ENHANCED β-GALACTOSIDASE ACTIVITY IN PROBIOTICS FOR IMPROVED BIOCONVERSION OF SOY ISOFLAVONES IN DAIRY AND SOY- BASED YOGHURT

A thesis submitted for the degree of DOCTOR of PHILOSOPHY

By

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I dedicate this PhD to my late father, Tek N Sah and late mother, Anatiya Devi
who believed so much in education
and
to the entire Prasad family

Abstract

The main aims of this project were to evaluate the suitability of whey as a medium for the production of β- galactosidase (β-gal) from *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (LB) and *Bifidobacterium animalis* Bb12 (BB12). The study was undertaken to examine and compare the influence of selected prebiotics, i.e. galactooligosaccharides (GOS), modified waxy maize starch (MWMS), inulin and β-glucan on the growth of selected organisms and on the physical attributes of yogurt and sogurt. The study also investigated the role of β-gal produced by LB and BB12 in biotransformation of isoflavone glycosides (IGs) to aglycones (IAs).

The production of β-gal by BB12 and LB and the effect of four different extraction methods i.e. sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatments on enzyme activity was investigated. Both organisms were grown in deproteinised whey containing yeast extract (3.0 g/L), peptone (5.0 g/L) and glucose (10.0 g/L) for 18 h, at 37 ºC for BB12 and at 45 ºC for LB. The optimum intracelluar β-gal activity on 15 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) assay was found at pH 6.8 for both organisms irrespective of the method of extraction used. Also, the effect of temperature on enzyme activity was studied at various temperatures (30, 35, 40, 45, and 50 °C). At 35 °C and 40 °C, Bb12 exhibited more intracellular β-gal activity extracted by sonication than other temperatures and extraction methods. However, LB showed more intracellular β-gal activity at both 35 °C and 45 °C when extracted by lysozyme-EDTA treatment. Among the four methods used for β-gal extraction, sonication gave the best result (6.80 Unit/mL) for BB12 while lysozyme-EDTA treatment was found to be the best (7.77 Unit/mL) for LB.

The effect of various carbons sources such as lactose, glucose and galactose and nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium

sulphate and MRS broth (control) on the growth of BB12 and LB and their effect on β-gal activity was also investigated. To investigate the effect of carbon source, both organisms were grown in a medium containing 3.5% (w/v) yeast extract, 0.03% (w/v) L-cysteine, 0.3% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$ and 4% (w/v) of either lactose, glucose or galactose as carbon source. Likewise, the medium for nitrogen source study contained 4% (w/v) lactose, 0.3% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $M_gSO_4.7H_2O$, 0.03% (w/v) L-cysteine and 3.5% (w/v) of either yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate or MRS broth. All these sugars were found to be suitable carbon sources to produce high β-gal activity; however, the maximum level of β-gal activity (73.66 Unit/mL by BB12 and 48.63 Unit/mL by LB) was produced in the presence of galactose.

The crude enzymes extracted from BB12 and LB were used at 0.1, 0.5, and 1.0 g/L levels to hydrolyse glycitin (an isoflavone glycosides IG) to its biologically active form (isoflavone aglycones; IA) in soymilk prepared from 4.0% (w/v) SPI supplemented with 2.0 % (w/v) D-glucose and incubated at 37 ˚C for BB12 enzyme and 45 ˚C for LB enzyme up to 12 h. A control sample was prepared without addition of crude enzyme. The biotransformation rate of glycitin was found to be directly related to the level of enzyme addition with 74.4% biotransformation obtained from 1.0 g/L BB12 enzyme and 75.25% from LB enzyme.

Furthermore, this study examined the influence of galactooligosaccharides (GOS) and modified waxy maize starch (MWMS) addition on the growth of ST and LB, and syneresis and firmness of low-fat yogurt during storage for 28 d at 4˚C. The control yogurt (CY) was prepared without any prebiotics addition. Incorporation of 2.0% (w/v) GOS improved the growth of LB and ST resulting in a shorter fermentation time and a significant ($p<0.05$) increase in proteolysis as measured by the absorbance value (0.728). Addition of GOS also

resulted in higher ($p<0.05$) concentration of lactic and acetic acids in comparison with that of MWMSY and the CY up to day 14, thereafter the product showed a decrease in lactic acid content in all three batches until the end of storage. The level of syneresis was the lowest (2.14%) in MWMSY as compared with that of GOSY (2.35%) and CY (2.53%). There was no statistically significant ($p>0.05$) difference in the firmness among the 3 types of yogurt.

This study also investigated the influence of galacto-oligosaccharides (GOS), modified waxy maize starch (MWMS) and inulin added to soymilk on the growth of starter culture, their proteolytic activity and organic acids production and on the syneresis, viscosity and firmness of soy yogurt during 28 days storage at 4 ˚C. All soy yogurts were prepared from soymilk with addition of 1% (w/v) glucose and 10% (w/v) sucrose that including 2% (w/v) prebiotics (GOS or MWMS or inulin) had a final total solids (TS) content of 22% (w/v). The control sample was prepared without addition of prebiotics. The survival of both organisms in all yogurts was investigated on day 1 and on weekly intervals during the shelf life of 28 days at 4 ˚C. The soy yogurt containing inulin showed better retention of culture viability (ST and LB) and lower pH than soy yogurts containing GOS, MWMS and the control. The concentration of lactic and acetic acids consistently increased throughout the storage period. The soy yogurt containing inulin had lower syneresis values compared to the control, MWMS and GOS.

Declaration

"I Laxmi Narayan Prasad, declares that the PhD thesis entitled "**ENHANCED β-GALACTOSIDASE ACTIVITY IN PROBIOTICS FOR IMPROVED BIOCONVERSION OF SOY ISOFLAVONES IN DAIRY AND SOY-BASED YOGHURT**" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Laxmi N Prasad Date:

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2. Prasad, L. N., M. M. Ayyash, and N. P. Shah. 2011. Effect of nitrogen sources on production of β-galactosidase from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey under different culture conditions. International Food Research Journal 18: 445-450.

3. Prasad, L. N., and N. P. Shah. 2011. Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842. International Food Research Journal. 22: 610-617.

4. Prasad, L. N., F. Sherkat, and N. P. Shah. 2012. Influence of Galactooligosaccharides and Modified Waxy Maize Starch on Probiotic Yogurt's Attributes. Journal of Food Science. M1-M7.

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Poster Presentations

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Table Of Contents

List Of Tables

List Of Figures

Abbreviation

µg = microgram

µL = microliter (s)

ANOVA = Analysis of variance

Bb12 = *Bifidobacterium animalis* Bb12

β-gal = β-galactosidase

β-glu= β-glucosidase

BSA = Bovine serum albumin

˚ C = degree Celsius

CWP = Cell wall protease

CFU = Colony forming unit

D or d = day (s)

EPS = Exopolysaccharide (s)

FAO = Food and Agriculture Organization for the United Nations

g = gram

GIT = Gastro-intestinal tract

GOS= Galactooligosaccharides

H_2SO_4 = sulphuric acid

HCl = Hydrochloric acid

HPLC = High Performance Liquid Chromatography

IA = Isoflavone Aglycones

IG = Isoflavone glycosides

H = hour(s)

HDL = high-density lipoprotein

kJ = kilojoules

L = litre

LAB = Lactic Acid Bacteria

LA = *Lactobacillus acidophilus*

LB = *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842

M17 = selective medium for *S. thermophilus*

M = Molar

Min = minute(s)

mL= millilitre

mm = millilitre

MRS = deMan Rogosa Sharpe

MW = molecular weight

nm = nanometer

OPA = o-phthaldialdehyde

Pa = Pascal

pH = Hydrogen ion concentration

p-NPG = p-nitrophenyl-β-D glucopyranoside

RPM = revolution per minute

RSM = Reconstituted Skim Milk Powder

SI = Soy Isoflavones

SM = Soymilk

SMP = Skim Milk Powder

SPI = Soy Protein Isolate

s = second(s)

Ssp. = subspecies

ST = *Streptococcus thermophilus*

TCA = Trichloroacetic acid

UV = Ultraviolet

v/v = volume per volume

WMS = Modified waxy maize starch

w/v = weight per volume

w/w = weight per weight

WPC = whey protein concentrate

1. Introduction

Phytoestrogens are plant-derived, phenolic compounds with a structural homology to human estrogens. Phytoestrogens found in soybeans consist of the di-phenolic, isomeric family of compounds named isoflavones (Setchell and Cassidy, 1999). Soybean and soy food-derived isoflavones are found in two chemical forms including aglycones and β-glucoside conjugates (IG). The biologically active, estrogen-like isoflavone isomers are the aglycone configurations (IA), genistein, daidzein and glycitein (Setchell and Cassidy, 1999). The structural differences of soy isoflavone aglycones and glucosides have been described in figure 2.1. In soybean and soy products isoflavones are mainly found as glucoside conjugates malonyl-, acetyl-, typically comprising about 83% of the total isoflavones. Aglycone isomers are able to bind to estrogen receptor sites and hence mimic the functions of estradiol in the human body (Setchell and Cassidy, 1999). From the reviews of epidemiological (Setchell, 1998) and small-scale human clinical studies (Anderson et al., 1995) isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders prevalent in Western societies. Comparatively, Asian populations with their high intake (50-70 mg/day) of soy-derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms and mortality from cardiovascular disease and cancer (Nagata et al., 1998).

To deconjugate IG to biologically active IA, the β-glucosidic linkage between the glycoside and aglycone moieties must be broken. Several workers have reported on the transformation of IG to IA by microbial fermentation (Sherkat et al., 2000 and 2001; Tsangalis et al., 2002; Otieno et al., 2006; Chien et al., 2006; Chun et al., 2007). In these studies, β-glucosidase (β-glu) produced by bifidobacteria was claimed to be responsible for the transformation of IG to aglycones. However, *Bifidobacterium* produces β-galactosidase (β-gal) in addition to β-glu (Shah and Jelen, 1990). The β-gal acts on the β-galactosidic bond,

regardless of the rest of the molecular structure. The β-gal was reported not to be strictly specific to the β- galactosidic bond. It was shown that β-gal could hydrolyze α- galactosidic bond in α-lactose (Huber et al., 1981). Therefore, it is possible that β-gal is also responsible for the biotransformation of IG to IA since the β-galactosidic and β-glucosidic bonds are relatively similar (Huber et al., 1981).

Recent research has shown that isoflavone aglycones (IA) in soy foods are absorbed more rapidly and thoroughly than their respective isoflavone glycosides (IG) in human's gastro-intestinal tract (Izumi et al., 2000). Intestinal microfloras play a key role in the metabolism and bioavailability of isoflavones, as they hydrolyse the glucoside components via β-glu activity in the jejunum, releasing the bioavailable bioactive aglycone forms. The enzymatic hydrolysis has been reported as a potentially useful method for its high specificity, yield, and productivity. Enzymatic hydrolysis has been achieved using microbial β-glu from *B. Subtilis, Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Escherichia coli* (Ismail and Hayes, 2005; Donkor et al., 2008; Kuo et al., 2008).

Izumi et al. (2000); Setchell et al. (2001); Otieno et al. (2006) evaluated enzymic potential of β-glu for biotransformation of phytoestrogens in soymilk and reported that β-glucosidic forms of daidzin, glycitin and genistin contributed to the highest concentration of isomers (83%) with a total of 3.26 mg 100 per mL. Moreover, the concentration of genistin was found to be the highest among the individual isomers at 2.19 mg per 100 mL. Chien et al. (2006) and Pham and Shah (2009) reported that there was a significantly higher biotransformation of isoflavone glycosides to aglycones in soymilk supplemented with skim milk powder (SMP) than that in un-supplemented soymilk.

Supplementation with SMP appeared to have the greatest stimulating effect on the biotransformation of IG to IA by *Lactobacillus* strains mainly as a result of the introduction of lactose in soymilk. Thus, *Lactobacillus* strains produced more β-gal as a positive response

of lactose in order to breakdown the lactose into galactose and glucose. Additionally, the same enzyme simultaneously breaks down the β-glucosidic bonds in isoflavone glycosides (Sherkat et al., 2000; Pham and Shah, 2009). SMP also contains milk proteins and it is known that *Lactobacillus* strains require casein for their growth (Vasiljevic and Jelen, 2003a).

Kluyveromyces lactis is the major commercial source of β-gal. Its natural habitat is the dairy environment and its β-gal has outstanding lactose-hydrolysing activity (Gekas and Lopez-Leiva, 1985). Commercial β-gal has been used to hydrolyze lactose to produce lactose-free milk products and has recently attracted interest for the production of galacto-oligosaccharides (GOS) from lactose via the transgalactosylation reaction (Onishi and Tanaka, 1995; Hung and Lee, 2002). Mitsuoka (1990) and Sako et al. (1999) reported that GOS stimulated the growth and establishment of bifidobacteria in the human intestine and suppressed potentially harmful bacteria such as Clostridia and *Bacteriodes* species in the gut and as such are now regarded as a prebiotic food ingredient.

Since β-gal or lactase is an intracellular enzyme, one of the major hindrances in effective production of this enzyme is the release of enzymes in sufficient quantities from the cells (Panesar et al., 2006). It has been established that the industrial application of β-gal is hampered by the difficulty and cost of extracting and purification of active enzyme in sufficient quantity from bacteria and yeast cells (Panesar et al., 2006). Thus, a major drawback in the use of whole cells as a source of β-gal is the poor permeability of the β-gal through the cell membrane. Different methods have been applied to increase lactase permeability through microbial cells (Panesar et al., 2006). It is important to evaluate the ideal method in terms of efficacy and enzyme yield so that the process could be scaled up to at commercial levels.

There is an increasing demand for functional foods in developed countries. The rapidly growing markets for functional or health-enhancing foods has emerged as a response to

patterns of health and disease, innovation in food and health related research, and globalization (Kotilainen et al., 2006). Functional Foods are “foods that meet consumer needs for general health and wellbeing, and the prevention of the risk of developing disease, and management of health conditions” (Sloan, 2005). Dairy products containing probiotics and prebiotics (synbiotics) are most popular in this category of foods. Probiosis can be defined as ‘the positive effect of consumption of fermented dairy products with live culture of lactic acid bacteria (LAB) on the equilibrium of intestinal microflora’ (Tomasik and Tomasik, 2003). During fermentation, LAB produces a range of metabolites (e.g. folic acid, vitamin B11) which have been associated with health-promoting properties. The most notable of these are the vitamin B11 and bioactive peptides released from food proteins (Tuohy et al., 2003).

Prebiotics are non-digestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial to health (Roberfroid et al., 1998). Prebiotics of proven efficacy include fructooligosaccharide (FOS), inulin, lactulose and galacto-oligosaccharides. These oligosaccharides have the ability to beneficially alter the gut microbiota thus having the potential to reduce the risk of carcinoma, stimulate immune response, alleviate symptoms of inflammatory bowel disease, modify serum triglycerides and cholesterol levels, enhance mineral absorption in the intestine and thereby reduce the risk of intestinal infectious diseases, non-insulin dependent diabetes, obesity aqnd osteoporosis (Roberfroid, 2000; Williams and Jackson, 2002; Tuohy et al., 2003; Shah, 2007).

The term synbiotics is used when referring to a product that uses prebiotics and probiotics in combination (Roberfroid et al., 1998). Microorganisms might also indirectly impart health-promoting characteristics in food through the production of bioactive metabolites during fermentation (Takano, 2002).

Among various ingredients used to replace fat in low-fat or non- fat foods, fibre-based mimetics provide especially positive physiological benefits since there is a growing

recognition for the role of dietary fibre in disease prevention (Jones, 1996). There has been a considerable interest in preparing yoghurt with prebiotics such as modified waxy maize starch (MWMS), GOS, inulin and β-glucan (Gibson and Wang, 1994a; Roberfroid et al., 1998).

Yogurt is traditionally considered to be a healthy food. It can be made more functional by the addition of probiotics and prebiotics. The product functionality is further enhanced by the release of bioactive peptides during lactic fermentation (Arunachalam, 1999; Shah, 2007). According to Food Standard Australia and New Zealand (FSANZ, 2002), yogurt is 'the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. However, in recent years yogurts containing probiotics have gained popularity. These products should contain *Lactobacillus* and *Bifidobacterium* species at 10^6 CFU per gram at the time of consumption (Arunachalam, 1999).

Live yoghurt cultures, which are the subject of the health claim, are sufficiently characterised in relation to the claimed effect, which includes lactose digestion. A cause and effect relationship has been established between the consumption of live yoghurt cultures in yoghurt and improved digestion of lactose in yoghurt in individuals with lactose maldigestion (EFSA, 2010). In order to claim the health benefit, the yoghurt should contain at least 10^8 CFU live starter microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) per gram (Labayen et al., 2001).

Most of the research works that have been conducted so far relate to the viscosity and textural properties, organic acid production, and proteolytic activity of low-fat yoghurt containing modified starch (Guven et al., 2005; Kip et al., 2006; Tarrega and Costell, 2006; Aryana et al., 2007). However, not much information is available on the application of GOS.

It is a natural prebiotic ingredient, structured as chains of galactose with a glucose end unit produced from glucose molecule transferred from a sucrose donor to a maltose acceptor by a glycosyl-transferase (Wang and Gibson, 1993).

Inulin and oligofructoses (OF) are natural food components belonging to a class of carbohydrates known as fructans, produced mainly from chicory and Jerusalem artichoke (Paseephol et al., 2008; Paseephol and Sherkat, 2009) which consist of a series of oligo- and polysaccharides of fructose with β (2→1) linkages, where the terminal sugar in most chains is glucose (Kaur and Gupta 2002).

The β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. These are a diverse group of molecules that can vary with respect to molecular mass, solubility and viscosity. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria.

The MWMS is used in yogurt to improve uniformity and stability as thickening agent and stabilizer. It improves smoothness and creaminess of dairy products as well as freeze-thaw stability of frozen desserts (Sprague, 1939).

Streptococcus thermophilus (ST) is used in the production of yogurt (Kilic et al., 1996) along with *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB). These two species are synergistic, and ST provides LB with folic acid and formic acid which it uses for purine synthesis (Sieuwerts et al., 2010). The ST has the unique ability is to break down casein for use by LB, while the proteolytic activity of LB produces stimulatory peptides and amino acids for use by ST (Leroy and De Vuyst, 2004)

Bifidobacterium strains are important probiotics used in the food industry. Bifidobacteria from human origin are able to utilize galactose, lactose and fructose as carbon sources (Mayo

et al., 2010).

The *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was selected based on previous evidence as a high β-gal producer (Vasiljevic and Jelen, 2003). The *B. animalis* ssp. *lactis* BB12 was found to possess the highest level of β-gal activity compared to others Bifidobacteria (Dechter and Hoover, 1998).

Aims of the project

The main aims of this project were:

- To evaluate the suitability of sweet whey as a medium for the production of β-gal from LB and BB12.
- To investigate the influence of selected prebiotics, i.e. GOS, MWMS, inulin and β-glucan on the growth of selected LAB.
- To study the role of β-gal produced by LB and BB12 in biotransformation of IG to IA

The specific objectives of the project were:

1. To examine the effect of various nitrogen and carbon sources on the production of β-gal by LB and BB12 in deproteinised whey.
2. To evaluate the suitability of sweet whey as a medium for the production of β-gal from LB and BB12.
3. To study the role of β-gal produced by LB and BB12 in biotransformation of IG to IA.
4. To examine the influence of GOS and MWMS on the growth of ST, LB and BB12, their proteolytic activity, yogurt pH and organic acid production and syneresis,

viscosity and firmness of low fat cow's milk yogurt.

5. To investigate the influence of prebiotics addition levels on changes in pH, viable counts of LAB, their proteolytic activity and organic acid production, and physical attributes of low-fat yogurt as well as soy yogurt.

With respect to the thesis contents, Chapter 1.0 contains introduction of this thesis. Chapter 2.0 presents the literature review on β-gal and β-glu and their application in food industry, enzyme extraction methods, effect of carbon and nitrogen sources on the production of β-gal, viable counts and changes in pH, the transformation mechanism of IG to IA, and the influence of GOS, MWMS, inulin and β-glucan on the growth of starter bacteria, ST and LB, proteolytic activity, organic acid production and textural attributes of skim milk yogurt.

Chapter 3.0 deals with the extraction of β-gal produced by BB12 and LB grown in whey, using sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatments. The suitability of whey as a medium for the production of β-gal from these organisms is also reported.

Chapter 4.0 focuses on the effect of carbon and nitrogen sources on growth of BB12 and LB and production of β-gal. This study also focused on the selection of micro-organisms which are capable of producing high level of β-gal in deprotenized whey.

Chapter 5.0 deals with the effect of nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate on the production of β-gal from BB12 and LB grown in deprotenized whey under different conditions.

Chapter 6.0 presents the conversion of isoflavone glycosides to aglycones in soymilk made from soy protein isolate (SPI) supplemented with 2.0 % (w/v) of D-glucose using crude enzyme extracted from BB12 and LB.

Chapter 7.0 focuses on the influence of GOS and MWMS on the growth and viability of ST and LB and their proteolytic activity and organic acid production in non-fat yogurt stored

at 4˚C for 28 days.

Chapter 8.0 investigates the effects of prebiotics addition to soymilk on the viable counts, proteolytic activity and organic acid production by LAB, and on physical attributes of soy yogurt

The overall conclusions and future research directions are included in Chapter 9.0 and references are listed in Chapter 10.0.

2. Literature Review

2.1 β-galactosidase (β-gal, EC 3.2.1.23)

2.1.1 Introduction

β-Galactosidase also known as lactase is a hydrolase enzyme that catalyzes the hydrolysis of lactose into monosaccharides. There are a variety of substrates available of β-gal which includes ganglioside GM1, lactosylceramides, lactose, and various glycoproteins (Kim and Rajagopal 2000). The β-gal enzyme hydrolyses lactose to galactose and glucose. The commercial use of this enzyme is to break down lactose in milk to make it suitable for people suffering from lactose intolerance. Lactase is also used in the conversion of whey into syrup used as sweetener in many products (Matthews, 2005). Kim and Rajagopal (2000) and Albayrak and Yang (2002) reported that the β-gal is expensive and not available in sufficient quantities for industrial application. Lactose is a hygroscopic sugar and causes many defects in refrigerated products such as crystallization resulting in sandy or gritty texture and deposit formation (Panesar et al., 2007). Thus, the application of β-gal for the hydrolysis of lactose in dairy industry has been the focus of many research and development activities, including the development of the enzyme immobilization technology (German, 1997).

The methods of recovery of β-gal activity are usually culture based, usually involving the incubation periods of 18 to 24 h. In addition to enzymatic activity, these techniques use growth at appropriate temperatures in the presence of inhibitors, combined with demonstration of enzymatic activity to selectively detect target organisms (Tryland and Fiksdal, 1998). As per the CAZy (carbohydrate active enzymes) database, this enzyme has been classified under the glycoside hydrolase 2 (GH 2) family of carbohydrate active enzymes. This enzyme has several applications in the food, dairy and fermentation industries. Technologically, lactose easily crystallizes at higher concentrations, which sets the limits to certain processes and application of this enzyme in the dairy industry (Mahoney, 1997). Its

application can help to solve problems related to the use of by-products from cheese manufacturing industries, avoiding serious pollution problems caused by the waste disposal. Furthermore, lactose hydrolysis provides several advantages: i) nutritional, because a significant fraction of the world population suffers from lactase deficiency; ii) technological, because glucose and galactose are sweeter and more soluble than lactose; and iii) environmental, associated with whey disposal. Furthermore, glucose and galactose are more readily fermented than lactose (Kumar et al., 1992).

2.1.2 Enzyme sources

The β-Gal is a widespread enzyme found in nature. The major industrial enzymes are obtained from *Aspergillus* ssp. and *Kluyveromyces* ssp. (Jurado et al., 2002). The β-Gal produced by *Kluyveromyces lactis* is one of the most extensively used enzymes in the formation of GOS during lactose hydrolysis (Jurado et al., 2002; Lee et al., 2003; Klewicki, 2007).

This enzyme can also be produced by a large number of bacteria. *Streptococcus thermophilus* and *Bacillus stearothermophilus* are considered as potential bacterial sources. The enzyme from *Escherichia coli* is used as a model for understanding the catalytic mechanism of β-gal but is not considered suitable for food use due to safety problems associated with the source organism (Finocchiaro et al., 1980; Joshi et al., 1989; German 1997). Microorganisms provide a number of advantages such as ease of handling, higher multiplication rate and higher enzyme activity over other available sources. As a result of commercial interest in β-gal production, a large number of microorganisms have been assessed as a potential source of this enzyme (Table 2.1).

Table 2.1: Bacterial sources of β-galactosidase

Alicyclobacillus acidocaldarius ssp.
Arthrobacter ssp.
Bacillus acidocaldarius
B. circulans
B. coagulans
B. subtilis
B. megaterum
B. stearothermophilus
Bacteriodes polypragmatus
Bifidobacterium bifidum
B. infantis
Clostridium acetobutylicum
C. thermosulfurogens
Corynebacterium murisepticum
Enterobacter agglomerans
E. cloaceae
Escherichia coli
Klebsiella pneumoniae
Lactobacillus acidophilus
L. bulgaricus
L. helveticus
L. kefiranofaciens
L. lactis
L. thermophilus
L. delbrueckii
Leuconostoc citrovorum
Pediococcus acidilacti
Propioionibacterium shermanii
Pseudomonas fluorescens
Lactococcus cremoris
Lactococcus lactis
S. thermophilus

Source: Finocchiaro et al. (1980); Gul-Guven et al. (2007)

Microorganisms normally produce two kinds of enzymes; extracellular, which are released into the growth medium; and intracellular, which are retained inside the cell wall. Glucose isomerase is an example of intracellular enzyme which converts glucose into fructose and is highly significant in the food industry. An intracellular enzyme must be activated by a specific molecule. Because it is inside the cell, an average signal molecule cannot pass through the cell wall and activate it. Craig et al. (2012) reported that presence of the protease

inhibitors had a minimal effect on the average and distribution of single molecule activities. The changes in activity can be largely explained in each molecule containing subunits of identical activity. However, hydrophobic materials such as steroid molecules can pass through the cell membrane (Gueguen et al., 1995).

Extracellular or exoenzymes are those enzymes that are completely dissociated from the cell and are found free in the growth medium. However, the division between these and cell wall or membrane-bound enzymes is often thin. Some enzymes may be membrane bound in young cells and released (as exoenzymes) as the culture enters stationary phase (Gueguen et al., 1995).

Tochikura et al. (1986) and Shah and Jelen (1990) investigated *Bifidobacteria*'s potential as an industrial strain for the production of β-gal. *Bifidobacteria* produce both β-gal and β-glu which play important roles in the hydrolysis of isoflavone glycosides to the bioavailable aglycones. According to Kulp (1975), the best commercial sources include: *E. coli; A. niger* and lactose fermenting yeasts *K. Fragilis* and *K. lactis.* Ramana and Dutta (1977) studied *S. thermophilus* grown in deproteinized whey for β-gal production, and found that supplementation of basal media with whey and whey permeate resulted in the enhancement of growth rate and enzyme activity in bacterial culture.

Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) so the enzyme derived from them might be used without the need for extensive purification (Vinderola and Reinheimer, 2003). *Bifidobacteria* are probiotic organisms and their β-gal is used in foods and food systems (Tochikura et al. 1986). The enzyme produced by *Bifidobacterium longum* CCRC 15708, *Bifidobacterium longum* B6 and *Bifidobacterium infantis* CCRC 14633 were found to produce the maximum β-gal with high specific activity (Hsu et al., 2005). *Bifidobacterium* ssp. along with *Lactobacillus* ssp. are mainly used in fermented foods as probiotics because of their potential health benefits. *Bifidobacteria* and *Lactobacillus*

delbrueckii ssp. *bulgaricus* have been recognised as a good source of β-gal enzyme (Hsu et al., 2005). Bifidobacteria exist mainly in the human gastrointestinal tract (Biavati and Mattarelli, 2001; Van den Broek et al., 2008) which is rich in carbohydrates and they make use of different glycosidase systems to digest these carbohydrates in order to survive and compete with other microorganisms.

2.1.3 Methods of β-gal extraction

This review aims at examining various mechanical and chemical enzyme extraction techniques and their applicability for different β-gal producing microorganisms. Amongst several methods for releasing the enzyme, only four methods, sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatments are discussed.

2.1.3.1 Sonication

Sonication is one of the most widely used methods for disruption of the bacterial cell walls (Engler 1985). The cells are subjected to ultrasonic vibrations by introducing ultrasound emitting tip into the cell suspension to disrupt the cells. The high-frequency oscillation causes a localized low-pressure region resulting in cavitation ultimately breaking open the cells. In principle, the high-frequency is generated electronically and the mechanical energy is transmitted to the sample via a metal probe that oscillates with high frequency. Under intense sonication enzymes are released from cell organelles as a result of cell disintegration. In order to prevent the denaturation of enzyme due to heat generation by sonication, the sample was kept below 4ºC. To maintain this temperature in the tank during sonication, crushed ice was added to water bath. At the end of a typical sonication cycle (10 min. total time, 30sec “on”, 30 sec “off”), the temperature in the water bath should not exceed 0°C.

Toba et al. (1990) and Sakakibara et al. (1994) compared three enzyme extraction techniques namely sonication, bead milling and high-pressure homogenizer and found sonication to be more effective for releasing β-gal. In addition, Berger et al. (1995) studied

two physical disruption methods to maximize the extraction of intracellular enzymes from *Thermus* species and found that the sonication was superior to glass-bead milling. Disruption of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was studied by Bury et al. (2001) who found contrary to previous reports concluded that sonication was the least effective method on the release of β-gal.

2.1.3.2 Lysozyme-EDTA treatment

Salasbury (1989) found that lysozyme is often used for lysis of peptidoglycan layers as it catalyses the hydrolysis of β 1-4-glycosidic bonds. The enzyme is commercially available at a reasonable cost, and is produced from egg-white preparations. Gram-negative bacteria are less susceptible than the Gram-positive ones as their outer layer made of peptidoglycan shields the cell from the effect of lysozyme. However, combining lysozyme with EDTA allows the disruption of the cell wall and subsequent attack on the peptidoglycan structure (Salasbury 1989). Therefore, lysozyme combined with EDTA is a very effective method for releasing β-gal from Gram-negative bacteria (Andrews and Asenjo, 1987; Geciova et al., 2002).

2.1.3.3 Toluene-acetone and SDS-chloroform methods

Organic solvents like acetone mainly act on the cell membrane by solubilising its phospholipids and by denaturing its proteins and enzymes. Some solvents like toluene are known to disrupt fungal cell walls. The limitations of using organic solvents are similar to those with detergents-stable protease, i.e. the need to remove these from products and the denaturation of proteins (Sikkema et al., 1995).

Several workers have reported on the permeabilization of microbial cells by organic solvents (Flores et al., 1994; Kondo et al., 2000; Panesar et al., 2007; Park et al., 2007). Flores et al. (1994) studied the permeabilization of *K. lactis* cells by chloroform, toluene and ethanol in relation to β-gal extraction. They found that the performance of those solvents was

dependent on the incubation time and temperature, and the concentration of both cells and solvents.

2.1.4 Isolation and characterization of β-gal

Isolation of β-gal enzyme was studied by a number of workers including Pisani et al. (1990); Phan Tran et al. (1998) and Vinderola and Reinheimer (2003) who reported that maximum β-gal enzyme was isolated from commercial strains of *L. delbrueckii* ssp. *bulgaricus* from Minas fresh cheese. These findings agree with those of Gheytanchi et al. (2010) who detected two strains of *L. delbrueckii* with high and rapid enzyme activities from Minas fresh cheese.

Several workers have reported the purification and characterisation of enzyme from various microorganisms including *Bacillus licheniformis*, *Lactobacillus acidophilus*, *Sulfolobus solfataricus*, *Enterobacter agglomerans*, *Thermotoga maritima*, *Alicyclobacillus acidocaldarius* ssp. *rittmannii* and *Arthrobacter psychrolactophilu*s (Pisani et al., 1990; Phan Tran et al., 1998; Kim et al., 2004; Nakagawa et al., 2006; Nguyen et al., 2006).

Purification of β-gal is simple because of its large molecular size. Wallenfels and Weil (1972) and Shukla (1975) reported various procedures of isolating and purifying the enzyme, whereas Mahoney and Whitaker (1978) reported on the isolation technique by pseudo-affinity chromatography.

2.1.5 Utilization of whey as medium for β-gal production

Whey has enormous therapeutic applications due to its composition in terms of proteins, lactose, minerals and other valuable milk nutrients. The disposal of whey has become a major problem for the dairy industry especially in developing countries where a comparatively insignificant part of whey is used for production of protein concentrates and a significant part of it is disposed off into the water streams causing severe water pollution concerns in Asian

countries.

The main options are treatment or bioconversion of whey into commercially important products such as ethanol or β-gal which finds an increasing use due to growing lactose intolerant population. Thus, microbial β-gal production is an important rout for whey utilization. Availability of carbohydrate reservoir (lactose) in whey and presence of other essential nutrients for the growth of microorganisms provided it is free from bacteriophage, makes the whey one of the most potent raw materials for the production of different bio-products through biotechnological means (Panesar et al., 2007). The activity and stability of enzyme is influenced by the type of strain and the growth medium composition (Jurado et al., 2002; Tari et al., 2007).

Oberoi et al (2008) reported that *K. lactis* M2 produced maximum enzyme activity (up to 8103 U/mg) when grown in whey. This yeast strain could find valuable application in the bioconversion of whey (Moeini et al., 2004). Oda and Nakamura (2009) reported that *K. marxianus* could convert lactose in a concentrated media containing 20% (w/v) sugar from cheese whey to ethanol. Saad (2004) demonstrated that *Aspergillus japonicus* when grown on cheese whey produced β-gal with 29.51 U/mg specific activity.

Tari et al. (2009) investigated β-gal production by ST and LB in a reconstituted skim milk (RSM) containing deproteinzed whey (5% w/v), corn steep liquor (4% w/v), potassium phosphate (2 % w/v) and peptone (2 % w/v) at 43˚C for 8 h. The symbiotic relationship produced 6.4 % and 39 % more β-gal activity than pure culture of these two strains.

Therefore, availability carbohydrate lactose in whey and presence of other essential nutrients such as milk proteins, water soluble vitamins and minerals for the growth of microorganisms makes the whey one of the most important raw materials for the production of enzymes through biotechnological process (Panesar et al., 2007).

2.1.6 Galacto-oligosaccharides (GOS) production

The β-gal enzyme plays an important role in bacterial physiology either by hydrolysing simple disaccharides (e.g. lactose) or complex GOS to simpler carbohydrates and hence supplying the cell with necessary energy and carbon sources (Hinz et al., 2004; Van den Broek et al., 2008). Although most β-gal has been shown to be active towards lactose or synthetic substrates (such as proplonyl and succinyl), some have shown preference to GOS with different levels of polymerisation (Hinz et al., 2004).

The GOS belongs to prebiotics, non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon (Roberfoid, 2000). This prebiotic occurs naturally in trace amounts in breast milk, cow's milk, honey, and a variety of fruits and vegetables (Sako et al., 1999). Much research is focussed upon microorganisms for the production of GOS by β-gal producing bacteria (Table 2.2).

Commercially available GOS in powder or liquid form are mixtures of several types of GOS (more than 50% w/v), lactose (20% w/v), glucose (20% w/v) and a small amount of galactose. The GOS is quite stable during long-term storage at room temperature even in acidic conditions (Urgell and Orleans, 2001). Therefore, GOS can be applied without decomposition in variety of foods. Major companies dealing with oligosaccharides production (including GOS) are in Japan (Urgell and Orleans, 2001). Recently, there is also increasing trend of GOS production in Europe. Besides lactulose and soybean oligosaccharides, all oligosaccharides are prepared by transglycosylation from mono and disaccharides or by controlled hydrolysis of polysaccharides (Rastall and Maitin, 2002). Rabiu et al. (2001) investigated several strains of *Bifidobacterium* to produce GOS from 30 % lactose solution.

Table 2. 2: Research reports on GOS production by β-gal producing bacteria

β-gal producers	GOS and by-products	References
E. cloacae	GOS, glucose, galactose	Lu et al. (2009)
B. indica L3	Heteropolysaccharide-7	Lu et al. (2009)
P. expansum F3	GOS, glucose, galactose	Li et al. (2009); Li et al. (2008)
Lactobacillus ssp.	β-D-Gal-(1→6)-D-Glc, β -D-Gal-(1→6)-D-Lac, β -D-Gal-(1→6)-D-Gal, β-D-Gal-(1→3)-D-Lac, β -D-Gal-(1→3)-D-Gal	Splechtna et al. (2007)
B. longum BCRC 15708	tri-, tetrasaccharides, lactose, galactose, glucose	Hsu et al. (2007)
G. stearothermophilus KVE39	Lactosucrose, β-D galactopyranosyl-(1→3)-β-Dgalactopyranosyl-(1→4)- D-glucopyranoside (3'-galactosyl-lactose)]	Placier et al. (2009)
L. reuteri	β -D-Gal-(1→6)-D-Glc, β -D-Gal-(1→6)-D-Gal, β -D-Gal-(1→3)-D-Gal β-D-Gal-(1→6)-D-Lac β-D-Gal-(1→3)-D-Lac	Splechtna et al. (2007a)
L. bulgaricus	Sialyllactose, 14 other oligosaccharides	Shene and Bravo (2007)
L. delbrueckii subsp. bulgaricus	Galactose, lactic acid , acetic acid, ethanol	Shene and Bravo (2007)
B. infantis	GOS, lactose, monosaccharides	Jung and Lee (2008)
Lactobacillus plantarum	β -D-Gal-(1→6)-D-Lac, β -D-Gal-(1→6)-D-Glc	Iqbal et al. (2010)
Bacillus circulans	*N*-acetylactosamine, N-acetylglucosamine	Kaftzik et al. (2002)

Source: Sako et al. (1999).

They obtained the maximum of GOS production (43.8 %) using *B. angulatum*, whereas the strain *B. pseudolongum* produced no more than 26.8% of GOS. Thus, β-gal plays a significant role in the production of GOS that can be used as food and feed for humans and animals. The GOS has become the focus of a great deal of attention in the field of functional foods, owing to their known health benefits and potential to improve the quality of many foods.

2.1.7 Application of β-gal in food

Microorganisms are considered to be the most appropriate source of β-gal for industrial applications. However, they differ in their optimum conditions for enzyme production. Therefore, there has been increasing interest in screening the microorganisms with adequate properties for industrial use, higher production capacity and less expensive purification methods of this enzyme. The β-gal enzyme is mainly used in the food industry to reduce the lactose concentration in milk products, with the aim of overcoming lactose intolerance, a worldwide problem (Scrimshaw and Murray, 1988).

Both the hydrolase and transferase activity of β-gal can be useful for industrial applications (Nakayama and Amachi, 1999). The hydrolysis of lactose to glucose and galactose in different food such as milk or whey is the most common industrial use due to various applications of lactose-reduced ingredients in the food and dairy industry (Greenberg and Mahoney, 1982; Gekas and Lopez-Leiva, 1985).

Culture medium supplementation with whey in presence of carbon and nitrogen source improves not only production of both enzymes but also enable them to be produced simultaneously. Owing to the immense potential for the use of β-gal in the food biotechnology, an attempt was made in the present study to examine the effect of various nitrogen and carbon sources on the production of β-gal by LB and BB12. The β-gal activity of a given microorganism depends on the characteristics of a medium. A rich medium is necessary to maximize the enzyme activity (Vasiljevic and Jelen, 2001). This high enzyme activity is necessary to hydrolyses lactose to galactose and glucose efficiently. The commercial use of this enzyme is to break down lactose in milk to make it suitable for people suffering from lactose intolerance. Lactase is also used in the conversion of whey into syrup used as sweetener in many products (Matthews, 2005).

2.2 Effect of Carbon Sources on the Growth and β-Gal Production by microorganisms

Several workers reported that the effects of carbon source in the activity of β-gal may vary and depend on the microorganisms used (Fiedurek and Szczodrak, 1994; Fekete et al., 2002). They further reported on the effect of lactose, glucose, galactose, acid and sweet wheys on β-gal activity. Siham et al. (2010) found that the growth of *Lactobacillus acidophilus* was higher in the culture that contained either glucose or galactose as the sole carbon source but the maximum β-gal activity (12.29 U/mL) was recorded with acid whey followed by sweet whey and lactose (9.83 and 8.6 U/mL, respectively). These results were similar to those reported by De Bales and Castillo (1979), Nahvi and Moeini (2004) and Eliwa and El-Hofi (2010). Concentration of carbon source in the medium was increased up to 4% (w/v) may affect the expression of β-gal by microorganisms (Inchaurrondo et al., 1998; Fiedurek and Szczodrak, 1994; Hsu et al., 2005). The β-gal activity increased as the concentration of acid whey in the medium increased up to 3.5% (w/v) giving 12.9 U/mL. The final viable population of *B. longum* was higher in cultures containing either 5% (w/v) lactose or glucose with the maximum β-gal activity (5.44 U/mL) detected with lactose followed by galactose and the lowest activity was observed with glucose Eliwa and El-Hofi (2010). In contrast, Kim and Rajagopal (2000) reported that *L. cripatus* grown in MRS broth containining galactose as the carbon source showed the maximum β-gal activity than the lactose and glucose.

However, Fiedurek and Szczodrak (1994) and Shaikh et al. (1997) demonstrated that lactose was the best carbon source which induced the maximum activity of β-gal by *Rhizomucor* ssp. while glucose was a poor inducer. A similar study by Nehad and Enas (2011) on optimization of β-gal activity using various carbon sources such as sucrose, fructose, glucose, galactose, lactose, xylose and starch found lactose to be a better carbon source for the activity of β-gal from *Bacillus licheniformis* than the other carbon sources

(Kumar, 2009).

Eggins and Pugh (1972) investigated on the effect of eight different types of fermentation media such as $(NH_4)_2SO_4$ (0.5 g/L), KCl (1.0 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), L-asparagine (0.5 g/L), $CaCl_2$ (0.1 g/L), yeast extract (0.5 g/L) with 1% carbon source (w/v) for the production of β-glu. $(NH_4)_2SO_4$ was found to be the best medium for the production of β-glu. These results were agreement with Grajek (1987) who reported that $(NH_4)_2SO_4$ as the best nitrogen source for the production of β-glu from *Sporotrichum thermophile*. Busto et al. (1995) reported that cellobiose is the best carbon source for the production of β-glu than those of lactose, maltose, and sucrose. However, Jager et al. (2001) and Rajoka et al. (2006) found wheat bran was the best substrate for the production of β-glu from different *Aspergillus* strains using solid state fermentation.

Supplementation of lactose in the medium produced the maximum β-gal activity (43.82 U/mL) in *L. reuteri* in comparions to other carbon sources. The presence of lactose in the media probably triggered β-gal activity via the lac operon mechanism (Miller and Reznikoff, 1978). This finding was in line with the findings of Akolkar et al. (2005) and Hsu et al. (2005), who showed that the presence of lactose in the media led to an enhancement of β-gal activity for bifidobacteria and *L. acidophilus*, respectively. On the other hand, Kim and Rajagopal (2000) found that galactose was better than lactose as an inducer of β-gal activity in *Lactobacillus crispatus*.

2.3 Effect of Different Nitrogen Sources on the Production of β-gal

Nitrogen sources play important role in the activity of β-gal enzyme. Shaikh et al. (1997); Ramana-Rao and Dutta (1977) and Hsu et al. (2005) reported a clear variation in the activity of β-gal ranging from 2 to 22 U/mL detected in the cultures with various nitrogen sources including peptone, yeast extract and ammonium sulphate.

Moeini et al. (2004) investigated the effect of different concentrations of ammonium

sulphate $(NH_4)_2SO_4$ in the medium. Enzyme activity (38.5 U/mL) was reported with increased concentration of $(NH_4)_2SO_4$ up to 3.0% (w/v), whereas a reduction in the activity (22. 9 U/mL) was recorded with further increase in $(NH_4)_2SO_4$ content in the medium. These results were in agreement with Eliwa and El-Hofi (2010) who reported that the $(NH_4)_2SO_4$ addition resulted in more β-gal activity. Ramana-Rao and Dutta (1997) examined the effect of various inorganic and organic nitrogen supplements and found that enzyme activity by inorganic nitrogen was much lower than that by organic nitrogen supplements. Proteose peptone (2.0%, w/v) stimulated enzyme activity almost twice as much as the other organic nitrogen sources. Among the mono-, di-, and tribasic sodium salts tested, monobasic salts (0.8%, w/v) tested, resulted in maximum enzyme activity. Similarly, Eliwa and El-Hofi (2010) studied fifteen different organic or inorganic nitrogen sources separately in the medium containing malt and yeast extract. The results showed that $(NH_4)_2 SO_4$ was the most suitable for the maximum activity of β-gal enzyme (190 U/mL) followed by ammonium triphosphate (130 U/mL). These data were in agreement with the results obtained by Manera et al. (2008) who used $(NH_4)_2SO_4$ for β-gal activity and studied the effect of concentration on enzyme level.

Most of the available literature suggests the optimal fermentation time ranged from 20-36 h (Mahoney et al., 1975). Pavani et al. (2011) evaluated the effect of different organic nitrogen sources on the activity of β-gal. The soya peptone showed the maximum β-gal activity (211 U/mL) followed by peptone (183 U/mL) while gelatin showed the least activity (31 U/mL). Similarly, Hayashi et al. (1993) reported that among the nitrogen sources tested, yeast extract was the best for the activity of β-gal by *A. niger*.

The β-gal is not only useful for industrial applications (Nakayama and Amachi, 1999) in hydrolysis of lactose to glucose and galactose in different foods such as milk or whey (Greenberg and Mahoney, 1982; Gekas and Lopez-Leiva, 1985) but it is also responsible for

the biotransformation of isoflavone glycoside to isoflavone aglycone since the β- galactosidic and β-glucosidic bonds are relatively similar.

Addition of yeast extract media produced significantly higher β-gal activity (35.43 U/mL) than the other nitrogen sources followed by tryptone (23.99 U/mL and peptone (21.43 U/mL). Both Bury et al. (2001) and Hsu et al. (2005) showed that yeast extract was effective in enhancing β-gal activity in *L. delbrueckii* ssp. *bulgaricus* and bifidobacteria.

2.4 Properties of isoflavones?

Isoflavones are organic compounds found in certain types of plants. These are called phyto-nutrients because scientific evidence shows the important roles they play in nutrition and health (Piskula et al., 1999). Some isoflavones are also known as phyto-estrogens because they are able to mimic the effects of estrogen in humans (Kaufman et al., 1997). Isoflavones are present in two main forms in plants. The isoflavone glycosides (IGs) that are poorly absorbed in the small intestine because of their greater molecular weight and higher hydrophilicity compared with isoflavone aglycones (IAs) (Brown, 1988; Chang and Nair, 1995). As a result, the IGs such as daidzin, genistin and glycitin are known to be less bioactive than their respective aglycones, daidzein, genistein and glycitein (Xu et al., 1994; Piskula et al., 1999).

Genistein has been shown to inhibit breast and prostate cancer cell growth (Brown, 1988). Daidzein has been found to enhance bone formation and to prevent and treat the osteoporosis. Genistein has been shown to prevent bone loss in animals (Brown, 1988). The chemical structure of isoflavones is very similar to that of estrogen. Because of this similarity in structure, they can interfere with the action of estrogen. The structure of soy isoflavone aglycones and glucosides is shown in Fig 2.1.

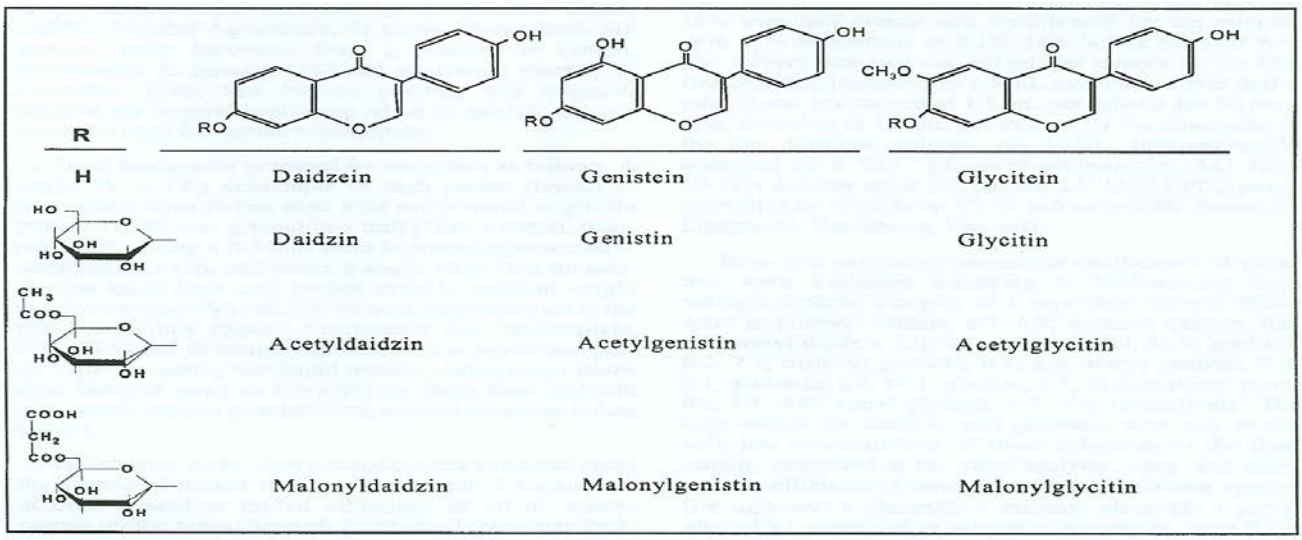

Figure 2. 1: Structure of soy isoflavone aglycones and glucosides

Adapted from King and Bignell (2000).

Table 2. 3:Type of isoflavone compounds found in nature

Forms of isoflavone	Isoflavone compounds	Formula	Molecular weight
Isoflavone aglycones	Daidzein	$C_{15}H_{10}O_4$	254
	Glycitein	$C_{16}H_{12}O_5$	284
	Genistein	$C_{15}H_{10}O_5$	270
	Biochanin A	$C_{16}H_{12}O_5$	284
	Formononetin	$C_{16}H_{12}O_4$	268
Isoflavone glycosides	Acetyl daidzein	$C_{23}H_{22}O_{10}$	416
	Acetyl glycitin	$C_{24}H_{24}O_{11}$	446
	Acetyl genistein	$C_{23}H_{22}O_{11}$	432
Acetyl glycosides	Acetyl daidzein	$C_{23}H_{22}O_{10}$	458
	Acetyl glycitin	$C_{24}H_{24}O_{11}$	488
	Acetyl genistein	$C_{23}H_{22}O_{11}$	474
Malonyl glycosides	Malonyl daidzin	$C_{24}H_{22}O_{12}$	506
	Malonyl glycitin	$C_{25}H_{24}O_{13}$	532
	Malonyl genistin	$C_{24}H_{22}O_{13}$	518

Source: King and Bignell (2000).

In nature, isoflavones are found in two forms. Glycosides (Genistin, Daidzin and Glycitin) have a sugar molecule attached to their structure and aglycones (Genistein, Daidzein, Glycitein, Biochanin A and Formononetin as shown in table 2.3) that are metabolites of the glycosides, without the sugar moiety and therefore can be absorbed from the gut (King and Bignell 2000). Del Monte et al. (2006) and Sacks et al. (2006) reported that in unprocessed soybeans the 6”-O-malonyl-β-glucosides of the isoflavones predominate and the 6”-O-acetyl-β glucosides are formed by decarboxylation of malonyl moiety; and that isoflavone β-glucosides and aglycones are formed from the corresponding malonyl-glucosides during the processing of soybeans or during sample preparation.

2.4.1 The sources of isoflavone

Isoflavones are found in soybeans, chick peas and other legumes. However, soybeans are unique because they have the highest concentration of these compounds (mg/100g). They are also found in trace amounts in cow’s milk, breast milk and in grains (Knight et al., 1998; Liggins et al., 2000a). Isoflavones are produced almost exclusively by the members of the *Fabaceae* (i.e., *Leguminosae,* or bean) family. Soymilk especially contains same 4 to 12 mg isoflavones per 100 mL (King and Bignell, 2000; Tsangalis et al., 2002) but is subject to considerable variation in content and composition (Murphy et al., 1999; King and Bignell, 2000). Isoflavones content of selected foods is presented in table 2. 4.

2.4.2 Dietary Intake of Isoflavones

Many workers have reported lower incidences of menopausal symptoms, breast cancer and possibly prostate cancer and osteoporosis in Japanese and Chinese populations who consumed soy (Messina et al. 1994; Horn-Rose et al., 2000).

Murphy et al. (1999); Boker et al. (2002) and Van Erp-Baart et al. (2003) reported that adult

population in Japan and China consumed between 25 and 50 mg isoflavones per day with less than 5% consuming 100 mg per day (Ho et al., 2000; Somekawa et al., 2001; Messina et al., 2006). People in the United States and Europe consume on average 3 mg isoflavones per day (Keinan-Boker et al., 2002).

Mazur and Adlercreutz (2000) reported that intakes of isoflavones in Western Europe are very low, but until recently no valid data was available. De Kleijn et al. (2001) estimated the isoflavone aglycones (genistein and daidzein) intakes in Caucasian postmenopausal women in the USA to be less than 1 mg per day.

Table 2. 4: Isoflavones content of selected foods and ingredients

Food/ingredients	Total isoflavones (mg/100 g)	Daidzein (mg/100 g)	Genistein (mg/100 g)
Soy flour, defatted	177.89	71.19	96.83
Soy flour, textured	148.61	59.62	78.90
Soy flour, full fat	131.19	57.47	71.21
Soybeans	128.34	46.46	73.76
Soy protein concentrate	102.07	43.04	55.59
Soy protein isolate	97.43	33.59	59.62
Natto	58.93	21.85	29.04
Soybean chips	54.16	26.71	27.45
Tofu, fried	48.35	17.83	28.00
Tempeh	43.52	17.59	24.85
Miso	42.55	16.13	24.56
Soybean sprouts	40.71	19.12	21.60
Tofu, soft	29.24	8.59	20.65
Tofu, silken	27.91	11.13	15.58

Soy infant formula	25.00	7.23	14.75
Tofu, firm	22.70	8.00	12.75
Soy hot dog	15.00	3.40	8.20
Okara	13.51	5.39	6.48
Soy protein alcohol	12.47	6.83	5.33
Bacon, meatless	12.10	2.80	6.90
Soy milk	9.65	4.45	6.06
Vegetarian berger	9.30	2.95	5.28
Soy cheese, Mozzarella	7.70	1.10	3.60
Soy cheese, Cheddar	7.15	1.80	2.25
Soy drink	7.01	2.41	4.60
Split peas	2.42	2.42	0.00
Shoyu	1.64	0.93	0.82
Pigeon peas	0.56	0.02	0.54
Clover sprouts	0.35	0.00	0.35
Peanuts	0.26	0.03	0.24
Navy beans	0.21	0.01	0.20
Mung bean	0.19	0.01	0.18
Granola bars	0.13	0.05	0.08
Chickpeas	0.10	0.04	0.06
Green tea	0.05	0.01	0.04
Broad beans	0.03	0.02	0.00
Cowpeas	0.03	0.01	0.02
Lima beans	0.03	0.02	0.01

Source: USDA IOWA (2002). State University, Data base on Isoflavones, Rel 1-3.

The small groups of soya food consumers had a higher intake level of 11 mg per day in the Netherlands and 6 mg per day in Ireland. However, even this intake is not as high as the intake in Asian countries (Nagata et al., 1997; Chen et al., 1999; Ho et al., 2000).

2.5 Bioconversion of isoflavone glucosides to aglycones

The bioavailability and bioactivity of soy isoflavonoids depend to some extent on the quantity consumed, chemical forms, and the physical properties of soy foods (King 1998; Hendrich and Fisher, 2001). In the process of bioconversion of isoflavones, β-glucosidase (β-glu) of intestinal microflora in lower bowel hydrolyse the IG to IA and promotes their absorption (Hendrich, 2002). Therefore, bacteria with β-glu activity are potentially important in the production of compounds with higher estrogenicity and better absorption, facilitating the bioavailability of isoflavones (Hur et al., 2000). Pham and Shah (2009 reported that the bacteria such as *Bifidobacterium* produced β-glu in addition to β-gal (Shah and Jelen, 1990). Regarding the specificity, β-gal is classed as a linkage specific enzyme. The enzyme acts on a particular type of chemical bond, which is β-galactosidic bond, regardless of the rest of the molecular structure. The β-gal was reported not to be strictly specific to the β-galactosidic bond. It was shown that β-gal could also hydrolyse the α-galactosidic bond (Huber, Hurlburt, & Turner, 1981). Therefore, it is possible that β-gal is also responsible for the biotransformation of IG to aglycones since the β-galactosidic bond and β-glucosidic bond are relatively similar.

2.5.1 Bioconversion of IG to IA and their metabolism in human (GIT)

Isoflavones compounds in non-fermented food products are predominantly presents in IG forms. The proportions of glucoside conjugates can vary significantly among different soy foods (Sherkat et al., 2000 and 2001; Wei et al., 2007). It has been proven that IGs do not cross the intestinal wall of healthy humans (Marotti et al., 2007). The IGs can be hydrolysed

by both intestinal mucosal and bacterial β-glu releasing the aglycones (Sherkat et al. 2000), which are then either absorbed directly or further metabolized by the intestinal microbiota in the large intestine into other metabolites, including equol (Setchell et al. 2000). However, due to variations in the member of intestinal bacteria through illnesses, diet or age (Hutt et al., 2006), the intestinal bacteria cannot always be relied upon for glucoside de-conjugation and releasing IAs.

