 cells mL^{-1} was added to each solution and

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incubated at 37°C. One milliliter of culture was taken out from each tube at 0, 3 and 12 h and dilutions prepared in sterile 9 mL, (0.85% saline) were plated. Plates were incubated at 37°C for 72 h (Mishra and Prasad, 2005).

2.6.3 Antimicrobial activity

To check the antimicrobial activity, the MRS agar plates were overlaid with 7 mL soft BHI agar (0.75 g 100 g^{-1} agar) inoculated with 20 µL of overnight active culture of indicator strains like *Listeria monocytogenes*, *Yersinia enterocolytica*, *Staphylococcus aureus*, *Enterobacter aerogenes* etc, as described in Materials and Methods. After solidification, wells of 5 mm in diameter made in agar plates were filled with 50 µL of 24 h old cell free broth which was neutralized to pH 6.5 centrifuged at 5000 rpm for 15 min. The diameter of zone of inhibition extending laterally around the well was measured and a clear zone of 1 mm or more was considered positive inhibition (Mishra and Prasad, 2005).

2.6.4 Cell surface properties of the isolates

2.6.5 Bacterial adhesion to hydrocarbons (BATH)

This test was assayed according to the procedure described by Rosenberg *et al.,* (1980). Cells were washed once with phosphate buffered saline and resuspended in the same buffer to an absorbance (A) of 0.5 at 600 nm. To this an equal volume of hexadecane or xylene was added. The two phases was thoroughly mixed by vortexing for 3 min. The aqueous phase was removed after 1h of incubation at room temperature and its A_{600} was measured. The percentage of affinity to hydrocarbons was reported as adhesion percentage using the formula

$$\mathbf{Ad\%} = \left[\frac{(\mathbf{A_0} - \mathbf{A})}{\mathbf{A_0}}\right] * \mathbf{100}$$

where A_0 and A are absorbance before and after extraction with organic solvents respectively.

2.6.6 Phase contrast and Scanning Electron Microscopy

Morphology of LAB and yeast *S. boulardii* was studied using phase contrast microscope. Overnight cultures of LAB incubated at 37°C were gram stained and yeast was stained with cotton blue. The structure was observed under Phase contrast Microscope (Olymus BX 40, Japan)

For conventional SEM, the culture grown on MRS stabs and YPD slants were gently removed and fixed in 0.1M potassium phosphate buffer (pH 7.3) containing 2% glutaraldehyde. The fine material was rinsed three times with 0.05 M potassium phosphate buffer and distilled water. After dehydration using ethanol (Asensio *et al.*, 2005), they were mounted on stub coated with gold and was observed under a Scanning Electron Microscope (LEO 435 VP).

2.7 Preparation of soymilk for fermentation studies

Whole soybeans were first cleaned, washed and soaked overnight in distilled water at room temperature for 12-14 h. After decanting the water, it was manually dehulled and the cotyledons were ground in a high speed blender for 3 min using distilled water (1:6 w/v). The resultant slurry was then filtered through double-layered cheesecloth to yield soymilk. Fifty milliliters of soymilk was dispensed into screw cap containers and autoclaved for 15 min at 121°C.

2.8 Preparation of soymilk for tofu

Soybean (200 g) was cleaned and soaked in excess water at room temperature (28 ± 2°C) for 12 -14 h. Hydrated soybean was washed once again to dehull the outer layer and ground with water (1:8 w/v) in a blender. The soybean slurry was indirectly heated in a water bath to avoid charring for 45 min at 85°C with constant stirring. The hot slurry was filtered through double-layered cheese cloth to separate soy milk from residue (okara) and the creamy layer formed was removed after cooling soy milk. The solid content of soy milk was determined using Abbe refractometer (American Optical Mode, 10450) and was adjusted to 9° Brix using water, which resulted in less foam production.

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2.8.1 Blanching of soybeans

Soybeans (200 g) were cleaned and soaked in an excess of water at room temperature (28 ± 2°C) for 12 -14 h.. Hydrated soybeans were washed in tap water and beans were dropped directly to the water to which 1% sodium bicarbonate solution was added and blanched at 85°C for 5, 10, 15 min in three different batches. Each batch was then processed by adding water (1:8 w/v). The soybean slurry was indirectly heated in water bath for 45 min at 85°C with constant stirring. The hot slurry was filtered through double layered cheese cloth to separate soymilk from residue (okara) and the fatty layer formed was removed after cooling the soymilk.

2.8.2 Solid content of soymilk

The solid content of soymilk prepared by the above method was determined using an Abbe Refractometer (American optical mode, 10450) as a degree of Brix and was adjusted to 7°, 8°and 9° Brix using distilled water.

2.8.3 Stirring and moulding of tofu

Soymilk was heated to 95°C for 20 min and cooled to 80°C with constant stirring at room temperature. Each coagulant was added to soymilk and stirred for 5, 10, 15 and 20 min in different batches. Milk was allowed to coagulate for 15 min, without disturbing. The coagulated milk was transferred to plastic porous mold lined with cheesecloth. The curd was pressed with 500, 700 and 1000 g weight and 1000 g initially for 15 min, and then reduced to 500 g weight for the next 15 min. At the end of pressing, the cloth was removed and the tofu was stored in water at 4°C (Sanjay *et al*., 2008).

2.9 Preparation of synthetic coagulants

Calcium sulphate and Magnesium chloride solution (0.2% w/v) were used as coagulants in the preparation of tofu samples. Each coagulant was dissolved completely in 10 mL of cold water and was used immediately. Calcium sulphate was not completely soluble in water, and hence saturated solutions of calcium sulphate obtained with the above concentrations were used.

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2.10 Extraction of natural coagulants

Twenty five grams (fresh weight) of each fruit viz., Garcinia, Tamarind, Lemon, Gooseberry and Passion fruit were soaked individually in 50 mL distilled water for 30 min and ground in a pestle and mortar. The slurries obtained were filtered using cheese cloth and the final volume of the individual extract was made up to 100 mL using distilled water. The extract from each fruit was used as a natural coagulant.

2.11 Preparation of tofu with $CaSO_4$ and $MgCl_2$

Tofu was prepared by coagulating soymilk using Calcium sulphate, and Magnesium chloride individually and in combination at different concentration levels from 0.2, 0.4, 0.5, 1.0 and 1.5% to understand the level of coagulant required.

Soymilk was heated to 95°C for 20 min and cooled to 80°C with constant stirring at room temperature. Coagulant was added to soymilk and stirred for 5 min. The soymilk-coagulant suspensions were allowed to stand undisturbed for a period of 15 min to ensure coagulation. The curd was gently transferred to a perforated mould (12.0 × 12.0 × 4.0 cm) lined with a double layer cheesecloth and pressed for 30 min using 1000 g weight initially for 15 min, and then reduced to 500 g weight for the next 15 min **(Fig. 2.1)**. After pressing, tofu and whey were weighed separately. After the separation of whey, tofu block was cut into pieces of $2.0 \times 2.0 \times 2.0$ cm^3. Tofu was transferred into a plastic bag and stored in a refrigerator till further analysis.

2.12 Preparation of tofu with natural coagulants

Soymilk (200 mL) was heated to 95°C for 20 min and cooled to 80°C with constant stirring at room temperature. For the preparation of tofu, 10 mL of 0.2% synthetic, 20 mL of 2% acidic solution of natural coagulants extract from each fruit obtained as mentioned earlier, was added to soymilk. After the addition of coagulant, soymilk was stirred for 5 min and allowed to coagulate for 15 min without disturbing. Coagulated milk was filtered through cheesecloth lined tofu mold. The curd was pressed with 1 kg weight for the first 20 min and later with 0.5 kg for the next 20 min **(Fig. 2.1)**. The yield of tofu was calculated as the weight of fresh tofu obtained from a specified

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amount of soymilk used for its preparation. The tofu cake was cooled in water and stored in a refrigerator (4°C).

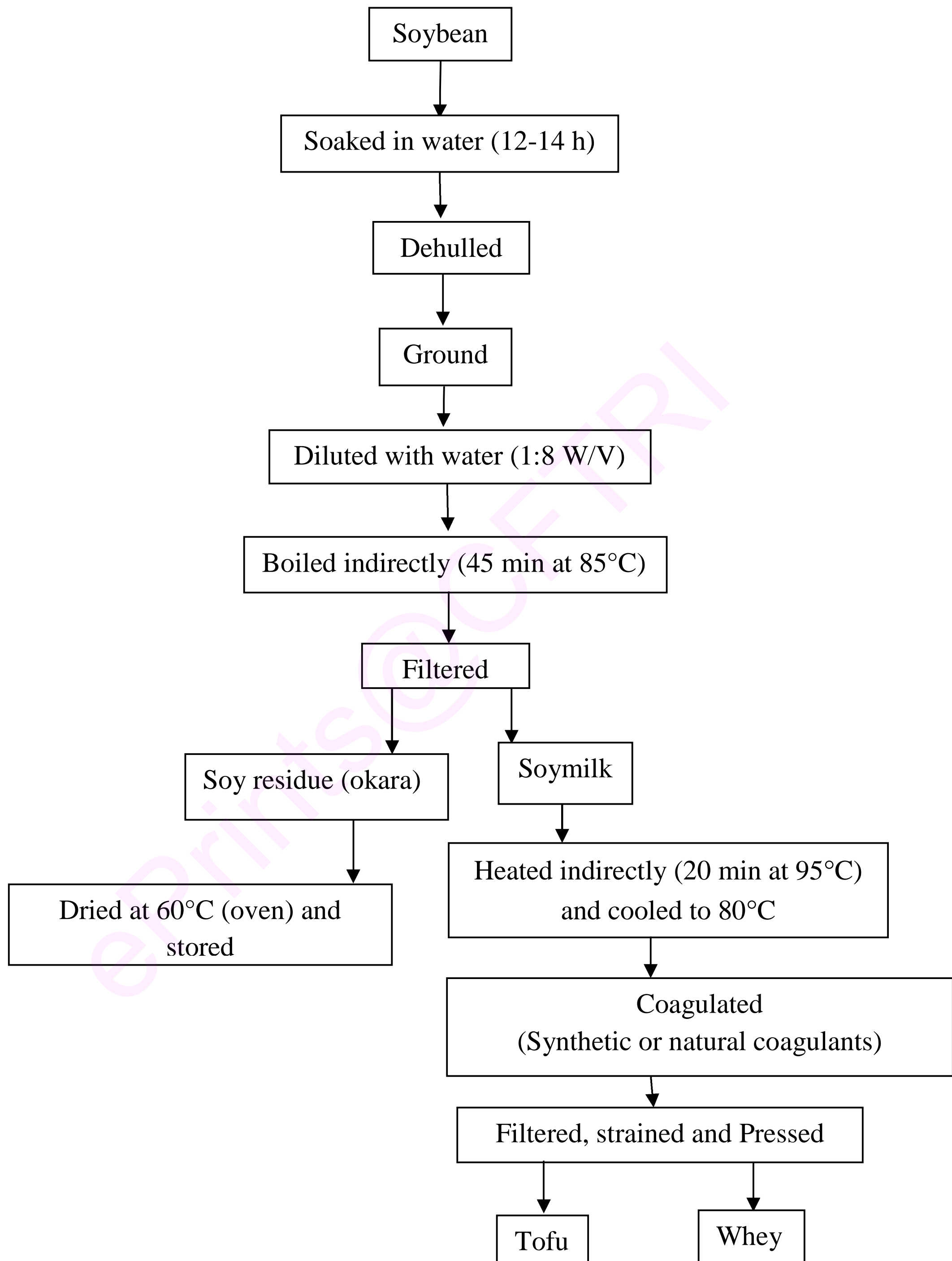

Fig. 2.1. Flow chart for Tofu preparation

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2.13 Microbiological analysis of tofu

Microbiology of soybeans, soaked soybeans, soy slurry, soymilk and tofu samples were analyzed for mesophilic aerobic bacteria, yeast and molds according to procedures outlined in the Compendium of Methods for the Microbiological Examination of Foods with some modifications (Downes and Ito, 2001). Ten grams of tofu sample was aseptically weighed, homogenized with 90 mL of 0.85% saline water (10^{-1} dilution), using a lab-blender at normal speed (2 min) and serially diluted further up to 10^{-10} dilutions with saline water. Appropriate dilutions were used for plating in duplicates. For pour-plating, 1mL of the dilution was taken. For spread-plating, 0.1 mL of the dilution was spread on the surface of a dried plate. After incubation, the colonies appearing on the selected plates were counted and calculated as colony forming units (CFU) per gram of sample.

2.13.1 Total count of mesophilic aerobic bacteria: Total count of mesophilic aerobic bacteria was enumerated by pour-plate method on Total plate count agar (TPCA) and incubated at 37°C (Bacterial Incubator with air circulation, Sri Rudran Instruments, Chennai, India) for 48 h.

2.13.2 Yeasts and molds: For Yeast and mold count, dilutions were spread plated on Potato dextrose agar PDA acidified to pH 3.5 with tartaric acid and were incubated at 30°C for 5 days.

2.13.3 Coliforms: Saline water (0.85%) containing 5 g of tofu was kept at room temperature (28°C) for 1 h for enumeration and pour plated on Eosin Methylene blue agar and the plates were incubated at 37°C for 48 h.

2.13.4 Presumptive Staphylococci: Selective enumeration was carried out by spread plates on Baird-Parker agar media. The plates were incubated at 37°C for 48 h. Typical black colonies with clear halos were counted.

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2.14 Proximate composition of Tofu

Moisture, protein (N X 6.25), fat, ash and crude fibre of tofu, okara and finished products were analyzed as per the Assocation of Official Analytical Chemists (AOAC 1990) procedures. Conversion factor of 6.25 was used to convert nitrogen into protein. Total carbohydrates were determined by difference method, by subtracting from 100, the sum of values for moisture, protein, fat and ash. All determinations were performed in duplicates.

2.15 Yield and Texture analysis of tofu

Yield of tofu was calculated as wet weight/gm of tofu 100 mL^{-1} of soymilk. Textural properties were measured by compression method using a Texture Analyzer (LLOYD texture instrument LR 5K. England) equipped with a 50 kg load cell. Tofu samples (1.5 cm cube) were cut from the central portion of tofu cake (5 cubes from one cake). Measurements were carried out using a weighing beam of 5 kg and a 5 cm diameter cylindrical plunger at a crosshead speed of 20 mm min^{-1}. Hardness, cohesiveness, springiness and chewiness were calculated from the texture profile analysis curve as described by Bourne (1968).

Textural parameters like hardness, cohesiveness, springiness and chewiness were analyzed using force time textural curve. Hardness is the force necessary to attain a given deformation of the material. Tofu with greater hardness means harder and firmer. Cohesiveness is related to work required to overcome the internal bonding of the material. Hence tofu with greater cohesiveness requires more work to break down the internal bonding. Springiness is described as the rate at which a deformed material recovers to its undeformed conditions after the deforming force is removed. Tofu with higher springiness possesses higher elasticity. Chewiness is defined as the energy required to masticate a solid food product to a state of readiness for swallowing and is instrumentally quantified as a product of hardness X cohesiveness X springiness. Therefore Tofu with greater chewiness is stiffer and harder to eat (Cai and Chang, 1997).

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2.16 Determination of antioxidant activity

2.16.1 DPPH free radical-scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was assessed according to Moon and Terao (1998). To 1.0 mL DPPH (500 µM in ethanol), 200 µg□ of lyophilized tofu or 200 µL aliquot of soycurd was added and the reaction mixture was made to 2.0 mL with Tris-HCl buffer (100 mM, pH 7.4). The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm using spectrophotometer (Schimadzu UV-160A, Japan). Reaction mixture without DPPH was used as control.

2.16.2 Inhibition of ascorbate autooxidation

The method described by Mishra and Kovachich, (1984) was used to determine the inhibition of ascorbate autooxidation. Lyophilized extract from tofu of 0.25 µg or 0.1 mL of soycurd was mixed with 0.1 mL of ascorbate solution (5.0 mM, Sigma) and 9.8 mL of 0.2 M phosphate buffer and placed at 37 °C for 10 min. The absorbance of this mixture was measured at 265 nm using spectrophotometer. Similar reaction mixture with distilled water in place of tofu was used as control. The ascorbate autooxidation inhibition rate of the sample was calculated as

$$\%\ \text{Inhibition effect} = \left[\frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} - 1\right] \text{X}100\%$$

2.16.3 Reducing activity

Reducing activity was determined according to the method of Oyaizu (1986). Lyophilized extract from tofu of 0.25 µg or 0.5 mL of soycurd was mixed with 0.5 mL of potassium ferricyanide (1.0%) and 0.5 mL of sodium phosphate buffer (0.02 M, pH 7). This was incubated at 50°C for 20 min and then 0.5 mL of trichloro acetic acid (10%) was added. The mixture was centrifuged at 780 g for 5 min and the supernatant was collected. The supernatant (1.5 mL) was mixed with 0.1% ferrichloride (0.2 mL) and the absorbance was measured at 700 nm using spectrophotometer. Similar reaction mixture wherein tofu was replaced by distilled water was used as control.

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2.17 Shelf life of tofu in Low density polyethylene pouches

Tofu was placed in a polyethylene pouches and stored at 4°C for 2 days. Samples were studied for microbial analysis at an interval of 3 days. All experiments were duplicated and analysis was done as described above.

2.18 Shelf life of tofu in different storage containers

Earthern pots and steel vessels are common storage containers, which were used to study the shelf life. Tofu pieces were immersed in earthern pots and steel vessels containing water which was replaced every day with fresh water. The shelf life of tofu was studied in these containers at 4°C (Refrigerator) for 12 days. Samples were analyzed for microbiological quality every 3 days. All experiments were done in duplicates.

2.19 Bioavailability of nutrients in tofu incorporated with lactic acid bacteria

Sterilized tofu was sprayed with the starter culture *L. casei* (1% of 16 h grown lactic acid bacteria containing approximately 7-8 CFU mL^{-1}) and incubated for 16 h at 37°C, freeze dried and lyophilized. The nutrient content of tofu like fat, protein, ash and isoflavones were examined.

2.20 Fermentation of Soymilk

Whole soybeans were washed and soaked overnight in distilled water. After decanting the water, soybeans were comminuted in a blender for 3 min using distilled water (1:6 w/v). The resultant slurry was then filtered through double-layered cheesecloth to yield soymilk. Soymilk (50 mL) was dispensed into screw cap containers and autoclaved for 15 min at 121°C. Sixteen-hour-old LAB and yeast suspensions, with the OD 600 of 1.0 (approximately 6-7 log and 7-8 log CFU mL^{-1}) in the ratio of 1:1 was inoculated to soymilk and incubated at 37°C for 48 h.

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2.21 pH and Titrable acidity

The pH of the fermented soymilk was measured using a pH meter (Cyberscan-Eutech Instruments, India). To determine titrable acidity, 10 g of sample was taken in a 100 mL conical flask to which 20 mL of distilled water was added. After adding 3-4 drops of phenophthalein, the contents were mixed well and titrated against 0.1 N NaoH to an end point of pale pink colour and expressed as % lactic acid produced (AOAC, 1984).

2.22 Enumeration of LAB

Viable cell counts of LAB were determined in duplicate by using pour plate method on MRS agar media with 2.5 mg L^{-1} Amphotericin B to inhibit the yeast growth (Himedia Mumbai, India). Viable cell count of *S. boulardii* was determined by the spread plate method on Potato dextrose agar medium. Ten grams each of fermented soymilk was added to 90 mL of sterile 0.85% saline (w/v) and vortexed for 30 sec. The resulting suspension was serially diluted with 9 mL of sterile saline. 1 mL of the appropriate dilution was used for selective enumeration by pour plate technique. The cell number of each organism was assessed by enumerating bacterial population on MRS agar after 12, 24 and 48 h of fermentation of soymilk. Plates containing 25 to 250 colonies were counted and recorded as colony forming units (CFU) per gram of fermented soymilk.

2.23 Determination of Polyphenols

Polyphenols was determined using Folin–Ciocalteu reagent (Singleton and Rossi, 1965). The sample (0.1 mL) was mixed with 0.9 mL of distilled water and was extracted for 2 h at room temperature on a mechanical shaker. To this, 1 mL of Folin–Ciocalteu reagent (1:2 dilution) and 2 mL of 10% Na_2Co_3 was added. The mixture was centrifuged at 20,000 g for 20 min, and the supernatant was decanted and filtered through Whatman No. 1 filter paper. The absorbance of the clear supernatant solution was measured at 765 nm (Shimadzu 160 UV A). Gallic acid was used as a standard. Each sample was analyzed twice with duplicates. Results were expressed as milligram Gallic acid equivalent per 100 g dry weight.

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2.24 Protein Hydrolysis

The degree of protein hydrolysis in soymilk during fermentation, expressed as contents of leucine amino equivalent, was determined according to the method described by Adler-Nissen (1979). Samples (225 μL) were mixed with 2.0 mL of 0.10% trinitrobenzensulfonic acid, followed by incubation in the dark for 60 min at 50°C. The reaction was quenched by adding 4.0 mL of 0.1 N HCL solution and the absorbance at 340 nm was measured with a spectrophotometer (Model 7800, Jasco and Tokyo, Japan). L-Leucine (Sigma, St. Louis, USA) was used as the standard to prepare a standard curve.

2.25 Assay for β–glucosidase activity

β–glucosidase activity was assayed by determining the rate of hydrolysis of p-NPG (Matsuda *et al*., 1994). Cells of 14-16 h old were harvested by centrifugation at 3000 rpm for 10 min at 4°C, washed twice with 0.1 M sodium phosphate buffer (pH 7.0) and resuspended in the same buffer. The enzyme activity was determined by incubating mixtures of 0.2 mL of substrate (p-NPG) in 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 mL of an enzyme solution at 37°C for 30 min. The reaction was stopped by adding 0.4 mL of 0.5 M sodium carbonate solution. The amount of p-nitrophenol released in the supernatant was measured at 405 nm using a spectrophotometer (Beckman, 4300 N, and Fullerton, USA). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from the substrate per min.

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2.26 Determination of mineral content in fermented soymilk by simulated digestion of the soy curd

2.26.1 Gastric digest

20 mL sample was suspended in 250 mL conical flask, 70 mL of water was added and pH was adjusted to 2 with 6 N HCl. The solution was kept at room temperature for 5 min and 3 mL of pepsin solution was added. The volume was made up to 100 mL with distilled water. It is then incubated at 37ºC for 2 h on a incubator shaker at 110 rpm. The gastric digest is stored at 0ºC for 90 min and titrable acidity was measured in an aliquot of 20 mL (Dennis *et al*., 1981).

2.26.2 Titrable acidity

The gastric digest is brought to room temperature and an aliquot of 20 mL is taken and 5 mL pancreatin bile mixture was added, titrated against 0.2 N sodium hydroxide till it reaches pH 7.5. Titrable acidity is defined as the amount of 0.2 N sodium hydroxide required to attain a pH to 7.5. The amount of sodium bicarbonate required to perform intestinal digest was calculated as per sodium hydroxide volume required for the titration. Amount of sodium bicarbonate required = Burette reading x Normality of sodium bicarbonate (0.1 N) x sodium bicarbonate molecular weight (Dennis *et al*., 1981).

2.26.3 Intestinal digestion

20 mL of gastric digest was taken in 100 mL flask and equilibrated at 37°C for 10 min. The segments of dialysis tube containing 25 mL of 0.1 M sodium bicarbonate (calculated from titrable acidity) was placed in a conical flask and incubated at 37ºC for 30 min or longer till pH reaches 5.0. Pancreatic mixture (5 mL) was added to the contents and incubated at 37ºC in an incubator shaker for 3 h or pH 7.5 is reached. The dialysis bag was removed and the volume was measured after thorough washing of dialysis bag with distilled water. The dialysate was acidified with 5 mL warm concentrated HCl and made up to 50 mL of volume with distilled water. Mineral concentrations were determined by atomic absorption spectrophotometry (Dennis *et al*., 1981) (ASC-6000, Shimadzu, AA-6701F, Atomic absorption flame emission spectrophotometer) in an acetylene-air flame at the following wavelengths: 422.7 nm (Ca), 248.3 nm (Fe) 285.2 nm (Mg), and 213.9 nm (Zn).

2.27 High Performance Liquid Chromatography (HPLC) analysis of Isoflavones

The stock solutions of each of the standard compounds of daidzin, genistin, daidzein and genistein was prepared by dissolving 1 mg of each in 10 mL of 80% aqueous methanol and were stored in the refrigerator (4°C). Each isoflavone standard solution was injected into the HPLC and the peak areas were determined.

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For HPLC analysis of isoflavones, the procedure of Chiou and Cheng, (2001) was used. One milligram of lyophilized tofu was taken in 10 mL centrifuge tube, 4 mL of methanol (100%) was added to it and the tube was screw capped. After vortexing, the tube was heated at 70°C for 30 min. During heating, the tubes were inverted by hand for agitation at 5 min intervals. The tubes were centrifuged at 20°C at 1800 g for 30 min. One milliliter of the sample was withdrawn from the middle layer, filtered through 0.45 µm and 20 µL of the solution was injected into HPLC system (Model LC 10A, Shimadzu, Japan) and the compounds were detected with UV detector (265 nm). A reversed-phase water C18 Column (Spherisorb ODS 2, 4.6×250 mm) with a gradient solvent system started with methanol 20% (solvent A) and Milli Q water 80% (solvent B) was progressed to 80% methanol and 20% Milli Q water within 16 min followed by holding for an additional 2 min. The flow rate was 1.0 mL min^{-1}.

2.28 Liquid chromatography-Mass spectrometry (LC-MS)

The active fractions collected from HPLC were lyophilized (5 mL) and dissolved in 100% methanol. Mass spectrum of the collected fraction was recorded on instrument HP 1100 MSD series (Palo Alto, CA) by electron spray ionization (ESI) technique with a flow rate of 0.2 mL min^{-1} on C-18 column and total run time of 40 min. Photo diode array was used as detector. These samples were subjected to molecular weight determination using mass spectrometric method by ESI negative fragmentation (LC-MS-Q- Tof ultima, Water's, UK). The conditions were Similar to HPLC (Degenhardt and Winterhalter, 2001).

2.29 HPLC analysis of B-vitamins

The HPLC method of Hou *et al.*, (2000) was followed to determine the content of niacin, riboflavin and thiamin. The mobile phase was prepared by mixing 390 mL HPLC grade methanol, 600 mL distilled deionized water and 10 mL of glacial acetic acid. Twenty-five mL each of Pentane sulfonic acid and Heptane sulfonic acid ion partitioning reagents were added and the solution was filtered through a 0.45 µm membrane and deaerated by vacuum. The flow rate and column temperature was 1 mL min^{-1} and 40°C respectively. The wavelength for the detector was 254 nm.

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2.30 Volatile compounds extraction from fermented soymilk

2.30.1 Simultaneous Steam Distillation and Extraction (SDE)

Likens and Nickerson type SDE apparatus (model 523010-000, Kontes, NJ) was used. Conditions for the extraction of samples were similar to those described by Chung (1999). Samples (60 g each) were taken in a 5 L round bottom flask. One milliliter of internal standard (IA), 2, 4, 6 - trimethylpyridine (10 μg mL^{-1}) and boiled distilled water (400 mL) was added to the sample flask. Redistilled dichloromethane (50 mL) was used as the extraction solvent. Each extraction was carried out for 2 h. Extracts were initially concentrated by a gentle stream of nitrogen gas of 99.99% purity to 15 mL, dried with 2.3 g of anhydrous sodium sulphate, and further concentrated to 0.05 mL and were kept in a freezer (-70°C) until further analyzed.

2.30.2 Gas chromatography (GC)

Sample of 5 µL was injected to Gas Chromatograph (Shimadzu Corporation, Japan): Capillary series gas chromatograph, equipped with a flame ionization detector. A BP1 column was used. oxygen flow of 300 mL min^{-1} and hydrogen flow of 30 mL min^{-1} was employed. GC oven conditions were initially at 35°C, programmed at 2°C min^{-1} until 195°C, and held for 90 min (Chung, 2000). Injector temperature was 10°C and detection temperature was 250°C. Helium carrier gas flow was at 30 cm s^{-1}.

2.30.3 Gas chromatography-Mass Spectrometry (GC-MS)

Five µL of extract was injected at splitless mode into a Turbomass gold mass spectrometer (Perkin Elmer International, Switzerland) coupled with gas chromatograph equipped with turbomass version-4 software with a polar capillary column (Supelcowax 10, 60 m length X 0.25 mm, i.d X 0.25 µm df, Supleco, Inc., Bellefonte, PA). The conditions were similar to GC. MS conditions were as follows: ion source temperature, 230°C: MS quadrupoles temperature, 106°C; electron multiplier, 1160 V; and scan rate, 6.52 scans s^{-1} (Chung, 2000).

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2.31 Screening for antimicrobial activity

Ponce *et al*., (2008) procedure was followed. An agar well diffusion assay was used for detection of antagonistic activity of pathogenic microorganisms. Cell-free-culture supernatants were maintained at pH 6.5 using NaOH as neutralizer to avoid inhibition by acid. MRS agar (1.5 g 100 mL^{-1} media) plates were overlaid with 5 mL of molten BHI (Brain Heart Infusion) agar (0.80 g 100 mL^{-1} media) inoculated with a 14-16 h culture (50 µL) of indicator strain. Wells of 8 mm in diameter were cut and cell-free culture supernatant (50 µL) of LAB strains was placed into each well. The plates were then incubated aerobically for 24 h at 37°C and were subsequently examined for zones of inhibition (6 mm clear or larger zones around the well were scored as positive inhibition).

2.32 Effect of temperature on antimicrobial compound

The thermal stability of the cell supernatant of the neutralized culture was determined by incubating at different temperatures such as 50°C for 15 min, 100°C for 15 min and 30 min and 121°C for 15 min. In all the cases, a positive control, consisting of freshly prepared cell-free supernatants was tested on parallel. The growth was monitored for 24 – 48 h and the antimicrobial activity was determined (Koji *et al*., 2005).

2.33 Effect of pH on antimicrobial compound

The sensitivity of the active supernatant to different pH values was estimated by adjusting the pH of culture-supernatant to pH 3.0, 5.0, 7.0, 9.0 with either 1 N HCl or NaOH. After 2 h of incubation at room temperature, the residual activity was assayed against indicator strains. Non-inoculated MRS broth whose pH values were adjusted to 3.0, 5.0, 7.0 and 9.0 were used as control (Koji *et al*., 2005).

2.34 Effect of proteases on antimicrobial compound

To determine the effects of proteolytic enzymes on bacteriocin, the cell supernatants of the culture, neutralized with 1 N NaOH were incubated with 20 µL of enzyme solutions at 35°C for 1 h. The enzymes protease, trypsin and papain was added to reach a final concentration of 10 mg mL^{-1} (Ammor *et al*., 2006). Samples with and without proteases were incubated for 24 h at 3°C. Residual activity of supernatants was

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determined by the agar-well diffusion method. The absence of inhibition zone in presence of the protease confirmed polypeptide nature of antibacterial substances (Lewus *et al*., 1991).

2.35 Extraction of crude antimicrobial compound

The method described by Burianek and Yousef (2000) was followed for chloroform extraction of bacteriocin. MRS broth (500 mL) was inoculated with 0.1% of 14-16 h culture of the bacteriocin-producing bacterium and incubated for approximately 18 h at 37°C. Cells were pelleted at 7100 g for 15 min in a refrigerated (12°C) centrifuge and the bacteriocin-containing supernatant fluid was collected. Chloroform (250 mL) was added to the supernatant fluid and stirred vigorously using a magnetic stirrer for 30 min, distributed to centrifuge tubes and centrifuged at 10,400 g (12°C) for 20 min. The sediment on the side and/or bottom, and the solids at the interface, were recovered by carefully pouring off the top aqueous layer and holding back the floating interfacial precipitate at the interface with a pipette, then pushing back the interfacial precipitate while pouring off the solvent, which resulted in the interfacial precipitate remaining in the tube. Tris buffer (0.1 mol L^{-1}, 5-10 mL, pH 7.0) was used to resuspend the contents of the tube (sediments, interfacial precipitate and remainders of chloroform and culture medium) and the mixtures were combined in a 50 mL Teflon tube. The combined mixture was centrifuged again at 12,100 g for l5 min and sediments were separated from the remaining chloroform and medium. The pellet was then freeze-dried. The freeze-dried material was resuspended in 1mL of 0.1 mol L^{-1} Tris buffer, pH 7, and assayed for bacteriocin activity against indicator organism.

2.36 Antibacterial activity of crude antimicrobial compound by agar diffusion method

The antimicrobial activity of crude bacteriocin extracted from the above method was screened against three indicator organisms such as *L. monocytogenes* Scott A, *E. coli* MTCC 108 and *S. aureus* FRI 722 by agar well diffusion method. BHI soft agar (w/v) was inoculated with 1% (v/v) of 14-16 h old culture as an indicator strain. Wells were punched in the agar plate and loaded with 50 µL of the chloroform and culture medium,

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interfacial precipitate and bottom sediment layers (The three layers from the top) and zone of inhibition was observed (Ponce *et al*., 2008).

2.37 Survival of indicator microorganisms in soymilk

The survival of indicator microorganisms was investigated by co-cultivating with the bacteriocin producing LAB. Three bacteriocin-producing LAB (*L. acidophilus, L. casei* and *P. acidilactici)* were co-cultivated with *L. monocytogenes and S. aureus* separately in different combinations. Cell concentrations of LAB and S.acerens were $10^7 \log_{10}$ and $10^5 \log_{10}$ CFU mL^{-1} respetively by three flasks containing 100 mL sterile soymilk were taken. To the first flask, 1.0% *S. aureus* was inoculated. The second flask was inoculated with 1.0% *L. acidophilus*, *L. casei* and *S. aureus* and the third flask contained *L. acidophilus*, *L. casei*, *P. acidilactici* and *S. aureus*. All the three flasks were incubated at 37°C for 20 h. Samples were taken for enumeration, at an interval of every 4 h for 20 h. Similar procedure was followed for *L. monocytogenes* with 5 to 6 $\log_{10}$ CFU mL^{-1}.

Enumeration of the cell counts of LAB was determined in duplicates by pour plate method on MRS agar media, *S. aureus* on Baird Parker agar medium for and *L. monocytogenes* on Listeria oxford agar medium. Fermented soymilk (10 g) was added to 90 mL of sterile 0.85% saline (w/v) and vortexed for 30 sec. The suspension was serially diluted in sterile 9 mL saline and 1 mL of the appropriate dilution was used for selective enumeration by pour plate technique. Viability was enumerated at an interval of 4 h to 20 h. Similarly soymilk was co-cultivated with *L. monocytogenes* and the count was enumerated by the above method. The inhibitory effect of the bacteriocin-producing LAB on the indicator organism was determined by comparing the viable count of indicator microorganism in the soymilk inoculated only with *S. aureus* and *L. monocytogenes* which served as controls.

2.38 Tricine-Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS PAGE)

The bacteriocin precipitated using chloroform extract as concentrated by lyophilization and suspended in 10 mM phosphate buffer. The apparent molecular size of bacteriocin was estimated by the Tricine Sodium dodecyl sulfate-polyacrylamide gel

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electrophoresis (Tricine-SDS PAGE). A standard protocol by Schagger and Von Jagow, (1987) was followed. The samples (5 µL) of bacteriocin and molecular weight standard were loaded to Tricine-SDS gel with tris-Tricine running buffer. Electrophoresis was performed in a vertical slab gel apparatus at a constant voltage (100 V). After electrophoresis, the gel was cut into two halves. One half of the gel, which was not stained, was fixed for 2 h in a mixture of 20% isopropanol and10% acetic acid. The gel was then washed in deionised water for 6 h with frequent changes. The gel was stained with silver stain for recovery. The other half of the gel was overlaid with 20 mL of BHI soft agar (0.80%) seeded with freshly grown indicator strain of *L. monocytogenes* Scott A. The plate was incubated at 37°C for 24 h and examined for the presence of zone of inhibition (Bhunia *et al*., 1987).

Molecular mass of the protein was determined by running the standards along the side of protein sample in Tris SDS-PAGE.

Based on the migration of standards, the molecular mass of the samples were determined.

2.39 Preparation of control idli batter

Rice soji (broken rice) and black gram dhal were purchased from local market in one lot for the entire study and they were taken in the ratio of 3:1 for the preparation of batter (Jama and Varadaraj, 1999). Dehusked split black gram dhal (30 g) was washed twice, soaked in 120 mL water for 4 h at room temperature (28 ± 2°C) and ground separately with required quantity of water into a batter of desirable consisitency, using an electrically operated blender. Rice soji (90 g) was washed with water and mixed with dhal batter. The batter was dispensed into commercially available metallized polyester polyethylene pouches of 20X18 cm^2 and heat sealed using a hand sealing machine (Quick seal, Sevana, India) and allowed to ferment for 14 h at room temperature. After every 2 h of fermentation, pH, acidity and the amount of CO_2 released were measured. At the end of 14 h, the fermented batter was distributed into hollow depressions in steaming pans and steam cooked for 10 min.

