Responses of Probiotic Lactic Acid Bacteria to Dietary Isoflavone

A

DISSERTATION REPORT

Submitted in partial fulfillment of the requirements for award of

the Degree Of **Master of Science in Biotechnology**

Under the guidance of:

Dr. Abhijit Ganguli

Associate Professor

Submitted by:

Ajay kumar sandhu

Roll no: 301001002

DEPARTMENT OF BIOTECHNOLOGY AND ENVIRONMENTAL SCIENCES

THAPAR UNIVERSITY

PATIALA – 147004 INDIA June, 200

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Response of Probiotic Lactic acid Bacteria to dietary isoflavone**" in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar university, Patiala, is an authentic record of my work during the period of six months from January, 2012 to June 2012, under the guidance of Dr. Abhijit Ganguli, Assoc. Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

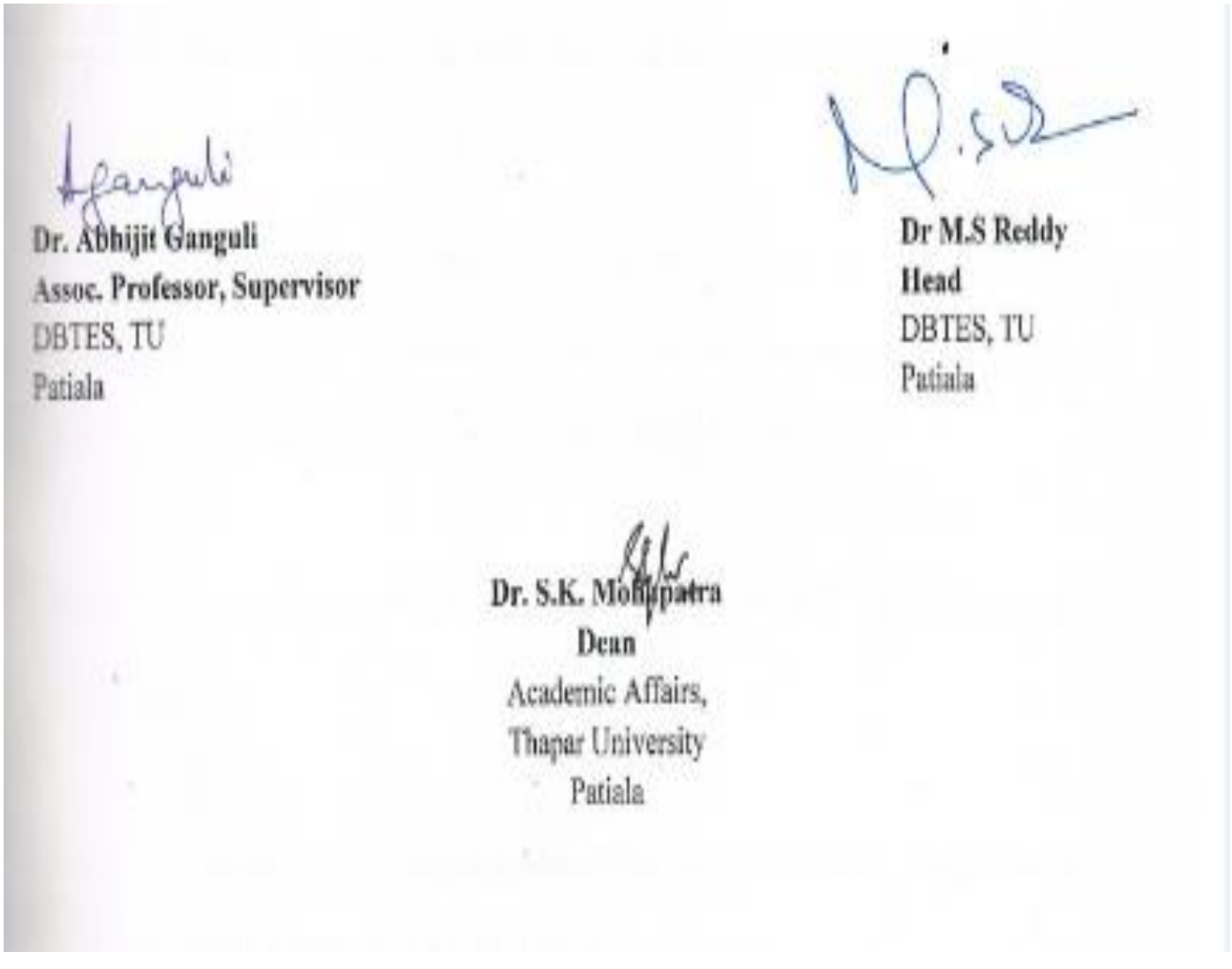
Ajay Kumar

AJAY KUMAR SANDHU

(3001001002)

CERTIFICATE

This is to certify that the thesis entitled "**Response of Probiotic Lactic acid Bacteria to dietary isoflavone**" submitted by Ajay Kumar Sandhu in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.



Dr. Abhijit Ganguli
Assoc. Professor, Supervisor
DBTES, TU
Patiala

Dr M.S Reddy
Head
DBTES, TU
Patiala

Dr. S.K. Mohapatra
Dean
Academic Affairs,
Thapar University
Patiala

ACKNOWLEDGEMENT

This project would not have been possible without the support of many people. First, I wish to express my deepest gratitude and regards to my adviser, Dr. Abhijit Ganguli, for his able guidance and inspiration throughout the research. His continued support led me to the right way.

I would also like to express my gratefulness and indebtedness to Dr. M. Sudhakara Reddy, Professor & Head and the entire faculty of the Department of Biotechnology and Environmental Sciences, Thapar University.

I would like to extend my sincere appreciation to the Ph.D. scholars Ms. Gurpreet Kaur Khaira, Ms. Seema Bhanwar, Ms Taranpreet Kaur for their invaluable support and advice during my research. I am thankful to my colleagues Abhishek Singh for guidance.

Special thanks to the laboratory staff for their kind cooperation and assistance in my experiments.

I would also like to extend my deepest gratitude to my family. Without their encouragement and support, I would not have a chance to be at Thapar University.

AJAY KUMAR SANDHU
(301001002)

CONTENTS

	Page no.
ABSTRACT	**1**
INTRODUCTION	**2**
REVIEW OF LITERATURE	**4**
SCOPE OF DISSERTATION	**13**
MATERIALS &METHODS	**14**
RESULTS & DISCUSSION	**22**
CONCLUSION	**37**
APPENDIX	**39**
REFERENCES	**43**

Abbreviation

RP-HPLC – Reverse phase- high performance liquid chromatography.

TLC- Thin layer chromatography.

UV –Ultraviolet

MRS- Man, Rogosa and Sharpe

PBS- Phosphate Buffer Solution

P.E – Petroleum ether

GAD-Glutamate decarboxylase

MATS-Microbial Adhesion of solvents.

pNPG- p- nitrophenyl β-d galacto pyranoside

oNPG- o-nitrophenyl β-d galacto pyranoside

CFU- Colony forming unit

Abstract

The objective of this study was to investigate the metabolic response of probiotic lactic acid bacteria *L.casei* towards isoflavones consumed through traditional Indian food. The tolerance of *L.casei* towards principle isoflavones (Daidzein, Genistein, Biochanin A, Formononetin, Coumestrol) were examined. Daidzein and Genistein showed higher tolerance to *L.casei*. The survival, growth and expression of probiotic attributes of *L. casei* in varying concentrations of daidzein in MRS medium and soya (containing daidzein) was monitored over a period of 24 hours; the fate of daidzein in both the combinations were then analyzed by High-performance liquid chromatography; Significant ($p<0.05$) quantities of Daidzein was utilized by the probiotic culture within 16 hours(28-37 C) from MRS media wheras daidzein at concentrations of 10-15µg/mg from Soya was completely utilized by *L.casei* over a period of 24 hours. Furthermore, the *L.casei* grown in daidzein tolerated artificial gastric juice, exhibited antagonistic activity against common enteric pathogens (*Salmonella typhimurum*), glutamate decaboxlation activity and cell surface hydrophobicity towards xylene . The β- galatosidase and β-glucosidase enzyme activities were observed in the presence of Daidzein exposed cultures suggesting their involvement in utilization of daidzein. The results of this study imply a possibility of incorporating the probiotic *L.casei* with food containing daidzein as the principle isoflavone to beneficialy impact health effects.

Key words: Isoflavones , *L.casei*, Soyabean

Introduction

Isoflavones are natural flavonoids , estrogen-related compound. Isoflavones are present in relatively large amounts in all soy products. Whole soy contain about 200 mg isoflavones per 100g (Chien *et al*., 2007).

Isoflavones are group of flavonoids compounds. Daidzein structurally belongs to the group of isoflavones. Daidzein and other isoflavone compounds, such as Genistein, are present in a number of plants and herbs like the Soy isoflavones are a group of compounds found in and isolated from the soybean. Of note, total isoflavones in soy beans are in general—37 percent daidzein, 57 percent genistein and 6 percent glycitein, according to USDA data. Soy germ contains 41.7 percent daidzein. Formononetin is an O-methylated isoflavone. It is found in a number of plants and herbs like the red clover. Along with other phytoestrogens, It predominantly occurs in leguminous plants and Fabaceae, particularly in beans, such as green beans, lima beans, soy. Coumestrol was first identified by E. M. Bickoff in alfalfa in (1957). It has since be found in a variety of legumes, soybeans, brussels sprouts, and spinach. Clover and soybeans have the highest concentrations. Biochanin A is an O-methylated isoflavone. It is a natural organic compound in the class of phytochemicals known as flavonoids. Biochanin A can be found in red clover in soy, in alfalfa sprouts, peanuts, chickpea (*Cicer arietinum*) and in other legumes.

In soy the most beneficial isoflavones are Genistein and Daidzein. Health benefits of isoflavones are that it eases menopause symptoms, reduce heart disease risk, protect against prostate problems, improve bone health, reduce cancer risk. The bioavailability of these isoflavones compounds depends upon the gut bacteria like lactic acid bacteria.

The objective of thesis, to developed the extraction process for isoflavones with high efficiencies from the soya sample. Then to developed the thin layer chromatography and HPLC procedure for the identification of isoflavones (Daidzein and Genistein). Probiotic activities of *L.casei* in the presence of isoflavones. Then to study the kinetics of *L.casei* with isoflavones. The quantitative and qualitative measurement of degraded isoflavones by *L.casei* through TLC and HPLC.

Intestinal microflora especially probiotic bacteria play a key role in the metabolism and bioavailabilty of isoflavones as they hydrolyse the glycoside components using their indigenous β-glucosidase and β-galactosidases in the jujenun, releasing the bioactive aglycone isoflavone form (Setchell *et al*., 2000). Aglycone forms have also been found to absorb faster and in higher amounts in human than their respective glycoside forms (Izumi *et al*., 2000). Intestinal bacteria are known to convert daidzein to its metabolite, equol, which is more potent estrogenic compound than its precursor (Setchell *et al*., 1999). Thus the use of probiotic bacteria to improve the biological activity of soy based products during processing formed an integral part of this study.

1. Review of literature

1.1 Extraction of isoflavones phytoestrogens

Wong (1962) for detecting isoflavones in clover developed a method in which fresh and rehydrated plant material was macerated and soaked in ethanol and filtered to give a crude extract. Petroleum ether was commonly employed to remove waxes and lipid material and the remaining mixture was evaporated and then further extracted with ethyl ether to isolate the compound of interest. Beck (1964) modified the pervious procedure after finding that bound isoflavones in the form of glycosidases were not released by alcohol extraction of intact plant material. Flavonoids commonly occur as glycosides in plant. Hydrolysis of the glycosides was effected by crushing plant material prior to alcoholic extraction, indicating that hydrolytic enzyme is present in the leaves. Francis *et al*.,(1965 b) compared treatments of clover leaves prior to alcoholic extraction and obtained maximum isoflavones yield if plant materials were crushed and allowed to stand a maximum for 10 min. Ohta *et al*., 1979 isolated Daidzein , genistein and there glucosidases ,daidzin and genistin by alcoholic extraction of soy flour product.