According to Setchell et al. (2000), IAs are absorbed directly from the gastrointestinal tract, whereas IGs require cleavage to IAs prior to absorption and this will not occur until IGs reach the microflora in the large intestine (Barnes, 1995; Day et al., 1998). Several workers reported that the large intestine and gut microflora play vital functions in the transformation of IGs to IAs (Kneifel et al., 2000; Tsangalis et al., 2002; Chien et al., 2006; Otieno et al., 2006; Farnworth et al., 2007; Donkor and Shah, 2008). The linkage of IAs to their sugar moieties is via β-glucosidic bond and the gut microflora including lactobacilli, bifidobacteria and bacteroides, *Enterococcus*, *Streptococcus*, and *Weissella* are able to generate β-glu to hydrolyse the β-glucosidic bonds (Chun et al., 2007). However, the degree of the transformation of IGs to IAs and IAs metabolism by the gut microflora directly depends on each individual consumer parameters such as age, gender, and diet (Frankenfeld et al., 2005). Furthermore, there is still a disagreement in the absorption of IAs and IGs. In the study of Setchell et al. (2000), IGs were not detected in plasma suggesting that IG were not able to be absorbed through the human gut wall. However, according Richelle et al. (2002), similar levels of plasma and urine pharmacokinetics were observed for the IA and IG enriched drinks.

Izumi et al. (2000) reported that IAs were absorbed much faster and in higher amounts than their IG counterparts. As a result, those isoflavones that were absorbed excreted in un-conjugated form in the faeces (Adlercreutz et al., 1995).

2.5.2 Metabolism of soy isoflavones by gut microflora

The biological and metabolic activities of soy isoflavones differ depending on their chemical form (Cassidy, 1996). The chemical form of the isoflavones and their metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than their glycoside conjugates (Setchell, 2000; Izumi et al., 2000). The acetyl and malonyl derivatives of genistin and daidzin are first metabolized to genistin and daidzin, which are then hydrolysed in the large intestine by bacteria resulting in the removal of the sugar moiety to liberate their respective aglycones, daidzein, and genistein (Izumi et al., 2000). The absorbed aglycones and their metabolites are readily conjugated in the liver with glucuronic acid and sulfate which circulate enterohepatically with potential metabolism and re-absorption in the intestine which are finally excreted mainly in the urine. The glucuronide fraction, the main conjugate representing up to 83% of isoflavones in humans, (Doerge et al. 2000) is considered biologically inactive (Cassidy, 1996), whereas the free and sulphated fractions that are present at much lower concentrations, are biologically active. Alternatively, daidzein may be further metabolized by inhabitant microflora in the gastrointestinal tract to equol and O-desmethylangolensin (ODMA) through their respective intermediates, dehydroequol and dihydrodaidzein. Berger et al. (2008) postulated that daidzein may be metabolized to 6'- hydroxy-*O*-desmethylangolensin through the intermediate equol (Fig. 2.2). Kulling et al. (2000) identified hydroxylated metabolites of both genistein and daidzein using liver microsomes which might be an important metabolic pathway *in vivo*. Heinonen et al. (1999) reported that metabolites of glycitein have been identified in human urine as 5'-OH-*O*-desmethylangolensin and 5'-methoxy-*O*-desmethylangolensin.

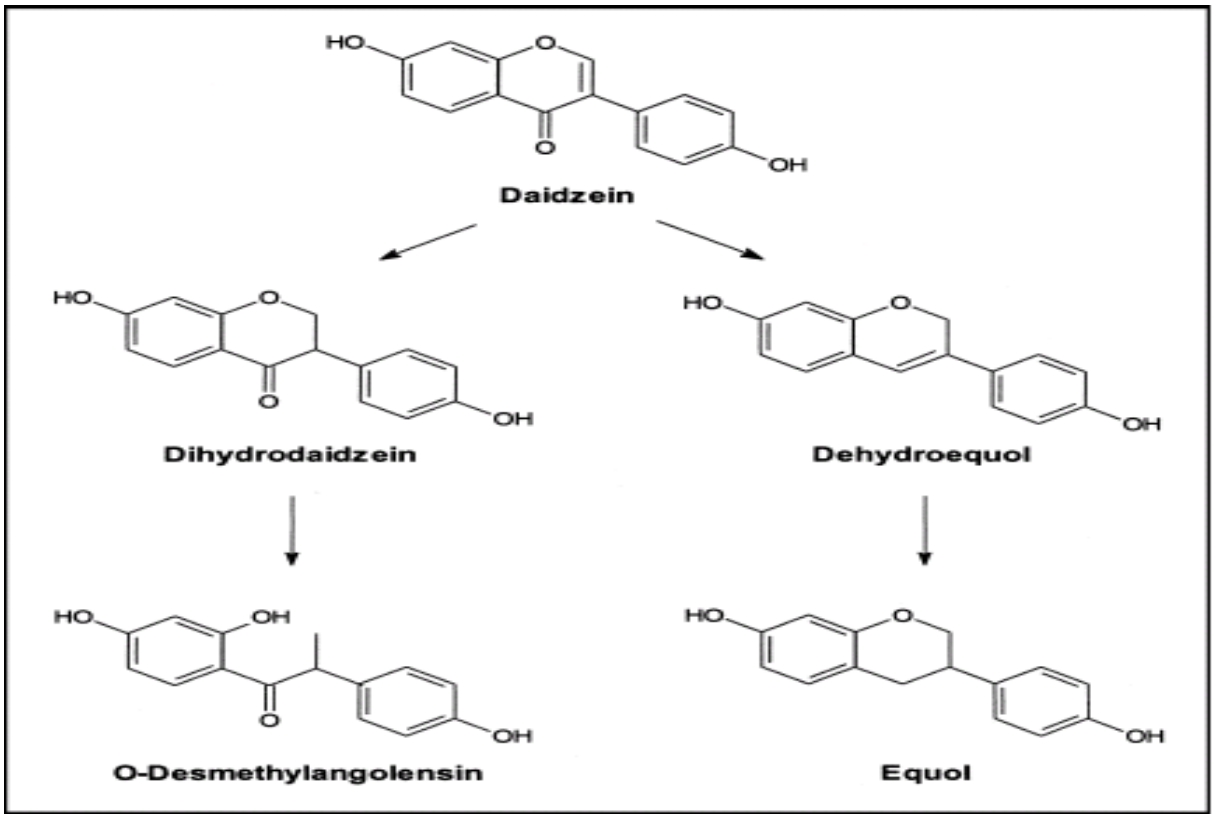

Figure 2. 2: The metabolism of daidzein

Adapted from Bayer et al. (2001)

The absorption and metabolism of soy isoflavones vary among subgroups in a population, based on age or gender, and among cultural groups. Metabolisms of isoflavones in infants (0-1 year) have been reported to vary because the gut microflora cannot hydrolyze the un-conjugated forms (Huggett et al. 1997). In addition, differences in metabolic pathways may arise due to different microflora, intestinal transit time, pH, or redox potential (Hendrich and Fisher, 2001) and factors that are influenced by diet, drugs (including antibiotics), bowel disease, surgery, and host immunity (Knight and Eden, 1995).

2.5.3 Factor affecting the bioavailability of soy isoflavones in human

Setchell et al. (2001) suggested that the clinical outcome of ingesting diets containing

isoflavones seems to be a tendency toward equol production. Furthermore, differences in the absorption rates between the glucosylated and aglycone forms of isoflavones were reported by Setchell et al. (2001) and Zubik and Meydani. (2003) who suggested that not all isoflavones can be considered the same if present in different types of foods.

2.5.3.1 Type of food

Cassidy et al. (2006) reported that the bioavailability of isoflavones was mainly influenced by the type of food. Tempeh contains mainly the aglycones daidzein and genistein. Isoflavones in soy milk are absorbed faster than others soy foods (Wang et al., 1990). The metabolism of aglycones seems to be strongly influenced by the diet. A high-carbohydrate diet which causes increased intestinal fermentation results in more phytoestrogens being transformed in equol (Wang and Murphy, 1994).

2.5.3.2 Gender

Lu and Anderson (1998) reported that differences were detected between genders, with an initially higher urinary excretion of isoflavone conjugates in women than in men. Several subsequent studies found no difference in the percentage of excreted through urine or the plasma pharmacokinetics of genistein or daidzein between genders (Zhang et al., 1999; Faughnan et al., 2004).

Over time, the urinary excretion of genistein and daidzein decreases and that of equol increases in women, whereas it remains constant in men. Faughnan et al. (2004) reported more equol production among postmenopausal women than among premenopausal women which suggests that both gender and age might be determinants for the intestinal metabolism of isoflavones.

Setchell et al. (2003) found no differences in the single-dose pharmacokinetics of either genistein or daidzein between pre- and postmenopausal women. However, a significant

difference was observed in plasma and urine levels of equol during the first months of life compared with adulthood which probably is due to an immature gut flora (Setchell, 1998).

2.5.4 Chemical hydrolysis of IGs

Delmonte et al. (2006) found that isoflavones were often present in food or dietary supplements in forms that could not be readily measured. Figure 2.3 shows the effect of acid and basic hydrolysis on isoflavone derivatives to break them down into compounds that are easier to quantify.

Basic hydrolysis breaks ester bonds, removing acid groups that are bound to the sugar moiety of the isoflavone glycosides. As a result, the 6”-*O*-malonyl-glucoside and the 6”-*O*-acetyl-β-glucoside isoflavone forms are converted to the respective β-glucosides. Acid hydrolysis breaks the bond between the isoflavone and the glycoside moieties, transforming all of the isoflavone derivatives into their aglycone forms. This procedure results in the quantification of isoflavones that are linked to sugars other than glucose (Delmonte et al. 2006).

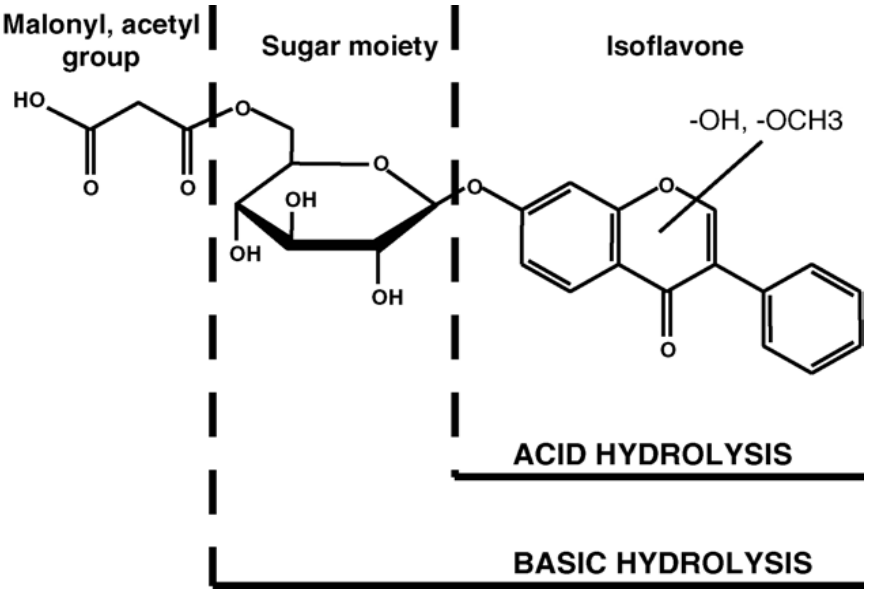

Figure 2. 3: Scheme of acid and alkaline hydrolysis of isoflavone derivatives

Adapted from Delmonte et al. (2006)

2.5.5 Microbial Bioconversion of IGs to IAs

Some microorganisms play major roles in the hydrolysis of β-glucoside isoflavones and enhance their absorption and bioavailability (Hur et al., 2000; Setchell et al., 2001). Biotransformation and the production of metabolites of isoflavones in the intestinal tract are highly dependent on the nature of intestinal microflora (Chang and Nair, 1995 and Omoni and Aluko, 2005) and that any change in intestinal microflora composition could affect the bioavailability of isoflavones. Some Lactobacilli (Choi et al., 1999) and Bifidobacteria (Jeon et al., 2002) are known to hydrolyse β-glucosides. Chun et al. (2007) demonstrated that fermentation of soymilk with lactic acid bacteria (LAB) such as *Lactobacillus paraplantarum*, *Enterococcus durans*, *Streptococcus thermophilus*, and *Weissela confusa* might make isoflavones more available to the human body by converting them efficiently into the aglycone form. The *L. paraplantarum* is very effective in the fermentation of soymilk due to its high capacity in isoflavone conversion. The LAB and probiotic organisms have been also used widely to produce lactic acid as the major metabolic end product of carbohydrate fermentation (Chun et al., 2007).

The most important microorganism used for the transformation of IGs to IAs are presented in table 2.5. They all are considered to have strong ability to produce β-glu (Tsangalis et al., 2002; Chien et al., 2006; Otieno et al., 2006). Apart from LAB, probiotic organisms such as bifidobacteria have also been used widely for the conversion.

Several studies have been attempting to enhance the isoflavone aglycones levels in soymilk by fermenting it with β-glu producing LAB (Scalabrini et al., 1998; Sherkat et al., 2000; Pyo et al., 2005; Chien et al., 2006; Otieno et al., 2006). These studies suggested that the enhancement of isoflavone aglycones levels before consumption of soy foods as well as the ingestion of viable bacteria could improve the bioavailability of isoflavones from soy foods.

Table 2. 5: Microorganisms used for bioconversion of IGs to IAs

Microorganisms	References
B. adolescentis	Marotti et al. (2007)
B. animalis	Otieno et al. (2006); Farnworth et al. (2007)
B. breve	Pyo et al. (2005); Marotti et al. (2007)
B. catenulatum	Marotti et al. (2007)
B. longum	Tsangalis et al. (2002); Farnworth et al. (2007)
B. pseudocatenulatum	Marotti et al. (2007)
E. durans	Chun et al. (2008); Rekha and Vijyalakshmi. (2010)
L. acidophilus	Sherkat et al. (2000); Rekha and Vijyalakshmi. (2010)
L. casei	Otieno et al. (2006a); Donkor et al. (2008)
L. delbrueckii ssp. *bulgaricus*	Farnworth et al. (2007); Rekha and Vijyalakshmi. (2010)
L. paracasei	Marotti et al. (2007)
L. paraplantarum	Chun et al. (2007); Rekha and Vijyalakshmi. (2010)
L. rhamnosus	Farnworth et al. (2007); Rekha and Vijyalakshmi. (2010)
S. thermophilus	Sherkat et al. (2000); Chien et al. (2006); Marotti et al. (2007)
W. confuse	Chun et al., (2007 and 2008); Rekha and Vijyalakshmi. (2010)

2.6 Yogurt

The Australian standards define low-fat yogurt as 'the yogurt prepared by culturing skim or low-fat cow's milk, resulting in a thickened, tangy yogurt and does not contain fruit or flavouring. It contains on an average 6.6% protein and 0.3% fat' (Food Standards Australia and New Zealand, 2006). Yogurts are available in a variety of textures, fat contents (low-fat, fat-free) and flavours (natural, fruit, cereal). The low-fat varieties of yogurt provide an array of important nutrients in significant amounts in relation to their energy and fat content, therefore making them a nutrient-dense food (McKinley, 2005).

A starter culture can be defined as ‘a microbial preparation of large number of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process’ (Leroy and De Vuyst, 2004).

2.6.1 The role of probiotics in yogurt

Probiotic foods are ‘food products that contain a living probiotic organism in adequate numbers, so that after their ingestion, the postulated effect is obtained, and is beyond that of usual nutrient supply (Saxelin et al., 2003). These bacteria are added to milk to ferment it into yogurt. Most bacteria with probiotic properties belong to the genera *Lactobacillus* and *Bifidobacterium*, which are common but non-dominant members of the indigenous microbiota of the human gastrointestinal tract (GIT). According to functional foods of Australia and New Zealand’s (2003) are considered to be any food or food component that may provide demonstrated physiological benefits or reduce the risk of chronic diseases, above and beyond basic nutritional functions. Some of the potential health benefits of functional foods containing probiotic bacteria include improved lactose utilization, antagonistic action towards enteric pathogens, colonization in gut, anti-carcinogenic and hypocholesterolemic effect, immune modulation, prevention of allergy and prevention of inflammatory bowel disease (Marteau, 2001; Gorbach, 2002). *Lactobacillus acidophilus*, *L. casei*, *L. paracasei* and *Bifidobacterium* species are predominantly used in yogurt (Holzapfel et al., 2001). Manufacturers of therapeutic fermented milk products commonly use five species of *Bifidobacterium* including *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* (Arunachalam, 1999).

Lactobacilli and *bifidobacteria* are the two of the main types of bacteria that reside in the small intestine. Some commercial strains of these groups of bacteria are used as probiotics. Both bacterial groups may play a role including both local and systemic immunity of the

intestine. Aside from providing a gut defense barrier, some strains of these two types of bacteria may secrete antimicrobial substrates to inhibit the growth of pathogens (Saavedra, 2007). Intestinal bacteria are also shown to improve the secretory immune function and the intestinal flora such as influencing secretory immunoglobulin A (sIgA) synthesis and sIgA precursors. sIgA is an immunoglobulin that is present in mucosal surfaces. Its function is mainly to protect against the attack of antigens, pathogens, toxins and viruses (Saavedra, 2007). Sazawal et al. (2006) showed that high bacteroid and *Clostridium* counts accompanied by low *Bifidobacterium* counts can lead to altered immune responses in infants. Premature infants are expected to have intestines that contain non-*bifidobacterium* species because of their frequent exposure to microbial environments and antibiotic use.

Some probiotic strains are sufficiently proteolytic to grow remarkably in milk, but others need growth stimulants. Mostly, *Bifidobacterium* species cannot ferment milk by themselves because they require low redox potential and the peptides generated from the breakdown of casein. Moreover, when co-cultured with lactobacilli, they become inhibited as the pH drops (Klaver et al., 1993). Several factors such as strain characteristics, food matrix, temperature, pH and accompanying bacteria affect the viability of probiotics (Fonden et al., 2000). Around 30% of the global population buy probiotic dairy products on a regular basis, representing a major part of the AUD 84.15bn global functional foods market, of which the US, Western Europe and Japan account for over 70% (Shah, 2007). In 2008, the global probiotics market (both food stuffs and supplements) was worth over AUD 15.54bn, or over 18% of the global functional foods market. Since 2003, the global probiotics market has more than doubled in value and is currently rising by almost 15% per annum. Although probiotics remain best suited to dairy products such as spoonable and drinking yogurts, probiotic products have emerged in products like breakfast cereals, infant formula and soft drinks. A list of probiotic yogurts and fermented milks that are commercially produced is presented in Table 2.6.

Table 2. 6: Probiotics dairy products available in the world market

Product	Country of origin	Organisms
Activia	USA	*B. animalis* DN173010
Activia (drinkable)	USA	*B. animalis* DN173010
Activia (stirred)	USA	*B. animalis* DN173010
Actimel	France	*L. casei defensis*
AB-yogurt	Denmark	*L. acidophilus, B. bifidum*
ACO- yogurt	Switzerland	*S. thermophilus, L. bulgaricus*
Batavito	Brazil	*L. casei*
Bifigurt	Germany	*B. longum, S. thermophilus*
Biograde	Germany	*L. acidophilus, B. bifidum*
Biokys	Slovakia	*B. bifidum, L. acidophilus*
Bulla Thick and Fruit	Australia	*L. acidophilus, B. bifidum*
Chamyto	Brazil	*L. johnsonii, L. helveticus*
Cultura-AB	Denmark	*L. acidophilus, B. bifidum*
LITE	Australia	*L. casei, L. acidophilus, B. bifidus*
Miru Miru	Japan	*L. acidophilus, L. casei*
No Fat Tamara	Australia	*L. casei, L. acidophilus, B. bifidus*
Ski	Australia	*L. acidophilus, B. bifidum*
Sofyl	Japan	*L. casei shirota*
Vaalia yogurt	Australia	*B. bifidum, L. acidophilus*

Source: Compiled from Vierhile (2006); Alhaj et al. (2007).

2.6.2 Characteristics of probiotic organisms

A synbiotic product containing the probiotic bacteria and prebiotics in a single food can

improve the survival of bifidobacteria during storage of the product and passage through the gastro intestinal tract (GIT) and also reduces the competition with microorganisms in the GIT. The combined use of two or more probiotic species is common in commercial probiotic foods, as these strains are believed to act synergistically on each other. Thus, the trend is to use yogurt bacteria as the main starter culture and probiotic bacteria as an adjunct culture (Lerayer, 2005).

The probiotic organisms (*L. acidophilus*, *Bifidobacterium* ssp. and *L. casei*) are typically characterized as Gram-positive, non-spore-forming, non-motile, catalase-negative bacteria that grow under anaerobic conditions. These strains are commonly used in many dairy products and easily available in the markets.

i) ***Lactobacillus acidophilus*** (LA) is a homo-fermentative species, fermenting lactose into lactic acid, which grows readily at low pH values (below pH 5.0) and has an optimum growth temperature of 37 °C. It is found primarily in the small intestine where it produces natural antibiotics called “lactocidin” and “acidophilin”. These bacteria increase our immune system against harmful *Candida albicans, Salmonella, E. coli*, and *Staphylococcus aureus* by attracting to the intestinal walls, as well as on the lining of the vagina, cervix, and urethra, thereby preventing other organisms from multiplying to the extent that they can cause infections (Ljungh and Wadstrom, 2006). This strain is commercially used in many dairy products, together with ST and LB in the production of acidophilus-type yogurt. Several strains of LA have been studied extensively for health effects. The LA is able to survive gastrointenstinal transit, since it is resistant to bile and low pH as well as digestive enzymes (Yuan-Kun, 2009).

ii) Lactobacillus casei (LC). This strain is acid tolerant, cannot synthesize porphyrins, and possess a strictly fermentative metabolism with lactic acid as the major metabolic end

product (Axelsson, 1998). These cells are rods of 0.7-1.1 × 2.0-4.0 μm, often with square ends, which tend to form chains. Bergey's Manual of Systematic Bacteriology recognizes four subspecies: *casei*, *pseudoplantarum*, *rhamnosus* and *tolerans*. The latest grouping of lactobacilli based on chemical-physiological criteria includes *L. casei* in the facultatively heterofermentative group. Hexoses are almost entirely converted into lactic acid via EMP pathway and pentoses are used by induced phosphoketolase, to produce lactic acid and acetic acid (Gobbetti, 2000).

iii) Bifidobacterium is a genus of Gram-positive, non-motile, often branched anaerobic bacteria. They are abundant, endo-symbiotic inhabitants of the gastrointestinal tract and the mouth of mammals and other animals (Sonomoto and Yokota, 2011). Bifidobacteria are one of the major groups of bacteria that make up the colonic flora in mammals. Bifidobacterium strains are considered as important probiotics used in the food industry. Different species and/or strains of Bifidobacteria may exert a range of beneficial health effects, including the regulation of intestinal microbial homeostasis, the inhibition of pathogens and harmful bacteria that colonize and/or infect the gut mucosa, the production of vitamins, and the bioconversion of a number of dietary compounds into bioactive molecules (Sonomoto and Yokota, 2011).

Bergey's Manual of Systematic Bacteriology (2001) identifies 24 species of *Bifidobacterium*, of which the types considered primarily from human origin are the species: *bifidum*, *longum*, *infantis*, *breve*, *adolescentis*, *angulatum*, *catenulatum*, *pseudocatenulatum* and *dentium*. They metabolize glucose exclusively by heterolactic fermentation by the fructose-6-phosphate shunt also know as bifid shunt, to form L(+) lactic acid and acetic acid in the molar ratio of 2:3 (Holt et al., 1994; Arunachalam, 1999; Gomes and Malcata, 1999; Hoover, 2000). Besides glucose, all bifidobacteria from human origin are also able to utilize

galactose, lactose and fructose as carbon sources. A proton symport has been identified as the lactose transport system for *B. bifidum* (Krzewinski et al., 1996). In some instances they are also able to ferment complex carbohydrates as reported by Crociani et al. (1994).

2.6.3 Characteristics of yogurt bacteria

i) ***Streptococcus thermophilus*** (ST) is a Gram-positive bacteria and a homo-fermentative facultative anaerobe (Courtin and Rul, 2003). This organism is one of the most widely used bacteria in the dairy industry. It grows best at 37 °C, is heterotrophic and generally fastidious, requiring simple carbohydrates as energy source and preformed amino acids as a nitrogen source. It ferments lactose homofermentative to give L (+) lactic acid as the principal product. Lactose is actively transported across the cell membrane of *S. thermophilus* by means of a membrane located enzyme galactoside permease. Inside the cell, the enzyme β-gal hydrolyses the lactose to glucose and galactose.

The glucose is metabolized to pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway (Robinson, 2000) and lactic dehydrogenase converts the pyruvate to lactic acid. In most strains of *S. thermophilus*, the galactose and lactic acid produced leave the cell and accumulate in the medium, but some strains possess a galactokinase, that converts the galactose to galactose-1-phosphate which is converted via the Leloir pathway to glucose-1-phosphate, which is further metabolized via the EMP pathway (Robinson, 2000; Zirnstein and Hutkins, 2000). The ST also produces exopolysaccharides that are essential to the texture of fermented reduced-fat dairy products to maintain similar characteristics to their full-fat counterparts (Amatyakul et al., 2006).

***ii) Lactobacillus delbrueckii* ssp. *bulgaricus* (LB):** *Lactobacillus delbrueckii* ssp. *bulgaricus* is also Gram-positive, but occurs in milk as chains of 3 to 4 short rods, each 0.5-0.8 × 2.0-9.0

μm, with rounded ends. The optimum growth temperature is 45 °C. Its basic metabolism is homofermentative, to give (D-) lactic acid at a level of 1.7-2.1% in milk. It converts hexoses into lactic acid via the EMP pathway (Teixeira, 2000). Although lactic acid is the major end product of fermentation, secondary end products such as acetaldehyde, acetone, acetoin and diacetyl can also be produced in very low concentrations. Like ST, LB can utilize lactose, fructose and glucose, and some strains can utilize galactose (Robinson, 2000).

The lactobacilli include over 25 unique species, and the first level of differentiation is based on end product composition: homofermentators being organisms that produce more than 85% lactic acid as their end product from glucose (e.g. *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*), and heterofermentators that produce approximately 50% lactic acid as the end product plus considerable amounts of carbon dioxide gas, acetate and ethanol (e.g. *L. brevis*, *L. casei*). Although they all produce lactic acid as a major end product they differ in the isomeric composition of lactic acid produced.

The starter culture used in yoghurt making process is usually a mixture of LB and ST. It is known for a long time that more rapid acid development is observed in mixed cultures of LB and ST than in the single strain cultures due to a symbiotic relationship between the two species of bacteria (Rasic and Kurmann, 1978). Tamime and Deeth. (1980) observed more rapid acid development in mixed cultures than in the single strain cultures due to the increase in number of streptococci. They also demonstrated active growth of ST in milk containing LB milk filtrate and concluded that LB provided essential growth requirements for stimulation of the ST. In symbiotic culture in milk, certain amino acids produced by LB were identified as valine, histidine, methionine, glutamic acid and leucine. Omission of the single amino acids from the mixture showed that valine was the most effective in stimulating acid production by ST (Higashio et al., 1977). A list of starter organisms used in fermented dairy products is presented in table 2.7.

Table 2. 7: Starter organisms used in fermented dairy products

Species[1]	Growth temperature		Lactic acid fermentation	
	Min (˚ C)	Opt (˚ C)	Lactic acid (%)	pH
Bifidobacterium (*bifidum, infantis*, etc)	22	37	0.1-0.4	4.5
Lb. acidophilus	27	37	0.3-1.9	4.2
Lb. brevis	8	30	1.2-1.5	-
Lb. casei ssp. *Lactis*	8	30	1.2-1.5	-
Lb. delbrueckii ssp. *Bulgaricus*	22	45	1.5-1.8	3.8
Lb. delbrueckii ssp. *Lactis*	18	40	1.5-1.8	3.8
Lb. helveticus	22	42	1.5-2.2	3.8
Lb. kefir	8	22	1.2-1.5	-
Lb. lactis ssp. *Cremoris*	8	22	0.5-0.7	4.6
Lb. lactis ssp. *Lactis*	8	30	0.5-0.7	4.6
Lb. lactis ssp. *lactis* biovar. *Diacetylactis*	8	22-28	0.5-0.7	4.6
Ln. mesenteroides ssp. *Cremoris*	4	20-28	0.1-0.2	5.6
Ln. mesenteroides ssp. *dextranicum*	4	20-28	0.1-0.2	5.6
S. thermophiles	22	40	0.6-0.8	4.5

Source: Knut (2001).

Soy yoghurt

Worsley et al. (2002) reported that twenty one per cent of Australian population consume soy based products such as soy bread, soy-based infant formula, soy-cheese and soy yoghurt. Among the soy food products, soy yogurt has received a lot of attention because of its health benefits. In addition to the high quality protein in soy yogurt, it also contains a considerable amount of isoflavone compounds, which have been known as natural substances to replenish

the female hormone oestrogen in order to relieve the menopausal symptoms (Hughes et al., 2003). Soy yogurt is made using soy milk, adding yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*), and sometimes adding sweeteners such as fructose, glucose, or sugar. The quality of soy yoghurt can be assessed through its chemical, microbiological and physical properties. In general, the chemical and microbiological parameters are controlled under food legislation of each country. For example, the Australian Food Standard Code (Standard 2.5.3, 2004) requires that the viable counts of yoghurt starter cultures be no less than 10^6 cfu/g of a product throughout the storage period.

2.7 Prebiotics

Prebiotics are non-digestible food ingredients that stimulate the growth and activity of bacteria in the digestive system. Prebiotic foods are food products that contain a prebiotic ingredient in adequate concentration, so that after their ingestion, the effect is obtained better than that of usual nutrient. Prebiotics essentially constitute non-digestible oligosaccharides which stimulate the growth of bifidobacteria (Roberfroid et al., 1998).

Prebiotic oligosaccharides are increasingly added to foods for health benefits. These include fructooligosaccharides (FOS), xylooligosaccharides (XOS), polydextrose and galactooligosaccharides (GOS). Few monosaccharides such as tagatose are also used sometimes as prebiotics. These oligosaccharides are absorbed in the small intestine. The most important prebiotics are β-glucans, fructans and mannans. Among the fructans, inulin and oligofructoses are more commonly used (Oliveira et al., 2009a).

Traditional dietary sources of prebiotics are soybeans, Jerusalem artichoke, jicama, chicory root, oats, un-refined wheat, un-refined barley and yacon. Some of the oligosaccharides that naturally occur in breast milk are believed to play an important role in the development of a healthy immune system in infants (Geier, 2006).

Roberfroid (2000); Williams and Jackson (2002) have focused on the difference between short-chain, long-chain, and full spectrum prebiotics. "Short-chain" prebiotics, e.g. oligofructose, contain 2-8 links per molecule, are typically fermented more quickly in the colon providing nourishment to the bacteria in that area. Longer-chain prebiotics, e.g. inulin, contain 9-64 links per molecule, and tend to be fermented more slowly, nourishing bacteria predominantly in the distal colon. Full-spectrum prebiotics provide the full range of molecular link-lengths from 2-64 links per molecule, and nourish bacteria throughout the colon, e.g. Oligofructose-Enriched Inulin (OEI) (Brigitta et al., 2001; Angelo et al., 2002). The majority of research works have concentrating on full-spectrum prebiotics, typically using OEI as the research material (Yoram et al., 1999; Brigitta et al., 2001; Hughes and Rowland, 2001; Angelo et al., 2002). Munjal et al. (2009) have shown the role of prebiotics in preventing and possibly stopping early stage colon cancer. It has been argued that many of these health effects derive from increased production of short-chain fatty acids (SCFA) by the stimulated beneficial bacteria.

2.7.1 Use of prebiotics in yogurt (Synbiotic yogurt)

2.7.1.1 Galactooligosaccharides (GOS)

The GOS are composed of galactose monomers linked together in a number of different structural arrangements. They usually consist of a number of β (1→6) linked galactopyranosyl units linked to a terminal glucopyranosyl residue via glycosidic bond. It has been found to selectively stimulate the growth and metabolic activity of beneficial bacteria in the colon (Wang and Gibson, 1993; Roberfroid et al., 1998; Gibson and Wang, 1994a). Wang and Gibson (1993) found that preparing yogurt with prebiotics including GOS resulted in improved textural attributes and water holding capacity. Guven et al. (2005) reported the GOSs are fermented by gut bacteria with decrease in pH (Wang and Gibson,

1993). According to Crittenden and Playne (1996), it is used in dairy-based gelling systems to modify the flow and texture properties of the final gel. The increase in viscosity due to the addition of GOS has been attributed to interactions between these oligosaccharides and dairy proteins and their water holding capacity (Fernandez-Garcia et al., 1998).

2.7.1.2 Modified waxy maize starch (MWMS)

Modified starch is a food additive that is prepared by treating native starch or starch granules enzymatically or chemically causing the starch to be partially degraded (Miyazaki et al., 2006). The purpose of the modifications is to enhance their properties particularly in specific applications such as to improve the water holding capacity, heat resistant behavior, binding capacity, minimizing the syneresis and improved thickening (Miyazaki et al., 2006). It is widely used for low-fat butter spread or margarine, low-fat mayonnaise, low-fat milk type products and low-fat ice cream (Sajilata and Singhal, 2004). Sprague (1939) reported the WWMS is used for improvement of smoothness, uniformity, stability and texture of dairy products. These starches are considered to be non-gelling starches and typically have a cohesive and gummy texture. Williams et al. (2004) reported that the addition of MWMS to yoghurt decreased the syneresis but developed a grainy texture. Incorporation of MWMS in milk has been used to increase growth of the probiotic organisms (Nielsen et al., 1991). Williams et al. (2004) reported that when modified starch is used (1% w/w), the viscosity of yogurt increased and the product developed a grainy texture. The increase in viscosity supports the earlier work by Lelievre and Husbands (1989) who also demonstrated increased viscosity of gelatinised maize starch and corn starch on the addition of caseinate.

2.7.1.3 Inulin

Inulin and oligofructose are used in food products especially to improve yogurt texture and

bioavailability of a variety of minerals such as calcium and iron and enhancing immune functions. Inulin is produced by many types of plants as a means of storing energy and is typically found in roots or rhizomes (Roberfroid, 2005). They belong to a class of fibers known as fructans. Inulin is a carbohydrate-derived dietary fibre, has a gelling capacity with water and is a functional food additive (Obrein et al., 2003).

2.7.1.4 β-glucan

The β-glucan is a polymer of D-glucose linked with glycosidic bonds at β (1→3), β (1→4), and β (1→6), and is typically found in the endosperm cell wall in oats and barley. It is commercially derived from oats, barley, mushrooms and some microorganisms. In microorganisms and mushrooms, these compounds are found to have a linear chain of D-glucose linked in the β (1→3) position with various sized D-glucose branches linked to the main chain by β (1→6) linkages. The β-glucan derived from cereals is a polymer of D-glucose with β (1→3) and β (1→4) linkages. It constitutes 1 % of wheat grains, 3-7% of oats and 5-11% of barley (Skendi et al., 2003).

The use of the β-glucan in health based food supplements is growing, not only because of its health benefits, but also because of its use as a processing aid. In cheese making, β-glucan affects the curd formation by reducing the curd cutting time and increasing curd yield (Tudorica et al., 2002). The β-glucan has been used in the manufacture of low-fat yogurt and ice cream to act as a fat mimetic by improving the mouth feels (Brennan and Cleary, 2005). It can be used as a structurally functional additive due to its thickening, stabilizing, and gelling properties (Burkus and Temelli, 1998). Skendi et al. (2003) also reported that β-glucan has a unique high viscosity that can provide desirable textural properties to yogurt.

With reference to further thesis content, chapter 3.0 conatains extraction and characterisation of β-galactosidase produced by *Bifidobacterium animalis* ssp. *lactis* Bb12

and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in Whey.

Chapter 3.0 Extraction and Characterisation of β-Galactosidase Produced by *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in Whey[1]

[1] A version of this chapter has been accepted in International Food Research Journal (Reference # 2012-119). Prasad, L. N., B. C. Ghosh, F. Sherkat, and N. P. Shah. 2012. Extraction and Characterisation of β-Galactosidase Produced by *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in Whey.

3.1 Introduction

β-Gal catalyzes the hydrolysis of lactose to glucose and galactose. This enzyme is used to hydrolyse milk lactose to combat the problems of lactose intolerance by individuals who are deficient in lactase (Artolozaga et al., 1998). Commercial β-gal is produced from bacteria (such as *Streptococcus thermophilus* and *Lactobaccillus lactis*); yeasts (such as *Kluyveromyces lactis* and *Kluyveromyces marxianus*) and moulds (such as *Aspergillus niger*, *Aspergillus candidus* and *Aspergillus oryzae* (Panesar et al., 2006; Zheng et al., 2006). The use of whole cells as a source of β-gal may appear as a good alternative, however, a major drawback is the poor permeability of cell wall membrane. Since β-gal is an intracellular enzyme, one of the major hindrances in effective production of this enzyme is its release in sufficient quantities from cells. Therefore, different methods have been applied to increase the permeability of microbial cell walls (Panesar et al., 2006).

Several workers have reported on the release of β-gal through permeabilization of microbial cells by organic solvents (Flores et al., 1994; Numanoglu and Sungur, 2004; Panesar et al., 2007; Park et al., 2007). Flores et al. (1994) studied the permeabilization of *K. lactis* cells by chloroform, toluene and ethanol to release β-gal enzyme. They found that the effectiveness of solvents was dependent on the incubation time, incubation temperature and concentration of both cells and solvents. Mechanical methods such as sonication, high-pressure homogenizer or glass bead mills have been traditionally used for the disruption of microbial cells (Geciova et al., 2000). The method of choice should be robust enough to disrupt cell membranes efficiently but gentle enough to preserve enzyme activity (Numanoglu and Sungur, 2004).

Sonication is one of the most widely used methods for disruption of the bacterial cell walls (Engler, 1985). Among the three methods, sonication, glass bead milling and high-pressure homogenizer, sonication was found by many workers to be more effective for releasing β-gal

(Toba et al., 1990; Sakakibara et al., 1994). Berger et al. (1995) compared two physical disruption methods for the extraction of intracellular β-gal enzyme from *Thermus* species and found that the sonication was superior to the glass bead milling. In contrast, Bury et al., (2001) studied the disruption of the cells of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and concluded that sonication was the least effective method on the release of β-gal.

Salasbury (1989) reported that lysozyme is often used for lysis of peptidoglycan layers as it catalyses hydrolysis of β 1-4-glycosidic bonds. The enzyme is commercially available at a reasonable cost, and is produced from egg-white preparations. Gram-negative bacteria are less susceptible than the Gram-positive ones as their outer layer made of peptidoglycan, is responsible for rigidity of bacterial cell wall and for determination of cell shape. It is made up of a polysaccharide backbone consisting of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues in equal amounts. However, combined lysozyme-EDTA treatment allows the disruption of the cell wall and subsequent attack on the peptidoglycan structure (Salasbury, 1989). Therefore, lysozyme-EDTA mixture is very efficient for releasing β-gal from Gram-negative bacteria cell walls (Andrews and Asenjo, 1987; Geciova et al., 2000).

Numanoglu and Sungur (2004) compared chemical (toluene, SDS-chloroform) and physical (glass bead mill) methods to facilitate the release of β-gal from *K. lactis* cells and found that the physical methods were better than chemical ones. This was in agreement with Fiedurek and Szczodrak (1994) who used three methods i.e. solvent and detergent extraction, freezing and thawing extraction, and mechanical disintegration to release the β-gal from *K. fragilis* cells and found that the highest yield was obtained by mechanical disintegration.

The lactic acid bacteria (LAB) require numerous growth factors such as whey, reconstituted skim milk (RSM) and MRS broth in addition to carbohydrate and nitrogen

sources in the growth medium (Stiles and Holzapfel, 1997) for the enzyme production. In search for a suitable and inexpensive medium that is readily available, whey appear as an attractive alternative to RSM (Gupta and Gandhi, 1995; Bury et al., 2000). The β-gal activity of a given microorganism depends on the characteristics of the medium. To maximize the enzyme activity, a rich medium is necessary. Therefore, sweet whey appears highly attractive mostly due to its relatively high lactose content which constitutes over 70% of the total solids in whey (Rhimi et al., 2007).

There are two types of whey; i) Sweet whey that is produced during the producing of rennet types or hard cheeses like Cheddar or Swiss cheeses. ii) Acid whey (also known as "sour whey") that is obtained during the production of acid type cheeses such as cottage cheese. Sweet whey is a rich source of whey proteins, lactose, enzymes, vitamins, bioactive compounds and some minerals (Agrawal et al., 1989; Joshi et al., 1989; Keerthana and Reddy, 2006). Many small-size cheese plants do not have proper treatment systems for the disposal of whey and the dumping of whey constitutes a significant loss of potential food as whey retains about 40-45% of total milk solids (Panesar et al., 2007). Its disposal as waste poses serious pollution problems for the surrounding environment (Carrara and Rubiolo, 1994; Dagbagli and Goksungur, 2008; Magalhaes et al., 2010a). Sweet syrup produced through lactose hydrolysis by β-gal can be used in dairy, confectionary, baking and soft drink industries (Mahoney, 1997; Rajakala and Selvi, 2006). Other applications of β-gal on whey could also include the production of biologically-active galacto-oligosaccharides from lactose hydrolysis (Boon et al., 2000; Albayrak and Yang, 2002).

The *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was selected based on previous evidence as a high β-gal producer (Vasiljevic and Jelen, 2003). The *B. animalis* ssp. *lactis* Bb12 was found to possess the highest level of β-gal activity compared to others Bifidobacteria (Dechter and Hoover, 1998). Therefore, the present study was undertaken to evaluate the

suitability of sweet whey as a medium for the production of β-gal from *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This study also evaluated physical and chemical methods of enzyme extraction from bacteria in terms of their efficacy and enzyme yield.

3.2 Materials and Methods

3.2.1 Micro-organisms

Pure culture of *B. animalis* Bb12 was obtained from Chr. Hansen, (Bayswater, VIC, Australia) and *L. delbrueckii* ssp. *bulgaricus* ATCC was obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80 ºC in 50/50 sterile MRS broth (Difco, Becton, Dickinson and Company, New Jersey, USA) and glycerol (MERCK Pty Ltd, Colchester Road, Kilsyth, Australia.

3.2.2 Culture growth conditions

The organisms were activated in two successive transfers in MRS broth supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) and incubated at 37 ºC for *B. animalis* ssp. *lactis* Bb12, and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. Activated organisms were grown in deproteinized sweet whey supplemented with yeast extract (3.0 g/L), peptone (5 g/L) and glucose (10 g/L). The sweet whey was deproteinized by heating at 85 ºC for 10 min after adjusting the pH to 4.5 using lactic acid. The heat-treated whey was cooled to room temperature and filtered through Whatman no. 1 filter paper. The pH of whey medium was then re-adjusted to 7.0 and sterilized at 121 ºC for 15 min then cooled to 30 ºC and inoculated aseptically with 1% of each organism and incubated at 37 ºC for *B. animalis* ssp. *lactis* Bb12 or 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h under anaerobic conditions.

3.2.3 Enzyme extraction

After 18 h of incubation, the cells were harvested by centrifuging at 10,000×*g* for 10 min at 4 °C. The supernatant was considered to be containing extracellular enzymes. The cell pellet was crushed and washed twice with a 0.03 M sodium phosphate buffer (pH 6.8) and centrifuged at 10,000×*g* for 10 min at 4 °C. The washed pellets were resuspended in 5 mL of 0.2 M phosphate buffer (pH 6.8) for intracellular enzyme extraction using four different cell disintegration methods listed below. The measure for efficacy of the extraction method is the yield of enzyme expressed in its units of activity under defined condition.

Sonication: The cell suspensions were sonicated for 30 min in ice bath using Sonirep 150 MSE (MSE Instruments, Crawley, UK) sonicator according to the method of Beccerra et al. (1998). The extract was then centrifuged at 15,000×*g* and 4 °C for 10 min and the supernatant containing the crude enzyme was stored at –20°C until used for enzyme assays. Ideally sample should be kept below 4°C. To maintain this temperature in the tank during sonication crushed ice was added to water bath. At the end of a typical sonication cycle (10 min., 30sec "on", 30 sec "off"), the temperature in the water bath should not exceed 0°C, which was confirmed by a thermometer.

Lysozyme-EDTA treatment: Lysozyme solution was prepared by dissolving 50 mg of lysozyme (Sigma Aldrich Pty Lim, Castle Hill NSW, Australia) in 1.5 mL of TE (Tris-EDTA; Ethylenediamine Tetra-acetic Acid) buffer containing 1 mM EDTA and 10 mM Tris-HCl, adjusted to pH 8.0. The lysozyme preparation was added to the cell suspension at the rate of 75 µL per mL, incubated for 30 min at room temperature then kept at -20 ^{0}C until enzyme activity measurement.

Toluene-acetone treatment: Ten millilitre of cell suspension was ground for 10 min in a pestle and mortar with 2.0 g alumina (Sigma Aldrich Pty Lim, Castle Hill NSW, Australia) and 0.1 mL of 9:1 mixture of toluene (BDH Chemical, Pty Limited, Kilsyth, Vic, Australia

with 99.5% purity) and acetone (Merck Pty Limited Kilsyth, Vic, Australia with 99% purity) solvents. The suspension was extended in 8 mL phosphate buffer and centrifuged at 15,000×*g* for 10 min at 4 °C (Mahoney *et al.,* 1975). The supernatant obtained was kept at -20^{0}C until used for enzyme assay.

S**odium Dodecyl Sulfate** (**SDS)-Chloroform treatment:** Permeabilization of cell membrane was carried out by vortexing 10 mL of the cell suspension in the presence of 100 µL chloroform and 50 µL 0.1% SDS solution for 30 min at room temperature (Mahoney et al., 1975). The suspension was centrifuged at 15,000×g for 10 min at 4 °C and the supernatant was kept at -20^{0}C until needed for the enzyme assay.

3.2.4 Enzyme assay

The β-Gal was determined as described by Hsu et al. (2005). The reaction mixture composed of 0.5 mL of supernatant containing extracted enzyme and 0.5 mL of 15 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After incubation for 10 min at 37 °C, 2.0 mL of 0.1 M sodium carbonate was added to the mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Helios R, Unicam Co., Cambridge, UK). One unit of β-gal was defined as the amount of enzyme that produced one micro-mol (µM) of *o*-nitrophenol per min under the assay condition (Vasiljevic and Jelen, 2001).

3.2.5 Effect of pH and temperature on β-Gal activity

The intracellular β-gal extracted by the above four methods were characterised for their optimum activity by incubating the enzyme in substrate of 15 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) adjusted at three levels of assay pH (4.5, 5.5 and 6.8) with 2N NaOH, or 3N HCL in 0.03 M sodium phosphate buffer for 10 min at 37 ºC. Similarly, the effect of temperature on enzyme activity was studied by incubating the enzyme in above

mentioned substrate at various temperatures (30, 35, 40, 45 and 50 ºC) for 10 min at pH 6.8.

3.2.6 Statistical analysis

All analyses were performed in triplicate and data were analyzed using Statistical Analysis System (SAS) software (SAS 1995) and one-way analysis of variance (ANOVA) at 5% confidence level. ANOVA data with a $P < 0.05$ were classified as statistically significant.

3.3 Results and Discussion

3.3.1 β-Gal production in whey and its extraction

The activity of β-gal from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and its extraction using various methods is shown in Table 3.1.

L. delbrueckii ssp. *bulgaricus* ATCC 11842 produced more ($p<0.05$) intracellular β-gal than *B. animalis* ssp. *lactis* Bb12 with all extraction methods, except sonication. There were significant ($p<0.05$) differences in β-gal levels extracted from each organism by the four extraction methods. Sonication method was found to be more effective for *B. animalis* Bb12 than the others methods, however, lysozyme-EDTA treatment was found to be more effective for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The maximum intracellular β-gal activity (7.77 Unit/mL) was obtained from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 by lysozyme treatment while the lowest activity (2.05 Unit/mL) was measured using toluene-acetone treatment. Similarly, this method resulted in the lowest activity (0.64 Unit/mL) from *B. animalis* Bb12 while the highest β-gal activity (6.80 Unit/mL) was obtained by sonication. However, lower intracellular β-gal activities (4.85 Unit/mL) and (1.58 Unit/mL) were obtained from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *B. animalis* Bb12, respectively by SDS-chloroform treatment. Toluene-acetone treatment was not as effective as the SDS-chloroform method. SDS is a non-ionic detergent which works by disrupting non-covalent bonds in proteins, thereby denaturing them, causing the molecules to lose their native shape (Panesar et al., 2006). Chloroform is also a common solvent because it is

relatively unreactive, miscible with most organic solvents, and conveniently volatile. It is an effective solvent for alkaloids in their base form and thus plant materials are commonly extracted with chloroform for pharmaceutical processing. Thus the action of SDS-chloroform mixture could be of synergistic nature resulting in efficient permeabilization of cell wall of yeast cells and subsequent release of the enzyme (Panesar et al., 2006).

Table 3. 1: Effects of extraction methods on intracellular β-gal activity from B. animalis ssp. lactis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842 grown in whey for 18 h at 37 ºC

Methods	**Lb ATCC 11842**	**Bb12**
	(Unit/mL)	**(Unit/mL)**
Sonication	3.09 ± 0.34^{Cb}	6.80 ± 0.35^{Aa}
Toluene-Acetone	2.05 ± 0.35^{Aa}	0.64 ± 0.06^{Db}
SDS-Chloroform	4.85 ± 1.14^{Ba}	1.58 ± 0.15^{Cb}
Lysozyme treatment	7.77 ± 2.78^{Aa}	3.96 ± 1.05^{Bb}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05).

Our findings agree with those of Berger et al. (1995) who found that sonication was more effective than high-pressure homogenization, glass bead milling and toluene-acetone treatments for the release of β-gal from *Thermus* species. However, our results are contrary to the finding by Bury et al. (2001) who concluded that sonication was the least effective method on the release of β-gal from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In our study, sonication method was found to be more effective for *B. animalis* Bb12, while lysozyme-EDTA treatment was more effective for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

3.3.2 Effect of pH on the activity of intracellular enzyme extracted from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

The optimum activity of the intracellular β-gal from *B. animalis* ssp. *lactis* Bb12 as extracted by four different methods and various assay pH levels (4.5, 5.5 and 6.8) is shown in Figure 3.1.

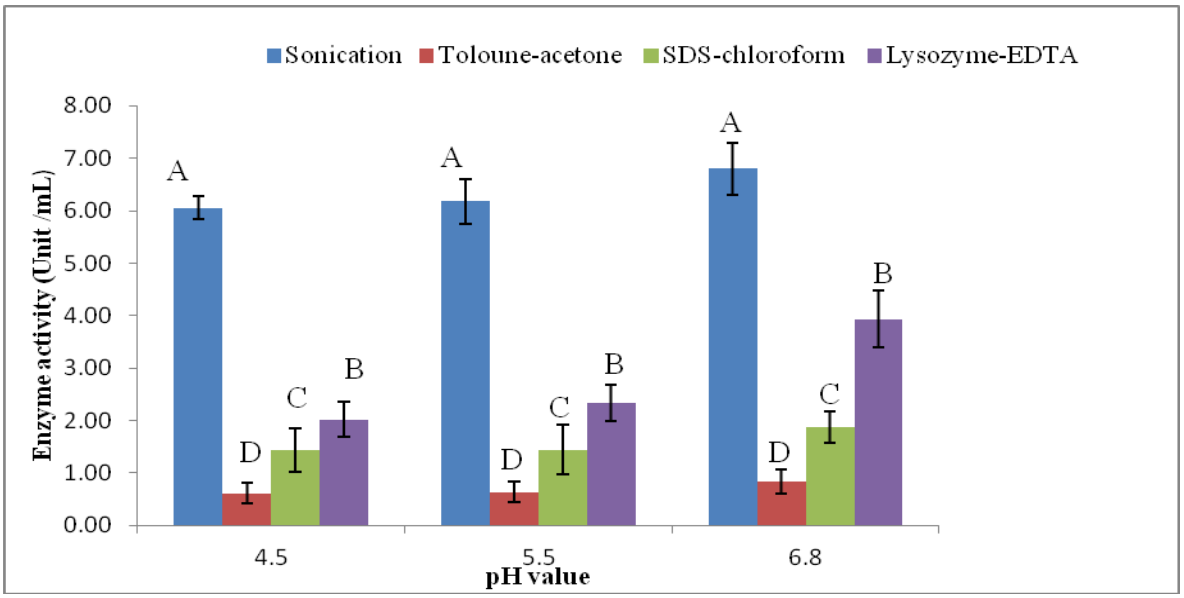

Figure 3. 1: Effect of medium pH at 37 ºC on the activity of intracellular β-gal enzyme extracted by four extraction methods from B. animalis ssp. lactis Bb12. Different capital letters between treatments (bar) at same pH are significantly different (P < 0.05).

The pH 6.8 was selected based on previous evidence as a high β-gal enzyme activity (Hsu et al., 2007). Among the four extraction methods employed for *B. animalis* ssp. *lactis* Bb12, sonication resulted in significantly ($p<0.05$) higher enzyme activity followed by lysozyme-EDTA treatment at pH 6.8. Enzyme from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 also showed (Figure 3.2) its maximum activity at pH 6.8 where lysozyme and SDS-chloroform treatments extracted more ($p<0.05$) enzyme than the other two methods. The enzyme activity at pH 6.8 was significantly higher ($p<0.05$) than at other pH levels for both organisms. Any

drop in pH value of assay medium resulted in a reduction on β-gal enzyme activity.

The maximum enzyme activity (7.77 Unit/mL) was obtained when *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was treated with lysozyme-EDTA mixture (Figure 3.2). Lower enzyme activities were found when SDS-chloroform (4.85 Unit/mL), sonication (3.09 Unit/mL) and toluene-acetone (2.05 Unit/mL) were employed. Therefore, our results revealed that at pH 6.8, β-gal activity was at its peak for both organisms.

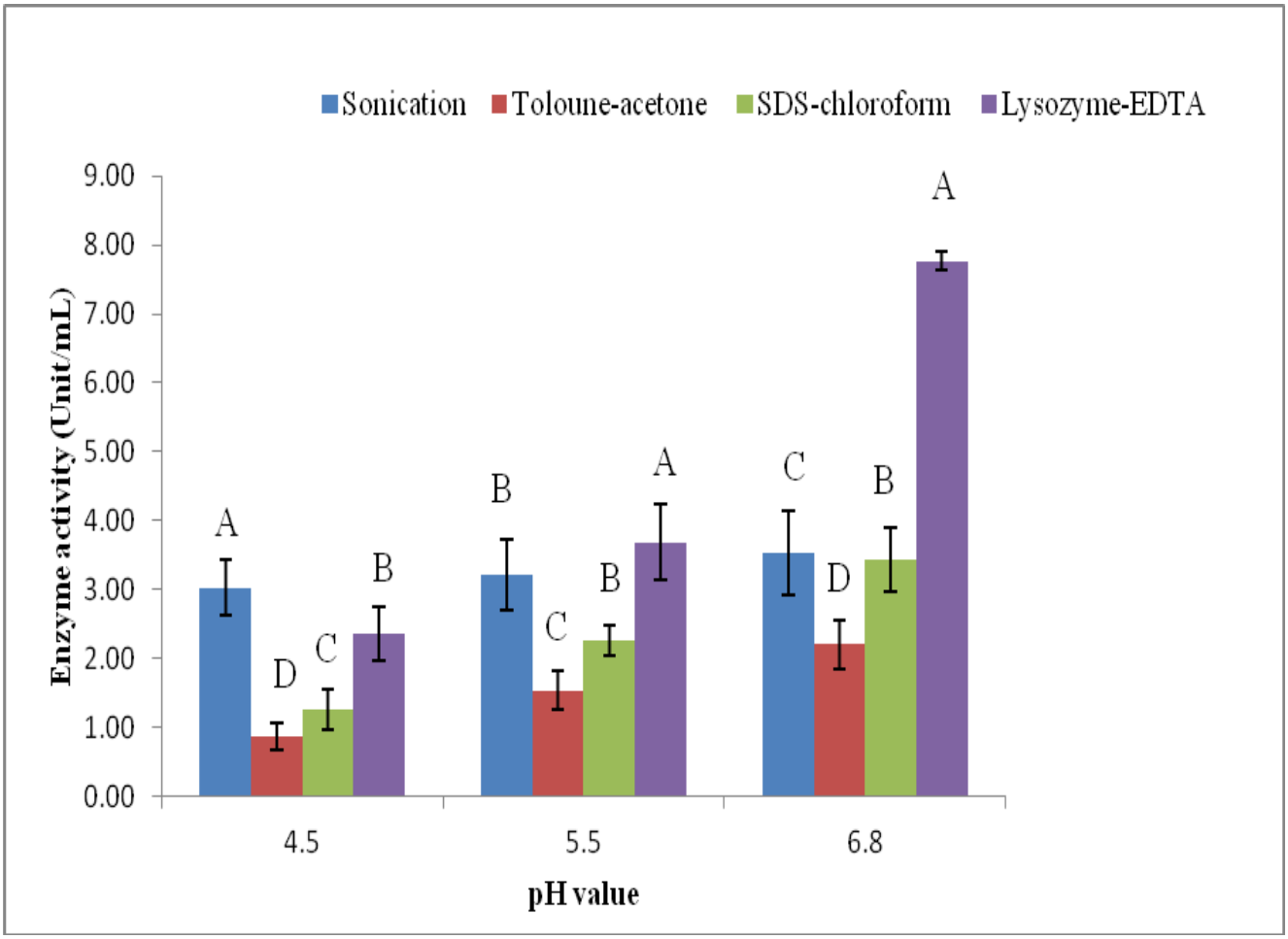

Figure 3. 2: Effect of medium pH at 37 ºC on the activity of intracellular β-gal enzyme extracted by four extraction methods from L. delbrueckii ssp. bulgaricus ATCC 11842. Different capital letters between treatments (bar) at same pH are significantly different (P < 0.05)

These findings agree with those of Greenberg and Mahoney (1982) and Nagy et al. (2001) who reported that β-gal enzyme activity was higher at pH 6.5 to 7.5 at 37˚C from *B. animalis,*

but it appeared to have detrimental effect as enzyme rapidly loose its activity outside this range. Various workers reported that β-gal activity was affected by metallic ions (Hung and Lee, 2000 and Kim et al., 2003). Moreover, Wang et al. (2004) also reported that the highest enzyme activity was observed in the pH range of 6.7 to 7.5.

