FUNCTIONAL ATTRIBUTES OF SOYMILK PROTEIN HYDROLYSATE

Megha Bhatnagar (123808)

Under supervision of Dr. Gunjan Goel

May-2017

Submitted in partial fulfillment of the degree of

Masters of Technology (Biotechnology)

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

CONTENT

CERTIFICATE

This is to certify that the project entitled "FUNCTIONAL ATTRIBUTES OF SOYMILK PROTEIN HYDROLYSATE" which is being submitted by **Megha Bhatnagar** in partial fulfillment for the award of degree of M.Tech in Biotechnology from Jaypee University of Information Technology is the record of candidate's own work carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any degree or diploma.

Dr. Gunjan Goel

Associate Professor

ACKNOWLEDGEMENTS

I owe a great thanks to many people who have been helping and supporting us during this project. My deepest thanks to Dr. Gunjan Goel for guiding and correcting us at every step of our work with attention and care. He has taken pain to go through the project and make necessary correction as and when needed. I would also thank Prof R.S Chauhan, Head of the department of Biotechnology for his support and guidance.

I would also like to thank Mr. Sampan Attri , Kavita Sharma and Deepak Sharma (Ph.D Scholars) of the Department of Biotechnology and Bioinformatics at Jaypee University of Information and Technology Waknaghat, Solan. They helped me throughout the project and instructed well to use the laboratory equipments, without their involvement it won't be easy to achieve results in my work.

Thanks and appreciation to the helpful people at college, for their support. I would also thank our Institution and my faculty members without whom this project would have been a distant reality. Finally I must express my heartfelt thanks to my family for providing me with support and continuous encouragement throughout my period of study and through the process of research. This accomplishment would not have been possible without them.

Megha Bhatnagar

ABSTRACT

Viability and metabolic activities are important characteristics of probiotic lactic microorganisms. They give rise to therapeutic benefits as well as increase physiological activity of cultured products through liberation of a number of bioactive peptide. Soy milk, an aqueous abstract of soybean, and its fermented product contain great biological properties and are found to be a good source of bioactive molecules such as peptides which can be utilized as a health enhancing ingredient in functional foods. A collection of nine strains of indigenous Lactic acid bacteria isolated from different fermented foods were evaluated for their impact of bioactive activities in fermented soymilk. . A 100 ml of soymilk was fermented by 1% of different lactic cultures and was incubated overnight for fermentation at 37ºC. Different lactic culures resulted in different degree of hydrolysis of soy protein ranging from 7.17 to 11.09% as estimated by o-phthaldialdehyde (OPA) method. Further the hydrolysate fraction as evaluated for its antioxidative and ACE inhibitory activities. The protein fraction from soymilk fermented by *L.rhamnosus GG, Brevibacilllus aydinogluensis, Brevibacillus thermoruber* showed the higher radical scavenging ranging from 67.09% to 62.57 % respectively activity as estimated by 2, 2'-azino- bis (3- ethylbenzothiazoline -6- sulphonic acid) or ABTS method. Strains *Lactobacillus paracasei* (CD4) and *Brevibacillus thermoruber* (HM34) gave maximum ACE inhibitory activity compared to other isolates.

CHAPTER 1

Introduction

"Soya" is a legume, known as *Glycine max*. For the past million years Soy has been grown in Asia and, recently, it is efficiently cultivated across the world. In the present days, the top world's manufacturers of soy are the United States, Brazil, Argentina, China and India. Soy milk is a plant based drink produced by manner of soaking dried soybeans and grinding them in water. Soy is one of the few plant which offers a entire protein as it incorporates all 8 amino acids which is critical for human health. Soy based liquids, known as soy milk is the aqueous extracts of complete soybeans and are a source of very good rate protein and balanced nutrients.

Soy-primarily based drinks are a legitimate alternative to cow milk, milk and dairy product are taken into consideration a balanced meals as they contain good source of vital amino acids, vitamins and calcium; but, their intake should be prevented in case of hypersensitive reaction to cow milk proteins or extreme intolerance to lactose. The consumer acceptability of soy-based totally liquids is affected by their bad taste although there may be an increased interest amongst clients. Soy-based totally beverages new formulations with different ingredients were sold in the marketplace and recently new technologies have allowed the development in their sensory characteristics, and their consumption is increasing worldwide , particular among the vegetarians.

Whole soybeans are tremendous source of protein and nutritional fibre. Soy protein is the simplest vegetable with a whole protein. Soy protein has lately attracted a number of interest due to its capability to lower LDL. Results from research have induced fitness professionals to request the authorities to officially provide a stamp of popularity of soy's ldl cholesterol-decreasing results. The Food and Drug Administration authorised the cholesterol-decreasing health claim for soy, indicating that every day intake of 25 grams of soy protein can help in lowering LDL cholesterol.

Functional soy milk may be taken into consideration as soymilk that includes bioactive additives and can also help to beautify fitness or decrease the danger of diseases. Soybean is a good source of phenolic compounds with antioxidant residences and has an excessive amount of isoflavones, a collection of phytoestrogens that have been suggested to probable decrease the risk of hormonal diseases and any type of age-associated illnesses.

It is proved that proteins from soymilk can yield bioactive peptides having opioid activity, antihypertensive, immunostimulating, mineral binding ability, cytomodulatory, antimicrobial and antioxidative activities in the human body. Bioactive peptides are the proteins which are synthesized inside the cell as huge prepropeptides, which are then cut and can be changed into dynamic items. They are generally 3 to 20 amino acids encoded inside the essential structure of a dietary protein. Several proteins create physiologically dynamic peptides in the process of gastrointestinal digestion and food material fermentation with the assistance of lactic acid bacteria.

Dietary proteins release bioactive peptides by either gastrointestinal digestion or by processing of the foods. According to bioactive peptides functional properties, they may be classified as antimicrobial, antithrombotic, antihypertensive, opioid, immunemodulatory, and anti oxidative. These peptides have huge importance in altering human health. The bioactive peptides created by soybeans comprise of unique and interesting medical advantages. Like in the inhibition of age-related endless issue, like, cardiovascular infection, cancer, obesity, and decrease in immune function. These peptides can likewise get absorbed by the gastrointestinal framework, in this way applying their activity on particular target organs.

Upon utilization, peptides with intense physiological activities might be liberated from Soymilk protein by the activity of proteolytic compounds in the gut and hence impact the real body's frameworks including endocrine, nervous, stomach related, cardiovascular and immune frameworks. Various health advantages of soymilk protein inferred bioactive peptides have been guaranteed for commercial interests in the environment of health sustaining-functional foods defined bioactive peptides as substances that can influence the natural procedures of the body capacities with useful impacts.

Lactobacillus strains are the most imperative starter cultures utilized as a part of traditional fermented soymilk producing. Their application predominantly originates from two critical properties: fast utilization of milk sugar prompting quick fermentation of soymilk as development medium, and exceedingly developed proteolytic framework equipped for providing basic amino acids required by a quickly growing organism. Various little and oligo-peptides with

various physiological capacities has been discharged from milk proteins through microbial proteolysis and has been very much perceived and evaluated.