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2.40 Preparation of okara fortified idli batter

Rice soji (90 g), black gram dhal (15 g) were taken and the batter was prepared as described above. To this 15 g of okara was ground to fine paste by adding little amount of water **(Fig. 2.2)**. This was allowed to ferment for 10 h at room temperature. Total acidity, pH, and the amount of CO_2 released were measured after every 2 h of fermentation time. At the end of fermentation period, the fermented batter was distributed into hollow depressions (moulds) pans and steam cooked.

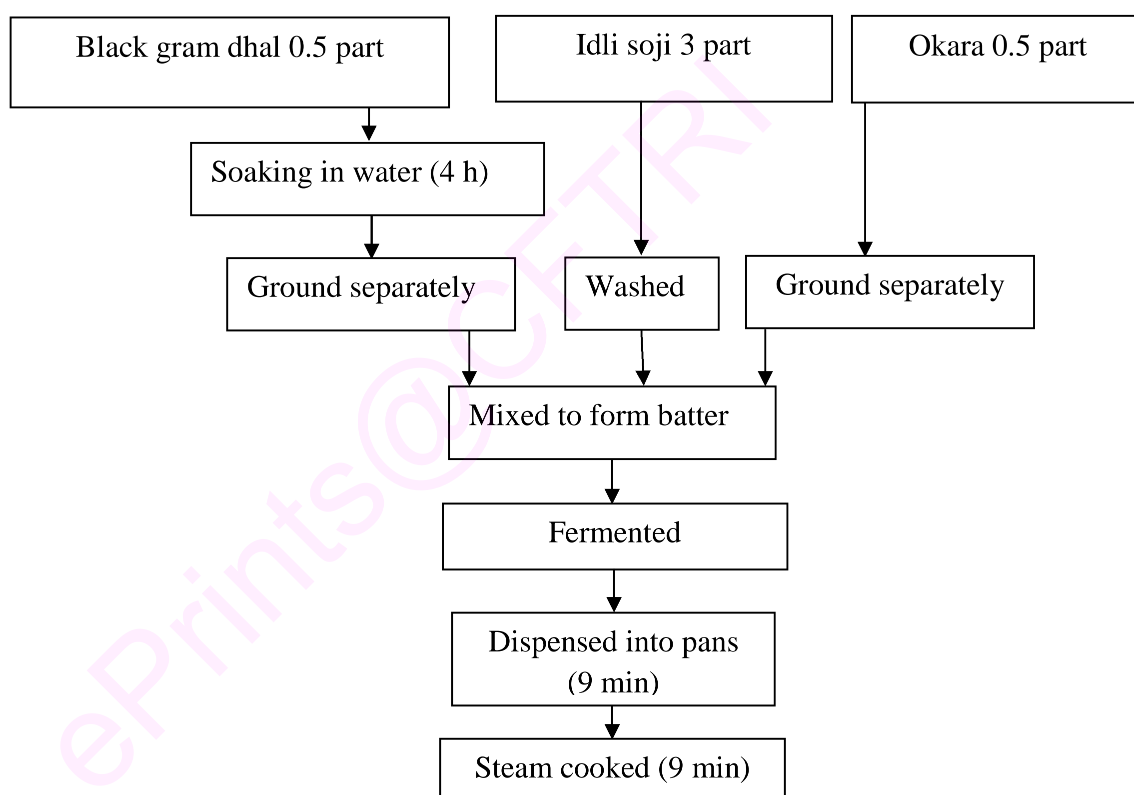

Fig. 2.2 Flow chart for idli preparation

2.41 Preparation of Vada

Gram dhal (100 g) is washed and soaked for 3-4 h. Green chillies (2%) ginger (0.5%), coriander and curry leaves (1%) were added to the soaked dhal and ground coarsely. To this salt (1.5%) and chopped onions (20%) were added. This mixture was divided into two portions. To one portion 20% of okara (dry basis) was added and the other portion was used as control. Flat round patties are made by pressing with moist palm and fried in oil till it becomes golden brown.

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2.42 Preparation of Tofu masala

Soy paneer (tofu) is cut into cubes and fried in 2-3 tb spoon oil. Oil (2 tb spoon) is heated in a vessel and cut onions (25%) were added, till they turned light brown in colour. To this ginger (0.5%) and garlic paste (0.5%) were added. Then chilli powder (3%), tomato puree (20%), turmeric powder (pinch), garam masala (1%) and salt (1%) were added. This was divided into two batches. To one of the batch, tofu cubes (20% supplementation) were added and the other batch was used as control. Both the batches were cooked till it reached semi solid consistency.

2.43 Microbiology of idli batter

The viable count of Lactic acid bacteria (LAB), mesophilic bacteria and yeast and molds of the fermented idli batter (control and okara fortified batter) was determined. Batter of 10 g was mixed with 90 mL of 0.85 % (w/v) sterile physiological saline in a 500 mL Erlenmeyer flask and placed on a rotary shaker at 120 rpm for 30 min. Ten fold serial dilutions were prepared and pour plated on MRS agar for the enumeration of LAB. Spread plate technique was employed to determine the counts of total mesophilic bacteria, yeast and molds using Nutrient agar (NA) and Potato dextrose agar (PDA) respectively.

2.44 Analysis of idli batter

2.44.1 Measurement of increase in batter volume during fermentation

A 50 mL of batter was transferred to a sterile measuring cylinder of 250 mL capacity, covered with aluminum foil and incubated at room temperature for 10 h. The volume of batter at 0 and 10 h of fermentation was recorded and the raise in batter volume was expressed as the % volume increase over the initial volume.

2.44.2 Measurement of CO_2

Amount of CO_2 released by the fermented batter was detected using CO_2 Analyzer (phi Dan sensor, Denmark).

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2.44.3 Extraction of volatiles of idli batter

The extraction of volatiles in idli batter was accomplished with methylene chloride. Idli batter of 1 g aliquots was suspended in 10 mL of methylene chloride and mechanically homogenized. The solvent layer was collected and dried over anhydrous sodium sulphate. The extraction procedure was repeated thrice and the solvent was centrifuged for 10 min at 5000 g at ambient temperature (28 ± 2°C) and concentrated to 1 mL volume. For analysis, 2 µl of sample was taken and injectd for GC/GC-MS (Agrawal *et al*., 2000)

2.44.4 GC/GC-MS conditions

The GC was carried out in a Shimadzu Model 14B (Shimadzu, Kyoto, Japan) gas chromatograph. The volatile constituents were separated using SE-30, 3 m (0.5 mm i.d) column with a flame ionization detector and carrier gas N_2 1 mL min^{-1}. The oven temperature was programmed from 40 to 220°C, at 4°C min^{-1}; injector and detector temperatures were kept at 250°C. The GC-MS analysis was carried in a Gas Chromatograph Mass Spectrometer Model QP-50 (Shimadzu, Kyoto, Japan) using a SE-30 Column (25 M X 0.32 mm) and helium (99.99%) as the carrier gas. The injector and detector temperatures were 250°C. The oven temperature was programmed from 100°C for 6 min holding , 100-150°C (4°C min^{-1}) and 150-220°C (8°C min^{-1})(Agrawal *et al*., 2000).

2.44.5 Texture analysis of the idli

The texture of idli was analyzed using texture analyzer TA–Hdi (Stable Microsystems, Surrey, U.K.) with a cross head speed of 0.5 mm s^{-1} and with 50% compression for hardness and stickiness parameters (Bharthi and Laxmi, 2008). Among the several textural parameters, hardness and stickiness were selected to represent the results because of their repeatability and reasonable variations. Measurements were performed in six replicates and the average was reported in Newtons.

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2.44.6 Sensory evaluation of the idli

Idli prepared with different batters were subjected to sensory evaluation by the method of Quantitative Descriptive Analysis (QDA), employing a trained panel. During initial session descriptors of the product were obtained by "Free choice profiling". Panelists were asked to describe the samples with as many spontaneous descriptive terms as they found applicable. The common descriptors chosen by more than one third of the panel was used in preparing a score card consisting 15 cm scale wherein 1.25 cm was anchored as low and 13.75 cm as high. The panelists were asked to quantify the perceived intensity of attributes by marking a vertical line on the respective scale and writing the code number of the sample. They were also asked to indicate the overall quality of the product on an intensity scale which was anchored at very poor, fair and very good to assess the liking or preference of the product. Sensory traits of control and okara fortified idli shows that panelists described a list of the following attributes namely appearance (buff) manual texture (Fluffiness, compactness, sponginess, firmness) oral texture (sticky) taste (beany, fermented, salty, sour, bitter after taste).

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2.45 Growth medium for *Monascus purpureus*

Monascus purpureus MTCC-410 was obtained from Institute of Microbial Technology (IMTECH) Chandhigarh, India. Stock cultures were maintained on potato dextrose agar slants at 4°C by periodical subculturing. Culture medium was prepared in two batches with 10 g rice taken in 500 mL conical flasks. Rice (10 g) was washed thoroughly with water, drained and 20 ml of distilled water (control) and 20 mL of whey was added separately to two different flasks, sterilized for 20 min at 115°C and cooled to room temperature. The flasks were inoculated with 1.0 mL *M. purpureus* spore suspension in 0.85% NaCl. Inoculated flasks were incubated at 30°C (Adolf Khuner Therm-Lab, Switzerland) for 11 days. The flasks were shaken periodically to ensure uniform mixing.

2.45.1 Extraction and Quantification of *M. purpureus* pigments

Pigments from rice fermented with *M. purpureus* were extracted using polar and non-polar solvents. The extraction was carried out at 30°C by keeping the flasks for 60

min on rotary shaker (110 rpm). The insoluble debris was removed by filtration and the absorbance of the supernatant was determined spectrophotometrically for quantifying the pigments. Optical density was determined at 375, 475 and 500 nm for yellow, orange and red pigments respectively. Pigment yield was calculated as OD Units using the formula

$$\text{OD Units} = \frac{\text{OD X Total Volume of Solvent X Dilution}}{\text{Red Rice (g)}}$$

2.46 Statistical analysis

Data were analyzed by Duncan's multiple range test using statistical package Statistica V 5.5. A significant level was defined as a probability of 0.05 or less. All treatments were done in triplicates.

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Chapter-III

Survival of probiotic isolates and their effect on food spoilage bacteria in soymilk

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C.R. Rekha and G. Vijayalakshmi (2010). Partial characterization of bacteriocin produced by Lactic acid bacteria and their effect on food borne pathogenic bacteria in soymilk. International Journal of Probiotics and Prebiotics (Accepted).

3.1 Introduction

Soybean is a legume rich in phenolics. It is consumed worldwide, most commonly in Asian countries, such as Japan, Korea and Indonesia (McCue and Shetty, 2004). Soybeans are processed into various forms of soy foods and tofu is widely accepted worldwide.

In addition to its popularity in Asian countries, tofu is gaining wide acceptance in the United States and other Western countries. Numerous epidemiological studies have demonstrated an association between the consumption of soybean and improved health, reduced risk for cancers such as prostate cancer, breast cancer, cardiovascular disease, and atherosclerosis (Yamakoshi *et al.*, 2000, Jenkins *et al.*, 2002; Yamamoto *et al.*, 2003). East Asian population, that readily consume soybean and its products, seem to have lower incidence of cancers and oxidation-linked old age diseases that are prevalent in western population who do not traditionally consume soybean foods (Sarkar and Li, 2003).

Soy protein is a popular food ingredient used for its nutritional and functional properties, especially after FDA allowed soy health claim in 1999 (Fukushima, 2001). Tofu is a non-fermented soybean curd and is highly nutritional. Gel-like soy food is one of the important traditional soy foods in the eastern world. The protein is as good as the protein derived from animal source, since it contains all the nine essential amino acids. Western countries have recently increased interest in the benefits of eating tofu to human health and the United States has increased tofu consumption substantially (Fukushima, 1981; Levinton, 1982; Wang, 1984).

Tofu is usually considered as a salt- or acid-coagulated water based gel, with soy lipids, proteins and other constituents trapped in the network (Kohyama *et al.,* 1995). Tofu making procedure involves soaking of beans, grinding, filtering, boiling, coagulating and moulding under pressure and pressed to form a soft whitish tofu (Rehbearger *et al.*, 1984) **(Fig. 3.1)**. Flavour, quality and texture of tofu are significantly influenced by the processing parameters. The factors that affect quality of tofu are

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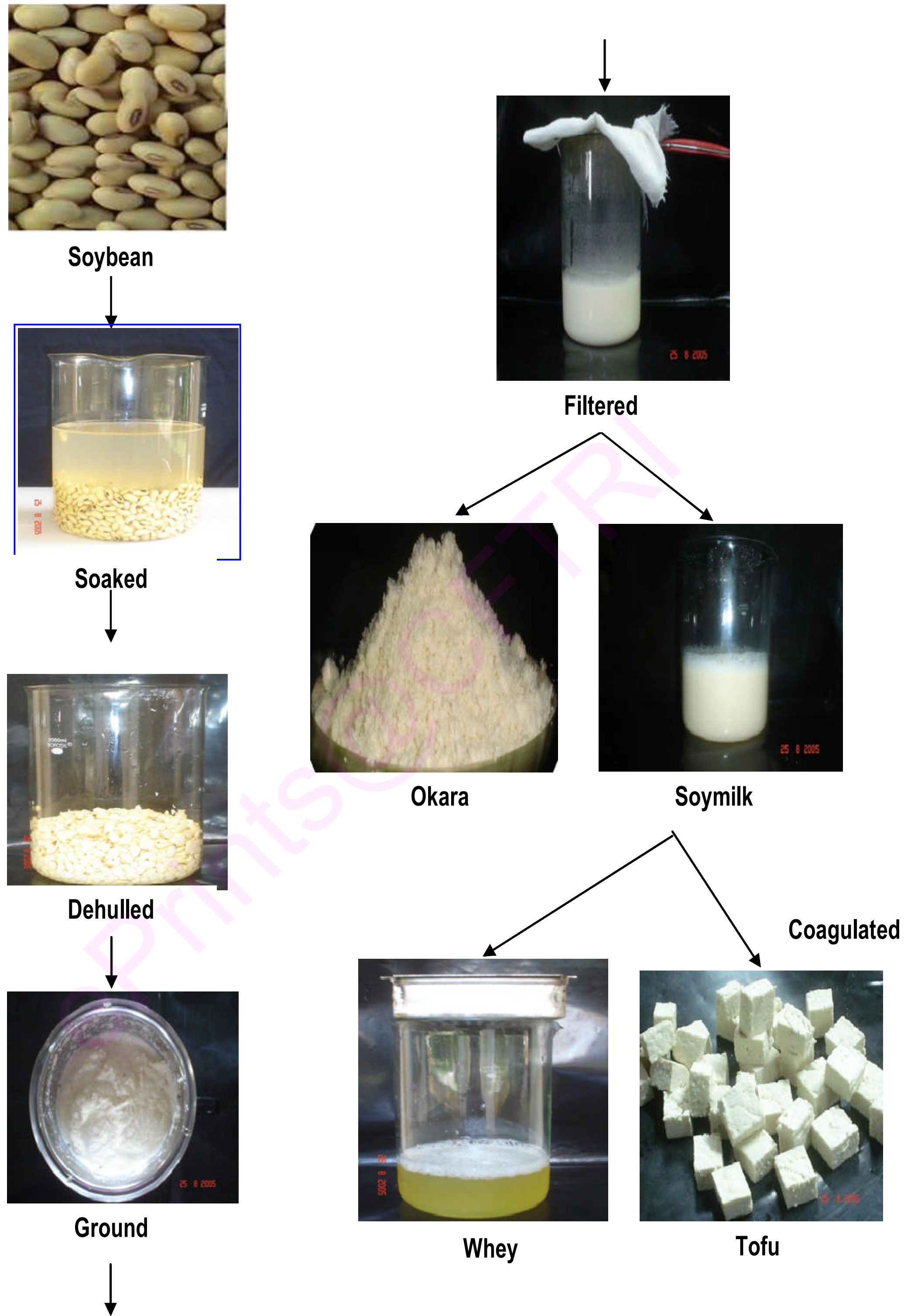

Fig. 3.1 Preparation of tofu

soybean cultivar (Sun and Breene, 1991), processing methods (Beddows and Wong,

1987) and types of coagulant used (Tsai *et al*., 1981, deMan, 1986, Lim *et al*., 1990). The amount of water needed to make soymilk is also important which affects the texture of tofu. Processing factors include soymilk heating rate, stirring time after adding the coagulant, stirring speeds, time for coagulation, temperature for coagulation, pressing time and weight (Hou *et al*., 1997).

The yield, moisture content, textural characteristics and colour of tofu are important to product quality and acceptability (Cai and Chang, 1997). Tofu processors desire a high quality product and high tofu yield. Tofu manufacturers determine acceptability from the yield and texture (Karim *et al*., 1999) as the taste of tofu is affected by its final texture (Kohyama and Nishinari, 1993; Jackson *et al*., 2002).

Based on the above literature, studies were carried out on the processing parameters like blanching of soybeans, solid content of milk, stirring times at fixed speed, pressing time and pressure on yield and texture of soft tofu. The possible sources of contamination during the processing of tofu were determined in order to evaluate the keeping quality of the finished products. The objective of our study was also to determine the effect of type and concentration of coagulants on the proximate composition of tofu in terms of texture and yield.

3.2 Influence of processing parameters on the quality of Soycurd (Tofu)

Coagulation of soymilk is the most important step in tofu making and the most difficult to control because it depends on complex relationships of many variables. Increasing coagulation temperature and rate of stirring immediately after adding coagulant makes tofu hard (Saio, 1979). Stirring method and mixing speed and time have a significant effect on tofu yield and quality (Shurtleff and Aoyagi, 1990). A complex interaction of several chemical factors takes place in making regular, soft and other tofu products.

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3.2.1 Effect of sodium bicarbonate blanched soybeans on the texture of tofu

Alkaline processing of soybeans and other legumes has received much attention. Nsofor *et al*., (1997), reports that blanching denatures soybean proteins, inactivates lipoxygenase enzyme that catalyzes the hydrolysis of unsaturated soybean oil resulting in the production of ketone and aldehydes responsible for the beany odour.

Soybeans were soaked in water at room temperature for 12-14 h. Beans were dropped directly to the boiling water to which 1% sodium bicarbonate solution was added and blanched at 85°C for 5, 10 and 15 min. Each batch was then processed by hand dehulling and ground in a high speed blender blender adding water (1.8 w/v). The soybean slurry was indirectly heated in water bath for 45 min at 85°C with constant stirring. The hot slurry was filtered through double layered cheese cloth to obtain soymilk. Tofu was prepared from soymilk as detailed in Materials and methods.

The texture of the tofu varied depending on the time of blanching (**Table 3.1**). Blanching of soybeans for 5 min, resulted in tofu with hard texture, while blanching for 10 min resulted in regular tofu. Tofu obtained with increased blanching time was not firm. As the blanching time increased, solid content decreased and in turn affected the tofu texture. Tofu prepared with the blanched soybeans had less beany flavor compared to unblanched.

Table 3.1 Effect of sodium bicarbonate blanched soybeans on the texture of tofu

Samples	Blanching time (min)	Tofu texture (N)
Control	_	Hard (7.8)c
$NaHCO_3$ (%)		
1.0	5	Hard (7.6)c
1.0	10	Regular (4.0)b
1.0	15	Soft (3.2)a

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

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3.2.2 Effect of solid content of soymilk on the texture of tofu

The quantity of water used to make soymilk is important because it affects soymilk solid content, quality and texture of tofu. Texture of tofu is an important quality attribute that affect product acceptability as tofu is bland. Tofu is of generally four basic types-soft, regular, firm and dry tofu according to its moisture content and firmness. Soft tofu has a very soft texture and it is difficult to maintain shape after cutting. It usually contains 87-90% moisture. Regular tofu is made somewhat firmer by pressing out more water, wherein the moisture ranges form 82-88%. Firm tofu has a firmer texture than soft and regular tofu, containing 76-81% moisture. Dry tofu is the firmest tofu and has a moisture content usually below76% (Cai and Chang, 1997).

Soymilk prepared by blanching the soybeans with 1% sodium bicarbonate for 10 min was adjusted to a solid content 7, 8 and 9° Brix using distilled water (Materials and Methods).

Low solid content in soymilk results in higher moisture content of tofu (Cai and Chang, 1997) which in turn produces tofu of soft texture. Increased solid content increases hardness of tofu as water retention in the tofu gel is low. The effect of solid content on tofu texture is shown in **Table 3.2**.

Higher solid content in soymilk produced tofu with greater hardness, cohesiveness, springiness and chewiness. Tofu made from soymilk of 9° Brix was harder, firm but elastic, chewier than tofu made from soymilk of lower solid content. Thus solid content of 7° Brix yielded soft tofu while hard tofu was obtained with solid content of 9° Brix.

Hardness of tofu with regard to tofu made with a solid content equivalent to 7, 8, and 9° Brix were 4.39, 5.89 and 6.00 N respectively. Cohesiveness ranged from 0.46 to 0.53 in tofu prepared with 7, 8, and 9° Brix respectively. Good springiness of 3.07 was retained in tofu made with a solid content of 9° Brix. Chewiness of tofu varied from 5.08 to 9.08. Hardness, cohesiveness, springiness and chewiness increased as the solid content increased.

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Table 3.2 Effect of total solid content of soymilk on the texture of tofu

Solid Content (°Brix)	Hardness[1] (N)	Cohesiveness[2]	Springiness[3] (mm)	Chewiness[4] (Nmm)
7	4.39a	0.46c	2.51a	5.08a
8	5.89b	0.49b	2.8ab	7.99b
9	6.00b	0.53a	3.07b	9.08b

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).
[1] Force necessary to attain a 25% deformation of the tofu**.** [2] Work done to break down the internal bonding of tofu. [3] Rate at which a deformed tofu recovers to its original condition. [4] Energy required to masticate solid tofu.

3.3 Effect of stirring time on tofu yield

Soybeans blanched with 1% sodium bicarbonate for 10 min with a solid content of 7° Brix was selected and the effect of stirring time on the yield of tofu was analyzed. Stirring speed and time has a significant influence on tofu yield and quality. Stirring is necessary to keep the coagulant suspended. Speed of stirring and time of stirring should be sufficient to maintain uniform distribution of coagulant in the soymilk.

Lower stirring time of 5 to 10 sec improved the yield of tofu. Increase in stirring time to 15 to 20 sec, lowered the tofu yield as expressed as fresh weight obtained from 100 mL soymilk. No significant difference in the yield of tofu was observed at stirring time ranging from 5-10 sec, but the yield decreased after 20 sec (**Table 3.3**).

Table 3.3 Effect of stirring time on the yield of tofu

Stirring time (sec)	Yield, g 100 mL^{-1} (wet wt)
5	22.5c
10	22.3c
15	21.8b
20	19.16a

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

3.4 Moulding of tofu on the yield and texture

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The pressure and time of pressing of bean curds affects the content and composition of tofu (Cai and Chang, 1999). Pressure applied can also affect the curd texture (Wang and Hesseltine, 1982). Thus tofu was prepared using soymilk with a solid content of 7° Brix, stirring time for 5 sec and blanching with 1% sodium bicarbonate for 10 min followed by molding and pressing to form the desirable texture.

During boiling the soymilk**,** coagulant is added and was allowed to coagulate for 15 min, without disturbing. The coagulated milk was then transferred to plastic porous mold, lined with cheesecloth. The curd was pressed with different weights and the yield was quantified.

The yield and texture of tofu varied with the weight applied and time used for pressing the curd **(Table 3.4)**. A weight of 700 and 1000 g for 30 min resulted in the yield of 22.5 and 22.3 g of tofu (wet wt) with harder texture. Pressing the tofu with a load of 1000 g initially for 15 min followed by 500 g for another 15 min, resulted (22.6 g 100 mL^{-1} of milk) firm tofu. Although the yield was same with the tofu pressed with 500 g weight, the texture was smooth puffy and was not firm.

Table 3.4 Moulding of tofu on the yield and texture

Weight (g) for 30 min	**Yield, g 100 mL^{-1} (wet wt)**	**Texture (N)**
500	22.6	Soft (3.8)a
700	22.5	Slightly harder (6.4)c
1000	22.3	Harder (7.8)d
1000 g wt for initial 15 min followed by 500 g	22.6	Regular (4.2)b

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

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Thus the optimum conditions for soft tofu were blanching of soybeans with 1% sodium bicarbonate for 10 min, soymilk with a solid content of 7° Brix, stirring time for 2-5 sec, and pressing the tofu with 1000 g initial weight for 15 min followed by 500 g.

3.5 Microbiological analysis of tofu during processing

Tofu is a highly perishable food even under refrigeration due to its relatively high pH (5.8 ± 6.2) and moisture content of 80 ± 88% (Lim *et al*., 1990; Shen *et al*., 1991). A number of methods like microwave treatment, coagulation with organic acid, pH adjustment of immersion solutions (Champagne *et al*. 1991; Pontecorvo and Bourne, 1978; Wu and Salunkhe, 1977), use of biopolymer chitosan (No and Meyers, 2004) and oyster shell powder (Kim, *et al.,* 2007) have been reported for extending the shelf-life of tofu. However, none of the above methods have been employed by commercial tofu manufacturers. Thus, there is need for a more practical and efficient method to improve the shelf life of tofu.

Boiling the soymilk eliminates most of the microorganisms but processing results in contamination of the finished product. Food borne pathogenic bacteria, such as Enterobacteriaceae, *Bacillus cereus* and *Staphylococcus aureus* were also found in commercial tofu (Rehberger *et al*., 1984; Van Kooij and De Boer, 1985; Ashraf *et al*., 1999). Tofu is often stored in the retail shops at 4°C for 15-20 days. Hence quality of tofu plays an important role.

3.5.1 Microbiology of raw materials

As tofu contains protein, it is susceptible to microbial growth. Boiling of soymilk before precipitation can effectively eliminate much of the initial load, but pressing the curds to form cakes and handling before packaging allows possible contamination.

The microbiology of tofu was studied during processing and to prevent further multiplication, a suitable packaging material was selected. The microbial load was monitored from the raw material to the end product **(Fig. 3.2)**. The samples were analyzed for aerobic mesophilic bacteria, yeast and molds, Coliforms and Staphylococci according to procedures outlined in the Compendium of Methods for the Microbiological Examination of Foods with some modifications (Materials and Methods). Presently there are no comprehensive standards regarding the bacteriological safety of tofu.

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Sample of 10 g of aseptically weighed, homogenized was mixed with 90 mL of 0.85% saline water (10^{-1} dilution) and serially diluted up to 10^{-10} dilutions. Appropriate dilutions were used for plating in duplicates. Total count of mesophilic aerobic bacteria was enumerated in pour-plates on plate count agar, yeast and molds were spread plated on potato dextrose agar. Coliforms and Staphylococci were enumerated on Eosin Methylene blue agar and Baird-Parker agar media. After incubation, the colonies appearing on the selected plates were counted and calculated as colony forming units (CFU) per gram fresh weight sample.

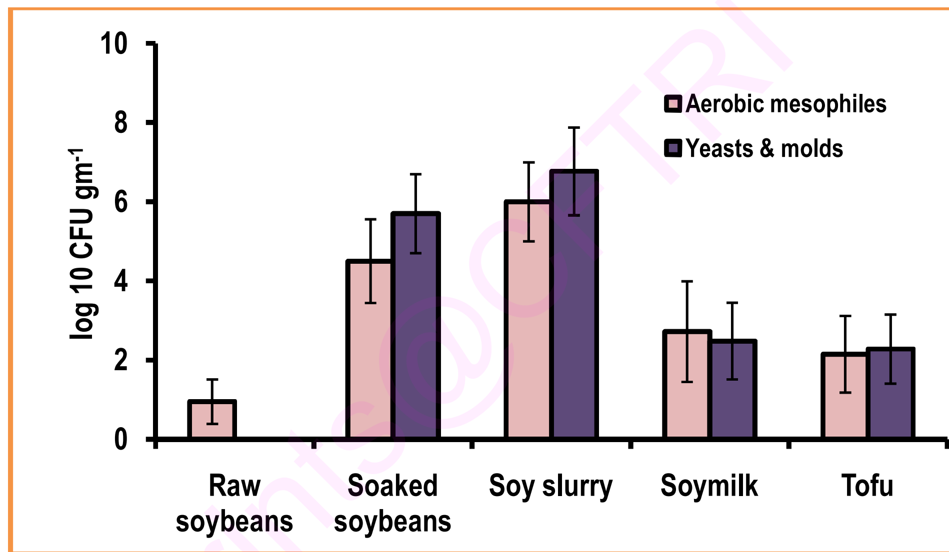

Fig. 3.2 Total aerobic mesophiles, yeast and mold count of raw materials to the final product

Raw soybean before soaking had an aerobic mesophilic bacterial load of 0.95 $\log_{10}$ CFU g^{-1} and yeast and molds were nil. During soaking the aerobic mesophilic bacterial count increased to 4.50 $\log_{10}$ CFU g^{-1} and yeasts and molds count were 5.70 $\log_{10}$ CFU g^{-1}. After grinding, the slurry contained a bacterial count of 6 $\log_{10}$ CFU g^{-1} and yeast and molds were 6.77 $\log_{10}$ CFU g^{-1}. Heating of soymilk reduced the count to 2.72 $\log_{10}$ CFU mL^{-1} of mesophilic bacteria and 2.48 $\log_{10}$ CFU g^{-1} of yeasts and molds.

Although boiled soymilk had lower bacterial and yeast and mold count, fresh tofu had a bacterial count of 2.15 $\log_{10}$ CFU g^{-1} and yeast and mold count was 2.28 $\log_{10}$ CFU

g^{-1}. Coliforms were not detected in any of the samples in the experiment. This may be due to the usage of distilled water.

3.6 Tofu with synthetic coagulants

A number of coagulants have been used in the preparation of tofu. Lu *et al.*, (1980) found calcium acetate and calcium chloride to be good coagulants. Of calcium salts, choice among most tofu makers is calcium sulphate (Wang and Hesseltine, 1982). Other coagulants used are magnesium sulphate and magnesium chloride for the preparation of tofu. Coagulation occurs due to the cross-linking of protein molecules in soymilk with the divalent cations (Prabhakaran *et al.*, 2006).

3.6.1 Tofu prepared using $MgCl_2$ and $CaSO_4$ individually on the quality of tofu

In the preparation of tofu (Materials and Methods) during boiling of soymilk, each coagulant ($MgCl_2$ and $CaSO_4$) was dissolved completely in 10 mL of cold water and was used immediately. Coagulant solution was poured to the milk without stirring and suspensions were allowed to stand undisturbed for a period of 10 min to ensure coagulation. The curd was pressed and yield of tofu and whey were weighed separately. After the separation of whey, tofu block was cut into pieces of $2.0 \times 2.0 \times 2.0$ cm^3. The tofu was transferred into a plastic bag and stored in a refrigerator till further analysis.

Proximate composition of tofu was analyzed following AOAC methods. Texture of the tofu was measured as per detailed in Materials and Methods.

When Calcium sulphate was used as a coagulating agent, it was observed that the moisture content of tofu samples varied depending on the concentration of the coagulant used **(Table 3.5)**. Calcium sulphate coagulated tofu (0.4%) was soft, retained high moisture and produced high yield when compared to other tofu prepared with different concentrations of the same coagulant.

Although highest protein content was observed when 0.4% $CaSO_4$ was used, there was marginal difference in the protein content with different concentrations which was comparable with the commercial product. The fat content was higher in tofu when 1% $CaSO_4$ was used as a coagulant.

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Table 3.5 Preparation of tofu with $CaSO_4$ and $MgCl_2$

Coagulants	w/v, %	Moisture, %	Protein, [a] %	Fat, [a] %	Texture (Hardness N)	Yield[b] %
$CaSO_4$	0.2	72.2±0.64ab	56.5±0.65ab	25.2±0.21a	5.83±0.042a	22.24±0.83ab
$MgCl_2$		68.5±0.28a	52.1±0.94a	25.9±0.81a	5.80±0.035ab	17.80±0.72a
$CaSO_4$	0.4	74.7±0.48b	58.9±0.21b	25.7±0.65a	4.90±0.102ab	25.32±0.68b
$MgCl_2$		71.2±0.35ab	56.7±0.57ab	27.8±0.32b	5.72±0.087ab	19.14±0.24ab
$CaSO_4$	0.5	70.5±0.23ab	56.9±0.56ab	25.8±0.34a	6.63±0.056ab	21.72±0.51ab
$MgCl_2$		73.5±0.79ab	58.6±0.35b	25.4±0.42a	4.66±0.098a	20.36±0.40ab
$CaSO_4$	1.0	72.8±0.48ab	56.1±0.66ab	27.3±0.24b	5.00±0.063a	22.20±0.58ab
$MgCl_2$		73.0±0.94b	58.3±0.80b	25.8±0.56a	4.81±0.044a	20.38±0.63ab
$CaSO_4$	1.5	73.5±0.89b	56.7±0.72ab	26.2±0.40ab	4.96±0.053a	23.30±0.19ab
$MgCl_2$		68.2±0.35a	53.9±0.54ab	26.1±0.38ab	6.22±0.048ab	17.54±0.18a
Commercial product		68.7±0.50a	58.2±0.80b	27.8±0.52b	7.78±0.090b	---

[a]g on dry weight basis, [b]g on wet weight basis. Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

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When magnesium chloride was used as a coagulant, rapid coagulation occurred compared to calcium sulphate. The yield ranged from 17.54 to 20.38 100 mL^{-1} of soymilk. Highest yield was seen in tofu coagulated with 0.5 and 1.0% concentration of magnesium chloride. With the same concentrations, the yield of tofu was more with $CaSo_4$ campared to $MgCl_2$.

The protein content was high when 0.5% of $MgCl_2$ was used individually which were comparable with the commercial product. When compared to $CaSO_4$, the tofu protein was high at 0.5 and 1.0% concentration of $MgCl_2$ **(Table 3.5)**. Higher amount of fat was observed when 0.4% $MgCl_2$ was used. The texture of tofu with 0.5% of $MgCl_2$ concentration was soft compared to other concentrations of the same coagulant and commercial tofu was hard compared to lab prepared tofu.

3.6.2 Combination of $CaSO_4$ and $MgCl_2$ as coagulants on the quality of tofu

Tofu making depends on complex interrelationships of many variables. Calcium and magnesium ions can be used to prepare tofu. However, the sites of cross-linking in the protein molecules may be different for both calcium and magnesium causing the latter to form a loose network. Hence we chose a combination of calcium sulphate and magnesium chloride for tofu coagulation at different concentration, to optimize the concentration of the coagulants.

Maximum yield, moisture, fat and soft textured tofu was obtained when 0.2% (1:1) of $CaSO_4$ and $MgCl_2$ were used. Protein content was not significant at different concentrations of $CaSO_4$ and $MgCl_2$ **(Table 3.6)**. The fat content was similar to the commercial product. Tofu was soft and firm when coagulated with different concentration of $CaSO_4$ and $MgCl_2$, whereas the commercial product was hard.