1.2 Identification techniques for isoflavones.

Thin layer chromatography

The thin layer chromatography method was developed by beck (1964). Beck tested 60 solvents and solvent mixture to resolve the estrogenic isoflavones from clover. Chloroform/methanol (89: 11,v/v) was found to be the only solvent system to give a satisfactory separation on silica gel plates. Formononetin and daidzein could be visualized as blue-white fluorescent spots under UV

light (257 nm). Genistein and biochanin A could be visualized as orange brown spot after spraying diazotinized sulphanilic acid.

1.3 Probiotic and Isoflavones

Isoflavones have activities against cancer cells (Adlercreutz *et al.,* 1992, Adlercreutz 1995), cardiovascular diseases Potter (1995) and osteoporosis (Kurzer *et al*., (1997) .When consumed, daidzein undergoes hydrolysis at the glycosidic bond by intestinal microflora before being absorbed in the gastrointestinal. Absorbed isoflavone aglycones are transformed to glucuronide in the liver and excreted through urine. The *β*-glycosidic bonds of isoflavone glucosides are also hydrolyzed during fermentation of soybean *Lactobacillus casei* Matsuda *et al.,* (1994). Soybean itself also contains this enzyme and hydrolyzes the isoflavone glucosides during the soaking and sprouting of the soybean Matsuura *et al.,* (1995). *β*-Glucosidase produced by the lactic acid bacterium, *Lactobacillus casei*, is cell associated. *β*-Glucosidases from soybean have been partially purified and characterized by Matsuura *et al.,* (1993, 1995). King *et al.*,(1996) reported that absorption of genistein was faster than genistin in humans but, in rats, the extent of absorption of genistein was similar for the glucoside and aglycone forms. Izumi *et al.* (2000) also reported that the isoflavone aglycones were absorbed faster and in greater amounts than their glucosides in humans. Urinary recovery of isoflavones was higher for aglycones than glucosides Hutchins *et al.,* (1995). Therefore it could be concluded that isoflavone aglycone-rich foods may be more effective than glucoside-rich foods in preventing chronic disease such as cancer.

Synbiotics refer to nutritional supplements combining probiotics and prebiotics in a form of synergism, hence synbiotics.

Using prebiotics and probiotics in combination is often described as synbiotic, but the United Nations Food & Agriculture Organization (FAO) recommends that the term "synbiotic" be used only if the net health benefit is synergistic. A further restriction is to require that the prebiotic be shown to increase the population and/or function of the probiotic it is paired with. Probiotic bacteria colonize the gut with all good microorganisms that help in proper digestion, they also avoid the adhering of pathogens to the intestinal tract. In prebiotic a fiber such as fructose oligosaccharide, galactose oligosaccharide etc. are consumed that is intended as a food for the microbes in the large intestine that promote the growth of intestinal microflora responsible for health benefits.

Isoflavones comprise a class of organic compounds, often naturally occurring, related to the isoflavonoids. Many act as phytoestrogens in mammals. Some are termed antioxidants because of their ability to trap singlet oxygen. Some isoflavones, in particular soy isoflavones, when studied in populations eating soy protein, have indicated that there is a lower incidence of breast cancer and other common cancers because of its role in influencing sex hormone metabolism and biological activity through intracellular enzymes, protein synthesis, growth factor actions, malignant cell proliferations, differentation and angiogenesis.

1.4 Asian and Indian diet with isoflavones

The asian diet contain highly processed foods made from legumes, such as tofu, retain most of their isoflavone content, with the exception of fermented miso, which has increased levels**.** Asians consume soybeans in many forms, including miso soup, tofu, natto (fermented soyabean) ,soy sauce, soya cheese, soya nuts, soya flour, barley miso, ice beans and tempeh. (Neil *et al*., 1993).

The Indian diet contain isoflavones include chick pea (biochanin A), peanut (genistein), soyabean (Daidzein and Genistein), spinach (coumestrol), green beans (formononetin)

1.5 Soy isoflavones

Soya (*Glycine max*) products are widely consumed by Indian and asian people. Isoflavones are present in relatively large amounts in virtually all soy products . Whole soya contain about 250 mg isoflavones per 100g. Soyabeans contain two types of isoflavones

Isoflavones are biologically-active, nonnutritive compounds that are present in relatively large amounts in soyabeans and soyfoods. Soyabeans contain two types of isoflavones; daidzein and genistein. These compounds are part of a larger group of plant chemicals, called flavonoids, that are common in many fruits, vegetables, and legumes. Soyabean are by far the most concentrated source of isoflavones in the Human diet.

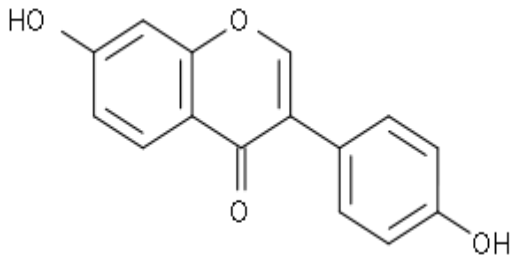
Soya isoflavones (Genistein and Daidzein) are one source of phytoestrogens in the human diet. Because most naturally occurring estrogenic substances show weak activity, normal

consumption of foods that contain these phytoestrogens should not provide sufficient amounts to elicit a physiological response in humans.

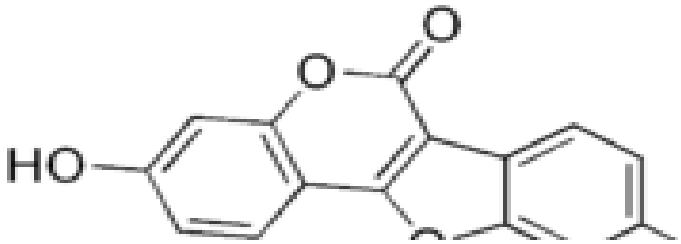
It is worth while to note that the asian populations with their high intake (50-70 mg/d) of soy derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms, cardiovascular disease and cancer (Nagata *et al*.,1998). The chemical form in which isoflavones occurs is an important consideration because it may influence the biological activity, the bioavailability and therefore the physiological effects of these dietary constituents.

1.6 Chemical structure of isoflavones. (Franke *et al*., 1996)

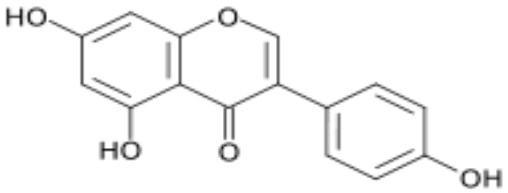
Structure of Daidzein



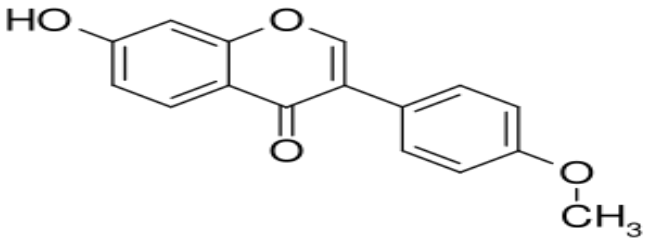
Structure of Coumestrol



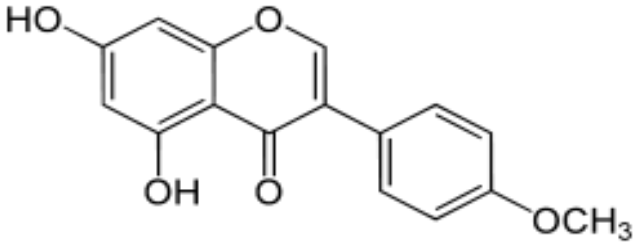
Structure of Genistein



Structure of Formononetin



Structure of Biochanin A



1.7 Probiotic Effects of Lactic acid bacteria

At present, much effort is given to the maintenance of health through various approaches such as exercise and natural foods. Use of probiotics, in demand for improved well-being, in humans and animals has been widely discussed (Fuller *et al.*, 1991). In recent years, the probiotic effects of Lactic Acid Bacteria have gained interest in terms of their functional aspect. Probiotic, a growth promoting agent, is obtained from living strains to maintain a balance of intestinal microflora by decreasing the growth of coliform baillus. It has been also expected as a new type of antibacterial substance for resolving some of the complications in the use of antibiotics and the resistance of microorganism to antibiotic substances. (Cho *et al*., 2000)

For commercial use, probiotics must satisfy safety as well as functional and technical issues, including viability, settlement, inhabitation, antibacterial agent creation, immunity hastening, antigenotoxic activation, pathogenic suppression, properties of organism, bacteriophage resistance and viability during the production process (Goldin *et al.*, 1977). Numerous studies have been carried out on the stabilization of gastrointestinal microflora, reduction of saprogenic products, prevention of degenerative disease, activation of immunity, mediation of anticancer activities, lowering of cholesterol, reduction of lactose intolerance, and suppression and prevention of constipation. For probiotics bacteria to be effective they must survive the harsh environments in the stomach (low pH) and intestinal track, which contain bile acid (Lee *et al.*, 2006).

1.8 Acid and bile tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Chou *et al*. 1998). After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. After traveling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract. Thus, strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health should also have the ability to resist the digestion process in the stomach and the intestinal tract. (Berrada *et al*. 2004) reported the time from entrance to release from the stomach to be 90 min. However, further digestive processes have longer residence times; hence, there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, which contain bile.

1.9 Microbial adhesion to solvents:

Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of glyco-proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Pelletier *et al*., 1997). It is known that only pronase- and pepsin-sensitive surface molecules are responsible for cell surface hydrophobicity in bacteria. In the previous studies, the autoaggregation ability of *L. acidophilus* M92 was also reduced by proteolytic treatment, while metaperiodate did not much

affect hydrophobicity As bacterial cells subjected to proteolytic attack weaken their autoaggregation ability, it is reasonable to consider proteins as mediators in the aggregation process.

1.10 Antagonistic Activity

Probiotic microorganisms are the first microorganisms to be encountered in the gastrointestinal tract (Bongaerts *et al*.,2001). Probiotics can balance intestinal bacteria by producing organic acid, bacteriocins, and antimicrobial peptides (Lee *et al*., 2006). This may lead to a competitive displacement of intestinal pathogens, the engagement of cell membrane receptors, which activate signaling events leading to cytokine synthesis, including interferons, and cell resistance to viral attack.

1. 11 β-glucosidase and β-galactosidase activity.

β-glucosidase and β-galactosidase activities of probiotic organisms. β-glucosidase activity is 15 times higher β-galactosidase activity found (Otieno *et al.*,2006). In soybeans, most isoflavones exist as glycoside, acetylglycoside, and malonylglycoside forms and, to a lesser extent, in the form of aglycones. However, it is recognized that the readily bioavilable isoflavones are aglycones rather than glycosides. The glucoside conjugates of isoflavones are converted into aglycones during soybean processing by the effect of β-glucosidase. β-glucosidase is considered to be key enzyme for the conversion of isoflavone form in soybean foods. We have shown β-glucosidase to be effective in converting isoflavone glycoside to aglycones (Pandjaitan *et al*., 2000a; 2000b). β-glucosidase has superior activity for hydrolyzing acetylglycoside and malonylglycoside isoflavone.

2. Scope of the thesis

Few Studies have evaluated the behavior / metabolic response of probiotic Lactic acid bacteria to isoflavones originating from traditional diets. Thus in this study an attempt was made to investigate the growth and expression of probiotic properties of strain of *L.casei*.