Our results showed that among the four extraction methods employed for *B. animalis* ssp. *lactis* Bb12, sonication resulted in significantly ($p<0.05$) higher enzyme activity followed by lysozyme-EDTA treatment at pH 6.8. Enzyme from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 also showed (Figure 3.2) its maximum activity at pH 6.8 where lysozyme and SDS-chloroform treatments extracted more ($p<0.05$) enzyme than the other two methods. The enzyme activity at pH 6.8 was significantly higher ($p<0.05$) than at other pH levels for both organisms. In a study performed by Kreft and Jelen (2000), the optimum pH for β-gal from *Streptococcus thermophilus* was found to be at pH 7.0. Enzymes from *Lactobacillus bulgaricus* and *Streptococcus thermophilus* would appear to be stable over a wider pH range whose stability decreased sharply below pH 6.0 and more gradually above 8.0 (Greenberg and Mahaney 1982). Shah and Jelen (1991) found that optimum temperature of β-gal from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 as 45-50 °C, Similarly, maximum activity for β-gal from *Streptococcus thermophilus* was determined at 55 °C (Greenberg and Mahoney 1982). It has been reported that the optimal temperature and pH of the β-gal produced by *K. lactis* ATCC 8583 were 37 °C and 7.0, respectively (Zhang et al., 2006) while the optimal temperature and pH of the β-gal produced by *K. fragilis* were 37 °C and 6.6 (Ding et al., 2001). Our results showed that among four extraction methods employed for *B. animalis* ssp. *lactis* Bb12, sonication resulted in significantly ($p<0.05$) higher enzyme activity followed by lysozyme-EDTA treatment at pH 6.8. Enzyme from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 also showed (Figure 3.2) its maximum activity at pH 6.8 where lysozyme and SDS-chloroform treatments extracted more ($p<0.05$) enzyme than the other

two methods. The enzyme activity at pH 6.8 was significantly higher ($p<0.05$) than at other pH levels for both organisms.

Due to low buffering capacity, the pH of a growth medium was lowered when organic acids were added. The inhibitory effect of low pH on the growth was compounded by the presence of organic acids in the medium. First, lowering the pH increases the concentration of un-dissociated acid and thus enhances the inhibitory effect for a given amount of acid (Narendranath et al., 2001). The greater inhibition would be expected as the pH is decreased. It is not clear how much of the growth inhibition observed was due to low pH and how much due to the level of un-dissociated acid. Considering the amount of biomass production in the presence of nutrients supplements, the yeast would have produced large quantities of metabolites to increase the buffering capacity of the medium. Nutrient supplements likely provided some of this buffering before the pH became too low for the organism to grow. This was verified by measuring the buffering capacities of the media (Imai and Ohno, 1995).

3.3.3 Effect of assay temperature on intracellular enzyme activity extracted from *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Based on the maximum enzyme activity results obtained for *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (Table 3.1), only the sonication and lysozyme-EDTA methods were chosen for the study of the effect of temperature on intracellular β-gal enzyme activity (Table 3.2).

The enzyme extracted from each organism was incubated at various temperatures (30, 35, 40, 45 and 50 ºC) for 10 min at pH 6.8. Intracellular β-gal enzyme extracted by sonication and lysozyme-EDTA treatment from *B. animalis* ssp. *lactis* Bb12 showed significantly ($p<0.05$) higher activity at 35°C and 40°C than other temperatures (Table 3.2), whereas, β-gal extracted from *L. delbrueckii* ssp. *bulgaricus* showed its maximum activity ($p<0.05$) at 35 to

45°C (Table 3.2).

There was a significant difference ($p<0.05$) in β-gal activity extracted from *B. animalis* BB12 by sonication method at assay temperatures 30°C, 45°C and 50°C while no such difference was observed at 35°C and 40°C. On the other hand, lysozyme-EDTA treatment showed a significant difference ($p>0.05$) in β-gal activity produced by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at 30°C, 35°C and 40°C but no difference was found at 45°C and 50°C.

Table 3. 2: Effect of assay temperature at pH 6.8 and extraction methods on intracellular β-gal activity extracted from B. animalis ssp. lactis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842

Assay	Lb ATCC 11842		Bb12	
Temp.	Sonication	Lysozyme	Sonication	Lysozyme
	(Unit/mL)	(Unit/mL)	(Unit/mL)	(Unit/mL)
30 ˚C	1.23 ± 0.01^{Ca}	4.29 ± 0.06^{Cb}	3.00 ± 0.05^{Ca}	2.01 ± 0.02^{Cb}
35˚C	2.36 ± 0.02^{Aa}	7.35 ± 0.19^{Ab}	6.68 ± 0.11^{Aa}	2.13 ± 0.05^{Bb}
40˚C	2.22 ± 0.03^{Ab}	7.36 ± 0.13^{Ba}	5.67 ± 0.13^{Aa}	2.27 ± 0.02^{Ab}
45˚C	1.93 ± 0.02^{Bb}	7.45 ± 0.08^{Aa}	3.82 ± 0.05^{Ba}	1.34 ± 0.05^{Db}
50˚C	1.40 ± 0.01^{Cb}	7.25 ± 0.04^{Aa}	2.87 ± 0.04^{Ca}	1.33 ± 0.02^{Db}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row for particular organism and particular temperature with the same lowercase superscripts are not significantly different ($P > 0.05$). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different ($P > 0.05$).

The maximum enzyme activity of 6.68 Unit/mL from *B. animalis* ssp. *lactis* Bb12 was obtained by sonication at 35 ºC whereas the maximum enzyme activity of 7.45 Unit/mL from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was obtained by lysozyme-EDTA treatment at 45 ºC (Table 3.2). Many workers have reported 37 to 45ºC as the optimum temperature range for maximum enzyme activity with different organisms (Tzortzis et al., 2005; Splechtna et al., 2006; Searle et al., 2009). The maximum β-gal enzyme activity from *S. thermophilus* (Somkuti and Steinberg, 1979), *B. infantis* HL96 (Hung and Lee, 2002) and *Penicillium chrysogenum* (Nagy et al., 2001) was obtained at 35-50 ºC.

Our results also revealed that β-gal extracted by sonication and lysozyme-EDTA treatment showed higher activity at temperature range of 35 to 45°C. Further increase in temperature beyond 50°C resulted in a reduction in enzyme activity. Most enzymes denatured rapidly at temperatures above 55˚ C (Bryan and Keith, 1981). Itoh et al. (1992); Cho et al. (2003) have shown that the activity of the enzyme reduced rapidly at or above 50 ºC with no activity detected beyond 60 ºC for 10 min.

3.4 Conclusion

Among the four extraction methods, sonication was found to be more effective for *B. animalis* ssp. lactis Bb12, whereas lysozyme-EDTA treatment was found to be more effective for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The enzyme activity at pH 6.8 was significantly higher ($P<0.05$) than at other pH levels for both organisms. The optimum temperature for the activity of enzyme obtained from *B. animalis* ssp. *lactis* Bb12 was found to be at 35 ºC whereas for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 it was 45 ºC. Deproteinised sweet whey was found to be a suitable medium for β-gal production, it should be possible to produce commercial amounts of β-gal using the two organisms reported in this study, however the enzyme extraction method need to be adapted to the strain used.

Chapter 4.0 Effect of carbon and nitrogen sources on growth of *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-galactosidase under different culture conditions[2]

[2] A version of this chapter has been published. Prasad, L. N., M. M. Ayyash, and N. P. Shah. 2011. Effect of carbon and nitrogen sources on growth of *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-galactosidase under different culture conditions. International Food Research Journal 18: 373-380

4.1 Introduction

In the manufacture of dairy products, β-galactosidase (β-gal) has been extensively used to hydrolyse lactose into glucose and galactose (Mahoney, 1998). *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 is particularly a promising microorganism for production of β-gal, which has a commercial importance in food and pharmaceutical industries (Somkuti et al., 1997). It was shown that β-gal could hydrolyse β- galactosidic bond in β-lactose (Huber et al., 1981). Lactose can be hydrolysed with β-gal to avoid lactose crystallization in frozen concentrated deserts and milk consumption by lactose-intolerant individuals can be improved (Kim and Rajagopal, 2000). In addition to this, lactose acts as a galactosyl donor and an acceptor to form di-, tri-, or higher galactooligosaccharides (Wallenfels and Weil, 1972; Prenosil et al., 1987). Furthermore, β-gal has been found in abundant in biological systems and micro-organisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Agrawal et al., 1989). *Bifidobacterium* and *Lactobacillus* have become organisms of interest for commercial production of β-gal (Shah and Jelen, 1990). Moreover, most of the β-gal are not accepted for food use, are costly and many are not available in adequate quantities for industrial application (Kim and Rajagopal, 2000; Albayrak and Yang, 2002).

Several studies have been conducted on β-gal enhancement *in vitro* by manipulation of growth parameters by Johnson et al. (1993) and Swallow (2003) who showed that unabsorbed lactose was hydrolysed to galactose and glucose by bacterial β-gal present in lactic acid bacteria. These monosaccharides were then available for bacterial fermentation by and colonic flora to short chain fatty acids with by-products of hydrogen and carbon dioxide, causing bloating in the small bowel and flatulence in the colon. A number of health benefits have been claimed for probiotic bacteria such as *L. acidophilus, and Bifidobacterium* spp. These benefits include anti-mutagenic effects, anti-carcinogenic properties, improvement in

lactose metabolism, reduction in serum cholesterol, and immune system stimulation (Fuller, 1989). Probiotic bacteria are the predominant members of the intestinal microflora and are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Vasiljevic and Shah, 2008). Due to their health benefits, bifidobacteria are widely used in dairy preparations in conjunction with *L. acidophilus* (Shah, 2007).

Stiles and Holzapfel (1997) reported that the fastidious nature of LAB requires numerous growth factors such as minerals and vitamins in addition to carbohydrate and nitrogen sources to be present in a growth medium to be used for the enzyme production. The formulation of a suitable inexpensive medium has been sought using readily available components such as whey and whey permeate, often with supplementation (Bury et al., 2000; Vasiljevic and Jelen, 2001).

The rate of enzyme enhancement depends upon the enzyme source, substrate concentration and reaction conditions (Cardelle-Cobas et al., 2008). Microbial β-gal has a prominent position in terms of their role in production of various industrially relevant products like biosensors, lactose hydrolyzed milk, ethanol. Marrakchi et al. (2008) have developed a biosensor associating two distinct enzymatic activities, that of the β-gal and that of the glucose oxidase, in order to apply it for the quantitative detection of lactose in commercial milk samples. Domingues et al. (2005) have investigated the constant production of extracellular heterologous β-gal and ethanol by a recombinant flocculating *S. cerevisiae*. Jointly with extracellular β-gal production, an ethanol productivity of 9 g/L h was obtained for the bioreactor fed with 50 g/L initial lactose concentration at 0.45 h^{-1} dilution rate. In addition to this, not many studies have been carried out recently for economical production of β-gal. Hence, selection of micro-organisms which are safe for human use and are capable of producing high level of β-gal becomes vital. In the present study, *B. animalis* Bb12 and *L.*

delbrueckii ssp. *Bulgaricus* ATCC 11842 were used for the production of β-gal under various growth conditions.

4.2 Materials and Methods

4.2.1 Microorganisms

Pure cultures of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80ºC in sterile MRS broth (50% w/v) and 50% glycerol.

4.2.2 Culture condition

The organisms were activated in two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) incubated at 37 ºC for *B. animalis* Bb12, and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The activated cultures were again inoculated into MRS broth and inoculated at 37 º C for *B. animalis* Bb12 and 45 º C for *L. delbrueckii* spp. *bulgaricus* ATCC 11842 for 18 h. For production of β-gal, 1 mL of culture was transferred to a medium that contained 4% lactose, 0.3% K_2HP0_4, 0.1% KH_2PO_4, 0.05% $M_gSO_4.7H_20$ and 0.03% L-cysteine. In order to examine the effect of various nitrogen sources on β-gal production, 3.5% of each of yeast extract, peptone, casein hydrolysate, tryptone or ammonium sulphate was added individually in the medium. MRS broth was used as a control. Similarly, 1 mL of culture was transferred to a medium that contained 3.5% yeast extract, 0.3% K_2HP0_4, 0.1% KH_2PO_4, 0.05% $M_gSO_4.7H_20$ and 0.03% L-cysteine. To examine the effect of various carbon sources on β-gal production, 4% of glucose, lactose or galactose was added individually in the medium. All fermentation experiments were carried out for 12 h and culture was maintained at 37 ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

4.3. Production of β-galactosidase

For production of β-gal, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 x g for 20 min at 10 ºC). The supernatant was discarded and cell pellets were collected. A total of 5 mL of 0.03 M sodium phosphate buffer (pH 6.8) was added and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. The β-Gal activity was then assayed according to the method of (Nagy et al., 2001). The reaction mixture consisted of 0.5 mL of enzyme source (cells treated with lysozyme) and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37 ºC, 2 mL of 0.1 M sodium carbonate was added to the reaction mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of enzyme that catalysed the formation of 1 µmol of o-nitrophenyl from ONPG per min under the assay condition.

The amount of enzyme present in a reaction was measured by the activity it catalysed. The relationship between activity and concentration was affected by many factors such as temperature, pH, etc. An enzyme assay must be designed so that the observed activity is proportional to the amount of enzyme present in order that the enzyme concentration is the only limiting factor. The enzyme solution was used to determine the effect of substrate concentration on β-gal activity at 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37 ºC, 2 mL of 0.1 M sodium carbonate was added to the reaction mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of

enzyme that catalysed the formation of 1 µmol of o-nitrophenyl from ONPG per min under the assay condition.

4.4. Determination of protein

Quantification of protein was based on the Lowry method for protein quantification as described by (Waterberg and Mathews, 1984). Bovine serum albumin (Sigma) was used as a standard. The total protein content of medium was determined after fermentation.

4.5. Enumeration of microorganisms

To enumerate *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, MRS agar supplemented with 1% (w/v) D-glucose was used. Peptone and water 0.15% (w/v) diluent was used to perform serial dilutions. Plates were incubated at 37 ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

4.6 Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter Merk Pty Limited, 207 Colchester Rd, Kilsyth 3137, Victoria, Australia after calibrating with fresh pH 4.0 and 7.0 standard buffers.

4.7. Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

4.8 Results and discussion

4.8.1 Effect of carbon source on production of protein and β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

The effect of different carbon sources on the production of protein by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in presence of lactose, glucose and galactose as the sole carbon source is shown in Figure 4.1.

B. animalis Bb12 produced higher ($p<0.05$) protein content than *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 with various carbon source including lactose, glucose and galactose. Statistically, *B. animalis* Bb12 had significantly different protein content with lactose and glucose; however, there was no significant difference ($p>0.05$) in protein content between glucose and galactose. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had no significant difference ($p>0.05$) in terms of protein content with various carbon source including lactose, glucose and galactose.

B. animalis Bb12 produced the highest ($p<0.05$) amount of protein (0.28 mg/mL) with lactose; however, this organism produced same level of protein content with galactose and glucose (0.24 mg/mL). The protein content increased ($p<0.05$) by 260.26, 105.08 and 218.42 percent in lactose, glucose and galactose, respectively at 12 h as compared with 0 h (data not shown). On the other hand, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced similar level of protein (0.11 mg/mL) with lactose, glucose and galactose. At 12 h, the protein content increased by 98.25, 85.25 and 51.39 percent in lactose, glucose and galactose, respectively as compared with 0 h (data not shown). The protein concentration gradually increased during incubation. However, the level remained lowest with glucose and galactose in both organisms.

The Lowry method determined the protein concentrations in the reactivity of the peptide nitrogen with the copper ions under alkaline conditions and the subsequent reduction of the

Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropoly-molybdenum blue by the copper-catalyzed oxidation of aromatic acids (Dunn, 13).

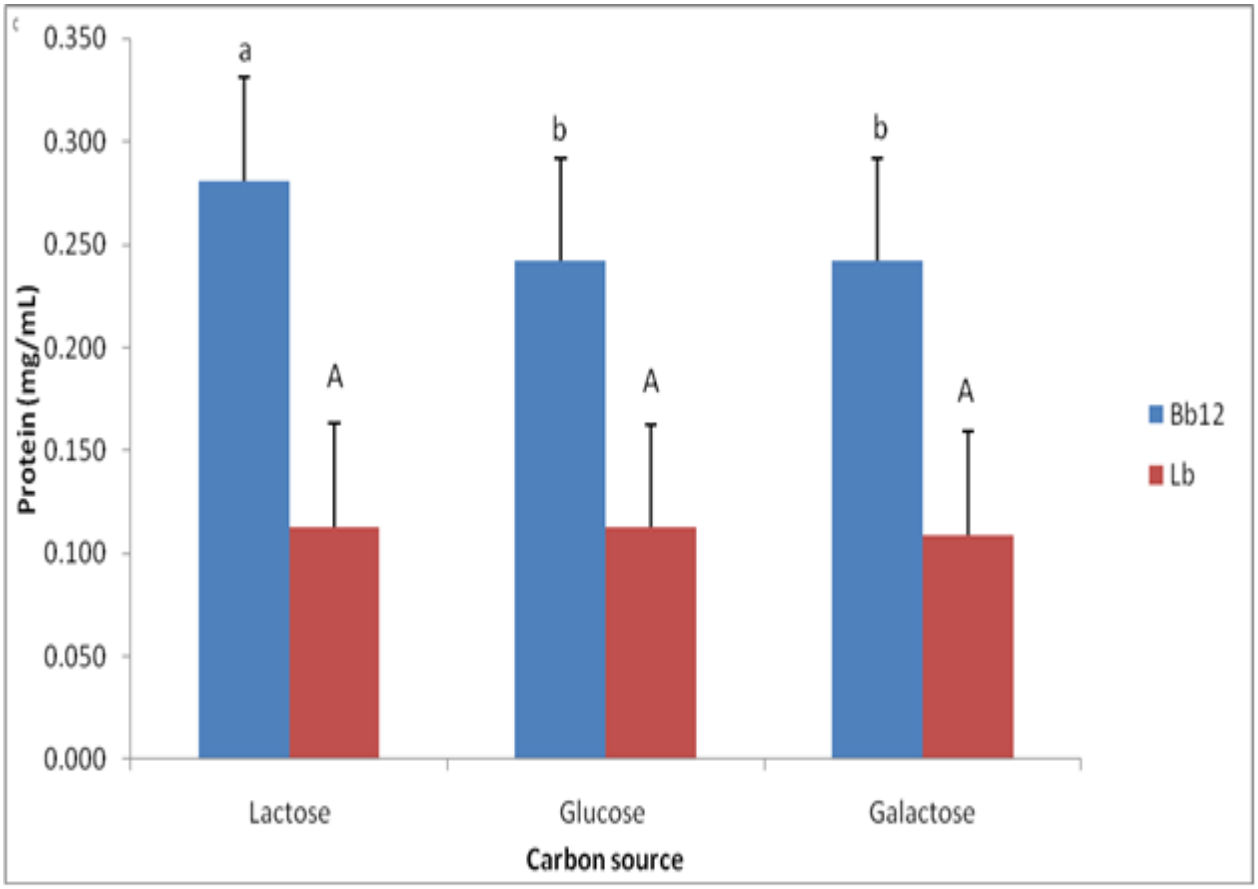

Figure 4. 1: Effect of carbon source on the protein production by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 3.5% yeast extract, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

A variety of compounds interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, sugars, salts, nucleic acids and sulphydryl reagents (Dunn, 1992). The Lowry method is sensitive to low concentrations of protein. Dunn (1992) suggested that the concentrations ranging from 0.10-2 mg of protein per mL while Price (1996) suggested that the concentrations of 0.005-0.10 mg of protein per mL. In general, protein content does not increase during the incubation period. However, the metabolites produced in culture play an important role in the physiological processes of the

microorganism in the medium containing nitrogen and carbon sources. The extent of secretion of the proteins varied from the micro-organisms tested and their nature of utilization of different carbon sources during incubation period (Rezende and Felix (1997). Our finding was also similar that protein concentration gradually increased during incubation.

However, Quan et al. (2002) reported that carbon metabolism was not only controlled by carbon-derived signals but also by the availability of nitrogen and other nutrients. Similarly, the metabolism of nitrogen, sulphur and phosphorus in bacteria was controlled by the carbon source because bacteria have the ability to utilise the nutrient supply and adapt their metabolism accordingly.

It is relatively easy to measure the protein content of a cell fraction; there may be a variable relationship between the protein content and a specific enzyme function. Extraction of an enzyme is accomplished by differential salt precipitation while proteins will precipitate together due to their solubility. To determine both protein content and enzyme activity requires two different procedures. It can be measured by the amount of protein, or we can kinetically measure the enzyme activity. Combining the two provide the specific activity (Brenner, 1961). According to Jurado et al. (2004); Tari et al. (2007), the enzyme activity is influenced by the type of strain, cultivation conditions (temperature, pH, incubation time) and the growth medium composition in presence of carbon and nitrogen sources. Siham et al. (2010) reported that the growth of *Lactobacillus* was higher in the culture that contained either glucose or galactose as the sole carbon source.

4.8.2 Effect of carbon sources on the β-galactosidase production

The influence of various carbon sources including lactose, glucose and galactose on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4.2. In general, both organisms produced higher ($p<0.05$) β-gal in galactose than lactose and glucose and lower ($p<0.05$) β-gal in glucose than lactose and galactose. *B.*

animalis Bb12 produced the highest ($p<0.05$) amount of β-gal with galactose (73.66 Unit/mL) followed by lactose (57.04 Unit/mL) and lowest activity with glucose (31.08 Unit/mL). The β-gal production increased ($p<0.05$) by 33.39, 27.43 and 95.02 percent in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). A similar pattern was seen with *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, which produced the highest ($p<0.05$) amount of β-gal (48.63 Unit/mL) with galactose followed by lactose (33.0 uni/mL) and lowest activity with glucose (28.9 Unit/mL). At 12 h, the β-gal production increased ($p<0.05$) by 28.65, 33.29 and 124.32 percent in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown).

Several workers reported that the effects of carbon source in the activity of β-gal may vary and depend on the microorganisms used (Fiedurek and Szczodrak, 1994; Fekete et al., 2002). They further reported on the effects of lactose, glucose, galactose, acid and sweet whey on β-gal activity. Siham et al. (2010) found that the growth of *Lactobacillus acidophilus* was higher in the culture that contained either glucose or galactose as the sole carbon source but the maximum β-gal activity (12.29 U/mL) was recorded with acid whey followed by sweet whey and lactose (9.83 and 8.6 U/mL, respectively). These results were similar to those reported by De Bales and Castillo (1979), Nahvi and Moeini (2004) and Eliwa and El-Hofi (2010). However, our results showed that deproteinised sweet whey was found to be a suitable medium for β-gal production. Thus it should be possible to produce commercial amounts of β-gal using the two organisms. *B. animalis* Bb12 produced the highest ($p<0.05$) amount of β-gal with galactose (73.66 Unit/mL) followed by lactose (57.04 Unit/mL) and lowest activity with glucose (31.08 Unit/mL). A similar pattern was seen with *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, which produced the highest ($p<0.05$) amount of β-gal (48.63 Unit/mL) with galactose followed by lactose (33.0 uni/mL) and lowest activity with glucose (28.9 Unit/mL).

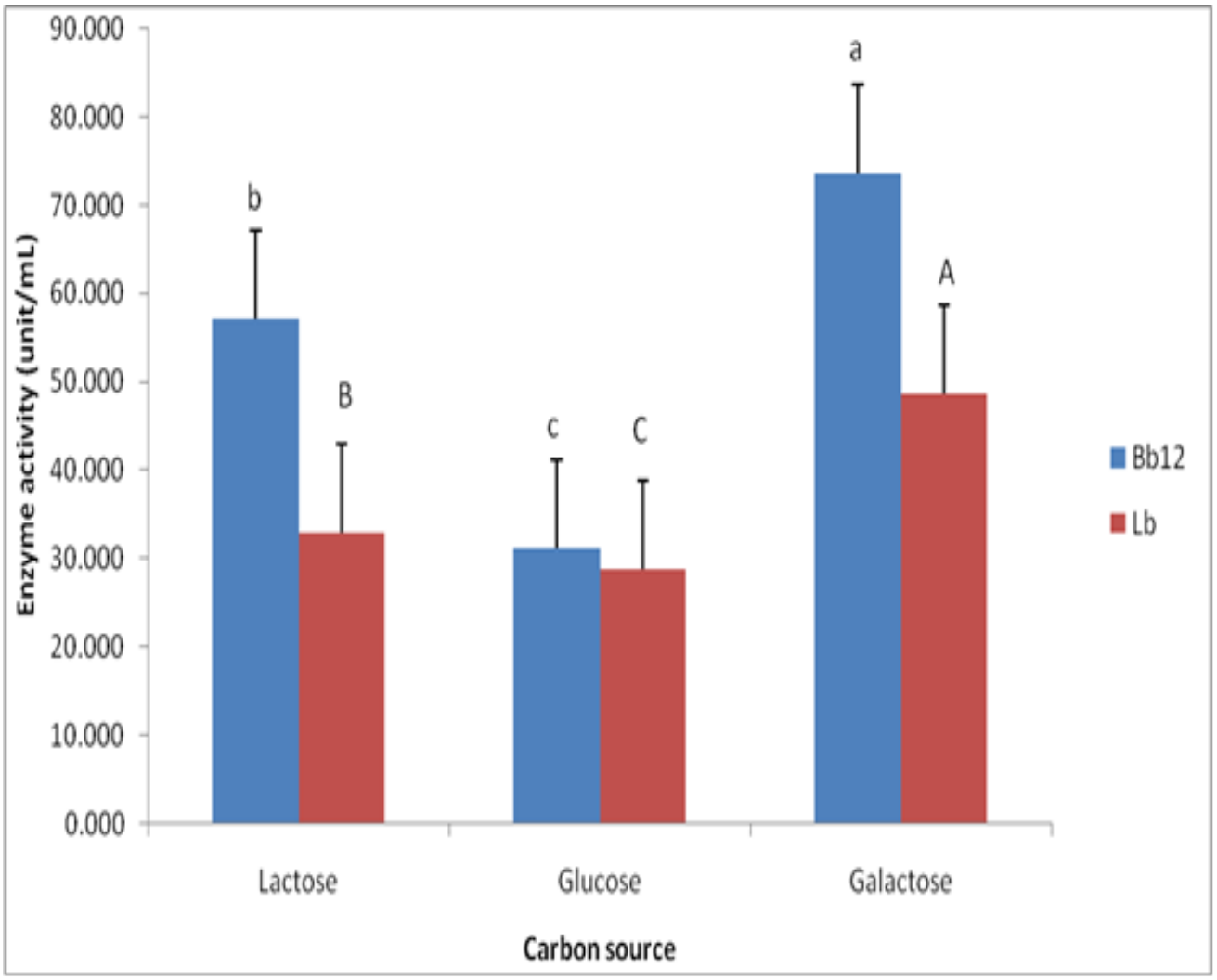

Figure 4. 2: Effect of carbon source on the β-galactosidase production by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 3.5% yeast extract, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

According to Bergey's Manual of Systematic Bacteriology (2001), *Bifidobacterium* metabolize glucose exclusively by heterolactic fermentation, by the fructose-6-phosphate shunt also know as bifid shunt, to form L(+) lactic acid and acetic acid in the molar ratio of 2:3 (Holt et al., 1994; Arunachalam, 1999; Gomes and Malcata, 1999; Hoover, 2000). Besides glucose, all bifidobacteria from human origin are also able to utilize galactose, lactose and fructose as carbon sources. A proton symport has been identified as the lactose transport system for *B. bifidum* (Krzewinski et al., 1996). In some instances they are also able

to ferment complex carbohydrates as reported by Crociani et al. (1994).

Lactobacillus delbrueckii ssp. *bulgaricus* is also a Gram-positive organism, but occurs in milk as chains of 3 to 4 short rods, each 0.5-0.8 × 2.0-9.0 μm, with rounded ends. It converts hexoses into lactic acid via the EMP pathway (Teixeira, 2000). Although lactic acid is the major end product of fermentation, secondary end products such as acetaldehyde, acetone, acetoin and diacetyl can also be produced in very low concentrations. Like ST, LB can utilize lactose, fructose and glucose, and some strains can utilize galactose (Robinson, 2000). The lactobacilli include over 25 unique species, and the first level of differentiation is based on end product composition: homofermentators being organisms that produce more than 85% lactic acid as their end product from glucose (e.g. *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*), and heterofermentators that produce approximately 50% lactic acid as the end product plus considerable amounts of carbon dioxide gas, acetate and ethanol (e.g. *L. brevis*, *L. casei*).

The enzymatic activity was expressed as units of β-gal activity per mL of concentrated cells. As presented in this thesis, enzyme activities obtained per mL Fig 4.2 and Fig 4.3 are that of cell concentrate rather cells suspension. Several workers reported that the effects of carbon source in the activity of β-gal may vary and depend on the microorganisms used (Fiedurek and Szczodrak, 1994; Fekete et al., 2002). They further reported on the effect of lactose, glucose, galactose, acid and sweet whey on β-gal activity. Siham et al. (2010) found that the growth of *Lactobacillus acidophilus* was higher in the culture that contained either glucose or galactose as the sole carbon source but the maximum β-gal activity (12.29 U/mL) was recorded with acid whey followed by sweet whey and lactose (9.83 and 8.6 U/mL, respectively). These results were similar to those reported by De Bales and Castillo (1979), Nahvi and Moeini (2004) and Eliwa and El-Hofi (2010).

A number of investigators have reported about the regulation of carbon source on β-

galactosidase biosynthesis in different micro-organisms (Fantes and Roberts, 1973; Montero et al., 1989; Fiedurek and Szczodark, 1994; Nikolaev and Vinetski, 1998; De Vries et al., 1999; Nagy et al., 2001a; Fekete et al., 2002). All these authors have reported that the role of carbon source in the biosynthesis of β-gal may vary and depend on the micro-organisms tested. Kim and Rajagopal (2000) reported that *L. criptus* grown in MRS broth containing galactose as carbon source showed the highest β-gal activity followed by moderate levels of enzyme production with lactose and significant activity with glucose or maltose. The expression of β-gal by micro-organisms may be affected by the amount of carbon source in the medium (Fiedurek and Szczodrak, 1994; Inchaurondo et al., 1998). According to Bergy's Manual of Systematic Bacteriology (1986), *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 does not ferment galactose, however, it appears that increasing fermentation period resulted in an increase in β-gal in all carbon sources (lactose, glucose and galactose) in both strains at 12 h incubation period. However, galactose produced highest β-gal in both strains (Figure 4.2). Akolkar et al. (2005) and Hsu et al. (2005), who reported that the carbon source containing lactose showed the maximum β-gal activity by bifidobacteria and *L.* ssp. respectively. However, Kim and Rajagopal (2000) reported that the carbon source containing galactose showed the maximum β-gal activity by *L.* ssp. This was also confirmed by our findings that galactose produced the maximum β-gal activity in both strains.

Supplementation of lactose in the medium produced the maximum β-gal activity (43.82 U/mL) in *L. reuteri* than any other carbon sources. The presence of lactose in the media probably triggered β-gal activity by the lac operon mechanism (Miller and Reznikoff, 1978). This finding was in line with the findings of Akolkar et al. (2005) and Hsu et al. (2005), who showed that the presence of lactose in the media led to an enhancement of β-gal activity for bifidobacteria and *L. acidophilus,* respectively. According to Bergy's Manual of Systematic Bacteriology (1986), *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 does not ferment galactose,

however our finding was similar to that of Murad (1998); Inchaurrondo et al. (1998) and Kim and Rajagopal (2000), who reported that the amounts of galactose in the medium may affect the activity of β-gal by micro-organisms. Therefore, galactose can be used efficiently in the production of β-gal activity by both organisms. McGinnis and Paigen (1973) reported that galactose was the most efficient inducer for the production of enzyme activity. This may be attributed that *De novo* synthesis occurs for synthesis of complex molecules from simple molecules such as sugars or amino acids.

4.8.3 Effect of carbon sources on the growth

Figure 4.3 demonstrates the effect of carbon source including lactose, glucose and galactose on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, *B. animalis* Bb12 as well as *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) final viable population in galactose than in lactose and glucose and lower ($p<0.05$) final viable population at glucose than the lactose and galactose. It was found that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significant different ($p<0.05$) the final viable population in various carbon sources. The amounts of carbon source in the medium may affect the expression of β-gal by micro-organisms (Fiedurek and Szczodrak, 1994; Inchaurrondo et al., 1998). The pattern showed an initial increase in viable population at the commencement of the fermentation followed by slower increasing trend towards the end of this process. Maximal population at 8.4 log CFU/mL, 7.8 log CFU/mL, 7.7 log CFU/mL in galactose, lactose and glucose, respectively was reached in *B. animalis* Bb12 (Fig 4.3).

Similarly, maximal population at 8.6 log CFU/mL, 7.8 log CFU/mL and 7.5 log CFU/mL in galactose, lactose and glucose, respectively, was reached in *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (Figure 4.3). The final viable population of the *B. animalis* Bb12 ranged from 6.92 to 8.43 log CFU/mL and the organism showed the highest ($p<0.05$) viable population of

8.43 log CFU/mL with galactose followed by lactose (7.82 log CFU/mL) and lowest with glucose (7.75 log CFU/mL).

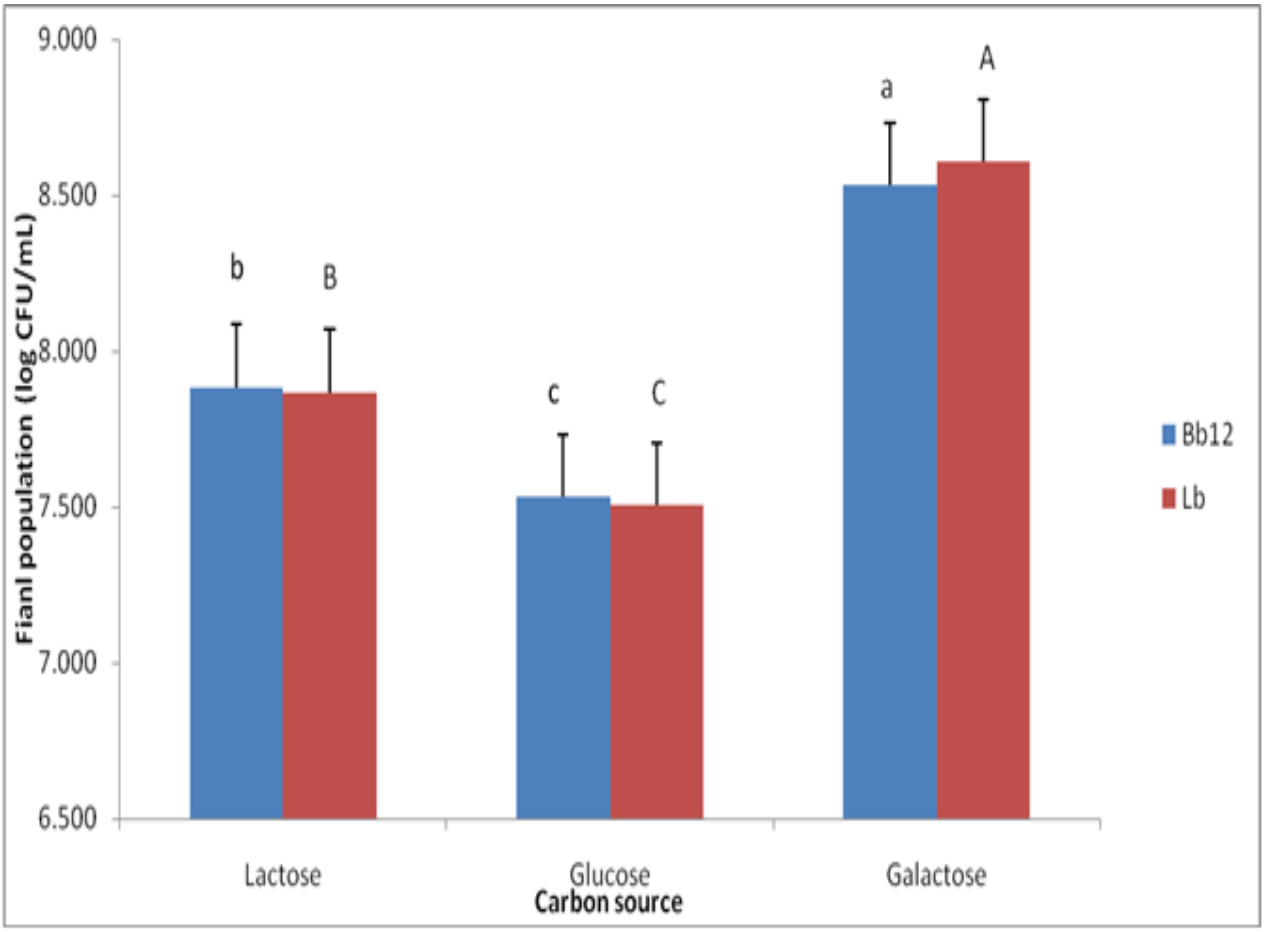

Figure 4. 3: Effect of carbon source on the final population by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842

Medium contained 3.5% yeast extract, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

The viable count increased ($p<0.05$) by 0.93, 0.82, and 1.23 log CFU/mL in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a similar trend. The final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.67 to 8.6 log CFU/mL and the organism exhibited the highest ($p<0.05$) viable population of 8.6 log CFU/mL with galactose followed by lactose (7.87 CFU/mL) and lowest with glucose (7.5 log

CFU/mL). At 12 h, the viable count increased ($p<0.05$) by 1.09, 0.83 and 1.82 log CFU/mL in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown).

4.8.4 Effect of carbon sources on pH

The effect of carbon source on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4.4. In general, pH value in glucose was lower ($p<0.05$) as compared with other carbon sources. The pH value of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was significantly ($p<0.05$) higher in lactose than that with glucose and galactose. However, the pH value in glucose and galactose are not significant different ($p>0.05$). On the other hand, *B. animalis* Bb12 showed no significant difference ($p>0.05$) in terms of pH values in various carbon sources. The decrease in pH by *B. animalis* Bb12 was lowest with glucose at 4.63 followed by galactose (4.80) and lactose (4.83). The pH value decreased ($p<0.05$) by 23.82, 25.20 and 19.60 percent in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). On the other hand, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with galactose (pH 4.59) followed by glucose (pH 4.63) and lactose (pH 5.13). At 12 h, the pH value decreased ($p<0.05$) by 18.70, 25.32 and 26.79 percent in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown). The drop in pH correlated with an increase in population of the two organisms. This finding was similar to that of Murad (1998) who reported that highest enzyme production by *L. bulgaricus* was obtained at pH 4.4. In the study, *B. animalis* Bb12 as well as *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) final viable population in galactose than those of other carbon sources. This finding was similar to that of Inchaurrondo et al. (1998), who reported that the amounts of galactose in the medium may affect the activity of β-gal by micro-organisms. Therefore, galactose can be used efficiently in the production of β-gal activity by both organisms. The effect of pH on cellular physiology was confirmed by other studies which showed that it

influenced acidification activity of lactic acid bacteria (Savoie et al., 2007) whereas Wang et al. (25) indicated that *Lactobacillus acidophilus* cells grown at optimal pH displayed a higher residual acidification activity than cells grown at lower pH control values. However, Schepers et al. (2002) and Savoie et al. (2007) demonstrated that this activity was higher when starters were produced without pH control or at low pH control values.

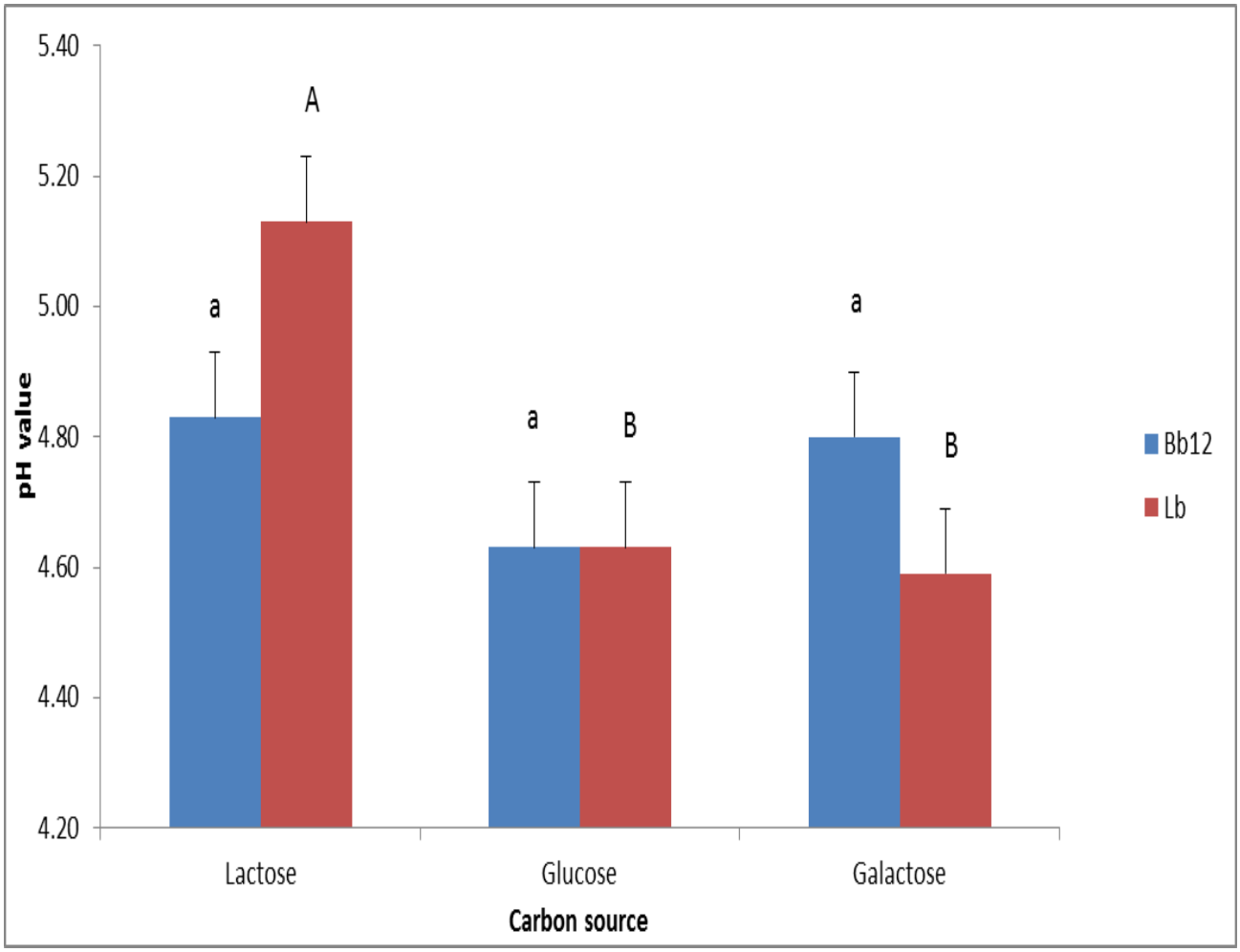

Figure 4. 4: Effect of carbon source on the pH value by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842

Medium contained 3.5% yeast extract, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

4.8.5 Effect of nitrogen source on production of protein by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Effect of nitrogenous substrates including yeast extract, peptone, casein hydrolysate,

tryptone, ammonium sulphate and MRS broth on production of protein by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4.5. In general, *B. animalis* Bb12 produced higher ($p<0.05$) protein in yeast extract, ammonium sulphate and MRS broth compared with other nitrogen sources. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) protein in MRS broth, tryptone and peptone than the other nitrogen sources. *B. animalis* Bb12 had significantly different protein content in yeast extract, peptone, casein hydrolysate and ammonium sulphate and there was no significant difference ($p>0.05$) between tryptone and MRS broth. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different protein content in yeast extract, peptone, tryptone and MRS broth and there was no significant difference ($p>0.05$) between casein hydrolysate and ammonium sulphate.

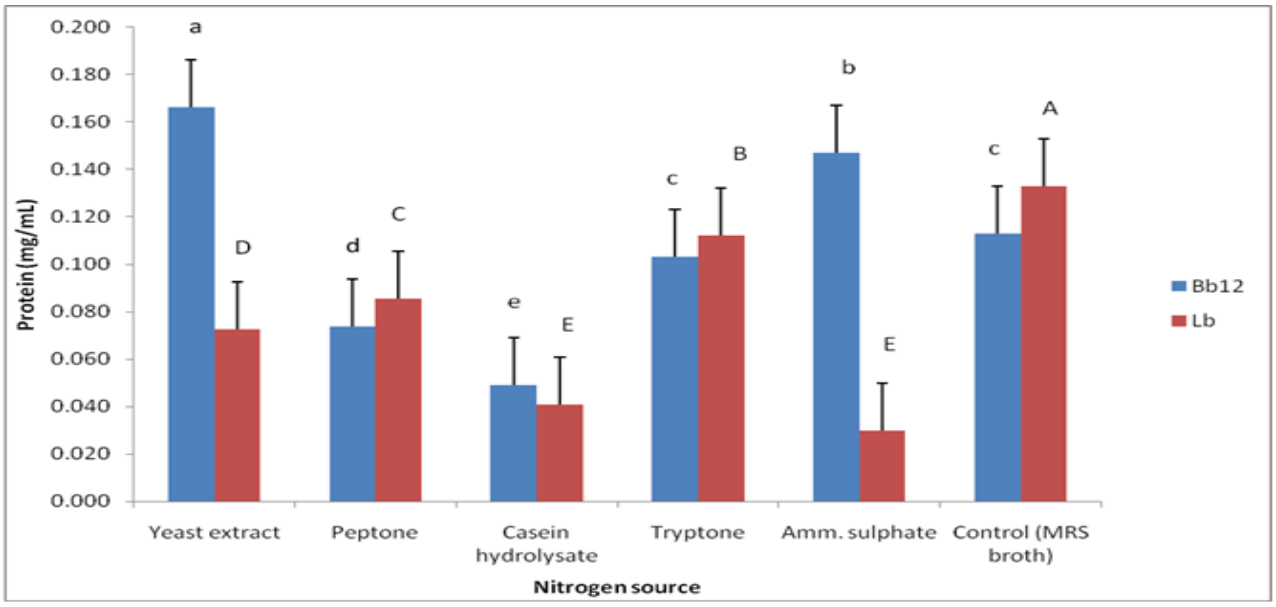

Figure 4. 5: Effect of nitrogen source on the protein production by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 4.0% lactose, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

Moreover, *B. animalis* Bb12 produced the highest amount of protein (0.17 mg/mL) with yeast extract followed by ammonium sulphate (0.15 mg/mL) and tryptone, casein hydrolysate gave

the lowest protein with MRS broth (Figure 5). The protein content increased in 12 h ($p<0.05$) by 172.13, 34.55, 133.33, 58.46, 161.36 and 156.82 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, as compared with 0 h (data not shown). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of protein (0.13 mg/mL) with MRS broth followed by tryptone (0.11 mg/mL) and peptone (Figure 4.5). At 12 h, the protein content increased ($p<0.05$) by 176.92, 177.42, 127.78, 322.22, 100.00 and 329.03 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively as compared with 0 h (data not shown). The protein concentration gradually increased during incubation. However, the level remained lowest with ammonium sulphate. MRS broth is usually used to grow *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 due to its nutrient contents, hence the organism grew best in MRS broth. Dunn (1985) reported that the medium containing ammonium sulphate, the ammonium ions influence the metabolism of certain amino acids in lactobacilli by their either α-ketoglutarate or glutamate. Thus, when ammonium sulphate was used, the inorganic nitrogen should first be converted to amino acids and then used for the synthesis of proteins which were needed as a growth factor for the organisms. Among different nitrogen sources, ammonium sulphate could be considered as a feasible and inexpensive alternative nitrogen sources for growth factor probiotic organisms (Arasaratnan et al., 1996).

4.8.6 Effect of nitrogen sources on the β-galactosidase production

The influence of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4.6. In general, *B. animalis* Bb12 produced higher ($p<0.05$) β-gal in MRS broth and tryptone compared with other nitrogen sources. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) β-gal production in casein hydrolysate and yeast extract than the

other nitrogen sources. Statistically, *B. animalis* Bb12 had significantly different β-gal production in yeast extract, peptone, casein hydrolysate and MRS broth and no significant difference ($p>0.05$) in ammonium sulphate, casein hydrolysate and tryptone. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different β-gal production in yeast extract, casein hydrolysate, ammonium sulphate and MRS broth and this was no significant difference ($p>0.05$) in β-gal production between peptone and tryptone. *B. animalis* Bb12 produced the highest amount of β-gal (51 Unit/mL) with MRS broth followed by tryptone (45 Unit/mL).

The β-gal production increased ($p<0.05$) by 45.32, 44.20, 81.86, 106.43, 94.51 and 104.03 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively at 12 h as compared with 0 h (data not shown). MRS broth provided optimum nutrients for this organism. Hence, the organism produced the highest level of β-gal. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of β-gal (50 Unit/mL) with casein hydrolysate followed by yeast extract (42 Unit/mL) and lowest activity (23 Unit/mL) with peptone (Figure 6). At 12 h, the β-gal production increased ($p<0.05$) by 106.30, 14.14, 122.17, 24.97, 60.07 and 43.93 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth respectively as compared with 0 h (data not shown). This may be attributed to the peptides and amino acids present in casein hydrolysate. According to Rao and Dutta (1979); Shaikh et al. (1997), nitrogen sources may affect microbial biosynthesis of β-galactosidase.

Millsap et al. (1996) reported that the composition of the fermentation medium was a major factor in determining the properties of microorganisms like *Lactobacillus*, the physicochemical properties and the protein composition of the bacterial cell wall remain largely unchanged if the carbohydrates were left out of the fermentation medium, although the bacterial growth was influenced. Regarding viability, the standard specifies that the sum

of microorganisms constituting the starter culture should be at least 10^7 CFU per g and that minimum count of other labelled microorganisms should be 10^6 CFU per g (Roy, 2005).

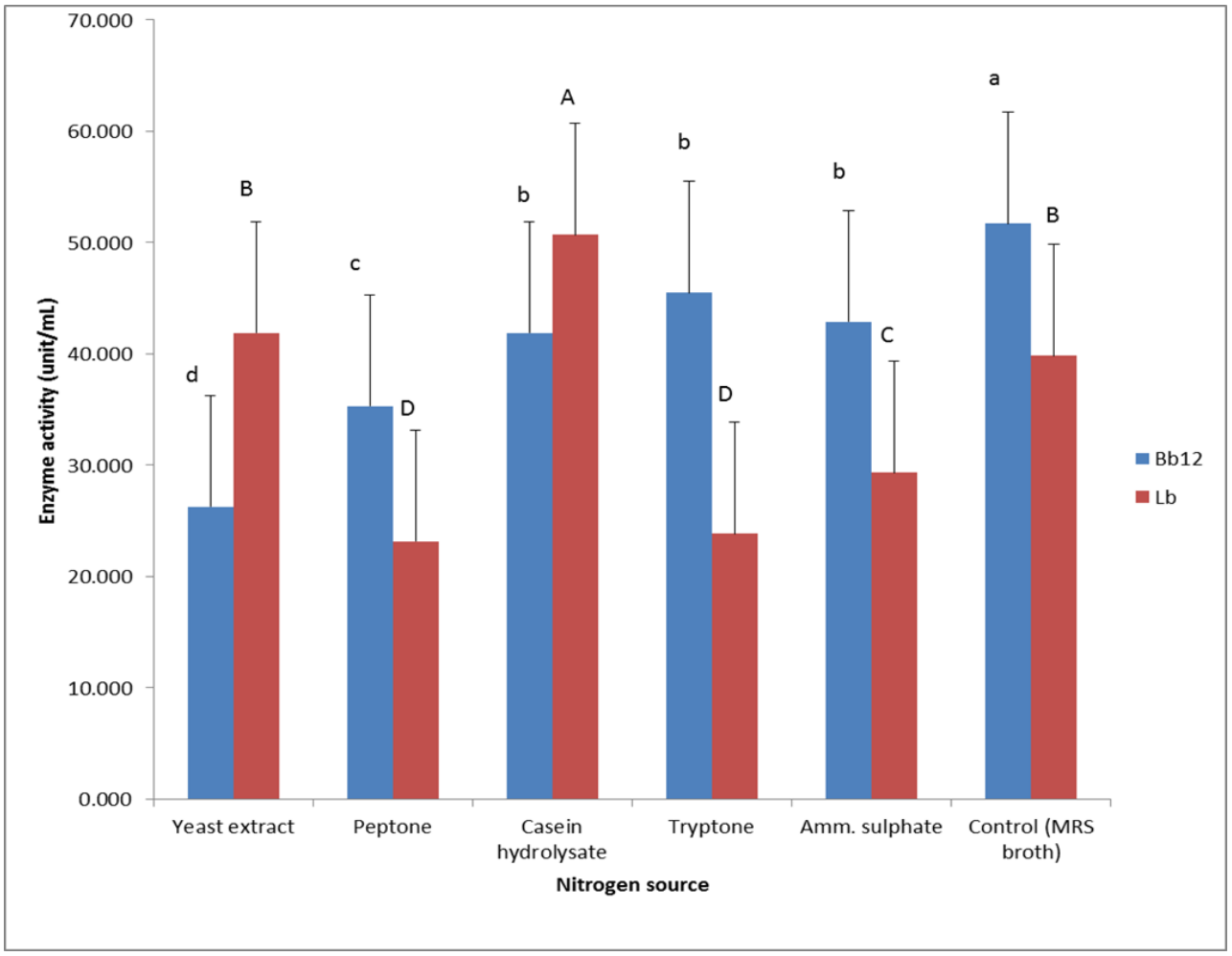

Figure 4. 6: Effect of nitrogen source on the β-galactosidase production by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 4.0% lactose, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

Some researchers have reported that yeast extract contains small peptides and vitamins that improved growth and β-gal activity at the 1.5-10% concentration level (Hsu *et al*., 2005; Vasiljevic and Jelen, 2002). Similarly, Jokar and Karbassi (2009) reported that the maximum β-gal activity was produced by *Lactobacillus delbruekii* when grown in permeate based medium enriched with a combination of yeast extract, whey powder as organic nitrogen sources. In the current study, the final viable population of the *B. animalis* Bb12 showed the

highest viable population of 8.5 log CFU/mL with MRS broth followed by casein hydrolysate. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed the highest viable population of 8.4 log CFU/mL with MRS broth. This may be attributed to the nutrients in addition to the nitrogen compounds present in MRS broth and casein hydrolysate.

Hsu et al. (2005) reported that the concentration of yeast extract in the medium was found to affect the activity of β-gal production by *Bifidobacterium*. The activity of β-gal increased upon increasing the yeast extract concentration up to 10.0% in the medium. Further increasing yeast extract content in the medium resulted in a sharp reduction in the activity of β-gal and a reduced the final population of the test organism. A considerable amount of β-gal was obtained in the medium containing 3.5% yeast extract. Yeast extract was found to support the highest production of β-gal by *Bifidobacterium*. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker, 1970).

4.8.7 Effect of nitrogen sources on the growth

Figure 4.7 demonstrates the effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, the viable counts were higher ($p<0.05$) in MRS broth and casein hydrolysate than others nitrogen source including yeast extract, peptone, tryptone and ammonium sulphate in both organisms (Figure 4.7). The viable counts of *B. animalis* Bb12 were significantly ($p<0.05$) lower in ammonium sulphate and tryptone; however, yeast extract and ammonium sulphate showed significantly ($p<0.05$) lower viable counts in *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The final viable population of the *B. animalis* Bb12 ranged from 6.8 to 8.5 log CFU/mL and the organism showed the highest viable population of 8.5 log CFU/mL at 12 h with MRS broth followed by casein hydrolysate at 8.2 log CFU/mL and lowest with ammonium sulphate at 7.6 log

CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 7.57, 18.63, 17.09, 3.23, 2.29 and 11.33 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, compared with 0 h (data not shown). However, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.6 to 8.4 log CFU/mL and the organism showed the highest viable population of 8.4 log CFU/mL at 12 h with MRS broth followed by casein hydrolysate at 8.2 log CFU/mL and lowest with peptone at 6.6 CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 7.67,14.78, 22.24, 18.70, 11.94 and 23.53 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, as compared with 0 h (data not shown). This may be attributed to the nutrients in addition to the nitrogen compounds present in MRS broth and casein hydrolysate. According to Rao and Dutta (1979 and Shaikh et al. (1997), nitrogen sources may affect microbial biosynthesis of β-galactosidase.