Calcium sulphate and magnesium chloride coagulants of 0.2% (1:1) ratio was best suitable coagulant for tofu preparation in terms yield, proximate composition and texture

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Table 3.6 Preparation of tofu with combination of $CaSO_4$ and $MgCl_2$ as coagulants

Coagulants ($CaSO_4$ & $MgCl_2$)w/v(%)	Moisture %	Protein[a] %	Fat[a] %	Texture (Hardness N)	Yield[b] %
0.2[c]	78.5±0.38b	56.3±0.80a	27.8±0.21b	4.11±0.098a	23.2±0.18b
0.4[c]	74.9±0.43a	56.4±0.42a	26.5±0.16ab	5.14±0.088ab	20.8±0.14a
0.5[c]	73.4±0.66a	56.5±0.88a	25.4±0.20ab	5.13±0.104ab	20.2±0.18a
1.0[c]	76.2±0.32ab	56.3±0.60a	24.5±0.22a	5.02±0.092ab	21.3±0.16ab
1.5[c]	76.5±0.50ab	56.4±0.56a	25.6±0.18ab	4.46±0.096ab	22.3±0.16ab
Commercial product		58.2±0.61a	27.8±0.20b	7.72±0.090b	---

[a]g on dry weight basis, [b]g on wet weight basis. [c](1:1) ratio of $CaSO_4$ & $MgCl_2$. Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

3.7 Influence of natural coagulants on the isoflavones and antioxidant activity of tofu

Researchers have postulated that the health benefit of soybean may be due to a specific group of phenolic compound, found uniquely within soybean, known as isoflavonoids. It may be due to its estrogenic effect or antioxidant activity (Lee *et al.,* 2004). Isoflavones are phytochemicals that exist in two basic categories, the aglycones and the glycosidic conjugates. The main glycosidic isoflavones are daidzin and genistin and the main aglycones are daidzein, and genistein (Batt *et al.,* 2003). However, it is the aglycone (glucoside-free) form of isoflavones that is metabolically active (Yuan *et al.,* 2003). It possess higher antioxidant activity and gets absorbed in the intestines faster than their glucoside bound form (Murota *et al.,* 2002; Rao and Muralikrishna, 2002; Setchell *et al.,* 2002). In addition, aglycones have been reported to be more stable than isoflavone glycosides during storage at different temperatures (Otieno *et al.,* 2006). Consequently, providing food products with aglycones would be considered as a novel trend for the food industry (Pham and Shah, 2007). According to Wang and Murphy

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(1994), each gram of tofu contains 0.532 mg of isoflavones. In another study, the total isoflavone content in raw tofu and cooked tofu was found to be 0.297 mg g^{-1} and 0.258 mg g^{-1} respectively (Franke *et al.,* 1999). Its original content in soybeans and extent of loss in whey during recovery of soybean curd determine variation of isoflavone contents in tofu products.

3.7.1 Preparation and proximate composition of tofu

Tofu was prepared using synthetic and natural coagulants **(Fig. 3.3)** as described in Materials and Methods. For the preparation of tofu, 10 mL of 0.2% synthetic, 20 mL of 2% acidic solution of natural coagulants extract from each fruit was added to soymilk. Tofu prepared using *Citrus limonum* had the highest moisture content (80.4%) followed by *Garcinia indica* (75.6%) and *Phyllanthus acidus* (75.3%), which is reflected in lower yield of whey **(Table 3.7)**. Whey from tofu prepared using the coagulants listed in Table 7, was clear indicating that the level of coagulants added was sufficient for complete coagulation of soy proteins. Highest yield of tofu was observed in tofu coagulated with *Garcinia indica* extract (22.5 %) followed by synthetic coagulant (21.3 %) and extract of *Passiflora edulis* (21.3 %) while the yield was less in tofu prepared with the extract of *Phyllanthus acidus* (15.6 %). Protein content was more in tofu coagulated with the extracts of *Garcinia indica* and *Tamarindus indica (*72.5%) followed by *Passiflora edulis* (70.0%), *Citrus limonum* (69.0%) *Phyllanthus acidus* (58.2%) and lowest was in the tofu prepared with synthetic coagulant (56.33%). Fat content was maximum in tofu prepared using *Citrus limonum* coagulant (27.8% db) and minimum in synthetic tofu (22.0% db).

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3.7.2 Antioxidant activity of tofu

Antioxidant property, specifically the radical scavenging activity, is important in foods and in biological systems for the ability to remove free radicals. Formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance (Min, 1998). Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in initiating oxidation reactions associated with aging (Wickens, 2001).

A

B

C

D

E

Fig. 3.3 Natural coagulants A. *Citrus limonum* B. *Tamarindus indica* C. *Garcinia indica* D. *Phyllanthus acidus* E. *Passiflora edulis*

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Table 3.7 Yield and proximate composition of tofu prepared using synthetic and natural coagulants

Coagulants	Yield, g 100 ml^{-1} soy milk	Moisture, %	Volume of whey 100 ml^{-1} of soy milk	Fat, % db	Protein, % db	Ash, % db
*Synthetic coagulant	21.3±0.16b	74.9±0.52ab	83±0.42a	22.0±0.21a	56.3±0.86a	2.3±0.03ab
Lemon	17.6±0.18ab	80.4±0.48b	82±0.68a	26.2±0.24ab	69.0±0.90b	2.6±0.02bc
Tamarind	21.0±0.14b	71.2±0.46a	85±0.50a	24.0±0.21ab	72.5±0.84b	2.0±0.08a
Garcinia	22.5±0.16b	75.6±0.34ab	82±0.64a	23.7±0.20ab	72.5±0.98b	2.8±0.02c
Gooseberry	15.6±0.16a	75.3±0.48ab	81±0.50a	26.0±0.18ab	58.2±0.40a	2.8±0.03c
Passion fruit	21.3±0.18b	72.1±0.56a	86±0.60a	27.8±0.20b	70.0±0.80b	2.6±0.04bc

db: dry basis. Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3). *($CaSO_4$ + $MgCl_2$)

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3.7.2.1 DPPH scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was assessed according to Moon and Terao (1998) (Materials and Methods). The free radical scavenging activities (FRSA) of tofu coagulated with natural coagulants were comparatively higher than the tofu with synthetic coagulant (Table 8). Tofu prepared with garcinia extract showed the highest FRSA (82.1%) and the least antioxidant activity was observed in synthetic coagulant (63%) **(Table 3.8)**.

3.7.2.2 Inhibition of ascorbate autooxidation

Lyophilized extract of tofu (0.25 µg) was mixed with 0.1 mL of ascorbate solution (5.0 mM) and 9.8 mL of 0.2 M phosphate buffer and placed at 37°C for 10 min. The absorbance of this mixture was measured at 265 nm using spectrophotometer.

Tofu prepared with synthetic coagulant showed lowest ability to inhibit ascorbate autooxidation **(Table 3.8)**. On the other hand, tofu prepared with natural coagulants significantly increased the inhibition rate of ascorbate autooxidation which may be due to polyphenols present in the fruits. Inhibition of ascorbate autooxidation ranged from 36.8-70.3% depending on the coagulants used. Tofu prepared with the extracts of *Passiflora edulis* exhibited the highest inhibition of ascorbate autooxidation (70.30%).

3.7.2.3 Reducing activity of tofu

Reducing activity of tofu is expressed as an equivalent amount of cysteine (µM). Tofu coagulated with the extracts of *Passiflora edulis* exhibited the highest reducing activity (0.565 µM) among the natural coagulants and lowest in synthetic coagulant tofu (0.485 µM) **(Table 3.8)**. In addition to the fruit extract with antioxidative activity, soybean isoflavones may also add to the antioxidative activity.

3.7.3 HPLC analysis of isoflavones

The procedure of Chiou and Cheng (2001) was followed for the quantification of Isoflavones. One milligram of lyophilized tofu was taken in 10 mL centrifuge tube, 4 mL of methanol was added to it and the tube was screw capped. After vortexing, the tube was heated at 70°C for 30 min. The tubes were centrifuged at 20°C at 15000 rpm for 30 min. One milliliter of the sample was withdrawn from the middle layer, filtered through

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0.45 µm and 20 µL of the solution was injected into HPLC system and the compounds were detected with UV detector (265 nm).

Table 3.8 Antioxidant activity of tofu coagulated with synthetic and natural coagulants

Coagulants used	**Antioxidant activity**		
	DPPH scavenging, %	Inhibition of ascorbate auto oxidation, %	Reducing activity equivalent cysteine, µM
Synthetic tofu	63.1±0.64a	36.9±0.42a	0.485±0.006a
Lemon	69.1±0.82a	37.3±0.34a	0.487±0.004a
Tamarind	80.4±0.57b	57.2±0.67c	0.540±0.003c
Garcinia	82.1±0.92b	45.3±0.56b	0.540±0.006c
Gooseberry	82.0±0.80b	57.8±0.45c	0.503±0.004b
Passion fruit	81.3±0.68b	70.3±0.80d	0.565±0.008d

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (n=3).

The HPLC chromatogram of isoflavones of tofu coagulated with *Citrus limonum* is shown in **Fig. 3.4 B**. The glycosidic isoflavone genistin and daidzin were abundant in soymilk but aglycones were in less concentration. In soymilk, the daidzin and genistin contents were 0.282 mg g^{-1} and 0.030 mg g^{-1}, respectively (dry weight). In contrast genistein and daidzein were quantitatively lesser in soymilk accounting to 0.003 mg g^{-1} and 0.007 mg g^{-1} respectively.

Tofu coagulated with synthetic and coagulants of plant origin had the isoflavone glycosidic content ranging from 0.310-0.471 mg g^{-1} of tofu and aglyconic content ranging from 0.308-0.430 mg g^{-1} of tofu. The differences between the two were not significant in glycosidic and aglyconic form of isoflavones **(Fig. 3.5)**. Basically tofu contains both glycosides and aglycones. The aglycones are generated due to the action of the soybean's native β-glucosidase during soaking of soybean for soymilk and tofu production. There is a decrease in daidzein compared to its conjugate daidzin. Genistein contributed to the greatest concentration of aglyconic form (0.235-0.334 mg g^{-1} of tofu)

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than daidzin (0.075-0.96 mg g^{-1} of tofu) in tofu coagulated with synthetic and coagulants of plant origin. There was an average of 47.4% of the original glycosides bioconverted into aglycones, which is represented in **Fig. 3.6**.

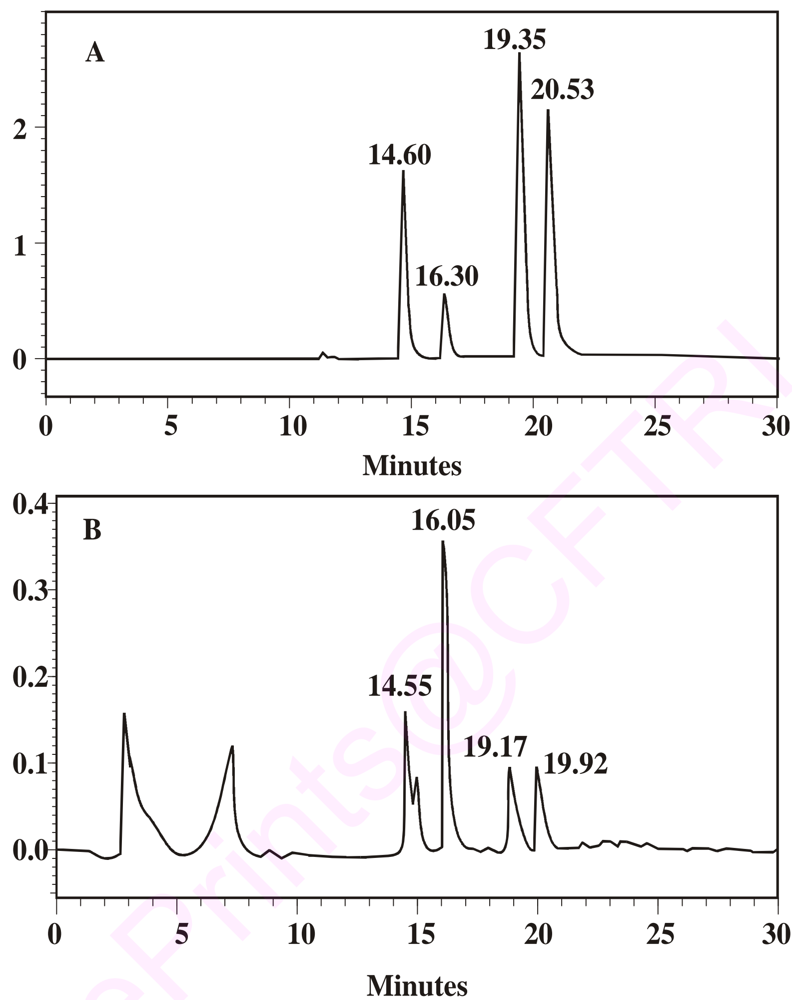

Fig. 3.4 HPLC chromatograms of isoflavones in tofu

A. HPLC chromatogram showing the retention time of standard isoflavones. Daidzin (14.60 min), Genistin (16.30 min), Daidzein (19.35 min) and Genistein (20.53 min).

B. HPLC chromatogram showing the retention time of isoflavones in *Citrus limonum* tofu: Daidzin (14.55 min), Genistin (16.05 min), Daidzein (19.17 min) and Genistein (19.92 min).

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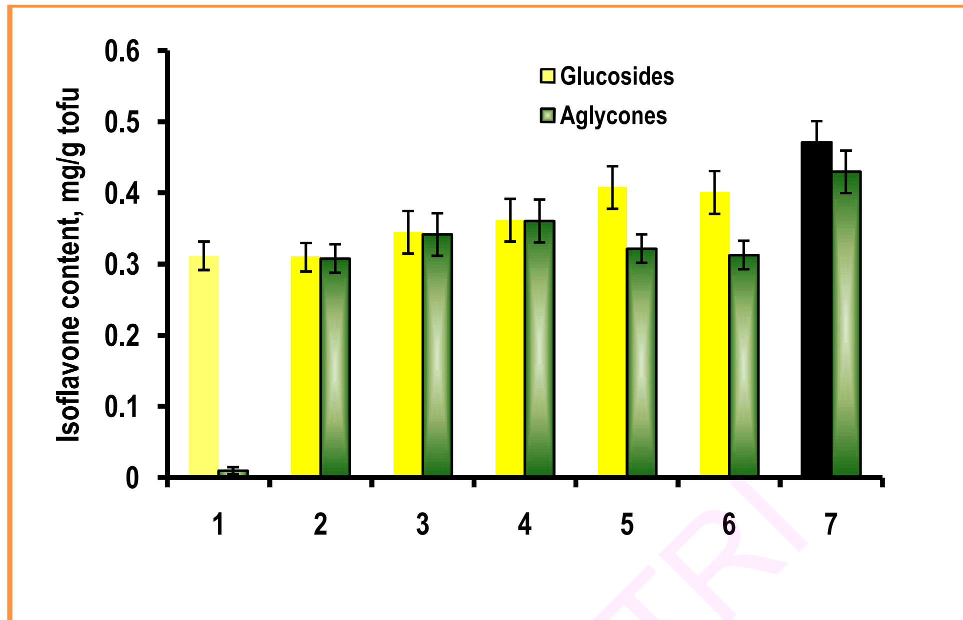

Fig. 3.5 Isoflavone content in soymilk and tofu coagulated with synthetic and natural coagulants (n=2) 1. Soymilk 2. Synthetic tofu 3. Lemon 4.Tamarind 5. Garcinia 6. Gooseberry 7. Passion fruit

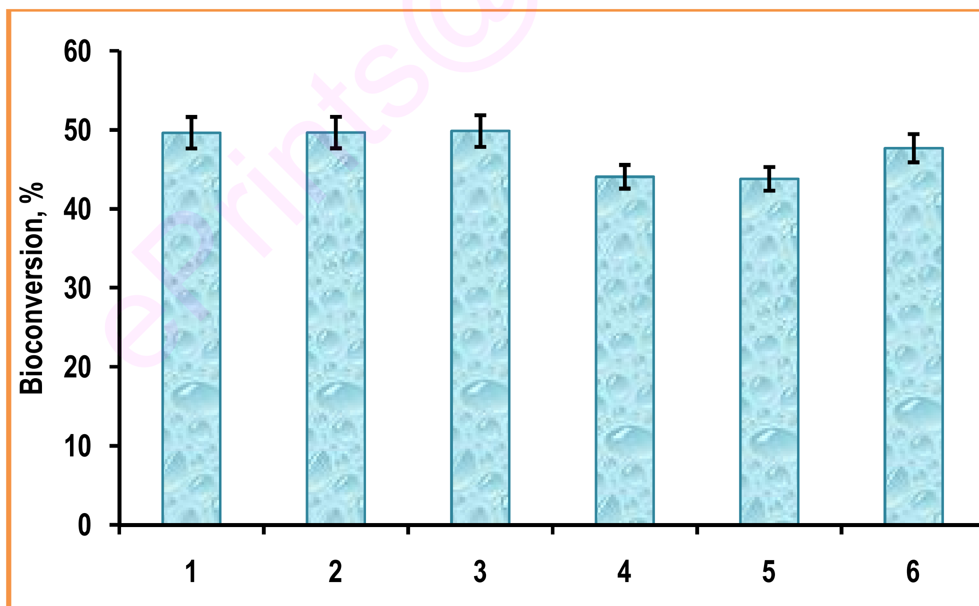

Fig. 3.6 Bioconversion of glycosides to aglycones in tofu coagulated with synthetic and natural coagulants (n=3). 1. Synthetic tofu 2. Lemon 3. Tamarind 4. Garcinia 5. Gooseberry 6. Passion fruit

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There was an increase in protein and antioxidant activity in tofu coagulated with natural coagulants. Fruit extracts, which are water soluble, rich sources of vitamins, carotenoids and other bioactive molecules, could be an alternative to synthetic coagulants in the preparation of tofu. Tofu prepared with lemon extract was the most preferred tofu which had a smooth, soft, but firm texture with whitish colour. The presence of more water soluble glycosidic isoflavones did not decrease the antioxidant potency of tofu. It is further contributed by the phytochemicals like polyphenols.

3.8 Shelf life of tofu stored in low density polyethylene pouches

The microbiology of tofu during processing and tofu stored in different storage containers like low density polyethylene pouches, earthern pots and stainless steel vessels were studied. The possible sources of contamination during the processing of tofu were determined in order to evaluate the keeping quality of the finished products.

Many manufacturers pack tofu in water packed sealed plastic pouches and store at 4°C in the refrigerator. Hence the microbiological study stored in low density polyethylene pouches was studied. The tofu prepared was placed in polyethylene pouches and stored at 4°C for 12 days and microbial analysis was done as described above. Samples were analyzed every 3 days.

Fig. 3.7 shows the changes in the aerobic mesophilic counts of tofu packed in Low-density polyethylene pouches for 12 days at 4°C. The initial aerobic mesophilic bacterial count of fresh tofu was 2.15 $\log_{10}$ CFU g^{-1}. There was a rapid increase until 12 days reaching a count of 8.02 $\log_{10}$ CFU g^{-1}. Molds and yeasts count which was initially 2.28 reached 3.46 $\log_{10}$ CFU g^{-1} on 12th day. With the increase in days, there was a gradual increase in yeast and molds count.

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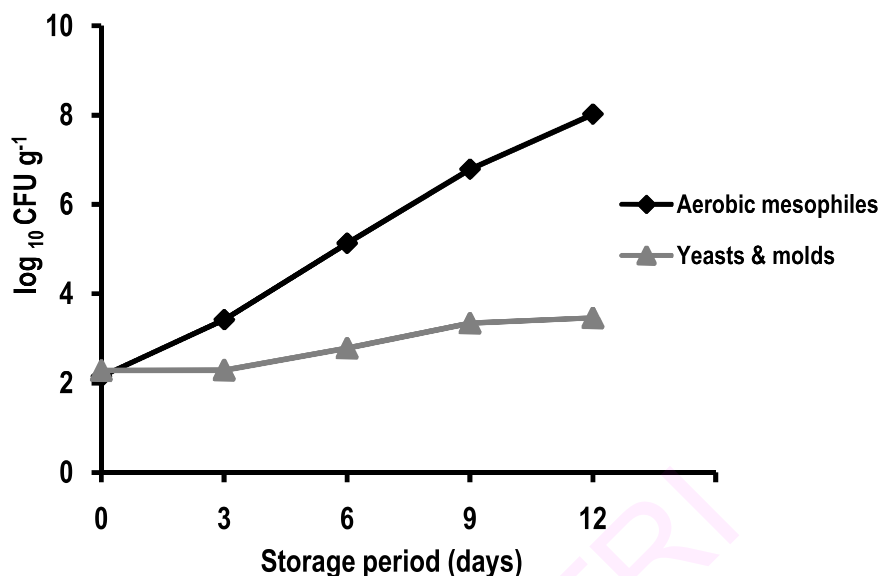

Fig. 3.7 Changes in the number of total bacterial and fungal count of tofu stored in low density polyethylene pouches at 4°C for 12 days

3.9 Shelf life of tofu stored in different storage containers

Earthern pots and steel vessels are common storage containers and these were used to study the shelf life. Tofu pieces were immersed in earthern pots and steel vessels containing water which was replaced every day with fresh water. The shelf life of tofu was studied in these containers at 4°C (Refrigerator) for 12 days at an interval of 3 days.

Earthern pots are traditionally used as storage containers. An earthern pot is a cooking pot made from clay which can be used on the stovetop, but more commonly they are used in the oven to make specialized dishes, and they may also be used in fires and buried pits for various regional foods. In our experiment, we used it for the storage of tofu.

Among various types of materials used for food contact surfaces of processing equipment, stainless steel is most widely employed because of its mechanical strength, corrosion resistance, longevity, and ease of fabrication (Holah and Thorpe, 1990). Cleaning and sanitation are very important as microbial cells on equipment surface could survive and cause cross-contamination leading to lowered shelf-life, food spoilage, and transmission of disease (Ortega *et al.,* 2009).

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The total aerobic mesophilic count and yeasts and molds count of storage containers is shown in **Fig. 3.8 & 3.9**. There was a gradual increase in aerobic mesophiles in tofu stored in earthern pots which reached 6.38 $\log_{10}$ CFU g^{-1} on 9th day and with further storage, there was only a marginal increase till 12th day (6.53 $\log_{10}$ CFU g^{-1}). In tofu stored in steel containers, the aerobic mesophilic count gradually increased upto 12 days.

The yeasts and molds count initially which was 2.28 $\log_{10}$ CFU g^{-1} in earthern pots reached 5.38 $\log_{10}$ CFU g^{-1} on 12 days of storage, whereas in steel containers, the count was higher compared to earthern pots, with a difference of 0.43 $\log_{10}$ CFU g^{-1}.

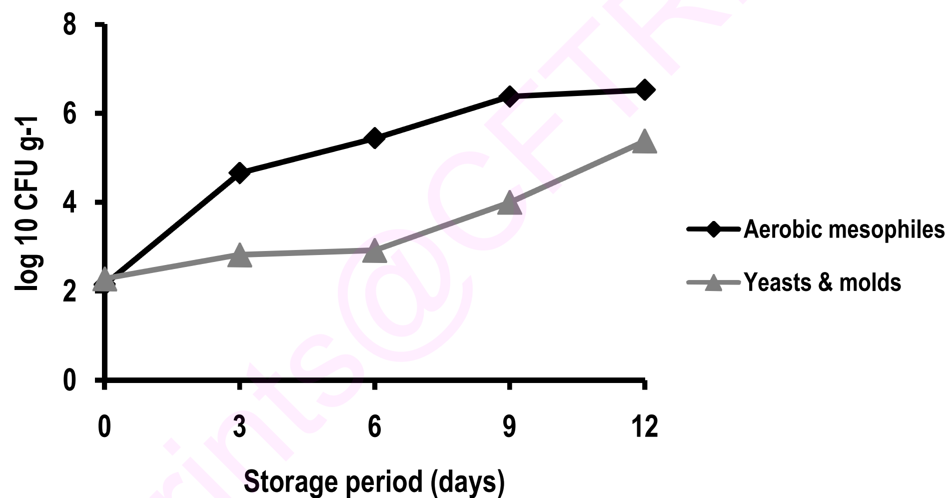

Fig. 3.8 Changes in the number of total bacterial and fungal count of tofu stored in earthern pots at 4°C for 12 days

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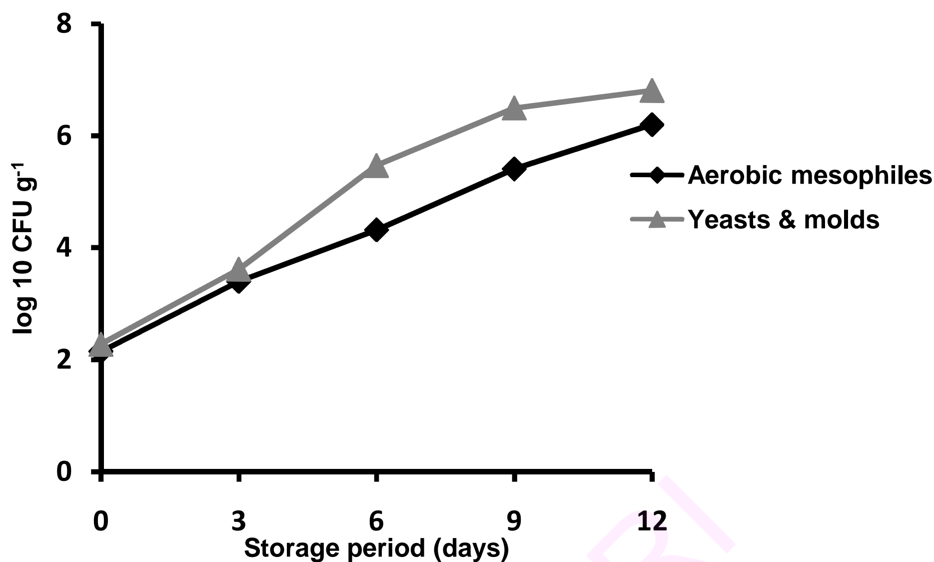

Fig. 3.9 Changes in the number of total bacterial and fungal count of tofu stored in steel vessels at 4°C for 12 days

Microbiological analysis of tofu thus revealed that although raw materials used for the preparation of tofu showed fluctuation in aerobic mesophilic count and yeasts and molds count, the count in fresh tofu was less due to boiling of soymilk. Most of the foods are susceptible to microbial growth due to longer storage time between preparation and consumption under improper temperature conditions (30-38°C). Thus the shelf life of tofu studied in low density polyethylene pouches revealed that tofu can be stored for 9 days, at 4°C. Steel containers and earthern pots were better than low density polyethylene pouches for tofu storage.

3.10 Bioavailability of nutrients in tofu with reference to lactic acid bacteria

Sterilized tofu was sprayed with the starter culture *L. casei* (1% of 16 h grown lactic acid bacteria containing approximately 7-8 CFU mL^{-1}) and incubated for 16 h at 37°C, freeze dried and lyophilized. The nutrient content of the tofu like fat, protein, ash and isoflavones were examined.

The proximate composition of control tofu and tofu fermented with lactic acid bacteria (*Lactobacillus casei*) are shown in the **Table 3.9**. The nutrient contents like protein, fat and ash were almost similar in both the tofu but the isoflavone content varied.

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In control tofu, glycosidic conjugates of isoflavone were more predominant than their aglycones. After inoculating lactic acid bacteria to the tofu, daidzin and genistin (glycosides) contents decreased and levels of their hydrolyzed counterparts daidzein and genistein (aglycones) increased significantly. It is obvious that increase of daidzein and genistein contents in tofu were enhanced by β-glucosidase from lactic acid bacteria.

Table 3.9 Proximate analysis and isoflavones of control and *Lactobacillus casei* inoculated tofu[a]

Samples	**(%) on dry wt basis**			**Isoflavones (μg g^{-1})**			
				Aglycones		**Glycosides**	
	Protein	Fat	Ash	Daidzin	Genistin	Daidzein	Genistein
Control tofu	53.11	25.00	5.29	4.02	3.57	0.05	0. 10
LAB tofu	53.77	27.06	5.10	0.47	0.08	0.82	1.31

[a]Data represent averages ± standard deviations of duplicate analyses of triplicate samples.

The conditions optimum for soft tofu were blanching of soybeans with 1% sodium bicarbonate for 10 min, solid content of 7° Brix, stirring time for 2-5 sec, and pressing the tofu with 1000 g initial weight for 15 min followed by 500 g. Microbiological analysis of tofu revealed that tofu can be stored for 9 days, at 4°C. Tofu made with calcium sulphate and magnesium chloride of 0.2% (1:1) ratio was best suitable coagulant for tofu preparation. Tofu prepared with lemon extract was the most preferred tofu which had a smooth, soft, but firm texture with whitish colour. Significant difference was not noticed between glycosidic and aglyconic form of isoflavones in the tofu prepared with synthetic and natural coagulants, but antioxidative activity was higher in tofu coagulated with natural coagulants. Tofu inoculated with lactic acid bacteria showed higher aglycones than glycosides.

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Chapter-IV

Utilization of tofu and byproducts in Indian traditional foods

C.R.Rekha, G. Vijayalakshmi, and Amudha Senthil. "A process for preparation of fermented okara (soy residue) based idli batter". Indian Patent, IPMD, New Delhi. **Patent Number 0809DEL2008**

C.R. Rekha and G. Vijayalakshmi (2010). Acceleration of fermentation of idli batter (a traditional cereal – legume based breakfast food) using soy residue okara. Journal of Food Science and Technology. (Article in press).

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4.1 Introduction

Soymilk is the aqueous extract of whole soybeans (*Glycine max*) which is considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for Lactose-intolerant population (Dhananjay *et al.,* 2006). Soybean is a rich source of isoflavone, which are reported to have beneficial estrogenic effects (Adlercreutz, 2002; Brouns, 2002; Cornwell *et al*., 2004) with potential bioactive antioxidant properties. Recently, several researchers have reported antioxidant and metal ion-chelating properties of isoflavones (Kao and Chen, 2006; Wang *et al.,* 2008). Hence Soy-based foods have been proposed as promising supplements to overcome existing protein calorie malnutrition problems (Khare *et al*., 1994).

Isoflavones are a unique subgroup of the flavonoids, one of the largest classes of plant phenolics with approximately five thousand member compounds. Isoflavones are phytochemicals that exist in two basic categories, the aglycones and the glycosidic conjugates (**Fig. 4.1**).

Isoflavones normally occur as glucoside-bound moieties called glycones (Rao and Muralikrishna, 2002). However, it is the aglycone (glucoside free) form of isoflavones that is metabolically active (Yuan *et al*., 2003). It has also been revealed that the β-glycosidic bond of isoflavone glycosides could be hydrolyzed during fermentation of soybean by a number of microorganisms, such as *Rhizopus oryzae*, *R. oligosporus, Saccharomyces rouxii, Bacillus subtilis, B. natto*, *Lactobacillus* and *Bifidobacteria*.

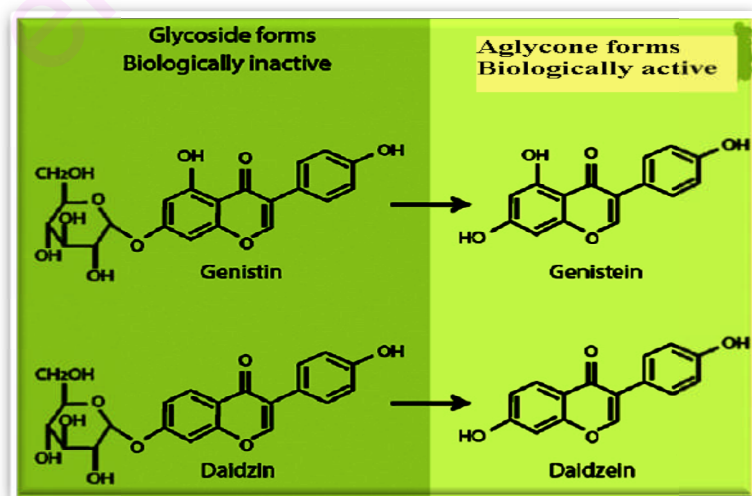

Fig. 4.1 Molecular structure of glycosidic and aglyconic forms of isoflavones

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Probiotics, live microbial cultures which when ingested in sufficient numbers, provide beneficial effects to the consumer beyond basic nutrition, may be traced to Metchnikoff (1907). Probiotic organisms include *Lactobacillus*, *Bifidobacteria* and *Saccharomyces*. However, the types of intestinal bacteria involved in isoflavone conversion to bioactive form and the effectiveness of this microbial biotransformation are not well known. *Lactobacillus* is a predominant member of the intestinal microflora. Hence, different isolates of β-glucosidase producing lactic acid bacteria (LAB) and biotransformation of isoflavone aglycones by these microorganisms in soymilk were studied.

The metabolic fate of soy isoflavones after consumption, as well as their biological activities, depends on their chemical structure (Marazza *et al*., 2009). After consumption, probiotic bacterial enzymes cleave the glycoside moieties from glycone isoflavonoids and release the biologically active health promoting aglycone isoflavones in the intestine. Interestingly, fermented soy foods are potentially rich in aglycone isoflavones due to the microbial bioprocessing during fermentation (McCue and Shetty, 2004). In humans, aglycones isoflavone are absorbed in greater amounts than their glycosides.

Most methods for the analysis of isoflavones are based on high performance liquid chromatography (HPLC). HPLC combined with Mass spectrometry (MS) is currently the most sensitive and selective analytical method for the rapid qualitative and quantitative analysis of known compounds and for the identification of unknown compounds from purified samples of natural products (Prasain *et al*., 2002).

In the present investigation evaluation of β-glucosidase activity of probiotic LAB and *S. boulardii* was carried out during soymilk fermentation in order to improve its nutritional quality in terms of aglycone isoflavone, genistein and daidzein, and to reduce the anti nutritional factors. The details are described in this Chapter.

4.2. Lactic acid bacteria and yeast

Lactic cultures used in this study were obtained from coworkers of Food Microbiology department, CFTRI. *S. boulardii* was isolated from the dietary supplement sachet '**Darolac'** obtained from local drug shop. Before use, for soymilk fermentation, they were confirmed for the genera and species and also for probiotic activities. The

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species level identification of isolates was carried out by conducting various biochemical tests as per Bergey's Manual (Mundt, 1986).

Lactobacilli are straight or curved rods occurring singly or in chains, sometimes in filaments. They are gram +ve, nonsporulating, catalase negative, non motile rod or cocci shaped microaerophilic to anaerobic bacteria. Pure cultures made from lactic acid bacterial isolates showed the cultural characteristics. Based on their morphology and fermentation characters, they were identified as follows:

Culture 1: *Lactobacillus acidophilus*: Rod shaped bacteria occurring singly or in pairs with rounded ends. Non motile, gram +ve, microaerophilic and ferments sucrose, raffinose and stachyose producing acid and no gas.

Culture 2: *Lactobacillus bulgaricus*: Slender rods with rounded ends, non motile, gram +ve colonies 2-3 mm in diameter, microerophilic, ferments sucrose, glucose, galactose.

Culture 3: *Lactobacillus casei*: Short or long rods occurring in short or long chains, non motile, gram +ve, acid form glucose, fructose mannose, galactose, maltose, lactose, microaerophilic, fermentes glucose, fructose, galactose etc.

Culture 4: *Lactobacillus plantarum*: Rods occurring singly or in short chains with rounded ends, non motile, microaerophilic, ferments sucrose and maltose.

Culture 5: *Lactobacillus helviticus*: Rods occurring singly and in chains, non-motile, gram +ve, acid produced from glucose, fructose, galactose and lactose, microaerophilic.

Culture 6: *Lactobacilllus fermentum*: Rods, variable in size, sometimes in pairs or chains, non motile and gram +ve, acid usually from glucose, fructose, sucrose and lactose.

To identify *Saccharomyces boulardii*, the Darolac sample was cultured on YPD medium (Yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, glucose 20 g l^{-1}, agar 20 g l^{-1}). *Saccharomyces boulardii* was purified and taxonomy was confirmed by staining and microscopic observations (Kuhle *et al.*, 2001).

For probiotic properties, various biochemical tests like acid and bile tolerance, antimicrobial activity against intestinal pathogens and ability to adhere and colonize the intestinal tract (Mishra and Prasad, 2005) were carried out as described in Materials and Methods. The morphology of the cultures was observed by microscopy as shown in **Fig. 4.2**.