3. Materials and Method

3.1 Reference standards and Materials

Reference standards for isoflavones namely (Daidzein, Genistein, Biochanin A ,Coumestrol ,Formononetin) were purchased from Sigma-Aldrich (USA). All standard were analytically HPLC graded and dissolved in HPLC graded Methanol. Soya bean samples were collected from local Patiala market.

3.2 Bacterial strain and culture media:

A strain of *Lactobacillus casei* isolated from vegetables and fermented beverages (fermented rice and Madhuca longifolia flowers) and characterized as potential new probiotic strains (Kumar *et al.* 2010) in this laboratory previously, was used in this study. The strain was stored as stock solutions in 20% (v/v) glycerol at - 70 C. Prior to all experiments, the culture was propagated in MRS medium at least thrice; purity of the strain was confirmed microscopically and by gram staining and catalase activity periodically.

3.3 Tolerance and growth of *Lactobacillus casei* in the presence of pure Isoflavones.

Overnight grown culture of *L.casei* on MRS broth. Treated with different concentration (30,20,15,10,5 µgm/ml) of isoflavones (Daidzein , Genistein , Biochanin A, Coumestrol, formononetin) dissolved in methanol. Performing the high throughput screening by growing the *L.casei* in MRS in microtite plate reader, shaking the plates after 30 min and measureing the O.D at 600 nm. *L.casei* Growth was measured as log cfu/ml on MRS agar media by plate count method.

3.4 Fate of isoflavones analysed by thin Layer Chromatography and HPLC.

Fate of isoflavones were analysed by thin layer chromatography by Beck (1964). The solvent system taken was chloroform: methanol (89: 11). The spraying reagent was concentrated ammonia. Formononetin and daidzein could be visualized as blue-white fluorescent spots under UV light (257 nm). Genistein and biochanin A could be visualized as orange brown spot after spraying diazotinized sulphanilic acid The HPLC method was same that was analyzed previously reported.

3.5 Minimal growth media

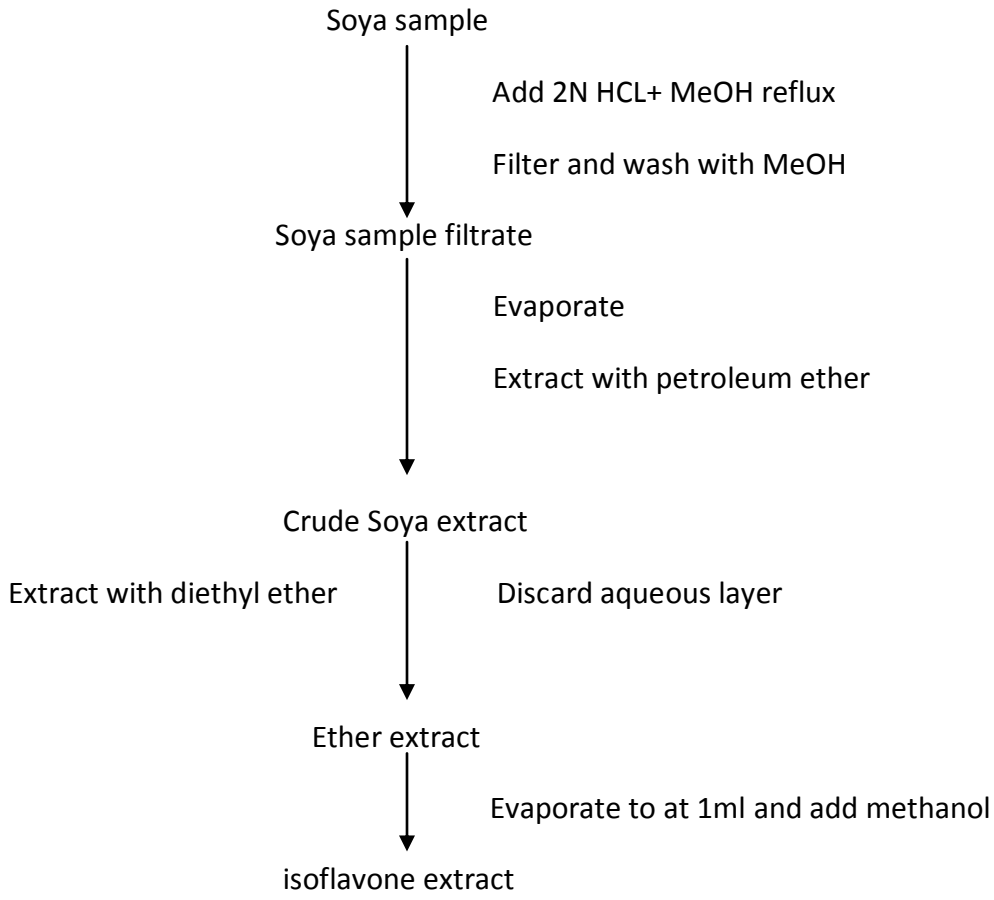
The growth of L.casei in basal minimal medium adopted from (Cerning *et al.*, 1994). The ability of *Lcasei* to grow in basal minimal media with glucose (control) and isoflavones as carbon source was examined. The minimal media contains KH_2PO_4 454mg/l, $Na_2HPO_4.12H_2O$ 1.19 g/l, NaCl 0.2 g/l, $MgSO_4.7H_20$ 0.1g/l, $CaCl_2.2H_2O$ 0.002g/l, $MnSO_4$ 0.004g/l, $FeSO_4$ 0.005 g/ml , peptone 0.002g/l .The control with glucose as carbon source and Daidzein, Genistein , Formononetin as sole carbon source with *L.casei* **.**

3.6 Extraction of isoflavones from Soya sample.

The method developed for isoflavones extraction from Soya sample was adapted from the extraction method reported by Wong (1962), Beck (1964), Francis *et al.*, (1965) for analysis of clover forage. Several modifications were made to these methods in part to account for small sample size in present study and to prepare the sample extract for subsequent analysis by High performance liquid chromatography.

Approximately 5 gm of Soya sample were ground with washed, dried sand using a mortar and pestle. The ground sample was allowed to stand for 10 min as per Francis *et al*., (1965) to permit hydrolysis of the phytoestrogens glycosides by the herbal drug sample glycosidases. The sample was then refluxed with 5 ml 2N HCL and 20 ml methanol for 30 min to further hydrolysis. After the 10 min cooling period, the Soya slurry was filtered through Whatman No.1 filter paper. The filter cake was washed with methanol and discarded. The filtrate was transferred to a 1000 ml round bottom flask with rinsing water. Evaporation to remove a portion of methanol in the filtrate was carried out under vacuum for 25 min using a rotary flash evaporator in conjunction with water bath at 30 C . This temperature allowed rapid removal of methanol yet limited the amount of heat applied to the sample. The remaining aqueous methanol extract was transferred to 250 ml separatory funnel, again rinsing the flask with water. Volume was adjusted to with water too a previously calibrated 18.5 ml mark. Lipids and chlorophyll pigments are removed by extraction with 3 × 20 ml portions of petroleum ether (b.p 37-58 C).After the addition of petroleum ether the mixture was shaken vigorously for 30 sec and allowed to stand for 10 min to permit the phases to settle and the pigments to transfer to the upper petroleum ether layer.

The final extraction of isoflavones from Soya sample was accomplished with 4 × 20 ml portion of diethyl ether .Each aqueous layer mixture was shaken for 30 sec and allowed to stand for 10 min before the removal of diethyl ether . The ether extract was combined and evaporated under vacuum at 32 C for 15 min to approximately 1 ml. The remaining residue was transferred into a 10 ml volumetric flask with HPLC graded methanol rinses and diluted to volume. Sample was stored in borosilicate glass vials at 5 C until analyzed.



3.7 Identification of principle isoflavones from Soya sample.

Thin layer chromatography

For detection of isoflavones method of Beck (1964) was used. Beck tested 60 solvents and solvent mixture to resolve the estrogenic isoflavones from clover. Chloroform /methanol (89: 11,v/v) was found to be the only solvent system to give a satisfactory separation on silica gel plates. Formononetin and daidzein could be visualized as blue-white fluorescent spots under UV light (257 nm). Genistein and biochanin A could be visualized as orange brown spot after spraying diazotinized sulphanilic acid.

3.8 Measurement of isoflavones by High performance liquid chromatography.

All HPLC analyses were performed using a Reverse phase column (C-18) octadecylsilane (4 mm I.D ×30 cm) by Murphy *et al*., (1978). Detector used was UV-VIS variable wavelength detector. Colum temperature was maintained at 30 ˚C. Detection of phytoestrogens was made at 254 nm (optical bandwidth 8 nm).

The methanol, water used for this purpose was HPLC graded. Separation was achieved by using a linear methanol-water gradient system at a flow rate of 1.0 ml/min. Methanol and water reservoirs each contained 1 % glacial acetic acid (v/v) and 0.01 M ammonium acetate HPLC grade by (Casteele *et al*. 1982). The gradient was programmed to increase from 53% to 58% reservoir B (methanol) over 30 min. total analysis time per sample, including column equilibration was 60 min. Preliminary peak identification was based on a comparison of retention times of phytoestrogens standards and unknown peaks in the sample extracts.

3.9 Real time study for fate of Daidzen in soya samples challenged with *L.casei.*

One gm of powdered soya sample containing daidzein was dissolved in Phosphate buffer solution freshly prepared. Overnight grown culture of *L.casei* was taken and dissolved in the sample containing PBS and incubate 37 C. The 0 and 3 hrs soya sample was taken, extracted with 3×20 ml petroleum ether till it settle down. Then remove the upper layer of Petroleum ether (P.E). Final extraction was done with 4×20 ml diethyl ether and discard aqueous layer. Dissolved the sample in methanol and kept in at 5 C until analyzed. Analysis of daidzein and formononetin was performed through thin layer chromatography (Beck 1964).

3.10 Tolerance of artificial gastric juice:

The ability of the *L.casei* with isoflavones (Daidzein and Genistein) to survive under gastric conditions was examined according to the method of Casey (2004). Overnight cultures were washed with phosphate-buffered saline (PBS), resuspended in synthetic gastric juice (pH 1.85 adjusted using HCl) and incubated at 37 C. The artificial gastric juice consisted of 3.5 gl-1 D-glucose, 2.05 g l-1 NaCl, 0.6 g l-1 KH_2PO_4, 0.11 g l-1 $CaCl_2$, 0.37 g l-1, KCl, 0.05 g l-1 Ox bile, 0.1 g l-1 lysozyme and 13.3 mg l-1 pepsin. Samples containing control ,daidzein and formonontin were withdrawn at regular intervals, serially diluted in PBS and enumerated on MRS agar to enumerate the viable cells.

3.11 Glutamate decarboxylase activity:

A rapid assay (Cotter *et al.*, 2001) was used to determine qualitatively the glutamate decarboxylase (GAD) activity in the isolate with isoflavones (daidzein and formononetin). One-milliliter volumes of overnight cultures in MRS were washed in quarter strength of Ringer's solution and resuspended in 0.5 ml of test reagent adjusted to pH 3.0 with 1 M HCl. The reagent consisted of 90 g l-1 NaCl, 1g L-glutamic acid, 0.3 g l-1 Triton X-100 and 0.05 g l-1 bromocresol green, made up in distilled water. The development of a blue coloration after 4 h of incubation at 37 C indicated a positive result.

3.12 Microbial adhesion to solvents:

Microbial adhesion to solvents (MATS) was measured according to the method of Rosenberg *et al*. (1980) with some modifications (Fontaine *et al*., 1996). *L.casei* treated with isoflavones were harvested in the stationary phase by centrifugation at 5000 g for 15 min, washed twice, and resuspended in 0.1 mol-1 KNO3 (pH 6.2) to approximately 108 CFU ml-1. The absorbance of the cell suspension was measured at 600 nm (A0). One milliliter of solvent was added to 3 ml of cell suspension.