In recent years it has been identified that dietary proteins supply a rich source of biologically active peptides. A variety of naturally formed bioactive peptides have been found in fermented dairy products, such as yoghurt, sour milk , cheese and many others. In particular, antihypertensive peptides have been identified from fermented soymilk. Bioactive peptides have the prospects to be used in the nutraceuticals, or as potent drugs with well pharmacological effects.

Based on above studies, the present study was designed with following objectives

a. Preparation and characterization of soy milk protein hydrolysate

b. Functional activities of soymilk protein hydrolysate

CHAPTER 2

Literature Review

2.1 Soybean:

Soybeans, is one of the most plentiful plant resources of dietary protein, which consists 36% to 56% degree of protein. They contain 35% carbohydrates, including soluble di- and oligosaccharides, 18% to 22% fats, including polyunsaturated fatty acids, phytosterols, phospholipids, minerals, B nutrients, and fiber in it. Recent studies shows that soy milk is an aqueous summary of soybean, and its fermented product comprise awesome biological houses and are recognized for the coolest supply of bioactive peptides which may be applied as a fitness improving component in practical ingredients.

Soybean,is an essential source of food proteins and has acquired increasing interest from public due to exceptionally healthy benefits. It includes the additives like isoflavones, saponins, proteins, and peptides. Soymilk also includes soy proteins which include higher quality of amino acid stability in comparison to animal proteins.

The amount of isoflavones in soy products varies with the kind of soybean we're using, the situation and vicinity wherein it is cultivated, and the way it is processed. Soy food, particularly soymilk, can be taken into consideration as an awesome substitution for all dairy product for people who have milk intolerance. Milk intolerance together with cow's milk protein allergy (CMPA), cow's milk protein intolerance (CMPI), and lactose intolerance is located on the high price inside the world, mainly determined more in children. CMPA can affect from 2 to 6% of children. Lunasin, Bowman-Birk inhibitor, lectin and 3-conglycinin are a number of the biologically active peptides and protein which might be present in soybean.

It is a excellent supply of nutritional peptides, having many activities like antihypertensive, anti cholesterol, and antioxidant, it additionally help in stopping most cancers. The processing of soy protein into peptides takes place in the GI tract which help in growing their wholesome results by exposing there active companies inside the amino acid chain. These may be powerful in many approaches like they help inside the prevention of age-associated continual disorders, together with cardiovascular disease, most cancers, obesity, and decreased immune function. Despite the ability anti-nutritive effects, a huge quantity of studies papers have suggested that soy meals have lot of health advantages within the area of ailment prevention. Soy foods help to prevent

cancer in a just like the mechanism of blood vessel protection. Formation of latest blood vessels is the crucial step for the growth of human cancers and facilitation of tumor mobile metastases. Soy isoflavone act as a chief protective additives, as it can without problems repair the structure of blood vessels and inhibit cell transformation and tumor cell proliferation.

Soybean is a totally beneficial supply of inhibitor of the angiotensin-converting enzyme. Many peptides produced via the hydrolysis of meals proteins indicates inhibitory interest against the enzyme which reduces the blood strain after there oral management. Daily utilization of these food containing such peptides might be effective in preserving blood pressure at the normal level.

2.2 Benefits of Soy Milk

- Soymilk is an outstanding supplier of protein and dietary fiber.
- It includes very little amount of saturated fats and do not contain cholesterol as well as lactose content.
- It is likewise one of the properly source of iron
- Soy protein may additionally enables in lowering the danger of positive cancers, which include breast most cancers, prostate in addition to lung most cancers.
- It consists of isoflavones which cope with many fitness troubles, helps in the the prevention of many type of cancers, coronary heart disorder, osteoporosis, antioxidant and so on.
- It doesn’t include galactose, so that it can easily replace breast milk in children with galactosemia.
- Who've lactose intolerance, or any form of milk allergy it is suitable for them.
- It additionally enables in combating the menopause symptoms.
- It additionally indicates anti-diabetic and anti-obesity property.

2.3 Soy Protein Characteristics

Soy proteins include two predominant fractions, glycinin and β-conglycinin, accounting for 40% and 30% of general protein, respectively. Generally soymilk includes approximately 2% fats, specifically in the shape of triglycerides, with a fatty acid composition of poly-and mono-unsaturated hydrocarbon chains. The texture and sensory fine of soy products such as soymilk and tofu get affected by the presence and concentration of unsaturated fats. 60% of overall lipids are located within the protein debris in the soymilk.

Soy proteins are the principle constituent in soymilk and play a major position in offering its processing functionality. Soy protein based totally elements are generally hired due to their emulsification activity and balance, fat absorption, hydration ability, colloidal stability, acid and heat gelation, adhesion/cohesion, thickening and foaming skills. For this reason, maximum of the fundamental studies on structure–function of soy proteins is completed on protein isolates.

Glycinin, often known as 11S, is the unmarried biggest fraction of soy protein, and it usually money owed for over 40% of general seed protein. The isomers which make up β-conglycinin are a mixture of β'and α ,α subunits, that are found in six specific mixtures. The heat balance of β-conglycinin is lower than that of glycinin, because of the dearth of disulfide bonds stabilizing the quaternary structure.

2.4 Fermentation Impact on soybean protein

Due to the presence of high amount of enzyme inhibitors inclusive of proteinase and trypsin inhibitors Soybean proteins is not fantastically digestible. Fermentation process is an green or ecofriendly method, which helps soybean protein to be easily digested. The proteolytic enzymes can be released during fermentation, via the microbial populations, which hydrolyzes the proteins into peptides and unfastened amino acids. Soybean fermented with proteolytic traces of *Bacillus subtilis* was found to boom free amino acid content10-20 folds. Efficiency of fermentation with respect to its functionality of the hydrolyzed product differs with specific cultures that is used for fermentation. It was found that there is a difference in volume of soybean protein hydrolysis which was fermented with unique lines of the equal species. It has also been

suggested that profile of critical amino acids remained unchanged after fermentation, rather fermentation accelerated the amount of smaller peptides (<20 kD), which have been now not obtained from the unfermented soybean. Composition of protein in soybean may also affect the production of bioactive peptides. Glycinin is the precursor of 95% of the peptides fashioned via soybean protein hydrolysis. the alternative important protein in soybean is B-conglycinin, which is located to be proof against proteolytic degradation even by means of the multi-enzyme systems. Soymilk fermented with particular lactic acid micro organism outcomes in peptides having particular fitness benefits.

Bacillus sp, results in alkaline fermentation of Soybean, whereas soymilk fermented by lactic acid bacteria results in acidic fermentation. Soymilk fermented with *Enterococcus faecium* lines have proven to possess antioxidant and Angiotensin I converting enzyme (ACE) inhibitory residences (Martinez-Villaluenga et al., 2012).

2.5 Features of Lactic acid bacteria

Lactic microorganisms have been utilized to mature or culture nourishments from atleast last 4000 years. They are utilized as a part of specific in fermented milk items from everywhere throughout the world. Lactic acid bacteria are accordingly superb representatives for a regularly microbial world. . They are not only of major economic significance, as well as of significant worth in keeping up and advancing human wellbeing.