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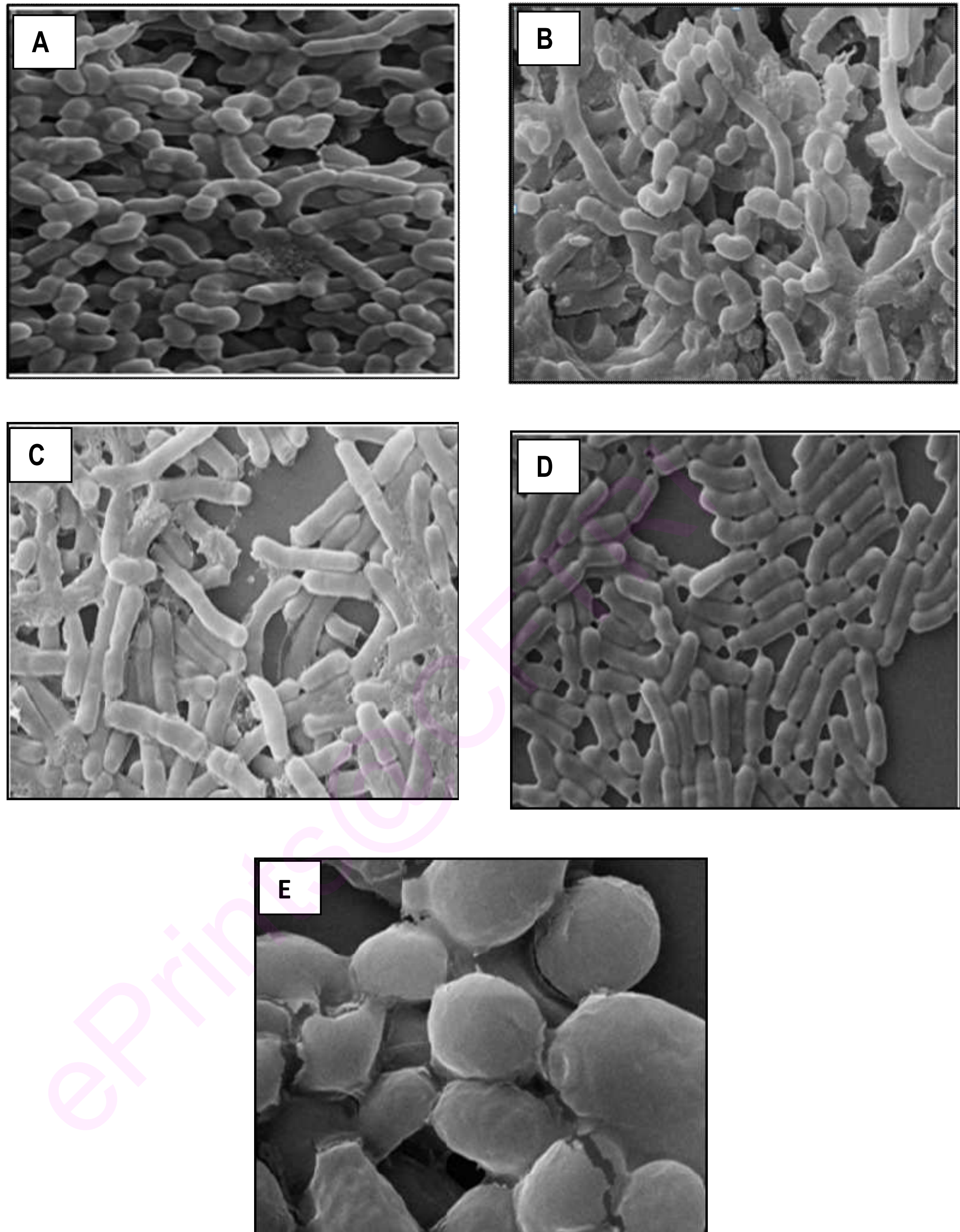

Fig. 4.2 Morphology of organisms

A. *Lactobacillus acidophilus* **B.** *Lactobacillus bulgaricus* **C.** *Lactobacillus casei* **D**. *Lactobacillus plantarum* **E.** *Saccharomyces boulardii* (SEM photographs)

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4.3 Nutritional quality and biomolecules of fermented Soymilk

Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. Isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders prevalent in current western civilizations (Beck *et al*., 2003). Isoflavones also have some health benefits such as prevention of certain types of cancer (Ravindranth *et al*., 2004), lowering the risk of cardiovascular disease (Goodman and Kritz, 2001) and improvement of bone health (Weaver and Cheong, 2005). Aglycone isomers are able to bind to receptor sites of estrogen and mimic the functions of estradiol in the human body (Setchell and Cassidy, 1999). In Asian countries, soy isoflavone intake is estimated to range from 20 to 100 mg/day (Brouns, 2002). These levels are much higher than those of Americans (Fukutake *et al*., 1996), whose mortality rate for breast, colon and prostate cancers and incidence of heart disease are greater than in the Asians (Brouns, 2002).

Beneficial effects of isoflavones have been related to their antioxidant activity (Pyo *et al*., 2005a). Foods containing antioxidants are used to reduce the oxidative damage related to ageing and diseases, such as atherosclerosis and cancer (Ana, 2004). The use of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyl anisole (BHA) are restricted as they are suspected to be carcinogenic. Therefore much attention is given to natural antioxidants.

Consumption of unfermented milk may also lead to digestive problems associated with the presence of raffinose and stachyose. Fermentation can overcome the problem and also improve acceptability. Fermentation improves bioavailability of isoflavones, assists in digestion of protein, provides more soluble calcium, enhance intestinal health and supports immune system.

It has been shown that the glycoside isoflavones are very poorly absorbed in the small intestine compared to aglycones because of greater molecular weight and higher hydrophilicity (Chang and Nair, 1995). Furthermore, the glycosides are known to be less bioactive than their respective aglycones (Xu *et al*., 1995). Probiotic lactic acid bacteria and *Saccharomyces boulardii* yeast, when grown in milk have the ability to convert the glycoside isoflavones into their respective aglycones without the supplementation of any nutritious sources.

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Growth of *S. boulardii* in association with lactic acid bacteria has been suggested to stimulate the growth of the probiotic lactic acid organisms and to assure their survival during shelf life. Low pH of yogurt and the ability of yeasts to utilize organic acids create a selective environment for yeast growth (Fleet and Main, 1987).

The present study describes the bioconversion of glycosidic isoflavones to aglyconic form of isoflavone and improvement in nutritional quality soymilk when fermented with probiotic yeast and lactic acid bacteria.

4.3.1 pH and Acidity of fermented soymilk

After confirming the taxonomy of lactic acid bacteria and yeast, the isolates were stored at 4°C for further use as inoculums to ferment soymilk. Soybeans after washing and soaking overnight, were ground and filtered to get fresh soymilk. Fifty milliliters of soymilk was dispensed into screw cap containers and autoclaved. Sixteen hour old LAB and yeast suspensions (2%), in the ratio of 1:1 (approximately 7-8 $\log_{10}$ CFU mL^{-1}) was used as inoculum as described in Materials and Methods and incubated at 37°C for 24 h. The pH and titrable acidity was monitored from 0 to 24 h at an interval of 8 h.

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Table 4.1 shows the titrable acidity and pH during fermentation of soymilk with different isolates of Lactic acid bacteria and yeast. The titrable acidity of soymilk increased from 0.11-0.14 % to 0.27-0.34% respectively with different combinations. The pH of soymilk fermented with Sb+Lp culture was highest (4.87) followed by Sb+La (4.83), while the others ranged from 4.78 to 4.80 at the end of 24 h fermentation at 37°C. An increase in TA of soymilk from 0.14% to 0.34% was observed after 24 h fermentation with Sb+Lc combination. Marginal increase was observed with Sb+La and Sb+Lb (0.28%) and Sb+Lp (0.30%). On the other hand, relatively lower TA of 0.27% was seen in curd fermented with Sb+Lh combination.

4.3.2 Fat, Protein and Ash

Fermented milk obtained was also analyzed for fat, protein and ash content. Total nitrogen was determined by Kjeldahal method. Nitrogen-to-protein conversion factor of 6.25 was used. Fat and ash were determined by AOAC procedures. The nutritional profile (% dry weight basis) of fermented soymilk is represented in **Fig. 4.3**.

Table 4.1 Changes in pH and titrable acidity (%) of soymilk fermented with LAB and *S. boulardii* for 24 h

Combinations	**Fermentation period (h)**							
	pH				TA (%)			
	0	8	16	24	0	8	16	24
Sb+La	6.55±0.09a	6.39±0.05a	5.00±0.03a	4.83±0.02a	0.13±0.03b	0.20±0.02b	0.25±0.02b	0.28±0.02b
Sb+Lb	6.56±0.02a	5.96±0.10a	5.42±0.04a	4.78±0.03a	0.11±0.00b	0.22±0.02b	0.26±0.02b	0.28±0.03b
Sb+Lc	6.53±0.00a	6.25±0.06a	5.24±0.02a	4.79±0.03a	0.14±0.03b	0.18±0.00b	0.30±0.00b	0.34±0.03b
Sb+Lp	6.56±0.04a	6.42±0.08a	5.16±0.02a	4.87±0.06a	0.11±0.01b	0.23±0.00b	0.28±0.01b	0.30±0.01b
Sb+Lh	6.54±0.02a	6.18±0.02a	5.38±0.00a	4.80±0.05a	0.13±0.01b	0.25±0.01b	0.26±0.01b	0.27±0.01b

Ta: Titrable acidity (% Lactic acid). Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (Sb- *Saccharomyces boulardii* La- *Lactobacillus acidophilus,* Lb- *Lactobacillus bulgaricus*, Lc- *Lactobacillus casei,* Lp- *Lactobacillus plantarum,* Lh- *Lactobacillus helviticus*).

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Fat content was higher in soymilk fermented with *S. boulardii* and *L. acidophilus* (23.17%) followed by *S. boulardii* and *L. helviticus* (22.50%), *S. boulardii* and *L. bulgaricus* (21.56%) and *S. boulardii* and *L. casei* (20.47%). The least was in *S. boulardii* and *L. plantarum* (10.81). Protein content was more in soymilk fermented with Sb+Lh (48.67%) followed by Sb+Lp (45.48%), Sb+La (45.12%), Sb+Lc (44.42%), and in Sb+Lb (42. 31%). The ash content was high in Sb+Lc (5.3%), and the least was in Sb+Lb 4.3%).

There was no significant difference in protein content of test curds when compared to control. The difference in fat content was more significant in some of the combinations. The texture, physical stability, flavor, and aroma of the soy yogurt were related to pH (Ankenman and Morr, 1996). In general, coagulation of sterilized soymilk occurs at pH 5.7 (Chou and Hou, 2000).

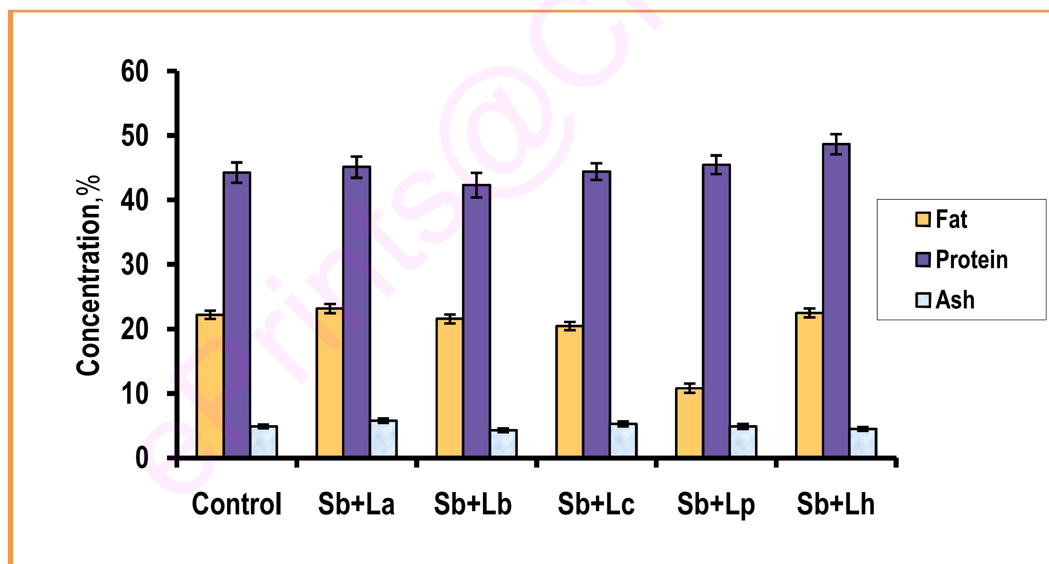

Fig. 4.3 Nutritional profile of soymilk and soymilk fermented with LAB and yeast *S. boulardii* (Sb- *Saccharomyces boulardii* La- *Lactobacillus acidophilus,* Lb- *Lactobacillus bulgaricus,* Lc- *Lactobacillus casei,* Lp- *Lactobacillus plantarum,* Lh- *Lactobacillus helviticus*).

4.3.3 Protein Hydrolysis

After determining the nutritional content of fermented soymilk, the proteolytic activity was carried out as probiotic organisms are rich in proteolytic activity.

The addition of probiotic organisms to soymilk results in increased free amino acid content. The degree of protein hydrolysis is expressed as content of leucine amino equivalent in soymilk after 24 h of fermentation (Kurmann and Rasic, 1991). It was determined according to the method described in Materials and Methods.

The proteolytic activity of fermented soymilk is shown in **Fig. 4.4**. The rate of protein hydrolysis ranged from 2.46 to 2.80 mmol l^{-1} with different strains of LAB and yeast *S. boulardii* combinations. The highest was seen in Sb+Lh (2.80) followed by Sb+Lp (2.69), Sb+La (2.66), and Sb+Lb (2.52); the least was in Sb+Lc (2.46) combination. Neverthless, the fermented soymilk showed higher proteolytic activity compared to control.

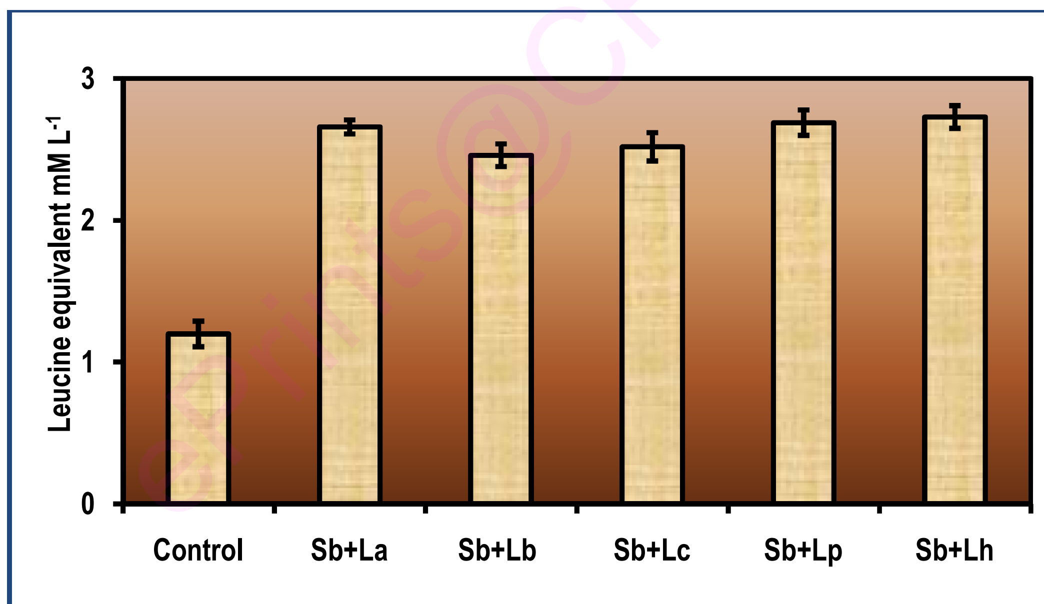

Fig. 4.4 Protein hydrolysis of soymilk and soymilk fermented with LAB and yeast *S. boulardii* (Sb, La, Lb, Lc, Lp & Lh: Expansions as given in Fig. 4.3).

4.3.4 Polyphenols

Polyphenolic compounds are secondary metabolites biosynthesized throughout the plant kingdom and found in foodstuffs derived from plant sources. These compounds can delay the onset of lipid oxidation and the composition of hydroperoxides in food

products as well as in living tissues. (Ana *et al.,* 2004). Polyphenols are present in considerable amount in soymilk.

Polyphenols was determined using Folin–Ciocalteu reagent using Gallic acid as a standard. The absorbance of the clear supernatant solution was measured at 765 nm as described in Materials and Methods.

The changes in polyphenol content are shown in **Fig. 4.5**. This content increased from 11.9 mg^{-1} to 26.58 mg^{-1} in various fermented milk, incubated for 24 h. Highest polyphenol content was seen in Sb+Lh curd followed by Sb+Lb curd. There was not much difference between Sb+La, Sb+Lc and Sb+Lp curd. The polyphenol content was higher in the soymilk fermented with Sb+Lh and Sb+Lb combinations compared to control.

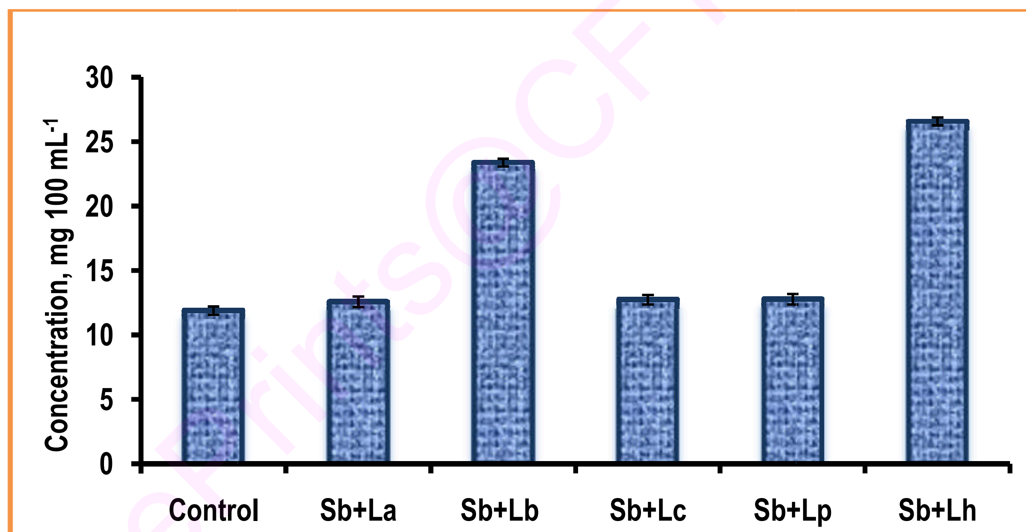

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Fig. 4.5 Polyphenol content of soymilk and soymilk fermented with LAB and yeast *S. boulardii* (Sb, La, Lb, Lc, Lp & Lh: Expansions as given in Fig. 4.3).

4.3.5 Antioxidant activity

4.3.5.1 DPPH Scavenging

Free radicals and reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen induce oxidative damage in lipids, proteins and DNA (Lee *et al*., 2004a). These radicals may cause oxidative damage by oxidizing biomolecules and

results in cell death and tissue damage. As synthetic antioxidants have restricted use in foods, the need for natural oxidants has greatly increased. Polyphenolic compounds have remarkable antioxidant activities which quench oxygen derived free radicals by donating a hydrogen atom or an electron to the free radicals (Ana *et al*., 2004).

Soybean contains antioxidant substances that are known for their potential bioactive antioxidant properties and radical scavenging capacity (Pyo *et al*., 2005). Fermented soybeans had increased polyphenols, so were used for the study. Antioxidant activity was determined by three different assays like DPPH scavenging activity, ascorbate autooxidation inhibition and reducing activity.

Methanol extract of the fermented soymilk was used for the analysis. There was a significant increase in DPPH scavenging activity in fermented milk when compared to unfermented soymilk under similar conditions **(Table 4.2)**. The milk fermented with Sb+Lh strain expressed highest radical scavenging activity (28.53%). This increased activity was about 21% over the control.

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4.3.5.2 Ascorbate autooxidation inhibition

Inhibition of autooxidation was studied using ascorbic acid. Though unfermented soymilk exhibited ability to inhibit ascorbate autooxidation, fermentation with yeast and LAB significantly increased the inhibition rate. The data depicted in Table 15 showed that the inhibition rate of fermented soymilk to inhibit ascorbate autooxidation ranged from 6.12–9.16% depending on the starter organisms used. Soymilk fermented with Sb+Lh exhibited a significantly higher inhibition rate of ascorbate autooxidation (9.16%) after 24 h of fermentation.

4.3.5.3 Reducing Activity

The reducing activity of soymilk was determined **(Table 4.2)** and expressed as an equivalent amount of cysteine (μM). Soymilk fermented with Sb+Lp exhibited the highest reducing activity (0.808) amongst soymilk fermented with different combinations. One milliliter of soymilk fermented with Sb+Lp showed the reducing activity equivalent to 0.808 μM cysteine.

Table 4.2 Antioxidant activity of fermented soymilk

Antioxidant activity			
Samples	DPPH scavenging (%)	Inhibition of ascorbate autooxidation (%)	Reducing (equivalent cysteine, µM)
Fresh Soymilk (Control)	7.21±0.09a	4.26±0.08a	0.727±0.004a
Fermented with			
Sb+La	15.98±0.32b	8.23±0.09c	0.747±0.005a
Sb+Lb	26.22±0.24c	6.12±0.02b	0.762±0.004a
Sb+Lc	26.68±0.20c	6.34±0.06b	0.776±0.003b
Sb+Lp	16.40±0.33b	8.80±0.08d	0.808±0.007b
Sb+Lh	28.53±0.20c	9.16±0.02d	0.751±0.006a

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (Sb, La, Lb, Lc, Lp & Lh: Expansions as given in Table 4.1).

4.3.6 Isoflavones

Quantification of isoflavones was found necessary in the light of increased DPPH scavenging activity, inhibition of ascorbate autooxidation and reducing activity explained above in fermented soymilk. The method of Chiou and Cheng (2001) was followed for the extraction of Isoflavones. Twenty microliter sample was taken for HPLC analysis as described in Materials and Methods.

The isoflavone concentration of both glycosidic forms and aglyconic forms were determined in both fermented and unfermented soymilk **(Table 4.3).** The HPLC chromatogram of isoflavones is shown in **Fig. 4.6**. The isoflavones genistin, daidzin, genistein, and daidzein were successfully separated and identified. The most abundant form of isoflavones in soybean and nonfermented soy foods are glycosides (genistin and daidzin), and in fermented foods, they are in the form of aglycones (genistein and

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Table 4.3 Isoflavone content of unfermented and fermented soymilk with LAB and yeast *S. boulardii* for 24 h

Samples	Glucosides (mg 100 mL^{-1})			Aglycones (mg 100 mL^{-1})			Total (mg 100 mL^{-1})
	Daidzin	Genistin	Sub-Total	Daidzein	Genistein	Sub-Total	
Soymilk (Control)	6.65	19.7	26.35	1.19	1.71	2.91[a]	29.26a
Fermented with							
Sb+La	0.08	0.56	0.64	5.90	22.63	28.53b	29.17a
Sb+Lb	0.12	0.68	0.80	6.94	28.40	35.34d	36.14c
Sb+Lc	0.07	0.43	0.50	6.29	23.28	29.57bc	30.07ab
Sb+Lp	0.14	0.66	0.80	6.39	24.78	31.17bc	31.97ab
Sb+Lh	0.19	0.72	0.91	6.98	29.60	36.58d	37.49c

Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (Sb, La, Lb, Lc, Lp & Lh: Expansions as given in Table 4.1).

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daidzein). Unfermented soymilk is rich in glycosides, and total glycoside in unfermented soymilk was 26.35 mg^{-1} (daidzin 6.65 mg 100 mL^{-1} and genistin 19.7 mg 100 mL^{-1}. In contrast, aglyconic form was quantitatively lesser in soymilk accounting of 2.91 mg 100 mL^{-1} (genistein 1.17 mg and daidzein 1.19 mg 100 mL^{-1}. Soymilk fermented with different cultures show decrease in glycosidic content ranging from 0.50 to 0.91 mg 100 mL^{-1} and increase in aglyconic content ranging from 28.53 to 36.58 mg 100 mL^{-1}.

The total concentration of isoflavone isomers (genistin, daidzin, ginistein, and daidzein) in soymilk was 29.26 mg 100 mL^{-1} after 24 h. The non-bioavailable biologically inactive glycoside forms (genistin and daidzin) contributed the greatest concentration of isomers (90%). But soymilk fermented with five combinations of LAB and yeast contained a total isoflavone content of 29.17–37.49 mg^{-1}, of bioactive aglycones (genistein and daidzein) after 24 h of fermentation. After 24 h of incubation, the concentration of aglycones in soymilk fermented with Sb+Lh was higher than that of other combinations (36.58 mg 100 mL^{-1}) followed by Sb+Lb—35.34 mg 100 mL^{-1}, Sb+Lp—31.17 mg^{-1}, Sb+Lc—29.57 mg^{-1}, and Sb+La—28.53 mg 100 mL^{-1}.

In this study, the significant bioconversion of the glycoside isoflavones into their corresponding aglycones during soymilk fermentation was because of cleavage of glycosyl bond by microbial fermentation. Thus, there was an average 14.2 fold increase in the concentration of aglycones in soymilk fermented with five different combinations of LAB and yeast *S. boulardii* with an average of 90% of the original glycosides (genistin and daidzin) bioconverted into aglycones (genistein + daidzein).

The genistein contributed to the greatest concentration of aglyconic form (3.13–9.55 mg^{-1} wet weight) than daidzein (1.84–3.98 mg^{-1}). This was possibly due to the higher content of genistin in the original soymilk compared with the other isomers.

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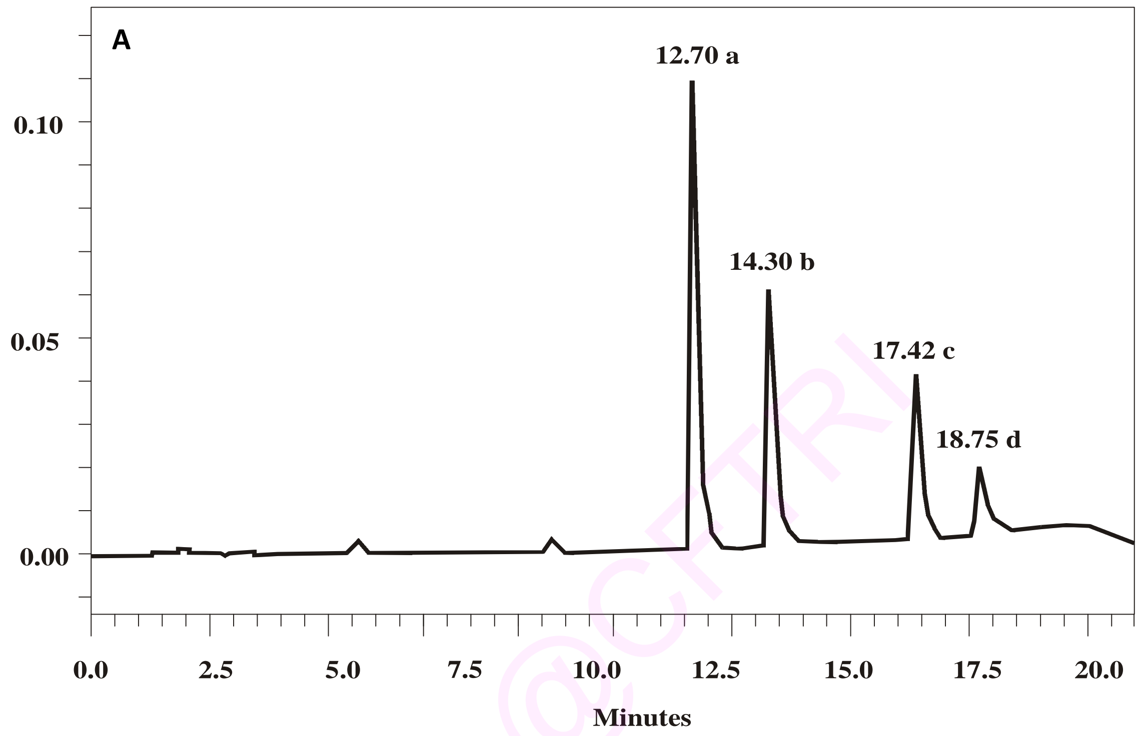

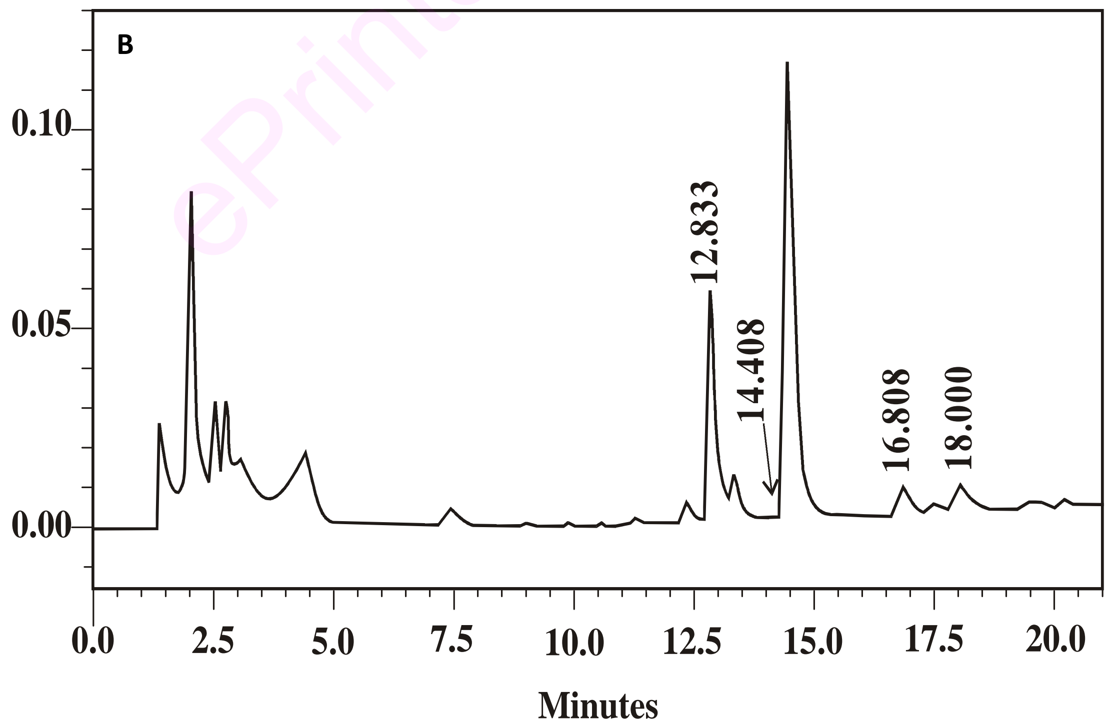

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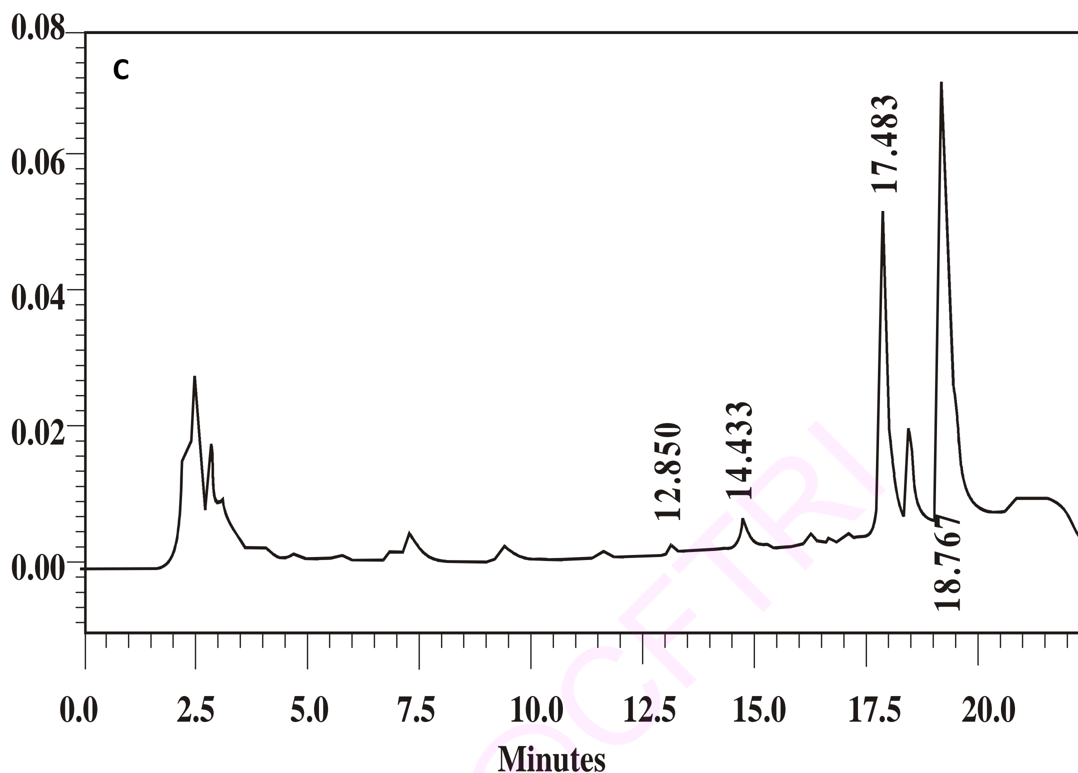

Fig. 4.6 HPLC chromatagrams of standard isoflavones, soymilk and fermented soymilk with Sb+La

A. HPLC chromatogram showing the retention time of standard isoflavones -Daidzin (12.70 min), Genistin (14.30 min), Daidzein (17.42 min) and Genistein (18.75 min).

B. HPLC chromatogram showing the retention time of isoflavone in soymilk (control): Daidzin (12.83 min), Genistin (14.40 min), Daidzein (16.80 min) and Genistein (18.00 min).

C. HPLC chromatogram showing the retention time of isoflavone in soymilk fermented with Sb+La combination: Daidzin (12.85 min), Genistin (14.43 min), Daidzein (17.48 min) and Genistein (18.76 min)

Thus there was an increase in antioxidant activity of fermented soymilk due to the significant bioconversion of the glycosidic form of isoflavones (genistin and daidzin) into their bioactive aglyconic form of isoflavones (genistein and daidzein). *S. boulardii* is capable of utilizing the yogurt constituents as growth substrates, and its application as a probiotic microorganism seems promising, as no gas and alcohol are produced. Hence,

growth of probiotic yeast in association with probiotic bacteria has been suggested for enhancing the viability of lactic acid bacteria.

4.4 Bioconversion of isoflavone glycosides to aglycones during soymilk fermentation

Probiotic microorganisms typically lactobacilli or *bifidobacteria* (Lourens-Hattingh and Viljoen, 2001) are increasingly incorporated into food as dietary adjuncts to benefit human health in order to increase immune functions, lower cholesterol, prevent diarrhea, ulcerative colitis and irritable bowel syndrome (Tang *et al*., 2007). It is shown that lactobacilli possess β-glucosidase activity and play a major role in the hydrolysis of glycosides during fermentation (Donkor and Shah, 2008). Although each group of probiotics has varying potential in the hydrolysis of isoflavones during fermentation, the hydrolytic action has been found to cause major increase in the concentration of bioactive isoflavone aglycones and concomitant decrease in the concentration of isoflavone glucosides.

S. boulardii, which is described as a biotherapeutic agent, is reported efficient in the prevention of recurrence of antibiotic-associated diarrhea and colitis in humans (Surawicz *et al*., 1989). It is used as a food additive in a limited number of cases such as in the fermentation of vegetable (Sindhu and Khetarpaul, 2003) and for incorporation into commercial yoghurts (Lourens-Hattingh and Viljoen, 2001). Importantly, probiotics possess β–glucosidases (Otieno *et al*., 2005) that can improve the biological activity of soymilk. Therefore, improving the bioavailability of isoflavones in soy foods may require enrichment of isoflavone aglycone prior to consumption and modulation of intestinal microflora through viable bacteria (Tsangalis *et al*., 2004).

The stability of LAB in fermented products is very important in probiotic dairy foods to increase nutritional value. Different methods have been used, under refrigerated conditions, to increase their stability. These are addition of prebiotics and nutrients, stress adaptation, use of protectants and microencapsulation. Interaction between yeasts and bacteria involve stimulation or inhibition. Yeasts are known to help in the stabilization of lactic acid bacterial population in cheese and yoghurt (Liu and Tsao, 2009).

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The presence of antinutritional factor, phytate, in soybeans may decrease the absorption of minerals leading to mineral deficiencies. It may chelate nutritionally important cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}, thus decreasing the dietary bioavailability of these nutrients (Wodzinski and Ullah, 1996). Since fermentation with microorganisms can remove or reduce phytic acids, fermenting soybean products has become important. In this regard, phytases of yeasts, Lactic acid bacteria (LAB) and *Bifidobacteria* have assumed significance (Hurrell, 2003) as they also carry a GRAS status (Oh and Lee, 2007; In *et al.*, 2008). Lactic acid fermentation is known to reduce the phytate content in plant-based foods and the role of the bacteria has been intensively studied for whole wheat bread making (Palacios *et al.*, 2008).

Based on the above literature, β–glucosidase producing probiotic bacteria and yeast were studied with respect to bioconversion of glycosides to aglycones.

4.4.1 Screening of high β-glucosidase activity producing bacteria and yeast strains

The types of intestinal bacteria involved in isoflavone conversion to bioactive form and the effectiveness of biotransformation are not well understood. Lactobacillus is a predominant member of the intestinal microflora. β–glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) comprise a heterogeneous group of enzymes that are able to cleave the β-glucoside linkages of di and /or oliogosaccharide or other glucose conjugates. Hence, different isolates of β-glucosidase producing lactic acid bacteria (LAB) and yeast *Saccharomyces boulardii* were analysed to study biotransformation of isoflavone aglycones in fermented Soymilk.

The LAB and *S. boulardii* were screened for β-glucosidase activity using the substrate p-NPG. The details are described in Materials and Methods.