After a 10-min preincubation at room temperature, the two-phase system was mixed by vortexing for 2-min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A1) was measured. The percentage of bacterial adhesion to solvent was calculated as (1 - A1/A0) * 100. Three different isoflavones were tested in this study.. *L.casei* adhesion to xylene reflects cell surface hydrophobicity or hydrophilicity.

3.13 β Galactosidase enzyme activity.

β-galactosidase activity was evaluated according to the method of (Otieno *et al.*, 2006) with some modifications. The *L.casei* was activated in rehydrated de Mann Rogosa Sharpe (MRS) broth at 37 C for 20 h twice followed by a third activation in Soya hydrolysate using an inoculum level of 5% (v/v). Of 20 ml aliquot extract, 1ml was aseptically drawn at 0, 3, 6, 9, 12, 15, 18 and 24 h of fermentation with *L.casei*. Exactly 1 ml of 15 mmol l) of o-nitrophenyl β-d galacto pyranoside (oNPG) in 0.03 mol l) of sodium phosphate buffer (pH 6.8) was added to the aliquot and incubated at 37C for 15 min. The reaction was stopped by adding 0.5 ml of cold 0.1 mol l) sodium carbonate. Absorbance was measured at 420 nm with a spectrophotometer. A unit

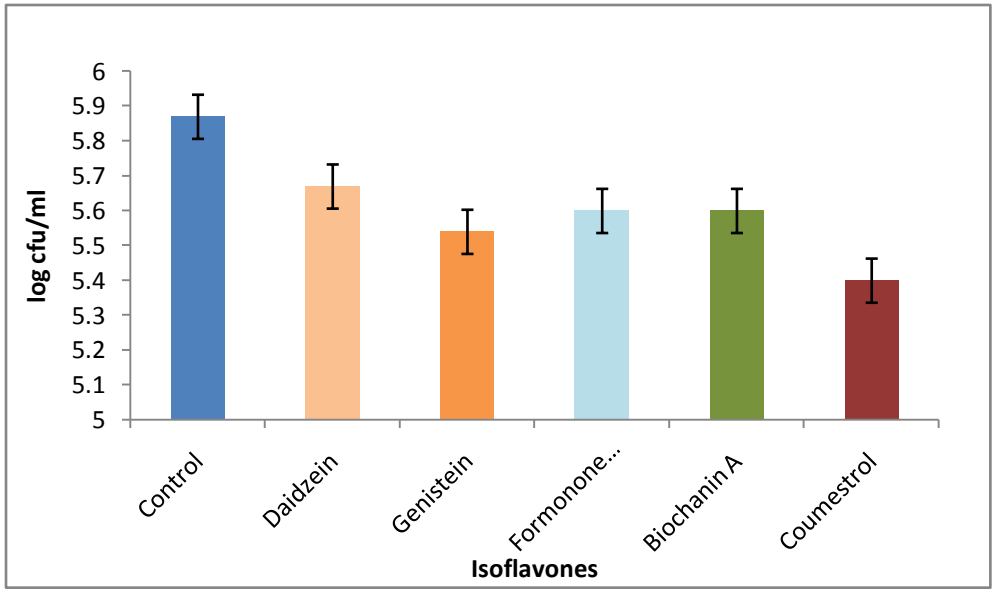
of β-galactosidase was defined as the amount of enzyme that catalyses the formation of 1 mol/l of o-nitrophenol per minute under the assay condition.

3.14 β-Glucosidase activity in the presence of isoflavones.

The determination of β-Glucosidase activity was evaluated according to the method (Otieno *et al*., 2006) with some modifications. The *L.casei* was activated in rehydrated de Mann Rogosa Sharpe (MRS) broth at 37 C for 20 h twice followed by a third activation in Soya hydrolysate using an inoculum level of 5% (v/v). The enzyme was determined in Soya extract with isoflavones by measuring the rate of hydrolysis of p-nitrophenyl-β-d-glucopyranoside (pNPG). Of the 20-ml aliquot, 1 ml was used for the determination of β-glucosidase activity according to the method by Otieno *et al*., (2005). Briefly, 1 ml of 5 mmol/ l of pNPG prepared in 100 mmol/ l of sodium phosphate buffer (pH 7.0) was added to 1 ml of aliquot and incubated at 37 C for 15 min (Scalabrini *et al*., 1998). Exactly 0.5 ml of 1 mol /l cold sodium carbonate was added to stop the reaction. The aliquots were then placed in 1.8-ml eppendorf centrifuge tubes followed by centrifugation (14000 g for 30 min). The amount of p-nitrophenol released was measured using a spectrophotometer at 420 nm.One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 mol of p-nitrophenol from the substrate pNPG per millilitre per minute under assay conditions.

4. Result and discussion

4.1 Tolerance of *L.casei* on MRS with isoflavones.



Bar graph indicated the growth of *L.casei* with isoflavones (Daidzein, Genistein, Biochanin A, Formononetin,Coumestrol) and control. *L.casei* tolerated the isoflavones dissolved in MRS media. To observe the different concentration of isoflavones on *L.casei* high throughput analysis performed.

4.2 Growth kinetics of *L.casei* in presence of isoflavones.

3.1 The growth kinetic of probiotic strain *Lactobacillus casei* in MRS supplemented with varying concentrations of isoflavones (Daidzein, Formononetin, Genistein, Biochanin A, Coumestrol) was evaluated over a period of 24 hours. Each experiment was performed in duplicate and result was the average of two readings. The highest cell number of test organism achieved was 6.68 Cfu/ml. The specific growth rate achieved was 0.54 /h. The *L.casei* tolerated different concentration of isoflavones provided in the MRS media.

Fig. 1 Growth kinetic of *L.casei* with Daidzein.

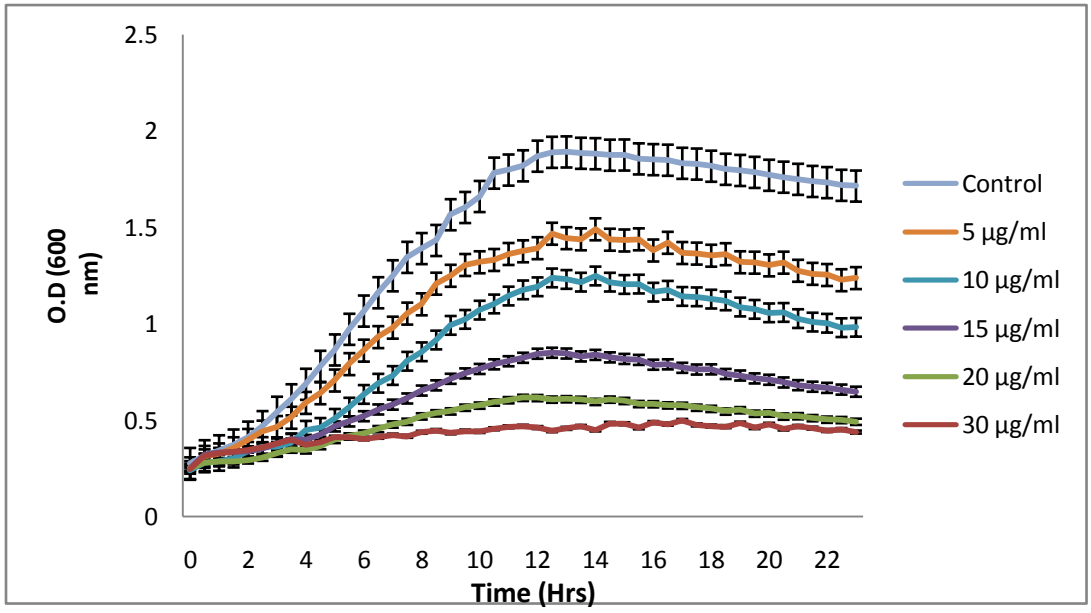


Fig. 2 Growth kinetic of *L.casei* with Formononetin.

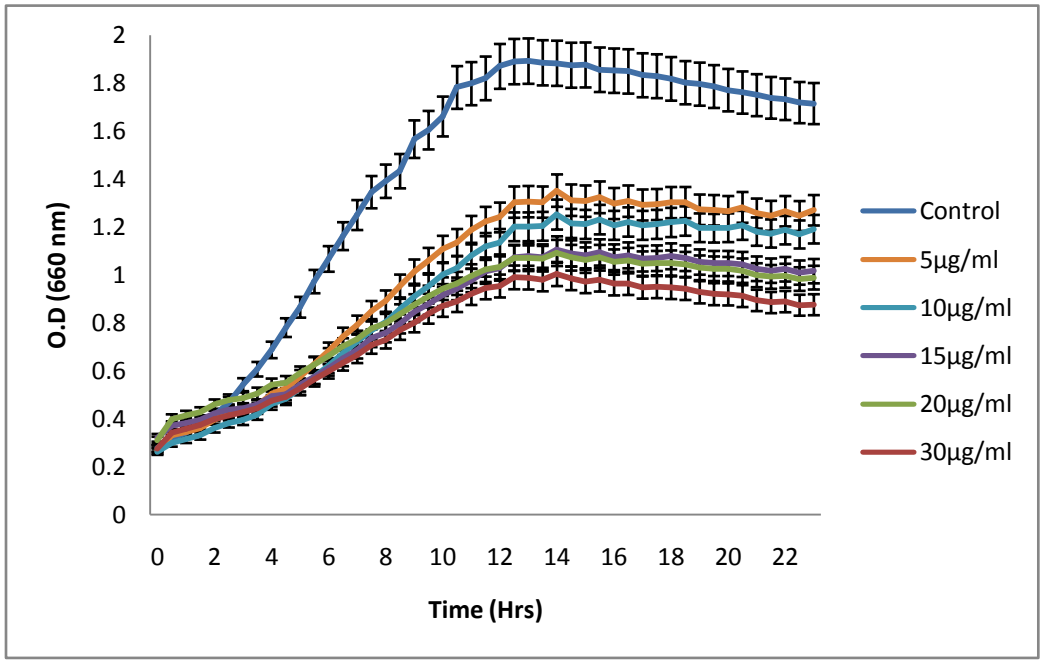


Figure 3. Growth kinetic of *L.casei* with Genistein.

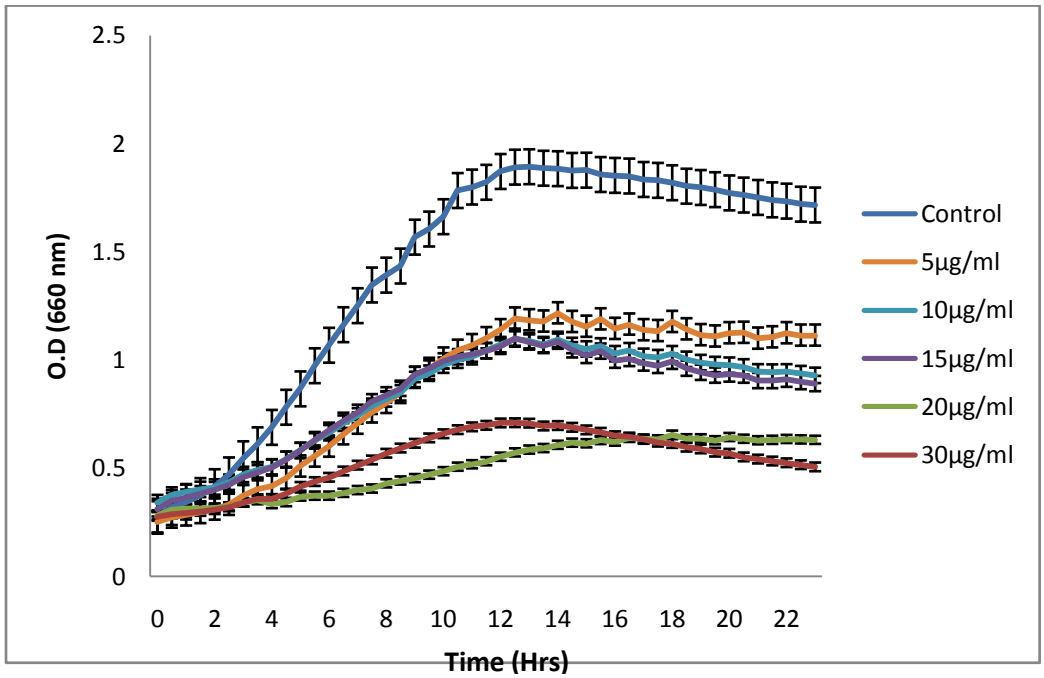


Fig. 4 Growth kinetic of *L.casei* with Biochanin A.