In the current study, it was confirmed that supplementation of MRS broth in the medium was the best nitrogen source for the maximum β-gal activity. It appeared that MRS broth not only enhanced the growth of selected organisms but also decreased pH value.

4.8.8 Effect of nitrogen sources on the pH

The effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4.8.

In general, pH value of MRS broth was lower ($p<0.05$) compared with other nitrogen sources for both organisms. The pH value of *B. animalis* Bb12 was significantly ($p<0.05$) higher in ammonium sulphate among other nitrogen sources.

Similar pH values were higher in ammonium sulphate, tryptone, casein hydrolysate and

peptone. These were significantly ($p<0.05$) higher compared with yeast extract and MRS broth.

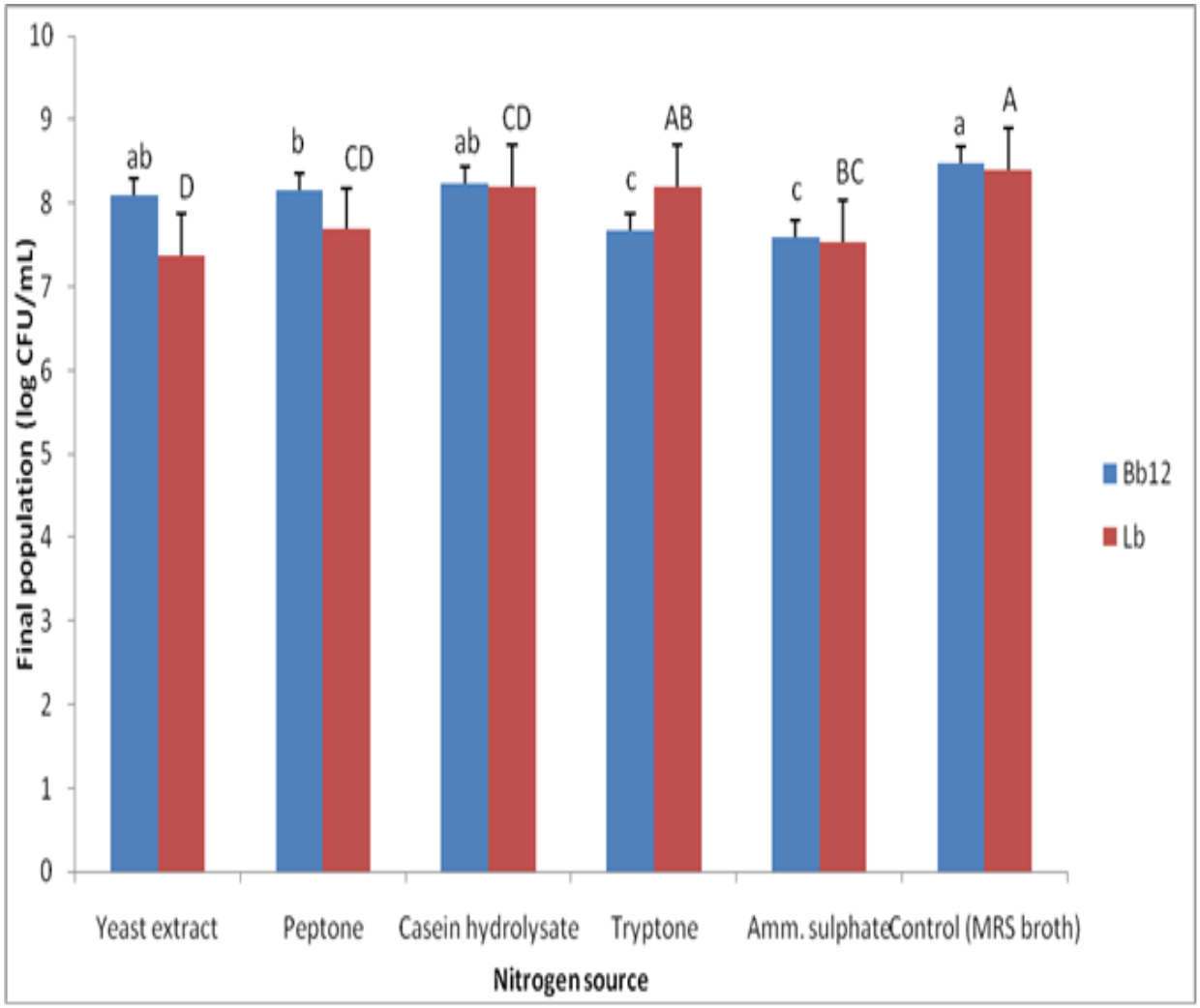

Figure 4. 7: Effect of nitrogen source on the final population by B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 4.0% lactose, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

The decrease in pH value by *B. animalis* Bb12 was lowest with MRS broth (4.24) followed by yeast extract (5.56) and highest with tryptone (6.69. The pH value decreased ($p<0.05$) by 33.85, 15.50 and 2.81 percent in MRS broth, yeast extract and tryptone, respectively at 12 h as compared with 0 h (data not shown). Similarly, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with MRS broth (pH 4.26) followed by yeast extract

(pH 5.27) and highest with tryptone (pH 6.18). The pH value decreased ($p<0.05$) by 35.16, 20.15 and 8.08 percent in MRS broth, yeast extract and tryptone, respectively at 12 h as compared with 0 h (data not shown). The drop in pH is correlated with increase in population of the two organisms in MRS broth.

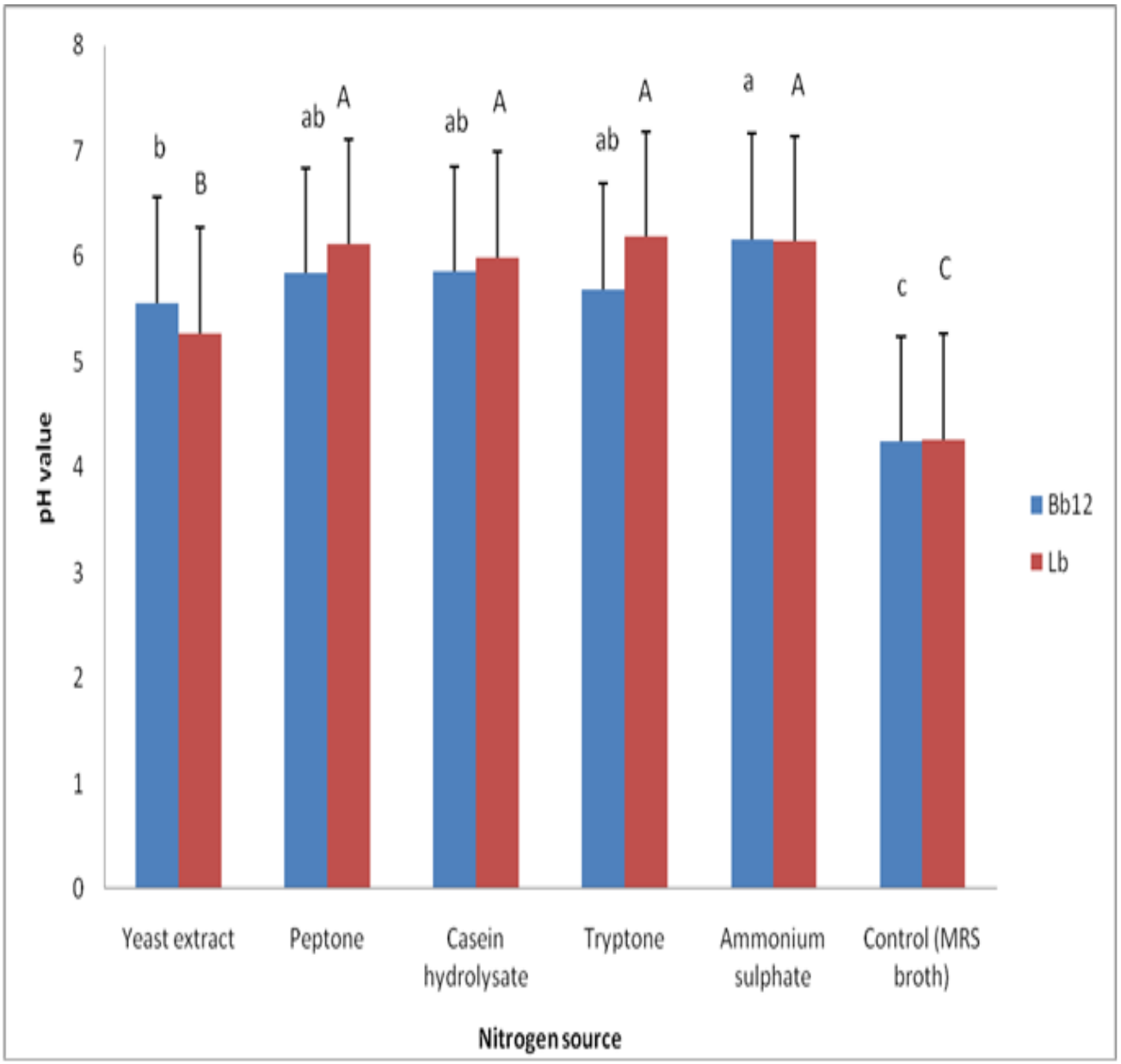

Figure 4. 8: Effect of nitrogen source on the pH value by Bifidobacterium animalis Bb12 and Lactobacillus delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 4.0% lactose, 0.3% K_2HPO4, 0.1% KH_2PO4, 0.05% $M_gSO4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$).

4.9 Conclusion

The results of this study demonstrated that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 are capable of producing high level of β-gal. A maximum of β-gal of 73.66 Unit/mL and 48.63 Unit/mL were produced by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, respectively in carbon source including lactose, glucose and galactose. Similarly, maximum β-gal at 51.6 Unit/mL and 50.7 Unit/mL were produced by *animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, respectively in nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Considering the high yield of β-gal with *B. animalis*, this organism may be a potential useful industrial strain for the production of β-gal.

Chapter 5.0 Effect of nitrogen sources on production of β-galactosidase from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey under different culture conditions[3]

[3] A version of this chapter has been published. Prasad, L. N., M. M. Ayyash, and N. P. Shah. 2011. Effect of nitrogen sources on production of β-galactosidase from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey under different culture conditions. International Food Research Journal 18: 445-450.

5.1 Introduction

The β-Gal, is one of important enzymes used in dairy industry for hydrolysis of lactose into glucose and galactose. β-Gal is found in abundance in biological systems and micro-organisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Agrawal et al., 1989). Since, there is a large numbers of sufferers from lactose intolerance population in different countries of the world, this makes the enzyme even more important. The need for low lactose milk is particularly important in food-aid programs as severe tissues dehydration, diarrhoea even death may result from feeding lactose containing milk to lactose in-tolerant children and adults suffering from protein-calories malnutrition. In addition, lactose has a low solubility, which results in crystal structure at concentrations above 11% (w/v), which prevents the utilization of concentrated whey in several food processes (Bansal et al., 2008). Dairy industry waste whey, contains lactose (5%), whey protein (0.8%), mineral and vitamins, which are essential components that have not been exploited for the cultivation of *B. animalis* Bb12 and *L. delbrueckii* ssp. b*ulgaricus* 11842 (Mahalakshmi et al., 2000). Whey represents about 85–95% of the milk volume and retains 55% of milk nutrients. It is, a by-product of the cheese making process, which is a rich source of milk proteins, water soluble vitamins, lactose and minerals. The composition and the type of whey produced at dairy plants depend on a type of cheese manufactured and process technology employed (Keerthana and Reddy, 2006). Most of milk plants do not have proper treatment system for the disposal of whey and the dumping of whey constitutes a significant loss of potential food and energy as whey retains about 55% of total milk nutrients. Its disposal as a waste also poses a serious pollution problems for the surrounding environment (Carrara and Rubiolo, 1994). To overcome this problem, a better alternative would be subjecting the whey to processes through which the value added products can be manufactured, which may contribute wholly or partially to the costs. Availability of

carbohydrate reservoir of lactose in whey and presence of other essential nutrient for the growth of microorganisms makes the whey one of the most potent raw materials for the production of different bio-products through biotechnological means (Panesar et al., 2007a). Membrane separation processes, such as ultrafiltration (UF), reverse osmosis (RO) and diafiltration (DF), are now industrially applied in the manufacture of whey powders and WPCs with protein contents of 30-80% (Etzel, 2004).

The disposable of whey remains a significant problem for dairy industries especially in developing countries where a relatively insignificant part of whey is used for production of whey protein concentrates and significant part of it disposed off into the water streams causing serious water pollution problems. Hence, problems associated with whey disposal, lactose crystallization and milk consumption by lactose- intolerant populations of the world have drawn the attention of several research workers. This has led to the selection of micro-organisms with view to high potentials for producing β-gal, the enzyme that hydrolyses lactose into its component monosaccharide units (Rao and Dutta, 1997). Therefore, it is important to evaluate the production of β-gal using whey by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* 11842 organisms in terms of effectiveness and enzyme production so that process could be scaled up the β-gal production. The enzyme β-gal catalyzes hydrolysis of whey lactose to glucose and galactose. The use of β-gal to avoid lactose crystallization in condensed and frozen dairy products raises its industrial importance and makes it suitable for avoiding whey disposal (Dagbagli and Goksugur, 2008). In this regards, not many studies have been carried out recently for economical production of β-gal. Hence, selection of micro-organisms which are safe for human use and are capable of producing high level of β-gal becomes vital. Thus, the present study was conducted to evaluate the effect of various nitrogen sources on the production of β-gal by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *B. animalis* Bb12 in deprotenized whey.

5.2 Materials and method

5.2.1 Micro-organisms

The pure cultures of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80ºC in sterile MRS broth (50% w/v) and 50% glycerol.

5.2.2 Culture condition

The organisms were activated in two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine for (Sigma Chemical Company, St. Louis, MO, USA) and incubated at 37 ºC for *B. animalis* Bb12, and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. Deproteinized whey was prepared by heating whey at 90 º C and pH 4.5 for 10 min, filtered through Whatman no. 1 filter paper to remove the coagulated protein, adjusted to pH 7.0 and sterilized at 121 º C for 15 min. For production of β-gal, the sterile whey was supplemented with 3.5% of each of nitrogen source individually including yeast extract, peptone, casein hydrolysate, tryptone or ammonium sulphate and inoculated with 1% of active culture of each organism. The various nitrogen sources were used in order to study their effect on β-gal production. Deproteinized whey was used as a control. All experiments were carried out for 24 h. The culture was maintained at 37 ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

5.3 Production of β-gal

For production of β-gal, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 x g for 20 min at 10 ºC). The

supernatant was discarded and cell pellets were washed twice with 5 mL of 0.03 M phosphate buffer, was added and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. β-Gal activity was then assayed according to the method of (Nagy et al., 2001). The reaction mixture consisted of 0.5 mL of enzyme source (cells treated with lysozyme) and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37 ºC, 2 mL of 0.1 M sodium carbonate was added to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of enzyme that catalysed the formation of 1 µmol of o-nitrophenyl from ONPG per min under the assay condition.

5.4 Enumeration of micro-organisms

To enumerate *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, MRS agar supplemented with 1% (w/v) D-glucose was used. Peptone and water 0.15% (w/v) diluent was used to perform serial dilutions. Plates were incubated at 37 ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

5.5 Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

5.6 Results and discussions

5.6.1 Effect of nitrogen source on the production of β-gal

The influence of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 5.1 and 5.2.

In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference ($p<0.05$) in β-gal in all nitrogen sources. *B. animalis* Bb12 produced higher ($p<0.05$) β-gal in peptone, yeast extract and casein hydrolysate compared with other nitrogen sources at 24 h (Figure 5.1). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) β-gal production in yeast extract and casein hydrolysate than the other nitrogen sources (Figure 5.2).

Statistically, *B. animalis* Bb12 had significantly different ($p>0.05$) β-gal production at 24 h between casein hydrolysate, ammonium sulphate and others nitrogen sources. However, significant difference ($p<0.05$) was found between casein hydrolysate, tryptone and other nitrogen sources; yeast extract, peptone, casein hydrolysate and other nitrogen sources; and peptone, casein hydrolysate, tryptone and other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different ($p>0.05$) β-gal production at 24 h between ammonium sulphate, whey and other nitrogen sources. However, significant difference ($p<0.05$) was found amongst yeast extract, whey and other nitrogen sources; yeast extract, peptone, casein hydrolysate, whey and rest of nitrogen sources; and whey between other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

B. animalis Bb12 produced the highest amount of β-gal (45.69 Unit/mL) with peptone followed by (43.44 Unit/mL) with yeast extract and lowest activity (33.0 Unit/mL) with whey

(control) at 24 h (Figure 5.1). The β-gal production increased ($p<0.05$) by 126.01, 149.40, 79.35, 64.63, 86.14, and 64.49 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, at 24 h as compared with 0 h (Figure 5.1).

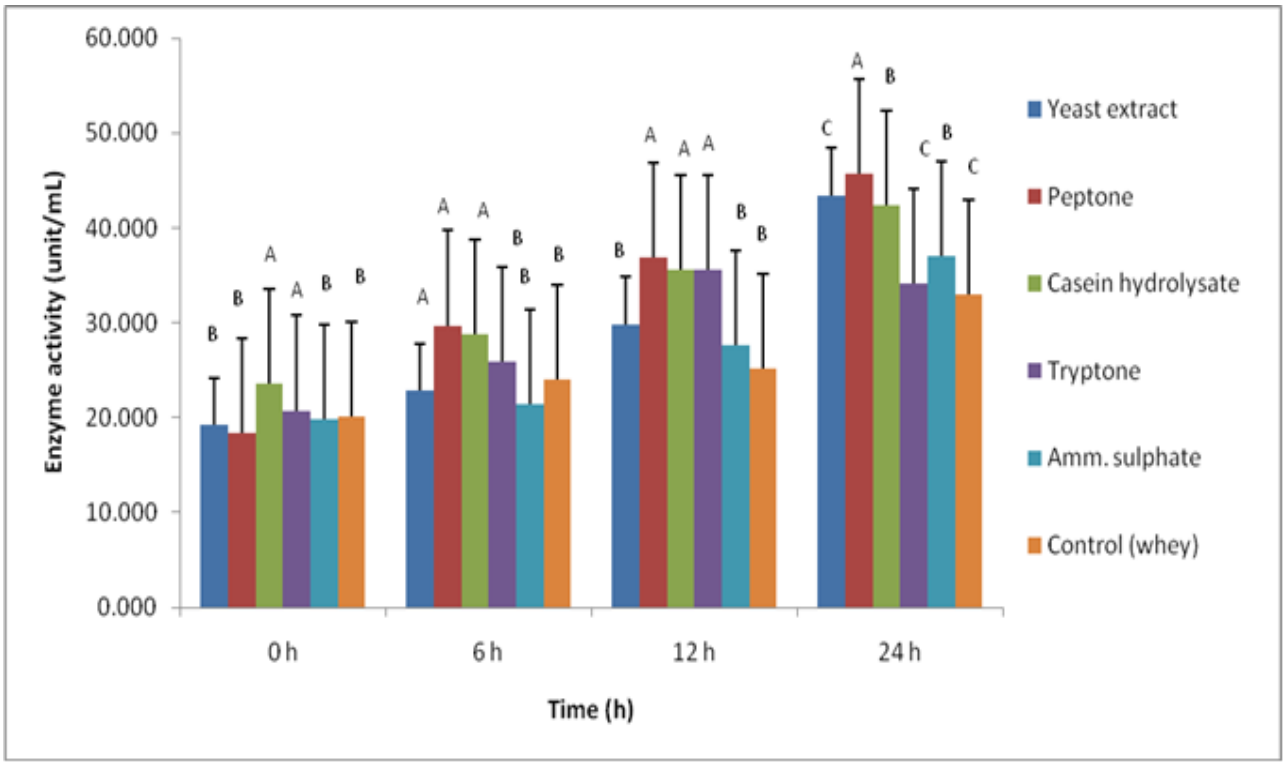

Figure 5. 1: Effect of nitrogen source on the β-galactosidase production by B. animalis Bb12

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$).

Peptone provided optimum nutrients for this organism. Hence, the organism produced the highest level of β-gal. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of β-gal (46.6 Unit/mL) with casein hydrolysate followed by yeast extract (46.25 Unit/mL) and lowest activity (31.8 Unit/mL) with whey (Figure 5.2). At 24 h, the β-gal production increased ($p<0.05$) by 90.09, 91.20, 78.90, 123.74, 65.06 and 86.33 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey respectively as compared with 0 h (Figure 5.2).

Our results are also in line with the results reported by (Mahoney et al., 1975), in which

maximum β-gal activity was achieved at about 22 h incubation period. Most of the available literature suggests the optimal fermentation time in the range of 20-36 h (Mahoney et al., 1975; Ku and Hang, 1992; Ranzi et al., 1987).

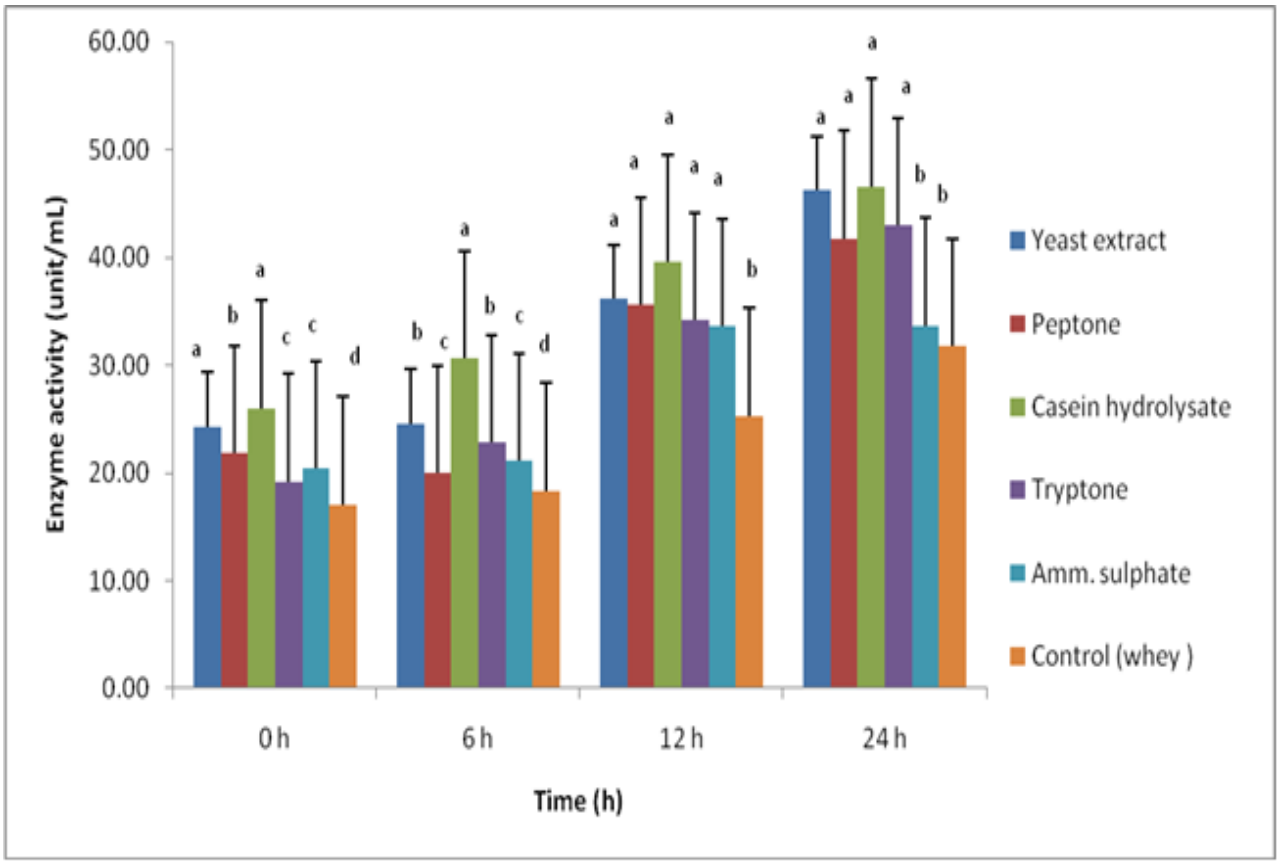

Figure 5. 2: Effect of nitrogen source on the β-galactosidase production by L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$).

Furthermore, Bury et al. (2001) study reveals maximum β-gal activity after 15-17 h of grown in whey in yeast extract medium. The β-gal activities of cultures grown with 0.2-0.8% yeast extract were approximately 2.5 times higher than for cultures grown without yeast extract in case of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker, 1970). According to Rao and Dutta (1979) and Shaikh et al., (1997), nitrogen sources may

affect microbial biosynthesis of β-gal. The production of β-gal activity and final population significantly increased as increased the incubation period up to 24 h. This trend is in agreement with the findings of Hsu et al. (2005) in their work on *Bifidobacteria*. The activity of β-gal increased upon extending the incubation period to 22 h. However, any further prolongation of fermentation resulted in a reduction in the activity of β-gal and a reduced final population of the tested organism.

5.6.2 Effect of nitrogen sources on the growth

Figures 5.3 and 5.4 demonstrate the effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significant difference ($p<0.05$) in the viable counts in all nitrogen sources.

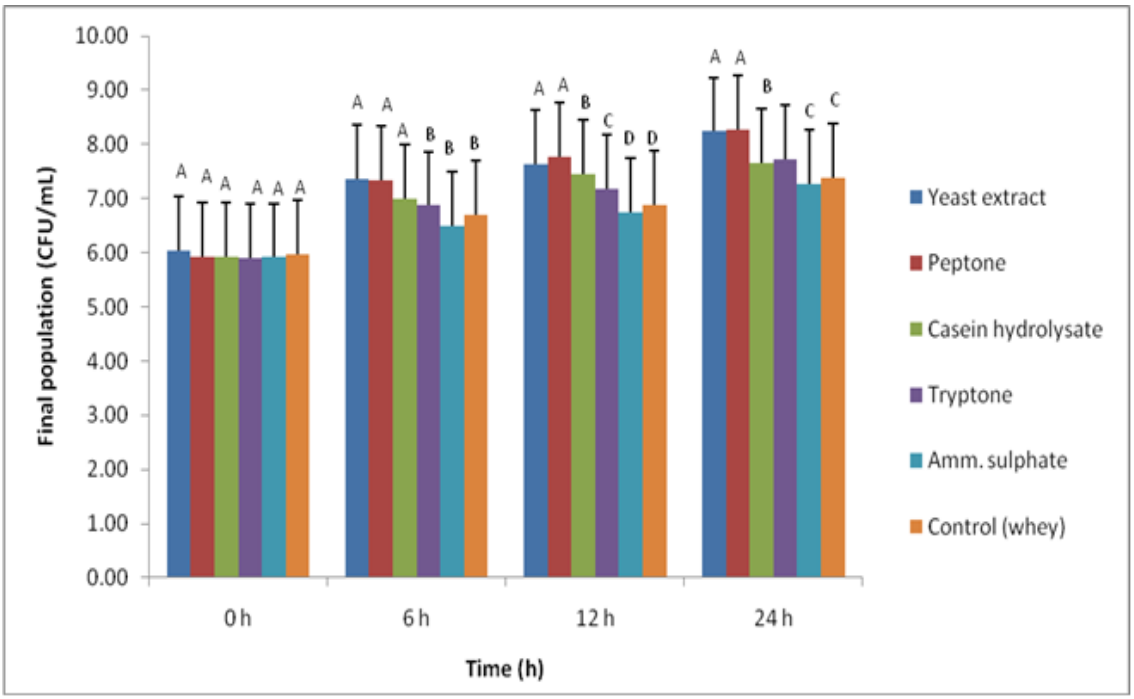

Figure 5. 3: Effect of nitrogen source on the viable population by B. animalis Bb12.

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters at each incubation time indicate significant difference ($p<0.05$).

The viable counts were higher ($p<0.05$) in yeast extract and peptone than others nitrogen source including yeast extract, peptone, tryptone and ammonium sulphate in both organisms (Figures 5.3 and 5.4). Statistically, *B. animalis* Bb12 had no significantly different ($p>0.05$) the viable count at 0 h in various nitrogen sources. However, significant difference ($p<0.05$) was found between yeast extract, peptone, casein hydrolysate and other nitrogen sources; yeast extract, casein hydrolysate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 6 h; 12 h; and 24 h, respectively.

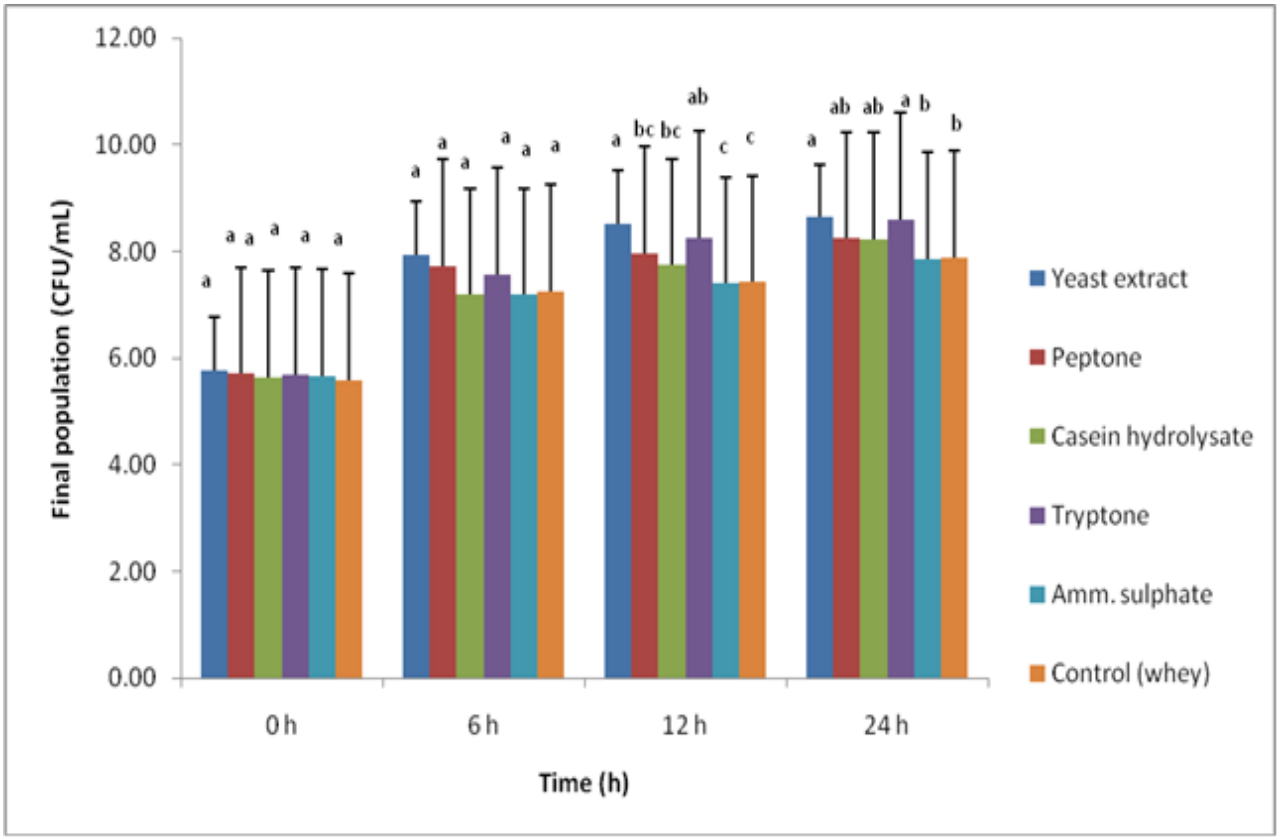

Figure 5. 4: Effect of nitrogen source on the viable population by L. delbrueckii spp. bulgaricus ATCC 11842.

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters at each incubation time indicate significant difference ($p<0.05$).

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed no significantly different ($p>0.05$) the viable count at 0 h and 6 h in various nitrogen sources including yeast extract,

peptone, casein hydrolysate, tryptone, ammonium sulphate and whey. However, significant difference ($p<0.05$) was found between yeast extract, tryptone and other nitrogen sources; yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen source; at 12 h and 24 h, respectively. The final viable population of the *B. animalis* Bb12 ranged from 5.91 to 8.27 log CFU/mL and the organism showed the highest viable population of 8.27 log CFU/mL at 24 h with peptone followed by yeast extract 8.24 log CFU/mL and lowest with ammonium sulphate 7.26 log CFU/mL. At 24 h, the viable count increased ($p<0.05$) by 36.42, 39.70, 29.01, 30.80, 22.84 and 23.83 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, compared with 0 h (Figure 5.3).

Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 5.59 to 8.64 log CFU/mL and the organism showed the highest viable population of 8.64 log CFU/mL at 24 h with yeast extract followed by tryptone 8.59 log CFU/mL and lowest with whey 7.87 CFU/mL. The viable count increased ($p<0.05$) by 49.48, 44.31, 46.10, 51.23, 39.05 and 40.97 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 5.4). Yeast extract provided optimum nutrients for this organism. Hence, the organism produced the highest viable count ($p<0.05$). The rate of growth by bacteria grown in whey can be increased by addition of whey protein concentrate (Bury et al., 1998), yeast extract (Gupta et al., 1995). However, effectiveness of the supplementation of these nutrients for the production of β-gal has not been studied to any great extent. Supplementation of nitrogenous sources especially yeast extract increases the amount of nutrients available to the bacteria, which could explain the increase in the viable population of the organisms. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker, 1970). According to Rao and Dutta (1979) and Shaikh et al., (1997), nitrogen sources may

affect microbial biosynthesis of β-gal.

Supplementation of nitrogenous sources especially yeast extract increases the amount of nutrients available to the bacteria, which could encounter for an increase in the viable population of the organisms. This may also be attributed to the presence of specific growth factors in addition to nitrogen compounds present in yeast extract (Bridson and Brecker, 1970). Kenneth (2009) also reported that a selective medium could also promote the growth of organisms after adjusting the physical conditions of a culture medium such as pH and temperature.

5.6.3 Effect of nitrogen sources on the pH

The effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 5.5 and 5.6. In general, pH value of the medium containing yeast extract and peptone was lower ($p>0.05$) compared with other nitrogen sources for both organisms.

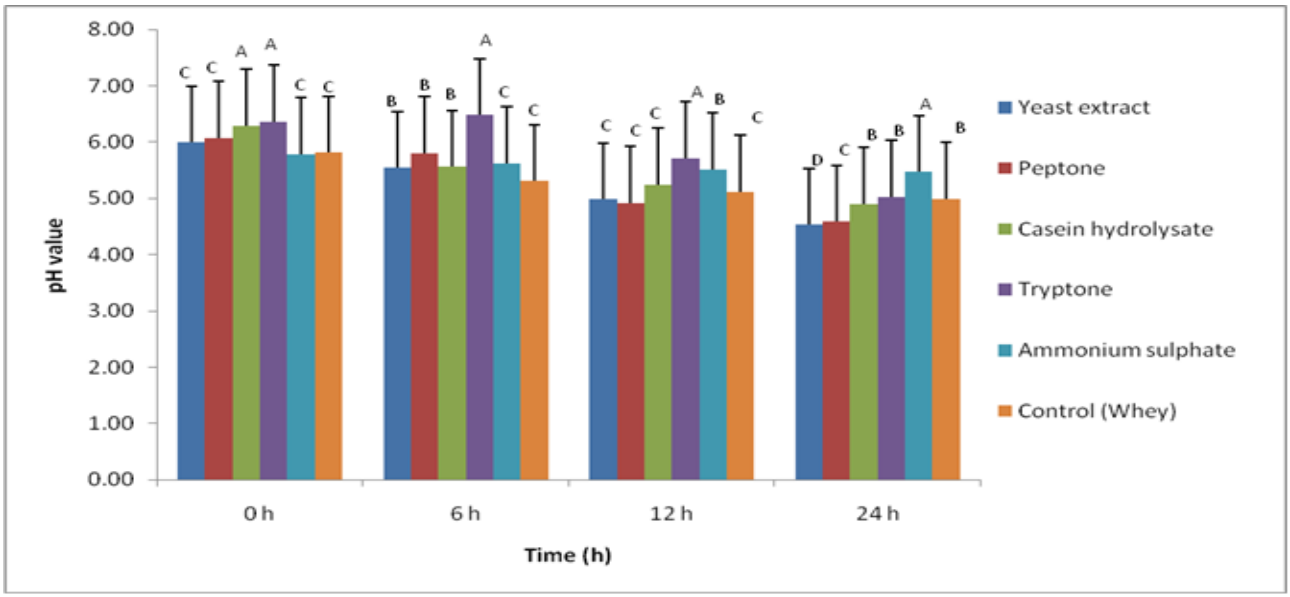

Figure 5. 5: Effect of nitrogen source on the pH by B. animalis Bb12.

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$).

The pH value was significantly ($p<0.05$) higher in tryptone among other nitrogen sources in both organisms. *B. animalis* Bb12 had significantly different ($p>0.05$) pH values at all fermentation times. However, significant difference ($p<0.05$) was found between yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources; yeast extract, tryptone, ammonium sulphate and other nitrogen sources; tryptone, ammonium sulphate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 0 h, 6 h; 12 h; and 24 h, respectively. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different ($p>0.05$) pH values at all fermentation times. The significant difference ($p<0.05$) was found at 0 h and 6 h in all nitrogen sources.

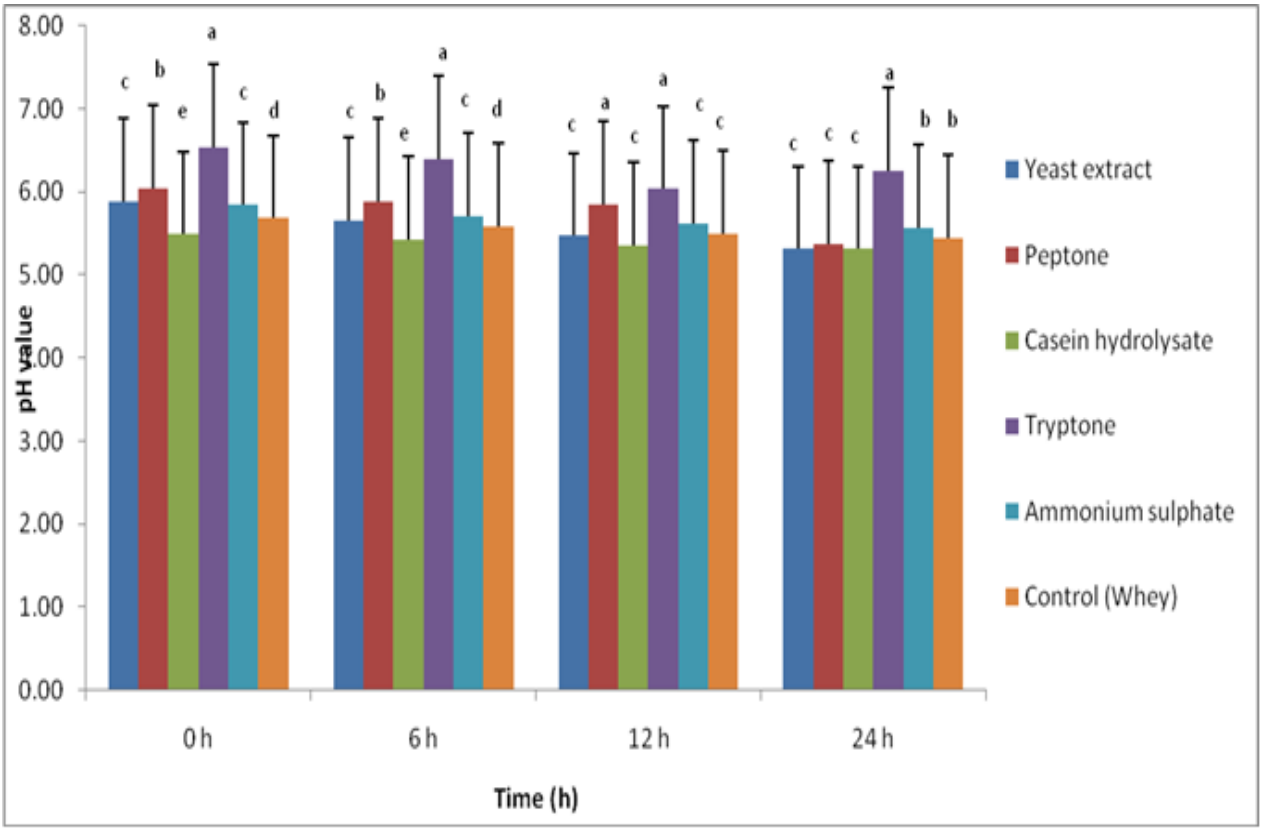

Figure 5. 6: Effect of nitrogen source on the pH by L. delbrueckii ssp. bulgaricus ATCC 11842.

Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$).

However, significant difference was recorded between yeast extract, peptone, ammonium sulphate and rest of nitrogen sources; yeast extract, peptone, casein hydrolysate, ammonium sulphate and other nitrogen sources at 12 h and 24 h, respectively.

The decrease in pH by *B. animalis* Bb12 was lowest with yeast extract (4.53) followed by peptone (4.59) and highest with ammonium sulphate (5.47). The pH value decreased ($p<0.05$ by 24.37, 24.51, 22.10, 20.91, 5.53 and 14.26 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 5). Similarly, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with yeast extract and casein hydrolysate (5.31) followed by peptone (5.37) and highest with tryptone (6.26). At 24 h, the pH value decreased ($p<0.05$ by 11.09, 3.10, 4.13, 4.62, and 4.23 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively as compared with 0 h (Figure 5.6). The drop in pH correlated with an increase in population of the two organisms.

The decline in pH was correlated with an increase in population of the two studied organisms. Similarly, Gerald et al. (1992) reported that while decreasing pH of the growth medium, the level of β-gal activity simultaneously increased with growth of organisms. The higher level of β-gal activity was observed in our study during lowering pH down to 5.5. At this pH the β-gal activity appeared to be at its optimum.

The production of lactic acid is influenced by the culture conditions such as the type of nitrogen and carbon sources as the nutritional requirements of lactic acid bacteria, and other parameters such as pH and temperature (Wee et al., 2006). The level of pH affects functioning of enzymes and transport of nutrients across the cell wall for the growth of organism (Klovrychev et al., 1979). Norton et al. (1993) studied on the effect of pH level on the culture performance of *L. delbrueckii* ssp. *bulgaricus* in a medium supplemented with

nitrogen sources and the organism showed the maximum viable population was obtained at pH 5.5.

In the current study, the various nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey were evaluated on the effect of pH level by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed the highest viable population of 8.64 log CFU/mL at 24 h with yeast extract and pH value of the medium containing yeast extract was lower ($P<0.05$) compared with other nitrogen sources. This finding was similar to that of Murad et al. (1992), who reported that yeast extract was considered to be an essential nutrient for the growth of *lactobacilli.*

5.7 Conclusion

The addition of nitrogen sources (especially yeast extract and peptone at 3.5%) in whey can increase the β-gal by these organisms. Whey medium supplemented with nitrogen sources could be suitable for fermentation for *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In addition, the high yield of β-galactosidase with *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 may be potential organisms for the production of β-gal.

Chapter 6.0 Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842[4]

[4] A version of this chapter has been published. Prasad, L. N. and N. P. Shah. 2011. Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842. International Food Research Journal. 22: 610-617.

6.1 Introduction

Soy protein isolate (SPI) is made from defatted soy meal by removing fat and carbohydrates, resulting in a product containing 90% protein. Soymilk made from soy protein isolate (SPI) has no undesirable flavour (Shurtleff and Aoyagi, 1984). However, soymilk made from SPI has reduced level of biologically active isoflavone due to losses during protein isolation (Wang and Murphy, 1996). Soymilk generally contains a total of 4 to 12 mg isoflavones per 100 g (King and Bignell, 2000; Tsangalis et al., 2002) but is subject to considerable variation in isoflavone content and composition (Murphy et al., 1999; King and Bignell, 2000).

The phytoestrogens found abundantly in soybeans consist of the di-phenolic, isomeric family of compounds named isoflavones. Soybean and soy-food derived isoflavones are found in 4 chemical forms, including aglycones, malonyl-, acetyl-, and glucoside conjugates. The biologically active, estrogen-like isoflavone isomers are the aglycone configurations of genistein, daidzein, and glycitein (Setchell and Cassidy, 1999). Aglycone isomers are able to bind to estrogen receptor sites and hence mimic the functions of estradiol in the human body (Setchell, 1998; Setchell and Cassidy, 1999). From reviews of epidemiological (Cassidy, 1996; Setchell, 1998) and small-scale human clinical studies, isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders common in current Western civilizations. Genistein has been demonstrated to promote the health of human beings by reducing the occurrence of specific chronic diseases, namely, cancer and atherosclerosis (Lee et al., 1991; Witztum, 1994). Daidzein and genistein have been documented to have beneficial effects on osteoporosis (Anderson et al., 1987). Soy isoflavones have also been shown to relieve menopause symptoms (Aldercreutz et al., 1992). Asian populations with their high intake (50 to 70 mg/d) of soy-derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms, and mortality

from cardiovascular disease and cancer. According to Murphy et al. (1999) and Tsangalis et al. (2002) of the total concentration of isoflavones in soymilk greater than 90% of the isomers exist as glucosidic forms. Izumi et al., (2000) found that aglycone forms were absorbed faster and in greater amounts than their glucosides in humans. Furthermore, Setchell et al., (2002) reported that isoflavone glucosides were not absorbed through the human gut wall, and their bioavailability required initial hydrolysis of the sugar moiety by intestinal β-glucosidases. This suggests that consuming isoflavone aglycone-rich soy foods may be more effective in preventing chronic diseases.

Tochikura et al. (1986) reported that probiotic microorganisms possess β-glu, β-gal and α-galactosidase which play an important role in the hydrolysis isoflavone glycosides to the bioavailable aglycones forms.

To deconjugate IG to biologically active IA, the β-glucosidic linkage between the β-glycoside and aglycones need to be broken. Several scientists have reported on the transformation of IG to IA by microbial fermentation (Tsangalis et al., 2002; Otieno et al., 2006). The bacteria such as *Bifidobacterium* produce β-galactosidase (β-gal) in addition to β-glu (Shah & Jelen, 1990). According to Shah and Jelen (1990), *Lactobacillus delbrueckii ssp. bulgaricus* ATCC 11842 and *S. thermophilus* have been found to produce very high level of β-gal. Therefore, the current study was directed towards production of the enzyme using low cost medium such as whey supplemented with affordable nitrogen sources. Our results were in line with the results reported by Mahoney et al. (1975) who showed that the maximum β-gal activity was achieved at about 22 h of the incubation period. Furthermore, Bury et al. (2001) study revealed that the maximum β-gal activity was obtained after 15-17 h of grown in whey in yeast extract medium. It was shown that β-gal could hydrolyse α- galactosidic bond in α-lactose (Huber et al., 1981). Therefore, it is possible that β-gal is also responsible for the biotransformation of IG to IA.

The β-gal has superior activity for hydrolysing acetyl-glycoside and malonyl-glycoside isoflavones. If β-gal can effectively convert acetyl-glycoside and malonyl-glycoside to their aglycones, it can lead to an enhancement of isoflavone aglycones is oy protein isolate. Probiotic microorganisms possess β-glu, β-gal and α-galactosidase (Tochikura et al., 1986), which play an important role in the hydrolysis isoflavone glycosides to the bioavailable aglycones forms.

Shah and Lankaputhra (2002) have reported that the genus *Bifidobacterium* constitutes a major part of the natural microflora of the human intestinal tract. Tsangalis et al. (2004) revealed that *B. animalis* Bb12 hydrolysed isoflavone glucosides into aglycones when grown in soymilk increasing the concentration of aglycones from 8 to 50% of total isoflavones. In human clinical studies, *B. animalis* Bb-12 has shown to effectively modulate intestinal microflora (Playne, 2002). LB grows well at elevated temperatures with a reported optimum 45 ºC. This organism is considered a high β-gal producer (Vasiljevic and Jelen, 2001). Our objective was to examine the effectiveness of crude enzyme extract from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in biotransformation of glycitin, an isoflavone glycoside (IG) to their aglycones in soymilk made from SPI.

6.2 Materials and Methods

6.2.1 Isoflavone compounds and other chemicals

Genistein, daidzein, and flavone were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Glycitin, Formononetin, and Biochanin A were obtained from Indofine Chemical Co. (Summerville, N.J., U.S.A.). Acetonitrile, methanol, ethanol, and phosphoric acid used for HPLC were of analytical grade. Soy protein isolate SUPRO 590 was from The Solae Co. (Chatswood, NSW, Australia).

6.2.2 Bacterial growth and media

The organisms were activated by two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) incubated at 37 ºC for *B. animalis* Bb12, and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The activated cultures were again inoculated into MRS broth and inoculated at 37 º C for *B. animalis* Bb12 and 45 º C for *L. delbrueckii* spp. *bulgaricus* ATCC 11842 for 18 h. The third transfer was carried out in 4% (w/v) SPI containing 2.0 % (w/v) D-glucose prepared as per Tsangalis et al., (2002). SPI, supplied by Sanitarium Health Food Co. (Cooranbong, N.S.W., Australia), was used in the production of soymilk at a ratio of 40 g per 1 L ultra-pure distilled water. For reconstitution, distilled water was heated to 40 °C prior to the addition of SPI powder, followed by heating the mixture at 50 to 60 °C for 30 min with stirring to disperse solid particles. After cooling to room temperature, the pH was adjusted to 6.7 using 5 M sodium hydroxide. The entire volume of soymilk was sterilized by autoclaving at 121 °C for 15 min. For production of extract crude enzyme, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 × g for 20 min at 10 ºC). The supernatant was discarded and cell pellets were collected. A total of 5 mL of 0.03 M sodium phosphate buffer (pH 6.8) was added and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme.

6.2.3 Enumeration of viable microorganisms

One millilitre sample was used for the enumeration of populations of *B. animalis* Bb12

and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. MRS agar supplemented with 1% (w/v) D-glucose was used for enumeration of the organisms. Peptone water at 0.15% (w/v) diluent was used to perform serial dilutions. One millilitre of serially diluted samples at 0, 6, and 12 h was aseptically spread onto the plates and incubated at 37 ◦C for *B. animalis* Bb12 and 45 ◦C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per gram of sample.

6.2.4 Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (Merk Pty Limited, Kilsyth, Vic, Australia) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

6.2.5 Isoflavone standard solution and calibration curves

Stock solutions of isoflavone standards such as glycitin, daidzein, genistein, biochanin A and formononetin were prepared by dissolving 1 g of crystalline pure compound in 10 mL of 100% methanol. Each solution was diluted with methanol (100%) to 5 working solutions at concentration ranging from 1 to 40 µg/mL in order to prepare a standard curve. Retention time and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavones.

6.2.6 Determination of isoflavone content

6.2.6.1 Extraction of isoflavones for HPLC analysis

The extraction of isoflavone aglycones and glucoside isomers and HPLC analysis was performed in triplicate based on Griffith and Collison, (2001) and Nakamura et al. (2001) with some modifications as described in Pham and Shah (2007). Briefly, 10 mL of methanol (80%, v/v) and 1 mL of acetonitrile (100%, v/v) were added to 1 g of freeze-dried sample with stirring using a vortex mixer (Chiltern Scientific, Auckland, New Zealand). The sample was freeze dried after harvesting the cells by centrifugation. The supernatant was discarded and cell pellets were collected. Extracted crude enzymes were frozen in the freezer at -20^{o}C before freeze-drying (Airvac Engineering Pty. Ltd., Rowville, VIC, Australia). All samples were spread in Petri-dishes and allowed to freeze up to 72 h at a temperature between -55 and -60^{o}C. In addition, 100 μL each of Carrez I and Carrez II solutions were added to the samples and mixed thoroughly. Furthermore, 100 μL of flavone (1 mg/mL) as the internal standard was added followed by thorough shaking. The samples were left in a water bath (model NB 6T-10935, Thermoline Australia) at 50◦C for 2 h until the proteins precipitated. The samples were then filtered through a Whatman No. 3 filter paper and a 0.45 μM Phenomenex nylon filter into an HPLC vial then injected into HPLC system within 4 h to avoid the degradation (Griffith and Collison, 2001). The HPLC system included an Alltech Alltima HP C18 HL (4.6 × 250 mm), a 5-μm particle size column and an Alltima HP C18HL (7.5 × 4.6 mm), a 5 μm guard column, Hewlett Packard 1100 series HPLC with an autosampler, a quaternary pump, a diode array ultraviolet detector, a vacuum degasser, and a thermostatically controlled column compartment. Mobile phase consisted of solvent A (water: phosphoric acid, 1000:1, v/v) and solvent B (water: acetonitrile: phosphoric acid, 200:800:1, v/v/v). The gradient was as follows: solvent A 100% (0 min) → 80% (5 min) → 0% (50 min) → 100% (55 min) → 100% (60 min). The flow rate was 0.8 mL/min. A diode array UV detector was set at 259 nm.

Isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). The moisture content of the freeze-dried soymilk samples was determined by AACC

40-40 (AACC 2000) methods. The biotransformation of IG to IA was defined as percentage of IG hydrolyzed and was calculated as follows:

Percent glycitin (IG) hydrolysis =

$$\frac{\text{initial glycitin} - \text{residual glycitin}}{\text{initial glycitin}} \times 100$$

6.2.7 Statistical Analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

6.3 Results and discussion

6.3.1 pH changes during incubation

The effect of change of pH in soy protein isolate during incubation as affected by growing *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 6.1 and 6.2. In general, pH value of 1.0 g/L crude enzyme extract (CEE) was lower ($p>0.05$) compared with other crude enzyme concentrations for both organisms.

The pH value was significantly ($p<0.05$) higher in control (without crude enzyme extract) and 0.1g/L of CEE than the others in both organisms. Both organisms showed no significantly different ($p>0.05$) pH values at 0 h and 6 h at different crude enzyme concentrations including control and a significant difference ($p<0.05$) was found at 12 h between 1.0 g/L and 0.5g/L CEE than the others. The decrease in pH by *B. animalis* Bb12 was lowest with 1.0 g/L (4.35) followed by 0.5 g/L (4.45) and the highest with the control (4.69). The pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82 percent in control,

0.1g/L, 0.5g/L and 1.0g/L CEE respectively, at 12 h as compared with 0 h (Fig. 6.1).

Similarly, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with 1.0 g/L (5.19) followed by 0.5 g/L (5.52) and highest with the control (5.86). At 12 h, the pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82% in control, 0.1g/L, 0.5g/L and 1.0g/L CEE respectively, at 12 h as compared with 0 h (Fig. 6.2). The drop in pH correlated with an increase in population of the two organisms (Tables 6.1 and 6.2).

6.3.2 Viable counts of *Lactobacillus* and *Bifidobacterium* during incubation

Tables 6.1 and 6.2 demonstrate the viable count of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in soymilk prepared from soy protein isolate. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) viable counts at 1.0 g/L enzyme concentration during the entire incubation. *B. animalis* Bb12 showed a significant difference ($p>0.05$) in the viable count at 0 h, 6 h and 12 h at different enzyme concentrations including control and a no significant difference ($p<0.05$) was found within crude enzyme concentrations at 0 h, 6 h and 12 h (Table 6.1).

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different viable counts at 0 h and 12 h; however, there was no significant difference ($p<0.05$) in viable counts between 0 and 6 h (Table 6.2). Moreover, there was no significant difference ($p<0.05$) within enzyme concentrations including control at 0 h, 6 h and 12 h. The final viable population of the *B. animalis* Bb12 ranged from 6.9 to 7.4 log CFU mL^{-1} and the organism showed the highest viable population of 7.4 log CFU mL^{-1} at 12 h with 1.0 g/L CEE followed by 0.5g/L of crude enzyme at 7.3 log CFU mL^{-1} and lowest with control at 6.9 log CFU mL^{-1}.