There was a wide variation in enzyme activity among the strains. Of the strains tested, five strains *L. bulgaricus, L. fermentum. L. acidophilus, L. casie and L. plantarum,* showed higher β-glucosidase activity than the others **(Fig. 4.7).** β-glucosidase activities of these cultures reached a maximum after 18-24 h of cultivation which corresponded to the exponential phase of growth. *L. plantarum* showed the highest (35 m U^{-1}) and *L. helviticus* lowest (11 m U^{-1}) activity.

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High β-glucosidase producing bacterial strains were selected for further study and were used as a functional starter culture for fermenting soymilk individually and in combination with *S. boulardii.*

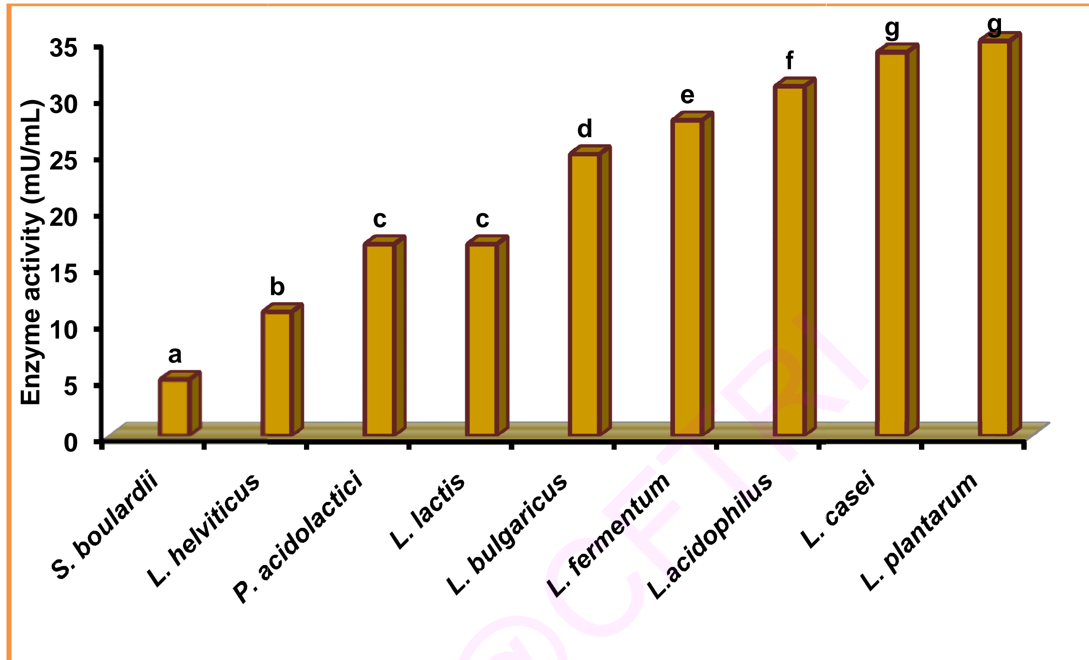

Fig. 4.7 β-glucosidase activity of isolates. Values with different letters were significantly different by Duncan's multiple range test (P ≤ 0.05).

4.4.2 Acid development during fermentation

The strains of LAB showing detectable levels of β-glucosidase activity were used for fermenting soymilk. LAB and yeast were activated in MRS broth and potato dextrose broth respectively. The LAB were multiplied in MRS broth at 37°C for 12–15 h, yeast at 30°C for 16 h and the suspended cells were used for fermentation (Materials & Methods).

The acid development and pH of soymilk fermented for 12, 24 and 48 h with five strains of LAB individually and in combination with *S. boulardii* is given in **Table 4.4.** Decrease in pH and increase in TA were observed during fermentation of soymilk. There was a decline in pH during fermentation at the end of 24 h fermentation and a marginal decrease at the end of 48 h from 24 h at 37°C. This is due to the production of lactic and acetic acids in the fermented soymilk. The initial pH of most of the samples ranged from 6.55 to 6.58 and reduced to 4.61 to 5.84 at the end of 24 h fermentation. Simultaneously TA increased from 0.11 to 0.13 and was 0.88 to 1.12% after 24 h fermentation. Increasing the time of fermentation over 24 h, resulted in a rapid increase in pH.

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Table 4.4 Changes in pH and titrable acidity (%) of soymilk fermented with LAB for 48 h

Culture	Fermentation period (h)							
	pH				TA (%)			
	0	12	24	48	0	12	24	48
La	6.57±0.04a	6.26±0.10a	5.84±0.02a	4.87±0.01a	0.14±0.01a	0.17±0.01a	0.62±0.03a	0.80±0.00 a
Lb	6.58±0.04a	6.30±0.08a	5.08±0.10a	5.79±0.12a	0.13±0.00a	0.18±0.00 a	0.92±0.00bc	0.76±0.00 a
Lc	6.57±0.03a	6.14±0.03a	5.66±0.12a	6.12±0.16a	0.13±0.02a	0.28 ±0.00ab	0.88±0.01b	0. 74±0.02a
Lp	6.55±0.16a	6.36±0.10a	5.17±0.20a	5.24±0.26a	0.13±0.01a	0.38±0.01b	0.90±0.01b	0.84 ±0.01a
Lf	6.57±0.05a	5.60±0.03a	4.61±0.03a	4.67±0.05a	0.11 ±0.01a	0.13±0.01 a	1.12c±0.02	0.86±0.02a

Ta: Titrable acidity (% Lactic acid). Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (La- *Lactobacillus acidophilus*, Lb- *Lactobacillus bulgaricus*, Lc- *Lactobacillus casei*, Lp- *Lactobacillus plantarum*, Lf- *Lactobacillus fermentum*).

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Table 4.5 Changes in pH and titrable acidity (%) of soymilk fermented with LAB and *S. boulardii* for 48 h

Combinations	Fermentation period (h)							
	pH				TA (%)			
	0	12	24	48	0	12	24	48
Sb+La	6.55±0.0a	4.94±0.12a	4.83±0.01a	4.16±0.04a	0.13±0.02b	0.27±0.0b	0.27±0.03b	0.30±0.02b
Sb+Lb	6.56±0.05a	5.30±0.02a	4.78±0.02a	4.89±0.03a	0.11±0.00b	0.25±0.0b	0.27±0.01b	0.27±0.01b
Sb+Lc	6.56±0.14a	5.10±0.09a	4.79±0.04a	4.87±0.04a	0.14±0.0b	0.27±0.02b	0.30±0.02b	0.27±0.02b
Sb+Lp	6.53±0.18a	5.01±0.02a	4.87±0.04a	4.88±0.12a	0.11±0.0b	0.27±0.02b	0.28±0.01b	0.21±0.03b
Sb+Lf	6.57±0.04a	4.92±0.01a	4.57±0.04a	4.38±0.04a	0.09±0.00b	0.32±0.01b	0.36±0.03b	0.32±0.04b

Ta: Titrable acidity (% Lactic acid). Values in the same column with different small letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (Sb- *S. boulardii* La- *Lactobacillus acidophilus* Lb- *Lactobacillus bulgaricus* Lc- *Lactobacillus casei* Lp- *Lactobacillus plantarum* Lf- *Lactobacillus fermentum*).

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This was most conspicuous in soymilk fermented with Lb, Lc, Lp and Lf but with soymilk fermented with La, wherein pH reduced to 4.87 as the fermentation period increased to 48 h. The acidity in soymilk fermented with La was maximum at 48 h (0.80%). In soymilk fermented with other bacteria, maximum acidity ranged from 0.90 to1.12% after 24 h.

The reduction in pH during fermentation caused soymilk coagulation, indicating enough acid development for curd formation. In general, coagulation of sterilized soymilk occurred and the pH of (Angeles and Marth, 1971) commercial yogurt ranged from 4.2 to 4.4 (Pinthong *et al*., 1980).

In soymilk fermented with different LAB along with *S. boulardii*, the pH ranged from 6.53 to 6.57 at 0 h and 4.57 to 4.87 after 24 h fermentation. In the samples TA increased from 0.09 to 0.14 to 0.27 to 0.36% at the end of 24 h fermentation. The highest acidity was seen in soymilk fermented with Sb+Lf combination (0.36%) and the lowest was in Sb+Lp combination (0.28%). Correspondingly the maximum pH was seen in Sb+Lp combination (4.87) and minimum in Sb+Lf combination (4.57) **(Table 4.5).** Increase in the acid development in soymilk was noticed with increase in fermentation time. However, at 24 h and at 48 h of fermentation, soymilk fermented with Sb+La combination showed marginal decrease in pH from 4.83 to 4.16 and increase in total acidity from 0.27 to 0.30%. In contrast, the soymilk fermented with other combinations like Sb+Lb, Sb+Lc, Sb+Lp and Sb+Lf, there was a marginal increase in pH and decrease in acidity after 24 h.

Soymilk fermented with LAB alone and with yeast showed a marked decrease in pH and a rapid increase in TA during the first 24 h fermentation. There was no further increase in pH or TA when fermentation was extended beyond 24 h.

4.4.3 Viability of bacteria and yeast during fermentation

Growth of β-glucosidase strains in soymilk during fermentation was assayed by enumerating the viable cell counts of LAB. Pour plate method on MRS agar media with 2.5 mg l^{-1} Amphotericin B and spread plate method on Potato dextrose agar medium were used to determine viable cell count of LAB and *S. boulardii* respectively. The experimental procedure is described in Materials and Methods.

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The growth pattern of LAB and in combination with yeast *S. boulardii*, during fermentation of soymilk is shown in **Table 4.6**. In soymilk fermented with Lb, Lc, Lp and Lf, the highest viable count reached after 24 h. In the case of La the highest cell count was observed at 48 h.

Table 4.6 Viable count of LAB during soymilk fermentation

	Viable count (log_{10} CFU g^{-1})				
Fermentation period (h)	La	Lb	Lc	Lp	Lf
0	6.90a ± 1.10	6.60a ± 1.18	6.82a ± 1.26	6.60a ± 1.32	6.84a ± 1.00
12	7.75a ± 1.24	7.92a ± 1.22	7.51a ± 1.14	8.50a ± 1.40	8.46a ± 1.13
24	8.04a ± 1.00	8.17a ± 1.11	8.67a ± 1.31	8.81a ± 1.26	8.93a ± 1.12
48	8.41a ± 1.32	8.06a ± 1.00	8.15a ± 1.22	8.63a ± 1.10	8.91a ± 1.20

Values in the same column with letters were not significantly different by Duncan's multiple range test ($p \leq 0.05$) (comparison on h of fermentation of each strain). (La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.4).

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In Soymilk fermented with LAB and *S. boulardii*, the highest viable count of bacteria occurred at 48 h for La (8.72 log_{10} CFU mL^{-1}) and marginal increase in growth was seen thereafter (48 h) **(Table 4.7)**. After 24 h, there was reduced growth of Lb, Lc, Lp and Lf which reflected a change to stationary growth phase. The growth of *S. boulardii* in all the fermented soymilk reached the maximum at 48 h of incubation which ranged from 7.57 to 7.87 log10 CFU mL^{-1}. The increase in viable count of *S. boulardii* ranged from 2.33, 2.42, 2.50, 2.58 and 2.71 log_{10} CFU mL^{-1} in Sb+Lc, Sb+La, Sb+Lp, Sb+Lb and Sb+Lf respectively.

4.4.4 β–glucosidase activity of fermented soymilk

β–glucosidase present in probiotic microorganisms play an important role in hydrolyzing β-glucosidic bond of glycosides releasing bioactive aglycone forms (Esaki *et al.*, 2004). Hence β–glucosidase activities of bacteria and yeast were further studied in relation to the experimental procedure during fermentation of soymilk.

Table 4.7 Viable count of LAB and *S. boulardii* during soymilk fermentation

Fermentation period(h)	Viable count (log_{10} CFU g^{-1})									
	Sb+La		Sb+Lb		Sb+Lc		Sb+Lp		Sb+Lf	
	La	Sb	Lb	Sb	Lc	Sb	Lp	Sb	Lf	Sb
0	6.60a±1.02	5.26a±0.88	6.69a±1.17	5.20a±0.74	6.43a±1.10	5.24a±0.83	6.79a±1.24	5.29a±0.96	6.71a±1.2	5.16a±0.64
12	7.73c±1.68	6.29abc±1.12	7.75c±1.70	5.97ab±1.24	7.69c±1.62	5.95ab±1.3	7.57bc±1.54	6.03ab±1.10	7.90c±1.85	5.73a±0.96
24	8.36e±1.96	6.70abcd±1.0	7.81cde±1.76	6.30a±1.10	7.60abcde±1.59	6.38ab±0.96	7.81bcde±1.76	7.54abc±1.48	8.02de±1.92	6.43abc±1.1
48	8.72b±1.84	7.68ab±1.64	6.74a±1.18	7.78ab±1.66	6.84a±1.02	7.57ab±1.48	7.20ab±1.23	7.79ab±1.56	7.07ab±1.38	7.87ab±1.6

Values in the same row with different letters were significantly different by Duncan's multiple range test ($p \leq 0.05$).
(Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.5).

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β–glucosidase was estimated as described in Materials and Methods. The soymilk fermented for 48 h at 37°C was used as the experimental source. β–glucosidase activity of soymilk fermented with the five isolates of LAB is shown in **Fig. 4.8**. There was a significant difference ($p < 0.005$) in β–glucosidase activity of the fermented soymilk. La showed 160 mU mL^{-1} enzyme activity after 48 h of fermentation, whereas other LAB strains showed maximum activity at 24 h of fermentation which ranged from 93 to 152 mU mL^{-1}. Higher enzyme activity may be due to the increased cell growth of LAB.

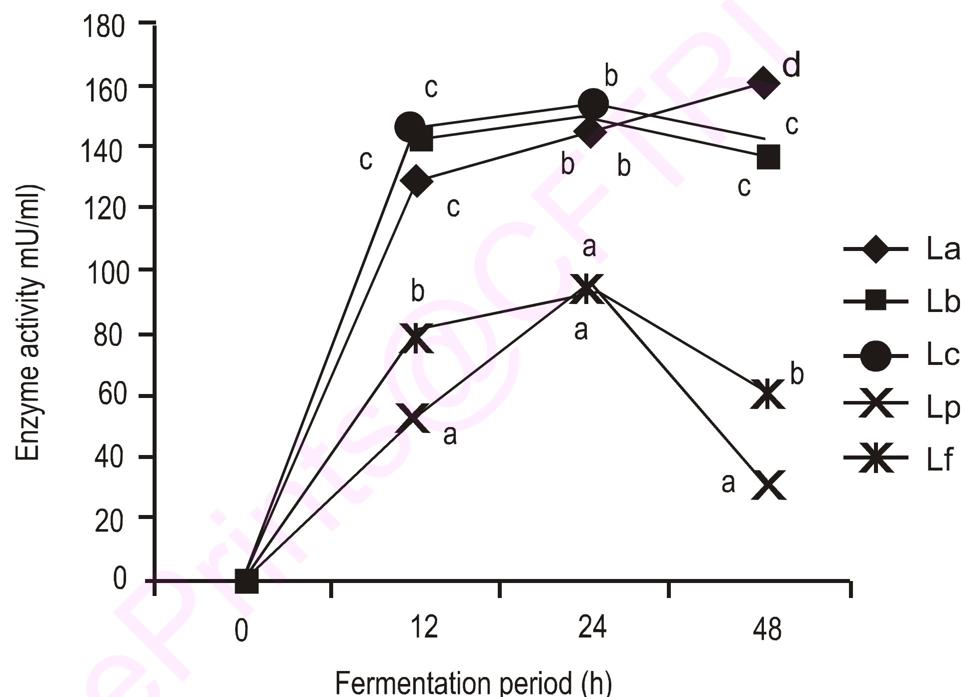

Fig. 4.8 β–glucosidase activity of fermented soymilk with LAB

Values with different letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.4).

β-glucosidase activity of soymilk fermented with LAB and *S. boulardii* are shown in **Fig. 4.9**. In these, β-glucosidase activity (Sb+Lb: 163 mU mL^{-1}, Sb+Lc: 169 mU mL^{-1}, Sb+Lp: 94 mU $mL^{-1}$and Sb+Lf: 99 mU mL^{-1}) increased gradually with fermentation time. Only, in soymilk fermented with Sb+La, the β-glucosidase activity increased after 24 h and at 48 h fermentation, 152 mU mL^{-1} activity was determined.

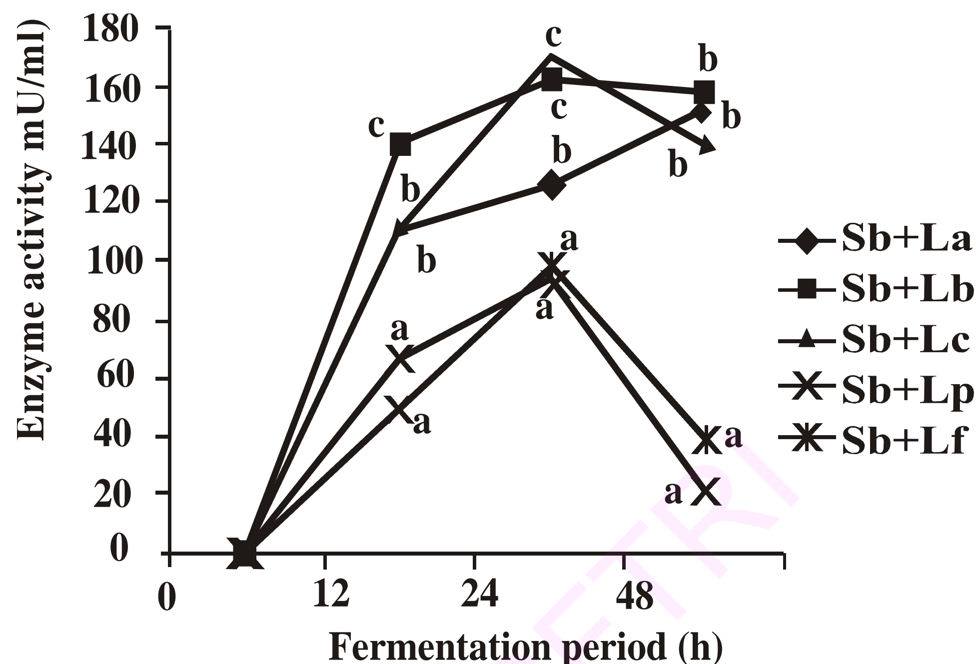

Fig. 4.9 β–glucosidase activity of LAB with *S. boulardii* in fermented soymilk
Values with different letters were significantly different by Duncan's multiple range test ($p \leq 0.05$). (Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.5).

4.4.5 Transformation of isoflavone of soymilk fermented with lactobacilli

To quantify the different isoflavones, hydrolysed by β–glucosidase producing strains in fermented soymilk, the enzymatic transformation of isoflavones was studied. All strains hydrolyzed the conjugated isoflavones, daidzin and genistin, present in soymilk to the respective unconjugated isoflavones, daidzein and genistein.

The procedure of Chiou and Cheng (2001) was followed for the analysis of isoflavones (Materials and Methods). Changes in the four isoflavone contents in soymilk were monitored up to 48 h fermentation at 37°C. The contents of isoflavone glycosides, in soymilk fermented with LAB or in combination with yeast, significantly decreased with fermentation ($p < 0.05$). Rate of hydrolysis of isoflavone glycosides varied with the types of cultures used.

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The elution profile of standard glycosidic (genistin and daidzin) and aglyconic isoflavones (daidzein and genistein) and these in soymilk fermented with Lc is shown in **Fig. 4.10 A & B**. The isoflavone isomers were eluted according to their polarity and hydrophobic interaction with the reverse-phase HPLC column. Glycosidic isoflavones eluted first, followed by aglycones. Among the glycosidic form of isoflavones, daidzin eluted first followed by genistin. The aglyconic form of isoflavone, daidzein eluted before genistein.

The total concentration of isoflavone isomers (genistin, daidzin, genistein, daidzein) in unfermented soymilk (control) was 29.26 mg 100 mL^{-1} after 24 h (**Table 4.8**). The glycosidic forms (genistin and daidzin) occurred in high concentrations (90.05%) with a total of 26.35 mg100 mL^{-1}. Genistin was the highest individual isomer (19.7 mg mL^{-1}) while the concentration of bioactive isoflavone aglycones was very low (2.91 mg 100 mL^{-1}).

The changes in the concentration of glycoside and aglycone isoflavone isomers in soymilk fermented by La, Lb, Lc, Lp and Lf cultures individually for 24 and 48 h at 37°C is shown in **Table 4.8**. Soymilk fermented with Lb, Lc, Lp and Lf cultures, showed aglycone to increase and decrease in glycosidic isoflavones. The β-glucosidase catalyzed hydrolysis of isoflavone ranged from 27.98 to 39.56, 27.98 to 32.49 mg 100 mL^{-1} at 24 and 48 h of soymilk fermented with different LAB. However, the concentration of biologically inactive isoflavones was significantly reduced, ranging from 0.35 to 0.92 mg mL^{-1}. The lowest glycosidic isoflavone (0.35 mg100 mL^{-1}) was in soymilk fermented with *L. plantarum* and highest (0.92 mg100 mL^{-1}) was in soymilk fermented with *L. casei*.

After 48 h fermentation, the daidzein and genistein content of isoflavone aglycones in soymilk fermented with *L. acidophilus* continue to increase from 27.95 to 30.74 mg100 mL^{-1}. With Lb, Lc, Lp and Lf, it decreased from 30.66, 38.64, 27.54 and 33.90 mg100 $mL^{-1}$to 28.38, 31.52, 26.99 and 22.19 mg 100 mL^{-1} respectively. Parallely, the glycosidic concentration of soymilk fermented with different strains of LAB was less ranging from 0.79 to 9.22 mg100 mL^{-1}.

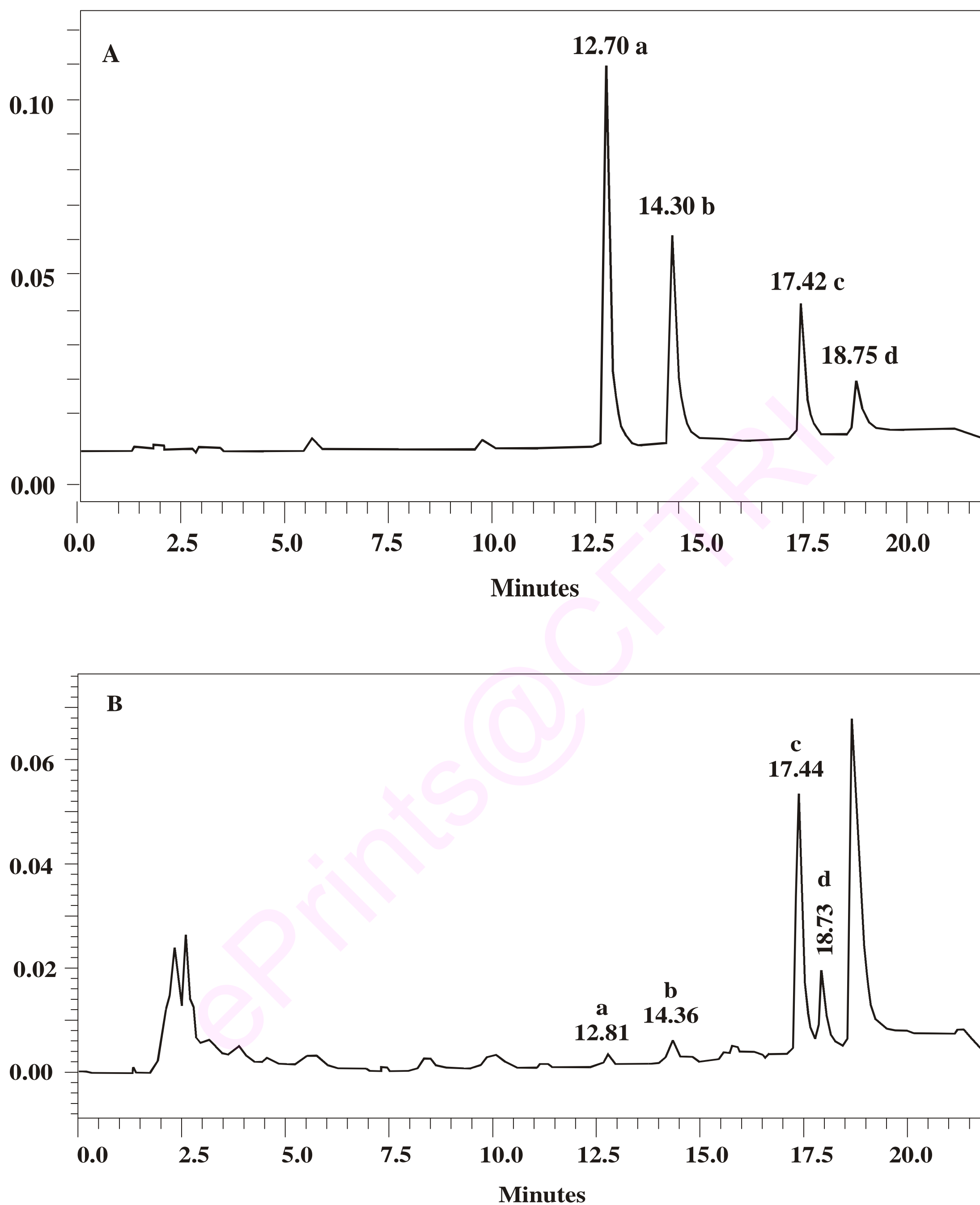

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Fig. 4.10 HPLC chromatograms of standard isoflavones and soymilk fermented with Lc

A. Daidzin (a), Genistin (b), Daidzein (c), Genistein (d).

B. Soymilk fermented with *L. casei* for 48 h at 37°C Daidzin (a), Genistin (b), Daidzein (c), Genistein (d).

Table 4.8 Isoflavone content of unfermented and fermented soymilk with LAB

Samples	Fermentation period (h)	Isoflavone content (mg100 mL^{-1}) Glucosides			Aglycones			Total (mg 100 mL^{-1})
		Daidzin	Genistin	Sub-Total	Daidzein	Genistein	Sub-Total	
Soymilk	24	6.65	19.7	26.35	1.19	1.71	2.91a	29.26ab
La	24	0.11[b](0.11)	0.59(0.68)	0.70(0.79)	5.97(6.57)	21.98(24.17)	27.95c(30.74c)	28.65a(31.53b)
Lb	24	0.11(0.19)	0.55(0.77)	0.66(0.96)	6.49(6.25)	24.17(22.13)	30.66c(28.38bc)	31.32b(29.34b)
Lc	24	0.13(0.19)	0.79(0.78)	0.92(0.97)	7.89(6.99)	30.75(24.53)	38.64e(31.52c)	39.56d(32.49b)
Lp	24	0.05(0.17)	0.30(0.82)	0.35(0.99)	7.38(6.09)	20.16(20.90)	27.54c(26.99b)	27.98a(27.98a)
Lf	24	0.07(1.49)	0.52(7.73)	0.59(9.22)	7.04(5.31)	26.86(16.88)	33.90d(22.19a)	34.49c(31.41b)

[b] Figures in parenthesis indicate the concentration after 48 h fermentation

Values in the same column with different letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (comparison on 24 and 48 h of fermentation with different strains).

(La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.4).

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In this study, significant glycoside isoflavone bioconversion to aglycones occurred during soymilk fermentation. As shown in **Fig. 4.11**, there appeared a correlation between β-glucosidase activity of strain used and bioconversion (%) of isoflavone glycoside to bioactive forms. Soymilk fermented with LAB showed maximum bioconversion after 24 h fermentation. The bioconversion in soymilk fermented with La, Lb, Lc, Lp and Lf at 24 h ranged from 97.55 to 98.43%.

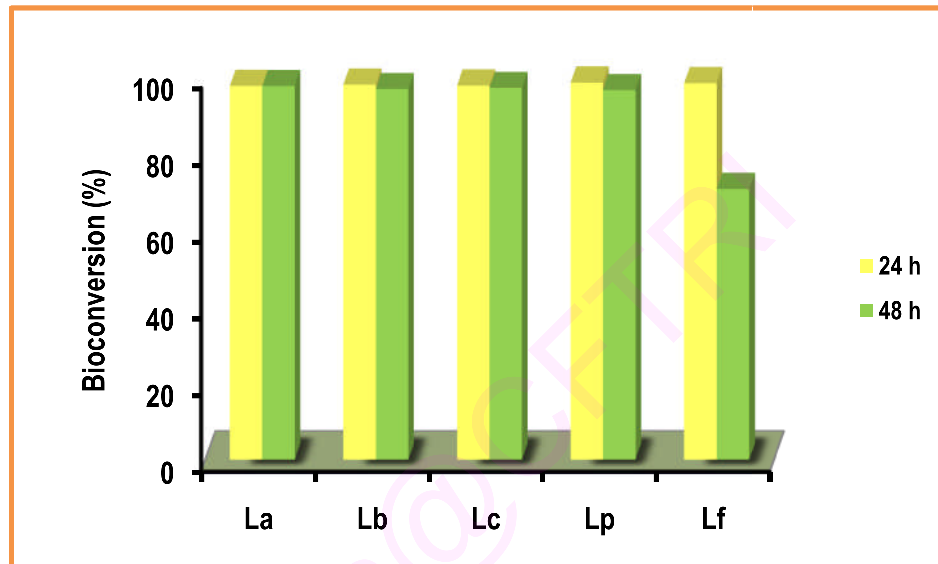

Fig. 4.11 Bioconversion of glycosides to aglycones in soymilk fermented with LAB (La, Lb, Lc, Lp & Lf: Expansions as given in Fig 4.8).

4.4.6 Transformation of isoflavone of soymilk fermented with lactobacilli and *S. boulardii*

The elution profiles of isoflavones of soymilk fermented with Sb+La is shown in **Fig. 4.12.** Changes occurred in the concentration of glycoside and aglycone isoflavone isomers in soymilk fermented by La, Lb, Lc, Lp and Lf in combination with *S. boulardii* for 24 and 48 h at 37°C **(Table 4.9)**. In these total isoflavones estimated were in the range 26.82 to 34.23 mg 100 mL^{-1}.

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Table 4.9 Isoflavone content of unfermented and fermented soymilk with LAB and yeast *S. boulardii* for 48 h

Source	Fermentation period (h)	Isoflavone content (mg100 mL^{-1})						Total (mg 100 mL^{-1})
		Glucosides			Aglycones			
		Daidzin	Genistin	Sub-Total	Daidzein	Genistein	Sub-Total	
Soymilk	24	6.65	19.7	26.35	1.19	1.71	2.91a	29.26a
Sb+La	24	0.08[b](1.19)	0.56(1.83)	0.64(3.02)	5.90(6.20)	22.63(25.01)	28.53b(31.21c)	29.17a(34.23c)
Sb+Lb	24	0.12(1.06)	0.68(1.26)	0.80(2.32)	6.94(5.45)	28.40(22.07)	35.34d(27.52bc)	36.14c(29.84ab)
Sb+Lc	24	0.07(0.88)	0.43(1.23)	0.50(2.11)	6.29(5.30)	23.28(20.04)	29.57bc(25.34bc)	30.07ab(27.45a)
Sb+Lp	24	0.14(1.43)	0.66(4.07)	0.80(5.5)	6.39(5.57)	24.78(20.69)	31.17bc(26.26bc)	31.97ab(31.76bc)
Sb+Lf	24	0.07(1.78)	0.42(8.22)	0.49(10.0)	6.72(4.32)	25.44(12.50)	32.16c(16.82a)	32.65b(26.82a)

[b] Figures in parenthesis indicate the concentration after 48 h fermentation

Values in the same column with different letters were significantly different by Duncan's multiple range test ($p \leq 0.05$) (comparison on 24 and 48 h of fermentation with different strains). (Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.5).

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After 48 h fermentation, the concentration of aglycones in soymilk fermented with Sb+Lb, Sb+Lc, Sb+Lp and Sb+Lf showed a decrease of 7.82, 4.23, 4.91 and 15.34 mg 100 mL^{-1}. After 48 h fermentation, the daidzein and genistein in soymilk fermented with Sb+La increased from 28.53 to 31.21 mg 100 mL^{-1}.

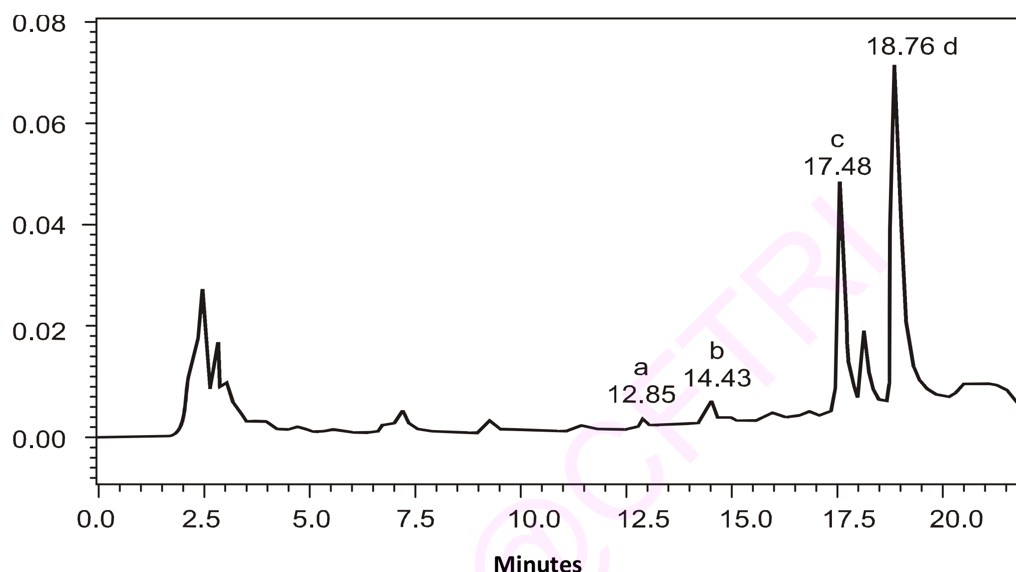


Fig. 4.12 HPLC chromatogram showing the elution profile of soymilk fermented with Sb+La. Daidzin (a), Genistin (b), Daidzein (c) Genistein (d).

Bioconversion of the glycoside isoflavones into their corresponding aglycones is shown in **Fig. 4.13**. The reduction in the content of β-glucosides (daidzin & genistin) and the increase in the content of their respective aglycones may be based on the hydrolytic reaction catalyzed by β glucosidase produced by each bacterial strain. Genistein and daidzein after 24 and 48 h of fermentation ranged from 97.49 to 98.49% and 62.71 to 92.31% respectively. All the combinations in fermented soymilk, showed highest bioconversion at 24 h rather than 48 h fermentation except Sb+Lf. Of the bioactive aglycone isomers, the concentration of genistein (12.50-25.01 mg 100 mL^{-1}) was significantly higher than daidzein (4.32-6.23 mg 100 mL^{-1}).