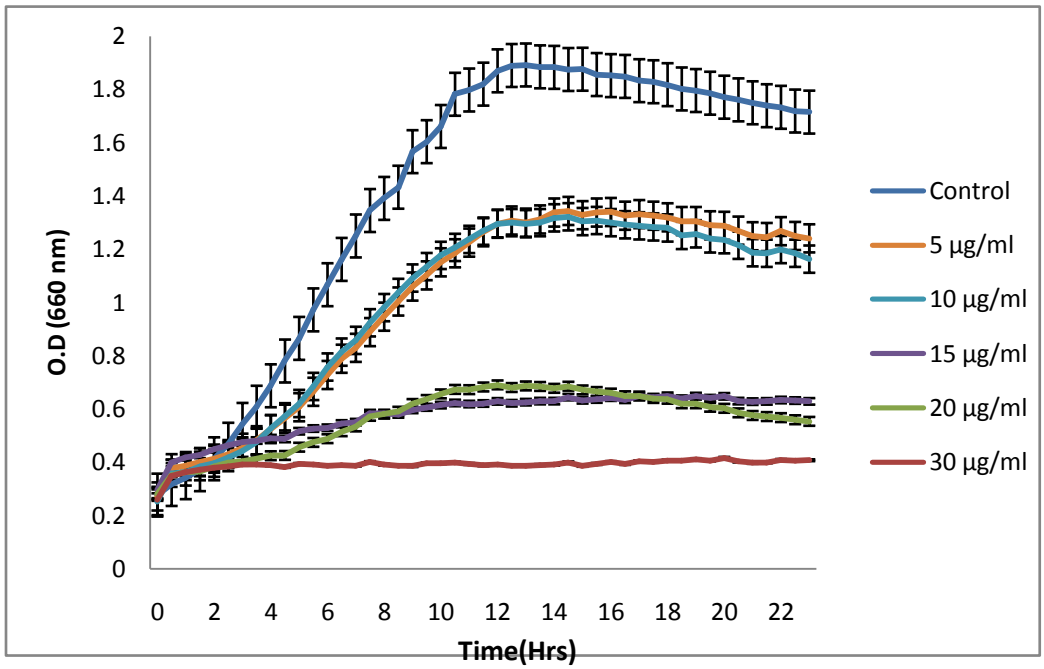
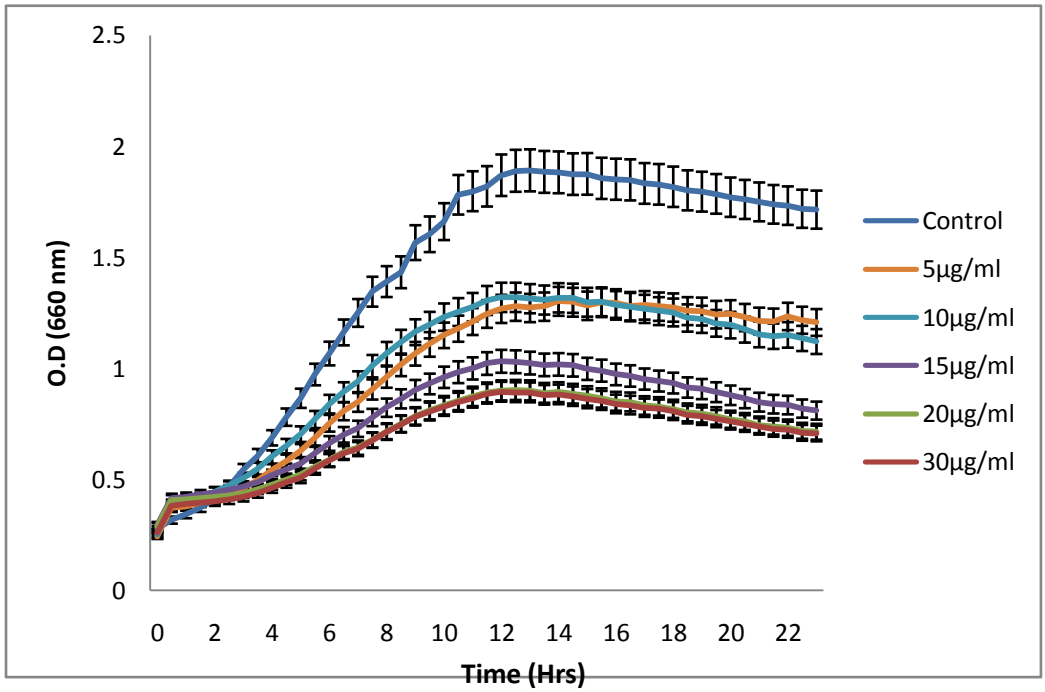


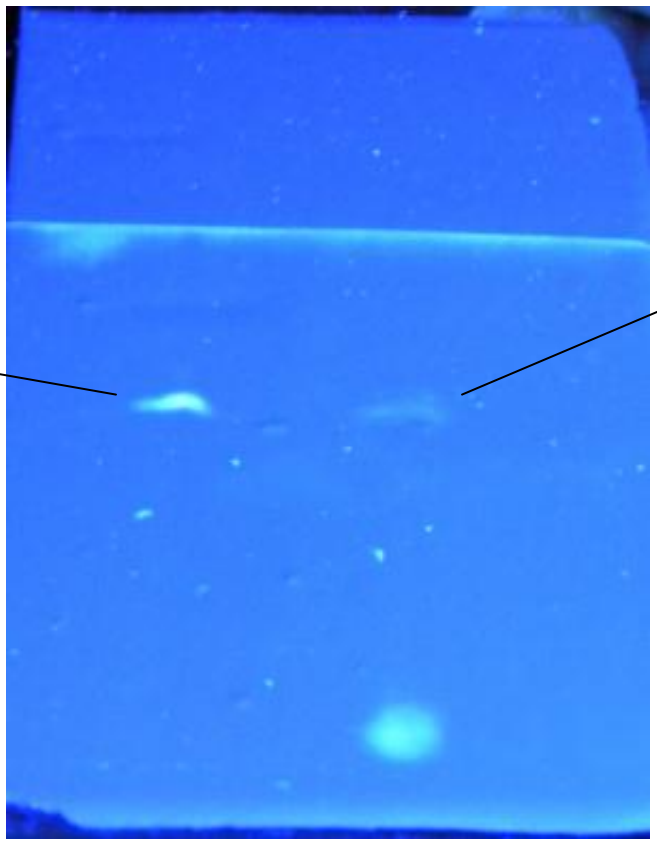
Fig. 5 Growth kinetic of *L.casei* with Coumestrol.



4.3 Fate of selected isoflavones on thin layer chromatography.

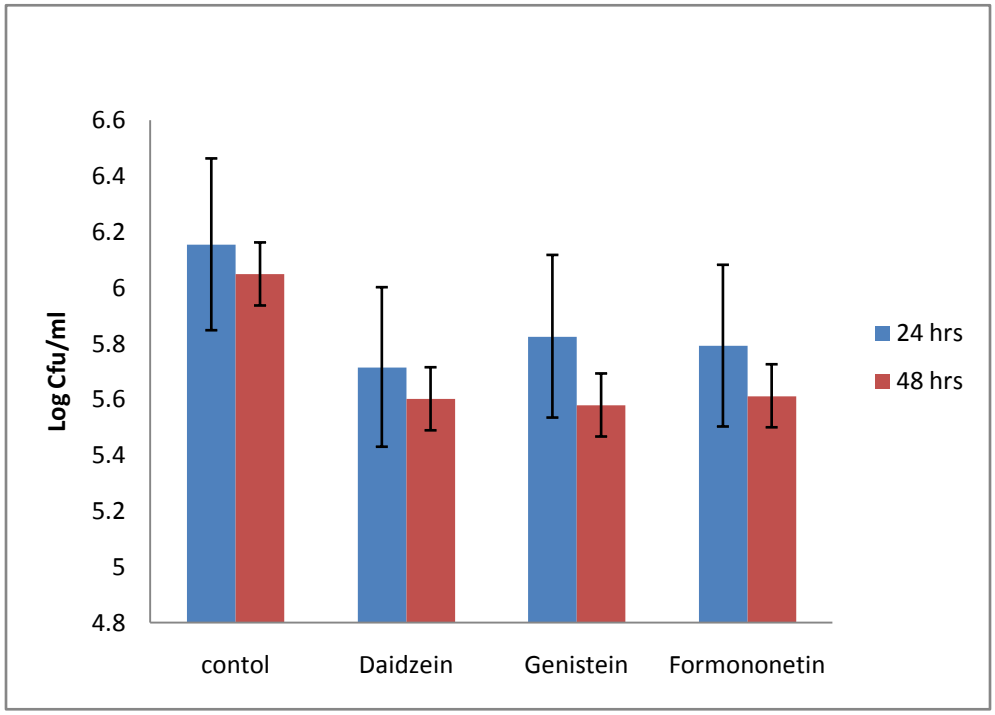
In order to ascertain the fate of isoflavones consumed by the *L.casei* on MRS media was analysed through thin layer chromatography as described by Beck (1964). The isoflavones degraded were extracted from *L.casei* on MRS media and compared with standard isoflavones on silica gel plates. The spot of degraded isoflavones under UV-VIS rays (260 nm). R.F value of Pure Daidzein 4.5; R.F value of degraded Daidzein by *L.casei* 4.4.

Standard daidzein
R.F= 4.4



Degraded Daidzein
R.F= 4.4

4.4 Utilization of *L.casei* with isoflavones in minimal media



Minimal media allowed the growth *L.casei* with glucose (control) and isoflavones (Daidzein, Genistein, fomononetin) during the time period of 24 and 48 hours. MRS media contain all the nutrients for growth where as basal minimal media contain minimum nutrients thus in basal minimal media isoflavones acted as carbon source for the growth of *L.casei*. Compared to Cerning *et al.* (1994) *L.casei* can grow using various carbon source including glucose in basal minimum media. A final viable population of the test organism ranging between 6.04 and 5.61 log CFU/ml was observed with glucose and isoflavones as carbon source. Specific growth ranged from 0.15 /min to 0.11 /min . These results indicated that *L.casei* utilized isoflavones as carbon source.