The lactic acid bacteria are characterized as Gram –positive cocci or rods having low-GC content. These are common metabolic and physiological characteristics shows that they are acid-tolerant in nature and are generally non-spore forming bacteria. Presence and absence of oxygen don't affect there growth. Spoiling plants and lactic products producing lactic acid as the major metabolic end product due to carbohydrate fermentation can be the source of LAB. Additional hurdle for spoilage and pathogenic microorganisms are Several strains of LAB producing proteinaceous, bacteriocins.

The drop in pH that happens as lactic acid is delivered by the microscopic organisms (bacteria) which is valuable in the preservation of nourishment in food. The brought down in pH restrains

the development of numerous other decay microorganisms which can cause spoilage of food . Plentiful development of the lactic acid bacteria, so the production of lactic acid, is similarly hindered by the low pH . The low pH condition drags out the time span of usability of food (e.g., pickles, yogurt, cheese) from contamination by bacteria that are normal in the kitchen or microorganisms that can develop at refrigeration temperatures. The drop in the oxygen level amid lactic acid fermentation is likewise an inhibitory component for potential food pathogens. Lactic acid bacteria create antibacterial compounds called bacteriocins. Bacteriocins act by punching openings through the layer that encompasses the bacteria. In this manner, bacteriocins activity is generally lethal to the bacteria.

Organoleptic and textural profile of any food item is due to lactic acid and other metabolic activity products. Lactic acid bacteria is more recognized by their generally recognized as Safe(GRAS) status due to their ubiquitous presence in fermented foods, their contribution to the intestinal microflora of human mucosal surfaces,and also in there importance in the industry.

2.6 Definition

A bioactive dietary substance is a food component that can affect biological processes or substrates and, hence, have give a good impact on body function or condition and ultimately health.

Bioactive peptides have been defined as specific protein fragments which gives a positive impact on body function or conditions and may result in ultimately influencing the health of person. Major body systems gets affected by the oral administration of bioactive peptides. The beneficial health effects may be classified into antimicrobial, anti oxidative, antithrombotic, and antihypertensive and many others.

2.6.1 Mechanisms for the production of bioactive peptides

Bioactive peptides derived from milk products, are usually composed of two- twenty amino acids which become active only after they get released from the precursor protein where they are being encrypted. These are the specific mechanisms which can be used to launch the encrypted bioactive peptides from the precursor proteins:

(a) enzymatic hydrolysis with the aid of the assist of digestive enzymes

(b) processing of food and

(c) proteolysis via enzymes derived from microorganisms or by the plant.

Fitz Gerald et al in 2004, reported that usually using gastrointestinal enzymes pepsin and trypsin many of the known bioactive peptides is produced. For example, Angiotensin-converting enzyme (ACE)-inhibitory peptides and calcium-binding phosphopeptides (CPPs), are most commonly produced by using trypsin.

Kilara & Panyam in 2003 reported that Other digestive enzymes and different enzyme combinations of proteinase which include alcalase, chymotrypsin, pancreatin, pepsin and thermolysin . Enzymes from different bacterial and fungal sources have also been used to generate bioactive peptides from various proteins.

Tsai et al in 2006 studied that when the Soymilk is fermented with the help of five different lactic acid bacteria (*Lactobacillus casei, Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus bulgaricus, Bifidobacterium longum*) and a protease, prozyme 6 it resulted in increase in soluble protein content which range freom 47 to 390 mg/g, free amino acid content increased from 91.3 to 214.4 mg/g, and increase in peptide content from 39.6 to 463.3 mg/g and inhibition of angiotensin I-converting enzyme (ACE) IC50 decreased from 9.28 to 0.66 mg powder/ml, respectively.

Surajit et al in 2012 reported that when Soymilk is fermented with probiotic lactobacilli (*Lactobacillus rhamnosus strain, L. casei*). *L. rhamnosus C6* strain showed maximum antioxidative activity and proteolytic activity as well as polyphenol content get reduced.

Sheng-Yao Wang et al, 2006 reported that the Oral administration of milk and soy milk kefirs to mice inoculated with sarcoma 180 tumor cells resulted in 64.8% and 70.9% inhibition of tumor growth, respectively, compared with control.

Table 1: Different Active peptide fragments which are generated from soybean storage protein

Source: a) Chen et al. (1995); b) Kawamura (1997); c) Tanaka et al. (1994); and d) Yoshikawa et al. (1993)

	Peptide fragments	Protein source
Antioxidant activities	VNPHQN	β-conglycinin
	LVNPHDHQN	β-conglycinin
	LLPHH	β-conglycinin
	LLPHHADADY	β-conglycinin
	VIPAGYP	β-conglycinin
	LQSGDALRVPSGTTYY	β-conglycinin
Inhibition of angiotensin-converting enzyme	FVIPAGY	α,,ά (β-conglycinin subunit)
	ASUDTLI	α,,ά (β-conglycinin subunit)
	DQTPRVI	A5A4B3 (glycinin subunit)
	YRILEF	ά (β-conglycinin subunit)
Promoting action of phagocytosis	MITLAIPVNKPGR	ά (β-conglycinin subunit)
	HCQRPR	A1aB1b (glycinin subunit)
	QRPR	A1aB1b (glycinin subunit)

Milk products which are fermented have evidently excessive nutritional value, and as an additional advantage many fitness-selling effects, consisting of development of metabolism of lactose, decrease in serum cholesterol and decrease in risk of cancer chance. The change of the protein existing inside the soybean to soy protein isolates is particularly incompetent and highly-priced, and as a result greater direct usage of soybeans in forms like soymilk or fermented product such as tofu, provide effective means of adding soy protein in the diet of the humans. The beneficial health outcomes associated with a few fermented dairy products may additionally, in part, be attributed to the discharge of bioactive peptide sequences at some stage in the fermentation method. Number of different peptides and peptide fractions, having bioactive homes have been remoted from fermented dairy product.

2.7 Benefits of Soymilk to Human Health:

Soymilk has been suggested as an opportunity to update cow's milk which contains lactose. Soymilk is an opportunity as soymilk it is wealthy in protein and does no longer contain lactose. Additionally, soymilk incorporates calcium, zinc, magnesium, iron and essential amino acids that is an extraordinary dietary fee for human food regimen (Sacks et al., 2006). The cholesterol-

lowering effect of soymilk has been recognized by way of most research. Past studies said that amino acid of proteins in soymilk contributes to hypocholesterolaemic effects. Past research have proven that these isoflavonoids in soymilk reduced the risks of cardiovascular illnesses because of their capacity to lessen cholesterol. Due to their estrogen mimetic residences, soybean phytoestrogens were found able to get rid of signs and symptoms of menopause (Murkies et al., 1995).