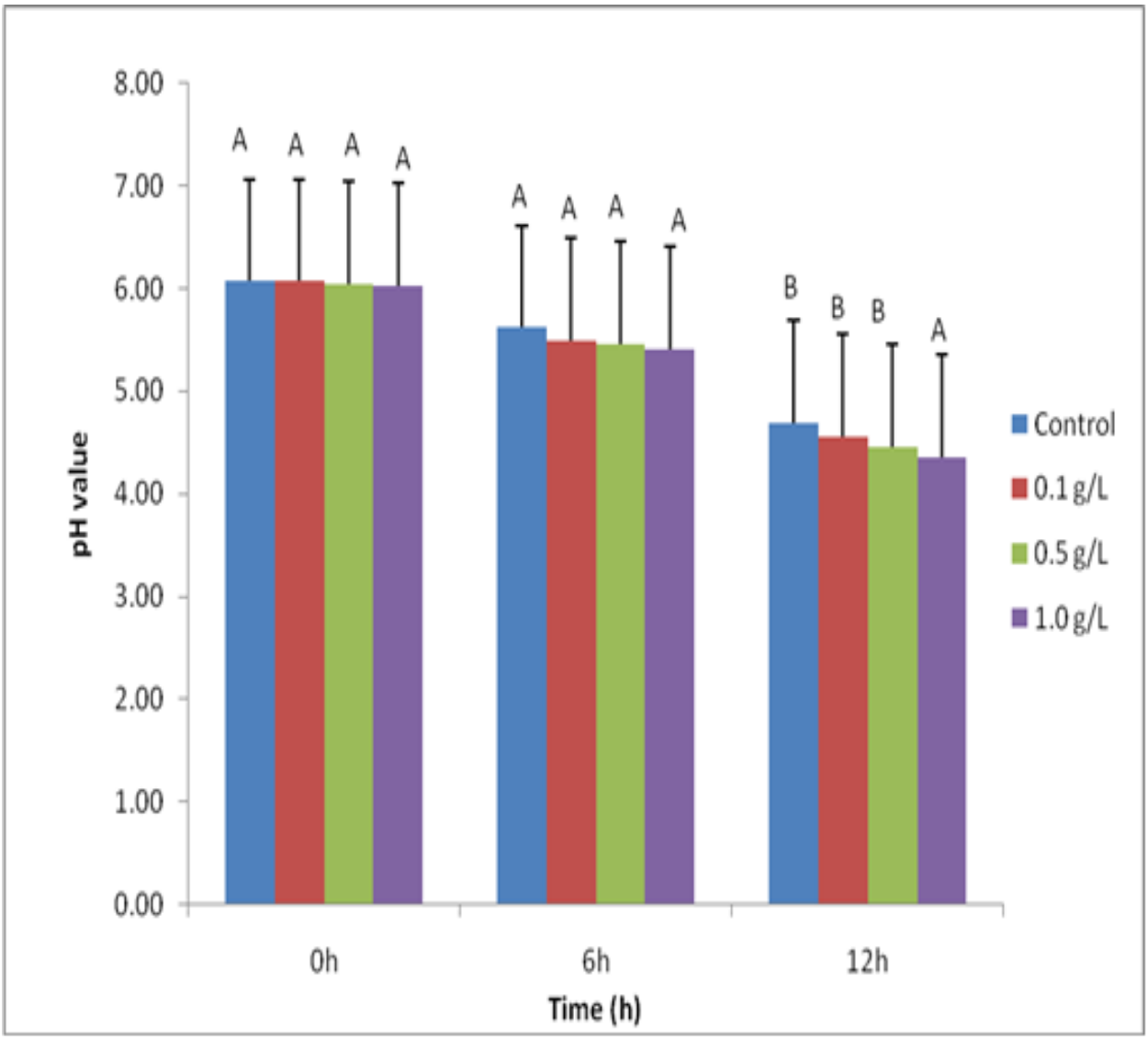

Figure 6. 1: pH value of soymilk during 12 h fermentation by B. animalis Bb12 at 37 ◦C. Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Different capital letters at each incubation time indicate significant difference ($p<0.05$)

At 12 h, the viable count increased ($p<0.05$) by 17.31, 20.36, 20.51 and 20.10% in control, 0.1g/L, 0.5g/L and 1.0g/L CEE, respectively, at 12 h as compared with 0 h (Table 6.1). Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.1 to 6.7 log CFU mL^{-1} and the organism showed the highest viable population of 6.7 log CFU mL^{-1} at 12 h with 1.0 g/L crude enzyme followed by 0.5 g/L crude enzyme 6.4 log CFU mL^{-1} and lowest with control 6.1 CFU mL^{-1}.

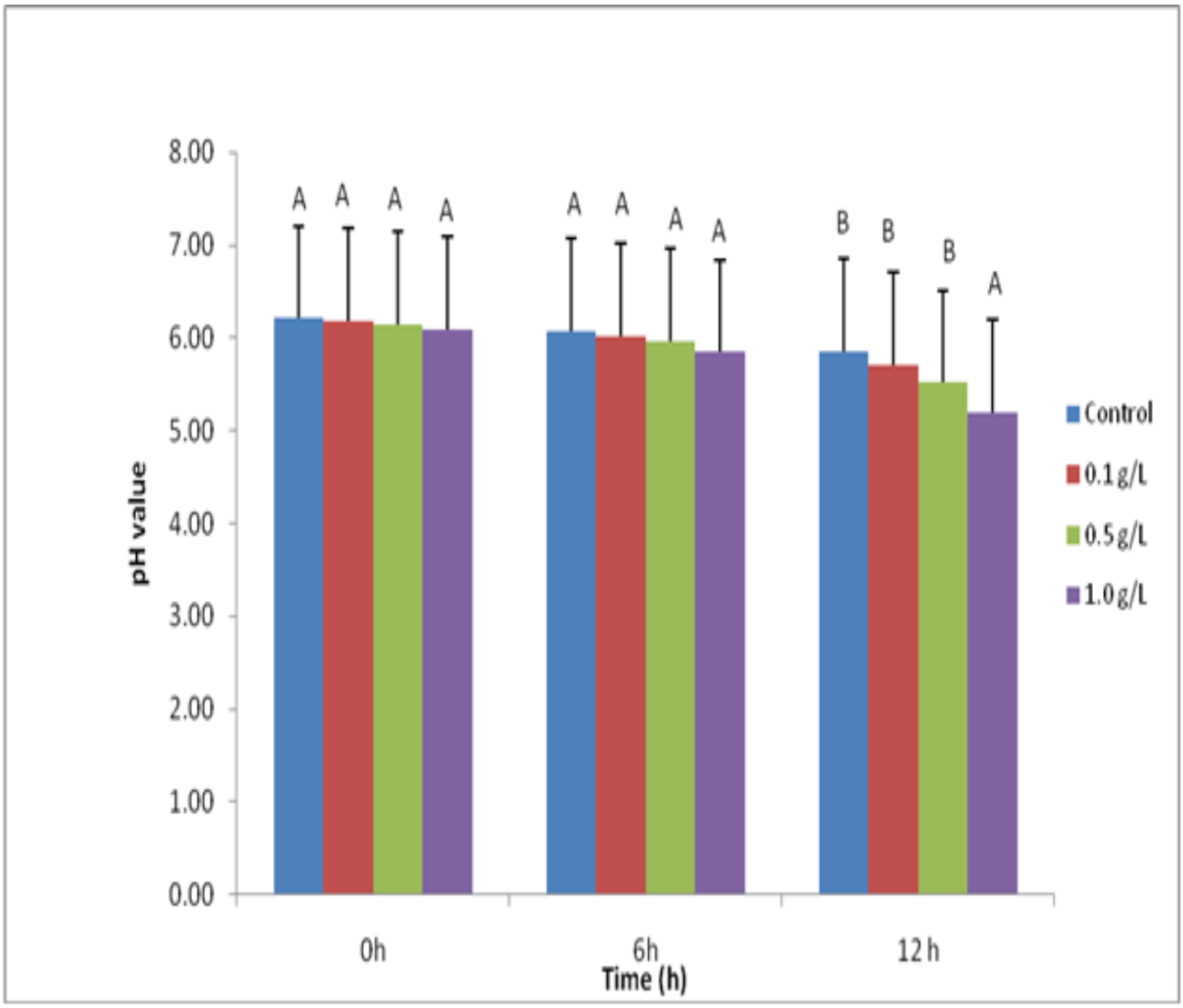

Figure 6. 2: pH value of soymilk during 12 h fermentation by L. delbrueckii ssp. bulgaricus ATCC 11842 at 45 ◦C. Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Bars indicate standard deviations. Different capital letters at each incubation time differed significantly ($p<0.05$)

The viable count increased ($p<0.05$) by 40.18, 40.44, 36.56, and 35.84% in control, 0.1g/L, 0.5g/L and 1.0g/L CEE, respectively, at 12 h as compared with 0 h (Table 6.2). Soymilk could not appeared to support the growth of *Bifidobacterium and Lactobacillus*, possibly due to the low amount (less than 1%) of simple carbon compounds in SPI, including sucrose, raffinose, and stachyose, which have been removed during processing (Nutrition Data, 2007).

Table 6. 1: Viable microbial counts (log CFU mL-1) of B. animalis Bb12 in soymilk during 12 h fermentation at 37 ◦C

	Enzyme concentration (g L^{-1})			
Time	Control	0.1	0.5	1.0
0h	5.9 ± 0.01^{Ab}	6.00 ± 0.02^{Ac}	6.1 ± 0.03^{Ac}	6.2 ± 0.06^{Ac}
6h	6.5 ± 0.03^{Ab}	6.4 ± 0.27^{Ab}	6.8 ± 0.03^{Ab}	6.9 ± 0.01^{Ab}
12h	6.9 ± 0.02^{Ba}	7.2 ± 0.01^{Aa}	7.3 ± 0.02^{Aa}	7.4 ± 0.03^{Aa}

Results are expressed as mean ± SE (n=3). Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme. Data were analysed by means of 1-way ANOVA. A-B Means values in the same row with the same capital superscripts are not significantly different (P > 0.05). a-c Mean values in the same column for a particular organism with the same lowercase letter are not significantly different (P > 0.05).

Table 6. 2: Viable microbial counts (log CFUmL-1) of L. delbrueckii ssp. bulgaricus ATCC 11842 fermentation at 37 ◦C in soymilk during 12 h

	Enzyme concentration (g L^{-1})			
Time	Control	0.1	0.5	1.0
0h	4.4 ± 0.03^{Ab}	4.4 ± 0.06^{Ab}	4.7 ± 0.04^{Ab}	4.9 ± 0.01^{Ab}
6h	6.0 ± 0.10^{Ba}	6.0 ± 0.22^{Ba}	6.3 ± 0.10^{Aa}	6.5 ± 0.09^{Aa}
12h	6.1 ± 0.19^{Ba}	6.2 ± 0.10^{Ba}	6.4 ± 0.21^{Aa}	6.7 ± 0.13^{Aa}

Results are expressed as mean ± SE (n=3). Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme. Data were analysed by means of 1-way ANOVA. A-B Mean values in the same column for a particular organism with the same capital superscripts are not significantly different (P > 0.05). a-b Mean values in the same column for a particular organism with the same lowercase letter are not significantly different (P > 0.05).

According to Shah (2006), the mild acidic condition of soymilk during fermentation (pH 6.15 to 6.80) was still in a favourable range for the growth of *Bifidobacterium* could be responsible for maintaining the viability of the probiotic organism. Supplementation of

carbon sources to soymilk especially D-glucose stimulated the growth of *Bifidobacterium*, which could explain why there was an increase in the viable population of the organisms (Briczinski et al., 2006). Based on previous reports, there has appeared to be a correlation between the level of cell population and β-glu activity of LAB and bifidobacteria in soymilk fermentation, indicating that it would be related to the culture growth (Esaki et al. 1994; Choi et al. (2002). Similarly, in the current study, we have found that there was a positive correlation between biotransformation of isoflavone glycosides to isoflavone aglycones and level of viable cells (CFU) in soymilk.

6.3.3 Biotransformation of IG to IA by *Lactobacillus* and *Bifidobacterium* in soymilk

Tables 6.3 and 6.4 show the biotransformation of IG (glycitin) to IA (daidzein and genistein) in soymilk by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and their hydrolytic potential during fermentation at 37 °C for *B. animalis* Bb12, and 45 °C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The concentration of glycitin was recorded in decreasing trend whereas concentrations of daidzein and genistein were in increasing trend in soymilk during hydrolysis by microbial enzymes. The percent of moisture content of freeze dried samples ranged from 1.78 to 2.2. The isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). There were no significant differences ($P > 0.05$) in the moisture contents of the freeze-dried samples. Therefore, it is believed that there was no effect of the moisture content on the quantification of isoflavone compounds.

In general, there was only one IG (glycitin) and four IA (daidzein, genistein, biochanin A and formononetin) were used to determine the quantification of IG and IA in the soymilk sample at 0 h, 6 h and 12 h. Isoflavone concentrations of glycitin, daidzein and genistein were detected in different CEE concentrations including 0.1g/litre, 0.5g/litre and 1.0g/litre and

control at entire incubation. Biochanin A and Formononetin were not detected in soymilk in different CEE concentration and control. This also suggests their glycosides forms (sissotrin and ononin, respectively) were not available in SPI.

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference in glycitin at 0 h, 6 h and 12 h; however, there was no significant difference in hydrolysis ($p>0.05$) between different CEE concentrations and control. Moreover, daidzein and genistein showed a significant difference ($p<0.05$) in both organisms in all incubations as well as different CEE concentrations and control at 0 h, 6 h and 12 h.

B. animalis Bb12 produced glycitin at 9.41, 11.80, 8.11 and 13.83 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 6.32, 8.42, 8.11 and 5.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h and 3.47, 4.98, 5.42 and 3.52 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of glycitin was found at 13.83 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 0 h. Similarly, daidzein was produced at 23.92, 22.64, 25.57 and 26.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 0 h; 25.23, 30.36, 45.49 and 42.33 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 48.05, 52.16, 49.97and 47.81 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of IA in daidzein was found 52.16 mg/100 g of freeze-dried in 0.1 g/litre of CEE at 12 h. Likewise, genistein was produced 17.60, 18.68, 20.71 and 20.42 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 25.26, 27.67, 31.71 and 29.22 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 6 h; and

Table 6. 3: Biotransformation of IG to IA in soymilk by B. animalis Bb12

Isoflavone(mg/ 100 g) of freeze dried sample	Enzyme concentrations (g L^{-1})											
	Control			0.1			0.5			1.0		
	0 h	**6 h**	**12 h**	**0 h**	**6 h**	**12 h**	**0 h**	**6 h**	**12 h**	**0 h**	**6 h**	**12 h**
Glycitin	9.41± 0.01[a]	6.32 ± 0.04[b]	3.47 ± 0.03[c]	11.80 ± 0.02[a]	8.42 ± 0.04[b]	4.98 ± 0.05[c]	13.23 ± 0.01[a]	8.11 ± 0.03[b]	5.42± 0.05[c]	13.83 ± 0.03[c]	5.23 ± 0.01[b]	3.53 ± 0.06[c]
% of glycitin (IG) hydrolysed	0	23.86	26.14	0.0	28.66	57.81	0.0	38.70	59.01	0.0	62.15	74.44
Daidzein	23.92 ± 0.02[c]	25.53 ±0.03[b]	48.05 ±0.08[a]	22.64 ±0.02[c]	30.36 ±0.02[b]	52.16 ±0.01[a]	25.57 ±0.01[c]	45.49 ±0.01[b]	49.97 ±0.01[a]	26.23 ±0.03[c]	42.33 ±0.01[b]	47.81 ±0.12[a]
Genistein	17.60 ±0.01[c]	25.26 ±0.01[b]	31.1 ±0.01[a]	18.68 ±0.03[c]	27.67 ±0.01[b]	32.43 ±0.03[a]	20.71 ±0.05[b]	31.71 ±0.05[a]	30.82 ±0.05[a]	20.42 ±0.03[c]	29.22 ±0.02[b]	35.31 ±0.01[a]

Results are expressed as mean ± SE (n=3). Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme Data were analysed by means of 1-way ANOVA. a-c Mean values at the same treatment and different time have same lowercase letters are not significantly different (P> 0.05).

Table 6. 4: Biotransformation of IG to IA in soymilk by L. delbrueckii ssp. bulgaricus ATCC 11842

Isoflavone(mg/ 100 g) of freeze dried sample	Enzyme concentration (g L^{-1})											
	Control			0.1			0.5			1.0		
	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
Glycitin	7.42± 0.04^{a}	6.04 ± 0.01^{b}	4.76 ± 0.06^{b}	9.15 ± 0.02^{a}	5.41 ± 0.07^{b}	4.56 ± 0.04^{c}	10.55 ± 0.03^{a}	4.51 ± 0.06^{b}	2.8 ± 0.03^{c}	14.26 ± 0.01^{a}	6.51 ± 0.03^{b}	3.53 ± 0.05^{c}
% of glycitin (IG) hydrolysed	0.0	18.55	35.80	0.0	40.88	50.16	0.0	57.22	73.46	0.0	54.34	75.23
Daidzein	21.93 ± 0.01^{b}	23.15 ± 0.01^{a}	19.2 ± 0.01^{c}	21.03 ± 0.01^{c}	24.04 ± 0.01^{b}	25.2 ± 0.01^{a}	20.14 ± 0.01^{c}	23.43 ± 0.01^{a}	22.8 ± 0.01^{b}	22.77 ± 0.01^{a}	21.50 ± 0.01^{c}	22.60 ± 0.01^{b}
Genistein	11.67 ± 0.01^{c}	14.04 ± 0.01^{b}	16.34 ± 0.01^{a}	14.59 ± 0.02^{b}	15.16 ± 0.28^{b}	17.4 ± 0.02^{a}	15.24 ± 0.01^{c}	16.38 ± 0.01^{b}	17.8 ± 0.01^{a}	15.46 ± 0.01^{c}	15.5 ± 0.02^{b}	17.56 ± 0.01^{a}

Results are expressed as mean ± SE (n=3). Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme Data were analysed by means of 1-way ANOVA. a-c Mean values at the same treatment and different time have same lowercase letters are not significantly different (P> 0.05).

31.1, 32.43, 30.82 and 35.31 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of IA in genistein was found 35.31 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 12 h.

On the other hand, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced glycitin 7.42, 9.15, 10.55, and 14.26 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 6.04, 5.41, 4.51, 6.51 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 4.76, 4.56, 2.8, and 3.53 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at12 h. The higher level of glycitin was found 14.26 mg/100 g of freeze-dried in 1.0 g/litre at 0 h. Likewise, daidzein was produced 21.93, 21.03, 20.14 and 22.77 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 23.15, 24.04, 23.43 and 21.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 19.2, 25.2, 22.8 and 22.60 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of daidzein was found 25.2 mg/100 g of freeze-dried in 0.1g/litre of crude enzyme at 12 h. Similarly, genistein was produced 11.67, 14.59, 15.24 and 15.46 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively at 0 h; 14.04, 15.16, 16.38 and 15.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 16.34, 17.4, 17.8 and 17.56 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of genistein was found 17.80 mg/100 g of freeze-dried in 0.5g/litre of CEE at 12 h.

The biotransformation of glycitin occurred higher in *B. animalis* Bb12 (74.44 %) followed by (62.15%) with 1.0 g/litre CEE at 12 h and 6 h, respectively and lowest percent hydrolysis (28.66%) with 0.1 g/litre of CEE at 6 h (Table 6.3). However, the biotransformation of glycitin

was higher for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (at 75.23%) followed by 73.46% with 1.0 g/litre and 0.5 g/litre CEE, respectively, at 12 h and lowest percent of hydrolysed (18.55%) with control at 6 h (Table 6.4). D-glucose appeared to have stimulating effect on the biotransformation by the organism at 12 h. The results suggest that D-glucose allowed the growth of these two organisms (data not shown). The biotransformation of glycitin might be a consequence of high level of viable cells in soymilk. During hydrolysis, the concentration of glycosides such as glycitin reduced while the concentration of aglycones such as genistein and daidzein increased in soymilk fermented by both organisms. The conversion of individual forms of isoflavone glycosides to respective aglycones has been reported by Otieno and Shah (2006a). In addition, low pH condition in soymilk may have also contributed to the increase in the biotransformation level. Delmonte et al., (2006) and Mathias et al., (2006) reported that some IG was partly hydrolyzed to IA in a low pH condition. At 1.0 g/L enzyme concentration in both organisms increased the concentration of aglycones such as genistein and daidzein. This appeared to be related between the level of viable microbial counts and different enzyme concentrations. Our results demonstrated that fermentation of soymilk with lactic acid bacteria or bifidobactera enhanced the content of aglycones, which might be able to bind to estrogen receptor sites and mimic the functions of estradiol. This finding was in agreement with the reports of Esaki et al. (2004) and Choi et al. (2002). Ismail and Hayes (2005) reported that the aglycone was more bioavailable than its respective glycoside, which has led to the development of aglycone enriched products. Delmonte et al., (2006) and Mathias et al., (2006) have also been carried out to determine the optimal processing conditions for the maximum bioconversion of isoflavone form IG to IAs by the application of β-glu. It was noted that the reaction was significantly affected by the amount of enzyme added (Ishihara et al., 2007). The conversion rate increased up to 84.5% at 0.02% enzyme concentration. Beyond this concentration there was no

substantial change in the conversion rate. However, in our study, the biotransformation of glycitin occurred at a higher bioconversion (74.44% by BB12 and 75.23% by LB) at 12 h at 1.0 g/L of crude enzyme. As the study aimed at examining the role of crude enzyme extracts particularly produced by *L. delbrueckii* ssp. *bulgaricus* ATCC 11482 and *Bifidobacterium*, our result thus showed that extracted enzyme enhanced the bioconversion of IG to IAs. However, bacteria such as *Bifidobacterium* produce β-galactosidase (β-gal) in addition to β-glu (Shah & Jelen, 1990). The β-gal acts on the β-galactosidic bond, regardless of the rest of the molecular structure. The β-gal was reported not to be strictly specific to the β- galactosidic bond. It was shown that β-gal could hydrolyse α- galactosidic bond in α-lactose (Huber et al., 1981). Therefore, it is possible that β-gal is also responsible for the biotransformation of IG to IA.

6.4 Conclusions

The result of this study demonstrated that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 are capable of hydrolysing glycitin to biologically active forms in soymilk (SM) prepared from soy protein isolate (SPI) and soymilk supplemented with 2.0 % (w/v) of D-glucose at different concentrations of CEE. The biotransformation rate of glycitin was found to be directly related to the level of enzyme addition with 74.4% biotransformation obtained from 1.0 g/L BB12 enzyme and 75.25% from LB enzyme. The increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of daidzein and genistein in fermented soymilk. Increased daidzein and genistein content in fermented soymilk is likely to improve the biological functionality of soymilk. The crude enzyme extract played a greater role in biotransformation. The biotransformation of glycitin occurred lower in *B. animalis* Bb12 (74.44 %) than the *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (75.23 %).

Chapter 7.0 Influence of Galactooligosaccharides and Modified Waxy Maize Starch on Yogurt's Attributes[5]

[5] A version of this chapter has been accepted in Journal of Food Science. Reference # JFS 2012-0662. Prasad, L. N., F. Sherkat, and N. P. Shah. 2012. Influence of Galactooligosaccharides and Modified Waxy Maize Starch on Probiotic Yogurt's Attributes.

7.1 Introduction

Commercial yogurt is typically produced by a starter culture consisting of *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) (Tamime and Marshall, 1997; Tamime and Robinson, 2007). During fermentation, starter culture bacteria produce proteolytic enzymes which hydrolyze milk proteins and lactase that converts lactose to lactic acid and decreases pH. The conversion of milk to yogurt is an aggregation of casein micelles into a gel structure at a pH of 4.6. The functionality of yogurt is further enhanced by the release of bioactive peptides due to proteolytic activities of the organisms used (Shah, 2007).

Textural and rheological properties are important attributes of yogurt for consumer acceptability. The texture of yogurt is influenced by various factors, e.g. quality and composition of milk and its fat and total solid content, presence of prebiotic ingredients heat treatment of milk, combination of the lactic acid bacteria used, acidification rate and storage time (Dello et al., 2004; Purwandari et al., 2007).

Prebiotics are non-digestible food ingredients that pass through the upper digestive system relatively intact and ferment in the lower colon, thus supporting the growth of colonic microbiota (Lamsal, 2012). There is a considerable interest in preparing yogurt with prebiotics such as inulin, modified waxy maize starch (MWMS) and galactooligosaccharides (GOS) (Tarrega and Costell, 2006; Paseephol et al., 2008 and Paseephol and Sherkat, 2009). These prebiotics are used at 2 to 5% (w/v) of yogurt formulation for improvement in texture and growth of probiotic organisms. Prebiotic like GOS is increasingly being recognized as useful dietary ingredient for the modulation of the colonic microbial balance. Konar et al. (2011) reported that GOS is a key food ingredient that has created better opportunities in the food industry. The GOS is a well known prebiotic compound and its incorporation as food ingredient is safe for manufacturers and

consumers.

Vivinal® GOS syrup is stable under low pH conditions and at high temperatures. Pasteurization and sterilizations at low pH do not affect Vivinal® GOS and the product will maintain its structure, appearance and content. Vivinal® GOS is produced through the enzymatic conversion of lactose (Matsumoto, 1993). It is prepared from edible lactose, isolated from sweet whey. The lactose is subjected to the action of β-gal, which increases the chain lengths by a series of transglycosylation reactions (Mcbain and Macfarlane, 2001).

The GOS is a mixture of octasaccharides composed of 1-7 galactose units linked to a glucose molecule at the reducing end. The major saccharide in the GOS fraction of Vivinal® is the trisaccharide O-β-D-galactopyanosyl-(1-4)-O-β-D-galactopyranosyl-(l-4)-β-D-glucose. The molecular weights of this oligosaccharides range between 342 (disaccharide) and 1,315 (octasaccharide) Daltons, with an average molecular weight of approximately 522 Daltons (Mcbain and Macfarlane, 2001).

It has been reported that GOS selectively stimulates the growth and metabolic activity of the beneficial bacteria in the colon (Roberfroid et al., 1998), where it is fermented and decreases the pH. According to Crittenden and Playne (1996), GOS is used in dairy-based gel systems to modify the flow and textural properties of the final gel.

Among available commercial prebiotics, the Vivinal® GOS is a natural prebiotic rich in galacto-oligosaccharides, structured as chains of galactose with a glucose, produced from glucose molecules transferred from a sucrose donor to a maltose acceptor by a glycosyl-transferase (Crittenden and Playne, 1996).

The modified starches are composed of units of amylase and amylopectin, mostly in amorphous regions on the surface of the granule that are modified without destroying the

granular nature of the starch. This modification improves acid stability, heat stability and shears stability, inhibits gel formation, and controls viscosity during processing (Tecante and Doublier, 1999). The MWMS is used for improving smoothness and texture of dairy products and is considered to be a non-gelatinizing starch that typically gives a cohesive and gummy texture in dairy gel systems (Sprague, 1939). It has also been used in milk to enhance the growth of yogurt cultures (Nielsen et al., 1991). Williams et al. (2004) reported that the addition of MWMS to yogurt decreased syneresis (spontaneous whey separation) but developed a grainy texture. Other reason for using MWMS is to improve the water binding and water holding capacity, heat resistant behavior, improve thickening and to minimize syneresis of yogurt (Miyazaki et al., 2006).

Syneresis is defined as the spontaneous separation of whey from protein matrix which then becomes visible as the surface whey. Ibrahim et al. (2004) reported that the addition of GOS reduced the extent of syneresis in yogurt towards the end of storage. It has been suggested that faster rate of acidification inhibits network rearrangement, thereby resulting in less whey separation (Castillo et al., 2006). Increasing yogurt solids content with skim milk powder is also reported to result in less syneresis (Puvanenthiran et al., 2002; Amatyakul et al., 2006).

This study was undertaken to examine and compare the influence of GOS and MWMS on the growth of ST and LB, their proteolytic activity, and organic acid production and syneresis and firmness of low-fat yoghurt.

7.2 Materials and Methods

7.2.1 Activation of starter cultures

The frozen pure culture of ST was obtained from Chr. Hansen (Bayswater, VIC, Australia) and LB was obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80º C in sterile medium composed of 50% (v/v) MRS broth (Difco, Becton, Dickinson and Company, New Jersey, USA) and 50% (v/v) glycerol. The LB culture was activated in MRS broth supplemented with 1% (w/v) yeast extract and 2% (w/v) glucose, whereas ST was first activated in M17 broth (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 10% (w/v) lactose then transferred (1% v/v) into 12% (w/v) sterile reconstituted skim milk (RSM). ST was incubated at 37˚ C, while LB was incubated at 45˚C for 18 h.

7.2.2 Preparation of yogurt batches

Three batches of yogurt mix were prepared using Skinny milk (10.5% total solids and 0.12% fat; Parmalat Foods Pty Ltd., Wahgunyah, Vic, Australia) and 1.5% (w/v) skim milk powder (Commercial Road, Koroit, VIC, Australia). The control yogurt (CY) was prepared from this standardized low-fat milk. The experimental batches (GOSY and MWMSY) were prepared from standardized low-fat milk added with 2% (w/v) prebiotic, either Vivinal® GOS (Great Ocean Ingredients, Warrnambool, Victoria, Australia) or MWMS (KF Specialty ingredients, Kings Park, NSW, Australia). All milk bases were were heated at 85˚C for 30 min, cooled in a cold water bath to 40˚C, inoculated with 2% (v/v) activated culture of ST and 1% (v/v) of LB, mixed thoroughly, dispensed into 50 mL pre-washed and sanitized cups, sealed, and incubated at 40˚C until the pH reached 4.5. The yogurt cups were refrigerated at 4˚C and stored for 28 days. Samples were taken on day one after overnight storage at 4˚C and on weekly intervals to monitor changes in pH, viable counts, proteolysis and lactic and acetic acids content, whey separation

and firmness. All the experiments were replicated 3 times.

7.2.3 Enumeration of cultures

Peptone water diluent was prepared by dissolving 1.5 g (w/v) of peptone (Oxoid, West Heidelberg, Australia) in 1 L of distilled water and autoclaving at 121°C for 15 min. Enumeration of starter culture, in freshly inoculated yogurt bases (0 h) and in yogurts stored at 4 °C was carried out by pour plate technique using MRS agar (Dave and Shah, 1996). Duplicate plates were placed in anaerobic jars (Becton Dickinson Microbiology System, Sparks, MD, USA) containing anaerobic gas generating kits (Oxoid Ltd., Hamshire, UK) and incubated at 37°C for 24 h for ST, and for 72 h at 45˚C for LB. Noteably, counting of both organisms was done together in the MRS medium and the cell counts were presented as their total numbers. Plates showing 25 to 250 colonies were counted and results were expressed as colony forming units (CFU) per gram of the inoculated sample.

7.2.4 pH measurement

The change in pH was measured at day 0 and on weekly intervals during 28 days of storage at 4˚C by using a pH meter (Model WTW, InoLab 720 Weilheim, Germany) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Samples were tempered at room temperature before pH measurement.

7.2.5 Determination of organic acids

The concentration of lactic and acetic acids in yogurt was determined by the high-performance liquid chromatography (HPLC) as described by Ramchandran and Shah (2008). Briefly, 40 μL of 15.5 M nitric acid and 500 μL of 0.01 M sulphuric acid were mixed with 1 g of yogurt sample

and centrifuged at 14,000 × *g* for 30 min. The supernatant thus obtained was filtered using a 0.45-μm-membrane filter (Millipore Corp., Bedford, Mass., USA) into HPLC vials. An aliquot of 10 μL of each sample was injected into HPLC system. The organic acids were separated in an Aminex HPX-87H, 300 × 7.8mm ion exchange column (Biorad Life Science Group, Hercules, Calif., U.S.A.) fitted with a guard column maintained at 65˚C at flow rate of 0.6 mL/min. The column was attached to a Varian HPLC (Varian Analytical Instruments, Walnut Creek, Calif., U.S.A.) fitted with a UV/Vis detector. Eluent containing 0.005 M sulphuric acid (MERCK Pty Ltd, Colchester Road, Kilsyth, Australia) was used as mobile phase for 25 min. The organic acids were detected at 210 nm. The retention times of lactic and acetic acids were compared with those of the standard working solutions of L (+) lactic acid and acetic acid prepared from a 10% (v/v) stock solution.

7.2.6 Determination of proteolytic activity in yogurt

The proteolysis was determined according to Church et al. (1983) in the filtrates of inoculated milks (time 0) and in non-fat yogurt samples. Fifty gram of each sample was centrifuged at 4,000 × *g* for 30 min at 4˚C. The supernatants thus obtained were filtered through a 0.45 μL membrane filter and stored at –20˚ C until assayed. A 150 µL aliquot of the filtrate was added to 3 mL of OPA reagent prepared according to Church et al. (1983), and vortexed for 10 s. The free amino acid content was determined by measuring absorbance at 340 nm within 2 min using a spectrophotometer (UV/VIS spectrophotometer, Ontario, Canada). The readings of samples at time 0 as well as the reagent blank were deducted from the corresponding readings of samples to obtain the free amino acids (measured by absorbance at 340 nm) released as a consequence of the proteolytic activity of the starter cultures during fermentation and storage.

7.2.7 Measurement of syneresis

Syneresis in yogurt samples was measured using a siphon method described by Amatyakul et al. (2006). A cup of yogurt with the lid was weighed and slanted at an angle of 45˚ to collect the surface whey. The collected whey was drawn out with a syringe to which a needle was attached. Thereafter, the cups with the lids were re-weighed and percent syneresis was calculated by dividing the weight of separated whey with the initial weight of the yogurt sample multiplied by 100.

7.2.8 Large deformation of yoghurt texture

The firmness of yogurt samples was determined using TA-XT plus Texture Analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK) attached to a Texture Exponent Software, with a P20 probe (diameter 20 mm) and 25 kg load cell. The speed of penetration was set at 1 mm s^{-1} and depth of penetration was 10 mm. The ratio of cup diameter to probe diameter was 3.5:1 (Amatyakul et al., 2006). The gel strength was expressed in grams, indicative of the force required to break the gel. The TA-XT plus texture analyser uses a probe to analyse characteristics such as firmness and smoothness of dairy products. This is an updated version that has the capability to test tough food samples with high accuracy. The measurements were performed as soon as the samples were removed from the refrigerator.

7.2.9 Statistical analysis

All tests and analyses were performed in triplicate and data were analyzed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using Statistical Analysis System (SAS, 1995). ANOVA data with a $P < 0.05$ were classified as statistically significant.

7.3 Result and discussion

7.3.1 Effect of prebiotic addition on pH change

The changes in pH during preparation of the 3 types of yogurts are presented in Table 7.1. The decrease in pH during the first 5 h of fermentation was maximum in the GOSY (4.62) followed by MWMSY (4.83) and the CY (4.89). However, GOSY reached the pH of 4.5 earlier than MWMSY and the CY (Table 7.1). Thus, incorporation of GOS appeared to improve the growth and activity of starter organisms that resulted in a shorter fermentation time. This is in agreement with the findings of Hardi and Slacanac (2000) and Ozer et al. (2005) who reported that the rate of pH decrease of fermented milk products increased by the addition of GOS.

Table 7. 1: Effect of prebiotic type on pH changes during incubation of milk inoculated with LB and ST at 40˚C.

	Type of milk bases		
Periods of incubation	**Control**	**GOSY**	**MWMSY**
0 h	6.40 ± 0.02^{A}	6.40 ± 0.02^{A}	6.39 ± 0.02^{A}
2 h	5.49 ± 0.20^{A}	5.36 ± 0.02^{A}	5.28 ± 0.03^{A}
4 h 30 min	4.92 ± 0.09^{A}	4.79 ± 0.11^{A}	4.89 ± 0.10^{A}
5 h	4.89 ± 0.02^{A}	4.62 ± 0.04^{B}	4.83 ± 0.02^{AB}
5 h 25 min	4.52 ± 0.02^{A}	4.48 ± 0.02^{A}	4.50 ± 0.03^{A}

Values shown are average of 3 replicates. Control: Yogurt prepared from non-fat milk and starter cultures (1 % of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2 % S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS. ^{ABC} Means in the same row (at same incubation time) with different alphabets are significantly different.

These results are similar to those reported by Guven and others (2005). The pH of all samples decreased steadily during storage without any significant ($P<0.05$) difference among samples. However, GOSY showed lower pH values than other samples throughout the storage period (Fig 7.1). The decrease in pH during storage was due to continuous conversion of lactose to lactic

acid and acetic acid. There was no significant difference (P > 0.05) in the pH values of supplemented and non-supplemented prebiotic yogurt at the end of the storage period (Fig. 7.1). In the current study, the pH of all samples decreased steadily during storage without any significant (P< 0.05) difference among samples (Fig. 7.1). However, the production of lactic acid continued until day 14 of storage and thereafter was reflected as a slight pH decrease in all yogurt samples. This is in agreement with Donkor et al. (2006) who concluded that the level and type of organic acids was found to have a more pronounced effect on the strain's variability than the pH value.

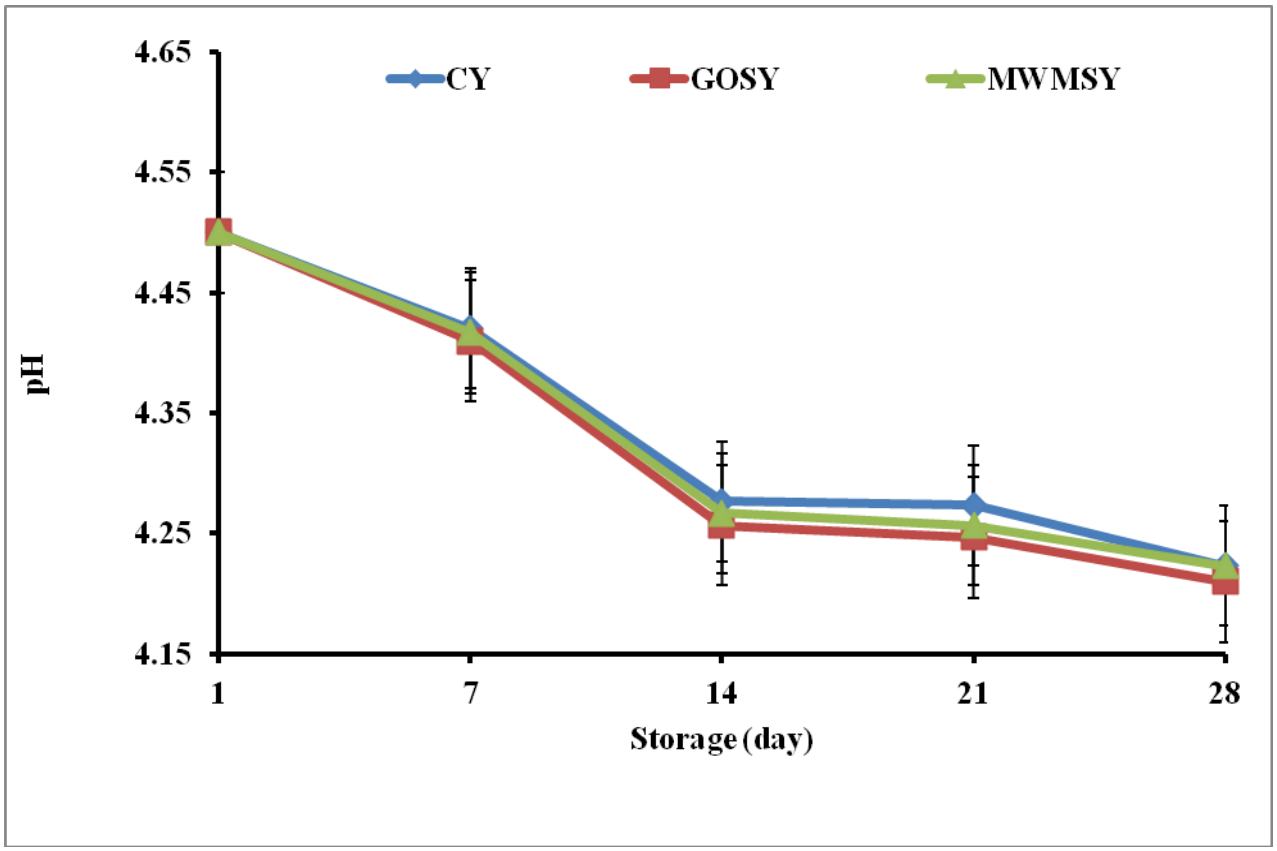

Figure 7. 1: Changes in pH of non-fat yogurts with and without prebiotic during storage at 4˚C.

CY (control): Yogurt prepared from non-fat milk and starter cultures (1% of L. delbrueckii ssp. bulgaricus ATCC 11842 and 2 % S. thermophilus S5). GOSY and MWMSY: Yogurt prepared from non-fat milk and 2% GOS or MWMS: Bars indicate standard deviations.

7.3.2 The viability of starter cultures

Figure 7.2 depicts the viable counts in log CFU per gram of ST and LB in yogurt with and without prebiotic during storage at 4˚C. Total count increased in all samples during incubation and storage and the maximum numbers were found at day 14 of storage and thereafter there was a slight decline in the counts until the end of storage. All three types of yogurt showed significant differences ($P<0.05$) in the viable count during the first 21 days but no significant difference ($P>0.05$) was found at the end of storage. However, GOSY consistently showed higher numbers ($P<0.05$) throughout the storage period. The maximum viable count of 6.95 log CFU per gram was observed in GOSY at day 14 followed by 6.84 and 6.73 log CFU per gram in MWMSY and the CY, respectively. This represented 16.56%, 12.52% and 14.62% increase in the counts of cultures in CY, GOSY, and MWMSY, respectively (Fig 7.2).

These findings agree with those of Ito et al. (1993b) and Vulevic et al. (2008) who reported that GOS was an excellent source for stimulating the growth of *Bifidobacterium* and *Lactobacillus*. Studies have shown that yoghurt bacteria (*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*) survive well in yoghurt throughout the storage period (Hamann and Marth, 1984; Rohm et al., 1990; Akalin *et al*., 2004). Moreover, a type of prebiotics also plays a significant role on the growth of the yoghurt organisms.

The GOS are carbohydrates with short chains of variable length made up of the sugars galactose and glucose. The GOS are considered prebiotics, which are defined as food compounds that are indigestible by humans but digestible by intestinal bacteria. Thus GOS serve as an energy source for beneficial bacteria that live in the human intestine (Roberfroid and Gibson, 2010). Similarly, Onishi and Tanaka (1997) concluded that GOS must be essential ingredient for stimulating the growth of intestinal microbiota incorporated in the culture medium. In the current study, the

control was prepared from non-fat milk and starter cultures (ST and LB) only and devoid of GOS and as such was compared with other prebiotics.

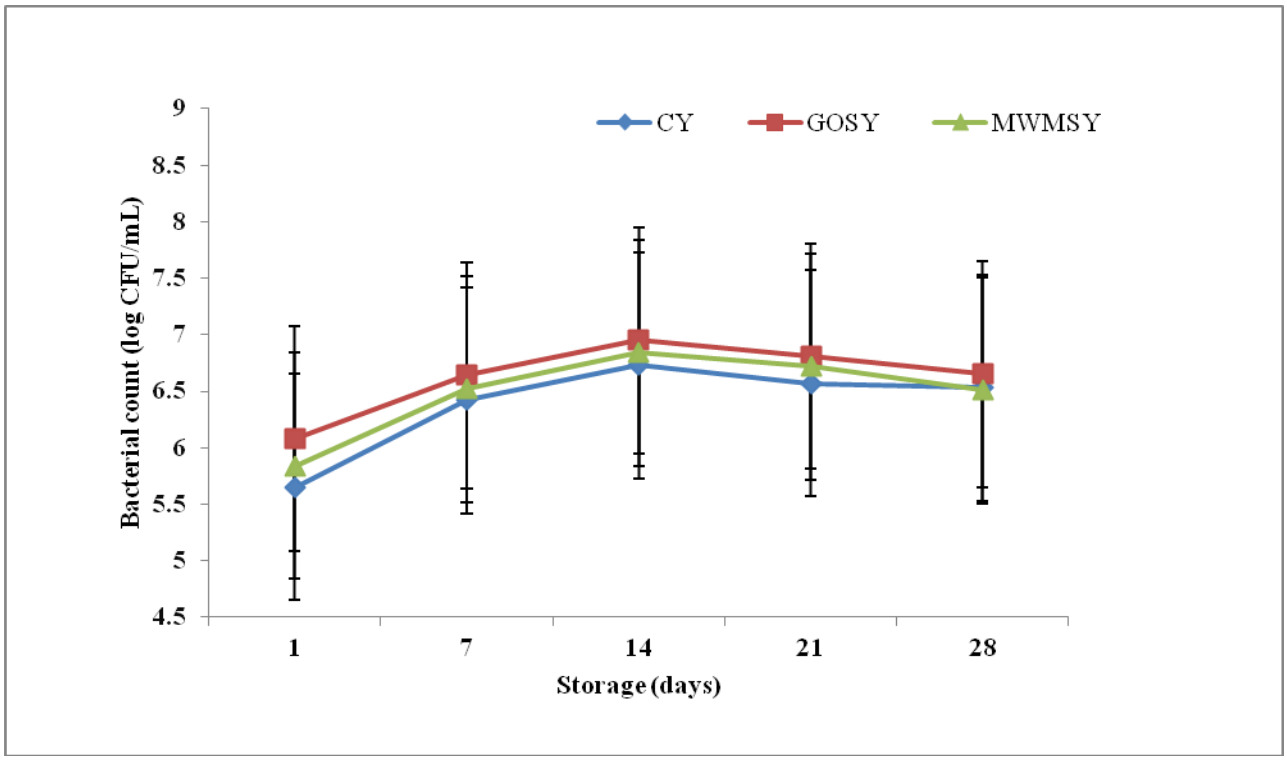

Figure 7. 2: Changes in viable counts of starter cultures grown in non-fat yogurts ± prebiotics during storage at 4˚C.

CY (control): Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% *S. thermophilus* S5). GOSY and MWMSY: Yogurts prepared from as above with the addition of 2% GOS or MWMS. Bars indicate standard deviations. Column bars with same color at different times having similar letters did not differ significantly.

Several factors have been identified in fermented milk that can affect their viability, such as the pH and acidity levels, temperature of incubation and/or the presence of oxygen (Kailasapathy and Ribka, 1997). This study showed that total count increased in all samples during incubation and storage. The maximum numbers (6.95 log CFU per gram) were found at day 14 of storage in comparision to 5.65 log CFU per gram found on 1 day. After two weeks, the cell counts steadily declined until the end of storage. The total count increased approximately by more than 1 log

during the storage. This finding was similar with those of Kurmann and Rasic (1991) who reported that the number was reached and maintained at the level of 6 log CFU/mL at the end of storage.

7.3.3 Production of organic acids

The concentration of acetic and lactic acids in the three types of yogurts during storage at 4˚C for 28 d is shown in Table 7.2.

Table 7. 2: Concentration of lactic acid and acetic acids (%) in non-fat yogurts with and without prebiotics stored at 4˚C.

	Storage period (days)				
	1	**7**	**14**	**21**	**28**
Lactic acid					
Control	1.28 ± 0.02^{Bb}	1.30 ± 0.02^{Ab}	1.32 ± 0.2^{Ac}	1.13 ± 0.03^{Cb}	1.05 ± 0.03^{Dc}
GOSY	1.34 ± 0.02^{Ba}	1.36 ± 0.05^{Ba}	1.39 ± 0.03^{Ca}	1.27 ± 0.04^{Db}	1.13 ± 0.04^{Ca}
MWMSY	1.29 ± 0.03^{Cb}	1.32 ± 0.02^{Bb}	1.37 ± 0.03^{Ab}	1.17 ± 0.04^{Da}	1.08 ± 0.03^{Eb}
Acetic acid					
Control	0.61 ± 0.04^{Cc}	0.67 ± 0.02^{Bc}	0.67 ± 0.03^{Bc}	0.72 ± 0.05^{Ac}	0.74 ± 0.04^{Ac}
GOSY	0.83 ± 0.02^{Aa}	0.84 ± 0.02^{Aa}	0.86 ± 0.03^{ABa}	0.87 ± 0.02^{Aa}	0.88 ± 0.02^{Aa}
MWMSY	0.72 ± 0.02^{Bb}	0.72 ± 0.03^{Bb}	0.74 ± 0.02^{Bb}	0.76 ± 0.03^{Ab}	0.77 ± 0.03^{Ab}

Values are the statistical means of 6 observations. Control: Yogurt prepared from non-fat milk and starter cultures (1 % of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2 % S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS; ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABCDE}Means in the same row with different alphabets are significantly different for a particular day of storage.

The production of lactic acid increased until day 14 of storage and thereafter there was a slight decrease in all yogurt samples. Addition of GOS resulted in a significantly ($p<0.05$) higher lactic acid production until day 14 of storage than the other two batches. Similarly, GOSY produced more ($p<0.05$) acetic acid during incubation than other batches (Table 7.2). There was a decline

in the amount of lactic acid in all three types of yogurt from day 14 to 28 (Table 7.2). However, the production of acetic acid gradually increased in all samples throughout the storage. Several workers have reported that the utilization of prebiotics by bacteria varied depending on the strain (Desai et al., 2004). Sako and Matsumoto (1999) have reported that incorporation of non-digestible oligosaccharides in fermented food products increased the acidity due to production of short chain fatty acids (SCFA). The concentration of acetic acid varied considerably in all yogurt batches (Table 7.2). In the current study, only 1 % of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2 % S. *thermophilus* S5) were added to the product with samples prepared from non-fat milk with addition of 2% GOS or MWMS. Donkor et al. (2007) reported that the amount of lactic and acetic acidsvaried in yogurt samples supplemented with different sources of carbon including inulin and Hi-maize. Our findings also showed that the production of acetic acid gradually increased in all samples throughout the storage. Several workers have reported that the utilization of prebiotics by bacteria varied depending on the strain (Desai et al., 2004). Sako and Matsumoto (1999) have reported that incorporation of non-digestible oligosaccharides in fermented food products increased the acidity due to production of short chain fatty acids (SCFA).

7.3.4 Syneresis

Table 7.3 shows the syneresis (%) in all 3 types of yogurt during storage at 4˚C for 28 days. All samples showed reduction in the amount of whey separation up to 4 weeks of storage at 4 °C, and there was significant difference ($p< 0.05$) in syneresis values in all yogurt batches at the end of storage.

Al-Kadamany et al. (2003) have also reported a decrease in the extent of syneresis in yogurts towards the end of the storage. This may be due to reduced permeability of serum through the protein gel (Amatayakul et al., 2006).

Table 7. 3: Syneresis (%) in non-fat yogurts with and without prebiotics during storage at 4˚C.

	% of syneresis during storage period (days)				
	1	**7**	**14**	**21**	**28**
Control	3.66 ± 0.03^{Aa}	3.63 ± 0.04^{Aa}	2.80 ± 0.08^{Ba}	2.53 ± 0.03^{Ca}	2.53 ± 0.04^{Ca}
GOSY	3.45 ± 0.02^{Ac}	3.44 ± 0.02^{Ab}	2.54 ± 0.02^{Bb}	2.39 ± 0.03^{Cab}	2.14 ± 0.02^{Cc}
MWMSY	3.54 ± 0.02^{Ab}	3.53 ± 0.03^{Ac}	2.60 ± 0.02^{Bb}	2.35 ± 0.02^{Cb}	2.35 ± 0.02^{Db}

Values are the statistical means of 6 observations, Control: Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2 % S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS; ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABCD}Means in the same row with different alphabets are significantly different for a particular treatment.

Syneresis has been related to slow shrinkage of the protein gel network that results in the loss of the ability to entrap all the serum phase (Lucey, 2002). It has been suggested that faster rate of acidification inhibits network rearrangement during whey expulsion thereby resulting in less syneresis (Castillo et al., 2006). It also could be due to re-absorption of whey back into the gel as the storage time progresses.

In our study, the amount of separated whey was found to be lower in the GOSY (2.14%) followed by MWMSY (2.35%) and CY (2.53%). Considering that GOSY reached the pH of 4.5 faster than the others (Table 7.1), it can be concluded that this could be one of the reasons for lower levels of whey separation. Yogurts containing added prebiotics are reported to show less syneresis (Puvanenthiran et al., 2002; Isleten and Karagul-Yuceer, 2006).

Our findings agree with those of Kalab et al. (1983) who found that the rate of syneresis decreased in all yogurt samples due to the bonds between the networks of milk gel becoming weaker.

7.3.5 Proteolytic activity of starter culture in yogurt

The proteolytic activity of starter culture in the 3 types of yogurts stored at 4˚C for 28 d is

presented in Figure 7.3. Changes in the extent of proteolysis and liberation of free amino acids were measured by absorbance at 340 nm in the fresh and stored yogurt samples. There was a significant ($p<0.05$) increase in proteolysis in all the yogurt samples during the entire storage period. Tourneur (1974) reported that higher degree of proteolysis may be due to peptidase activity of organisms during storage period. Proteolytic activity may have increased because the organisms are capable of acting on peptides liberated from casein by the rods, and more peptides and amino acids are liberated than the bacteria can utilize. Proteolysis in GOSY was significantly ($p<0.05$) higher than those of MWMSY and the CY throughout the storage period. This could have an influence on the higher survival rate of yogurt culture grown in GOS substrates (Hernandez-Hernandez et al., 2012).

The maximum proteolytic capability (0.728) was observed in GOSY whereas only (0.583) and (0.530) were found in the MWMSY and the CY respectively on day 28. All yogurts showed an increasing trend in the amount of free amino acids during the storage period. The ability of LAB to grow to high cell densities in milk is dependent on a proteolytic system that can liberate essential amino acids from casein-derived peptides (Christensen et al., 1999). Proteinase and peptidases constitute the primary enzymes in LAB responsible for proteolysis of caseins as a source of amino acids and nitrogen for LAB (Shihata and Shah, 2000). Cruz et al. (2012) reported that higher concentrations of glucose oxidase (750 to 1,000 mg Kg^{-1}) and storage period had an influence on characteristics of probiotic yogurt and resulted in more post acidification and higher proteolysis.

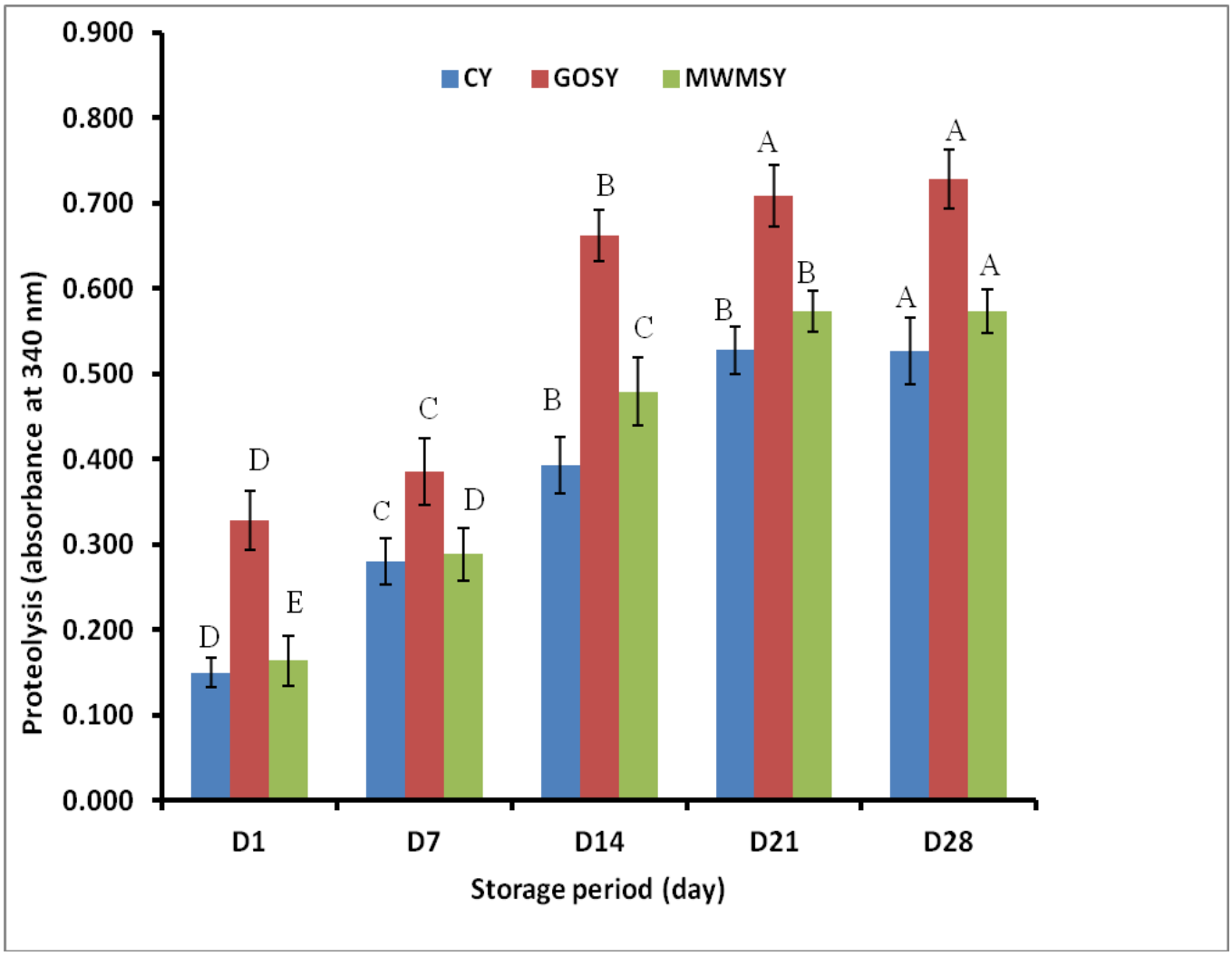

Figure 7. 3: Progressive proteolysis of the non-fat yogurts with and without prebiotics during storage at 4˚C

CY (control): Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% *S. thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS: Bars indicate standard deviations. Column bars with same color at different times having similar letters did not differ significantly.

The proteolytic system of LAB can contribute to the liberation of health-enhancing bioactive peptides from milk (Wouters et al., 2002). This may improve absorption in the intestinal tract; stimulate the immune system and exert antihypertensive effects. The proteolytic system of LAB may allow the manufacture of suitable starter cultures that generate desirable texture and flavour characteristics in the fermented products (Caplice and Fitzgerald, 1999). They acidify the food,

resulting in a tangy lactic acid taste, frequently exert proteolytic and lipolytic activities, and produce aromatic compounds from amino acids upon further bioconversion (van Kranenburg et al., 2002). For example, an expression of certain peptidases of *Lactobacillus* ssp. improved the sensory quality of cheese (Guldfeldt et al., 2001).