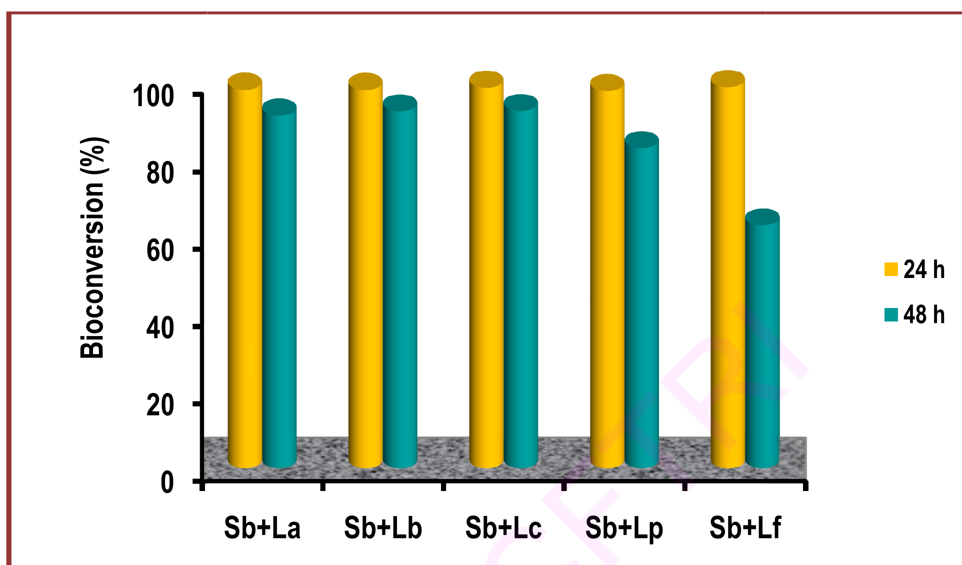
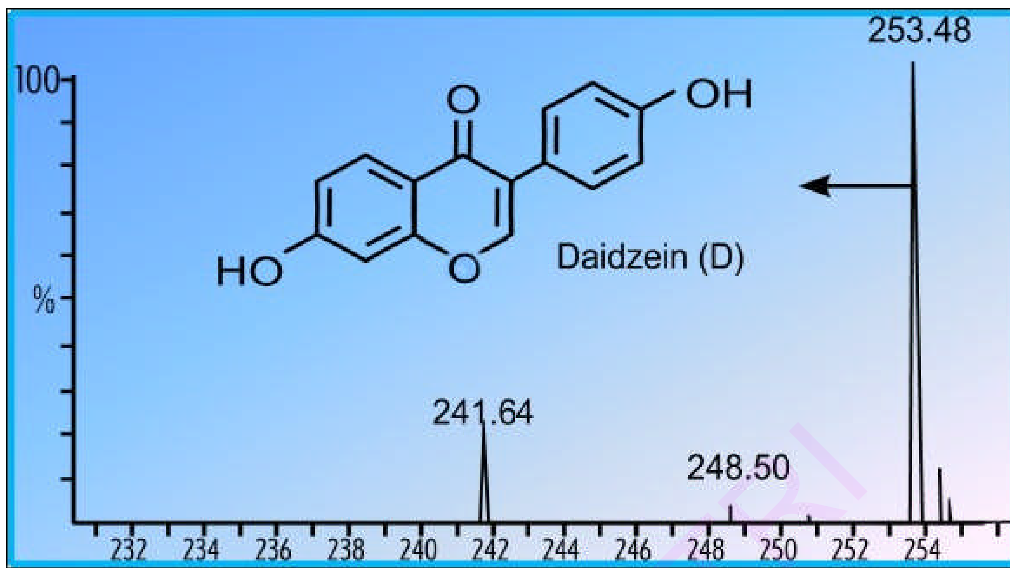


Fig. 4.13 Bioconversion of glycosides to aglycones in soymilk fermented with LAB and *S. boulardii*. (Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Fig 4.9).

4.4.7 Mass spectrometric analysis of isoflavones

The identification of isoflavones in fermented and unfermented soymilk is necessary to generate information for *in vivo* and *in vitro* studies and for the authentification of isoflavones in soy based fermented milk. So the fractions collected after HPLC were subjected to MS/MS analysis for further confirmation. The mass spectrometry of the aglyconic isoflavones, namely daidzein and genistein, confirmed by ESI negative fragmentation mode $[M-H]^-$, is described in **Fig. 4.14**. The two isoflavone glycosides, daidzin and genistin, were identified by comparison of the retention time and mass spectral data with those of standards. The molecular weight, mass and MS fragments of daidzein were 254, 255, 248.50,242 and genistein were 270, 271, 265.66, 256.70. The specific fragments at *m/z* 254, 270 for daidzin and genistin was confirmed by their MS/MS spectral data.



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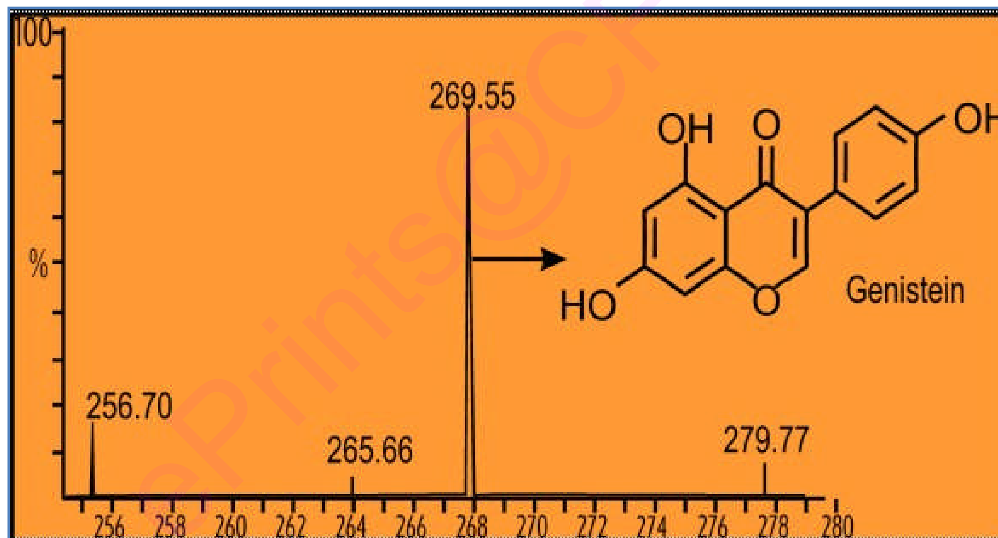

Fig. 4.14 ESI-MS/MS profile of aglycone isoflavones
A. daidzein B. genistein in fermented soymilk

4.4.8 Bioavailability of minerals in soymilk fermentation by LAB along with *S. boulardii*

The presence of antinutritional factor phytate in soybeans may decrease the absorption of minerals leading to mineral deficiencies. It may chelate nutritionally important cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}, thus decreasing the dietary

bioavailability of these nutrients (Wodzinski and Ullah, 1996). Since fermentation with microorganisms can remove or reduce phytic acids, fermenting soybean products have emerged increasingly significant. Lactic acid fermentation was known to reduce the phytate content in plant-based foods and has been most intensively studied in whole wheat bread making (Palacios *et al*., 2008).

The gastric digest of the sample was prepared and was titrated against 0.2 N sodium hydroxide till it reached pH 7.5 and was further subjected to intestinal digestion. The amount of sodium bicarbonate required to perform intestinal digest was calculated as per sodium hydroxide volume required for the titration. Intestinal digestion was carried out as detailed in Materials and Methods. Mineral concentrations were determined by atomic absorption spectrophotometry (Dennis *et al*., 1981) in an acetylene-air flame at the following wavelengths: 422.7 nm (Ca), 248.3 nm (Fe), 285.2 nm (Mg) nm and 213.9 nm (Zn).

Mineral availability of unfermented soymilk and soymilk fermented with diffennent strains of LAB and *S. boulardii* is shown in **Table 4.10**. Increase in calcium and magnesium levels was consistent in soymilk fermented with all combinations and also in yeast fermented milk compared to control. Highest increase in calcium bioavailability was observed in soymilk fermented with Sb+Lp (502%) and Sb+Lc (482%) combination and the lowest was seen in Sb+La (242%). The magnesium level was highest in soymilk fermented with Sb+Lh and Sb alone. In contrast iron content decreased in soymilk fermented with all combinations of LAB ranging from 39.22 to 25%. There was no significant difference in the zinc levels except in soymilk fermented with Sb+Lf combination and Sb alone, where the content was 121.70 and 86.63%.

4.4.9 Changes of B-vitamin content in soymilk during fermentation

Different researchers have reported that fermentation of soymilk increases the nutritional value by increasing the vitamins. It was reported by Hailong and Liang (2009) that the contents of niacin, riboflavin and thiamin increased when soymilk was fermented with the basidiomycete *Ganoderma lucidum* WZ02. It has been observed that in the preparation of fermented soybean products like Natto and Tempeh, most of the B-complex vitamins except thiamin increased.

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Table 4.10 Mineral availability of unfermented and fermented soymilk with LAB and yeast *S. boulardii*

Cultures	Calcium[a]	Iron[a]	Magnesium[a]	Zinc[a]
Soymilk (control)	5.40 ± 0.2 (0)	6.21 ± 0.2(0)	346.22 ± 18 (0)	22.33 ± 2 (0)
Sb+ La	18.50 ± 2 (+242.8)	4.49 ± 0.2 (-27.8)	359.52 ± 17 (+3.84)	24.15 ± 2 (+8.15)
Sb+ Lb	25.91 ± 1 (+380.15)	4.62 ± 0.3 (-25.7)	364.53 ± 20 (+5.29)	32.27 ± 3 (+44.5)
Sb+ Lc	31.43 ± 1 (+482.36)	4.59 ± 0.1 (-26.2)	368.94 ± 15 (+6.56)	23.66 ± 2 (+5.95)
Sb+ Lp	32.53 ± 1 (+502.72)	4.33 ± 0.2 (-30.3)	366.31 ± 23 (+5.80)	54.99 ± 4 (+146.2)
Sb+ Lh	28.73 ± 2 (+432.3)	3.77 ± 0.1 (-39.22	371.98 ± 20 (+7.44)	28.95 ± 3 (+29.62)
Sb+ Lf	26.74 ± 2 (+395.46)	4.11 ± 0.3 (-33.71)	366.21 ± 16 (+5.77)	121.70 ± 8 (+445)
Sb	24.10 ± 1 (+346.56)	4.04 ± 0.3 (-34.9)	375.73 ± 22 (+8.52)	86.63 ± 6 (+287)

[a](mg 100 g^{-1}) Values are means ± Standard error for triplicate analysis. Figures in parenthesis indicate % increase (+) or decrease (-) during fermentation. (Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.5).

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Soymilk fermented with lactobacilli and *S. boulardii* was hydrolyzed in order to study the nutritive value in terms of B-vitamins. The HPLC method of Hou *et al*., (2000) was followed to determine the content of niacin, riboflavin and thiamin (Materials and Methods).

The vitamin content of soymilk fermented with different combinations of LAB and *S. boulardii* and only yeast for 24 h is presented in **Table 4.11**. The increase of riboflavin and niacin content and decrease in thiamin was observed during fermentation. The riboflavin content in soymilk fermented with different combinations of LAB and yeast ranged from 0.43 to 0.67 mg 100 mL^{-1}. The highest was in soymilk fermented with Sb alone and among combinations Sb+Lb combination (0.67 mg 100 mL^{-1}) was highest compared to unfermented soymilk (0.36 mg 100 mL^{-1}) during 24 h of fermentation. The content of niacin was maximum in soymilk fermented with Sb+Lc 0.98 mg 100 mL^{-1} and minimum in soymilk fermented with Sb+Lp combination. In contrast the content of thiamin in fermented soymilk decreased, compared to control.

Table 4.11 Changes in B-Vitamin content of unfermented and fermented soymilk with LAB and yeast *S. boulardii* [a]

Cultures	Thiamin	Riboflavin	Niacin
Soymilk	2.17	0.36	0.10
Sb+La	2.10	0.57	0.83
Sb+Lb	2.30	0.67	0.78
Sb+Lc	2.60	0.63	0.98
Sb+Lp	1.60	0.63	0.73
Sb+Lh	1.50	0.43	0.77
Sb+Lf	1.70	0.50	0.97
Sb	1.60	12.84	0.73

[a]Data represent averages ± standard deviations of duplicate analyses of triplicate samples. (Sb, La, Lb, Lc, Lp & Lf: Expansions as given in Table 4.5).

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4.4.10 Volatile compounds of fermented soymilk

Beany flavor and aroma is limiting the acceptance of soy products. A variety of volatile compounds such as alcohols, aldehydes, ketones, pyrazene and furans have been reported to contribute beany flavor in soy (Vara-ubol *et al*., 2003). Most of the researchers report hexanol, hexanal, pentanal as compounds associated with green beany odour of the soybean extract.

Though many attempts have been made to eliminate the beany odour in soy products, heat, acid and enzymatic treatments have greatly reduced the flavor. Supercritical CO_2 extraction and addition of flavour compounds have also been used to remove or mask the beany odour (Blagden and Gilliland, 2005). Each of these processes can mask or remove the beany odour but the processes interfered with protein functionality.

One possible solution to overcome the odour is to use LAB for fermentation. Although the mechanism is not fully understood, research has revealed that certain bacterial strains can reduce the volatile compounds that are responsible for the beany flavor (Lee, 2001; Saide, 2001).

Volatile compounds of soymilk fermented with lactic acid bacteria individually and in combination with yeast were extracted using simultaneous steam distillation and extraction apparatus and extracts were analyzed by GC/MS. Identification of compound and confirmation were done by camparing the retention time and mass spectra with those of authentic standards. Quantification of compounds in each sample was determined by the standard curve method using the peaks of a specific fragment (a compound) with internal standards.

Tables 4.12 and 4.13 lists the volatile compounds identified in soymilk fermented with different strains of lactic acid bacteria and yeast. These compounds were grouped into esters, aldehydes, alcohols, hydrocarbons and acetals.

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Table 4.12 Volatile compounds of soymilk fermented with Lactic acid bacteria

Compound	Retention time	Concentration (%)
Aldehydes		
Isovaleraldehyde	3.05	2.81
Pentanal	3.17	2.95
Hydrocarbons		
Butane 2-3 dimethyl	3.47	14.19
2-methyl pentane	3.77	40.64
2,3 dimethyl pentane	3.99	51.02
3-methyl pentane	4.02	54.89
2 propanol-1-isopropanyl	5.71	2.63
Alcohol		
2-heptanol-3 methyl	5.74	7.24
Acetals		
Cyclobutane 1-2,dicyclopropyl	17.12	2.96
Others		
n-pentyl furan	16.62	1.93

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Table 4.13 Volatile compounds found in soymilk fermented with Lactic acid bacteria and yeast *S. boulardii*

Compound	Retention time	Concentration (%)
Alcohols		
2 butane-1,4 diol	4.00	19.50
1 methyl-1 octanol	5.71	3.79
2 methyl 2 pentanol	11.54	0.76
Isopentane alcohol	13.57	0.70
2-heptanol,6-amino, 2methyl	14.57	0.59
n-hexanol	17.87	0.98
7 octene-4-ol	20.64	0.27
Acetals		
Cyclopropane isopropyl	3.78	70.00
Cyclobutane 1-2 dicyclopropyl	13.28	57.53
Hydrocarbons		
2 methyl pentane	3.43	2.41
2,3 dimethyl pentane	3.75	7.12
3-dodecene(Z)	14.20	3.01
3-Tetradecene(Z)	20.33	2.98
Acids		
Hexadecnoic acid	26.47	13.97
pentanoic acid,1-undecyl ester	28.84	54.5
Esters		
Linalyl acetate	15.54	2.20

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The gas chromatographic separation of the total volatile distillate in the carbowax column is shown in **Fig. 4.15**. The results revealed the presence of 3-dodecene(Z), 2-heptanol,6-amino,2-methyl, 3-Tetradecene(Z), Hexadecanoic acid, pentanoic acid,1-undecyl ester as the volatile compounds in the fermented soymilk.

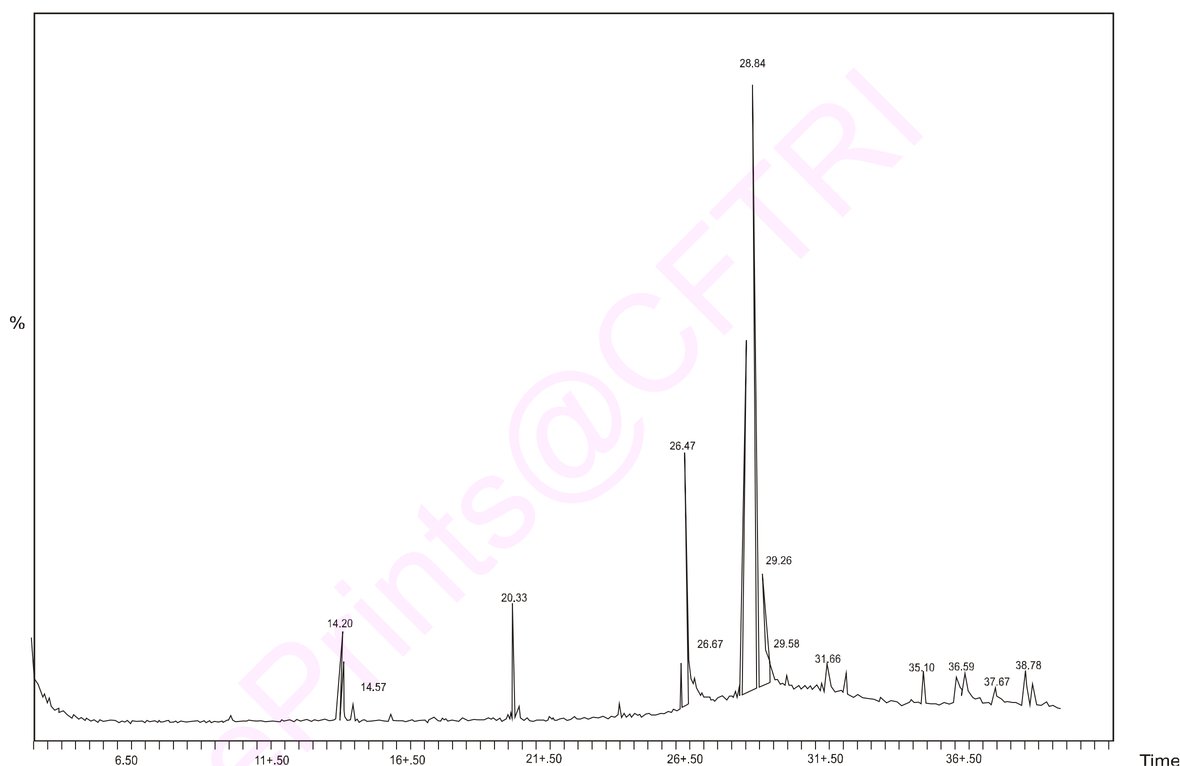

Fig. 4.15 GC-MS analysis of soymilk fermented with Sb+Lb

The results of the investigation showed that all the isolates of LAB and *S. boulardii* during fermentation of soymilk enriched bioactive isoflavones, enhanced viability of LAB strains, decreased antinutrient phytic acid, increased the mineral bioavailability and decreased the beany odour.

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5.1 Introduction

Soymilk is a low-cost substitute for dairy milk for the poor in developing countries. Being free of cholesterol and lactose, it is also a suitable food for lactose-intolerant consumers, vegetarians and milk-allergy patients (Chou and Hou, 2000). It is a popular soft drink in oriental countries. They are available in markets in packed cartons after pasteurization. The etiological agents of concern in soy milk are mainly *Listeria monocytogenes* and *Staphylococcus aureus* which act as post pasteurization contaminants.

L. monocytogenes is a widely recognized food-borne pathogen, known to survive under adverse conditions of temperatures and pH (Carrasco *et al*., 2006). This pathogen has been found in a wide range of foods including soymilk (Ferguson and Shelef, 1990) which can survive and grow at refrigerated temperature. Thus the storage of soymilk in refrigerator cannot successfully prevent the growth of psychrotrophic *L. monocytogenes*. Listerosisis is commonly associated with food products that support the growth of *L. monocytogenes* to levels in excess of 100 CFU g^{-1} (Chen and Hoover 2003).

Dairy products are known vehicles of staphylococcal poisoning. *S. aureus* is a facultative anaerobe, non motile, Gram positive food borne pathogen. It is readily destroyed in milk during pasteurization. The staphylococcal enterotoxins are relatively heat stable and are not easily inactivated in foods during cooking (Castro *et al*., 1986; Mossel and Vn Netten 1990; Dudrikova *et al*., 1998). To prevent this possible contamination or to eliminate these food borne pathogens, the application of bacteriocins produced by LAB to food products are reported by Ryan *et al*., (1996) and the inhibitory effects of bacteriocins produced by lactobacilli have also been described (Leisner *et al*., 1996; Ennahar *et al*., 1998).

Bacteriocins are small, ribosomally synthesised, extracellularly released, antibacterial peptides or proteins produced by LAB strains, that inhibit Gram positive bacteria, particularly closely related species (Cotter *et al*., 2005). Some of these inhibit food spoilage and food-borne pathogenic bacteria like *Bacillus, Clostridium, Staphylococcus* and *Listeria.*

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation because they are (i) generally recognised as safe substances, (ii) not active and nontoxic to eukaryotic cells, (iii) become inactivated by

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digestive proteases, having little influence on the gut microbiota, (iv) usually pH and heat-tolerant, (v) have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane, no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Galvez *et al*., 2007). Because of their potential applications, bacteriocins can be used in food fermentation processes in order to prevent contamination and spoilage (De Vuyst, 2000).

LAB are traditionally used as starter cultures for the fermentation of foods and beverages because, of their contribution to flavour, aroma development and retardation of spoilage (Gilliland, 1986). During fermentation, acid production lowers the pH and creates an environment that is unfavourable to pathogens and spoilage organisms. The preservative effect is not only due to acidic conditions, but also to antimicrobial compounds like hydrogen peroxide produced, ethanol, diacetyl, carbon dioxide, besides bacteriocin (De Vuyst and Vandamme, 1994).

Bacteriocin producing species have been identified in most of the LAB genera, *Lactococcus, Steptococcus, Lactobacillus, Leuconostoc* and *Pediococcus* as well as several *Enterococcus* sps (Jack *et al.,* 1995). The bacteriocins of Lactobacillus species belong to the class II bacteriocins, which are small and heat-stable, membrane-active peptides. Some of them are inhibitory towards food spoilage and food borne pathogenic bacteria (Zamfir *et al.,* 1999).

The present study was undertaken to determine the survival of food borne pathogenic bacteria *Listeria. monocytogenes* and *Staphylococcus aureus* in soymilk in presence of probiotic LAB. Efforts were also made to partially purify and characterize the bacteriocin produced by the lactic acid bacteria.

5.2 Screening for antimicrobial activity of LAB

An agar-well assay was used for the detection of antimicrobial activity. MRS agar plates were overlaid with 5 mL BHI (Brain Heart Infusion) soft agar inoculated with an overnight culture (50 mL) of indicator strain. Wells of 8 mm were cut and cell-free

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culture supernatant from selected LAB strains was placed into each well. After incubating the plates under aerobic conditions for 24 h at 37°C, they were examined for

zones of inhibition. Antimicrobial activity of the culture supernatants of nine LAB genera against indicator organisms, is presented in **Table 5.1**. Before assay, the culture supernatants of all the isolates of LAB, were adjusted to pH 6.5 to rule out the activity due to the production of organic acids.

The supernatant from three LAB, *L. acidophilus, L. casei* and *P. acidilactici* were selected for further study, as it showed the broadest spectrum by inhibiting all the indictor microorganisms and interaction among themselves were also tested by the above method and were chosen for further investigations.

The LAB strains selected, suggested the characterization of the antimicrobial compound of the cell free culture supernatant.

5.2.1 Effect of temperature on the antimicrobial compound

The effect of temperature on the stability of antimicrobial compound in the cell-free culture broths of LAB was determined by incubating at 50°C, 100°C and 121°C for 15 min and 30 min. For 50°C and 121°C treatment the culture broth was incubated at the temperature for 15 min only. Positive controls consisted of untreated samples. Antimicrobial activity was recorded after 24 and 48 h.

All the three isolates namely *L. acidophilus, L. casei* and *P. acidilactici* were stable after a treatment at 50°C for 15 min, 100°C for 15 min and 30 min. The cell-free extracts treated to 121°C were not active against *S. aureus* and *E. coli*. However they were active against *L. monocytogenes* **(Table 5.2 and Fig. 5.1).** The results appearing indicated the presence of antimicrobial compounds in the cell-free extracts of which are stable to autoclaving was active against *L. monocytogenes*.

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Table 5.1 Agar-well assay for antibacterial activity of LAB culture broth

Producer Strains	**Indicator strains (Inhibition zone in mm)** [a]								
	L. monocytogenes	*Y. enterocolitica*	*S. aureus*	*E. faecalis*	*P. aeruginosa*	*E. aerogenes*	*B. cereus*	*E. coli*	*S. Typhi*
L. acidophilus	25	++[b]	23	22	19	22	19	22	26
L. bulgaricus	12	++	19	18	15	17	15	12	19
L. casei	21	++	18	18	16	18	12	11	16
L. plantarum	-	++	-	13	-	-	-	-	-
L. helviticus	22	++	14	-	-	-	10	-	-
L. amylophilus	-	++	-	-	-	-	-	-	-
S. thermophilus	-	++	16	-	-	-	-	-	-
P. acidilactici	20	++	14	17	-	12	10	11	19
L. lactis	20	++	13	17	-	12	11	12	17

[a]Antimicrobial activities were performed in two independent experiments and average was taken.
[b]Complete inhibition Well (8 mm) containing 50 uL cell free culture broth

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Table 5.2 Effect of temperature on the activity of antimicrobial compounds of *L. acidophilus*, *L. casei and P. acidilactici**

Strains	Indicator organisms				
	Time of thermal treatment (min)	Temperatures (°C)	*S. aureus*	*E. coli*	*L. monocytogenes*
L. acidophilus	15	50	+	+	++
	15	100	+	+	++
	30	100	+	+	++
	15	121	_	_	+
L. casei	15	50	+	+	++
	15	100	+	+	+
	30	100	+	+	+
	15	121	_	_	+
P. acidilactici	15	50	++	++	++
	15	100	++	+	++
	30	100	++	+	+
	15	121	_	_	+

*Data represent averages ± standard deviations of duplicate analyses of triplicate samples. Diameter of the inhibiton zone: + inhibition zone (6-8 mm): ++ inhibition zone (9-12 mm): - no inhibition zone

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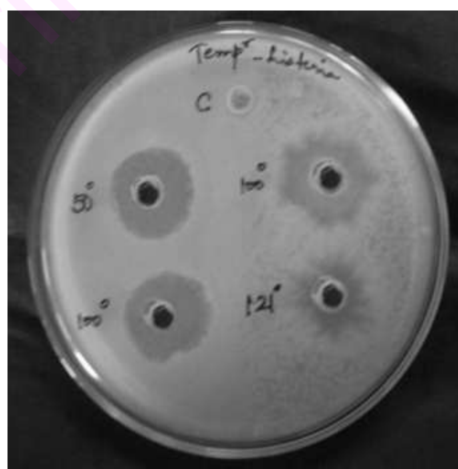

Fig. 5.1 Effect of temperature on the activity of antimicrobial compound
C: Control Test organism: *L. monocytogenes*

5.2.2 pH stability of the antibacterial compound

The stability of the pH of cell-free culture broths was studied, by adjusting the pH to 3.0, 5.0, 7.0 and 9.0 with 1 N HCl or NaOH. It is was allowed to stand at room temperature for 2 h and the residual activity was assayed against indicator strains by the agar well-diffusion method. Extracts consisted of uninoculated MRS broth adjusted to pH as described earlier.

The antimicrobial compound was stable at pH 3, 5 and 7. However maximum activity was measured when the cell free culture broth was maintained at pH 3 and 5. There was no inhibition at pH 9 **(Table 5.3 and Fig. 5.2).**

Table 5.3 Effect of pH on the activity of antimicrobial compound*

Strains	Indicator organisms			
	pH	*S. aureus*	*E. coli*	*L. monocytogenes*
L. acidophilus	3	+	++	++
	5	+	+	++
	7	+	+	++
	9	-	-	-
L. casei	3	+	+	++
	5	+	+	++
	7	+	+	++
	9	-	-	-
P. acidilactici	3	+	++	++
	5	+	+	++
	7	+	+	+
	9	-	+	-

*Data represent averages ± standard deviations of duplicate analyses of triplicate samples. Diameter of the inhibiton zone: + inhibition zone (6-8 mm): ++ inhibition zone (9-12 mm): - no inhibition zone.

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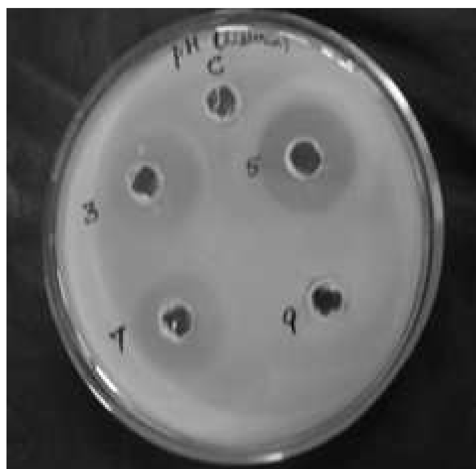

Fig. 5.2 Effect of pH on the activity of antimicrobial compound
C: Control Test organism: *L. monocytogenes*

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5.2.3 Effect of proteases on the antimicrobial compound

To determine the effects of proteases on the antimicrobial compound present in cell free extracts, the pH was brought to 6.5 and treated with 10 mg mL^{-1} protease, trypsin and papain. Cell free culture broth without proteases served as control. The samples were incubated for 24 h at 37°C for protease activity and inhibiting activity was determined by the agar-well diffusion assay. The absence of inhibition zone in presence of the proteases was also confirmed.

Our results showed that the antibacterial activities the three isolates namely *L. acidophilus, L. casei* and *P. acidilactici* was lost after 2 h of treatment with protease, trypsin and papain **(Table 5.4).** Proteases destroyed antimicrobial activity of all cell-free supernatant **(Fig. 5.3 A & B).** Antimicrobial activity was observed in the control, consisting of untreated supernatants from the respective LAB strains. Since the protease treated samples showed no inhibition zones, it appeared that the antimicrobial compound was a polypeptide.

Table 5.4 Effect of proteases on the activity of antimicrobial compound*

Strains	Protease	Indicator organisms		
		S. aureus	*E. coli*	*L. monocytogenes*
L. acidophilus	Control[a]	12.3[b]	9.8	8.5
	Trypsin	-[c]	-	-
	Pepsin	-	-	
	Papain	-	-	-
L. casei	Control	9.2	8.3	7.6
	Trypsin	-	-	-
	Pepsin	-	-	-
	Papain	-	-	-
P. acidilactici	Control	11.2	9.4	8.7
	Trypsin	-	-	-
	Pepsin	-	-	-
	Papain	-	-	-

*Data represent averages ± standard deviations of duplicate analyses of triplicate samples. [a]Control samples consisting of freshly prepared cell supernatants without treatment. [b]Inhibition halos (mm) by the agar-well diffusion assay. [c]No inhibition zone

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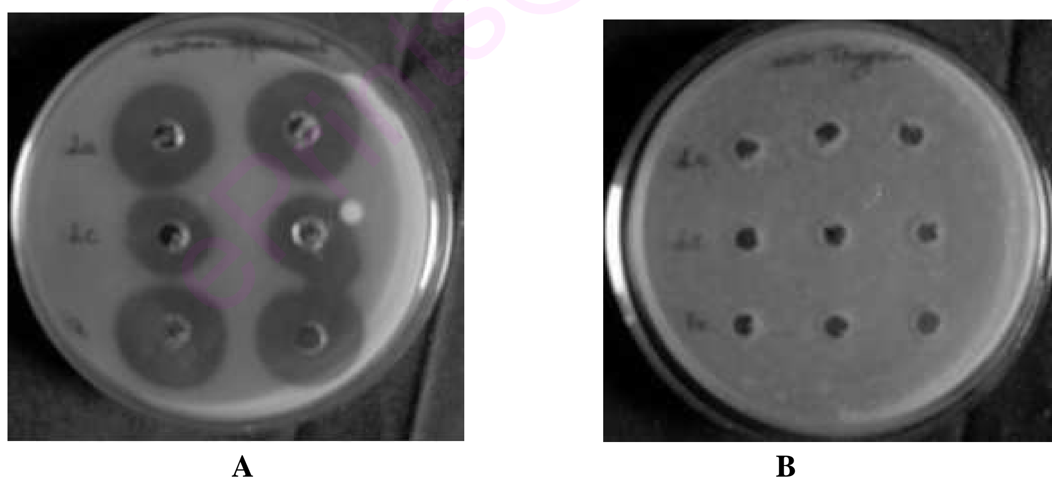

A B

Fig. 5.3 Effect of proteases on the activity of antimicrobial compound. **A** Cell free culture broth treated to proteases showing no inhibition zones **B** Controls

5.3 Chloroform extraction

Based on preliminary experiments, the antimicrobial compound was extracted in chloroform as described in Materials and Methods.

Although the chloroform layer did not have antimicrobial activity, the precipitate at the interface between the chloroform and the culture supernatant fluid showed antimicrobial activity. This precipitate was isolated and dissolved in phosphate buffer for assay. Therefore, rapid and efficient separation of antimicrobial compound from culture supernatant fluid by chloroform seems feasible. The total activity was greater in the precipitates dissolved in the crude culture broth **(Fig. 5.4).** Hence the precipitate was used as crude antimicrobial compound.

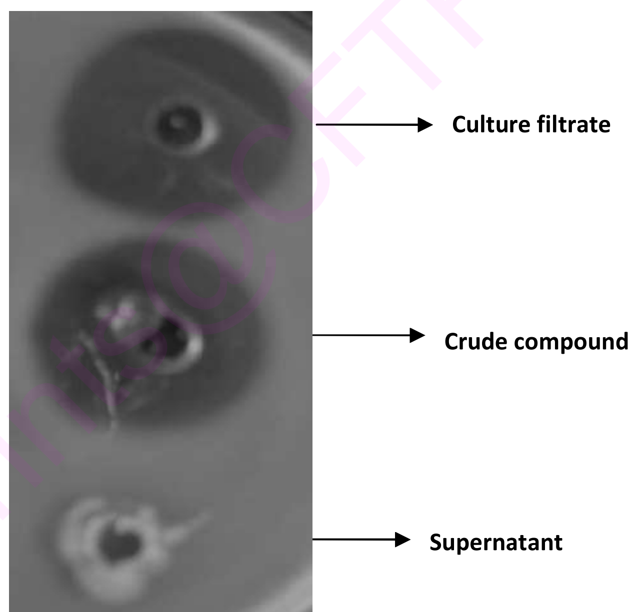

Fig. 5.4 Antibacterial activity of the crude antimicrobial compound

5.4 Tricine SDS-PAGE

The crude antimicrobial compound concentrated by lyophilization and suspended in 10 mM phosphate buffer was used for electrophoresis. The apparent molecular mass was estimated by the Tricine Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE). The procedure for the SDS-PAGE is as described in Materials and Methods.

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After electrophoresis, the gel was cut into two halves. One half of the gel was stained with silver. The other half of the gel was overlaid with 20 mL of BHI soft agar (0.80%) seeded with freshly grown indicator strain of *L. monocytogenes* Scott A. The plate was incubated at 37°C for 24 h and examined for the presence of zone of inhibition (vide Materials and Methods).

Protein in the crude separated by Tris SDS-PAGE revealed an antimicrobial low-molecular size peptide band ~ 6.5 KDa **(Fig. 5.5, Lane 2 & 3).** The apparent molecular mass was estimated to be a 4.0 to 4.5 kDa peptide. When the gel was overlaid with BHI soft agar containing *L. monocytogenes* Scott A as indicator strain, the protein band showed a zone of growth inhibition corresponding to the band **(Fig. 5.5, Lane 4) .**

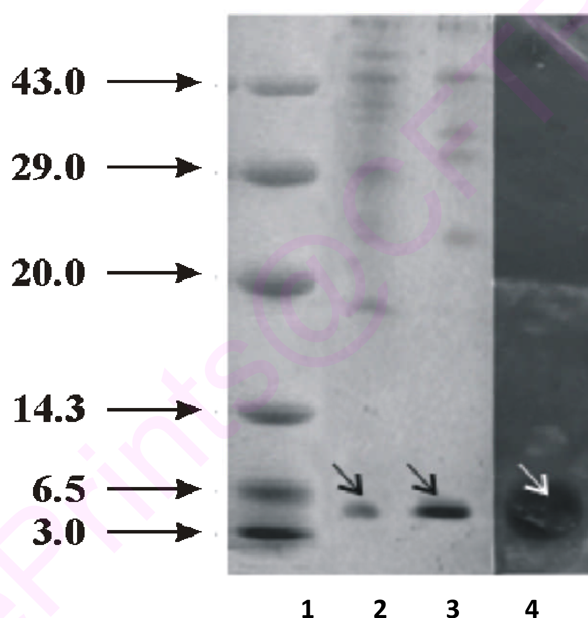

Fig. 5.5 Tricine SDS-PAGE profiles of partially purified bacteriocin

Lane 1 : Molecular weight markers (silver stain)

Lane 2 & 3 : Crude antibacterial compound of *L. acidophilus* and *P. acidilactici* (silver stain)

Lane 4 : Zymogram showing antibacterial activity of the protein (arrow).

The assay was performed as described in Materials and Methods.

5.5 Survival of *S. aureus* in soymilk fermented with LAB

Bacteriocinogenic strains are bacteria producing bacteriocin. These adjuncts, co-cultures in combination with a starter culture, or as protective cultures (especially in the case of nonfermented foods) can be used directly as starter cultures. When used as starter cultures, such bacteriocinogenic strains must be able to carry out the desired fermentation process, optimally, besides being able to produce bacteriocin to afford protection (Hikmate *et al*., 2007). Thus their use in food preservation is now being approved in several countries (Delves-Broughton *et al.,* 1996; Gomez *et al*., 1997).