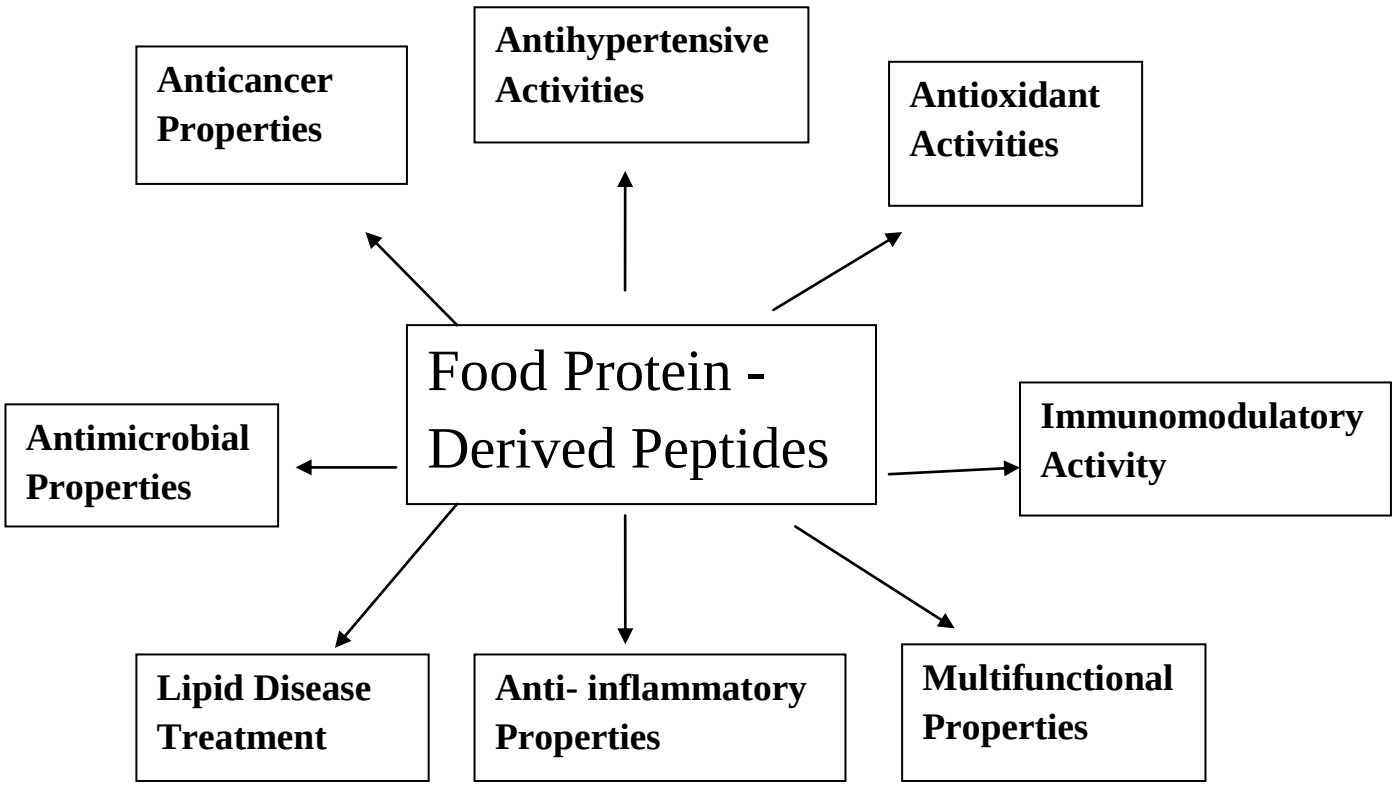

FIG1: Food protein-derived peptides Bioactive properties related to the prevention of disease and human health

2.8 Future perspectives

Consumers are getting more and greater interested in wholesome meals. Soy beans and soy product have gained substantial attention for their capacity function in improving fitness hazards consisting of threat elements for cardiovascular disease. These advantages are attributed to soy

protein and soy isoflavones. Soybeans are most people source of isoflavones in human food. Besides soy proteins and isoflavones, soybeans are very flexible and a wealthy source of crucial nutrients, i.e. Polyunsaturated fats, fiber, vitamins and minerals.

Providing customers with statistics about the health advantages associated with soy elements even might also enhance the perceived sensory great of soy products. As a appropriate opportunity for probiotic dairy products, Soy and experimental cancer in animal research. Soy-based totally ingredients that include probiotic lines can be extra considered. Soy beverages and yogurts might be the subsequent meals category for which the healthful bacteria will make their mark.

Fermented products of dairy and different food item having bioactive molecules might have the ability of providing huge number of health advantages to purchasers. That is why there is a essential need to carry out further fundamental research to make it clear, why those peptides have physiological consequences, Business product are actually getting commercially used having bioactive peptides in it. Food and pharmaceutical corporations are actively running for grabbing good advantage of bioactive molecules in nutrients of human and in health promotion.

Molecules which are bioactive have the ability for use inside the making of practical food items, cosmetics and as powerful capsules having well defined pharmacological effects. With the upward push of the issue raised by consumer about the harmful results of chemical preservatives and the increasing desire for herbal and safe components, bioactive substances derived from milk may also have excessive cost in food maintenance and nutraceuticals.

Appropriate molecular research is needed to look over the mechanisms via which these special molecules exert their activities. In the end this research may be supportive in understanding, avoiding and treating lifestyle associated illnesses which include cardiovascular disease, cancers, osteoporosis, stress and obesity.

CHAPTER 3

Materials and Methods

3.1 Chemicals

Sodium dodecyl sulphate(SDS), Di-sodium tetradecahydrate, O-phthalaldehyde, Serine, Dithiothriol, Sodium bicarbonate, Sodium Potassium tartarate, Copper sulphate, Folin & Ciocalteu's phenol reagent, Bovine serum albumin, ABTS, ethanol, dH_2O, acrylamide, Tris, TEMED, APS.

3.2 Procurement of Milk & Cultures

Amul skimmed milk was obtained from market. The pure lactic cultures were obtained from the lab, JUIT.

a) *Lactobacillus rhamnosus* (from NDRI karrnal)

b) *Brevibacilllus aydinogluensis* (BTM9)

c) *Lactobacillus paracasei* (CD4)

d) *Lactobacillus gastricus* (BTM7)

e) *Brevibacillus thermoruber* (CD13)

f) *Brevibacillus thermoruber* (HM29)

g) *Brevibacillus thermoruber* (HM34)

h) *Weissella confuse* (CD1)

i) *Lactobacillus* sp. 78

3.3 Maintenance of lactic culture

9 lactic cultures were maintained by inoculating each culture individually in MRS broth (de Man, Rogosa and Sharpe**)** and were incubated at 37^{o}C for 24 hrs.

3.4 Propagation of cultures

An aliquot of inoculums of different lactic cultures was added to 20ml of sterilized skimmed milk and was incubated for 24hrs at 37˚C in shaking incubator.

After incubation the tubes were checked for setting of the curd.

3.5 Preparation of soymilk

- 50g of Soybeans were taken and soaked in the clean water overnight.
- Washing and Dehusking (removal of the cover of the beans) were done.
- Water was added 10 times the weight of the soybeans and grounded into the paste.
- Slurry was sieved through the muslin cloth and soymilk was further analysed.