7.3.6 Large deformation of yoghurt texture

The firmness of the 3 batches of yogurt during storage at 4˚C for 28 d is presented in Table 7.4. The firmness was measured on day one and during 28-days of cold storage, using the penetration force (g) to break the gels. The maximum firmness was recorded on day one in GOSY (50.16 g) followed by MWMS (45.30 g) and the CY (38.68). By the end of storage the firmness of all yogurts was increased wher GOSY still showed higher firmness (69.82 g) than MWMSY (65.45 g) and the CY (64.66 g). Bozanic et al. (2001) found that the firmness of yogurt improved upon the addition of prebiotic. According to Tamime and Robinson (1999), the primary aim of adding these prebiotic to the milk base is not only to enhance and maintain the yogurt texture and consistency but also to improve the general appearance and mouth feel. In our study, the addition of GOS and MWMS marginally improved in the texture of yogurts which is in agreement with findings of Jawalekar et al. (1993). Therefore, GOS can be added to dairy applications such as yogurts, buttermilk and dairy-based drinks due to its excellent solubility. After the addition of GOS, structure of yoghurt was found to be smoother and creamier (Sangwan et al., 2011).

Table 7. 4: Firmness (g) of non-fat yogurts with and without prebiotics during storage at 4˚C.

	Storage period (days)				
	1	7	14	21	28
Control	38.68± 1.78^{Ca}	46.52± 1.05^{Bb}	63.12± 0.96^{Aa}	62.65± 0.65^{Ab}	64.66± 0.26^{Aa}
GOSY	50.16± 1.78^{Aa}	56.80± 1.55^{Aa}	64.85± 2.42^{Aa}	67.85± 0.80^{Aa}	69.82± 1.67^{Aa}
MWMSY	45.30± 3.33^{Bb}	55.25± 0.78^{Aa}	64.13± 1.12^{Aa}	64.14± 0.34^{Ab}	65.43± 1.23^{Ab}

Values are the statistical means of 6 observations. Control: Yogurt prepared from non-fat milk and starter cultures (1 % of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2 % S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS: ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABC}Means in the same raw with different alphabets are significantly different for a particular day of storage.

7.4 Conclusions

The supplementation of yogurt with prebiotics improved the retention of viability of *S. thermophilus* M5 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in all batches of yogurt during cold storage especially in the presence of GOS. The GOS was found to be a better growth stimulant of selected organisms than MWMS. Addition of GOS produced the highest lactic and acetic acids than those of MWMSY and the CY. However, the production of acetic acid increased in all samples throughout the storage. Furthermore, improved proteolysis was found in the presence of GOS as compared to that of MWMS. The extent of syneresis was lower in GOSY compared to the MWMSY and the CY. Our study confirmed that supplementation of milk with GOS was the best of the two prebiotic ingredients tested for a better yoghurt body. It appears that GOS not only enhanced the growth of selected organisms but also improved proteolysis of the yogurt. Therefore, addition of 2% GOS could provide functional properties in production of yogurt.

Chapter 8.0 The effects of prebiotics addition to soymilk on the viable counts, proteolytic activity and organic acid production by LAB, and on physical attributes of soy yogurt[6]

[6] A version of this chapter has been submitted to Journal of Food Sciencefor publication. Reference # Manuscript JFDS 2012- 1390. Prasad, L. N., F. Sherkat, and N. P. Shah. 2012. The effects of prebiotics addition to soymilk on the viable counts, proteolytic activity and organic acid production by LAB, and on physical attributes of soy yogurt.

8.1 Introduction

Soybeans are excellent source of protein and used for oil extraction and for the production of soy flour, protein concentrates and isolates (Liu, 1999). The use of soy ingredients in foods is receiving significant attention from the food industry and consumers because of its role as a functional food. Several studies have confirmed the role of soy in reducing serum cholesterol and lowering the risk of heart disease (Anderson et al., 1995; Kennedy, 1995; Sirtori et al., 1997; Jacobson et al., 1998). Soy-based foods may provide additional benefits for the consumer due to their hypolipidemic, anticholesterolemic and anti-atherogenic properties (Messina et al., 1994; Lopez-Lazaro and Akiyama, 2002).

Production of soy yogurt has been studied by several workers (Buono et al., 1990; Cheng et al., 1990; Lee et al., 1990; Nsofor et al., 1996; Favaro Trindale et al., 2001, Sherkat et al., 2001). The commonly used starters for yogurt, LB and ST, produce adequate amounts of acid in soy yogurt (Nsofor et al., 1992). According to Kamaly (1997), the enrichment of soy milk with carbohydrates (lactose, sucrose and glucose) stimulates growth of bacteria and acid production. Murti et al. (1993) showed that ST is capable of fermenting lactose, sucrose, glucose and fructose, whereas LB ferments lactose, glucose, fructose and galactose. The ST produces L+ and LB produces D- lactic acid, which helps in the protein gel formation. Supplementation with either MWMS or inulin has been shown to affect on firmness and viscosity of yogurt as well as the growth of starter cultures (Radke-Mitchell and Sandine, 1986).

Soy milk-based yogurt has emerged as a popular alternative to traditional dairy-based yogurts due to their reduced level of cholesterol and saturated fat (Sarkar, 2006). Furthermore, incorporation of probiotic bacteria as dietary adjuncts has increased the consumption of probiotic products in Asia and Europe (Donkor et al., 2007). Prebiotics have been used to promote the growth and activity of beneficial microorganisms in vitro and in the

large intestine (Fuller and Gibson, 1997).

An important physiological role is attributed to soy oligosaccharides which could meet the standards of a prebiotic. Prebiotics have been used to promote the growth and activity of beneficial microorganisms in the large intestine (Gibson and Roberfroid, 1995; Fuller and Gibson, 1997). Some lactic acid bacteria (LAB) have been reported to grow slowly or poorly in soymilk (Mital et al., 1974) and produce low levels of organic acids (Liu, 1997). Therefore, to improve the growth of probiotic bacteria and production of organic acid, soy milk needs to be supplemented with various prebiotics such as GOS, MWMS or inulin in combination with glucose and sucrose (Roberfroid et al., 1998; Chou and Hou, 2000; Tsangalis and Shah, 2004).

Fooks et al. (1999) suggested the use of prebiotics as stabilizing agents in soy milk based products. Once again, prebiotics are considered non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon especially *Lactobacillus* and *Bifidobacterium* ssp. (Gibson and Roberfroid 1995). In general, supplementation of prebiotics to a soy based growth medium supported the growth of lactic acid bacteria (Fooks et al., 1999). Additionally, Siok-Koon and Min-Tzeliong (2010) reported that the supplementation of prebiotics enhanced *in-vitro* antihypertensive effect and production of bioactive aglycones in probiotic-fermented soymilk. Therefore, this soymilk could potentially be used as a dietary therapy to reduce the risks of hypertension and hormone-dependent diseases such as breast cancer, prostate cancer and osteoporosis.

Among available commercial prebiotics, Vivinal® GOS is a natural prebiotic ingredient rich in galacto-oligosaccharides, structured as chains of galactose with a glucose, produced from glucose molecules transferred from a sucrose donor to a maltose acceptor by a glycosyl-transferase (Crittenden and Playne, 1996). The soybean oligosaccharides (raffinose and

stachyose) are well known α-galactosyl sucrose derivatives extracted from soybean (Boever et al., 2000). Rycroft et al. (2001), studying a comparative *in vitro* evaluation of soybean oligosaccharides has found a comparable effect in soymilk addition to other oligosaccharides. According to Tamime and Robinson (1999), the primary aim of adding GOS and inulin to the milk base is not only enhancement and maintaining the yogurt texture and consistency but also improvement of general appearance as well as mouthfeel. The addition of GOS had a remarkable improvement in the texture of yogurts in the current study, which was in accordance with the findings of Jawalekar et al. (1993).

The MWMS is used to improve the structure and stability of processed food products due to its thickening and gelling properties after gelatinisation. This prebiotic is also used to improve the water holding capacity, binding capacity and minimizing the syneresis (Miyazaki et al., 2006).

The inulin is a natural food component belonging to a class of carbohydrates known as fructans. It is mainly produced from chicory roots and consists of a series of oligo- and polysaccharides of fructose with β (2→1) linkages, where the terminal sugar in most chains is glucose. It increases the growth, stability and the proteolytic activity of yogurt bacteria; the level of organic acids and firmness of yogurt (Kaur and Gupta, 2002). Roberfroid et al. (1998), Capela et al. (2006) and Aryana et al. (2007) observed that chicory based inulins were the favoured carbon source for *Lactobacollus* strains, hence increasing the growth performance and the viability during storage.

Soy yogurt may have a slight beany soy taste when made directly from freshly prepared soy milk. Soymilk is a good medium for growing yogurt culture in presence of sucrose and glucose which are fermented by these cultures (Kamaly 1997). Importantly, the growth of yogurt culture in soymilk is governed by the presence of fermentable sugars. Different

workers have observed an increase in acid production after addition of simple sugars such as lactose, sucrose, glucose, and galactose, and/or enrichment with milk solids (Pinthong et al., 1980; Karleskind et al., 1991).

Hence, the aims of the present study were to examine the influence of GOS, MWMS and inulin on soy yogurt's pH, the growth of ST and LB and their proteolytic activity, organic acid production, syneresis, viscosity and firmness of low-fat soy yogurt.

8.2 Materials and methods

8.2.1 Activation of starter cultures

The frozen pure culture of ST was obtained from Chr. Hansen (Bayswater, VIC, Australia) and LB was obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80º C in sterile medium composed of 50% (w/v) MRS broth (Difco, Becton, Dickinson and Company, New Jersey, USA) and 50% (w/v) glycerol. The LB culture was activated in MRS broth supplemented with 1% (v/v) yeast extract and 2% (w/v) glucose whereas ST was first activated in M17 broth (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 10% (w/v) lactose then transferred (1% v/v) into 4% (w/v) sterile soymilk prepared from soy protein isolate (SPI). The ST was incubated at 37˚ C, while LB was incubated at 45˚C for 18 h.

8.2.2 Preparation of soy yogurt batches

Three batches of soymilk blends were prepared using So Good Soymilk (9.0% total solids including 0.1% fat, 3.6% protein, 5.1% carbohydrate and 0.2% sodium and calcium; Australian Health Nutrition Ltd., Berkeley Vale, NSW, Australia). The control yogurt (CY) was prepared from the above soymilk supplemented with 1.0% (w/v) glucose (Merck,

Darmstadt, Germany) and 12.0% (w/v) sucrose (ASTRAL, Gymea, NSW, Australia) without any prebiotic. The experimental batches (GOSY or MWMSY or IY) contained onlty 10% sucrose and 1% glucose plus 2% (w/v) prebiotic, either Vivinal GOS (Great Ocean Ingredients, Warrnambool, Victoria, Australia) or MWMS (KF Specialty ingredients, Kings Park, NSW, Australia) or Chikory inulin (Miller Street, QLD, Australia), followed by heating at 85˚C for 30 min. The milk bases were then cooled in a cold water bath to 40˚C, inoculated with 5% (v/v) activated cultures of ST and LB (2:1), mixed thoroughly and aseptically dispensed into 100 mL pre-washed and sanitized yogurt cups sealed and incubated at 40˚C until the pH reached 4.5. The yogurts were then refrigerated at 4˚C and stored for 28 days. Samples were taken on day one and at weekly intervals to monitor changes in pH, viable counts, proteolysis and lactic and acetic acids levels, syneresis, viscosity and gel firmness. The day 1 analysis was carried out after overnight storage at 4˚C. All the experiments were replicated 3 times.

8.2.3 Enumeration of cultures

Peptone water diluent was prepared by dissolving 15 g of peptone (Oxoid, West Heidelberg, Australia) in 1 L of distilled water and autoclaving at 121°C for 15 min. ST agar media was prepared by dissolving 15 g of bacteriological agar (Oxoid Ltd, Basingstoke, Hampshire, England) and 37.25 g M17 broth in 950 mL distilled water, followed by sterilizing at 121 °C for 15 min and adding 50 mL of filter- sterilised lactose solution (10% w/v).

Enumeration of ST and LB, in freshly inoculated milk bases (0 h) and in yogurts stored at 4 °C was carried out by pour plate technique using M17 agar and MRS agar (Dave and Shah, 1996). Duplicate plates were placed in anaerobic jars (Becton Dickinson Microbiology System, Sparks, MD, USA) containing anaerobic gas generating kits (Oxoid Ltd., Hamshire, UK) at 37°C for 24 h for ST and for 72 h at 45˚C for LB. Plates showing 25 to 250 colonies

were counted and results were expressed as colony forming units (CFU) per gram of the inoculated sample.

8.2.4 pH measurement

Samples were taken on day one and at weekly intervals to monitor changes in pH during 28 days of storage at 4˚C by using a laboratory pH meter (Model WTW, InoLab 720 Weilheim, Germany) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Samples were tempered at room temperature before pH measurement. The pH was also monitored during incubation of milk bases at 40 ˚C.

8.2.5 Determination of organic acid

Determination of organic acids was carried out according to Shah and Ravula (2000). Samples were taken on day one and at weekly intervals to determine the organic acids during 28 days of storage at 4˚C. Briefly, 3 mL yoghurt samples were mixed with 50 µL of 15.5 M nitric acid (Merck Pty Ltd., Kilsyth, VIC., Australia) and 1.0 mL of 0.01M sulphuric acid (Merck Pty Ltd., Kilsyth, Vic., Australia). The resulting mixture was centrifuged at 14,000 x g for 30 min using an Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Australia) for removal of coagulated proteins. The supernatant was filtered through a 0.20 µm membrane filter (Schleicher and Schuell GmbH, Dassel, Germany) into an HPLC vial. The separation of organic acids was achieved using a Varian HPLC (Varian Analytical Instruments, CA, USA) fitted with an Aminex HPX - 87H, 300 x 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, USA). The mobile phase was 0.01M sulphuric acids with a flow rate of 0.6 mL/min. The organic acids were detected at 210 nm. The retention times of lactic and acetic acids were compared with those of the standard working solutions of L (+) lactic acid and acetic acid prepared from 10% (v/v) stock solutions.

8.2.6 Proteolysis determination

The proteolytic activity of cultures was determined according to Church et al. (1983) and Shihata and Shah (2000). Yogurt samples (2.50 mL) were diluted with 5 mL of 0.75% trichloroacetic acid (Merck Pty Ltd., Kilsyth, Vic., Australia) and the mixture was vacuum filtered using an Advantec # 231 filter paper (MFS. Inc., CA, USA). An aliquot of 150 µL of the filtrate was added to 3 mL of OPA reagent (The OPA solution was made by combining the following reagents and diluting to a final volume of 50 mL with MilliQ water: 25 mL of 100 mM sodium tetra-borate; 2.5 mL of 20% (w/v) sodium dodecyl sulphate (SDS); 40 mg of OPA (dissolved in 1 mL of methanol); and 100 µL of β-mercaptoethanol) that was prepared according to Church et al. (1983) and the absorbance of the solution was measured spectrophotometrically (UV/VIS spectrophotometer, Ontario, Canada) at 340 nm after 2 min at room temperature. The readings of samples at 0 h as well as the reagent blank were deducted from the corresponding readings of soy yogurt samples to obtain the free amino acids contents released as a consequence of the proteolytic activity of the starter cultures during fermentation and storage.

8.2.7 Syneresis measurement

Syneresis in yogurt samples was measured using a siphon method described by Amatyakul et al. (2006). A cup of yogurt with the lid was weighed and slanted at an angle of 45˚ to collect the surface whey with a syringe to which a needle was attached. Thereafter, the cups with the lids were weighed and percent whey syneresis was calculated by dividing the weight of separated whey with the initial weight of the soy yogurt sample multiplied by 100.

8.2.8 Determination of viscosity

The viscosities were determined according to Aryana (2003). The viscosity of the soy yogurt batches was measured on day 1 and on weekly intervals during storage at 4˚C.

Samples were tested using Brookfield Viscometer (Stoughton, Mass., USA) attached with a helipath stand and a LV-4 spindle. The data were acquired using the Wingathers software (Brookfield Engineering Lab Inc., Stoughton, MA). The yogurt was gently stirred for 1 min before reading was taken. All determinations were repeated three times on the same sample. The readings were expressed in Pa.s.

8.2.9 Large- scale measurement of yogurt texture

The firmness of yogurt samples was determined using TA-XT plus Texture Analyser (Burns Bay Rd, Lane Cove, NSW, Australia) attached to a Texture Exponent Software with a P20 probe (diameter 20 mm) and 25 kg load cell. The speed of penetration was set at 1 mm s^{-1} and depth of penetration was 10 mm. The ratio of cup diameter to probe diameter was 3.5:1 (Amatyakul et al., 2006). Gel firmness was expressed in grams, indicative of the force required to break the gel. The measurements were performed as soon as the samples were removed from the refrigerator. Three readings were taken per sample.

8.2.10 Statistical analysis

All tests and analyses were performed in triplicate and data were analyzed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using Statistical Analysis System (SAS, 1995). ANOVA data with a $P < 0.05$ were classified as statistically significant.

8.3 Results and discussion

8.3.1 Organic acids

The mean concentration (mg mL^{-1}) of acetic and lactic acids in the four types of yogurts during storage at 4˚C for 28 days is shown in Table 8.1. The results showed that the concentration of lactic acid in all types of yogurts was substantially higher than that of acetic

acid after fermentation and during 28 days storage at 4 ˚C (Table 8.1).

The concentration of lactic and acetic acids in the soy yogurt sample containing prebiotics increased significantly ($P < 0.05$) during the first two weeks of storage and thereafter increased gradually in lactic and acetic acids until 28 days. Similar results were reported by Fernandez-Garcia et al., (1998) in yogurts supplemented with prebiotics. The amount of acetic acid was slightly higher in the samples containing prebiotics compared to CY (Table 8.1).

In our study, the addition of inulin resulted in more lactic and acetic acids production than other treatments throughout the storage time. The increase in organic acid content could be attributed to the fermentation of carbohydrates as energy supply of lactic acid bacteria. The organic acid production was found to depend on the choice of carbon substrate (Ohleyer et al., 1985).

8.3.2 Effect of prebiotic addition on pH change

The initial pH of soy milk ranged from 6.57 to 6.65 and pH of soy yogurt at the end of fermentation dropped to 4.49 to 4.56 (Table 8.2).

The pH drop (4.49) was earlier (30 min) in IY than the others (Table 8.2). Hardi and Slacanac (2000) reported that the rate of pH drop in fermented milk products increased by inulin addition. However, Guven et al (2005) reported that inulin addition did not affect the pH of soy yogurt. The IY and MWMSY showed significant difference ($P < 0.05$) in pH values between 21 and 28 days but no significant ($P > 0.05$) difference was found on days 1, 7 and 14 of storage. The change in pH during fermentation was found to vary with the types of prebiotic. In this study, IY consistently showed lower pH values than the other samples throughout the storage period (Fig. 8.1).

Table 8. 1: Concentration of lactic acid and acetic acids (mg L-1) in soymilk yogurts with and without prebiotics during 4-week storage at 4˚C

	Storage period (days)				
	1	**7**	**14**	**21**	**28**
Lactic acid					
CY	132±5.55^{Bb}	167±7.23^{Ac}	173±8.29^{Ab}	189±6.11^{Ab}	191±12.20^{Ab}
GOSY	169±10.82^{Ba}	195±4.91^{ABb}	214±6.69^{Aab}	202±3.48^{ABab}	204±19.66^{Ab}
MWMSY	182±6.57^{Ba}	228±6.94^{Aa}	238±41.06^{Aa}	251±57.38^{Aa}	266±2.73^{Aa}
IY	144±6.49^{Cb}	175±8.95^{BCbc}	243±11.67^{ABCa}	284±7.97^{Aa}	273±24.21^{ABa}
Acetic acid					
CY	9±1.76^{Bb}	13±2.31^{ABb}	15±1.73^{ABb}	16±1.53^{Ab}	18±2.65^{Ac}
GOSY	17±3.21^{Bab}	25±5.78^{Aab}	29±4.63^{Aab}	27±2.96^{Aab}	28±4.62^{Abc}
MWMSY	25±6.43^{Ba}	30±5.51^{Aa}	31±4.84^{Aa}	34±7.94^{Aa}	36±4.62^{Aab}
IY	22±2.33^{Ba}	22±4.06^{Bab}	35±5.81^{ABa}	39±4.91^{ABa}	44±1.45^{Aa}

Values are the statistical means of 6 observations. Control: Yogurt prepared from soymilk and starter cultures (1 % LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: ^{abc} Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABC} Means in the same row with different alphabets are significantly different for a particular day of storage.

8.3.3 Changes in the counts of yogurt bacteria

Changes in the viable counts (log CFU g^{-1}) of ST and LB in soy yogurt with and without prebiotics supplementation during fermentation and over the storage period of 28 days are presented in Figures 8.2 and 8.3. Among the prebiotics tested, the best retention of ST counts was observed in IY (8.90 log CFU mL^{-1}) followed by MWMSY (8.77 log CFU mL^{-1}), GOSY (8.61 log CFU mL^{-1}) and the CY (8.48 log CFU mL^{-1}). Similarly, viable counts of LB was observed in IY (8.76 log CFU mL^{-1}) followed by MWMSY (8.62 log CFU mL^{-1}), GOSY (8.60 log CFU mL^{-1}) and the CY (8.47 log CFU mL^{-1}). The retention of the viability of ST was better than that of LB in all types of yogurt.

Table 8. 2: Effect of prebiotic type on pH changes in inoculated soymilk bases at 4˚C.

Types of yogurt mixes				
Periods of incubation	**CY**	**GOSY**	**MWMSY**	**IY**
0 h	6.65 ± 0.02^{Aa}	6.61 ± 0.02^{Aab}	6.60 ± 0.03^{Aab}	6.57 ± 0.03^{Ab}
4.0 h	5.12 ± 0.07^{Bab}	4.91 ± 0.02^{Bb}	5.40 ± 0.03^{Ba}	4.89 ± 0.29^{Bb}
6.0 h	4.84 ± 0.05^{Ca}	4.75 ± 0.02^{Cab}	4.62 ± 0.08^{Cb}	4.59 ± 0.03^{Cb}
6.5 h	4.79 ± 0.03^{Ca}	4.57 ± 0.03^{Db}	4.55 ± 0.04^{Cb}	4.52 ± 0.06^{Cb}
7.0 h	4.65 ± 0.13^{CDa}	4.52 ± 0.02^{Da}	4.52 ± 0.03^{Ca}	4.49 ± 0.04^{Ca}
7.5 h	4.56 ± 0.02^{Da}	4.50 ± 0.02^{Da}	4.49 ± 0.03^{Ca}	4.47 ± 0.04^{Ca}

Values shown are average of 3 replicates. Control: Yogurt prepared from soy milk and starter cultures (1 % LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin. ^{abc} Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABCD} Means in the same row (at same incubation time) with different alphabets are significantly different for a period of fermentation.

The total count of ST and LB increased in all samples during incubation and storage with the maximum numbers found at 14 days of storage and thereafter there was a slight decrease in the counts until the end of storage. These findings agree with those of Dave and Shah (1997) and Paseephol et al. (2008) and Paseephol and Sherkat (2009) who reported higher stability of ST and LB in yogurt during storage time. Hwa et al. (1974) reported that addition of glucose and sucrose in soymilk showed better retention of ST and LB during fermentation and storage period.

The IY showed significant difference ($P < 0.05$) in the viable count of ST during the first two weeks but no significant difference ($P > 0.05$) was found between third and fourth weeks of storage. However, IY consistently showed higher number ($P<0.05$) in both organisms throughout the storage period compared to other samples. The addition of inulin was helpful in improving the growth of ST and LB during fermentation and their survival during storage time. These results are consistent with the findings of Aryana and McGrew (2007) who reported that the increase in *L. casei* counts with the addition of inulin was found to be more

than the other prebiotics.

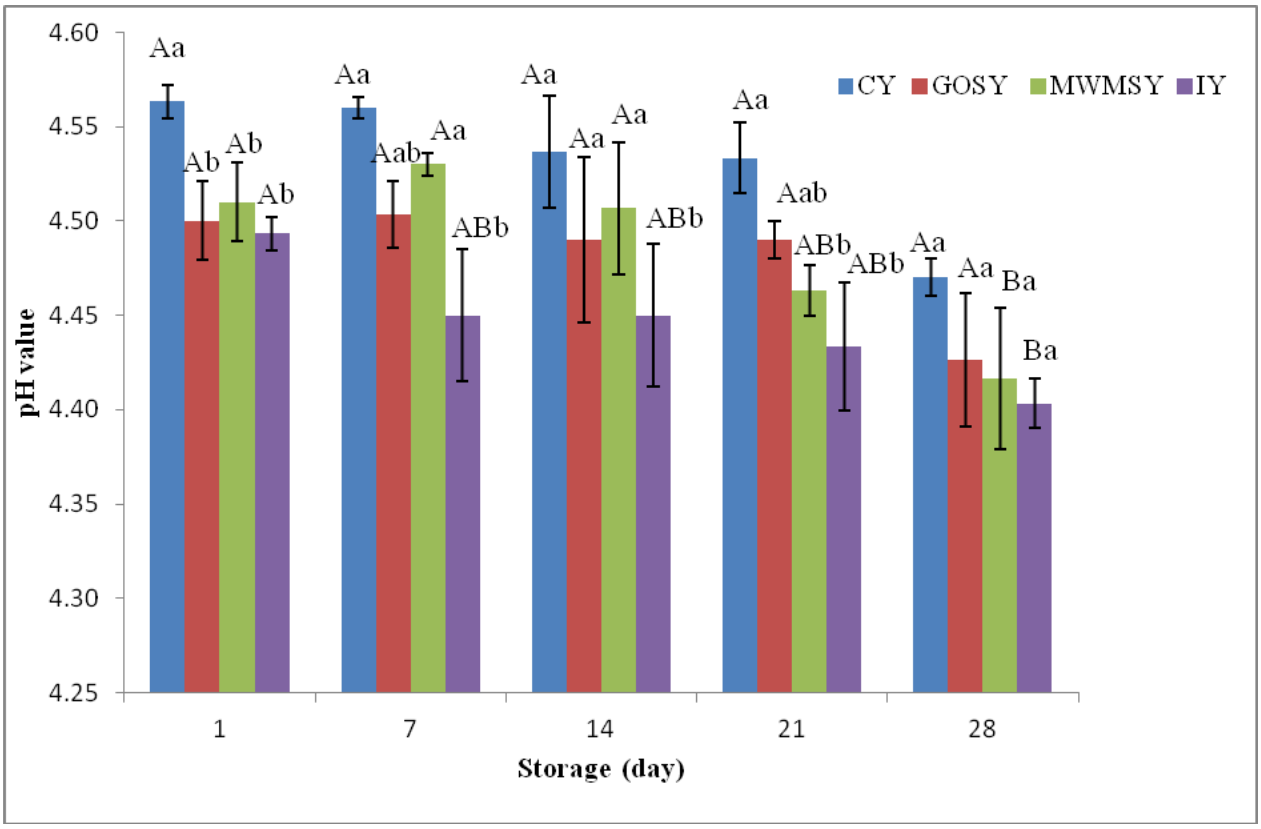

Figure 8. 1: Changes in pH of soy yogurts with and without prebiotic during storage at 4˚C.

CY (control): Yogurt prepared from soymilk in yogurt and starter cultures (1% LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in glucose and sucrose and 2% GOS or MWMS or Inulin: Bars indicate standard deviations

Donkor et al. (2007) showed that supplementation with prebiotics such as inulin and lactulose enhanced the viability of probiotics. Yeo and Min-Tze Liong (2010) reported that FOS and inulin were two most commonly used prebiotics and have been reported to be favourable carbon sources for the growth of LAB. However, there have been contradictory reports regarding the use of prebiotics to stimulate the growth of LAB strains. Heubner et al. (2007) reported that *L. paracasei* 1195 grew better in MRS medium containing 10 g/L FOS and 10 g/L inulin than in glucose medium, whereas Kaplan and Hutkins (2000) reported that three *Lactobacillus* strains and one *Bifidobacterium* strain were unable to ferment FOS. In the

present study, supplementation with inulin showed the highest 8.90 CFU ml^{-1} and 8.76 CFU ml^{-1} by ST and LB, respectively. These findings are in agreement with Sghir et al. (1998) who demonstrated that inulin was selectively fermented not only by bifidobacteria but also by lactic acid bacteria.

8.3.4 Proteolytic activity

The proteolytic activity of starter culture in four types of soy yogurts stored at 4˚C for 28 days is presented in Figure 8.4. The proteolysis was measured by the amount of free amino acids as absorbance at 340 nm in the fresh and stored yogurt samples. The OPA- based spectrophotometric detection of released amino groups resulting from proteolysis of soymilk proteins was used as direct indicator of proteolytic activity. There was a significant ($P < 0.05$) increase in the amount of free amino acids as a result of the LAB proteolytic activity in all soy yogurt samples during the first two weeks but declined during the last two weeks of storage. These results were similar to those of Radke-Mitchell and Sandine (1984) who reported that ST does not produce substantial amino acids and free peptide which is not enough to promote its growth during end of storage.

Stronger proteolysis was detected in IY than in GOSY, MWMSY and the CY. The maximum proteolytic capability was observed in IY (absorbance value of 0.486) whereas only 0.291, 0.274 and 0.187 were found in the GOSY, MWMSY and the CY respectively on day 28. Shihata and Shah (2000) reported that proteinase and peptidases constitute the primary enzymes in LAB responsible for proteolysis in yogurt proteins as a source of amino acids and nitrogen for LAB.

This study showed that ST and LB were proteolytic during their growth in soy yogurt and that the degree of proteolysis increased significantly ($P < 0.05$) with the storage time. This increased proteolytic activity may have improved the level of growth factors in the form of

free NH_3 groups and peptides.

8.3.5 Syneresis

The syneresis values are presented in Table 8.3. The extent of syneresis was significantly lower in samples containing prebiotics compared to CY due to interference of prebiotics in the shrinkage of the gel network (Lucey, 2002; Hammes and Hertel, 2002).

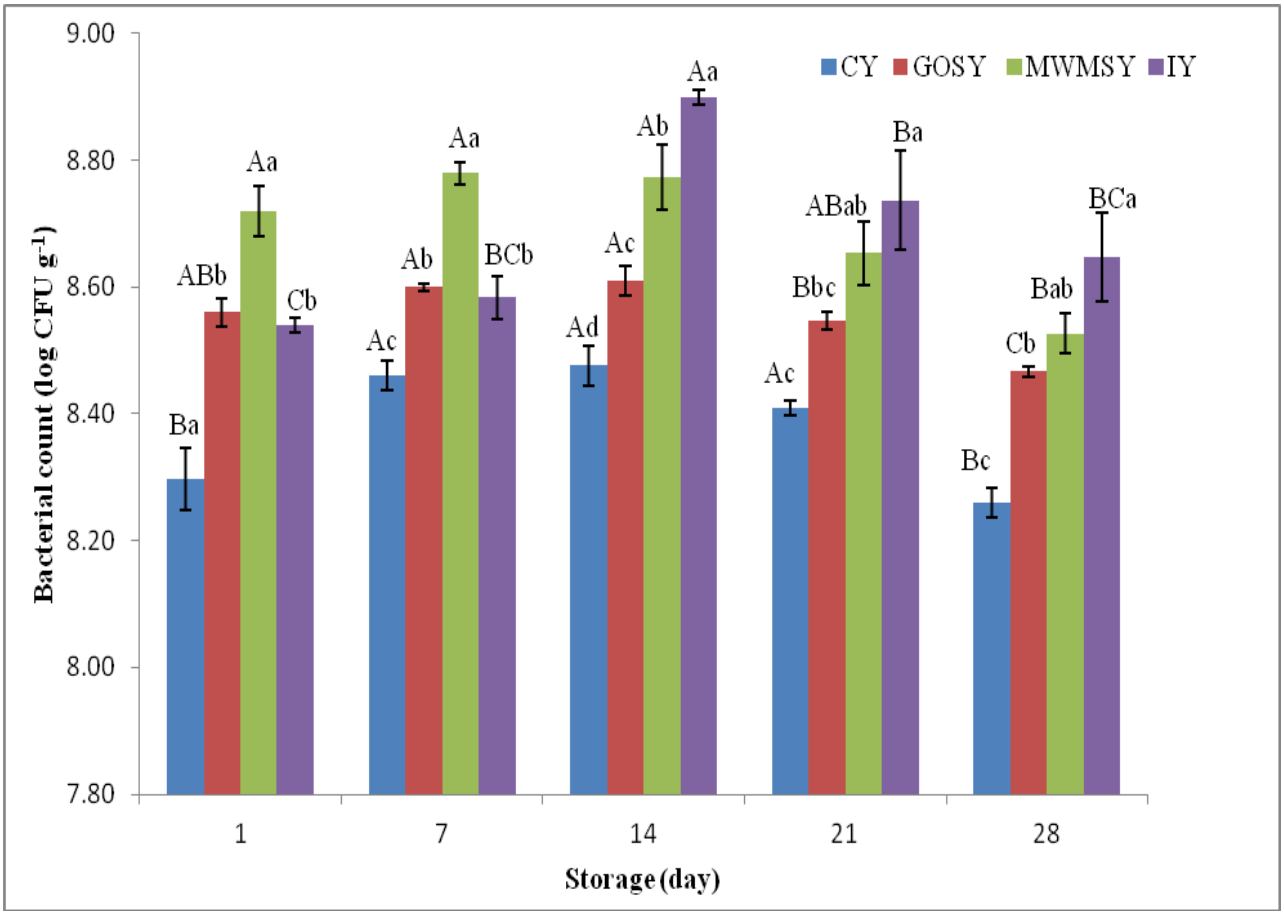

Figure 8. 2: Changes in the viable counts of ST grown in soymilk with and without prebiotics during storage at 4˚C.

CY (control): Yogurt prepared from soymilk and starter cultures (1% LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: Bars indicate standard deviations. Column bars with same colour at different times having similar letters did not differ significantly.

This may also be explained by improved water holding capacity of the treatments containing prebiotics, specially the inulin. The role of inulin was not only to improve water

holding capacity in yoghurt but it also served as a preferred carbon source for *Lactobacillus* strains, hence improved their growth performance during storage (Roberfroid et al., 1998; Capela et al., 2006; Aryana et al., 2007). There was a significant difference ($p<0.05$) in the level of syneresis between the CY and the IY but no significant difference was observed between the GOSY and MWMSY during the first three weeks of storage. The IY had lower syneresis values compared to the CY and the MWMSY and GOSY.

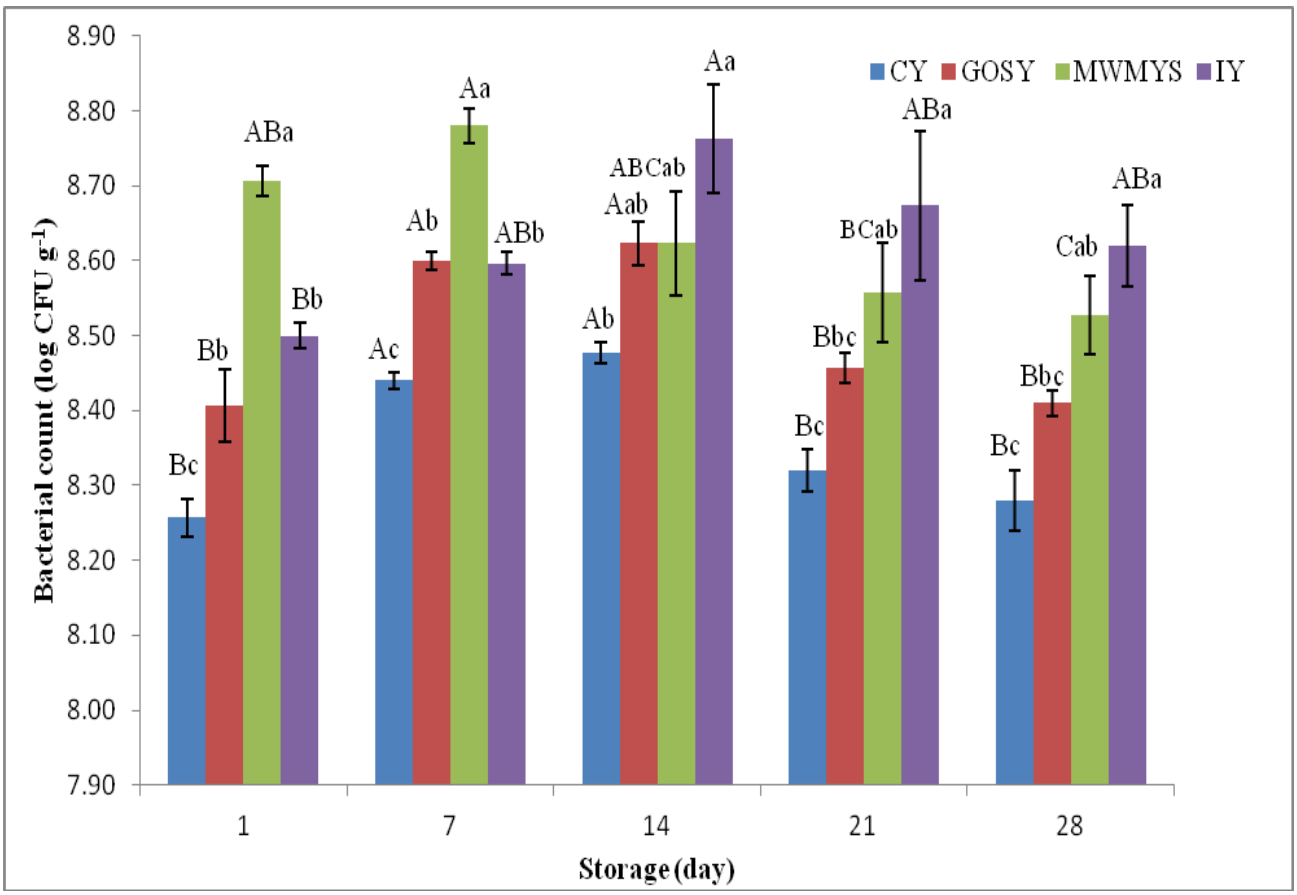

Figure 8. 3: Changes in the viable counts of LB grown in soymilk with and without prebiotics during storage at 4˚C.

CY (control): Yogurt prepared from soymilk and starter cultures (1% LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: Bars indicate standard deviations. Column bars with same colour at different times having similar letters did not differ significantly.

The highest syneresis was observed in the CY (4.63%) followed by GOSY (3.89%), MWMSY (3.16%) and the lowest in the IY (2.95%) at day 28 as shown in Table 8.3. This

may be due to addition of inulin possibly having a better water-holding capacity than other prebiotics (Deis, 2001).

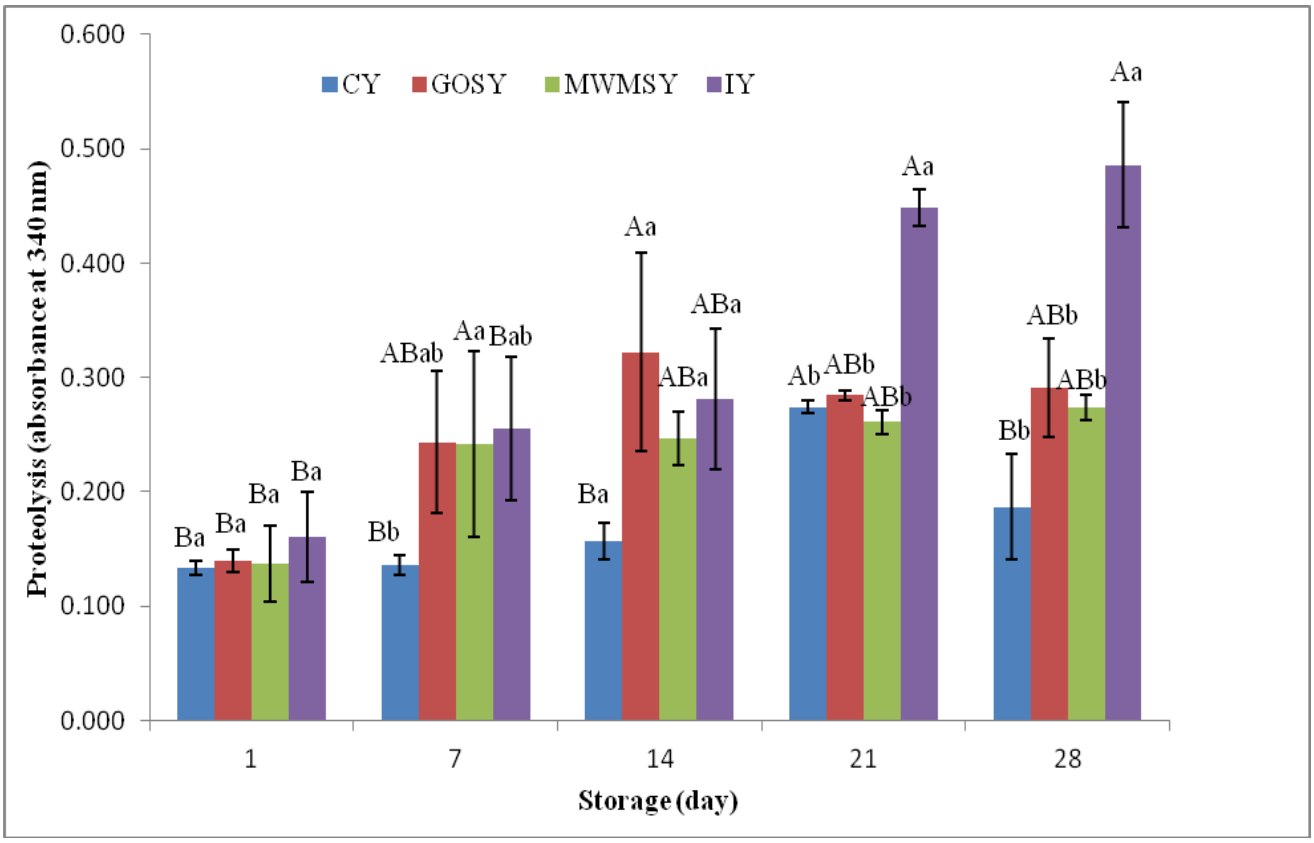

Figure 8. 4: Progressive proteolysis of soy yogurt with and without prebiotics during storage at 4˚C.

CY (control): Yogurt prepared from soymilk and starter cultures (1% LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: Bars indicate standard deviations. Column bars with same colour at different times having similar letters did not differ significantly.

8.3.6 Viscosity

The viscosity of all batches of yogurt with or without prebiotic supplementation stored at 4˚C is presented in Table 8.4. The addition of inulin resulted in higher viscosity compared with the GOSY, MWMSY and the CY.

Viscosity in all batches was found to increase during storage and maximum increased was at 28 days of storage. The highest viscosity value was observed in the IY (5.44 Pa.s) followed by GOSY (4.29 Pa.s), MWMSY (3.13 Pa.s) and the lowest in the CY (2.87 Pa.s) at 28 days.

Table 8. 3: Syneresis (%) in soy yogurts with and without prebiotics during storage at 4˚C

	Storage period (days)				
	1	**7**	**14**	**21**	**28**
CY	2.62 ± 0.27^{Ca}	3.24 ± 0.02^{BCa}	4.35 ± 0.37^{ABa}	4.47 ± 0.70^{Aa}	4.63 ± 0.24^{Aa}
GOSY	2.59 ± 0.21^{Ba}	3.15 ± 0.51^{Aa}	3.84 ± 0.64^{Aab}	3.88 ± 0.45^{Aab}	3.89 ± 0.19^{Aab}
MWMSY	2.02 ± 0.03^{Ba}	2.79 ± 0.05^{ABa}	2.93 ± 0.14^{Ab}	2.97 ± 0.37^{Aab}	3.16 ± 0.58^{Ab}
IY	2.22 ± 0.02^{Aab}	2.77 ± 0.06^{Aa}	2.90 ± 0.37^{Ab}	2.73 ± 0.28^{Ab}	2.95 ± 0.31^{Ab}

Values are the statistical means of 6 observations. Control: Yogurt prepared from soymilk and starter cultures (1 % LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: ^{abc} Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABCD}Means in the same row with different alphabets are significantly different for a particular treatment.

These results were similar with those of Bouzar et al. (1997) who reported viscosity increase during storage, possibly due to increasing hydration. Also, Hauly et al. (2005) reported that soy yogurt supplemented with inulin had a higher viscosity than the non-supplemented yogurt. The viscosity increase in these samples has been attributed to interaction between these polysaccharides and dairy proteins (Fernandez- Garcia et al., 1998; Syrbe et al., 1998 and Sodini et al., 2002).

According to Weinbreck et al. (2003a) and Ye et al. (2006), when milk proteins interact with polysaccharides at low pH, they may form soluble or insoluble complexs depending on the nature and the extent of interactions. During manufacturing processes, these interactions between proteins and polysaccharides may determine relationship between structure and properties of food (Tolstoguzov, 1997). Anne et al. (1999) reported that the best correlation was found between viscosity and texture. Viscosity is critical in the texture development of yoghurt. It may also be a crucial attribute in defining mouth feel, flavour release and refreshing quality of the products (Ramesh, 2006). However, sensory assessment of the product has not been included in this study.

8.3.7 Large scale measurement of yogurt texture

The firmness (g) of all batches of yogurt during storage at 4˚C for 28 d is presented in Table 8.5. There was significant ($p < 0.05$) difference in the firmness among all batches of yogurt at the end of storage time. The maximum firmness was recorded in the IY (57.00 g) followed by MWMSY (51.33 g), GOSY (33.00 g) and the CY (26.00 g). Bozanic et al. (2001) found that the firmness of yogurt improved upon the addition of prebiotic.

Table 8. 4: Viscosity (Pa.s) of soy yogurts with and without prebiotics during 4-week storage at 4˚C.

	Storage period (days)				
	1	**7**	**14**	**21**	**28**
CY	2.39 ± 0.15^{Bb}	2.99 ± 0.07^{Ab}	2.87 ± 0.05^{Ab}	2.12 ± 0.08^{Cb}	2.87 ± 0.46^{Cc}
GOSY	3.11 ± 0.25^{ABb}	3.14 ± 0.17^{Bb}	3.94 ± 0.30^{Aab}	$2.20.25^{Cb}$	3.13 ± 0.09^{Bbc}
MWMSY	5.42 ± 0.72^{Aa}	4.13 ± 0.02^{Ba}	4.88 ± 0.80^{Aa}	4.26 ± 0.70^{Ba}	4.29 ± 0.43^{Bab}
IY	2.17 ± 0.67^{Cb}	4.60 ± 0.47^{ABa}	5.06 ± 0.18^{ABa}	5.12 ± 0.27^{ABa}	5.44 ± 0.56^{Aa}

Values are the statistical means of 6 observations. Control: Yogurt prepared from soymilk and starter cultures (1 % LB and 2 % ST). GOSY, MWMSY and IY: Yogurt prepared from soymilk in with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABC}Means in the same raw with different alphabets are significantly different for a storage day.

Table 8. 5: Firmness (g) of soymilk yogurts with and without prebiotics during 4-week storage at 4˚C.

	Storage period (days)				
	1	**7**	**14**	**21**	**28**
CY	22.00 ± 0.58^{Ac}	22.33 ± 4.84^{Ac}	25.00 ± 1.73^{Ab}	25.67 ± 1.76^{Ac}	26.00 ± 1.73^{Cb}
GOSY	26.67 ± 0.88^{ABbc}	25.50 ± 3.18^{Bbc}	30.00 ± 5.20^{Aab}	30.50 ± 3.76^{Abc}	33.00 ± 0.04^{Bb}
MWMSY	41.33 ± 3.67^{Aa}	47.00 ± 5.13^{Aa}	46.67 ± 3.93^{Aa}	48.00 ± 9.53^{Aab}	51.33 ± 5.86^{Aa}
IY	35.00 ± 5.33^{Bab}	38.00 ± 4.73^{Bab}	46.33 ± 8.95^{ABa}	51.50 ± 6.08^{ABa}	57.00 ± 8.33^{Aa}

Values are the statistical means of 6 observations. Control: Yogurt prepared from soymilk and starter cultures (1 % LB and 2 % ST). Yogurt prepared from soymilk with addition of glucose and sucrose and 2% GOS or MWMS or Inulin: ^{abc}Means in the same row with different alphabets are significantly different within a particular treatment. ^{ABCDE}Means in the same column with different alphabets are significantly different for a particular day of storage.

According to Tamime and Robinson (1999), the primary aim of adding these prebiotic to the

milk base is not only enhancement and maintaining the yogurt texture and consistency but also improvement of general appearance as well as mouthfeel. The addition of GOS and MWMS had a remarkable improvement in the texture of yogurts which is in accordance with the findings of Jawalekar et al. (1993). There are few studies on the texture of soy yogurt containing prebiotics. In our study, results were similar with those of Radke-Mitchell (1986) and Donkor et al. (2008) who confirmed that supplementation with either MWMS or inulin resulted in a better body and viscosity of yogurt.

8.4 Conclusion

This study showed that soymilk supplementation with prebiotics improved the viability of yogurt organisms. The growth of ST and LB was higher in soy yogurt in presence of inulin than those of MWMS, GOS and the control. However, supplementation with 2% inulin resulted in the retention of viability of ST was better than that of LB in all types of yogurt. The viable counts of ST in the yogurt containing prebiotics and the CY were stable with >8 log CFU mL^{-1} throughout storage time while the numbers of LB was slightly lower than the ST. It appears the inulin not only enhanced the growth of selected organisms but also increased the lactic and acetic acids production throughout the storage time and decreased syneresis values compared to control, MWMS and GOS. This may also be explained by improved water holding capacity of the treatments containing prebiotics, specially the inulin. The use of yogurt culture resulted in the appreciable proteolytic activity likely improving the growth of selected organisms. Our study confirmed that supplementation of soymilk with inulin resulted in a better body and viscosity of soy yogurt. The maximum viability of both organisms was found in the soy yogurt containing inulin suggesting IY could have enhanced prebiotics properties compared to GOSY and MWMSY. Therefore, addition of 2% inulin could provide functional properties in production of soy yogurts.

Chapter 9.0 Overall Conclusions and Future Reseaarch Direction

9.1 Conclusions

This study investigated the production of β-Galactosidase (β-gal) by *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and the effect of four different extraction methods i.e. sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatment on enzyme activity from these organisms. Among the four extraction methods, sonication was found to be more effective for BB12, whereas lysozyme-EDTA treatment was found to be more effective for LB. The enzyme activity at pH 6.8 was significantly higher ($P<0.05$) than at other pH levels for both organisms. The optimum temperature for the activity of enzyme obtained from BB12 was found to be 35 ºC whereas for LB it was 45 ºC. Deproteinised sweet whey was found to be a suitable medium for β-gal production, it should be possible to produce commercial amounts of β-gal using the two organisms reported in this study; however the enzyme extraction method need to be adapted to the strain used.

The results of this study demonstrated that BB12 and LB are capable of producing high level of β-gal. A maximum of β-gal 73.66 Unit/mL and 48.63 Unit/mL were produced by Bb12 and LB, respectively in media contained galactose as carbon source. Similarly, maximum β-gal 51.61 Unit/mL and 50.73 Unit/mL were produced by BB12 and LB in MRS broth containing casein hydrolysate as nitrogen source. Results may justify that these strains are suitable for the production of β-gal enzyme. Considering the high yield of β-gal by BB12, it may be considered as a potentially useful industrial strain. Application of this enzyme for GOS production in whey needs to be further investigated.

The study confirmed that BB12 and LB are capable of hydrolysing glycitin to biologically active forms in soymilk prepared from soy protein isolate (SPI) and soymilk supplemented with 2.0 % (w/v) of D-glucose at different concentrations of crude enzyme extract (CEE). The increased cell growth resulted in higher enzyme activity, which

subsequently produced increased concentration of daidzein and genistein in fermented soymilk. This is likely to improve the biological functionality of soymilk.

The supplementation of yogurt with prebiotics improved the retention of viability of *S. thermophilus* M5 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in all batches of yogurt during cold storage especially in the presence of GOS. The GOS was found to be a better growth stimulant of selected organisms than MWMS. Addition of GOS produced the highest levels of lactic and acetic acids than those of MWMSY and the CY. However, the production of acetic acid increased in all samples throughout the storage. Furthermore, improved proteolysis was found in the presence of GOS as compared to that of MWMS. The amount of syneresis was lower in GOSY compared to the MWMSY and the CY. Our study confirmed that GOS was the best prebiotic ingredients for a improved yoghurt body. The increased proteolytic activity in GOSY may have resulted in improved level of growth factors such as NH_3 groups and peptides. Therefore, addition of 2% GOS could provide functional properties in the resulting yogurt.

This study showed that soymilk supplementation with prebiotics improved the viability of yogurt organisms. The growth of ST and LB in soy yogurt was higher in presence of inulin than in the presence of MWMS, GOS or in the control sogurt. However, supplementation with 2% inulin resulted in better retention of viability of ST than that of LB in all types of sogurt. The viable counts of ST in the sogurt containing prebiotics and the CY were stable with >8 log CFU g^{-1} throughout storage time while the numbers of LB was slightly lower than the ST. The use of yogurt culture resulted in the appreciable proteolytic activity likely improving the growth of selected organisms. Our study confirmed that supplementation of soymilk with inulin resulted in a better body and viscosity in soy yogurt. The maximum viability of both organisms was found in the soy yogurt containing inulin suggesting IY could have enhanced prebiotics properties compared to GOSY and MWMSY. Domagała et al.

(2005) examined the influence of storage time of yoghurt with the 2% prebiotic addition. They observed that the viscosity of examined yoghurts decreased during storage time. However, Tamime and Robinson (2007) reported that higher contents of solids in the yoghurt promote greater viscosity of final products.

9.2 Future Research Directions

This research has raised some interesting questions that need to be addressed in further research.

The study shows that there is appreciable production of intracellular β-gal from *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 using sonication and lysozyme-EDTA enzyme extraction methods. This suggests that these organisms can be employed as potential producers of intracellular β-gal which may have applications in both food industries and biotechnologies. Further studies will need to be conducted by researchers for further enhancement of enzyme production.

The study confirmed that addition of GOS enhanced the growth of selected organisms and increased proteolytic activity in yogurt, potentially opening opportunities for its use as nutrient supplement or a food ingredient. The GOS are available commercially as mixtures of galactose-based oligosaccharides of varying degrees of polymerization and linkage configuration with glucose, galactose and lactose. The purity of ingredient has an effect on it potential applications. Therefore, future research should be carried out on purity of GOS as food ingredient.

Inulin as carbohydrate- based fat replacer has gelling capacity with water and gives a fat-like mouthfeel. The present study also reveals the maximum viability of ST and LB was found in the soy yogurt containing inulin suggesting IY could have enhanced prebiotics properties. Therefore, further studies will need to be conducted by researchers for high yielding inulin production on dry weight basis.

This study shows that BB12 and LB are capable of hydrolysing glycitin to biologically active forms in soymilk prepared from soy protein isolate (SPI) and soymilk supplemented at different concentrations of CCE. Therefore, further research should be carried out to explore more sources of enzymes which are able to hydrolyse IGs to IAs and also the

biotransformation of IGs to IAs in soy yogurt fermented by the combination of yogurt starter and probiotic organisms during storage period.

10. References

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11. Appendix

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Extraction and characterisation of β-galactosidase produced by *Bifidobacterium animalis* spp. *lactis* Bb12 and *Lactobacillus delbrueckii* spp. *bulgaricus* ATCC 11842 grown in whey

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Abstract

This study investigated the production of β-Galactosidase (β-gal) by *Bifidobacterium animalis* ssp. lactis Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and the effect of four different extraction methods i.e. sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatment on enzyme activity from these organisms. Both organisms were grown in deproteinised whey containing yeast extract (3.0 g/L), peptone (5.0 g/L) and glucose (10.0 g/L) for 18 h, at 37 ºC for *B. animalis* ssp. *lactis* Bb12 and at 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The optimum intracelluar β-gal activity on 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) assay was at pH 6.8 for both organisms irrespective of the method of extraction used. Also, the effect of temperature on enzyme activity was studied at various temperatures (30, 35, 40, 45, and 50°C). At 35°C and 40°C, *B. animalis* ssp. *lactis* Bb12 exhibited more intracellular β-gal activity extracted by sonication than other temperatures and methods. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed more intracellular β-gal activity at 35°C and 45°C when extracted by lysozyme-EDTA treatment. Among the four methods used for β-gal extraction, sonication gave the best result (6.80 Unit/mL) for *B. animalis* ssp. *lactis* Bb12 while lysozyme-EDTA treatment was found to be the best (7.77 Unit/mL) for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

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Introduction

β-Gal; lactase, EC 3.2.1.23) catalyzes the hydrolysis of lactose to glucose and galactose. This enzyme is used to hydrolyse milk lactose to combat the problems of lactose intolerance by individuals who are deficient in lactase (Artolozaga *et al*., 1998). Commercial β-gal is produced from bacteria (such as *Streptococcus thermophilus* and *Lactobaccillus lactis*); yeasts (such as *Kluyveromyces lactis* and *Kluyveromyces marxianus*) and moulds (such as *Aspergillus niger*, *Aspergillus candidus* and *Aspergillus oryzae* (Panesar *et al*., 2006; Zheng *et al*., 2006). Since β-gal is an intracellular enzyme, one of the major hindrances in effective production of this enzyme is its release in sufficient quantities from cells. The use of whole cells as a source of β-gal may appear as a good alternative, however, a major drawback is the poor permeability of cell wall membrane. Therefore, different methods have been applied to increase their permeability of microbial cell walls (Panesar *et al*., 2006).