4.5 Detection of isoflavones through thin layer chromatography from Soya sample.

Figure 1. The isoflavones were extracted from soya sample and analysed by thin layer chromatography R.F value of Daidzein 0.45 and R.F value of Soya sample 0.45

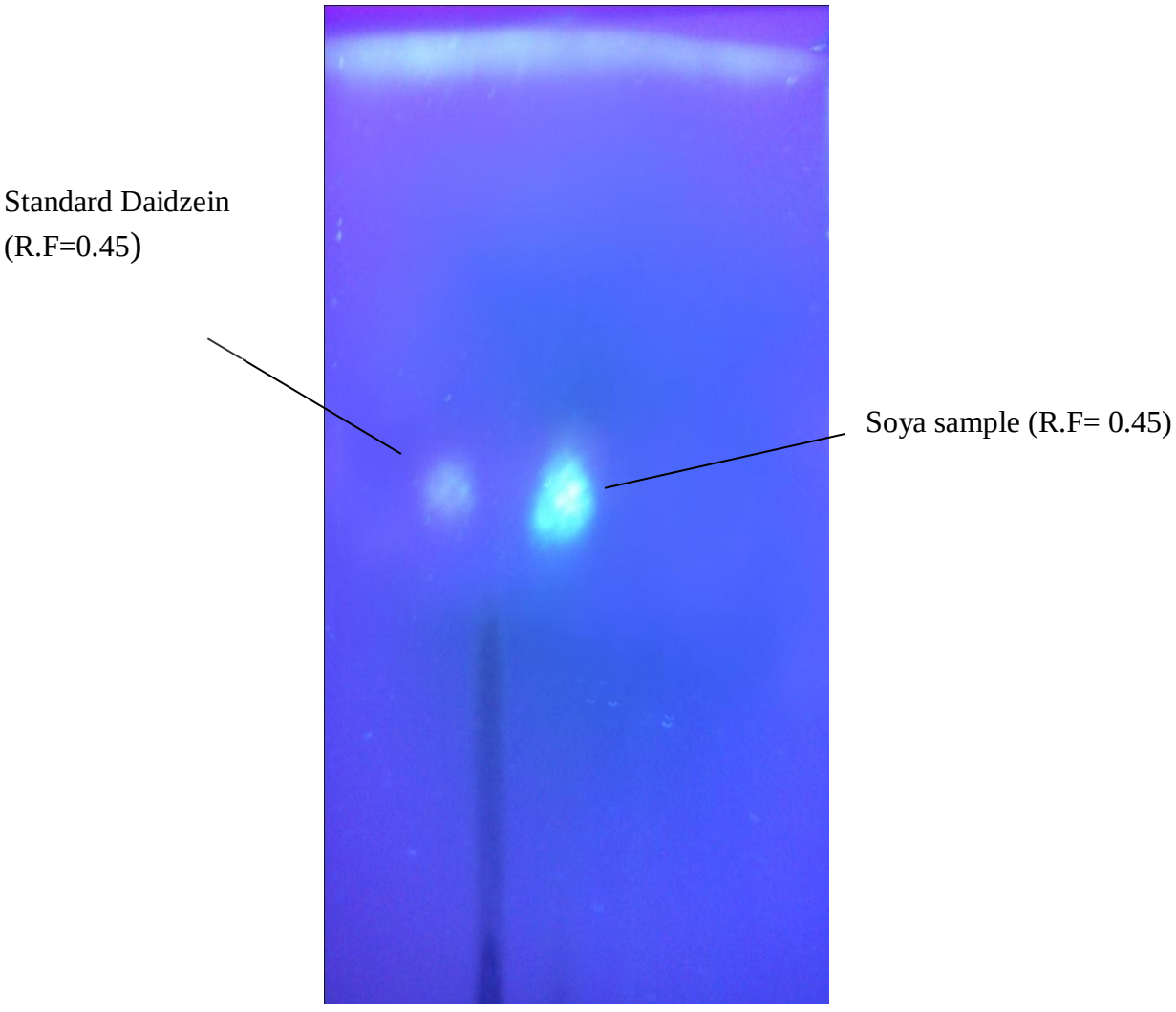
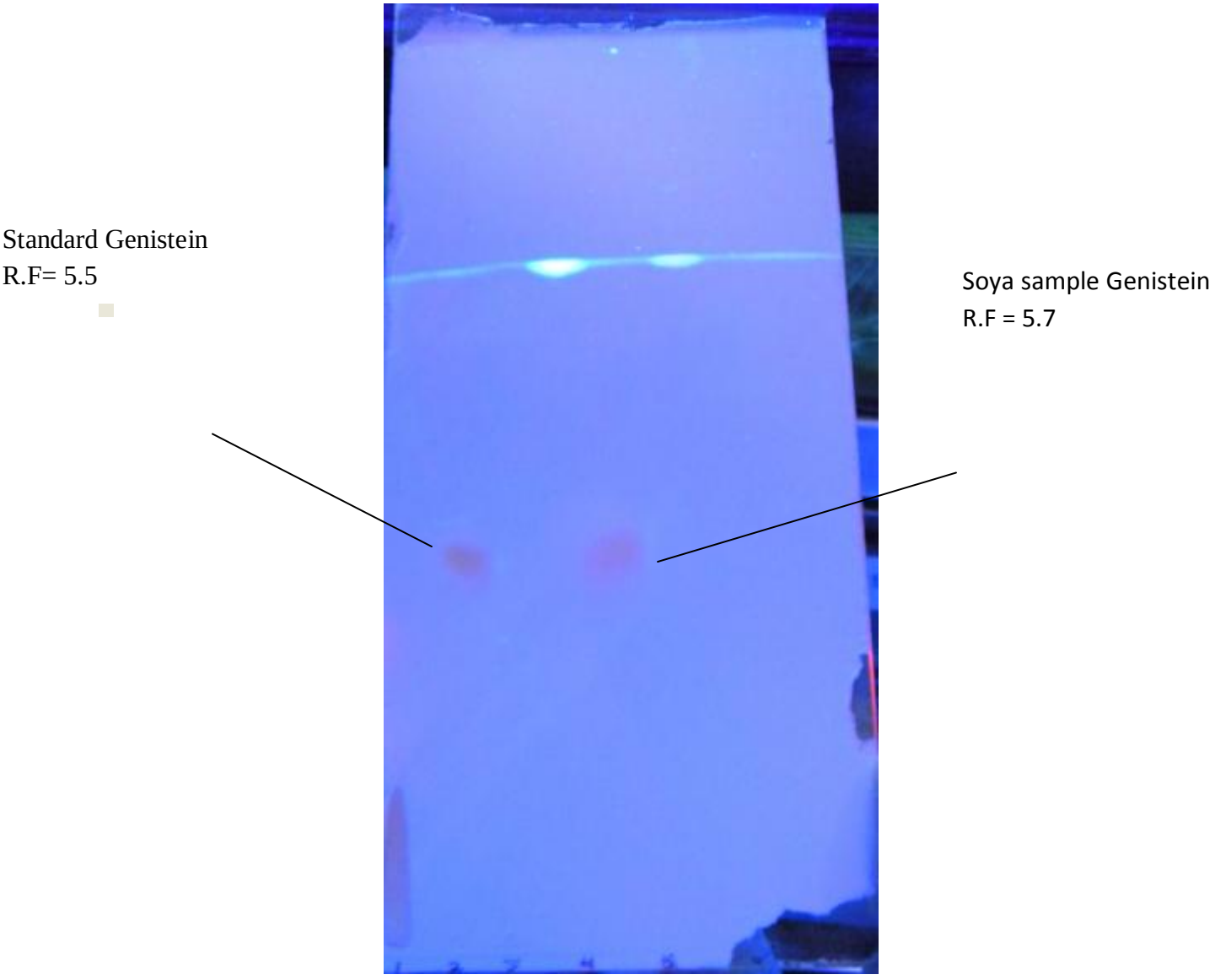


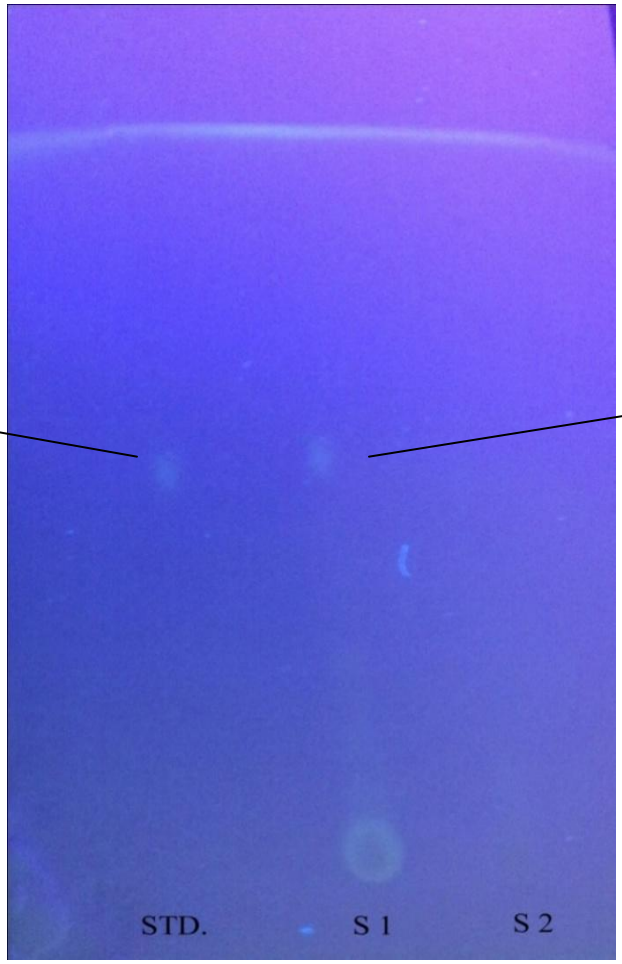
Figure 2. Thin layer chromatography Spot of Genistein from Soya sample .R.F value of Pure Genistein 5.5; R.F value of Soya sample 5.6.



4.6 Real time analysis of soya sample with *L.casei.*

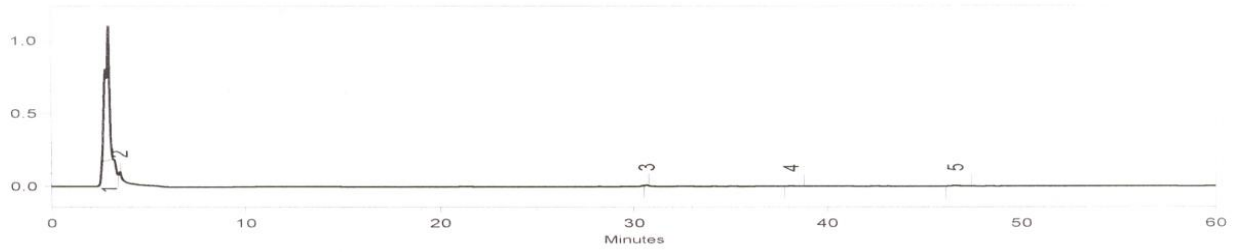
Soya sample was treated with *L.casei* dissolved in the PBS Solution and inoculated with overnight cultures of *L.casei*, the sample solution was incubated at 370c. Viable counts of *L.casei* , β glucosidase, β-galactosidase activities and residual daidzein was monitored hourly for upto 3 hours by TLC.