Flowchart

50g of soybean

↓

Soaked in clean water overnight

↓

Washing and dehusking was done

↓

Rinse in water (10 times the weight of soybean) and ground into paste

↓

Slurry sieved through muslin cloth

↓

Boil the soy milk and analyzed.

3.6 Preparation of fermented soymilk

A 100 ml of soymilk milk was added in flask and sterilized 121 ˚C.

The flask were inoculated with 9 different lactic cultures and were incubated for 24hrs at 37 ˚C. Fermentation was observed next day.

3.7 Processing of fermented soymilk

Fermented soymilk were taken and centrifugation was done at 7000rpm for 15 min.

The supernatant of the samples was taken and was freeze dried or lyophilized to obtain powder form.

3. 8 ANALYSIS

3.8.1 pH : pH of fermented soymilk was estimated with the help of pH meter.

3.8.2 Moisture Content Estimation: About 20 ml of soymilk sample weighed in an empty clean and dry petri-dish and then the sample was dried in a oven with temperature of 100ºC for 24 hours. The sample were cooled in a desiccator and weight measurement were done in the petridish .

Moisture content (%) = (M1−M) – (M2−M)/ (M1−M) × 100

Where:

M = Empty moisture dish weight

M1 = Moisture dish and sample weight

M2 = Dessicated sample weight

(M1 – M) – (M2 – M) = Loss of weight

(M1 – M) = Weight of the sample

3.8.3 Ash Content Estimation: 2 ml of milk sample was taken in empty clean and dried crucible and was kept in a muffle furnace at 500°C - 600°C for atleast 3 hours. After the stipulated time the crucible was removed from the muffle furnace and was left for cooling in a desiccator, After cooling crucible was weighed.

Ash content (%) = {A2−A}/ {A1−A} × 100

A = Empty crucible (weight in gm)

A1 = Sample and crucible (weight in gm)

A2 = Ash and crucible (weight in gm)

3.8.4 Estimation of Protein content (lowry's assay):

Standard – BSA (1 mg/ml)

0.2, 0.4, 0.6, 0.8 and 1ml of working standard were made.

Dilutions of was done for the sample.

5ml of lowry reagent was measured and added into the sample and vortexed. The samples were kept at 37°C for about 15 min. 0.5 ml of folin phenol reagent (1N) was added to each test tube and was incubated for 30 min in dark and absorbance was taken at 750 nm.

3.8.5 Fat Content Measurement:

Using UV Spectrophotometric method the fat content of the Soymilk was measured. For that 30 μL soymilk sample was added to the test tube containing 3 mL of ethanol(absolute) and is placed at −20°C. All vials were stored for atleast 1 hour at −20°C.

For the separation, centrifugation was done at 13,000 rpm for 20 min. Sample absorbance is taken at 208 nm.

3.9 Estimation of Proteolytic activity:

Proteolytic activity of cultures used in production of product which was assessed by measuring liberated amino acids and peptides using o-phthaldialdehyde (OPA) method.

The OPA reagent preparation:

For reagent preparation 7.620g of di- Na tetraborate decahydrate and 200 mg Na-dodecyl-sulfate were weighed and were solubilized in 150 mL water (deionized). Thereafter 160 mg of o-phthaldialdehyde 97% was separately weighed and dissolved in a test tube containing 4 ml of ethanol.

In the above solution 176mg of dithiothreitol (DTT) 99% was weight and added.

The volume of the reagent was raised up to 200 ml with the addition of deionized water.

Procedure

Standard: Serine 1 mg/ml

Serine standard was made using dilutions 10, 20, 50, 100µL. 3ml of OPA reagents was added to the test tube and was mixed thoroughly and was kept for 2 min. Optical density was measured at 340 nm using the spectrophotometer.

Sample measuring: 0.05 gm of lyophilized protein samples was solubilized in 1 ml of distilled water. Following dilutions of samples were made (10, 20, 50, 100 µL). 3ml of OPA reagents was added and vortexed. The solution was slowed to kept for exactly 2 min before being read at 340nm in the spectrophotometer.

Serine-NH = OD of sample – OD of blank / OD of standard – OD of blank * 0.9516 meqv/L *0.004 * 100/ Y * P

where serine-NH2 = meqv serine NH2/g protein; Y = g sample used; P = protein % in sample; 0.004 is the sample volume in liter (L), h tot for soy is given as 7.80 .

Degree of hydrolysis = h / h tot * 100 %

3.10 Estimation of protein content in lyophilized sample:

Reagent preparation: Lowry A – 2% sodium carbonate in 0.1 N sodium hydroxide.

Lowry B – 1% copper sulphate in distilled water (100ml)

Lowry C- 2% sodium potassium tartarate

Lowry solution- 49 ml A + 0.5 ml B + 0.5 ml C

Folin solution (2N) to Distilled water (1:1)

BSA standard was prepared (1mg/ml). dilutions was made 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard was pipetted into a series of test tube. Preparation of lyophilized sample was done by taking 50mg of sample was measured and was mixed in 1ml of distilled water. 5 ml of lowry solution was measured and mixed in above sample, vortexed and kept at 37ºC for 15 mins. Add 0.5 ml of folin phenol reagent (1N) and incubate it for 30 min in dark to measure absorbance at 750 nm.

Sample measuring: 50 mg of lyophilized protein samples in 1 ml of distilled water, and dilutions were made.Add 5 ml of lowry solution and was vortexed Then the solution was kept at 37ºC for about 15 min. 0.5 ml of folin phenol regent (1N) was added and left for incubation it for 30min in dark and absorbance was taken at 750nm.

3.11 Estimation of Anti-oxidative activity:

ABTS method

The scavenging activity was estimated according to procedure Pellegrino et al. (1993).

ABTS (7mM in water) was prepared by mixing an stock solution with potassium per sulphate (2.45mM) in an equal amount of quantities and left to stand for 12-16 hours at room temperature in dark until reaching a stable oxidative state. 80% ethanol was used to dilute the ABTS solution to set the optical density of 0.80 ± 0.05 at 734 nm. 100µL sample was pipetted in the test tube, 2.9 ml of ABTS solution was measured and mixed with above sample, The solution was kept at room temperature for 30 min in dark condition.

The absorbance was determined at 734 nm.

Scavenging effect (%) = (1 - (Absorbance of sample / Absorbance of control)) x 100

3.12 Determination of Angiotensin Converting Enzyme inhibitory (ACE) Activity:

According to method of Zhang et al. (2009) the ACE activity was determined.

To 50 µL of a sample solution (lyophilized sample dissolved in PBS) equal amount of 1 U of ACE solution were added. 50 µL of a 5 mmol/L substrate (HHL) solution in 1 mol/L phosphate buffer at pH 8.3 was mixed to the above solution containing sample and the enzyme. It was incubated at 37 °C for 30 min, 150 µL of 1 mol/L HCl was added to stop the reaction. Hippuric acid was extracted with the help of 1 mL of ethyl acetate. Centrifuge the above solution and transfer 0.5 mL of the organic phase to a fresh ependorff, Then evaporate the solution in a water bath at 100°C. 3 mL deionised water was added to the residue containing hippuric acid and it was measured using a UV visible spectrophotometer at 228 nm against blank which is deionised water.