Bacteriocins of LAB's can be antagonistic to various food borne pathogens such as *S. aureus*, *L. monocytogenes*, *Clostridium botulinum* and others. Bacteriocin-like compounds produced by LAB and their bactericidal mode of action were suggested application as additives in soymilk fermented products for the inhibition of food-borne toxigenic strains. Thus survival of *S. aureus* was investigated when co-cultivated with LAB's in this study.

Three bacteriocin-producing LAB's (*L. acidophilus* B4496, *L. casei* B1922 and *P. acidilactici* K7*)* were co-cultivated with *S. aureus* separately in different combinations. Cell concentrations of LAB and *S. aureus* were 10^7 $\log_{10}$ CFU mL^{-1} and about 10^5 $\log_{10}$ CFU mL^{-1} respectively. Three flasks containing 100 mL sterile soymilk were taken. To the first flask, 1.0% *S. aureus* was inoculated. The second flask was inoculated with 1.0% *L. acidophilus*, *L. casei* and *S. aureus* were added and the third flask contained *L. acidophilus*, *L. casei*, *P. acidilactici* and *S. aureus*. All the three flasks were incubated at 37°C for 20 h. Samples were taken for enumeration at an interval of 4 h for 20 h.

Cell counts of LAB were determined by pour plate method on MRS agar media for LAB and Baird Parker agar medium for *S. aureus*. Fermented soymilk (10 g) was added to 90 mL of sterile 0.85% saline (w/v) and vortexed for 30 sec. The suspension was serially diluted in sterile 9 ml saline and 1 ml dilution was used for viability determination. The inhibitory effect of the bacteriocin-producing LAB on the indicator organism was determined by comparing the viable count of *S. aureus* in soymilk inoculated only with *S. aureus*, which served as the control.

The viable count of *S. aureus* in fermented milk at the end of 16 and 20 h fermentation was 4.84 and 4.14 $\log_{10}$ CFU mL^{-1} respectively. Maximum growth was

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recorded after 16 h fermentation **(Fig. 5.6).** When it was co-cultivated with *L. casei* and *L. acidophilus* the CFU of *S. aureus* was 1.54 and 0.37 log_{10} decrease after 16 and 20 h respectively. With *L. casei, L. acidophilus* and *P. acidilactici*, no growth of *S. aureus* was recorded after 8 h fermentation. The results suggested a bacteriocidal effect against *S. aureus*.

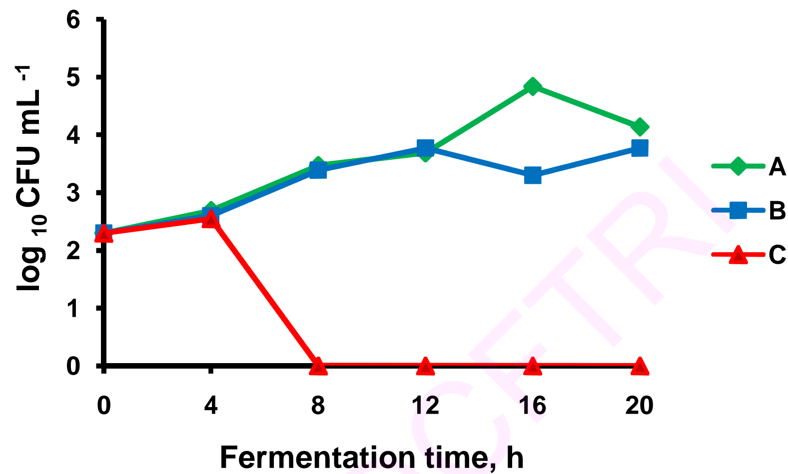

Fig. 5.6 Viability of *S. aureus* in presence of *L. acidophilus, L. casei* and *P. acidilactici* **A.** *S. aureus* (control) **B.** *S. aureus* viability in presence of *L. acidophilus* & *L. casei* **C.** *S. aureus* viability in presence of *L. acidophilus, L. casei* & *P. acidilactici*

5.6 Survival of *L. monocytogenes* in soymilk fermented with LAB

In this experiment, bacteriocin producing LAB's were used for soymilk fermentation with *L. monocytogenes.* Counts were obtained by spread plate on Listeria oxford agar medium.

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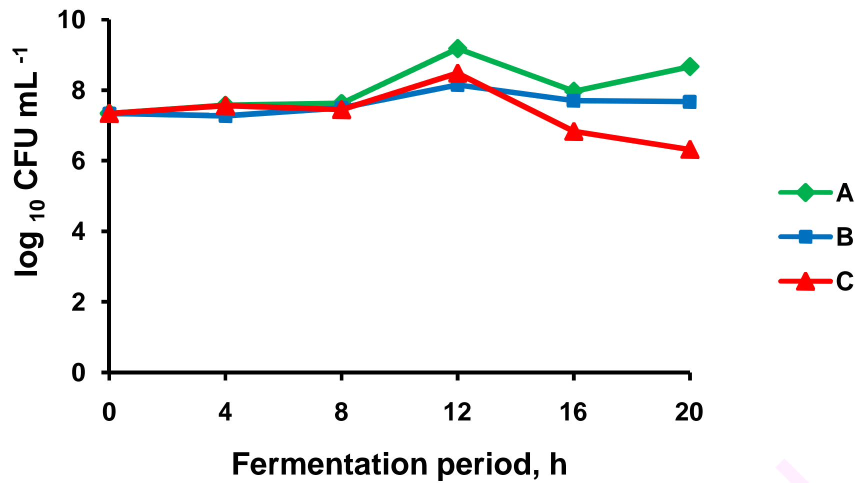

Fig. 5.7 Viability of *L. monocytogenes* in presence of *L. acidophilus, L. casei* and *P. acidilactici* **A**. *L. monocytogenes* (control) **B.** *L. monocytogenes* viability in presence of *L. acidophilus* & *L. casei* **C.** *L. monocytogenes* viability in presence of *L. acidophilus, L. casei* & *P. acidilactici*

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The results show that *L. monocytogenes*, was not fully eliminated **(Fig. 5.7),** although a difference of 2.35 log was determined at the end of 20 h fermentation. *L. casei* and *L. acidophilus* did not affect the growth of *L. monocytogenes*. When *L. monocytogenes* was cultured along with *P. acidilactici* there was 1.14 and 2.35 log_{10} CFU mL^{-1} reduction after 16 and 20 h fermentation.

6.1 Introduction

Annually India produces about 6.5 million tons of soybeans (Bhatnagar, 2002). Soybean is the richest source of protein (40%) with balanced contents of all essential amino acids among different legumes. It also contains many of the essential nutrients including cholesterol free fat, rich in polyunsaturated fatty acids, dietary fiber, vitamins and minerals. Though rich in various valuable nutrients and its vast production, it has remained under utilized in the country's traditional foods due to beany flavour that has poor sensory acceptability among the Indian consumers. India is a non-traditional soybean producing and consuming country.

During the manufacture of tofu, coagulation of soymilk with salts, acid or enzymes generate a liquid by-product called ''tofu whey'' and solid by-product "okara".

Tofu whey is a good source of carbohydrates mainly sucrose, raffinose and stachyose, as well as protein. Coagulating agent, magnesium also contributes to the nutrition (Kohyama *et al.,* 1995). It being low cost, disposal of the by-products constitutes an environmental and industrial problem (Penas *et al.,* 2006). Nguyen Thi *et al*., (2003) used tofu whey as a growth medium for the production of *Lactobacillus paracasei ssp. paracasei* LG3 and proposed that it can used as starters for the fermentation of soy product. Partially demineralized and pre-treated tofu whey was used as growth medium for *L. plantarum.* (Ben Ounis *et al.,* 2008).

Okara, a by-product of tofu, during soymilk or soy protein manufacturing, putrifies very quickly due to its high water activity (O'Toole, 1999). Therefore, its industrial use is not common and it is considered a by-product (Surel and Couplet, 2005). Fresh okara contains about 1.5% lipid, 7.0% sugars, 1.5% fiber and 0.4% ash by weight (O'Toole, 1999). It also contains about 20 to 27% protein (dry basis) and 52 to 58% dietary fiber (Ohno *et al*., 1996; Chan and Ma 1999). The high nutritive quality and superior protein efficiency ratio, suggested that it has a potential source of low cost vegetable protein for human consumption (Kasai *et al.,* 2004; O'Toole, 1997). Other components that are present in okara include, isoflavones, lignans, phytosterols, coumestans, saponins and phytates. These compounds have various physiological and therapeutic functions such as antioxidant activity, prevention of cardiovascular diseases and effective chemopreventive agents for certain types of cancer.

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Wet okara was utilized in the preparation of low-fat beef patties in different levels and found that the addition of okara reduced the cholesterol content of the samples by about 6–56% in uncooked beef patties and 9–42% in cooked beef patties. It increased the pH and can be used up to 22.5% for the production of cheaper and healthier beef patties. (Turhan *et al.,* 2007). According to Rinaldi *et al.,* (2000), wet okara can successfully be used to make and enrich extruded wheat products. Genta *et al*., (2002) used okara to make a soy candy and found that the lowest addition level, 18.3% (based on 100% formulation) of okara was the most acceptable and preferred by the judges. These studies showed that okara was a good source of protein for human health. Waliszewski *et al.,* (2002) studied the effect of okara supplementation on certain physical, chemical and sensory properties of corn tortilla and recommended its use in dried form, up to 10%, for tortilla enrichment.

Amudha *et al.,* (2002) reported dough characteristics and quality of fried savoury and sweet snacks prepared by blending wheat flour and defatted soy flour. An increase in protein content of fried savoury snacks from 20.75 to 27.50% and from 15.75 to 21.75% in sweet snack was reported with increase use of soy flour.

The Soybean Processing and Utilization Centre (SPU) at the Central Institute of Agricultural Engineering, Bhopal, India, has developed 19 soyproducts, 20 soybean processing equipments and pilot scale production facilities for full fat soyflour (FFSF), partially defatted soyflour (PDSF), soy-milk, soypaneer, soyfortified biscuits, soy-ice cream and other products (Swaminathan and Chadha, 2006).

In this study, tofu and okara (soy residue) were used for the preparation of traditional fermented foods like idli, vada and also tofu masala. Whey obtained during tofu preparation was also utilized for the natural pigment production by solid-state fermentation with *Monascus purpureus*.

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6.2 Acceleration of fermentation of idli batter using soy residue okara

Idli is a traditional steam cooked, popular fermented breakfast food, especially in Southern parts of India. The process of fermentation of ingredients is essential which determines the quality of idli. Idli is prepared by steaming the mixture of fermented rice (*Oryza sativa*) and black gram (*Phaseolus mungo*) batter in the ratio of 3:1. It makes an important contribution to the diet as a source of protein, calories and vitamins, especially

B-complex vitamins, compared to the raw unfermented ingredients (Reddy *et al*., 1982). Hence, the present study was conducted to reduce the fermentation period of idli batter from a conventional 14 h to 10 h by adding okara.

6.2.1 Effect of okara fortification on pH and titrable acidity

Rice soji and black gram dhal were taken in the ratio 3:1. Dehusked split black gram dhal (30 g) was washed twice, soaked in 120 mL water for 4 h at room temperature (28 ± 2°C) and ground separately in an electricity operated blender with required quantity of water, into a batter of desirable consisitency. Rice soji (90 g) was washed with water and mixed with dhal batter (control). The batter was allowed to ferment for 14 h at room temperature. For the preparation of okara fortified idli batter, rice soji, black gram dhal and okara were taken in the ratio 3:0.5:0.5. Okara was ground to fine paste with water, mixed with the batter and packed in metalized polyester-LDPE pouches and sealed. This was allowed to ferment for 10 h at room temperature. After every 2 h of natural fermentation, pH and acidity were measured.

The changes in pH and acidity of okara fortified and unfortified idli batter are represented in **Fig. 6.1.** The pH of control batter at 0 h was 6.32 which decreased to 4.51 at the end of 14 h of fermentation. The initial pH of okara fortified batter was 6.30 and 4.53 at 14 h of fermentation. With the progress of fermentation, increase in the acid content of batter during fermentation was observed in both control and okara fortified idli batter. Acidity increased from 0.18 to 0.64 in control batter and from 0.15 to 0.43 in okara fortified batter over a period of 14 h fermentation **(Fig. 6.1)**. Although pH of the control batter and okara batter was almost similar, the acidity was significantly different. The increase in acidity was seen within 2 h of natural fermentation in control batter and after 6 h of natural fermentation in okara fortified batter.

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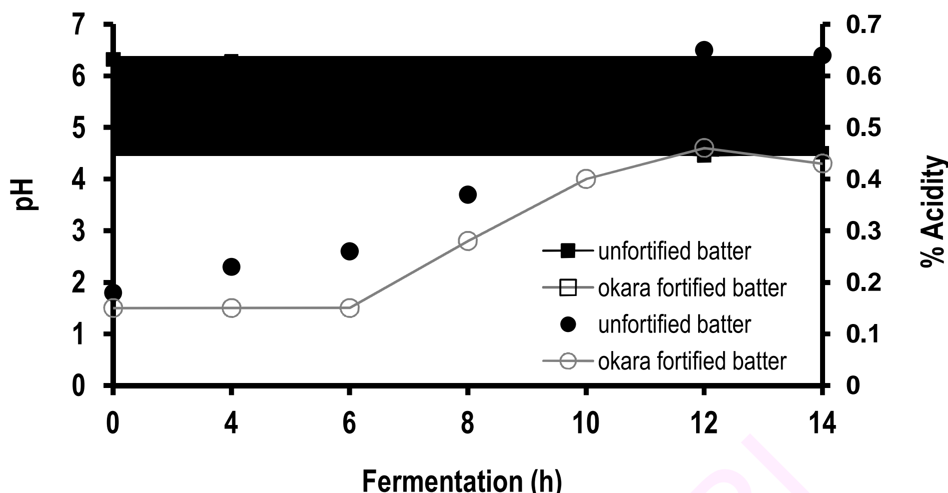

Fig. 6.1 pH and total acidity of control and okara fortified idli batter

6.2.2 Increase in batter volume during fermentation

Batter (50 mL) was transferred to a sterile measuring cylinder and increase in volume was recorded as described in Materials and Methods and the raise in batter volume was expressed as the % volume increase over the initial volume.

There was a noticeable change in batter volume during fermentation. At the end of 10 h fermentation, 20% raise in unfortified batter and 55% raise in okara fortified batter were recorded. An increase of 35% raise in batter volume was observed in okara fortified batter compared to unfortified batter. The increase in volume was due to the CO_2 production by yeast during fermentation and is a measure of their metabolic activity.

6.2.3 Effect of okara on CO_2 release during fermentation

Carbon-di-oxide released during batter fermentation was quantified using CO_2 Analyzer (phi Dan sensor, Denmark). The batter during fermentation, was analyzed from 0 to 14 h **(Fig. 6.2).** At the end of 10 h fermentation the % of CO_2 release in control batter was 4.0 while increase CO_2 release was quantified in (33.6%) okara fortified batter. At the end of 14 h fermentation, % CO_2 release was 19.7 and 35.5 in control and okara fortified batter, respectively.

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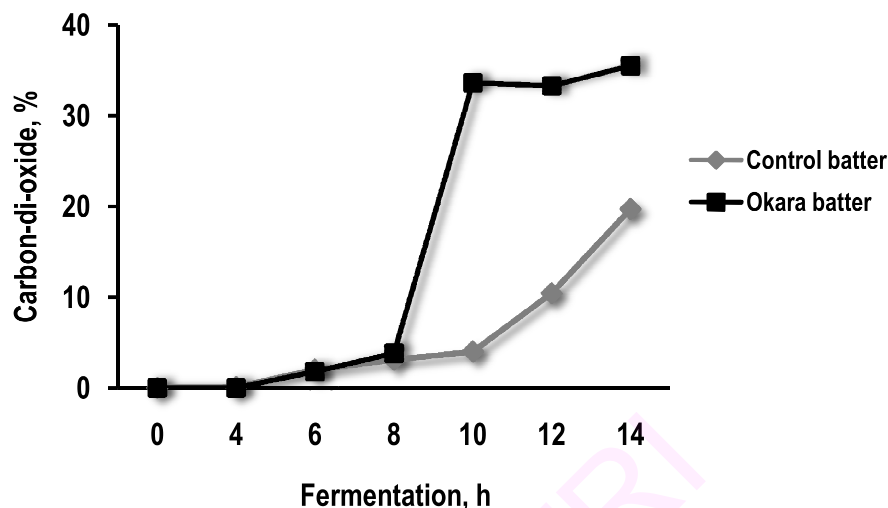

Fig. 6.2 Carbondioxide released during batter fermentation

6.2.4 Microbiology of fermented batter

To determine viable count of Lactic acid bacteria, mesophilic bacteria and yeast and molds of fermented 'idli' batter (control and okara fortified batter), 10 g samples were homogenized with 90 mL sterile 0.85% NaCl, for 2 min in a shaker at normal speed. Ten fold serial dilutions were prepared and pour plated on MRS agar for the enumeration of LAB. Spread plate technique was employed to determine the counts of total mesophilic bacteria, and yeast and molds using Nutrient agar (NA) and Potato dextrose agar (PDA) respectively.

Results of the microbial analysis are described in **Table 6.1.** It appeared that bacteria and yeasts fermented the batter. The counts of natural mesophilic bacteria and LAB showed a progressive increase in their counts with increase in time. There was a significant increase in yeast and mold counts in okara fortified batter compared to control. There was two log increase in LAB count in control batter compared to okara fortified batter.

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Table 6.1 Microbiological profile of control and okara fortified batter during fermentation[a]

	Lactobacillus count (log_{10} CFU g^{-1})[b]		Yeasts and Mould count (log_{10} CFU g^{-1})[b]		Mesophilic bacteria count (log_{10} CFU g^{-1})[b]	
Fermentation period(h)	0	10	0	10	0	10
Control batter	5.81±0.35	8.66 ±1.85	8.00 ±1.66	9.00 ±1.72	8.30 ±1.40	8.65 ± 1.65
Okara fortified batter	6.80±0.88	7.69 ±1.23	8.54 ±1.56	10.34 ±1.82	8.30 ±1.36	9.47 ± 1.22

[a]Data represent averages ± standard deviations of duplicate analyses of triplicate samples. [b]Colony forming units per gram

6.2.5 GC/GC-MS analysis of control and okara fortified batter

Volatile compounds were extracted with methylene chloride. Idli batter fermented for 10 and 14 h (1 g) was suspended in 10 mL methylene chloride and mechanically homogenized. The extraction procedure was repeated thrice and centrifuged for 10 min at 5000 g at ambient temperature. The solvent layer was collected, dried over anhydrous sodium sulphate and concentrated to 1 mL volume. For analysis, 2 µL sample was used for GC/GC-MS.

The idli batter prepared with and without okara, when subjected to GC/GC-MS analysis, revealed the presence of acids and ester which was identified based on the retention times and comparing the mass fragmentation pattern of the standard compounds **(Table 6.2).** Control batter and okara fortified batter showed the presence of same volatile compounds like acids and esters but the percentage varied **(Fig. 6.3).** Except 9-octadecenoic acid (E), okara fortified batter had lesser amount of acids and ester compared to control batter.

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Table 6.2 Flavour compounds of idli batter

Compounds	Retention time,		%	
	1	2	1	2
n-hexadecanoic acid	26.56	26.61	39.27	32.23
Hexadecanoic acid ethyl ester	26.90	26.92	1.49	1.41
9,12-octadecadienoic acid (Z,Z)	28.82	28.93	17.55	13.66
9-octadecenoic acid (E)	28.95	29.06	31.69	43.86
Octadecanoic acid	29.34	29.41	6.92	4.92

1. Control batter 2. Okara fermented batter

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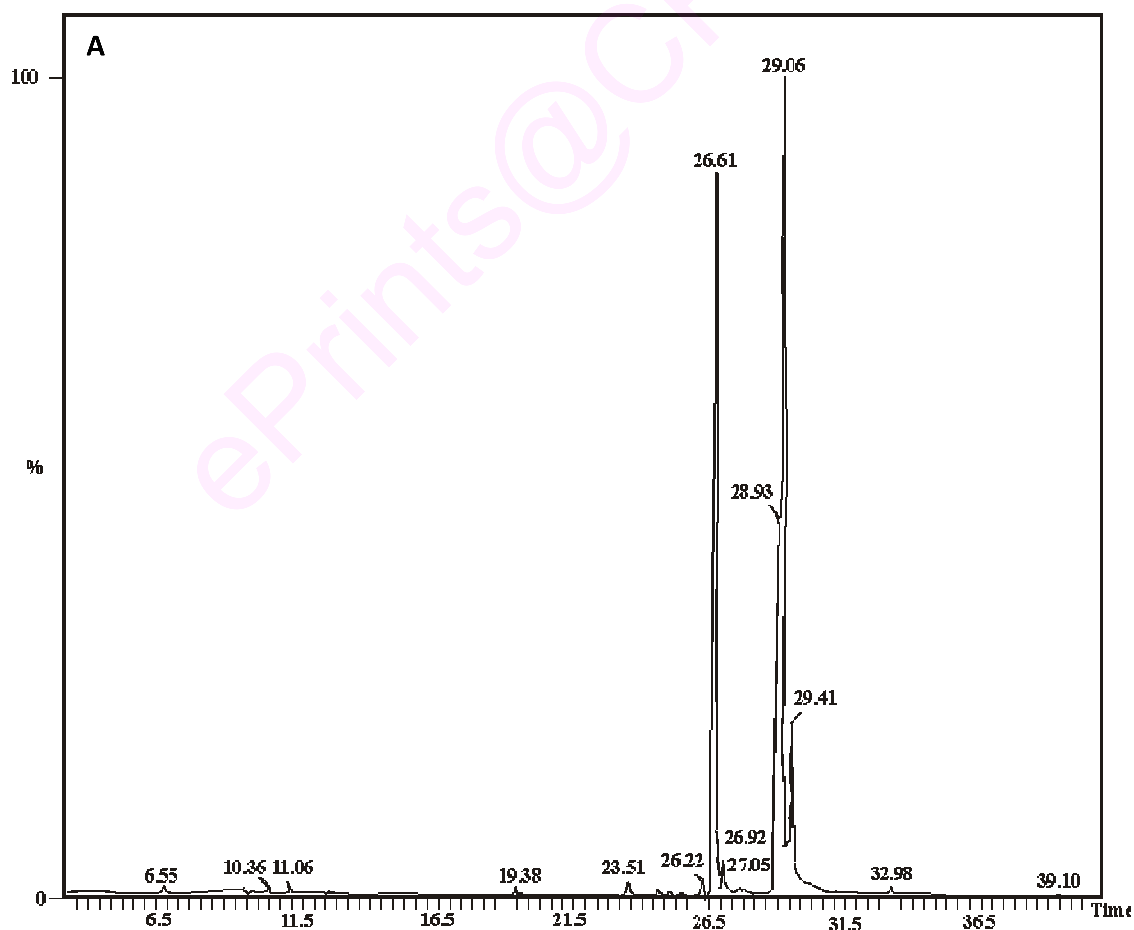

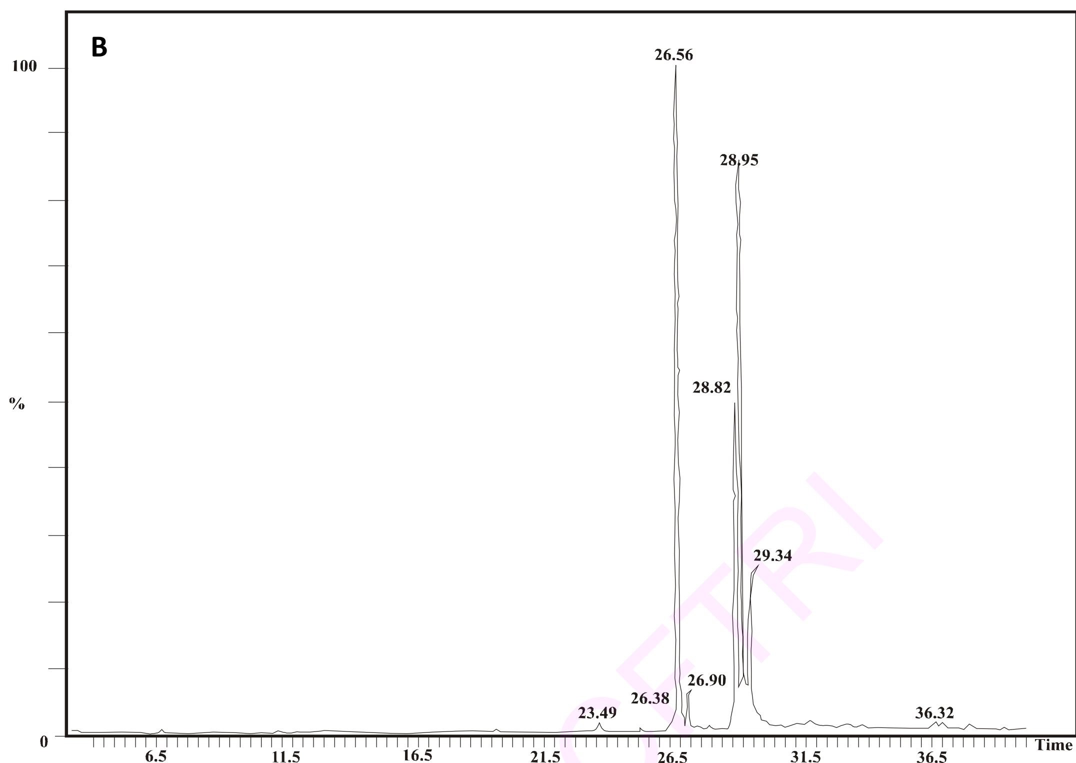

Fig. 6.3 GC-MS chromatogram of idli batter

A. Okara batter **B.** Control batter

6.2.6 Texture of idli

The texture of 'idli' was analyzed using texture analyzer (Materials and Methods). Among the several textural parameters, hardness and stickiness were selected to represent the results because of their repeatability and reasonable variations. Measurements were performed in six replicates and the average was recorded in Newton units.

Table 6.3 shows the result of textural parameters of idlis prepared using control and okara fortified batter. Idli had a circular shape of approximately 7-10 cm diameter (depending on the mould size) flat with lower and upper surface bulging, so that the product is thick at the center (2-4 cm) and tapering towards periphery **(Fig. 6.4).** Hardness was measured as the peak force during compression in the first cycle. Hardness of traditional idli was 33.05 Newton and idli prepared with okara substituted batter fermented for 10 h idli was 24.4 Newton, respectively.

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6.2.7 Sensory evaluation of idli

'idli' prepared with different batters were subject to sensory evaluation by the method of Quantitative Descriptive Analysis (QDA) as described in Materials and Methods. The scores of sensory evaluation of idli's prepared with control and okara fortified idli batter are presented in **Table 6.3.**

Table 6.3 Texture and sensory profile of idli's prepared

Parameter	Type of batter used for idli	
	Control	**Okara fortified**
A) Texture analysis	33.05a	24.40
Stickiness, (N. s)	0.13a	0.14a
Adhesiveness, (N. s)	0.053b	0.050b
B) Sensory attributes		
Buff	6.65	6.91
Fluffiness	7.88	7.98
Compactness	7.23	7.81
Sponginess	7.07a	7.41b
Firmness	5.96	6.17
Sticky	3.63a	5.67b
Beany	2.67a	3.64b
Fermented	6.98	6.99
Salty	5.91b	5.00a
Sour	4.28	4.31
Bitter after taste	2.73	3.18

Mean scores in the same row with different small letters differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test

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Fig. 6.4 Idli prepared with okara fermented batter

6.3 Utilization of Tofu whey as a partial substrate for growth and pigment production of *M. purpureus*

Natural colours are extracted from plants, plant products or microorganisms. Natural pigments are replaced by synthetic colours, to avoid undesirable toxic effect. Hence there is a growing interest for the development of food pigments from natural sources.

Pigments of the fungus *M. purpureus* have been used for many years as a natural food colourant and a health food, for centuries (Kim *et al.,* 2002a). It produces six major pigments, orange; rubropunctatin, monascorubrin, red; rubropunctamine, monascorubramine and yellow; monascine and ankaflavine (Pastrana *et al.,* 1995). Some of the compounds are applicable as food additives or pharmaceuticals (Kraiak *et al.,* 2000). They are also exploited to produce molecules such as antibiotics, enzymes, and organic acids (Hajjaj *et al.,* 2000). For Monascus pigments, solid state fermentation in rice has been carried out (Johns and Stuart, 1991). Several factors influence the pigment production. These include gaseous environments (Hamdi *et al.,* 1996), agitation, aeration (Hajjaj *et al.,* 2000) and source of carbon and nitrogen (Pastrana *et al.,* 1995).

In this study, rice procured from local market was used as the substrate for growing *Monascus purpureus* MTCC-410. Stock cultures were maintained on potato

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dextrose agar slants at 4°C by periodical subculturing. Culture medium was prepared in two batches with 10 g rice taken in 500 mL conical flasks. Rice (10 g) was washed thoroughly with water, drained and 20 mL distilled water (control) and 20 mL whey added separately to two different flasks and sterilized for 15 min at 121°C. The flasks were inoculated with 1.0 mL *M. purpureus* spore suspension prepared form slants using 0.85% NaCl. Inoculated flasks were incubated at 30°C for 11 days.

Pigments from rice fermented with *M. purpureus* were extracted using polar and non-polar solvents as described in Materials and Methods. Extraction was carried out at 30°C by keeping the flasks for 60 min on rotary shaker (110 rpm). Insoluble debris was removed by filtration and the absorbance of the supernatant was determined spectrophotometrically for quantifying the pigments. Optical density was determined at 375, 475 and 500 nm for yellow, orange and red pigments respectively. Pigment yield was calculated as OD Units using the formula

$$\text{OD Units} = \frac{\text{OD X Total Volume of Solvent X Dilution}}{\text{Red Rice (g)}}$$

The pigments produced by *M. purpureus* grown in rice cooked with water and whey is shown in **Table 6.4 and Fig. 6.5.** The total yield of pigments in these was much higher (649.06 OD units) compared to rice cooked with water alone (485.77 OD units). There was an increase of 163.29 OD units in rice cooked with whey compared to control.

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Table 6.4 Polyketides by *M. purpureus* using whey as a partial substrate for solid state fermentation

		Fractions						Total
		I Fraction	II Fraction	III Fraction	IV Fraction	V Fraction	VI Fraction	
Rice + water	*Pigment yield	18.87	31.56	336.50	18.96	59.18	20.70	485.77 (100%)
	Red (%)	5.90	18.10	24.30	45.40	48.10	37.60	179.40 (36.93%)
	Orange (%)	13.50	24.20	27.70	29.70	37.70	31.60	164.40 (33.84%)
	Yellow (%)	80.50	57.50	47.80	27.70	14.10	37.60	265.20 (54.59%)
Rice + whey	*Pigment yield	152.37	193.86	93.06	194.64	6.54	8.59	649.06 (100%)
	Red (%)	12.01	24.26	26.06	35.43	43.57	27.70	169.03 (26.04%)
	Orange (%)	26.93	43.60	44.00	34.69	33.33	30.03	212.58 (32.75%)
	Yellow (%)	61.05	32.13	29.92	29.86	23.08	42.25	218.29 (33.63%)

*(OD U g^{-1} Moldy rice))

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Fig. 6.5 *M. purpureus* fermented rice A. control B. cooked in whey

6.4 Vada and tofu masala supplemented with okara and tofu

6.4.1 Preparation of Vada

Vada is a South Indian snack. Vada can vary in size and shape, though most are comparable to the Western doughnut **(Fig. 6.6).** For its preparation, gram dhal (100 g) was washed and soaked for 3-4 h. Green chillies (2%) ginger (0.5%), coriander and curry leaves (1%) were added to the soaked dhal and ground coarsely. To this, salt and chopped onions were added. The mixture was divided into two portions. To one portion 20% okara (on dry weight basis) was added and the other portion was used as control. Flat round patties were made by pressing with moist palm and fried in oil till golden brown.

6.4.2 Preparation of tofu masala

Soy paneer (tofu) cut into cubes, was fried in oil. To oil, heated in a vessel cut onions (25%) were added, till they turned light brown in colour. Ginger (0.5%) and garlic paste (0.5%) were added. Chilli powder (3%), tomato puree (20%), turmeric powder, garam masala (1%), and salt (1%) were added to the above. The preparation was divided into two halves. To one half, tofu cubes (20% supplementation) were added and the other half was used as control. Both the batches were cooked till it reached semi solid consistency **(Fig. 6.6).**

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Moisture, protein (N X 6.25), fat, ash and crude fibre of tofu, okara and finished products were analyzed by following the procedure described by Assocation of Official Analytical Chemists (AOAC 2000). The factor of 6.25 was used to convert nitrogen into protein. Total carbohydrate was determined by difference method, by subtracting from 100, the sum of values for moisture, protein, fat and ash. All determinations were performed in duplicates.

The proximate composition of tofu, okara and vada and tofu masala supplemented with okara and tofu is given in **Table 6.5.** The effects of supplementation on the proximate composition revealed significant changes. The moisture content in vada increased by 3.13% compared to control due to the retention of more moisture during cooking because of their ability to bind water. The protein content in vada containing okara and tofu masala increased by 3.57 and 3.76% but, minerals and carbohydrate decreased.

Fig. 6.6 Vada and tofu masala

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Table 6.5 Nutritional composition (% dry wt basis) of soy fortified products[a]

Parameter	Tofu	Okara	Vada (control)	Vada (okara)	Tofu masala (control)	Tofu masala (Tofu)
Moisture	23.60	20.24	57.41	60.54	24.22	21.94
Fat	34.66	13.18	21.98	30.17	31.20	39.25
Protein	49.52	22.02	18.58	22.15	13.39	17.15
Ash	8.46	4.80	7.09	5.80	14.06	12.68
Crude fiber	0.25	16.76	3.34	5.30	8.40	6.50
Carbohydrate	7.11	43.24	49.01	36.58	32.95	24.42

[a]Data represent averages ± standard deviations of duplicate analyses of triplicate samples.

The above results showed improved protein content due to soy addition in Indian traditional foods.

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DISCUSSION

Soybean with 40% good quality protein and 20% oil content takes the most predominant position in combating protein-calorie malnutrition. Its nutritional value is much superior to most of the legumes (Alok *et al.,* 2006). It contains all the essential amino acids required for humans or animals and digestibility is about 84%. It also contains many minor substances, which are biologically active, non nutritive components such as soy lecithin, vitamin E, isoflavones, phytosterols, phytates, trypsin inhibitors, and prebiotic oligosaccharides which act as anticarcinogenic and hypocholesterolemic compound (Tripathi and Misra, 2005).

Soymilk and tofu are the common nonfermented soy foods. Soymilk is a highly economical rich beverage substituting cow's milk and nutritionally comparable to cow's milk. It is a good source of niacin, riboflavin, iron, potassium, calcium, magnesium and phosphorous. Tofu is an inexpensive, dietary source of protein, lipids and versatile substitute with bland taste and porous texture. Since it is cholesterol free, rich source of protein, minerals and PUFA (Pant, 1996) tofu is used as substitute for milk paneer.

Lactic acid bacteria ferment soymilk converting undesirable oligosaccharides into digestable monosaccharides and improve digestion, inhibit harmful bacteria of gastrointestinal tract, make bioavailabe milk constituents etc., (Mathur *et al.,* 2000).

Processing for quality Tofu

Tofu, a non-fermented soybean curd is a nutritious and digestible product with an important role as a source of high quality protein. The flavour, quality and texture of tofu are influenced by its processing parameters. Studies were carried out on the processing parameters like soaking of beans, solid content of milk, thermal treatment of milk, coagulating method, coagulation time and molding on the texture and quality of tofu.

Blanching time determines the texture of tofu **(Table 3.1)** since it is important for denaturing protein, destroying antinutritional factors and reducing beany flavor. Lipoxygenase enzyme catalyzes the hydrolysis of unsaturated soybean oil, resulting in the production of ketones and aldehydes responsible for the beany flavor (How and Morr, 1982). Lim *et al*., (1990) reported that beany flavor which is due to enzyme lipoxygenase, was inactivated when tofu was prepared with hot grind method. Soybeans

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were blanched with 1% sodium bicarbonate for 5, 10 and 15 min. As the blanching time increased, the solid content of soymilk decreased and the texture of the tofu was softer. Nsofor *et al.,* (1997) reported that lower starch and total solids contents was observed in the blanched samples. The solid content of milk determines the texture of tofu. Blanching reduced the solid content and produces soft textured tofu. Nelson *et al*., (1976), stated that presoaking and blanching whole soybeans in 0.5% $NaHCo_3$ improved the tenderization of soybeans. Kuntz (1977) showed that bicarbonate blanched soy beverages were less chalky and had less mouth drying which was comparable to blanched with tap water.