Several workers have reported on the release of β-gal through permeabilization of microbial cells by organic solvents (Flores *et al*., 1994; Numanoglu and Sungur, 2004; Panesar *et al*., 2007; Park *et al*., 2007). Flores et al. (1994) studied the permeabilization of *K. lactis* cells by chloroform, toluene and ethanol to release β-gal enzyme. They found that the effectiveness of solvents was dependent on the incubation time, incubation temperature and concentration of both cells and solvents. Mechanical methods such as sonication, high-pressure homogenizer or bead mills have been traditionally used for the disruption of microbial cells (Geciova *et al*., 2000). The method of choice should be robust enough to disrupt cell membranes efficiently but gentle enough to preserve enzyme activity (Numanoglu and Sungur, 2004).

Sonication is one of the most widely used methods for disruption of the bacterial cell walls (Engler,

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1985). Among the three methods, sonication, bead milling and high-pressure homogenizer, sonication was found to be more effective for releasing β-gal (Toba *et al*., 1990; Sakakibara *et al*., 1994). Berger *et al*. (1995) compared two physical disruption methods for the extraction of intracellular β-gal enzyme from *Thermus* species and found that the sonication was superior to the glass-bead milling. Bury *et al*. (2001) studied on the disruption of cells of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 who concluded that sonication was the least effective method on the release of β-gal.

Salasbury (1989) found that lysozyme is often used for lysis of peptidoglycan layers as it catalyses hydrolysis of β 1-4-glycosidic bonds. The enzyme is commercially available at a reasonable cost, and is produced from egg-white preparations. Gram-negative bacteria are less susceptible than the Gram-positive ones as their outer layer made of peptidoglycan, is responsible for rigidity of bacterial cell wall and for determination of cell shape. It is made up of a polysaccharide backbone consisting of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues in equal amounts. However, combining lysozyme-EDTA treatment allows the disruption of the cell wall and subsequent attack on the peptidoglycan structure (Salasbury, 1989). Therefore, lysozyme-EDTA mixture is very efficient for releasing β-gal from Gram-negative bacteria cell walls (Andrews and Asenjo, 1987; Geciova *et al*., 2000).

Numanoglu and Sungur (2004) compared chemical (toluene, SDS-chloroform) and physical (glass bead mill) methods to facilitate the release of β-gal from *K. lactis* cells and found that the physical method was better than chemical ones. This was in agreement with Fiedurek and Szczodrak (1994) who used three methods such as solvent and detergent extraction, freezing and thawing extraction, and mechanical disintegration to release the β-gal from *K. fragilis* cells and found that the highest yield was obtained by mechanical disintegration.

The lactic acid bacteria (LAB) requires numerous growth factors such as whey, reconstituted skim milk (RSM) and MRS broth in addition to carbohydrate and nitrogen sources in a growth medium (Stiles and Holzapfel, 1997) to be used for the enzyme production. In search for a suitable and inexpensive medium is readily available components such as whey appear as an attractive alternative to RSM (Gupta and Gandhi, 1995; Bury *et al*., 2000). The β-gal activity of a given microorganism depends on the characteristics of a medium. To maximize the enzyme activity, a rich medium is necessary. Therefore, sweet whey appears highly attractive mostly due to relatively high lactose content. Lactose constitutes over 70% of the total solids in whey (Rhimi *et al*., 2007).

There are two types of whey; i) Sweet whey is produced during the producing of rennet types or hard cheeses like Cheddar or Swiss cheeses. ii) Acid whey (also known as "sour whey") is obtained during the production of acid types cheeses such as cottage cheese. Sweet whey is a rich source of whey proteins, lactose, enzymes, vitamins, bioactive compounds and minerals (Agrawal *et a*l., 1989; Joshi *et al*., 1989; Keerthana and Reddy, 2006). Many small-size cheese plants do not have proper treatment systems for the disposal of whey and the dumping of whey constitutes a significant loss of potential food as whey retains about 40-45% of total milk solids (Panesar *et al*., 2007). Its disposal as waste poses serious pollution problems for the surrounding environment (Carrara and Rubiolo, 1994; Dagbagli and Goksungur, 2008; Magalhaes *et al*., 2010a). Sweet syrup produced through lactose hydrolysis by β-gal can be used in dairy, confectionary, baking and soft drink industries (Mahoney, 1997; Rajakala *et al*., 2006). Other applications of β-gal could also include the production of biologically-active galacto-oligosaccharides from lactose hydrolysis (Boon *et al*., 2000; Albayrak and Yang, 2002).

The *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was selected based on previous evidence as a high β-gal producer (Vasiljevic and Jelen, 2003). The *B. animalis* ssp. *lactis* Bb12 was found to possess the highest level of β-gal activity compared to others Bifidobacteria (Dechter and Hoover, 1998). Therefore, the present study was undertaken to evaluate the suitability of sweet whey as a medium for the production of β-gal from *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This study also evaluated physical and chemical methods of enzyme extraction from bacteria in terms of their efficacy and enzyme yield.

Materials and Methods

Micro-organisms

Pure culture of *B. animalis* Bb12 was obtained from Chr. Hansen, (Bayswater, VIC, Australia) and *L. delbrueckii* ssp. *bulgaricus* ATCC was obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80ºC in 50/50 sterile MRS broth (Difco, Becton, Dickinson and Company, New Jersey, USA) and glycerol (MERCK Pty Ltd, Colchester Road, Kilsyth, Australia.

Culture growth conditions

The organisms were activated in two successive transfers in MRS broth supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) and incubated at 37ºC for *B. animalis* ssp. *lactis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. Activated organisms were grown in deproteinized sweet whey supplemented with yeast extract (3.0 g/L), peptone (5 g/L) and glucose (10 g/L). The sweet whey was deproteinized by heating at 85ºC for 10 min after adjusting the pH to 4.5 using lactic acid. The heat-treated whey was cooled to room temperature and filtered through Whatman no. 1 filter paper. The pH of whey medium was then re-adjusted to 7.0 and sterilized at 121ºC for 15 min then inoculated aseptically with 1% of each organism and incubated at 37 ºC for *B. animalis* ssp. *lactis* Bb12 or 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h under anaerobic conditions.

Enzyme extraction

After 18 h of incubation, the cells were harvested by centrifuging at 10,000×g for 10 min at 4°C. The supernatant was considered to be containing extracellular enzymes. The cell pellet was crushed and washed twice with a 0.03 M sodium phosphate buffer (pH 6.8) and centrifuged at 10,000×g for 10 min at 4°C. The washed pellets were resuspended in 5 mL of 0.2 M phosphate buffer (pH 6.8) for intracellular enzyme extraction using four different cell disintegration methods listed below:

Sonication: The cell suspensions were sonicated for 30 min in ice bath using Sonirep 150 MSE (MSE Instruments, Crawley, UK) sonicator according to the method of Beccerra et al. (1998). The extract was then centrifuged at 15,000×g and 4°C for 10 min and the supernatant containing the crude enzyme was stored at –20°C until used for enzyme assays.

Lysozyme-EDTA treatment: Lysozyme solution was prepared by dissolving 50 mg of lysozyme (Sigma Aldrich Pty Lim, Castle Hill NSW, Australia) in 1.5 mL of TE (Tris-EDTA; Ethylenediamine Tetraacetic Acid) buffer containing 1 mM EDTA and 10 mM Tris-HCl, adjusted to pH 8.0. The lysozyme preparation was added to the cell suspension at the rate of 75 µL per mL, incubated for 30 min at room temperature then kept at -200C until enzyme activity measurement.

Toluene-acetone treatment: Ten millilitre of cell suspension was ground for 10 min in a pestle and mortar with 2.0 g alumina (Sigma Aldrich Pty Lim, Castle Hill NSW, Australia) and 0.1 mL of 9:1 mixture of toluene (BDH Chemical, Pty Limited, Kilsyth, Vic, Australia with 99.5% purity) and acetone (Merck Pty Limited Kilsyth, Vic, Australia with 99% purity) solvents. The suspension was extended in 8 mL phosphate buffer and centrifuged at 15,000×g for 10 min at 4°C (Mahoney et al., 1975). The supernatant obtained was kept at -20^{0}C until used for enzyme assay.

Sodium Dodecyl Sulfate (SDS)-Chloroform treatment: Permeabilization of cell membrane was carried out by vortexing 10 mL of the cell suspension in the presence of 100 µL chloroform and 50 µL 0.1% SDS solution for 30 min at room temperature (Mahoney et al., 1975). The suspension was centrifuged at 15,000×g for 10 min at 4 °C and the supernatant was kept at -20^{0}C until needed for the enzyme assay.

Enzyme assay

The β-Gal was determined as described by Hsu *et al*. (2005). The reaction mixture was composed of 0.5 mL of supernatant containing extracted enzyme and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After incubation for 10 min at 37°C, 2.0 mL of 0.1 M sodium carbonate was added to the mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Helios R, Unicam Co., Cambridge, UK). One unit of β-gal was defined as the amount of enzyme that produced one micro-mol (µM) of o-nitrophenol per min under the assay condition.

Effect of pH and temperature on β-Gal activity

The intracellular β-gal extracted by four different methods were characterised for their optimum activity by incubating the enzyme in substrate of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) adjusted at three levels of assay pH (4.5, 5.5 and 6.8) with 2N NaOH, or 3N HCL in 0.03 M sodium phosphate buffer for 10 min at 37ºC. Similarly, the effect of temperature on enzyme activity was studied by incubating the enzyme in above mentioned substrate at various temperatures (30, 35, 40, 45 and 50ºC) for 10 min at pH 6.8.

Statistical analysis

All analyses were performed in triplicate and data were analyzed using Statistical Analysis System (SAS) software (SAS, 1995) and one-way analysis of variance (ANOVA) at 5% confidence level. ANOVA data with a $P < 0.05$ were classified as statistically significant.

Results and Discussion

β-Gal production in whey and its extraction

The activity of β-gal from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and its extraction using various methods is shown in Table 1. *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced more ($p<0.05$) intracellular β-gal than *B. animalis* ssp. *lactis* Bb12 with all extraction methods, except sonication. There were significant ($p<0.05$) differences in β-gal levels extracted from each organism by the four extraction methods. Sonication method was found to be more effective for *B. animalis* Bb12 than the others methods, however, lysozyme-EDTA treatment was found to be more effective for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The maximum intracellular β-gal activity (7.77 Unit/mL) was obtained from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 by lysozyme treatment while the lowest activity (2.05 Unit/mL) was measured using toluene-acetone treatment. Similarly, this method resulted in the lowest activity (0.64 Unit/mL) from *B. animalis* Bb12 while the highest β-gal activity (6.80 Unit/mL) was obtained by sonication. However, lower intracellular β-gal activities (4.85 Unit/mL) and (1.58 Unit/mL) were obtained from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *B. animalis* Bb12, respectively by SDS-chloroform treatment. Toluene-acetone treatment was not as effective as the SDS-chloroform method. SDS is a non-ionic detergent which works by disrupting non-covalent bonds in proteins, thereby denaturing them, causing the molecules to lose their native shape (Panesar *et al*., 2006). Chloroform is also a common solvent because it is relatively unreactive, miscible with most organic liquids, and conveniently volatile. It is an effective solvent for alkaloids in their base form and thus plant materials are commonly extracted with chloroform for pharmaceutical processing. Thus the action of SDS-chloroform mixture could be of synergistic nature resulting in efficient permeabilization of cell wall of yeast cells and subsequent release of the enzyme (Panesar *et al*., 2006).

Our findings agree with those of Berger *et al*. (1995) who found that sonication was more effective than high-pressure homogenization, bead milling and toluene-acetone treatments for the release of β-gal from *Thermus* species. However, our results are contrary to the finding by Bury *et al*. (2001) who concluded that sonication was the least effective method on the release of β-gal from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Therefore in our study, sonication method was found to be more effective for *B. animalis* Bb12, while lysozyme-EDTA treatment was more effective for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

Table 1. Effects of extraction methods on intracellular β-gal activity from *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey for 18 h at 37 ºC

Methods	Lb ATCC 11842 (Unit/mL)	Bb12 (Unit/mL)
Sonication	3.09±0.34^{A}	6.80±0.35^{A}
Toluene-Acetone	2.05±0.35^{D}	0.64±0.06^{D}
SDS-Chloroform	4.85±1.14^{C}	1.58±0.15^{C}
Lysozyme treatment	7.77±2.78^{B}	3.96±1.05^{B}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different ($P > 0.05$). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different ($P > 0.05$)

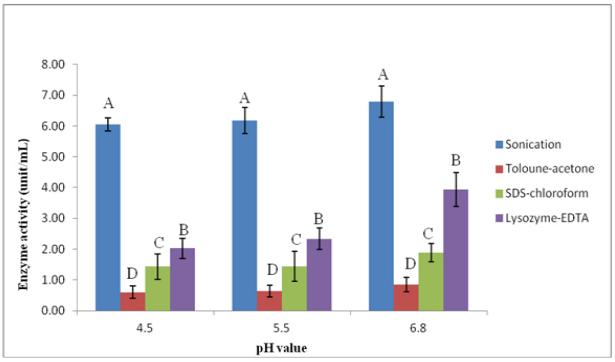

Figure 1. Effect of growth medium pH at 37 ºC on the activity of intracellular β-gal enzyme extracted by four extraction methods from *B. animalis* ssp. *lactis* Bb12. Bars indicate standard deviations. Different letters within each type of treatment indicate a significant difference ($p<0.05$). Mean values for a particular extraction method with same uppercase letters are not significantly different ($P>0.05$)

Effect of pH on the activity of intracellular enzyme extracted from B. animalis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842

The optimum activity of the intracellular β-gal from *B. animalis* ssp. *lactis* Bb12 as extracted by four different methods and various assay pH levels ranging from (4.5, 5.5 and 6.8) is shown in Fig 1. The pH 6.8 was selected based on previous evidence as a high β-gal enzyme activity (Hsu *et al*., 2007). Among the four extraction methods employed for *B. animalis* ssp. *lactis* Bb12, sonication resulted in significantly ($p<0.05$) higher enzyme activity followed by lysozyme-EDTA treatment at pH 6.8. Enzyme from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 also showed (Fig 2) its maximum activity at pH 6.8 where lysozyme and SDS-chloroform treatments extracted more ($p<0.05$) enzyme than the other two methods. The enzyme activity at pH 6.8 was significantly higher ($p<0.05$) than at other pH levels for the both organisms. Any drop in pH value of assay medium resulted in a reduction on β-gal enzyme activity by test organisms.

The maximum enzyme activity (7.77 Unit/mL) was obtained when *L. delbrueckii* ssp. *bulgaricus*

Table 2. Effect of assay temperature at pH 6.8 and extraction methods on intracellular β-gal activity extracted from *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

	Lb ATCC 11842		Bb12	
Incubation Temp.	**Sonication** (Unit/mL)	**Lysozyme** (Unit/mL)	**Sonication** (Unit/mL)	**Lysozyme** (Unit/mL)
30 ˚C	1.23 ± 0.01^{Ca}	4.29 ± 0.06^{Cb}	3.00 ± 0.05^{Ca}	2.01 ± 0.02^{Cb}
35˚C	2.36 ± 0.02^{Aa}	7.35 ± 0.19^{Ab}	6.68 ± 0.11^{Aa}	2.13 ± 0.05^{Bb}
40˚C	2.22 ± 0.03^{Ab}	5.86 ± 0.13^{Ba}	5.67 ± 0.13^{Aa}	2.27 ± 0.02^{Ab}
45˚C	1.93 ± 0.02^{Bb}	7.45 ± 0.08^{Aa}	3.82 ± 0.05^{Ba}	1.34 ± 0.05^{Db}
50˚C	1.40 ± 0.01^{Cb}	7.25 ± 0.04^{Aa}	2.87 ± 0.04^{Ca}	1.33 ± 0.02^{Db}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05)

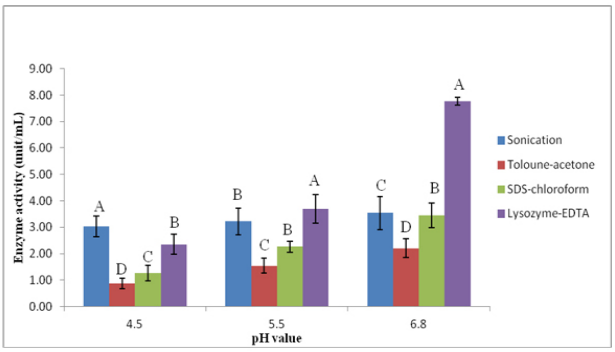

Figure 2. Effect of growth medium pH at 37 ºC on the activity of intracellular β-gal enzyme extracted by four extraction methods from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 Bars indicate standard deviations. Different letters within each type of treatment indicate a significant difference (p<0.05). Mean values for a particular extraction method with same uppercase letters are not significantly different (P>0.05)

ATCC 11842 was treated with lysozyme-EDTA mixture (Fig 2). Lower enzyme activities were found when SDS-chloroform (4.85 Unit/mL), sonication (3.09 Unit/mL) and toluene-acetone (2.05 Unit/mL) were used. Therefore, our results revealed at pH 6.8, β-gal activity was found to be at its peak for both organisms.

These findings agree with those of Greenberg and Mahoney (1982); Nagy *et al*. (2001) who reported that β-gal enzyme activity was found to be higher at pH 6.5 to 7.5 at 37˚C from *B. animalis*, but it appeared to be detrimental effect as enzyme is rapidly loose its activity at lower and higher of this range. Various workers reported that β-gal activity was affected by metallic ions (Hung and Lee, 2000; Kim *et al*., 2003). Moreover, Wang *et al*. (2004) also reported that the highest enzyme activity was observed in the pH range of 6.7 to 7.5.

Effect of temperature on intracellular enzyme activity extracted from B. animalis ssp. lactis Bb12 and L. delbrueckii ssp. bulgaricus ATCC 11842

Based on maximum enzyme activity results obtained for *B. animalis* ssp. *lactis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (Table 1), only the sonication and lysozyme-EDTA methods were chosen for the study on the effect of temperature on intracellular β-gal enzyme activity extracted from these organisms (Table 2). Subsequently, the enzyme extracted from each organism was incubated at various temperatures (30, 35, 40, 45 and 50ºC) for 10 min at pH 6.8.

Intracellular β-gal enzyme extracted by sonication and lysozyme-EDTA treatment from *B. animalis* ssp. *lactis* Bb12 showed significantly (p<0.05) higher activity at 35°C and 40°C than other temperatures (Table 2), whereas, β-gal extracted from *L. delbrueckii* ssp. *bulgaricus* showed its maximum activity (p<0.05) at 35 to 45°C (Table 2). There was a significant difference (p<0.05) in β-gal production by *B. animalis* Bb12 assay temperatures at 30°C, 45°C and 50°C using sonication method while no such difference was observed at 35°C and 40°C. However, lysozyme treatment showed a significant difference (p>0.05) in β-gal production by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at 30°C, 35°C and 40°C but no difference at 45°C and 50°C.

The maximum enzyme activity of 6.68 Unit/mL from *B. animalis* ssp. *lactis* Bb12 was obtained by sonication at 35 ºC whereas the maximum enzyme activity of 7.45 Unit/mL from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was obtained by lysozyme-EDTA treatment at 45 ºC (Table 2).

Many workers have reported 37 to 45ºC as the optimum temperature range for maximum enzyme activity with different organisms (Tzortzis *et al*., 2005; Splechtna *et al*., 2006; Searle *et al*., 2009). The maximum β-gal enzyme activity from *S. thermophilus* (Somkuti and Steinberg, 1979), *B. infantis* HL96 (Hung and Lee, 2002) and *Penicillium chrysogenum* (Nagy *et al*., 2001) was obtained at 35-50 ºC. Our results also revealed that β-gal extracted by sonication and lysozyme-EDTA treatment showed higher activity at temperature range of 35 to 45°C. Further increase in temperature beyond 50°C resulted in reduction in enzyme activity. Most enzymes denatured rapidly at temperatures above 55˚ C (Bryan and Keith, 1981). Itoh *et al*. (1992); Cho *et al*. (2003) have shown that the activity of the enzyme reduced rapidly at or above 50 ºC with no activity detected beyond 60 ºC for 10 min.

Conclusion

Among the four extraction methods, sonication was found to be more effective for *B. animalis* ssp. *lactis* Bb12, whereas lysozyme-EDTA treatment was found to be more effective for *L. delbrueckii*

ssp. *bulgaricus* ATCC 11842. The enzyme activity at pH 6.8 was significantly higher ($P<0.05$) than at other pH levels for both the organisms. The optimum temperature for the activity of enzyme obtained from *B. animalis* ssp. *lactis* Bb12 was found to be at 35ºC whereas for *L. delbrueckii* ssp. bulgaricus ATCC 11842 it was 45ºC. Deproteinised sweet whey was found to be a suitable medium for β-gal production, it should be possible to produce commercial amounts of β-gal using the two organisms reported in this study, however the enzyme extraction method need to be adapted to the strain used.

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Effect of carbon and nitrogen sources on growth of *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-galactosidase under different culture conditions

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Abstract: In this study, the effect of various carbon sources such as lactose, glucose and galactose and nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth (control) on growth of *Bifidobacterium animalis* BB12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* and production of β-galactosidase (β-gal) by these organisms were evaluated. The medium for carbon source contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 4% of lactose, glucose or galactose was supplemented as a carbon source. Similarly, the medium for nitrogen source contained 4% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 3.5% of yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate or MRS broth as a nitrogen source was added. In general, lactose, glucose and galactose were found to be suitable for β-galactosidase production. The highest level of β-gal activity of 73.66 unit/mL was produced by *B. animalis* Bb12 and 48.63 unit/mL by *L. delbrueckii* spp. *bulgaricus* ATCC 11842 in the presence of galactose as the carbon source. The strains were able to utilize a wide range of nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone and ammonium sulphate. *B. animalis* Bb12 produced the highest level of β-gal in MRS broth and yeast extract produced the highest level of β-gal by *L. delbrueckii* spp. *bulgaricus* ATCC 11842.

Keywords: β-Galactosidase, *Bifidobacterium*, *Lactobacillus*, β-galactosidase, carbon and nitrogen sources

Introduction

In the manufacture of dairy products, β-galactosidase (β-gal) has been extensively used to hydrolyse lactose into glucose and galactose (Mahoney, 1998). *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 is particularly a promising microorganism for production of β-gal (Vasiljevic *et al*., 2005), and has a commercial importance in food and pharmaceutical industries (Somkuti and Holsinger, 1997). It was shown that β-gal could hydrolyse β-galactosidic bond in β-lactose (Huber *et al*., 1981). Lactose can be hydrolysed with β-gal to avoid lactose crystallization in frozen concentrated deserts and milk consumption by lactose-intolerant individuals can be improved (Shah, 1993; Shah *et al*., 1993; Kim and Rajagopal, 2000). In addition to this, lactose acts as a galactosyl donor and an acceptor to form di-, tri-, or higher galactooligosaccharides (Wallenfels and Weil, 1972; Prenosil *et al*., 1987). Furthermore, β-gal has been found in abundant in biological systems and micro-organisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Agrawal *et al*., 1989). *Bifidobacterium* and *Lactobacillus Bifidobacterium* have been used for their probiotic properties (Shah, 2007). *Bifidobacterium* and *Lactobacillus* have become organisms of interest for commercial production of β-gal (Shah and Jelen, 1990). Moreover, most of the β-gal are not accepted for food use, are costly and many are not available in adequate quantities for industrial application (Kim and Rajagopal, 2000; Albayrak and Yang, 2002). In addition to this, not many studies have been carried out recently for economical production of β-gal. Hence, selection of micro-organisms which are safe for human use and are capable of producing high level of β-gal becomes vital. In the present study, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used for the production of β-gal under various growth conditions in various carbon and nitrogen sources.

Materials and Methods

Micro-organisms

Pure cultures of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at -80ºC in sterile MRS broth (50% w/v) containing 50% glycerol.

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Culture condition

The organisms were activated in two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) incubated at 37ºC for *B. animalis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The activated cultures were again inoculated into MRS broth and inoculated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* spp. *bulgaricus* ATCC 11842 for 18 h. For production of β-gal, 1 mL of culture was transferred to a medium that contained 4% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.03% L-cysteine. In order to examine the effect of various nitrogen sources on β-gal production, 3.5% of each of yeast extract, peptone, casein hydrolysate, tryptone or ammonium sulphate was added individually in the medium. MRS broth was used as a control. Similarly, 1 mL of culture was transferred to a medium that contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.03% L-cysteine. To examine the effect of various carbon sources on β-gal production, 4% of glucose, lactose or galactose was added individually in the medium. All fermentation experiments were carried out for 12 h and culture was maintained at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

Production of β-galactosidase

For production of β-gal, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 x g for 20 min at 10ºC). The supernatant was discarded and cell pellets were collected. A total of 5 mL of 0.03 M sodium phosphate buffer (pH 6.8) was added to the cell pellets and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0) was used to release the enzyme from the organisms. β-Gal activity was determined according to the method of (Nagy *et al*., 2001). The reaction mixture consisted of 0.5 mL of crude enzyme (cells treated with lysozyme) and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37ºC, 2 mL of 0.1 M sodium carbonate was added to the reaction mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of enzyme that catalysed the formation of 1 µmol of o-nitrophenol from ONPG per gram of sample per min under the assay condition.

Determination of protein

Lowry method was used for protein quantification as described by (Waterberg and Mathews, 1984). Bovine serum albumin (Sigma) was used as a standard.

Enumeration of micro-organisms

For enumeration of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, MRS agar supplemented with 1% D-glucose (w/v) was used. Peptone and water 0.15% (w/v) diluent was used to perform serial dilutions. Plates were incubated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (Merk Pty Limited, 207 Colchester Rd, Kilsyth 3137, Victoria, Australia) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

Statistical analysis

All experiments were replicated three times. All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

Results and Discussion

Effect of carbon source on production of protein and β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

The effect of different carbon sources on the production of protein by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in presence of lactose, glucose and galactose as the sole carbon source is shown in Figure 1. In general, *B. animalis* Bb12 produced higher ($p<0.05$) protein content than *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 with various carbon source including lactose, glucose and galactose. Statistically, *B. animalis* Bb12 had significantly different protein content with lactose and glucose; however, there was no significant difference ($p>0.05$) in protein content between glucose and

galactose. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had no significant difference ($p>0.05$) in terms of protein content with various carbon source including lactose, glucose and galactose. *B. animalis* Bb12 produced the highest ($p<0.05$) amount of protein (0.28 mg/mL) with lactose; however, this organism produced similar level of protein content with galactose and glucose (0.24 mg/mL). The protein content increased ($p<0.05$) by 260.26, 105.08 and 218.42 percent in lactose, glucose and galactose, respectively at 12 h as compared with 0 h (data not shown). On the other hand, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced similar level of protein (0.11 mg/mL) with lactose, glucose and galactose. At 12 h, the protein content increased by 98.25, 85.25 and 51.39 percent in lactose, glucose and galactose, respectively as compared with 0 h (data not shown). The protein concentration gradually increased during incubation. However, the level remained lowest with glucose and galactose in both organisms.

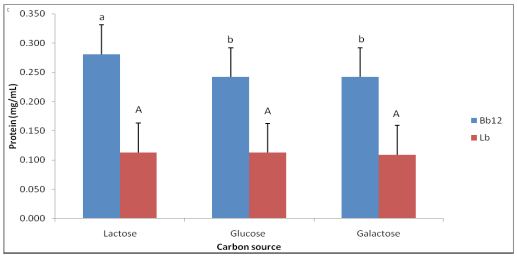

Figure 1. Effect of carbon source on the protein production by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Effect of carbon sources on the β-galactosidase production

The influence of various carbon sources including lactose, glucose and galactose on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 2. In general, both organisms produced higher ($p<0.05$) β-gal in galactose than lactose and glucose and lower ($p<0.05$) β-gal in glucose than that in lactose and galactose. *B. animalis* Bb12 produced the highest ($p<0.05$) amount of β-gal with galactose (73.66 unit/mL) followed by lactose (57.04 unit/mL) and lowest activity with glucose (31.08 unit/mL). The β-gal production increased ($p<0.05$) by 33.39, 27.43 and 95.02 percent in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). A similar pattern was seen with *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, which produced the highest ($p<0.05$) amount of β-gal (48.63 unit/mL) with galactose followed by lactose (33.0 uni/mL) and lowest activity with glucose (28.9 unit/mL). At 12 h, the β-gal production increased ($p<0.05$) by 28.65, 33.29 and 124.32 percent in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown).

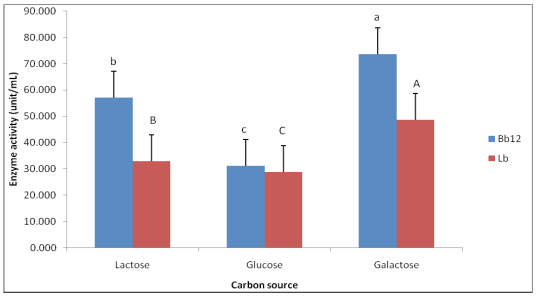

Figure 2. Effect of carbon source on the β-galactosidase production by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

A number of investigators have reported about the regulation of carbon source on β-gal biosynthesis in different micro-organisms (Fantes and Roberts 1973; Montero *et al*., 1989; Fiedurek and Szczodrak, 1994; Nikolaev and Vinetski, 1998; de Vries *et al*., 1999; Nagy *et al*., 2001; Fekete *et al*., 2002). All these authors have reported that the role of carbon source in the biosynthesis of β-gal may vary and depend on the micro-organisms tested. Kim and Rajagopal (2000) reported that *L. criptus* grown in MRS broth containing galactose as a carbon source showed the highest β-gal activity followed by moderate levels of enzyme production with lactose and a significant activity with glucose or maltose. The expression of β-gal by micro-organisms may be affected by the amount of carbon source in the medium (Fiedurek and Szczodrak, 1994; Incharurondo *et al*., 1998). According to Bergy's Manual of Systematic Bacteriology (John, 1986), *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 does not ferment galactose, however, it appears that increasing fermentation period resulted in an increase in β-gal in all carbon sources (lactose, glucose and galactose) in both strains at 12 h incubation period. However, galactose produced highest β-gal in both strains (Figure 2).

Effect of carbon sources on the growth

Figure 3 demonstrates the effect of carbon source including lactose, glucose and galactose on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, *B. animalis* Bb12 as well as *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) final viable population in galactose than in lactose and glucose and lower ($p<0.05$) final viable population at glucose than the

lactose and galactose. It was found that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had a significant difference ($p<0.05$) in the final viable population in various carbon sources. The amounts of carbon source in the medium may affect the expression of β-gal by micro-organisms (Fiedurek and Szczodrak, 1994; Inchaurrondo *et al*., 1998). The pattern showed an initial increase in viable population at the commencement of the fermentation followed by slower increasing trend towards the end of this process. A maximal population of 8.4 log CFU/mL, 7.8 log CFU/mL, 7.7 log CFU/mL in galactose, lactose and glucose, respectively, was reached in *B. animalis* Bb12 (Figure 3). Similarly, maximal population at 8.6 log CFU/mL, 7.8 log CFU/mL and 7.5 log CFU/mL in galactose, lactose and glucose, respectively, was reached in *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (Figure 3). The final viable population of the *B. animalis* Bb12 ranged from 6.92 to 8.43 log CFU/mL and the organism showed the highest ($p<0.05$) viable population of 8.43 log CFU/mL with galactose followed by lactose (7.82 log CFU/mL) and lowest with glucose (7.75 log CFU/mL). The viable count increased ($p<0.05$) by 13.42, 11.82 and 17.65 percent in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a similar trend. The final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.67 to 8.6 log CFU/mL and the organism exhibited the highest ($p<0.05$) viable population of 8.6 log CFU/mL with galactose followed by lactose (7.87 CFU/mL) and lowest with glucose (7.5 log CFU/mL). At 12 h, the viable count increased ($p<0.05$) by 16.08, 12.44 and 26.84 percent in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown).

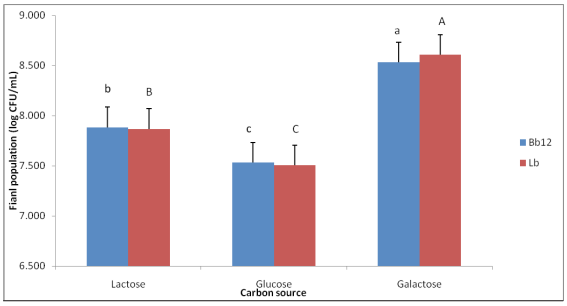

Figure 3. Effect of carbon source on the final population by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Effect of carbon sources on pH

The effect of carbon source on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 4. In general, pH value in glucose was lower ($p<0.05$) as compared with other carbon sources. The pH value of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was significantly ($p<0.05$) higher in lactose than that with glucose and galactose. However, the pH value in glucose and galactose were not significant different ($p>0.05$). On the other hand, *B. animalis* Bb12 showed no significant difference ($p>0.05$) in terms of pH values in various carbon sources. The decrease in pH by *B. animalis* Bb12 was lowest with glucose at 4.63 followed by galactose (4.80) and lactose (4.83). The pH value decreased ($p<0.05$) by 23.82, 25.20 and 19.60 percent in lactose, glucose and galactose, respectively, at 12 h as compared with 0 h (data not shown). On the other hand, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with galactose (pH 4.59) followed by glucose (pH 4.63) and lactose (pH 5.13). At 12 h, the pH value decreased ($p<0.05$) by 18.70, 25.32 and 26.79 percent in lactose, glucose and galactose, respectively, as compared with 0 h (data not shown). The drop in pH correlated with an increase in population of the two organisms.

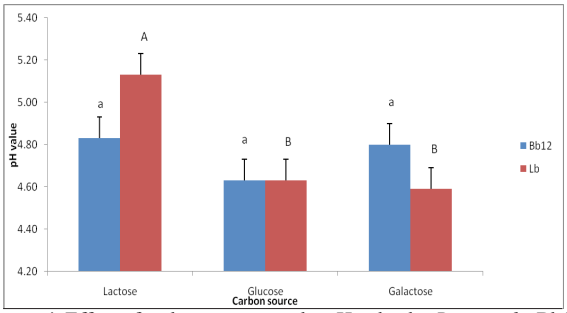

Figure 4. Effect of carbon source on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 4% of various carbon sources including lactose, glucose and galactose. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Effect of nitrogen source on production of protein and β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Effect of nitrogen sources on the protein content

Effect of nitrogenous substrates including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on production of protein by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 5. In general, *B. animalis* Bb12 produced higher ($p<0.05$) protein in yeast extract, ammonium sulphate and MRS broth MRS broth compared with other nitrogen sources. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) protein in

MRS broth, tryptone and peptone than the other nitrogen sources. *B. animalis* Bb12 had significantly different protein content in yeast extract, peptone, casein hydrolysate and ammonium sulphate and there was no significant difference ($p>0.05$) in protein content between tryptone and MRS broth. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had a significantly different protein content in yeast extract, peptone, tryptone and MRS broth and there was no significant difference ($p>0.05$) between casein hydrolysate and ammonium sulphate. Moreover, *B. animalis* Bb12 produced the highest amount of protein (0.17 mg/mL) with yeast extract followed by ammonium sulphate (0.15 mg/mL) and tryptone, casein hydrolysate gave the lowest protein with MRS broth (Figure 5). The protein content increased in 12 h ($p<0.05$) by 172.13, 34.55, 133.33, 58.46, 161.36 and 156.82 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, as compared with 0 h (data not shown). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of protein (0.13 mg/mL) with MRS broth followed by tryptone (0.11 mg/mL) and peptone. (Figure 5). At 12 h, the protein content increased ($p<0.05$) by 176.92, 177.42, 127.78, 322.22, 100.00 and 329.03 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively as compared with 0 h (data not shown). The protein concentration gradually increased during incubation. However, the level remained lowest with ammonium sulphate. MRS broth is usually used to grow *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 due to its nutrient contents, hence the organism grew best in MRS broth.

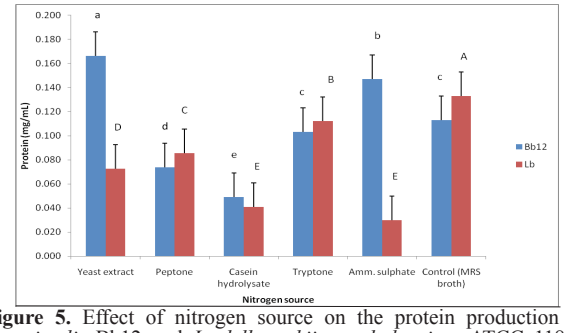

Figure 5. Effect of nitrogen source on the protein production by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 4.0% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Effect of nitrogen sources on the β-galactosidase production

The influence of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 6. In general, *B. animalis* Bb12 produced higher ($p<0.05$) β-gal in MRS broth and tryptone compared with other nitrogen sources. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) β-gal production in casein hydrolysate and yeast extract than the other nitrogen sources. Statistically, *B. animalis* Bb12 had significantly different β-gal production in yeast extract, peptone, casein hydrolysate and MRS broth and no significant difference ($p>0.05$) in ammonium sulphate, casein hydrolysate and tryptone. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different β-gal production in yeast extract, casein hydrolysate, ammonium sulphate and MRS broth and this was no significant difference ($p>0.05$) in β-gal production between peptone and tryptone. *B. animalis* Bb12 produced the highest amount of β-gal (51.69 unit/mL) with MRS broth followed by tryptone (45.48 unit/mL). The β-gal production increased ($p<0.05$) by 45.32, 44.20, 81.86, 106.43, 94.51 and 104.03 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively at 12 h as compared with 0 h (data not shown). MRS broth provided optimum nutrients for this organism. Hence, the organism produced the highest level of β-gal. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of β-gal (50.7 unit/mL) with casein hydrolysate followed by yeast extract (42.0 unit/mL) and lowest activity (23.15 unit/mL) with peptone (Figure 6). At 12 h, the β-gal production increased ($p<0.05$) by 106.30, 14.14, 122.17, 24.97, 60.07 and 43.93 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth respectively as compared with 0 h (data not shown). This may be attributed to the peptides and amino acids present in casein hydrolysate. According to Rao and Dutta (1979) and Shaikh and *et al*., 1997), nitrogen sources may affect microbial biosynthesis of β-gal.

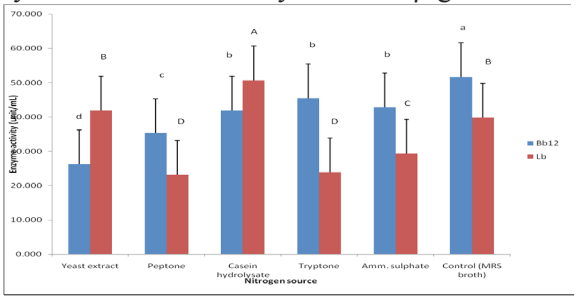

Figure 6. Effect of nitrogen source on the β-galactosidase production by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 4.0% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Effect of nitrogen sources on the growth

Figure 7 demonstrates the effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, the viable counts were higher ($p<0.05$) in MRS broth and casein hydrolysate than other nitrogen source including yeast extract, peptone, tryptone and ammonium sulphate in both organisms (Figure 7).

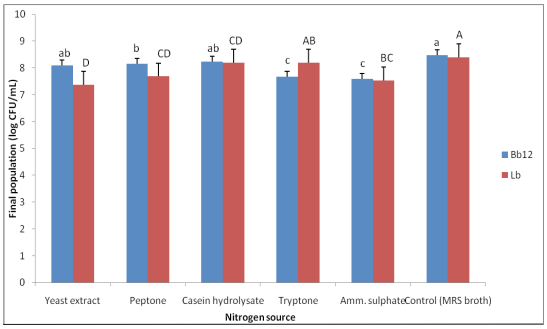

Figure 7. Effect of nitrogen source on the final population by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 4.0% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

The viable counts of *B. animalis* Bb12 were significantly ($p<0.05$) lower in ammonium sulphate and tryptone; however, yeast extract and ammonium sulphate showed significantly ($p<0.05$) lower viable counts for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The final viable population of the *B. animalis* Bb12 ranged from 6.8 to 8.5 log CFU/mL and the organism showed the highest viable population of 8.5 log CFU/mL at 12 h with MRS broth followed by casein hydrolysate at 8.2 log CFU/mL and lowest with ammonium sulphate at 7.6 log CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 7.57, 18.63, 17.09, 3.23, 2.29 and 11.33 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, compared with 0 h (data not shown). However, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.6 to 8.4 log CFU/mL and the organism showed the highest viable population of 8.4 log CFU/mL at 12 h with MRS broth followed by casein hydrolysate at 8.2 log CFU/mL and lowest with peptone at 6.6 CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 7.67,14.78, 22.24, 18.70, 11.94 and 23.53 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth, respectively, as compared with 0 h (data not shown). This may be attributed to the nutrients in addition to the nitrogen compounds present in MRS broth and casein hydrolysate.

Effect of nitrogen sources on the pH

The effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth on the pH value by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figure 8. In general, pH value of MRS broth was lower ($p<0.05$) compared with other nitrogen sources for both organisms. The pH value of *B. animalis* Bb12 was significantly ($p<0.05$) higher in ammonium sulphate among other nitrogen sources. Similar pH values were higher in ammonium sulphate, tryptone, casein hydrolysate and peptone. The decrease in pH value by *B. animalis* Bb12 was lowest with MRS broth (4.24) followed by yeast extract (5.56) and highest with tryptone (6.69. The pH value decreased ($p<0.05$) by 33.85, 15.50 and 2.81 percent in MRS broth, yeast extract and tryptone, respectively, at 12 h as compared with 0 h (data not shown). Similarly, the decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with MRS broth (pH 4.26) followed by yeast extract (pH 5.27) and highest with tryptone (pH 6.18). The pH value decreased ($p<0.05$) by 35.16, 20.15 and 8.08 percent in MRS broth, yeast extract and tryptone, respectively, at 12 h as compared with 0 h (data not shown). The drop in pH is correlated with increase in population of the two organisms in MRS broth.

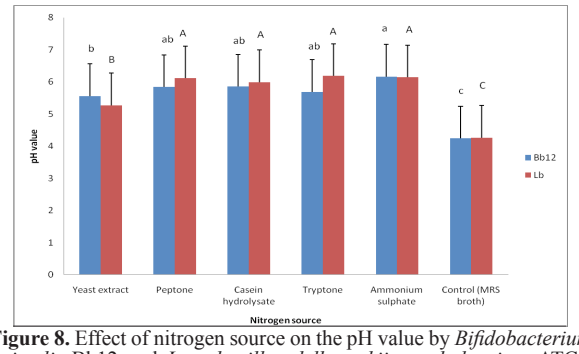

Figure 8. Effect of nitrogen source on the pH value by *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 4.0% lactose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.03% L-cysteine and 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Determinations were made after a 12-h cultivation. Bars indicate standard deviations. Different letters within each type of determination indicate a significant difference ($p<0.05$)

Conclusion

The results of this study demonstrated that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 are capable of producing high level of β-gal. A maximum of β-gal of 73.66 unit/mL and 48.63 unit/mL was produced by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, respectively in carbon source including lactose,

glucose and galactose. Similarly, maximum β-gal at 51.6 unit/mL and 50.7 unit/mL was produced by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, respectively in nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and MRS broth. Considering the high yield of β-gal with *B. animalis*, this organism may be a potential useful industrial strain for the production of β-gal.

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Effect of nitrogen sources on production of β-galactosidase from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey under different culture conditions

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Abstract: The present study examined the effect of various nitrogen source such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey (control) on the growth of *B. animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 and production of β-galactosidase (β-gal) grown in deproteinized whey by these organisms. The organisms were grown in deprotenized whey (control) and that containing 3.5% of yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate was supplemented as a nitrogen source and β-gal activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The strains were able to utilize a wide range of nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone and ammonium sulphate in deproteinized whey. In general, yeast extract and peptone were found to be more suitable for β-galactosidase production. The highest level of β-gal activity at 45.69 unit/mL was produced with peptone followed by yeast extract at 43.44 unit/mL by *B. animalis* Bb12 and 46.6 unit/mL was produced with casein hydrolysate followed by yeast extract at 46.25 unit/mL by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This study showed that whey medium supplemented with nitrogen sources was suitable for fermentation by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-gal.

Keywords: β-galactosidase, *Bifidobacterium*, *Lactobacillus*, β-galactosidase, nitrogen sources, deprotenized whey

Introduction

β-Galactosidase (β-gal), is an important enzyme used in dairy industry for hydrolysis of lactose into glucose and galactose. β-Gal is found in abundant in biological systems and micro-organisms such as yeasts and molds and bacteria still remain the only commercially exploited sources (Agrawal, Garg and Dutta, 1989). Since, a large number of people suffer from lactose intolerance in different countries of the world (Shah, 1993), this makes the enzyme even more important. The need for low lactose milk is particularly important in food-aid programs as severe tissues dehydration, diarrhoea even death may result from feeding lactose containing milk to lactose in-tolerant children and adults suffering from protein-calories malnutrition. In addition, lactose has a low solubility which results in crystal structure at concentrations above 11% (w/v), which prevents the utilization of concentrated whey in several food processes (Bansal *et al*., 2008). Dairy industry waste whey contains lactose (5%), whey protein (0.8%), mineral and vitamins, which are essential components that have not been exploited for the cultivation of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* 11842 (Mahalakshmi *et al*., 2000). Whey contains about 6.66% of important milk nutrients (Bansal and *et al*., 2008). The disposable of whey remains a significant problem for dairy industries especially in developing countries where a relatively insignificant part of whey is used for production of whey protein concentrates and significant part of it disposed off into the water streams causing serious water pollution problems. Hence, problems associated with whey disposal, lactose crystallization and milk consumption by lactose- intolerant populations of the world have drawn the attention of several research workers. This has led to the selection of micro-organisms with view to high potentials for producing β-gal, the enzyme that hydrolyses lactose into its component monosaccharide units (Rao and Dutta, 1997). Therefore, it is important to evaluate the production of β-gal using whey by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* 11842 organisms in terms of effectiveness and enzyme production so that process could be scaled up. In this regard, not many studies have been carried out recently for economical production of β-gal. Hence, selection of micro-organisms which are safe for human use and are capable of producing high level of β-gal becomes

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vital. Thus, the present study was conducted to evaluate the effect of various nitrogen sources on the production of β-gal by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *B. animalis* Bb12 in deprotenized whey.

Materials and Methods

Micro-organisms

The pure cultures of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The stock cultures were stored at -80ºC in sterile MRS broth (50% w/v) and 50% glycerol.

Culture condition

The organisms were activated in two successive transfers in *lactobacilli* MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) for growing *Bifidobacterium* and incubated at 37ºC for *B. animalis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. Deproteinized whey was prepared by heating whey at 90ºC and pH 4.5 for 10 min, filtered through Whatman no. 1 filter paper to remove the coagulated protein, adjusted to pH 7.0 and sterilized at 121ºC for 15 min. For production of β-gal, the sterile deprotenized whey was supplemented with 3.5% of each of nitrogen source individually including yeast extract, peptone, casein hydrolysate, tryptone or ammonium sulphate and inoculated with 1% of active culture of each organism. The various nitrogen sources were used in order to study their effect on β-gal production. Deproteinized whey was used as a control. All experiments were carried out for 24 h. The culture was maintained at 37ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

Production of β-galactosidase

For production of β-gal, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 x g for 20 min at 10ºC). The supernatant was discarded and cell pellets were washed twice with 5 mL of 0.03 M phosphate buffer. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. β-Gal activity was then assayed according to the method of (Nagy *et al*., 2001). The reaction mixture consisted of 0.5 mL of enzyme source (cells treated with lysozyme) and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37ºC, 2 mL of 0.1 M sodium carbonate was added to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of enzyme that catalysed the formation of 1 µmol of o-nitrophenyl from ONPG per min per gram under the assay condition.

Enumeration of micro-organisms

To enumerate *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, MRS agar supplemented with 1% (w/v) D-glucose was used. Peptone and water 0.15% (w/v) diluent was used to perform serial dilutions. Plates were incubated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

Results and Discussion

Effect of nitrogen source on the production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Effect of nitrogen sources on the β-galactosidase production

The influence of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 1 and 2. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference ($p<0.05$) in β-gal production in all nitrogen sources. *B. animalis* Bb12 produced higher ($p<0.05$) β-gal in peptone, yeast extract and casein hydrolysate compared with other nitrogen sources at 24 h (Figure 1). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) β-gal production in

yeast extract and casein hydrolysate than the other nitrogen sources (Figure 2). Statistically, *B. animalis* Bb12 showed significantly different ($p>0.05$) β-gal production at 24 h in casein hydrolysate and ammonium sulphate and others nitrogen sources. However, significant difference ($p<0.05$) was found between casein hydrolysate, tryptone and other nitrogen sources; yeast extract, peptone, casein hydrolysate and other nitrogen sources; and peptone, casein hydrolysate, tryptone and other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

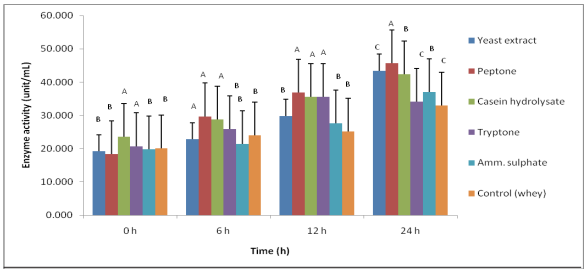

Figure 1. Effect of nitrogen source on the β-galactosidase production by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

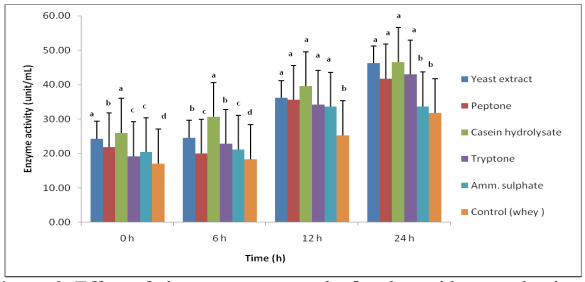

Figure 2. Effect of nitrogen source on the β-galactosidase production by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed significantly different ($p>0.05$) β-gal production at 24 h between ammonium sulphate, whey and other nitrogen sources. However, significant difference ($p<0.05$) was found amongst yeast extract, whey and other nitrogen sources; yeast extract, peptone, casein hydrolysate, whey and rest of nitrogen sources; and whey between other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

B. animalis Bb12 produced the highest amount of β-gal (45.69 unit/mL) with peptone followed by (43.44 unit/mL) with yeast extract and lowest activity (33.0 unit/mL) with whey (control) at 24 h (Figure 1). The β-gal production increased ($p<0.05$) by 126.01, 149.40, 79.35, 64.63, 86.14, and 64.49 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, at 24 h as compared with 0 h (Figure 1). Peptone provided optimum nutrients for this organism. Hence, the organism produced the highest level of β-gal. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of β-gal (46.6 unit/mL) with casein hydrolysate followed by yeast extract (46.25 unit/mL) and lowest activity (31.8 unit/mL) with whey (Figure 2). At 24 h, the β-gal production increased ($p<0.05$) by 90.09, 91.20, 78.90, 123.74, 65.06 and 86.33 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey respectively as compared with 0 h (Figure 2).

Our results are also in line with those reported by (Mahoney *et al*., 1975), in which maximum β-gal activity was achieved at about 22 h of incubation period. Most of the available literature suggests the optimal fermentation time in the range of 20-36 h (Mahoney *et al*., 1975; Ku *et al*., 1992; Ranzi *et al*., 1987). Furthermore, the study by Bury *et al*., (2001) reveals a maximum β-gal activity after 15-17 h of growth in yeast extract medium. The β-gal activities of cultures grown with 0.2-0.8% yeast extract were approximately 2.5 times higher than for those grown without yeast extract in case of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker, 1970). According to Rao and Dutta (1979) and Shaikh *et al*., (1997), nitrogen sources may affect microbial biosynthesis of β-gal.

Effect of nitrogen sources on the growth

Figures 3 and 4 demonstrate the effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference ($p<0.05$) in the viable counts in all nitrogen sources. The viable counts were higher ($p<0.05$) in yeast extract and peptone than others nitrogen source including yeast extract, peptone, tryptone and ammonium sulphate in both organisms (Figures 3 and 4). Statistically, *B. animalis* Bb12 showed no significantly difference ($p>0.05$) in the viable count at 0 h in various nitrogen sources. However, significant difference ($p<0.05$) was found between yeast extract, peptone, casein hydrolysate and other nitrogen sources; yeast extract, casein hydrolysate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 6 h; 12 h; and 24 h, respectively. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed no significantly difference

(p>0.05) in the viable count at 0 h and 6 h in various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey. However, a significant difference (p<0.05) was found between yeast extract, tryptone and other nitrogen sources; yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen source; at 12 h and 24 h, respectively. The final viable population of the *B. animalis* Bb12 ranged from 5.91 to 8.27 log CFU/mL and the organism showed the highest viable population of 8.27 log CFU/mL at 24 h with peptone followed by yeast extract at 8.24 log CFU/mL and lowest with ammonium sulphate at 7.26 log CFU/mL. At 24 h, the viable count increased (p<0.05) by 36.42, 39.70, 29.01, 30.80, 22.84 and 23.83 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, compared with 0 h (Figure 3). Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 5.59 to 8.64 log CFU/mL and the organism showed the highest viable population of 8.64 log CFU/mL at 24 h with yeast extract followed by tryptone 8.59 log CFU/mL and lowest with whey 7.87 CFU/mL. The viable count increased (p<0.05) by 49.48, 44.31, 46.10, 51.23, 39.05 and 40.97 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 4).

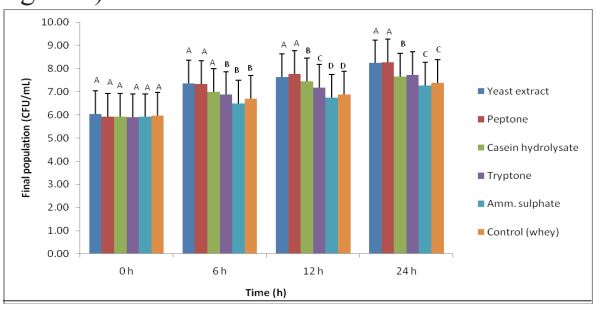

Figure 3. Effect of nitrogen source on the viable population by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference (p<0.05)

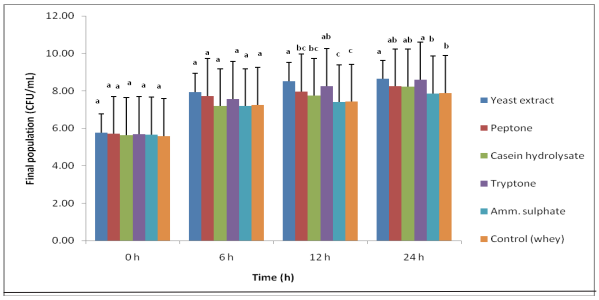

Figure 4. Effect of nitrogen source on the viable population by *L. delbrueckii* spp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference (p<0.05)

Yeast extract provided optimum nutrients for this organism; hence, the organism produced the highest viable count (p<0.05). The bacterial grown in whey can be increased by addition of whey protein concentrate (Bury and *et al*., 1998), yeast extract (Gupta *et al*., 1995). However, effectiveness of the supplementation of these nutrients for the production of β-gal has not been studied to a great extent. Supplementation of nitrogenous sources especially yeast extract increases the amount of nutrients available to the bacteria, which could explain why there was an increase in the viable population of the organisms. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker 1970).

Effect of nitrogen sources on the pH

The effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the pH of media as affected by growing *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 5 and 6. In general, pH value of the medium containing yeast extract and peptone was lower (p>0.05) compared with other nitrogen sources for both organisms. The pH value was significantly (p<0.05) higher in tryptone among other nitrogen sources in both organisms. *B. animalis* Bb12 had significantly different (p>0.05) pH values at all fermentation times. However, a significant difference (p<0.05) was found between yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources; yeast extract, tryptone, ammonium sulphate and other nitrogen sources; tryptone, ammonium sulphate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 0 h, 6 h; 12 h; and 24 h, respectively. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different (p>0.05) pH values at all fermentation times. The significant difference (p<0.05) was found at 0 h and 6 h in all nitrogen sources. However, significant difference was recorded between yeast extract, peptone, ammonium sulphate and rest of nitrogen sources; yeast extract, peptone, casein hydrolysate, ammonium sulphate and other nitrogen sources at 12 h and 24 h, respectively.

The decrease in pH by *B. animalis* Bb12 was lowest with yeast extract (4.53) followed by peptone (4.59) and highest with ammonium sulphate (5.47). The pH value decreased (p<0.05 by 24.37, 24.51, 22.10, 20.91, 5.53 and 14.26 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 5). Similarly, decrease in pH by *L.*

delbrueckii ssp. *bulgaricus* ATCC 11842 was lowest with yeast extract and casein hydrolysate (5.31) followed by peptone (5.37) and highest with tryptone (6.26). At 24 h, the pH value decreased ($p<0.05$ by 11.09, 3.10, 4.13, 4.62, and 4.23 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively as compared with 0 h (Figure 6). The drop in pH correlated with an increase in population of the two organisms.