Inhibition Equation: ACE inhibitory activity (%) = [(X-Y)/(X-Z)] *100

X is the absorbance measured with the enzyme and substrate without the inhibitory sample,

Y is the absorbance measured with the enzyme, substrate and inhibitory sample and Z is the absorbance measured with the substrate without enzyme and inhibitory sample.

The ACE inhibitory activity was also expressed as IC50, which was expressed as the amount of sample needed to inhibit 50% of the original ACE.

3.13 SDS PAGE:

Tris Hcl (1 M, pH 6.8) : 7.88 g in 50 ml distilled water

Running buffer (10 X ,pH 8.3) : 30 g Tris base, 144 g glycine, 10 g SDS

Acrylamide (30%): 30 g acrylamide and 0.8 g in 100 ml distilled water

Staining solution: 200 ml methanol, coomassie blue 0.5 g, 250 ml water, 50 ml glacial acetic acid.

Destaining solution: 250 ml water, 200ml methanol, 50 ml glacial acetic acid.

Sample buffer: 1.25 ml of 0.125 M Tris Hcl (6.8), 4ml of 4% SDS, 2ml of 20% Glycerol, 475 µl Beta mecaptoethanol added freshly.

12% gel (Resolving)

Distilled Water	3.3 ml
30% acrylamide	4.0 ml
Tris (pH 8.8, 1 M)	2.5 ml
10% SDS	0.1 ml
10%APS	0.1 ml
TEMED	0.04 ml

4% gel (Stacking)

Distilled Water	3.4 ml
30% acrylamide	0.8 ml
Tris (pH 6.8, 1M)	0.63 ml
10% SDS	0.05 ml
10%APS	0.05 ml
TEMED	0.005 ml

PROTOCOL:

1. Polyacrylamide gel was prepared according to standard protocol.

2. Sample was loaded and gel was run at 25 mA in 1x SDS Running Buffer.

3. Gel was removed, and staining solution was added and was kept overnight.

4. Destain solution was added and, kept on rocker for 1 hour.

5. Bands were observed.

CHAPTER 4

Results and Discussion

4.1 Preparation of Soymilk:

Soymilk was successfully prepared. 50 g of soybean was soaked overnight overnight, next day dehusking was done and 10 times weight of soybean water was added to ground soybean into paste. Slurry was sieved through muslin cloth and the aqueous solution was obtained known as Soymilk. It was further autoclaved and used for further studies.

Soaking of soybean

Dehusking

Sieving

Soymilk

Soymilk is a plant milk produced by soaking dried soybeans and grinding them in water. 50g of soybeans was taken which yields to 500ml of soymilk. It was further analyzed with various different methods like estimation of moisture content, ash content, pH, fat and protein content.

Analysis of soymilk was done to check the quality of the milk. pH of processed soymilk is generally reported to be 7.30 which is neutral in nature. The pH of the soymilk made in the lab was measured with the calibrated digital pH meter and was resulted to be 6.65 which is near to the neutral condition. Moisture content or water content is the quantity of water contained in the material. Soymilk contains a good amount of moisture content in it. The moisture content was directly measured using volume of the material and a drying oven. 20ml of soymilk was weighed and kept for 24hr in the oven to calculate moisture content which results as 93.74%

Ash content is the mineral content present in the sample, which is measured in muffle furnace at 600ºC. The average ash percentage in soymilk sample was calculated as 0.30%

Protein and fat content in soymilk was calculated as 2.6% and 3% respectively.

FIG 2: Soymilk

4.2 ANALYSIS OF SOYMILK

1) pH	**6.65**
2) MOISTURE CONTENT	**93.74%**
3) ASH CONTENT	**0.30%**
4) PROTEIN CONTENT	**2.6%**
5) FAT CONTENT	**3%**

Propagation of cultures:

After inoculation of lactic cultures were maintained at 37ºC for 24hrs in the sterilized skimmed milk the tubes and were checked for curdling. Further these different cultures were transferred to different flask of soymilk. Fermented soymilk was then centrifuged and supernatant was freeze dried or lyophilized.

FIG 3: Curdling of Soymilk

4.3 Protein content of soy milk hydrolysate :

Protein content of soymilk fermented by different lactic cultures are shown in the table. There is decreased in protein content when the sample gets fermented by different LAB. BSA standard curve is shown in the figure 4 which is used for determining the concentration of protein in each sample.

Table 2 : Protein content of soymilk fermented by different LAB

LAB Strains	Protein content (mg/ml)
Lactobacillus paracasei (CD4)	5.168 mg/ml
Lactobacillus gastricus (BTM7)	4.549 mg/ml
Brevibacilllus aydinogluensis (BTM9)	4.822 mg/ml
Brevibacillus thermoruber (CD13)	4.568 mg/ml
Brevibacillus thermoruber (HM29)	5.120 mg/ml
Brevibacillus thermoruber (HM34)	4.422 mg/ml
Weissella confuse (CD1)	6.415 mg/ml
L.rhamnosus GG (LRGG)	4.447 mg/ml
Lactobacillus sp. 78	4.384 mg/ml
soymilk	7.320 mg/ml

As from the results obtained, it is clear that ,Soymilk contain high protein content 7.320 mg/ml and soymilk fermented by *Weissella confuse* (CD1) and *Lactobacillus paracasei*(CD4) yield maximum protein content ranging from 5.168 mg/ml to 6.415 mg/ml.

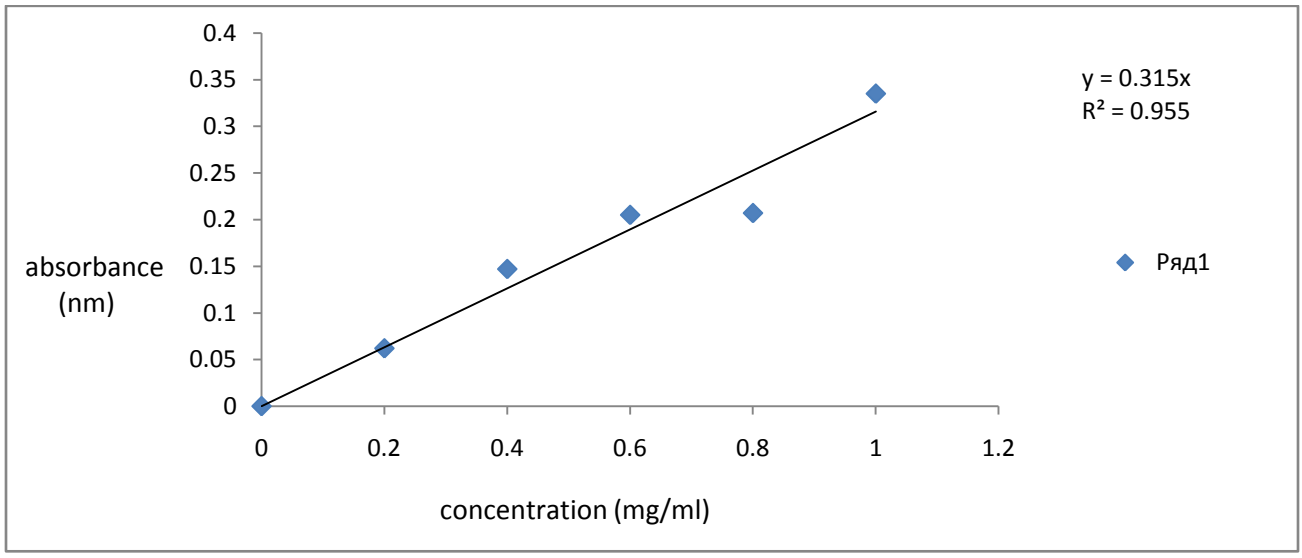

FIG 4: Standard curve of BSA

4.4 Degree of Hydrolysis of soy milk hydrolysate

Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. OPA is based on the measurement of amino groups generated from hydrolysis. It is the amino acid and pepties produced due to the breakdown of soymilk protein by LAB.