Fig 1.Shows the daidzein levels indicating complete utilization at the end of incubation period. Thin layer chromatographic analysis of soya sample inoculated with *L.casei*. Std: standard daidzen, R.F=0.44 , S1: soya sample in 0 hrs, R.F=0.45 and S2 soya sample after 3 hrs

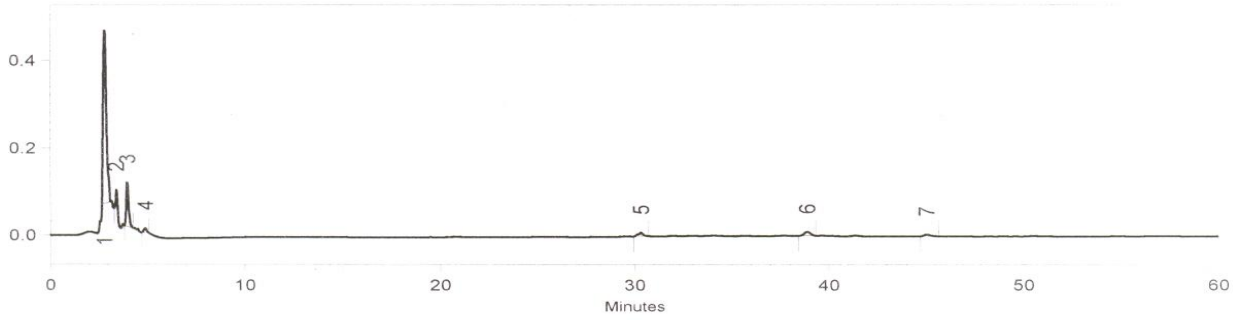


4.7 HPLC analysis of Daidzein and Soya extract .

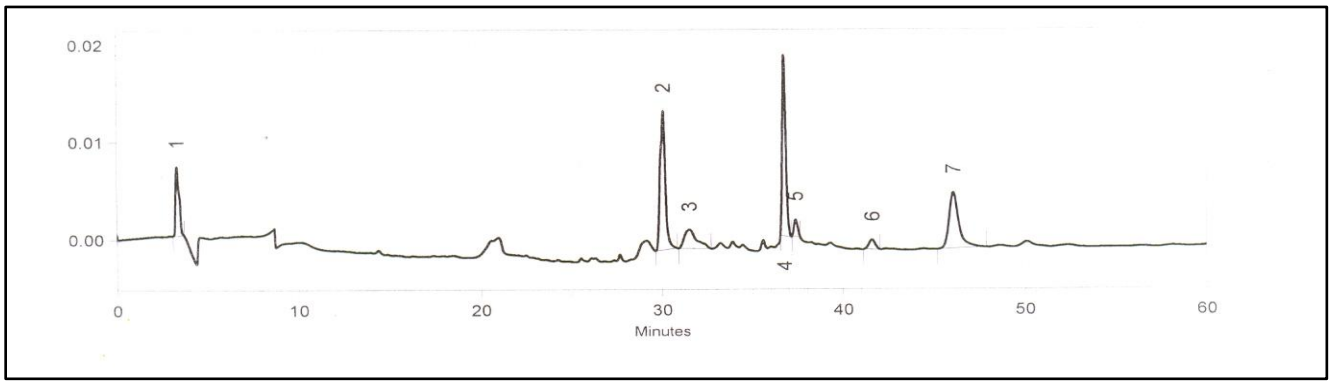
RP- HPLC analysis of standard Daidzein .



Quantitative analysis of Soya Sample through RP-HPLC analysis. The amount of Daidzein present in the soya sample is 15mg per gm of extract.

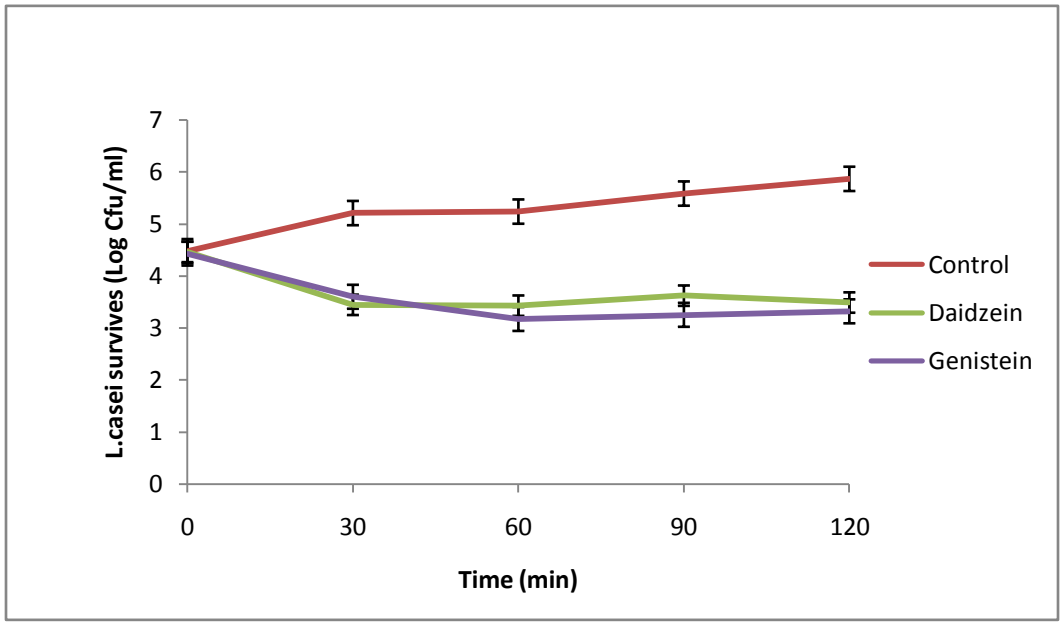


The RP- HPLC analysis of degraded Daidzein by *L.casei*. The amount degraded by *L.casei* was 99.23%.



4.8 Expression of probiotic characterstics.

Tolerance to artifical gastric juice.



To understand whether *L.casei* grown in Daidzein/Genistein retained the probiotic attributes. *L.casei* grown with isoflavones was subjected to artificial gastric juice tolerance. Small intestine and colons of humans and animals contain relatively high concentrations of bile acid, which can inhibit growth of many bacteria. Final viable population of the test organism ranged between 5.8 to 3.4 log CFU/ml was observed. A decrease in 2 log cfu/ml was observed(Fig 4.6). Casey *et al*. *(*2004) reported that *L.casei* also survived the gastric juice treatment upon growth in isoflavones.

4.9 Microbial Adhesion to Solvents (MATS):

MATS method was used to evaluate the hydrophobic/ hydrophilic cell surface properties of *L.casei* and compare them with the cell surface properties of *L.casei* grown with isoflavones . The hydrophobicity observed (Fig 4.9) was in the order- Control > Daidzein > Genistein > Formononetin . The percentage adhesion with Xylene was found to be 57% Control, Daidzein 45.6 % , Genistein 35.12 %, Formononetin 37.9 % respectively. The fact that a high percentage of *L.casei* cells adhered to xylene, a polar solvent, demonstrated hydrophobic cell surface of this strain. The results indicated that *L.casei* was less hydrophobic with some isoflavones.

4.10 Glutamate Decarboxylase (GAD) activity:

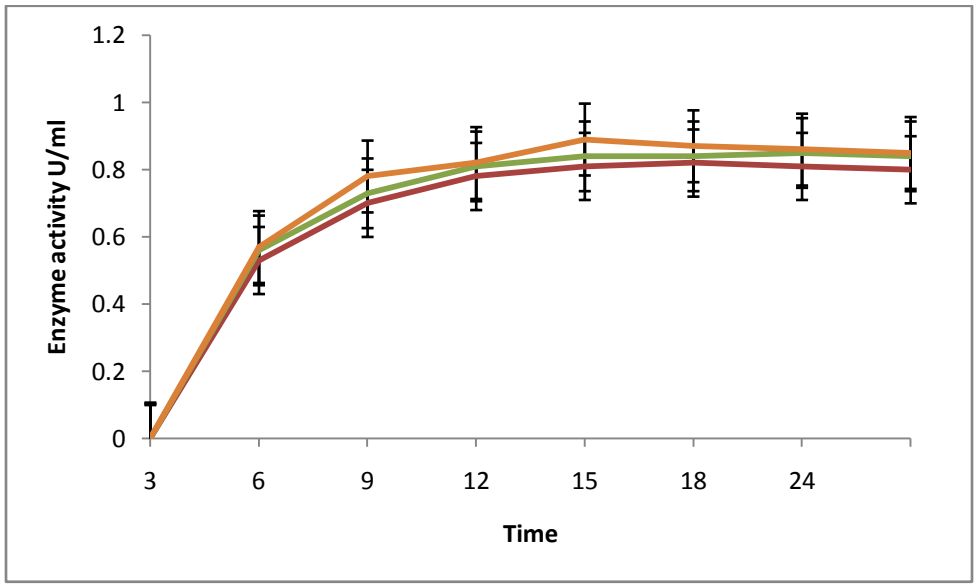
Among gram-positive bacteria, GAD acid resistance system is the only amino acid decarboxylation system that has been associated with acid response. The GAD system as an acid defense mechanism has been described for *L.lactis* among lactic acid bacteria, although GAD activity was also detected in *L.casei* with isoflavone- daidzein. A strong GAD activity by *L.* casei with isoflavones indicated the presence of this acid defense mechanism in this strain . The results agreed with those reported by Cotter *et al*., 2001 who showed that *L.lactis* retain GAD activity upon growth in isoflavones.

4.11 Antagonistic activity:

Fig 1. Shows the Antagonistic activity against *Salmonella typhimurium* by *L.casei*.

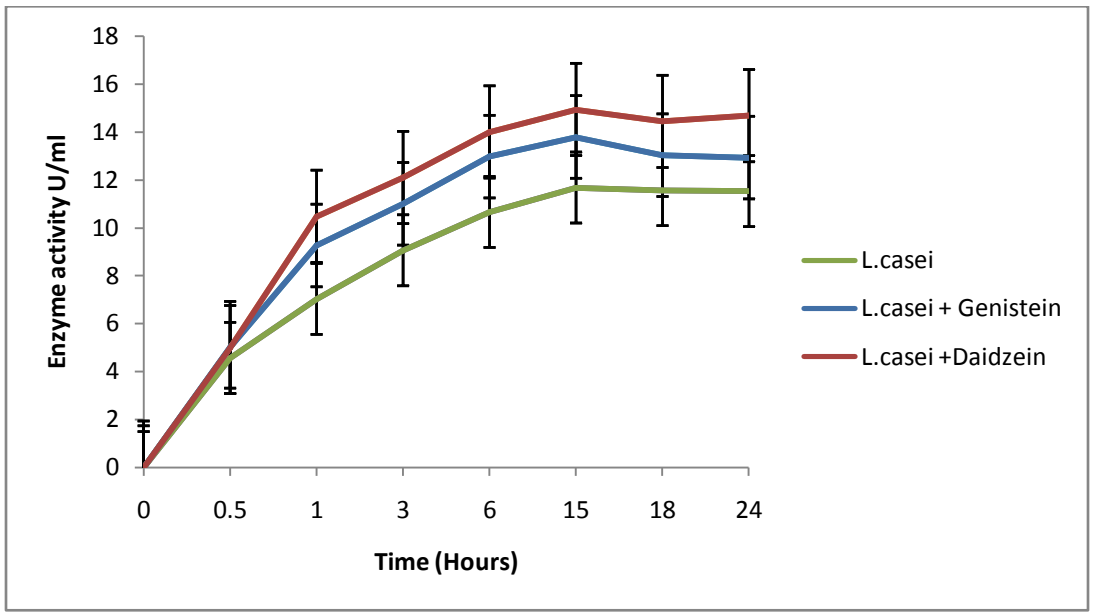
Inhibition zone was observed which may be attributed to the production of organic acid and bacteriocins (Lee *et al.* 2006) by *L.casei* . No significant difference in inhibition by cells in isoflavones were observed.antagonistic effects may lead to a competitive displacement of intestinal pathogens like *Salmonella typhimurium* thus beneficially affecting the host.Casey *et al*., 2004 showed similar results where antagonistic activity of *L.casei* remained unaffected following growth in isoflavones.

4.12 β-galactosidase activity of *L.casei* in the presence of isoflavones. Each experiment was performed in triplicate, Result was the average of three observations.



β-galactosidase enzyme is responsible for bioconversion of isoflavones glycosidase to isoflavones aglycone. The activity of β-galactosidase is 15 times lower than β-glucosidase. Compared with observation of Oteino *et al*. (2006) there was less variation in the activity of β-galactosidase enzyme. As shown, there was a significant increase in the enzyme activity after 6 h of fermentation for *L.casei*. Peak activity after 15 h .