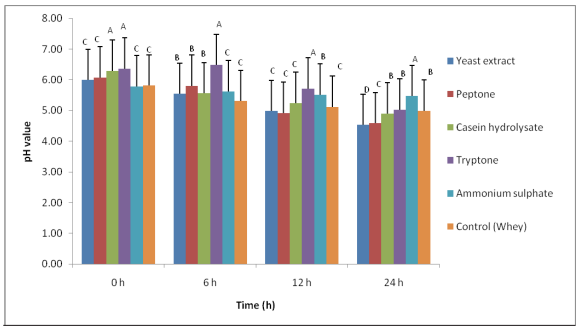

Figure 5. Effect of nitrogen source on the pH by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

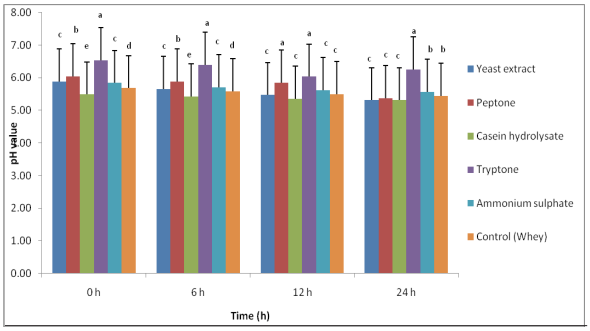

Figure 6. Effect of nitrogen source on the pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

Conclusion

The addition of nitrogen sources (especially yeast extract and peptone at 3.5%) in deprotenized whey can increase the β-gal by these organisms. Whey medium supplemented with nitrogen sources could be suitable for fermentation for *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In addition, due to high yield of β-galactosidase with *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, these organisms could be potential sources for the production of β-gal.

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Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842

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Abstract: Crude enzyme extracts from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5, and 1.0 g/L to hydrolyse glycitin (isoflavone glycosides) to its biologically active form (isoflavone aglycones; IA) in soymilk (SM) prepared from soy protein isolate (SPI) supplemented with 2.0% (w/v) of D-glucose. Enumeration of microbial populations, measurement of pH and quantification of isoflavones was carried out at 0 h, 6 h and 12 h of fermentation. The quantification of isoflavone compounds in SM was carried out using HPLC. The biotransformation of glycitin was higher at the enzyme level of 1.0 g/L from *B. animalis* Bb12 at 12 h than that at 0.5 g/L or 0.1 g/L, and the level of biotransformation was 74.4%, while 75.23% of glycitin was biotransformed with the enzyme extracted from *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at the same level of enzyme. The decrease in pH by *B. animalis* Bb12 was lowest with 1.0 g/L and highest with the control (4.69). Similarly, the decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with 1.0 g/L (5.19) and highest with the control (5.86). The final viable population of the *B. animalis* Bb12 ranged from 5.94 to 7.49 log CFU/mL and that of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 4.42 to 6.70 log CFU/mL and the organisms showed the highest viable population of 6.70 log CFU/mL at 12 h with 1.0 g/L crude enzyme.

Keywords: Aglycones, glycosides, soy protein isolate, biotransformation, isoflavones, *Lactobacillus, Bifidobacterium*, extracted crude enzyme

Introduction

Soy protein isolate (SPI) is made from defatted soy meal by removing fat and carbohydrates, resulting in a product containing 90% protein. Soymilk made from soy protein isolate (SPI) has no undesirable flavour (Shurtleff and Aoyagi, 1984). However, soymilk made from SPI has reduced level of biologically active isoflavone due to losses during protein isolation (Wang and Murphy, 1996). Soymilk generally contains a total of 4 to 12 mg isoflavones per 100 g (King and Bignell, 2000; Tsangalis *et al.,* 2002) but is subject to considerable variation in isoflavone content and composition (Murphy *et al.,* 1999; King and Bignell, 2000).

The phytoestrogens found abundantly in soybeans consist of the di-phenolic, isomeric family of compounds named isoflavones. Soybean and soy-food derived isoflavones are found in 4 chemical forms, including aglycones, malonyl-, acetyl-, and glucoside conjugates. The biologically active, estrogen-like isoflavone isomers are the aglycone configurations of genistein, daidzein, and glycitein (Setchell and Cassidy, 1999). Aglycone isomers are able to bind to estrogen receptor sites and hence mimic the functions of estradiol in the human body (Setchell, 1998; Setchell and Cassidy, 1999). From reviews of epidemiological (Cassidy, 1996; Setchell, 1998) and small-scale human clinical studies, isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders common in current Western civilizations. Genistein has been demonstrated to promote the health of human beings by reducing the occurrence of specific chronic diseases, namely, cancer and atherosclerosis (Lee *et al.,* 1991; Witztum, 1994). Daidzein and genistein have been documented to have beneficial effects on osteoporosis (Anderson *et al.,* 1987). Soy isoflavones have also been shown to relieve menopause symptoms (Aldercreutz *et al.,* 1992). Asian populations with their high intake (50 to 70 mg/d) of soy-derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms, and mortality from cardiovascular disease and cancer. According to Murphy *et al.* (1999) and Tsangalis *et al.* (2002) of the total concentration of isoflavones in soymilk greater than 90% of the isomers exist as glucosidic forms. Izumi *et al.* (2000) found that aglycone forms were absorbed faster and in greater amounts than their glucosides in humans. Furthermore, Setchell *et al.* (2002) reported that isoflavone glucosides were not absorbed through the human gut wall, and their bioavailability required initial hydrolysis of the sugar moiety by intestinal β-glucosidases. This suggests that consuming isoflavone aglycone-rich soy foods may be more effective in preventing chronic diseases.

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β-Galactosidase (β-gal) is an essential enzyme used for effective conversion of isoflavone glycoside to aglycones (Pandjaitan *et al.*, 2000). β-Gal has superior activity for hydrolysing acetyl-glycoside and malonyl-glycoside isoflavones. If β-gal can effectively convert acetyl-glycoside and malonyl-glycoside to their aglycones, it can lead to an enhancement of isoflavone aglycones in soy protein isolate. Shah and Lankaputhra (2002) have reported that the genus *Bifidobacterium* constitutes a major part of the natural microflora of the human intestinal tract. Tsangalis *et al.* (2004) revealed that *B. animalis* Bb12 hydrolysed isoflavone glucosides into aglycones when grown in soymilk increasing the concentration of aglycones from 8 to 50% of total isoflavones. In human clinical studies, *B. animalis* Bb-12 has shown to effectively modulate intestinal microflora (Playne, 2002). Our objective was to examine the effectiveness of crude enzyme extract from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in biotransformation of glycitin, an isoflavone glycoside (IG) to their aglycones in soymilk made from SPI.

Materials and Methods

Isoflavone compounds and other chemicals

Genistein, daidzein, and flavone were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Glycitin, Formononetin, and Biochanin A were obtained from Indofine Chemical Co. (Summerville, N.J., U.S.A.). Acetonitrile, methanol, ethanol, and phosphoric acid used for HPLC were of analytical grade. Soy protein isolate SUPRO 590 was from The Solae Co. (Chatswood, NSW, Australia).

Bacterial growth and media

The organisms were activated by two successive transfers in lactobacilli MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) incubated at 37ºC for *B. animalis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The activated cultures were again inoculated into MRS broth and inoculated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* spp. *bulgaricus* ATCC 11842 for 18 h. The third transfer was carried out in 4% (w/v) SPI containing 2.0% (w/v) D-glucose (Prahran Health Foods, Prahran, Vic., Australia) prepared as per Tsangalis *et al*., (2002). For production of extract crude enzyme, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 × g for 20 min at 10ºC). The supernatant was discarded and cell pellets were collected. A total of 5 mL of 0.03 M sodium phosphate buffer (pH 6.8) was added and vortexed thoroughly. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. Extracted crude enzymes from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were used at 0.1, 0.5 and 1.0 g/L and the control was without crude enzyme.

Enumeration of viable microorganisms

One millilitre sample was used for the enumeration of populations of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. MRS agar supplemented with 1% (w/v) D-glucose was used for enumeration of the organisms. Peptone and water at 0.15% (w/v) diluent was used to perform serial dilutions. One millilitre of serially diluted samples at 0, 6, and 12 h was aseptically spread onto the plates and incubated at 37°C for *B. animalis* Bb12 and 45°C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (Merk Pty Limited, Kilsyth, Vic, Australia) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

Isoflavone standard solution and calibration curves

Stock solutions of isoflavone standards such as glycitin, daidzein, genistein, biochanin A and formononetin were prepared by dissolving 1 g of crystalline pure compound in 10 mL of 100% methanol. Each solution was diluted with methanol (100%) to 5 working solutions at concentration ranging from 1 to 40 µg/mL in order to prepare a standard curve. Retention time and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavones.

Determination of isoflavone content

Extraction of isoflavones for HPLC analysis

The extraction of isoflavone aglycones and glucoside isomers and HPLC analysis was performed in triplicate based on Griffith and Collison (2001) and

Nakamura and *et al.* (2001) with some modifications as described in Pham and Shah (2007). Briefly, 10 mL of methanol (80%, v/v) and 1 mL of acetonitrile (100%, v/v) were added to 1 g of freeze-dried sample with stirring using a vortex mixer (Chiltern Scientific, Auckland, New Zealand). In addition, 100 μL each of Carrez I and Carrez II solutions were added to the samples and mixed thoroughly. Furthermore, 100 μL of flavone (1 mg/mL) as the internal standard was added followed by thorough shaking. The samples were left in a water bath (model NB 6T-10935, Thermoline Australia) at 50°C for 2 h until the proteins precipitated. The samples were then filtered through a Whatman No. 3 filter paper and a 0.45 μM Phenomenex nylon filter into an HPLC vial then injected into HPLC system within 4 h to avoid the degradation (Griffith and Collison, 2001). The HPLC system included an Alltech Alltima HP C18 HL (4.6 × 250 mm), a 5-μm particle size column and an Alltima HP C18HL (7.5 × 4.6 mm), a 5 μm guard column, Hewlett Packard 1100 series HPLC with an autosampler, a quaternary pump, a diode array ultraviolet detector, a vacuum degasser, and a thermostatically controlled column compartment. Mobile phase consisted of solvent A (water: phosphoric acid, 1000:1, v/v) and solvent B (water: acetonitrile: phosphoric acid, 200:800:1, v/v/v). The gradient was as follows: solvent A 100% (0 min) → 80% (5 min) → 0% (50 min) → 100% (55 min) → 100% (60 min). The flow rate was 0.8 mL/min. A diode array UV detector was set at 259 nm.

Isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). The moisture content of the freeze-dried soymilk samples was determined by AACC 40-40 (AACC 2000) methods. The biotransformation of IG to IA was defined as percentage of IG hydrolyzed and was calculated as follows:

$$\text{Percent glycitin (IG) hydrolysis} = \frac{\text{initial glycitin} - \text{residual glycitin}}{\text{initial glycitin}} \times 100$$

Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

Results and Discussion

pH changes during incubation

The effect of change of pH in soy protein isolate during incubation as affected by growing *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 1 and 2. In general, pH value of 1.0 g/L crude enzyme extract (CEE) was lower ($p>0.05$) compared with other crude enzyme concentrations for both organisms. The pH value was significantly ($p<0.05$) higher in control (without crude enzyme extract) and 0.1g/L of CEE than the others in both organisms. Both organisms showed no significantly different ($p>0.05$) pH values at 0 h and 6 h at different crude enzyme concentrations including control and a significant difference ($p<0.05$) was found at 12 h between 1.0 g/L and 0.5g/L CEE than the others. The decrease in pH by *B. animalis* Bb12 was lowest with 1.0 g/L (4.35) followed by 0.5 g/L (4.45) and the highest with the control (4.69). The pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82 percent in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE respectively, at 12 h as compared with 0 h (Figure 1). Similarly, decrease in pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lowest with 1.0 g/L (5.19) followed by 0.5 g/L (5.52) and highest with the control (5.86). At 12 h, the pH value decreased ($p>0.05$) by 5.64, 7.81, 10.15 and 14.82% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE respectively, at 12 h as compared with 0 h (Figure 2). The drop in pH correlated with an increase in population of the two organisms (Tables 1 and 2).

Table 1. Viable microbial counts (log CFU/mL) of *B. animalis* Bb12 in soymilk during 12 h fermentation at 37◦C

	Enzyme concentrations (g/L)			
	Control	0.1 g/L	0.5 g/L	1.0 g/L
0 h	5.9 ± 0.01^{b}	6.0 ± 0.02^{c}	6.1 ± 0.03^{c}	6.2 ± 0.06^{c}
6 h	6.5 ± 0.03^{b}	6.4 ± 0.27^{b}	6.8 ± 0.03^{b}	6.9 ± 0.01^{b}
12 h	6.9 ± 0.02^{a}	7.2 ± 0.01^{a}	7.3 ± 0.02^{a}	7.4 ± 0.03^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05).

Table 2. Viable microbial counts (log CFU/mL) of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 fermentation at 37◦C in soymilk during 12 h

	Enzyme concentration (g/L)			
	Control	0.1 g/L	0.5 g/L	1.0 g/L
0 h	4.4 ± 0.03^{b}	4.4 ± 0.06^{b}	4.7 ± 0.04^{b}	4.9 ± 0.01^{b}
6 h	6.0 ± 0.10^{a}	6.0 ± 0.22^{a}	6.3 ± 0.10^{a}	6.5 ± 0.09^{a}
12 h	6.1 ± 0.19^{a}	6.2 ± 0.10^{a}	6.4 ± 0.21^{a}	6.7 ± 0.13^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Mean values in the same column for a particular organism with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P > 0.05).

Viable counts of Lactobacillus *and* Bifidobacterium *during incubation*

Tables 1 and 2 demonstrate the viable count of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in soymilk prepared from soy protein isolate. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

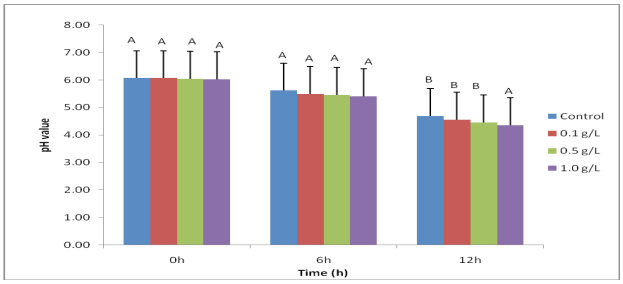

Figure 1. pH value of soymilk during 12 h fermentation by *B. animalis* Bb12 at 37◦C

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

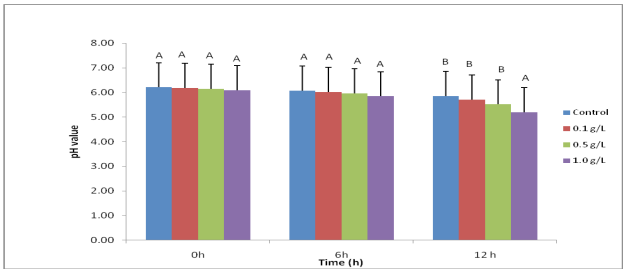

Figure 2. pH value of soymilk during 12 h fermentation by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 at 45◦C

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

produced higher ($p<0.05$) viable counts at 1.0 g/L enzyme concentration during the entire incubation. *B. animalis* Bb12 showed a significant difference ($p>0.05$) in the viable count at 0 h, 6 h and 12 h at different enzyme concentrations including control and a no significant difference ($p<0.05$) was found within crude enzyme concentrations at 0 h, 6 h and 12 h (Table 1). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different viable counts at 0 h and 12 h; however, there was no significant difference ($p<0.05$) in viable counts between 0 and 6 h (Table 2). Moreover, there was no significant difference ($p<0.05$) within enzyme concentrations including control at 0 h, 6 h and 12 h. The final viable population of the *B. animalis* Bb12 ranged from 6.9 to 7.4 log CFU/mL and the organism showed the highest viable population of 7.4 log CFU/mL at 12 h with 1.0 g/L CEE followed by 0.5 g/L of crude enzyme at 7.3 log CFU/mL and lowest with control at 6.9 log CFU/mL. At 12 h, the viable count increased ($p<0.05$) by 17.31, 20.36, 20.51 and 20.10% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE, respectively, at 12 h as compared with 0 h (Table 1). Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 6.1 to 6.7 log CFU/mL and the organism showed the highest viable population of 6.7 log CFU/mL at 12 h with 1.0 g/L crude enzyme followed by 0.5 g/L crude enzyme 6.4 log CFU/mL and lowest with control 6.1 CFU/mL. The viable count increased ($p<0.05$) by 40.18, 40.44, 36.56, and 35.84% in control, 0.1 g/L, 0.5 g/L and 1.0 g/L CEE, respectively, at 12 h as compared with 0 h (Table 2). Soymilk could not appeared to support the growth of *Bifidobacterium and Lactobacillus*, possibly due to the low amount (less than 1%) of simple carbon compounds in SPI, including sucrose, raffinose, and stachyose, which have been removed during processing (Nutrition Data, 2007). According to Shah (2006), the mild acidic condition of soymilk during fermentation (pH 6.15 to 6.80) was still in a favourable range for the growth of *Bifidobacterium* could be responsible for maintaining the viability of the probiotic organism. Supplementation of carbon sources to soymilk especially D-glucose stimulated the growth of *Bifidobacterium*, which could explain why there was an increase in the viable population of the organisms (Briczinski *et al.*, 2006).

Biotransformation of IG to IA by Lactobacillus *and* Bifidobacterium *in soymilk*

Tables 3 and 4 show the biotransformation of IG (glycitin) to IA (daidzein and genistein) in soymilk by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and their hydrolytic potential during fermentation at 37°C for *B. animalis* Bb12, and 45°C for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. The concentration of glycitin was recorded in decreasing trend whereas concentrations of daidzein and genistein were in increasing trend in soymilk during hydrolysis by microbial enzymes. The percent of moisture content of freeze dried samples ranged from 1.78 to 2.2. The isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). There were no significant differences ($P > 0.05$) in the moisture contents of the freeze-dried samples. Therefore, it is believed that there was no effect of the moisture content on the quantification of isoflavone compounds.

In general, there was only one IG (glycitin) and four IA (daidzein, genistein, biochanin A and formononetin) were used to determine the quantification of IG and IA in the soymilk sample at 0 h, 6 h and 12 h. Isoflavone concentrations of glycitin, daidzein and genistein were detected in different CEE concentrations including 0.1 g/litre, 0.5 g/litre and 1.0 g/litre and control at entire incubation. Biochanin A and Formononetin were not detected in soymilk in different CEE concentration and control. This also suggests their glycosides forms (sissotrin and ononin, respectively) were not available in SPI.

Statistically, *B. animalis* Bb12 showed a significant difference ($p<0.05$) in the glycitin and

daidzein at 0 h, 6 h and 12 h and there was no significant difference between different CEE concentrations; however genistein showed a significant difference ($p<0.05$) at both incubations as well as different CEE concentrations and control at 0 h, 6 h and 12 h. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference in glycitin at 0 h, 6 h and 12 h; however, there was no significant difference in hydrolysis ($p>0.05$) between different CEE concentrations and control. Moreover, daidzein and genistein showed a significant difference ($p<0.05$) in both organisms in all incubations as well as different CEE concentrations and control at 0 h, 6 h and 12 h.

B. animalis Bb12 produced glycitin at 9.41, 11.80, 8.11 and 13.83 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 6.32, 8.42, 8.11 and 5.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h and 3.47, 4.98, 5.42 and 3.52 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of glycitin was found at 13.83 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 0 h. Similarly, daidzein was produced at 23.92, 22.64, 25.57 and 26.23 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 0 h; 25.23, 30.36, 45.49 and 42.33 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 48.05, 52.16, 49.97and 47.81 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of IA in daidzein was found 52.16 mg/100 g of freeze-dried in 0.1 g/litre of CEE at 12 h. Likewise, genistein was produced 17.60, 18.68, 20.71 and 20.42 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 25.26, 27.67, 31.71 and 29.22 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 6 h; and 31.1, 32.43, 30.82 and 35.31 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of IA in genistein was found 35.31 mg/100 g of freeze-dried in 1.0 g/litre of CEE at 12 h.

On the other hand, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced glycitin 7.42, 9.15, 10.55, and 14.26 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively at 0 h; 6.04, 5.41, 4.51, 6.51 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 4.76, 4.56, 2.8, and 3.53 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at12 h. The higher level of glycitin was found 14.26 mg/100 g of freeze-dried in 1.0 g/litre at 0 h. Likewise, daidzein was produced 21.93, 21.03, 20.14 and 22.77 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 0 h; 23.15, 24.04, 23.43 and 21.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 19.2, 25.2, 22.8 and 22.60 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE respectively, at 12 h. The higher level of daidzein was found 25.2 mg/100 g of freeze-dried in 0.1g/litre of crude enzyme at 12 h. Similarly, genistein was produced 11.67, 14.59, 15.24 and 15.46 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively at 0 h; 14.04, 15.16, 16.38 and 15.5 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 6 h; and 16.34, 17.4, 17.8 and 17.56 mg/100 g of freeze-dried samples in control, 0.1 g/litre, 0.5 g/litre and 1.0 g/litre of CEE, respectively, at 12 h. The higher level of genistein was found 17.80 mg/100 g of freeze-dried in 0.5 g/litre of CEE at 12 h.

The biotransformation of glycitin occurred higher in *B. animalis* Bb12 (74.44%) followed by (62.15%) with 1.0 g/litre CEE at 12 h and 6 h, respectively and lowest percent hydrolysis (28.66%) with 0.1 g/litre of CEE at 6 h (Table 3). However, the biotransformation of glycitin was higher for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (at 75.23%) followed by 73.46% with 1.0 g/litre and 0.5 g/litre CEE, respectively, at 12 h and lowest percent of hydrolysed (18.55%) with control at 6 h (Table 4). D-glucose appeared to have stimulating effect on the biotransformation by the organism at 12 h. The results suggest that D-glucose allowed the growth of these two organisms (data not shown). The biotransformation of glycitin might be a consequence of high level of viable cells in soymilk. During hydrolysis, the concentration of glycosides such as glycitin reduced while the concentration of aglycones such as genistein and daidzein increased in soymilk fermented by both organisms. The conversion of individual forms of isoflavone glycosides to respective aglycones has been reported by Otieno and Shah (2006a). In addition, low pH condition in soymilk may have also contributed to the increase in the biotransformation level. Delmonte *et al.* (2006) and Mathias *et al.* (2006) reported that some IG was partly hydrolyzed to IA in a low pH condition.

Table 3. Biotransformation of IG to IA in soymilk by *B. animalis* Bb12

Isoflavone(mg/ 100 g) of freeze dried sample		Control			Enzyme concentrations (g/L) 0.1			0.5			1.0		
		0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
	Glycitin	9.41± 0.01^{a}	6.32 ± 0.01^{b}	3.47 ± 0.01^{c}	11.80± 0.01^{a}	8.42 ± 0.01^{b}	4.98 ± 0.01^{c}	13.23 ± 0.01^{a}	8.11 ± 0.01^{b}	5.42± 0.01^{c}	13.83 ± 0.01^{c}	5.23 ± 0.01^{b}	3.53 ± 0.01^{c}
	% of glycitin (IG) hydrolysed	**0**	**32.86**	**63.14**	**0.0**	**28.66**	**57.81**	**0.0**	**38.70**	**59.01**	**0.0**	**62.15**	**74.44**
	Daidzein	23.92 ± 0.02^{c}	25.53± 0.03^{b}	48.05 ± 0.08^{a}	22.64± 0.02^{c}	30.36 ± 0.02^{b}	52.16 ± 0.01^{a}	25.57 ± 0.01^{c}	45.49 ± 0.01^{b}	49.97 ± 0.01^{a}	26.23 ± 0.03^{c}	42.33 ± 0.01^{b}	47.81 ± 0.12^{a}
	Genistein	17.60 ± 0.01^{c}	25.26± 0.01^{b}	31.1 ± 0.01^{a}	18.68± 0.03^{c}	27.67 ± 0.01^{b}	32.43 ± 0.03^{a}	20.71 ± 0.05^{b}	31.71 ± 0.05^{a}	30.82 ± 0.05^{a}	20.42 ± 0.03^{c}	29.22 ± 0.02^{b}	35.31 ± 0.01^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05).
Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

Table 4. Biotransformation of IG to IA in soymilk by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Isoflavone(mg/ 100 g) of freeze dried sample		control			Enzyme concentration (g/L) 0.1			0.5			1.0		
		0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
	Glycitin	7.42± 0.02^{a}	6.04 ± 0.01^{b}	4.76 ± 0.01^{b}	9.15± 0.02^{a}	5.41± 0.00^{b}	4.56 ± 0.01^{c}	10.55± 0.01^{a}	4.51 ± 0.00^{b}	2.8 ± 0.01^{c}	14.26 ± 0.01^{a}	6.51 ± 0.00^{b}	3.53 ± 0.01^{c}
	% of glycitin (IG) hydrolysed	**0.0**	**18.55**	**35.80**	**0.0**	**40.88**	**50.16**	**0.0**	**57.22**	**73.46**	**0.0**	**54.34**	**75.23**
	Daidzein	21.93 ± 0.01^{b}	23.15 ± 0.01^{a}	19.2 ± 0.01^{c}	21.03± 0.01^{c}	24.04± 0.01^{b}	25.2 ± 0.01^{a}	20.14± 0.01^{c}	23.43 ± 0.01^{a}	22.8 ± 0.01^{b}	22.77 ± 0.01^{a}	21.50 ± 0.01^{c}	22.60 ± 0.01^{b}
	Genistein	11.67 ± 0.01^{c}	14.04 ± 0.01^{b}	16.34 ± 0.01^{a}	14.59± 0.02^{b}	15.16± 0.28^{b}	17.4 ± 0.02^{a}	15.24± 0.01^{c}	16.38 ± 0.01^{b}	17.8 ± 0.01^{a}	15.46 ± 0.01^{c}	15.5 ± 0.02^{b}	17.56 ± 0.01^{a}

Results are expressed as mean ± SE (n=3). Data were analysed by means of 1-way ANOVA. Means values in the same row with the same lowercase superscripts are not significantly different (P> 0.05).
Mean values in the same column for a particular organism with the same uppercase letter are not significantly different (P> 0.05).

Conclusions

The result of this study demonstrated that *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 are capable of hydrolysing glycitin to biologically active forms in soymilk (SM) prepared from soy protein isolate (SPI) and soymilk supplemented with 2.0% (w/v) of D-glucose at different concentrations of CEE. The increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of daidzein and genistein in fermented soymilk. Increased daidzein and genistein content in fermented soymilk is likely to improve the biological functionality of soymilk. The crude enzyme extract played a greater role in biotransformation. The biotransformation of glycitin occurred lower in *B. animalis* Bb12 (74.44 %) than the *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (75.23 %).

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Influence of Galactooligosaccharides and Modified Waxy Maize Starch on Some Attributes of Yogurt

Laxmi N. Prasad, Frank Sherkat, and Nagendra P. Shah

Abstract: This study examined the influence of galactooligosaccharides (GOS) and modified waxy maize starch (MWMS) addition on the growth of starter cultures, and syneresis and firmness of low-fat yogurt during storage for 28 d at 4 °C. The control yogurt (CY) was prepared without any prebiotics. Incorporation of 2.0% (w/v) GOS improved the growth of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 resulting in a shorter fermentation time. There was a significant ($P < 0.05$) increase in proteolysis in yogurt made with GOS (GOSY) as measured by absorbance value (0.728). Addition of GOS resulted in higher ($P < 0.05$) concentration of lactic and acetic acids in comparison with that of MWMSY and the CY up to day 14, thereafter, the product showed a decrease in lactic acid content in all 3 batches until the end of storage. The level of syneresis was the lowest (2.14%) in MWMSY as compared with that of GOSY (2.35%) and CY (2.53%). There was no statistically significant ($P > 0.05$) difference in the firmness among the 3 types of yogurt.

Keywords: firmness, galacto-oligosaccharides, lactic and acetic acids, modified waxy maize starch, proteolytic activity, spontaneous whey separation

Introduction

Commercial yogurt is typically produced by a starter culture consisting of *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) (Tamime and Marshall 1997; Tamime and Robinson 2007). During fermentation, starter culture bacteria produce proteolytic enzymes which hydrolyze milk proteins and lactose and converts lactose to lactic acid and decrease pH. The conversion of milk to yogurt is an aggregation of casein micelles into a gel structure at a pH of 4.6. The functionality of yogurt is further enhanced by the release of bioactive peptides due to proteolytic activities of the organisms used (Shah 2007).

Textural and rheological properties that are important attributes of yogurt for consumer acceptability. The texture of yogurt is influenced by various factors, for example, quality and composition of milk and its fat and total solid content, heat treatment of milk, combination of the lactic acid bacteria used, acidification rate and storage time (Dello and others 2004; Purwandari 2007).

Prebiotics are nondigestible food ingredients that pass through the upper digestive system relatively intact and are fermented in the lower colon, producing short chain fatty acids that support the growth of colonic microbiota (Lamsal 2012). There is a considerable interest in preparing yogurt with prebiotics such as inulin, modified waxy maize starch (MWMS) and galactooligosaccharides (GOS; Tarrega and Costell 2006; Paseephol and others 2008 and 2009). These prebiotics are used at 2–5% (w/v) of yogurt formulation for improvement in texture and growth of probiotic organisms. Prebiotics such as GOS are increasingly being recognized as useful dietary tools for the modulation of the colonic microbial balance. Konar and others (2011) reported that GOS is a key food ingredient that has created better opportunities in the food industry. The GOS is a well known prebiotic compound and its incorporation as food ingredient is safe for manufacturers and consumers.

Vivinal® GOS syrup is stable under low pH conditions and at high temperatures. Pasteurization and sterilizations at low pH do not affect Vivinal® GOS and the product will maintain its structure, appearance and content of GOS. Vivinal® GOS is produced through the enzymatic conversion of lactose (Matsumoto 1993). It is prepared from edible lactose, isolated from sweet whey. The lactose is subjected to the action of β-gal, which increases the chain lengths by a series of transglycosylation reactions (Mcbain and Macfarlane 2001).

The GOS is a mixture of di-to-octasaccharides and composed of 1–7 galactose units linked to a glucose molecule at the reducing end. The major saccharide in the GOS fraction of Vivinal® is the trisaccharide O-β-D-galactopyanosyl-(1–4)-O-β-D-galactopyranosyl-(l-4)-β-D-glucose. The molecular weights of the oligosaccharides range between 342 (disaccharide) to 1,315 (octasaccharide) Daltons, with an average molecular weight of approximately 522 Daltons (Mcbain and Macfarlane 2001).

It has been reported that GOS selectively stimulates the growth and metabolic activity of the beneficial bacteria in the colon (Roberfroid and others 1998), where it is fermented and decreases the pH. According to Crittenden and Playne (1996), GOS is used in dairy-based gel systems to modify the flow and textural properties of the final gel.

The modified starches are composed of units of amylase and amylopectin, mostly in amorphous regions on the surface of the granule that are modified without destroying the granular nature

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of the starch. This modification improves acid stability, heat stability and shear stability, inhibits gel formation, and controls viscosity during processing (Tecante and Doublier 1999). The MWMS is used for improving smoothness, and texture of dairy products and is considered to be a nongelatinizing starch that typically gives a cohesive and gummy texture in dairy gel systems (Sprague 1939). It has also been used in milk to enhance the growth of yogurt cultures (Nielsen and others 1991). Williams and others (2004) reported that the addition of MWMS to yogurt decreased syneresis (spontaneous whey separation) but developed a grainy texture. Other reason for using MWMS is to improve the water binding and water holding capacity, heat resistant behavior, improve thickening and to minimize syneresis of yogurt (Miyazaki and others 2006).

Syneresis is defined as the spontaneous separation of whey from protein matrix which then becomes visible as the surface whey. Ibrahim and others (2004) reported that the addition of GOS reduced the extent of syneresis in yogurt towards the end of storage. It has been suggested that faster rate of acidification inhibits network rearrangement, thereby resulting in less whey separation (Castillo and others 2006). Increasing yogurt solids content with skim milk powder is also reported to result in less syneresis (Puvanenthiran and others 2002; Amatyakul and others 2006).

Among available commercial prebiotics, the Vivinal® GOS is a natural prebiotic rich in galacto-oligosaccharides, structured as chains of galactose with a glucose, produced from glucose molecules transferred from a sucrose donor to a maltose acceptor by a glycosyl-transferase (Crittenden and Playne 1996).

This study was undertaken to examine and compare the influence of GOS and MWMS on the growth of ST and LB, their proteolytic activity, and organic acid production and syneresis and firmness of low-fat yoghurt.

Materials and Methods

Activation of starter cultures

The frozen pure culture of ST was obtained from Chr. Hansen (Bayswater, VIC, Australia) and LB was obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The purity of the cultures was confirmed by Gram staining. The stock cultures were stored at −80 °C in sterile medium composed of 50% (v/v) MRS broth (Difco, Becton, Dickinson and Company, New Jersey, USA) and 50% (v/v) glycerol. The LB culture was activated in MRS broth supplemented with 1% (w/v) yeast extract and 2% (w/v) glucose, whereas ST was first activated in M17 broth (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 10% (w/v) lactose then transferred (1% v/v) into 12% (w/v) sterile reconstituted skim milk (RSM). ST was incubated at 37 °C, while LB was incubated at 45 °C for 18 h.

Preparation of yogurt batches

Three batches of yogurt mix were prepared using Skinny milk (10.5% total solids and 0.12% fat; Parmalat Foods Pty Ltd., Wahgunyah, Vic, Australia), 1.5% skim milk powder (Commercial Road, Koroit, VIC, Australia). The control yogurt (CY) was prepared from the standardized low-fat milk. The experimental batches of yogurts (GOSY and MWMSY) were prepared from standardized low-fat milk added with 2% (w/v) prebiotic, either Vivinal® GOS (Great Ocean Ingredients, Warrnambool, Victoria, Australia) or MWMS (KF Specialty ingredients, Kings Park, NSW, Australia). All the yogurt mixes were heated at 85 °C for 30 min, cooled in a cold water bath to 40 °C, inoculated with 2% (v/v) activated culture of ST and 1% (v/v) of LB, mixed thoroughly, dispensed into 50 mL prewashed and sanitized cups, sealed, and incubated at 40 °C until the pH reached 4.5. The yogurt cups were refrigerated at 4 °C and stored for 28 days. Samples were taken on day one after overnight storage at 4 °C and on weekly intervals to monitor changes in pH, viable counts, proteolysis and lactic and acetic acids content, whey separation and firmness. All the experiments were replicated 3 times.

Media preparation for enumeration of starter cultures

Peptone water diluent. Peptone water diluent was prepared by dissolving 1.5 g (w/v) of peptone (Oxoid, West Heidelberg, Australia) in 1 L of distilled water and autoclaving at 121 °C for 15 min.

The ST agar

ST agar was prepared by dissolving 15 g of bacteriological agar (Oxoid Ltd, Basingstoke, Hampshire, England) and 37.25 g M17 broth in 950 mL distilled water, followed by sterilizing at 121 °C for 15 min and adding 50 mL of 10% (w/v) filter-sterile lactose solution.

Enumeration of cultures

Enumeration of starter culture, in freshly inoculated yogurt bases (0 h) and in yogurts stored at 4 °C was carried out by pour plate technique using MRS agar, respectively (Dave and Shah 1996). Duplicate plates were placed in anaerobic jars (Becton Dickinson Microbiology System, Sparks, MD, USA) containing anaerobic gas generating kits (Oxoid Ltd., Hamshire, UK) and incubated at 37 °C for 24 h for ST, and for 72 h at 45 °C for LB. Plates showing 25 to 250 colonies were counted and results were expressed as colony forming units (CFU) per gram of the inoculated sample.

pH measurement

The change in pH was measured at weekly intervals during 28 days of storage at 4 °C by using a pH meter (Model WTW, InoLab 720 Weilheim, Germany) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Samples were tempered to room temperature before pH measurement.

Determination of organic acids

The concentration of lactic and acetic acids in yogurt was determined by the high-performance liquid chromatography (HPLC) as described by Ramchandran and Shah (2008). Briefly, 40 μL of 15.5 M nitric acid and 500 μL of 0.01 M sulphuric acid were mixed with 1 g of yogurt sample and centrifuged at 14,000 × *g* for 30 min. The supernatant thus obtained was filtered using a 0.45-μm-membrane filter (Millipore Corp., Bedford, Mass., USA) into HPLC vials. An aliquot of 10 μL of each sample was injected into HPLC system. The organic acids were separated in an Aminex HPX-87H, 300 × 7.8 mm ion exchange column (Bio-rad Life Science Group, Hercules, Calif., USA) fitted with a guard column maintained at 65 °C at flow rate of 0.6 mL/min. The column was attached to a Varian HPLC (Varian Analytical Instruments, Walnut Creek, Calif., USA) fitted with a UV/Vis detector. Eluent containing 0.005 M sulphuric acid (MERCK Pty Ltd., Colchester Road, Kilsyth, Australia) was used as mobile phase for 25 min. The organic acids were detected at 210 nm. The retention times of lactic and acetic acids were compared with those of the standard working solutions of L (+) lactic acid and acetic acid prepared from a 10% (v/v) stock solution.

Determination of proteolytic activity in yogurt

Proteolysis was determined according to Church and others (1983) in the filtrates of inoculated milks (0 h) and in nonfat yogurt samples. Fifty grams of each sample were centrifuged at 4,000 × *g* for 30 min at 4 °C. The supernatants thus obtained were filtered through a 0.45 μL membrane filter and stored at −20 °C until assayed. A 150 μL aliquot of the filtrate was added to 3 mL of OPA reagent was prepared according to Church and others (1983) and vortexed for 10 s. The free amino acid content was determined by measuring absorbance at 340 nm within 2 min using a spectrophotometer (UV/VIS spectrophotometer, Ontario, Canada). The readings of samples at time 0 as well as the reagent blank were deducted from the corresponding readings of samples to obtain the free amino acids (measured by absorbance at 340 nm) released as a consequence of the proteolytic activity of the starter cultures during fermentation and storage.

Measurement of syneresis

Syneresis in yogurt samples was measured using a siphon method described by Amatyakul and others (2006). A cup of yogurt with the lid was weighed and slanted at an angle of 45 ° to collect the surface whey. The collected whey was drawn out with a syringe to which a needle was attached. Thereafter, the cups with the lids were re-weighed and percent syneresis was calculated by dividing the weight of separated whey with the initial weight of the yogurt sample multiplied by 100.

Large-scale measurement of yogurt texture

The firmness of yogurt samples was determined using TA-XT plus Texture Analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK) attached to a Texture Exponent Software, with a P20 probe (diameter 20 mm) and 25 kg load cell. The speed of penetration was set at 1 mm/s and depth of penetration was 10 mm. The ratio of cup diameter to probe diameter was 3.5:1 (Amatyakul and others 2006). The gel strength was expressed in grams, indicative of the force required to break the gel. The TA-XT plus texture analyzer uses a probe to analyze characteristics such as firmness and smoothness of dairy products. This is an updated version that has the capability to test tough food samples with high accuracy. The measurements were performed as soon as the samples were removed from the refrigerator.

Table 1–Effect of prebiotic type on pH change during incubation of inoculated milk bases at 40 °C.

Periods of incubation	Type of milk bases		
	Control	GOSY	MWMSY
0 h	6.40 ± 0.02[a]	6.40 ± 0.02[a]	6.39 ± 0.02[a]
2 h	5.49 ± 0.20[a]	5.36 ± 0.02[a]	5.28 ± 0.03[a]
4 h 30 min	4.92 ± 0.09[a]	4.79 ± 0.11[a]	4.89 ± 0.10[a]
5 h	4.89 ± 0.02[a]	4.62 ± 0.04[b]	4.83 ± 0.02[a,b]
5 h 25 min	4.52 ± 0.02[a]	4.48 ± 0.02[a]	4.50 ± 0.03[a]

Values shown are average of 3 replicates. Control: Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from nonfat milk with addition of 2% GOS or MWMS.
[abc]Means in the same row (at same incubation time) with different alphabets are significantly different.

Statistical analysis

All tests and analyses were performed in triplicate and data were analyzed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using Statistical Analysis System (SAS 1995). ANOVA data with a $P < 0.05$ were classified as statistically significant.

Result and Discussion

Effect of prebiotic addition on pH change

The changes in pH during preparation of the 3 types of yogurts are presented in Table 1. The decrease in pH during the first 5 h of fermentation was maximum in the GOSY (4.62) followed by MWMSY (4.83) and the CY (4.89). However, GOSY reached the pH of 4.5 earlier than MWMSY and the CY (Table 1). Thus, incorporation of GOS appeared to improve the growth and activity of starter organisms that resulted in a shorter fermentation time. This is in agreement with the findings of Hardi and Slacanac (2000) and Ozer and others (2005) who reported that the rate of pH decrease of fermented milk products increased by the addition of GOS.

These results are similar to those reported by Guven and others (2005). The pH of all samples decreased steadily during storage without any significant ($P < 0.05$) difference among samples. However, GOSY showed lower pH values than other samples throughout the storage period (Figure 1). The decrease in pH during storage was due to continuous conversion of lactose to lactic acid and acetic acid. There was no significant difference

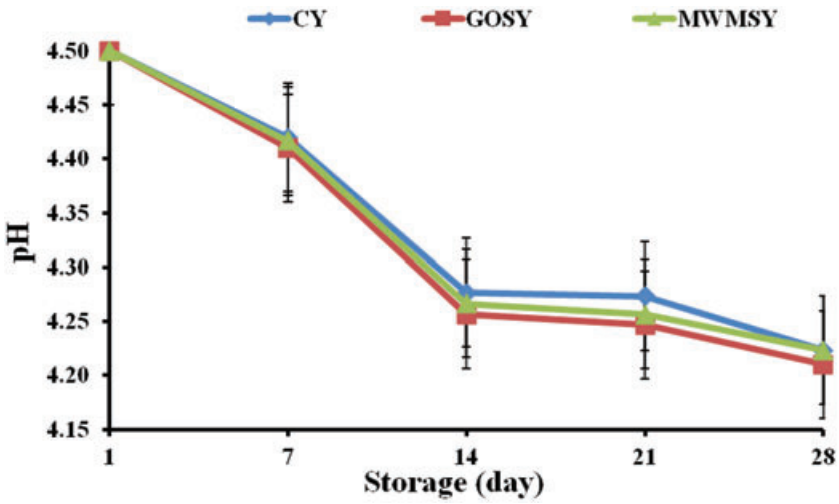

Figure 1–Changes in pH of nonfat yogurts with and without prebiotic during storage at 4 °C. Control: Yogurt prepared from nonfat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% *S. thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk and 2% GOS or MWMS: Bars indicate standard deviations.

($P > 0.05$) in the pH values of supplemented and nonsupplemented prebiotic yogurt at the end of the storage period (Figure 1).

Determination of the viability of starter cultures

Figure 2 depicts the viable counts in log CFU per gram of ST and LB in yogurt with and without prebiotic during storage at 4 °C. Total count increased in all samples during incubation and storage and the maximum numbers were found at day 14 of storage and thereafter there was a slight decline in the counts until the end of storage. All 3 types of yogurt showed significant differences ($P < 0.05$) in the viable count during the first 21 days but no significant difference ($P > 0.05$) was found at the end of storage. However, GOSY consistently showed higher numbers ($P < 0.05$) throughout the storage period. The maximum viable count of 6.95 log CFU per gram was observed in GOSY at day 14 followed by 6.84 and 6.73 log CFU per gram in MWMSY and the CY, respectively. This represented 16.56%, 12.52%, and 14.62% increase in the counts of cultures in CY, GOSY, and MWMSY, respectively (Figure 2). These findings agree with those of Ito and others (1993b) and Vulevic and others (2008) who reported that GOS was an excellent source for stimulating the growth of *Bifidobacterium* and *Lactobacillus*.

Production of organic acids

The concentration of acetic and lactic acids in the 3 types of yogurts during storage at 4 °C for 28 d is shown in Table 2. The production of lactic acid increased until day 14 of storage and, thereafter, there was a slight decrease in all yogurt samples. Addition of GOS resulted in a significantly ($P < 0.05$) higher lactic acid production until day 14 of storage than the other 2 batches. Similarly, GOSY produced more ($P < 0.05$) acetic acid during incubation than other batches (Table 2). There was a decline in the amount of lactic acid in all 3 types of yogurt from day 14 to 28 (Table 2). However, the production of acetic acid gradually increased in all samples throughout the storage. Several workers have reported that the utilization of prebiotics by bacteria varied depending on the strain (Desai and others 2004). Sako and Matsumoto (1999) have reported that incorporation of nondigestible oligosaccharides in fermented food products increased the acidity due to production of short chain fatty acids (SCFA). The concentration of acetic acid varied considerably in all yogurt batches (Table 2).

Syneresis

Table 3 shows the syneresis (%) in all 3 types of yogurt during storage at 4 °C for 28 days.

All samples showed reduction in the amount of whey separation up to 4 weeks of storage at 4 °C, and there was a significant difference ($P < 0.05$) in syneresis values in all yogurt batches at the end of storage. Al-Kadamany and others (2003) have also reported a decrease in the extent of syneresis in yogurts towards the end of the storage. This may be due to reduced permeability of serum through the protein gel (Amatayakul and others 2006). Syneresis has been related to slow shrinkage of the protein gel network that results in the loss of the ability to entrap all the serum phase (Lucey 2002). It has been suggested that faster rate of acidification inhibits network rearrangement during whey expulsion thereby resulting in less syneresis (Castillo and others 2006). It could also be due to re-absorption of whey back into the gel as the storage time progresses.

In our study, the amount of separated whey was found to be lower in the GOSY (2.14%) followed by MWMSY (2.35%) and CY (2.53%). Considering that GOSY reached the pH of 4.5 faster than the others (Table 1), it can be concluded that this could be one of the reasons for lower levels of whey separation. Yogurts containing added prebiotics are reported to show less syneresis (Puvanenthiran and others 2002; Isleten and Karagul-Yuceer 2006).

Our findings agree with those of Kalab and others (1983) who found that the rate of syneresis decreased in all yogurt samples due to the bonds between the network of milk gel becoming weaker.

Proteolytic activity of starter culture in yogurt

The proteolytic activity of starter culture in the 3 types of yogurts stored at 4 °C for 28 d is presented in Figure 3. Changes in

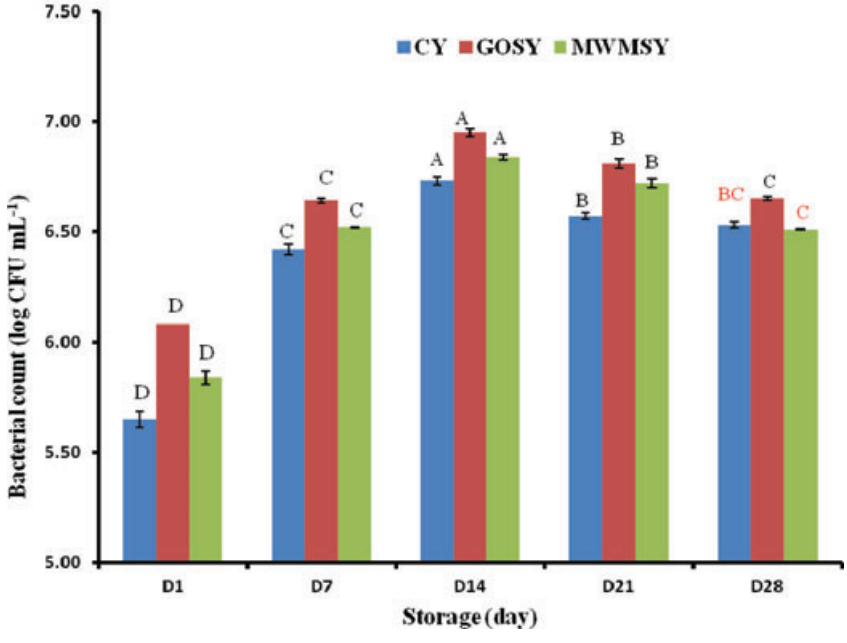

Figure 2–Changes in viable counts of starter cultures grown in non-fat yogurts with and without prebiotics during storage at 4 °C. Control: Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% *S. thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS: Bars indicate standard deviations. Column bars with same color at different times having similar letters did not differ significantly.

Table 2–Concentration of lactic acid and acetic acids (%) in nonfat yogurts with and without prebiotics stored at 4 °C.

	Storage period (days)				
	1	7	14	21	28
Lactic acid					
Control	1.28 ± 0.02^{Bb}	1.30 ± 0.02^{Ab}	1.32 ± 0.2^{Ac}	1.13 ± 0.03^{Cb}	1.05 ± 0.03^{Dc}
GOSY	1.34 ± 0.02^{Ba}	1.36 ± 0.05^{Ba}	1.39 ± 0.03^{Ca}	1.27 ± 0.04^{Db}	1.13 ± 0.04^{Ca}
MWMSY	1.29 ± 0.03^{Cb}	1.32 ± 0.02^{Bb}	1.37 ± 0.03^{Ab}	1.17 ± 0.04^{Da}	1.08 ± 0.03^{Eb}
Acetic acid					
Control	0.61 ± 0.04^{Cc}	0.67 ± 0.02^{Bc}	0.67 ± 0.03^{Bc}	0.72 ± 0.05^{Ac}	0.74 ± 0.04^{Ac}
GOSY	0.83 ± 0.02^{Aa}	0.84 ± 0.02^{Aa}	0.86 ± 0.03^{ABa}	0.87 ± 0.02^{Aa}	0.88 ± 0.02^{Aa}
MWMSY	0.72 ± 0.02^{Bb}	0.72 ± 0.03^{Bb}	0.74 ± 0.02^{Bb}	0.76 ± 0.03^{Ab}	0.77 ± 0.03^{Ab}

Values are the statistical means of 6 observations. Control: Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS; ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABCDE}Means in the same row with different alphabets are significantly different for a particular day of storage.

Table 3–Syneresis (%) in nonfat yogurts with and without prebiotics during storage at 4 °C.

	% of syneresis during storage period (days)				
	1	7	14	21	28
Control	3.66 ± 0.03^{Aa}	3.63 ± 0.04^{Aa}	2.80 ± 0.08^{Ba}	2.53 ± 0.03^{Ca}	2.53 ± 0.04^{Ca}
GOSY	3.45 ± 0.02^{Ac}	3.44 ± 0.02^{Ab}	2.54 ± 0.02^{Bb}	2.39 ± 0.03^{Cab}	2.14 ± 0.02^{Cc}
MWMSY	3.54 ± 0.02^{Ab}	3.53 ± 0.03^{Ac}	2.60 ± 0.02^{Bb}	2.35 ± 0.02^{Cb}	2.35 ± 0.02^{Db}

Values are the statistical means of 6 observations, Control: Yogurt prepared from non-fat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from non-fat milk with addition of 2% GOS or MWMS; ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment.
^{ABCD}Means in the same row with different alphabets are significantly different for a particular treatment.

the extent of proteolysis and liberation of free amino acids were measured by absorbance at 340 nm in the fresh and stored yogurt samples. There was a significant ($P < 0.05$) increase in proteolysis in all the yogurt samples during the entire storage period as compared to the control. Ramchandran and Shah (2009) reported that higher degree of proteolysis may be due to peptidase activity of organisms during storage period. Proteolytic activity may have increased because the organisms are capable of acting on peptides liberated from casein, and more peptides and amino acids are liberated than the bacteria can utilize. Proteolysis in GOSY was significantly ($P < 0.05$) higher than those of MWMSY and the CY throughout the storage period. This could have an influence on the higher survival rate of yogurt culture grown in GOS substrates (Hernandez-Hernandez and others 2012).

The maximum proteolytic capability (0.728) was observed in GOSY whereas only (0.583) and (0.530) were found in the MWMSY and the CY, respectively, on day 28. All yogurts showed an increasing trend in the amount of free amino acids during the storage period. The ability of LAB to grow to high cell densities in milk is dependent on a proteolytic system that can liberate essential amino acids from casein-derived peptides (Christensen and others 1999). Proteinase and peptidases constitute the primary enzymes in LAB responsible for proteolysis of caseins as a source of amino acids and nitrogen for LAB (Shihata and Shah 2000). Cruz and others (2012) reported that higher concentrations of glucose oxidase (750 to 1,000 mg/kg) and storage period had an influence on characteristics of probiotic yogurt and resulted in more postacidification and higher proteolysis.

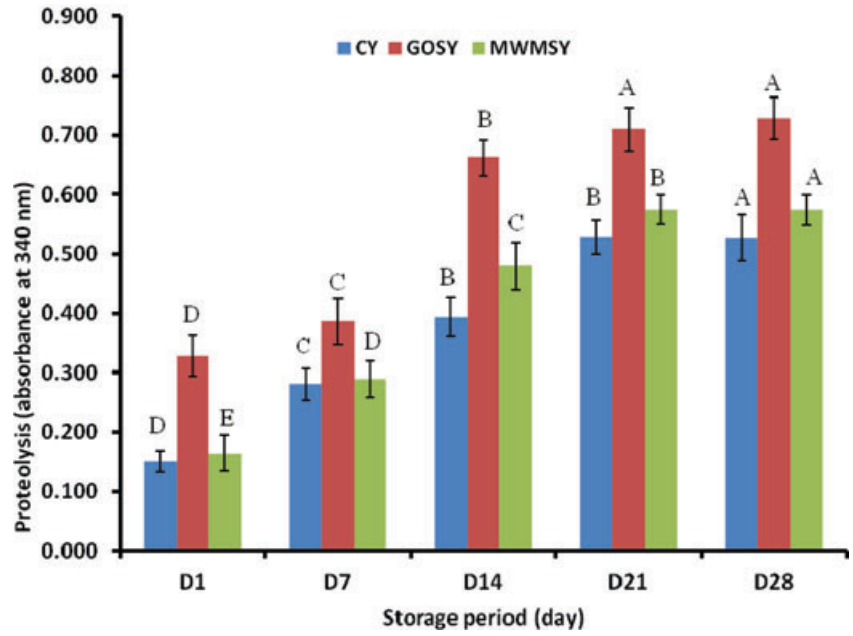

Figure 3–Progressive proteolysis of the nonfat yogurts with and without prebiotics during storage at 4 °C. Control: Yogurt prepared from nonfat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% *S. thermophilus* S5). GOSY and MWMSY: Yogurt prepared from nonfat milk with addition of 2% GOS or MWMS: Bars indicate standard deviations. Column bars with same color at different times having similar letters did not differ significantly.

Table 4–Firmness (g) of nonfat yogurts with and without prebiotics during storage at 4 °C.

	Storage period (days)				
	1	7	14	21	28
Firmness (g)					
Control	38.68 ± 1.78^{Ca}	46.52 ± 1.05^{Bb}	63.12 ± 0.96^{Aa}	62.65 ± 0.65^{Ab}	64.66 ± 0.26^{Aa}
GOSY	50.16 ± 1.78^{Aa}	56.80 ± 1.55^{Aa}	64.85 ± 2.42^{Aa}	67.85 ± 0.80^{Aa}	69.82 ± 1.67^{Aa}
MWMSY	45.30 ± 3.33^{Bb}	55.25 ± 0.78^{Aa}	64.13 ± 1.12^{Aa}	64.14 ± 0.34^{Ab}	65.43 ± 1.23^{Ab}

Values are the statistical means of 6 observations. Control: Yogurt prepared from nonfat milk and starter cultures (1% of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and 2% S. *thermophilus* S5). GOSY and MWMSY: Yogurt prepared from nonfat milk with addition of 2% GOS or MWMS: ^{abc}Means in the same column with different alphabets are significantly different within a particular treatment. ^{ABC}Means in the same row with different alphabets are significantly different for a particular day of storage.

Yogurt texture

The firmness of the 3 batches of yogurt during storage at 4 °C for 28 d is presented in Table 4. The firmness was measured on day 1 and during 28-days of cold storage, using the penetration force (g) to break the gels. The maximum firmness was recorded on day 1 in GOSY (50.16 g) followed by MWMS (45.30 g) and the CY (38.68). By the end of storage the firmness of all yogurts was increased whereas GOSY still showed higher firmness (69.82 g) than MWMSY (65.45 g) and the CY (64.66 g). Bozanic and others (2001) found that the firmness of yogurt improved upon the addition of prebiotic. According to Tamime and Robinson (1999), the primary aim of adding these prebiotic to the milk base is not only to enhance and maintain the yogurt texture and consistency but also to improve the general appearance and mouth feel. In our study, the addition of GOS and MWMS improved in the texture of yogurts which is in agreement with findings of Jawalekar and others (1993). Therefore, GOS can be added to dairy applications such as yogurts, buttermilk and dairy-based drinks due to its excellent solubility. After the addition of GOS, structure of yoghurt was found to be smoother and creamier (Sangwan and others 2011).

Conclusions

The supplementation of yogurt with prebiotics improved the retention of viability of *S. thermophilus* M5 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in all batches of yogurt during cold storage especially in the presence of GOS. The GOS was found to be a better growth stimulant of selected organisms than MWMS. Addition of GOS produced the higher levels of acetic acids than MWMSY and CY. However, the production of acetic acid increased in all samples throughout the storage period. Furthermore, increased proteolysis was found in the presence of GOS as compared to MWMS. The amount of syneresis was lower in GOSY compared to the MWMSY and the CY. Supplementation of milk with GOS was a better prebiotic ingredient than MWMS to enhance yogurt body. It appears that GOS not only enhanced the growth of selected organisms but also increased proteolysis of the yogurt. Therefore, addition of 2% GOS could provide functional properties in production of yogurt.

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