Table : Degree of Hydrolysis of soy milk hydrolysate

LAB Strains	**Degree of hydrolysis (%)**
Lactobacillus paracasei (CD4)	7.17
Lactobacillus gastricus (BTM7)	9.90
Brevibacilllus aydinogluensis (BTM9)	9.60
Brevibacillus thermoruber (CD13)	8.88
Brevibacillus thermoruber (HM29)	8.82

Brevibacillus thermoruber (HM34)	9.55
Weissella confuse (CD1)	7.16
L.rhamnosus GG (LRGG)	10.83
Lactobacillus sp. 78	11.09

As from the results obtained, it is clear that all the lactic cultures used in this study have limited protein hydrolytic activity which was obtained in the range of 7.17 to 11.09%. This might be due to lower peptidase acitivities in these lactic strains. Additionally the degree of hydrolysis is strain specific as the isolate 78 isolated from the fermented product bhatroo possessed the maximum degree of hydrolysis whereas all the other isolates were from the different dairy sources.

4.5 ACE Inhibitory activity:

Proteolytic strains of Lactobacillus have been used to produce bioactive peptides including Antihypertensive peptides in soymilk. In this study nine strains were cultured individually to produce fermented soymilks. The result indicated that the strains *Lactobacillus paracasei* (CD4) and *Brevibacillus thermoruber* (HM34) gave maximum ACE inhibitory activity compared to other isolates. Reports from Kitts and Weiler 2003, stated that soybean fermented by Protease from *Bacillus subtilis* yield Antihypertensive IC50 = 26.5 whereas Kimura and others 2000, reported *Bacillus natto* or *B. subtilis*, for the fermentation of soybean which yeilds several ACE inhibitory peptides, such as Val-Ala-His-Ile-Asn-Val-Gly-Lys or Tyr-Val-Trp-Lys, were isolated. However, these studies had used Bacillus species for proteolytic acitivities.

As from the results obtained, it is clear that all the lactic cultures used in this study have limited ACE activity which was obtained in the range of 6.90 to 41.66%. Additionally the ACE activity is produced from the *Lactobacillus paracasei* (CD4) and *Brevibacillus thermoruber* (HM34) isolated from the fermented dairy sources, while others do not posses such activities. It may be due to different LAB activities.

Table 4: ACE inhibitory activity of soymilk hydrolysate

LAB Strains	Inhibitory activity %
Lactobacillus paracasei (CD4)	41.66
Brevibacillus thermoruber (HM34)	6.90
Lactobacillus gastricus (BTM7)	-
Brevibacilllus aydinogluensis (BTM9)	-
Brevibacillus thermoruber (CD13)	-
Weissella confuse (CD1)	-
L.rhamnosus GG (LRGG)	-
Lactobacillus sp. 78	-
Brevibacillus thermoruber (HM29)	-

4.6 Anti-oxidative activity:

For the survival of human's cells, Oxidative metabolism is very important. This activity may lead to of free radicals generation which causes oxidative changes in the body. Free radicals are responsible for many disorders like diabetes, arthritis, atherosclerosis. By Inhibiting free radicals generation many serious diseases of the body can be prevented.

ABTS can be used to measure antioxidative property. It is also commenly used method by the food industry and agricultural. In this method, ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in color and absorbs light at 734 nm. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically at 734 nm.

The scavenging effect was measured by:

Antioxidant activity (%) = (1 - (Absorbance of the sample / Absorbance of the control)) x 100

Amino acids, like Tyr, Met, His, Lys, and Trp, are usually accepted as a good source of antioxidants. It is reported that if at the C-terminus there are Tripeptides having Trp or Tyr residues it will have strong radical scavenging Activities. Chen and others 1998, studied that During the process of hydrolysis, the structure of soyprotein will be changed which will lead to exposure of more active amino acid R group. Therefore, soybean peptides will show higher amount of antioxidant activity than that of intact protein. It is reported by Liu JR, Chen MJ, Lin CW 2005, that Native and heated soy protein isolate when hydrolyzed using different types of enzymes resulted in different range of degrees of hydrolysis 1.7%–20.6% and also resulted in high antioxidant activity which is ranging from 28% to 65%.

As from the results obtained, it is clear that all the lactic cultures used in this study yeild good antioxidative activities which was obtained in the range of 67.09 to 62.57%. This might be due differences in these lactic strains. High Antioxidant activity were seen in all the isolates from the different dairy sources. *L.rhamnosus GG* (LRGG) and *Brevibacilllus aydinogluensis* (BTM9) range the maximum activity.

Table 5: Anti oxidative Activities of soy milk hydrolysate

LAB Strains	***Antioxidant activity***
Lactobacillus paracasei (CD4)	60.83%
Lactobacillus gastricus (BTM7)	46.69%
Brevibacilllus aydinogluensis (BTM9)	65.15%
Brevibacillus thermoruber (CD13)	62.57%
Brevibacillus thermoruber (HM29)	61.64%
Brevibacillus thermoruber (HM34)	40.20%
Weissella confuse (CD1)	58.16%

L.rhamnosus GG (LRGG)	67.09%
Lactobacillus sp. 78	55.85%
soymilk	18.42%

4.7 SDS PAGE :

SDS PAGE size (sodium dodecyl sulfate polyacrylamide gel electrophoresis) technique is used to separate proteins according to their size. Distinct peptide bands which one obtain, helps to have a qualitative measure of the peptide profile. For the hydrolysates, this technique is beneficial as it can also be used to check whether intact protein present or not in the sample.