Solid content of the soymilk was adjusted by adding the required amount of water during grinding and its effect on the texture of the tofu was studied. Significant correlations were found among soymilk solid content and textural attributes. Higher solid content produced tofu with greater hardness, cohesiveness, springiness and chewiness **(Table 3.2)**. This is in accordance with the report by Cai and Chang (1997). Therefore, tofu made from soymilk Brix of 7° was less hard, less cohesive, less springy and less chewy due to significantly higher moisture content. Lim *et al*., (1990) and Shen *et al*., (1991) also reported that increased total solids of soymilk were associated with increased tofu hardness, springiness and chewiness. Watanabe *et al*., (1964) reported that boiling soybean slurry for more than 20 min not only reduced the total solids recovery and tofu yield but also affected the tofu texture.

Stirring is necessary to keep the coagulant suspended. Stirring speed and time are important to maintain uniform distribution of coagulant in the soymilk. Stirring speed and time had a significant influence on tofu yield and quality **(Table 3.3)**. This is in correlation with the report by Shih *et al*., (1997) that stirring time of 5 to11.3 sec at 285 rpm was optimum for soft tofu. It is very important to mix soymilk with coagulant in a short period of time to homogeneity to produce a high yield of tofu because, prolonged stirring may break the curd (Hou *et al*., 1997; Cai and Chang, 1999). The relationship between stirring speed and volume of tofu was investigated by Watanabe *et al*., (1964) who found that increasing stirring speed decreases tofu volume (Wang and Hesseltine, 1982). Longer stirring time destroys the structure of gel and results in lesser tofu yield,

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coarser texture and lower sensory overall score (Cai and Chang, 1998). Stronger mixing increases the hardness and reduces the gross weight and moisture content of the curds.

Pressure applied to expel the whey during preparation of tofu, significantly influenced the texture of tofu **(Table 3.4)**. Increase in weight applied decreased the yield, as the water was drained off from the tofu. This in turns affected the texture of tofu (Wang and Hesseltine 1982; Cai and Chang, 1999). As the weight increased to press the tofu curd, the higher was the hardness of the tofu.

Microbiological analysis and quality of tofu during processing and storage

The aerobic mesophiles, yeasts and molds gradually increased during processing soybeans to soy slurry. Due to boiling soymilk for tofu preparation, **(Fig. 3.2)** the microbial count decreased. Fresh tofu had a bacterial count of 2.15 log_{10} CFU g^{-1} and yeast and mold count was 2.28 log_{10} CFU g^{-1}. These counts were much lower to the report by Anbarasu and Vijayalakshmi, (2007) since tofu was prepared hygienically. *E. coli* and *S. aureus* were not detected as reported by Szabo *et al.*, 1989; Rehberger *et al.*, 1984; Anbarasu and Vijayalakshmi, 2007.

Microbiologically, fresh tofu stored in low density polyethylene pouches at 4°C for 12 h had an aerobic mesophilic bacterial count of 2.15 log_{10} CFU g^{-1}**(Fig. 3.7)**. The count was much lower than reported by Kim *et al.,* (2007). With the increase in days, there was a gradual increase in yeast and molds count reaching to 1.18 log_{10} CFU g^{-1} on 12th day. Kim *et al.,* (2004) and Anbarasu and Vijayalakshmi, (2007) reported that the mesophilic count of tofu rapidly increased until 10 days of storage and thereafter the increase was slow.

Kim and Lee (1992) reported tofu spoilage when viable counts of microorganisms were above 107 CFU mL^{-1}. However, Pontecorvo and Bourne, (1978) reported that freshly prepared tofu that had 3.0 X 10^4 CFU g^{-1} microbiological count reduced to 1.12 X 10^3 CFU g^{-1} after 10 days of storage at 24°C in 4% NaCl and 10% lemon juice. Since tofu stored in earthern pots and steel containers at 4°C showed low growth **(Fig.3.8 and 3.9)** the spoilage was slow.

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Tofu prepared using $MgCl_2$ and $CaSO_4$ coagulants

Tofu prepared with $MgCl_2$ and $CaSO_4$ differed significantly. Moisture content of tofu varied depending on the concentration of the calcium sulphate. It was probably due to the differences in gel network influenced by different anions and ionic strengths of concentration of coagulants towards the water holding capacity of soy protein gels. The high moisture content accounted for a higher tofu yield since tofu yield and moisture contents can be correlated (Cai *et al.,* 1997). Tofu made with 0.4% calcium sulphate was soft, retained high moisture and produced high yield when compared to tofu prepared with different concentrations of the same coagulant **(Table 3.5)**. Similar results were reported by Prabhakaran *et al.,* (2006). The decrease in the yield of tofu with increasing calcium sulphate concentration could be due to increasing synergesis and loss of whey from the curd as more bonding occurred thus making the protein matrix denser and compact (Sun and Breene, 1991).

Tofu prepared with 0.4% $CaSO_4$ showed highest protein recovery as well **(Table 3.5)**. This may be due to more whey proteins retained in it during pressing. The coagulation and pressing processes removes some carbohydrates which result in protein content increase. The cause of difference in protein recovery may be related to the effect of coagulants on cross linking of glycinin and β-conglycinin (Cai and Chang, 1999).

The type and concentration of the coagulant determines the texture of the tofu. When calcium sulphate was used as a coagulant, firm but not hard tofu was obtained **(Table 3.5)**. It has been reported that the coagulant concentration and type of anion might affect the hardness of tofu (Wang and Hesseltine, 1982; Sun and Breene, 1991; Prabhakaran *et al*., 2006).

When 0.2% $CaSO_4$ and $MgCl_2$ were used in combination (1:1 ratio), the yield, moisture and fat of tofu were high **(Table 3.6).** The yield, solid recovery, protein recovery and textural quality were optimal at 0.02 N $CaSo_4$ for 5 soybean varieties as shown by Nong and William (1991). Texture of tofu was smooth and soft with different concentration of $CaSO_4$ and $MgCl_2$, compared to commercial product. Schaefer and Love (1992) stated that the amount of coagulant influences the tofu texture. According to Tsai *et al.,* (1981), when coagulant concentrations were higher than 0.030 N, the

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texture of tofu became coarser and harder. Calcium salt concentrations ranging from 0.1 to 0.5% were suitable for soybean curd preparation (Lu *et al.,* 1980).

Likewise, in tofu prepared using coagulants of plant origin, *Citrus limonum, Garcinia indica, Tamarindus indica, Phyllanthus acidus* and *Passiflora edulis* **(Fig. 3.2)** moisture content and whey varied between 71.2 to 80.4% and 81 to 86 ml 100^{-1} respectively **(Table 3.7)**. In such tofu, significantly higher antioxidant activity was observed **(Table 3.8)**. The increase in antioxidative activity may be due to the polyphenolic compounds present in fruit extracts like Garcinol in *Garcinia*, Eriocitrin in *Citrus limonum* (Yoshiaki *et al*., 1997; Fumio *et al*., 2000; Lee *et al*., 2004a). It is reported by Martinello *et al*., (2006) that the crude tamarind extract is rich in polyphenols (34.02 nmol mL^{-1}) and flavonoids (35.5 µg mL^{-1}), which are well known antioxidants. The carotenoids which give characteristic colour to the yellow passion fruit rind, flesh and juice are important source of provitamin A and antioxidant activity (Stephen *et al*., 2003). *Phyllanthus acidus* contains flavonoids like Kaempferol and 2, 3-dihydroxy benzoic acid (DHBA) and other bioactive compounds (Li and Wang, 2004) which possess antioxidative activities.

Bioavailability of nutrients in tofu with reference to lactic acid bacteria

The nutrient contents like protein, fat and ash were almost similar in control and lactic acid bacteria inoculated tofu, but the isoflavone content varied. Glycosidic isoflavone were more than aglycones in control tofu but in tofu inoculated lactic acid bacteria glucosides were less than aglycones **(Table 3.9)**. Thus it appeared that increase of daidzein and genistein contents in tofu were due to β-glucosidases of lactic acid bacteria. Apparently isoflavone glycosides are the predominant isomeric forms in nonfermented soymilk and require the bacterial enzyme for hydrolytic deconjugation into a bioavailable aglycone (Murphy *et al.,* 1999; King and Bignell, 2000).

Nutritional quality and biomolecules of fermented Soymilk

Soymilk was fermented with five isolates of probiotic lactic acid bacteria individually and in combination with probiotic yeast *Saccharomyces boulardii*.

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Nutritional profile like fat, protein, ash, pH, acidity, polyphenol and protein hydrolysis were analyzed.

An increase in titrable acidity and decrease in pH was observed during soymilk fermentation for 24 h **(Table 4.1)** which was due to acid production during fermentation. With further incubation, titrable acidity decreased **(Table 4.4 and 4.5)**. The decrease in acidity after 24 h fermentation reflects a change from exponential to stationary growth phase, wherein the population decreased after 24 h fermentation. The concentration of mono and oligosaccharides of soymilk might have restricted the proliferation of these strains beyond 24 h of incubation. In contrast to our reports, Hou *et al*., (2000) observed increase in acetic and lactic acid contents increased with fermentation time when fermented with *B. longum* B6 and *B. infantisa* CCRC 14643 for 48 h. The texture, physical stability, flavour and aroma of the soy yogurt are related to pH (Ankenman and Morr, 1996). In general, coagulation of sterilized soymilk occurs at pH 5.7 (Chou and Hou, 2000). Previous research has shown that a common problem with soymilk yogurt is low acidity and flavour intensity (Karleskind *et al*., 1991). The optimum pH of soymilk yogurt is reportedly 4.2 to 4.3 (Oberman, 1985). The marked difference in acid development could be due to the difference in the *Lactobacillus* spp but the reduction in pH was sufficient to cause coagulation.

There was no significant difference in protein content of test curds when compared to control **(Fig. 4.3)**. The protein originating from *Lactobacillus helviticus* may have contributed to the increased content of protein in fermented milk. Similar finding was reported by Hou *et al*., (2000) in *Bifidobacteria*. The difference in fat content was more significant in some of the combinations.

Probiotic organisms are rich in proteolytic activity. The addition of probiotic organisms to soymilk result in increased free amino acid content **(Fig. 4.4)**. The degree of protein hydrolysis is expressed as content of leucine equivalent in soymilk after 24 h fermentation. Similar observation was reported by Kurmann and Rasic (1991) in yogurt fermented with *B. fifidum.*

Polyphenol content increased from 11.9 mg^{-1} to 26.58 mg^{-1} in various fermented by milk, incubated for 24 h **(Fig. 4.5)**. Fermentation with different probiotics organisms

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resulted in polyphenol increase. This observation contradicted the findings of Sindhu and Khetarpaul (2003) wherein they reported reduction in polyphenol content.

The antioxidant activity was determined using different methods like DPPH free radical-scavenging assay, inhibition of ascorbate autooxidation and measurement of reducing activity. The activities varied with the starter cultures used. Nevertheless, the activities were significantly higher than those found in unfermented soymilk. There was 21% increase in DPPH scavenging activity in fermented milk (**Table 4.2**). These results suggested that each extract might react as free radical scavenger and contribute hydrogen from phenolic hydroxyl groups, thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids. Fermentation of soymilk with yeast and lactic acid bacteria significantly increased the inhibition rate of ascorbate autooxidation (**Table 4.2**). Liberation of aglyconic form of genistein and daidzein through the catalytic action of β-glucosidase during fermentation (Chien, 2004) and the presence of intracellular antioxidants of starter organism may account for the increased inhibition of ascorbate autoxidation inhibition found with the fermented soymilk. The intracellular antioxidants, peptides of starter organism (Wang *et al*., 2006) and the hydrogen-donating ability (Yang *et al*., 2000) may have contributed to the increased reducing activity of soymilk after fermentation.

Enzymic transformation of Isoflavones

Reduced growth of LAB between 24 and 48 h fermentation **(Table 4.6 and 4.7)** indicates a diminishing nutritive supply which is strongly supported by Scalabarini *et al*., (1998), who found that stachyose and raffinose content of soymilk reduced after 24 and 48 h fermentation with *Bifidobacteria*, to at least half the original concentration. The cell growth in soymilk fermentation depends upon the cultures and fermentation period (Jiyeon *et al.,* 2008).

β-glucosidase activity was highest at 24 or 48 h of incubation which corresponds to the exponential phase of bacterial growth and decreased during stationary phase **(Fig. 4.7 and 4.8**). With further fermentation, the β-glucosidase activity decreased. Similar observation was reported for *L. plantarum* KFRI 00144 and *L .delbrukeckii* subsp *Lactis* KFRI 01181 by Pyo *et al*., (2005a). This can be related to differences in β-glucosidase activity of each bacterial strain.

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There appeared to be correlations (r=0.96) between β–glucosidase activity and growth characteristics during fermentation of soymilk. Donkor and Shah (2008) reported a similar correlation between growth of microorganisms in soymilk and β–glucosidase activity. Increased cell growth might have resulted in higher enzyme activity. Thus β–glucosidase activity is strain dependent and varies among the organisms (Donkar and Shah, 2008).

Isoflavones are predominantly found in soybeans and nonfermented soyfoods as biologically inactive glycoside conjugates. In unfermented soymilk, glycosides were the predominant isomeric forms and were higher compared to aglycones **(Table 4.8)**. In contrast, soymilk fermented with different isolates showed increase in aglyconic content **(Table 4.9)**. Soymilk requires bacterial induced hydrolytic deconjugation for transformation into a bioavailable aglycone (King and Bignell, 2000). Of the aglyconic forms of isoflavones, genistein was highest than daidzein. Similar observation was reported by Murphy *et al*., (1999) and Tsangalis *et al*., (2003). This was apparently due to higher content of genistin in the original soymilk compared with the other isomers. The isoflavone aglycones were absorbed faster and in greater amounts by humans than their glucosides (Shimada *et al*., 1992).

The aglycone isoflavones are more stable and may be due to their structural composition and molecular arrangements (Otieno and Shah, 2007). There appeared to be relationship between β-glucosidase activity of LAB, their growth characteristics in soymilk and bioactive isoflavones transformation. Hydrolytic cleavage of the glycoside moiety depends on the type of LAB which indirectly reflects the capability of β-glucosidase production in soymilk (Chun *et al*., 2007). Increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of isoflavone aglycones in fermented soymilk compared to unfermented soymilk.

Isoflavone aglycone rich products may be more effective than glycoside rich products in preventing chronic disease such as coronary heart disease and cancer (Izumi *et al*., 2000). Thus the significant bioconversion of the glycoside isoflavones into their corresponding aglycones improved the nutritional quality of fermented soymilk.

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Minerals and Vitamins

Phytic acid, the major antinutritional factor which blocks the availability of minerals in soybean decreases during fermentation due to phytase enzyme. LAB and yeast are the major source of the enzyme as it hydrolyzes phytate into myo-inositol and phosphate during fermentation (Nam and Man, 2009). There was an increase in calcium and magnesium levels and a decrease in iron in fermented soymilk as reported by Lopez *et al*., (2000) in whole wheat flour, where phytic acid was degraded by LAB which led to the increase in Ca and Mg availability **(Table 4.10)**. In contrast to our report, Svanberg *et al*., (1993) found that lactic fermentation of maize or sorghum can shift a "low iron bioavailability" diet into an "intermediate to high bioavailability" diet. The phytic acid content was reduced in pearl millet fermented with mixed cultures of *Saccharomyces diastaticus*, *S. cerevisiae*, *Lactobacillus brevis* and *L. fermentum* at 30°C for 72 h. A significant reduction of phytate content (around 50%) of its initial value was reported by Nam and Man (2009) at the end of 18 h soymilk fermentation with *L. mesenteroides*.

Vitamin content also varied with fermentation wherein there was an increase in riboflavin and niacin content and a decrease in thiamin **(Table 4.11)**. Deguchi *et al*., (1985) found that thiamin, nicotinic acid and folic acid varied widely among different species or strains. The decrease in thiamin content may be due to the consumption by the organism itself. In contrast increase in thiamin and decrease in niacin content was observed in soymilk fermented with either *B. infantis* CCRC 14633 or *B. longum* b6 (Hou *et al*., 2000). Yeast might increase the vitamins content in soymilk fermentation. Different researchers have reported that fermentation of milk increases the nutritional value by increasing the vitamins. Hailong and Liang (2009) reported that the contents of niacin, riboflavin and thiamin increased when soymilk was fermented with the basidiomycete *Ganoderma lucidum* WZ02. It has been observed that in the preparation of fermented soybean products like natto and tempeh, most of the B-complex vitamins increased except thiamin.

Volatile compounds of fermented soymilk

In soymilk, hexanal was the major volatile component contributing to the disagreeable aroma of soybean milk (Huang *et al.,* 2004). The typical green beany flavor of soy can probably be due to a mixture of many compounds. Pentanal and 2-pentyl

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furan can arise from linoleic acid by the catalytic action of lipoxygenase enzyme in soybean. 2-pentyl furan was described as possessing a characteristic beany or grassy odour and predominantly responsible for the reversion flavour of soybean oil. Methanol, acetaldehyde, ethanol and hexanal, the four volatile components found in soymilk could be reduced by fermentation (Blagden and Gilliland, 2005). The concentration of each component varied among batches of soymilk and also among the samples of fermented soymilk (Chung, 1999).

Although fermented soymilk retained the characteristic beany flavor due to the presence of n-hexanol and 2-pentyl furan, their concentration and some of the esters and alcohols were able to mask the undesirable flavour. Though there was not much variation in compounds between the soymilk fermented with LAB and LAB in combination with yeast, the concentration of the volatile compounds varied **(Table 4.12 and 4.13)**.

Antimicrobial compound of LAB

Antimicrobial activity of the culture supernatants of nine LAB genera exhibited varying degrees of inhibitory activity against different indicator organisms **(Table 5.1).** The antimicrobial activity of LAB may be due to the presence of different inhibitory substances (Zheng and Slavik, 1999) or may be due to the production of bacteriocin or bacteriocin-like compounds (Gonzalez *et al.,* 2007).

Antimicrobial compounds of *L. acidophilus, L. casei* and *P. acidilactici* were stable when treated at 50°C, 100°C and at 121°C (**Table 5.2 and Fig. 5.1**). All isolates showed maximum inhibitory activity against *L. monocytogenes*. Losteinkit *et al.,* (2001) working with a bacteriocin N 15 produced by *E. faecium* isolated from nuka (Japanese rice-bran paste) reported that thermal resistance of bacteriocins produced by *L. lactis* and *E. faecium*, did not elicit any loss of antimicrobial activity against *L. monocytogenes* and *Staphylococcus aureus* after treated at 100°C for 60 min. A similar observation was made in four isolates of LAB namely *Lactococcus, Lactobacillus*, *Pediococcus* and *Leuconostoc* by Belgacem *et al.,* (2008). These were stable after treatment at 60°C for 30 min, 100°C for 15 min and even at autoclaving temperature (121°C for15 min).

Supernatants containing the antimicrobial compound of the above isolates were stable at pH 3, 5 and 7. No inhibition zone was found when the supernatants were treated

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to pH 9 **(Table 5.3 and Fig. 5.2)**. Bacteriocins generated by lactic acid strains are generally stable at acid or neutral pH, indicating that these substances are well adapted to the environment produced by bacteria (Vignolo *et al.,* 1995). Campos *et al.,* (2006) studying the effect of pH range in the antimicrobial activity of extracellular extracts found that bacteriocins from *L. lactis* was also effective against *L. monocytogenes* at pH value of 6.5. Interestingly, the supernatant produced by *E. canis* exhibited a broader pH range (2.0 to 9.0) of activity against *E. coli*. Lee *et al.,* (1999) reported similar results for a bacteriocin produced by *L. lactis subsp. lactis* H-559. In addition, Noonpakdee *et al.,* (2003) analyzing nisin produced by *L. lactis* from nham, a traditional Thai fermented sausage, reported that bacteriocin was active over a wide pH range (2.0 to 10.0). It was reported by Mentes *et al.,* (2005) that the bacteriocins produced by *Lactobacillus* strains have optimal activities at pH 3 to 4. Thus the inhibition of indicator organisms is presumbly due to the production of bacteriocin.

Proteinases destroyed antimicrobial activity of cell-free supernatant **(Table 5.4 and Fig. 5.3)**. Similar result was reported by Losteinkit *et al*., (2001). This further indicated the production of polypeptide. When partially purified, it showed a molecular mass corresponding to ~ 4.0 kDa peptide. The bacteriocin-like compound produced by *L. acidophilus* as well as bactericidal mode of action suggests that they may have application for the inhibition of food-borne pathogenic bacteria (Zouhaier *et al*., 2008).

Bacteriocins have a bactericidal or bacteriostatic effect on other closely related sps (Garneau *et al*., 2002). *S. aureus* was affected completely by *P. acidilactici*, however in the presence of *L. acidophilus* and *L. casei* , not much reduction was found **(Fig. 5.6 and 5.7)**. *L. monocytogenes* was not affected by *P. acidilactici* compared to *S. aureus*. The mode of action of the bacteriocin was bacteriostatic against *L. monocytogenes.* Not all strains of *L. monocytogenes* show the same degree of sensitivity to antilisterial bacteriocins (Martinez *et al*., 2005).

Bacteriocin is produced during or at the end of exponential growth. The antimicrobial activity may be due to pH of the medium or changes in the composition of the medium, in turn influencing the antimicrobial activity (Mataragas *et al*., 2003). Bacteriocins generally which are cationic peptides target the cell membrane (Abee *et al*., 1995) and interact with the anionic lipids (of gram +ve bacteria) in the membrane and

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forms pores in the phospholipid bilayer (Chen and Hoover, 2003). This in turn affects the energetic status of the cell.

The use of bacteriocin-producing LAB could play a role in controlling colonization by pathogenic and spoilage bacteria in food processing facilities (Ammor *et al*., 2006). This bacteriocin like substance, resistant to heat may be used as biopreservative in food products.

Utilization of tofu and byproducts in soybased functional foods

People are becoming health conscious and thereby demand of speciality foods is increasing. Soybean has tremendous potential to be transformed into a number of such foods suiting to the requirements of people (Greenberg and Hartung, 1998).

Use of okara

Okara and whey are two byproducts of tofu. Okara was substituted in Indian traditional food like idli and vada and different factors like pH, acidity, raise in batter volume, Co_2 production, microbiological analysis, texture and sensory studies of the batters were studied.

The pH of control and okara substituted batter was almost similar but the acidity was significantly different **(Fig. 6.1)** apparently due to buffering effect caused by the higher content of soluble proteins, amino acids and also free fatty acids of the beans (Annan *et al*., 2005). The role of LAB is to reduce the pH of the batter to an optimum level (4.4 to 4.5) for yeast activity. This accounts for fall in pH and rise in acid content of batter as the natural fermentation progresses (Soni and Sandhu, 1990).

There was 35% raise in volume in okara fortified batter compared to control batter. The increase in volume, due to the CO_2 production by the yeast, during natural fermentation measured the metabolic activity. This is also because of combined contribution of both heterofermentative lactobacilli and non LAB (Thyagaraja *et al*., 1992). Since both leavening and acid development are required for 'idli' (Susheelamma and Rao, 1978), determination of the end point of the 'idli' fermentation became arbitrary. However, the use of different ingredients in different proportions resulted in raise in volume besides reduction of fermentation period.

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The increase in CO_2 production was very significant in okara fortified batter within 10 h of natural fermentation **(Fig. 6.2)**. This was due to the presence of more yeast. It has also been reported earlier that yeasts were responsible for more than 50% of the CO_2 for the increased volume of batter (Venkatasubbaiah *et al*., 1984).

It was seen that the bacterial counts were higher in the okara fortified batter than that of control batter. There was two log increase in LAB count in control batter compared to okara fortified batter (**Table 6.1**). This was due to the growth of LAB in sourdough and has been found to be enhanced by the presence of amino acid (Gobetti *et al.,* 1994). There was only one log increase in the okara batter.

'idli' fermentation is a mixed auto fermentation (Soni *et al.,* 1985). Organisms present in the ingredients as well as the environmental contaminants determine the type of the organisms involved in the natural fermentation. Lewis *et al*., (1955) have reported a number of wild yeast combined with different LAB in the fermenting batters. The fact that both bacteria and yeast participate in the fermentation has been shown by Desikachar *et al*., (1960) using Penicillin G and Chlorotetracycline as competitive inhibitors. Venkatasubbaiah *et al*., (1984) observed the involvement of both bacteria and yeasts in 'idli' batter fermentation.

From the above results it can be seen that initial natural fermentation was a 'free for all' with lactic and non-LAB growing together. A lag period for both lactic and non-LAB existed. The metabolic activities resulting in decrease in pH and, increase in acidity and batter volume were negligible during this period. When both lactic and non-LAB reached the end of log phase, LAB established as the main flora and the number of surviving non-LAB decreased. The decrease in non-LAB may be due to the antagonistic action of LAB, which is known to exert an inhibitory action by the production of lactic acid, hydrogen peroxide and bacteriocins, as well as decreasing the pH making the medium unfavorable for the growth of non-LAB (Thyagaraja *et al*., 1992). This is in correlation with the control batter, wherein the lactic count was more than in okara fortified batter. But in okara fortified batter, the non lactic count (yeast, mold and other bacterial count) was higher than LAB. This observation is in accordance with Sarkar *et al*., (1994), who have reported progressive increase in the count of *Bacillus subtilis, Enterococcus faecium* and *Candida parapsilosis* in fermented soybeans. Similarly, in

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okara fortified batter there was an increased count of bacteria and yeast and lesser count of lactic count compared to control batter. As there was less number of LAB, there was no antagonistic activity of LAB and increase in yeast count resulted in an increase in CO_2 %, resulting in soft and spongy texture of 'idli'.

The texture analysis indicated that the control 'idli' offered more resistance to compression than that of okara fortified 'idli'. Thus the okara substituted samples were softer and easy to bite compared to control samples (soft texture of 'idli's is a desirable quality). This is due to the microbes present in okara especially yeasts, which produced CO_2 during natural fermentation resulting in a softer product and partial substitution of black gram with okara might have contributed for accelerated natural fermentation. On the other hand the values for stickiness in case of okara 'idli' were relatively low. The stickiness of traditional 'idli' was 0.13 Newton and okara substituted 'idli' was 0.14 Newton. The adhesiveness of traditional and okara substituted 'idli' did not have significant difference and was 0.053 and 0.050 Newtons, respectively **(Table 6.3)**.

It has been reported by Soni and Arora (2000) that yeast involved in the fermentation not only contribute towards gas production which results in good texture but also towards the sensory qualities of the 'idli'. The difference in sensory quality of control and okara containing samples was significant in some of the attributes viz sponginess, sticky, beany, salty and overall quality **(Table 6.3)**. Natural fermentation of okara fortified batter for 10 h resulted in 'idli' with more sponginess and fluffy texture when compared to the control. This probably is due to more yeast growth when compared to the naturally fermented batters (Bharti and Laxmi, 2008).

The idli batter prepared with and without okara, when subjected to GC-MS analysis, revealed the presence of acids and esters **(Fig. 6.3)**. The flavor present in idli batter is due to the presence of the above compounds in the raw materials and microorganisms which bring about fermentation. The volatile compounds like acids and esters **(Table 6.2)** concentration varied in both the batters. Except 9-octadecenoic acid (E), okara fortified batter had lesser amount of acids and ester compared to control batter. Usually esters occur as flavouring agents in microbial fermentation. As the cell counts of the microorganisms increases, the intensity of esters also increases (Agrawal *et al.,* 2000).

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Parmar *et al.,* (2005) reported that the addition of tofu, okara and soy flour solids to maize chapattis enhanced the crude protein, crude fat and ash contents, proportionate to the levels of supplementation. Goel *et al*., (1999) reported that the oil content of deep fat fried noodle-like product prepared from blends of corn starch with soy protein increased with an increase in the content of protein. Oil absorption of extruded snack prepared from blends of starch and inactivated soya flour increased with the increase in soya flour (Ahamed *et al.,* 1997). Amudha *et al.,* (2002) have reported that with the increase in soya flour content, raise in the ash content from 2.88 to 3.86% in savoury and 1.23 to 1.96% in sweet snacks was observed. Similar increase in protein content was observed by Cheman *et al.,* (1992) and Gandhi *et al.,* (2000) in rice soy snack and chapatti containing varied levels of soya flour. The higher crude fibre in vada could be due to the addition of fibre rich okara. Thus okara and tofu can be added to traditional foods to mask the beany flavor of soy and also to increase protein content.

Use of whey

Silveira *et al.,* (2008) used several agro waste by-products and reported that *M. purpureus* produced more pigment in grape waste, followed by soybean bran. Low pigmentation was observed in *M. purpureus* when grown in industrial fibrous soybean residue, rice hulls and cheese whey. Similarly, broken rice ‘‘canjica’’ (Matter and Luchese, 1998) and gluten free effluent from wheat milling (Domınguez- Espinosa and Webb, 2003) have been used for pigment production by *M. purpureus*. Jack fruit seed has also been used for pigment production from *M. purpureus* by Babitha *et al.,* (2006).

Tofu whey consists of 5.1 g L^{-1} protein and 6.4 g of stachyose, 1.6 g raffinose, 11.3 g sucrose, 1.1 g fructose and 1.2 g glucose per liter (Nguyen Thi *et al.,* 2003). Increased pigment production by *M. purpureus* was seen in rice cooked with whey compared to rice cooked with water **(Table 6.4 and Fig. 6.5)**.

Thus the results of the study described in this thesis show the process parameters standardized for preparation and storage of tofu, β-glucosidase activity during soymilk fermentation and utilization of the by-products.

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SUMMARY

This thesis describes the preparation of non fermented soy based tofu, its quality in terms of microbiology and isoflavones, bioconversion of isoflavone glycosides to aglycones in soymilk fermented with different probiotic strains of lactic acid bacteria and yeast, their effect on food spoilage bacteria and utilization of tofu and its byproducts in Indian foods.

Processing parameters significantly influenced the yield and texture of tofu. The optimum conditions for soft tofu were blanching of soybeans with 1% sodium bicarbonate for 10 min, solid content of 7° Brix with natural and synthetic coagulant, stirring time for 2-5 sec and pressing the tofu with 1000 g initial weight for 15 min followed by 500 g resulted in soft textured tofu with less beany flavour.

Magnesium chloride of 0.5%, Calcium sulphate of 0.4% and 1.0% coagulated tofu had higher yield, moisture and was soft textured. Calcium sulphate and Magnesium chloride in combination of 0.2% (1:1 ratio) was best suitable coagulant for tofu preparation in terms of yield, proximate composition and texture.

Tofu prepared using coagulants of plant origin (*Citrus limonum, Garcinia indica, Tamarindus indica, Phyllanthus acidus,* and *Passiflora edulis*) showed an increase in protein and antioxidant activity compared to synthetic coagulant. Coagulants such as fruit extracts, which are water soluble, rich sources of vitamins, carotenoids and other bioactive molecules, could be an alternative to synthetic coagulants in the preparation of tofu. Tofu prepared with *Citrus limonum* extract was the most preferred tofu which had a smooth, soft, but firm texture with whitish colour.

Most of the foods are susceptible to microbial growth due to longer storage time between preparation and consumption under improper temperature conditions (30–38°C). Thus the shelf life of tofu stored in low density polyethylene pouches was stable for 9 days, at 4°C. Steel containers and earthern pots were better than low density polyethylene pouches for tofu storage.

Soymilk fermented with *Lactobacillus acidophilus* B4496 (La), *Lactobacillus bulgaricus* CFR2028 (Lb), *Lactobacillus casei* B1922 (Lc), *Lactobacillus plantarum* B4495 (Lp) and *Lactobacillus fermentum* B4655 (Lf) individually and with combination of yeast *Saccharomyces boulardii* produced β-glucosidase enzyme, which hydrolyzed

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isoflavone β-glucosides to aglycones in significant level ($p<0.05$) in the fermented soymilk. This hydrolysis depended on the growth and viability of each organism and β-glucosidase activity, which was strain dependent. *L. acidophilus* showed maximum growth, glucosidase activity and bioconversion at 48 h, whereas the other LAB showed similar observation at 24 h of fermentation.

Fermentation reduced antinutrient like phytic acid and increased the nutritional profile, mineral bioavailability and B-complex vitamins. The antioxidant activities varied with the starters used but, nevertheless, were significantly higher than those found in unfermented soymilk.

Yeast sps *S. boulardii* is capable of utilizing the yogurt constituents as growth substrates, and its application as a probiotic microorganism seems promising, as no gas and alcohol are produced. Yeasts have the ability to utilize organic acids, thereby increasing the pH of the environment. Thus, growth of probiotic yeast in association with probiotic bacteria has been suggested for enhancing the viability of lactic acid bacteria.

The antimicrobial activity of ten probiotic Lactic acid bacteria were screened against nine pathogenic food borne bacteria, of which *L. acidophilus* B4496, *L. casei* B1922 and *P. acidilactici* K7 were selected. The antimicrobial compound of these LAB were stable at 121°C for 15 min, pH at 3,5 and 7 and the antimicrobial activity was lost after 2 h of treatment with protease, trypsin and papain enzymes. The molecular mass of the bacteriocin was 4.0 to 4.5 kDa peptide. The survivability of food borne pathogens like *L. monocytogenes* Scott A and *S. aureus* FRI 722 when co-cultivated with probiotics lactic acid bacterial isolates in soymilk, *P. acidilactici* was found to be most efficient isolate against *S. aureus* as it completely inhibited the growth. The mode of action of the bacteriocin was bactericidal against *S. aureus* FRI 722, and was bacteriostatic against *L. monocytogenes* Scott A.

Highly nutritious, soy-based products are widely consumed throughout the world. In the manufacture of tofu, the by-products okara and whey are obtained. Okara contains about 4.7% protein (wet-weight basis) or 25.4–28.4% (dry basis) with high nutritive quality and superior protein efficiency ratio, suggesting a potential source of low cost vegetable protein for human consumption. Hence okara was used in idli and Vada - a

south Indian breakfast and snack preparation. The liquid by-product tofu whey was utilized for the cultivation of natural colours of *Monascus purpureus.*

The addition of soy residue okara to the ‘idli’ batter accelerated the natural fermentation rapidly and shortened the fermentation time. Fortification of okara to ‘idli’ batter has a beneficial effect in terms of higher amount of gas production and leavening during natural fermentation. Increase in CO_2 production (33.6%) resulted in soft and spongy ‘idli’ compared to control sample. Reduction in the fermentation time of the ‘idli’ batter is of great commercial significance for large scale ‘idli’ production.

By this study LAB in combination with yeast *S. boulardii* has great potential for the enrichment of bioactive isoflavones which has got its application in food and therapeutical application.

The results are discussed in relevance to literature pertinent to this investigation.

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List of Publications

1. **C.R.Rekha** and G. Vijayalakshmi (2008). Biomolecules and Nutritional Quality, Of Soymilk Fermented with Probiotic Yeast and Bacteria. *Appl Biochem Biotechnol* **151(2-3):** 452-463.
2. **C.R. Rekha** and G. Vijayalakshmi (2010). Influence of natural coagulants on the isoflavones and antioxidant activity of tofu. *J Food Sci Technol* **47(4):** 387–393.
3. **C.R. Rekha** and G. Vijayalakshmi (2010). Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *J Appl Microbiol* (Article in press- doi:10.1111/j.1365-2672.2010.04745.x
4. **C.R. Rekha** and G. Vijayalakshmi (2010). Acceleration of fermentation of idli batter (a traditional cereal – legume based breakfast food) using soy residue okara. *J Food Sci Technol* (Article in press).
5. **C.R. Rekha** and G. Vijayalakshmi (2010). Partial characterization of bacteriocin produced by Lactic acid bacteria and their effect on food borne pathogenic bacteria in soymilk. *Int J probiotics prebiotics* (Accepted).
6. **C.R. Rekha** and G. Vijayalakshmi (2010). Isoflavone phytoestrogens in soymilk fermented with β-glucosidase producing probiotic Lactobacillus bacteria. *Int J Food Sci nutr* (Accepted).
7. **C.R. Rekha** and G. Vijayalakshmi (2010). Influence of processing parameters on the quality of soycurd (tofu). *J Food Sci Technol* (Under review).
8. **C.R. Rekha** and G. Vijayalakshmi (2010). Microbiological analysis and quality of tofu during processing and storage using $CaSO_4$ and $MgCl_2$ as coagulants. *J Food Quality* (Communicated).

Patent

C.R.Rekha, G. Vijayalakshmi and Amudha Senthil. "A process for preparation of fermented okara (soy residue) based idli batter". Indian Patent, IPMD, New Delhi.

Patent Number 0809DEL2008.

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