4.13 β-glucosidase activity in *L.casei* in the presence of isoflavones.



Each experiment was performed in triplicate. Result was the average of three observation. β-glucosidase activity was for the period of 24 hours. β-glucosidases hydrolyse the predominant isoflavone glycosides into isoflavone aglycones. β-glucosidase activity was more than 15 times higher than β-galactosidase activity Otieno *et al*., (2006) . β-glucosidase is chief enzyme responsible for the bioconversion on isoflavones glycosidase to aglycones. Peak activities occurring after 15 h for *L.casei*. Compared to Oteino *et al*., 2006 there was less variation in the β-glucosidase activity. There was a significant increase in the enzyme activity after 1 h of fermentation for *L.casei* with isoflavones. The culminating peak activity after 15 h.

5. Conclusion

The study focused on interaction of isoflavones (daidzein and genistein) present in soyabean with *L.casei* (probiotic). Isoflavones are sufficiently consumed and degraded by *L.casei*. The *L.casei* tolerates the artificial gastric juice and bile secreation. β-glucosidase enzyme activity is increase in *L.casei* . Therefore the probiotic characteristic remain unaltered. The bioavailability of isoflavones may be increase by use of *L.casei* in the diet. The potential health benefits related to isoflavones like eases menopause symptoms, reduce heart disease risk, protect against prostate problems, improve bone health, reduce cancer may increase by using *L.casei* in diet containing isoflavones.

6. APPENDIX

7.1 Man Rogosa Sharpe (MRS) Broth

Component	Amount (g/l)
Proteose peptone	10
Beef Extract	10
Yeast Extract	5
Dextrose	20
Polysorbate 80 (Tween 80)	1
Ammonium citrate	2
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2
Sodium acetate	5

7.3 Artificial Gastric Juice (pH 1.85)

Component	Amount (g/l)
d- glucose	3.5
Sodium chloride	2.05
Potassium dihydrogen phospahte	0.6
Calcium chloride	0.11
Potassium chloride	0.37
Porcine bile	0.05
Lysozyme	0.1
Pepsin	13.3 mg

7.4 Phosphate Buffered Saline (PBS) {10 x, pH 6.8}

Component	Amount (g/l)
Sodium chloride	80
Potassium chloride	2
Disodium hydogen phosphate	14.4
Potassium dihydrogen phosphate	2.4

7.5 Ringer's solution (Full Strength)

Component	Amount (g/l)
Sodium chloride	9
Potassium chloride	0.42
Anhydrous Calcium chloride	0.24
Sodium bicarbonate	0.2

7.6 Minimal Media Composition

Component	Amount (g/l)
KH2PO4	454mg/l,
Na2HPO4.12H2O	1.19 g/l,
NaCl	0.2 g/l,
MgSO4.7H20	0.1g/l,
CaCl2.2H2O	0.002g/l,
MnSO4	0.004g/l,
FeSO4	0.005 g/ml ,
Peptone	0.002g/l .

Poster presented in ICFNP (JNU, New Delhi)

FB-O-23

REMOVAL OF HIGH LEVELS DAIDZEIN FROM HERBAL PREPARATIONS BY AN INDIGENOUSLY ISOLATED PROBIOTIC *LACTOBACILLUS CASEI*

Ajay K.Sandhu[1], Sutapa B. Neogi[2] and A.Ganguli[1]

[1]Department of Biotechnology and Environmental Sciences, Thapar University, Patiala-147004, Punjab, INDIA
[2]Indian Institute of Public Health & Hygiene, New Delhi

The objective of this study was to investigate the metabolic response of probiotic lactic acid bacteria towards isoflavones consumed through traditionally prepared herbal formulations. Several such spurious herbal formulations with claimed efficacy abound in the Indian market and are currently prescribed to rural population. The principal aglycone isoflavone characterized was daidzin at concentrations of 10-15µg/mg of formulation or tablet. The survival, growth of a probiotic strain *Lactobacillus casei* in MRS supplemented with low and high concentrations of daidzein and varying concentrations of herbal formulations(containing daidzein and simulated as per dosage) was evaluated over a period of 24 hours; the fate of daidzein in both the combinations were then analyzed by High-performance liquid chromatography. The *L.casei* strain exhibited excellent tolerance to a high range of daidzein (150µg/ml) and both survival and growth remained unaffected. Significant ($p<0.05$) quantities of Daidzein was utilized by the probiotic culture within 16 hours(28-370C) from MRS media. However rates of daidzein utilization was slower in conbinations containing herbal formulations, though approximately 91% utilization could be observed by 24 hours. The results of this study suggest the possibility of probiotic intervention through foods for possibly alleviating the toxic effects of high levels of phytoesterogens (foetus, brain and reproductive organs) administered through spurious herbal formulations. Key words: Phytoesterogens, probiotics, *L.casei*, herbal formulations.

7. References

1. Adlercreutz ., Mousavi Y., Clark J, Hockerstedt K, Hamalainen E, Wahala K, Makela T, Hase T (1992) Dietary phytoestrogens and cancer: in vitro and in vivo studies. J. Steroid Biochem. Mol. Biol.41: 331–337.
2. Adrian A. F. and Laurie J.C. (1996). Daidzein and genistein concentrations in human milk after soy consumption. Clinical Chemistry 42:6 55-964.
3. Beck A.B. (1964). The oestrogenic isoflavones of Subterranean clover. Aust. J. Agric. Res. 15:223.
4. Casey P.G., Casey G.D., Gardiner G.E., Tangney M., Stanson C., Ross R.P., Hill C. and Fitzgerald G.F. (2004) .Isolation and characterization of anti-*Salmonella* lactic acid 46 bacteria from the porcine gastrointestinal tract. Letters in Applied Microbiology 39, 431-438.
5. Casteele K.V., Geiger H. and Sumere C.F.V (1982). Separtion of flavonoids by reverse-phase high-performance liquid chromatography. J. chromatog. 240: 81.
6. Cerning J., Renard C. M. G. C., Thibualt J.F, Bouillanne C., Landon M.,Desmazeaud M.,and Topisirovic L.(1994) Carbon Source Requirements for Exopolysaccharide Production by *Lactobacillus casei* CG11 and Partial Structure Analysis of the Polymer.Applied and Environmental microbiology, P. 3914-3919 0099-2240/94/$04. Vol. 60, No. 11.
7. Coward L., Neil C. B., Kenneth D. R. S. and Barnes S. (1961) .Genistein, Daidzein, and Their & Glycoside Conjugates: Antitumor Isoflavones in Soybean Foods from American and Asian Diets. J. Agric. Food Chem. 1993, 41.

8. Donkor O.N. and Shah N.P. (2008). Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. J Food Sci.;73(1):M15-20.
9. Fontaine, M.N.B., Rault J. and vanoss C.J. (1996). Microbial adhesion to solvents:a novel method to determine the electron-donor/ electron-acceptor or Lewis acid-base properties of microbial cells. Colloids and Surfaces 7, 47–53.
10. Handley P.S.H., Wyatt D.W.S., Brown J.E., Doran C.R. and Gibbs A.C.C. (1987). A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrilar and fimbriate strains of *Streptococcus salivarius*. Journal of General Microbiology 133, 3207–3217.
11. Hutchins A.M., Slavin J.L., Lampe J.W. (1995) Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. J. Am. Diet Assoc. 95: 545–551.
12. Izumi T, Piskula M.K., Osawa S, Obata A, Tobe K, Saito M, Kataoka S, Kubota Y, Kikuchi M (2000) Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. J. Nutr. 130: 1695–1699.
13. Kayode R. M. O., Akinyosoye F. A. and Arotupin D. J. (2005). Comparative evaluation of the bacteria isolated from decomposing cow milk and soybean milk. Agric. Res. & Dev 2005 Vol 4 (2): 122-138.
14. King R.A., Broadbent J.L., Head R.J. (1996) Absorption and excretion of the soy isoflavone genistein in rats. J. Nutr. 126: 176–182

15. Kumar M., Ghosh M., Ganguli A. (2010).Mitogenic response and probiotic characteristics of lactic acid bacteria isolated from indigenously pickled vegetables and fermented beverages. World J Microbiol Biotechnology 28:703–711.
16. Kurzer M.S., Xu X (1997) Dietary phytoestrogen. Annu. Rev. Nutr.17: 353–381.
17. Lee, S.H. and M.J. No (1997). Viability in artificial gastric and bile juice and antimicrobial activity of some lactic acid bacteria isolated from Kimchi. Korean Journal of Applied Microbiology and Biotechnology. 28, 279-284.
18. Markham K.R. (1982). Detection of flavonoid Indentification by Reverse-phase HPLC. Academic press Ink, Newyork .
19. Matsuda S, Norimoto F, Matsumoto Y, Ohba R, Teramoto Y,Ohta N, Ueda S (1994)
20. Matsuura M, Obata A (1993) β-Glucosidases from soybeans hydrolyze daidzin and genistin. J. Food Sci. 58: 144–147.
21. Matsuura M, Sasaki J, Murao S (1995) Studies on β-glucosidases from soybeans that hydrolyze daidzin and genistin: isolation and characterization of an isozyme. Biosci. Biotech. Biochem. 59:1623–1627.
22. Naim M., Gestetner B., Zilkah S., Birk Y. and Bondi A. (1974). Soybean Isoflavones Characterization, Determination, and Antifungal Activity. J. Agr. Food Chem.. Vol. 22, No. 5, 1974.
23. Otieno D.O. and Shah N.P. (2007). Endogenous β-glucosidase and β-galactosidase activities from selected probiotic micro-organisms and their role in isoflavone biotransformation in soymilk. Journal of Applied Microbiology ISSN 1364-5072 .
24. Pandjaitan N., Hettiarachchy N. and Ju Z., Y. (2000a).Enrichment of genistein in soy protein concentrate with beat β-glucosidase. Journal Food Science 65, 403-407.

25. Pandjaitan N., Hettiarachchy N., Ju Z. Y., Crandall P., Seneller C. and Ombek, D. (2000b). Enrichment of Genistein in soy protein concentrate with hydrocolloids and β-glucosidase. Journal Food Science 65, 591-595.
26. Pauline S.H., Derek W.S.H, Janet E.W., Christopher J.(1997) . A Comparison of the Adhesion, Coaggregation and Cell-surface Hydrophobicity Properties of Fibrillar and Fimbriate Strains of *Streptococcus salivarius* .Journal of General Microbiology, 133, 3207-321 **7.**
27. Rojas M., Ascencio F., Patricia A. and Conway L. (2002).Purification and Characterization of a Surface Protein from *Lactobacillus fermentum* 104R That Binds to Porcine Small Intestinal Mucus and Gastric Mucin. Applied and Environmental Microbiology 68, 2330–2336.
28. Rosenberg M., Gutnick D. and Rosenberg E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters 9, 29–33.
29. Scalabrini P., Rossi M., Spettoli P. and Matteuzi, D. (1998) Characterisation of *Bifidobacterium* stains for use in soymilk fermentation. J Food Microbiol 39, 213–219
30. Solubilization of a novel isoflavone glucoside-hydrolyzing β-glucosidase from *Lactobacillus casei* subsp. rhamnosus. J. Ferment. Bioeng. 77: 439–441.
31. Wev L. and Shah N.P. (1995). Survival of *Lactobacillus acidophilus* and *Bifidobacterium spp*. in the presence of acid and bile salts. Cultured Dairy Products J. 30: 2-7.Chou L. and Weimer B. (1998). Isolation and Characterization of Acid- and Bile Tolerant Isolates from Strains of *Lactobacillus acidophilus*. J Dairy Sci 82:23–31

32. Wong E. (1962). Detection and estrimation of oestrogenic constituents in red clover .J. Sci. Food Agric.,vol. 13.