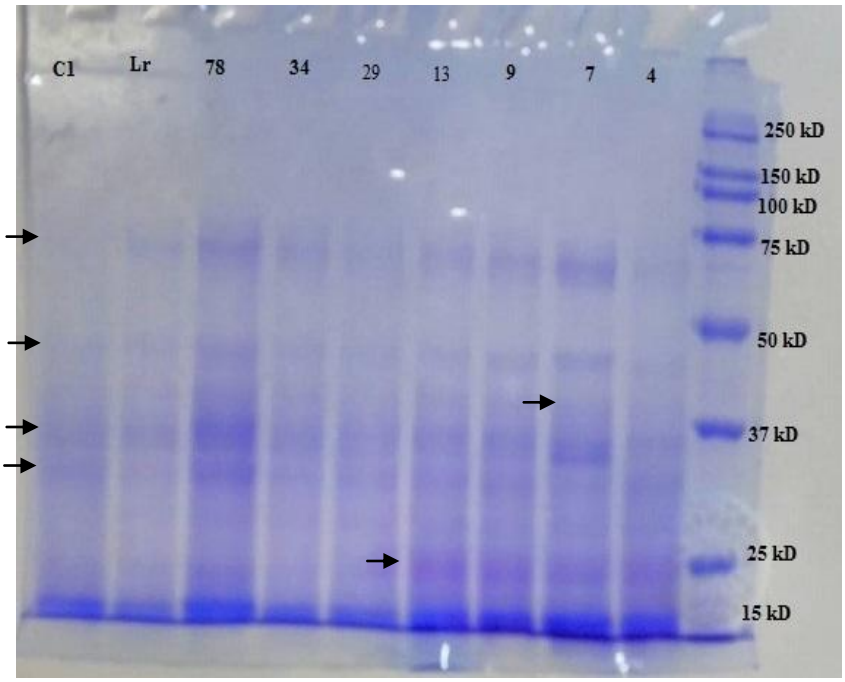

FIG 6: SDS-PAGE analysis of soyprotein hydrolysate hydrolysates

The SDS-PAGE analysis indicated that all the strains resulted in almost similar pattern of proteins with a range of 70Kda, 45Kda, 35 Kda, 30Kda, where as the cultures *Lactobacillus paracasei* (CD4), *Lactobacillus gastricus* (BTM7), *Brevibacilllus aydinogluensis* (BTM9), *Brevibacillus thermoruber* (CD13) with a protein band of 25Kda.

CONCLUSION

Several bioactive molecules in protein hydrolysate are reported in the present study from fermented soymilk by different lactic acid cultures. The LAB when inoculated in the soy milk have the ability to alter the antioxidative activity of the soy milk. Practically, for the development of any type of functional soy based food products with different health benefits Lactobacilli cultures can be used. LAB depends on proteolytic system which allows them to degrade the soymilk proteins for growth. OPA method is used for degree of proteolysis which resulted in 11.09 % to 7.16 %. *Weissella confuse* (CD1) and *Lactobacillus paracasei* (CD4) yield maximum protein content ranging from 6.415 mg/ml to 5.16 mg/ml. The protein hydrolysate fraction from soymilk fermented by *L.rhamnosus GG, Brevibacilllus aydinogluensis* (BTM9), *Brevibacillus thermoruber* (CD13) showed the higher radical scavenging ranging from 67.09% to 62.57% respectively activity as estimated by 2,2'-azino-bis(3- ethylbenzothiazoline -6-sulphonic acid) or ABTS method. Strains *Lactobacillus paracasei* (CD4) and *Brevibacillus thermoruber* (HM34) gave maximum ACE inhibitory activity compared to other isolates.

In conclusion, the indigenous isolated LAB have potential to enhance the functional attributes of soymilk via their metabolic activities.

References

[1] D.O. Forcato, M.P. Carmine, G.E. Echeverría, R.P. Pécora, S.C. Kivatinitz. (2005). Milk Fat Content Measurement by a Simple UV Spectrophotometric Method: An Alternative Screening Method. *J. Dairy Sci.*. 88 (2), 478–481.

[2] Liamngee K., Terna T.P., Bem A.A., Orpin J.B., Mzungu I., Obaje M. and Anum, T. (2003). Microbial Analysis of Soybean Milk. IOSR Journal Of Environmental Science. *Toxicology And Food Technology* . 3 (3), 97-104.

[3] Korhonen H and Pihlanto A. (2003). Food-derived Bioactive Peptides.*Current Pharmaceutical Design*. 9 (1), 1297-1308.

[4] Shahidi F , Zhong Y . (2008). Bioactive Peptides. *Journal of International*. 91 (4), 914-926.

[5] Moller N, Elisabeth K, . (2008). Bioactive peptides and proteins from foods: indication for health effects . *European Journal of Nutrition* . 47 (4), 171–182.

[6.] Hartmann , Meisel. (2007). Food derived peptides with biological activity: Current Opinion in Biotechnology . *Current Opinion in Biotechnology* . 18 , 163-169.

[7] Sharma S, Singh, Rana S. (2012). Bioactive Peptides: A Review. *INT.J. BIOAUTOMATION*. 15 (4), 223-250.

[8] Liamngee K., Terna T.P., Bem A.A. (2013). Microbial Analysis of Soyabean Milk. *IOSR Journal Of Environmental Science, Toxicology And Food Technology*. 3 (3), 97-104.

[9] Shilpa V, Subrota H, Deepika Y. (2011). Biofunctionality of Probiotic Soy Yoghurt. *Food and Nutrition Sciences*. 2 (5), 502-509.

[10] Janet C, Javier A . (2012). Use of Proteomics and Peptidomics Methods in Food Bioactive. *Peptide Science and Engineering*. 4 (4), 224–243.

[11] Wu J. Ding X. (2001). Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J Agric Food Chem* . 49 (1), 501-506.

[12] Gibbs BF, Zougman A, Masse R, Mulligan C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented foods. *Food and Research International*. 37 (2), 123-131.

[13] Johnson, W. K. and Snyder, H. E. . (1978). Soymilk Comparison of processing method, Yield And Composition . *Journal of food science* . 43 ,349-52 .

[14] Onuorah, C. E.,*Adejare, A. O. and Uhiara N. S.. (2007). Comparative physico-chemical evaluation of soymilk and soya cake produced by three different methods. *Nigerian Food Journal*. 25 (2), 28-38.

[15] Gesinde, A. T. Ovawove, O. M. and adebisi, a . (2008). Comparative Studies on the Quality and Quantity of Soymilk from Differen Varieties of soybean . *Pakistan Journal of Nutrition* . 7 (1), 157-160.

[16] O. E. Adeleke, B. A. Adeniyi and A. A. Akinrinmisi. (2000). Microbiological Quality of Local Soymilk: A Public Health Appraisal. *African Journal of Biomedical Research* . 3 (1), 89 - 92 .

[17] Akeem, A. A., uduma, E. O. Christian, C. O. and Emmanuel. (2001). Evaluation of Microbiology Quality of some soybean Milk products Consumed in Nigeria. *Prime Research on medicine* . 1 (2), 25-30.

[18] Shilpa Vij, Subrota Hati, Deepika Yadav. (2011). Biofunctionality of Probiotic Soy Yoghurt. *Biomedical & Life Sciences*. 2 (5), 502-509 .

[19] D. Fukushima. (2001). Recent Progress in Research and Technology on Soybeans. *Food Science and Technology Re-search*. 7 (1), 8-16.

[20] M. Messina and S. Barnes. (1991). The Role of Soy Products in Reducing the Risk of Cancer. *Journal of the National Cancer Institute*. 83 (8,), 541-546.