BIOACTIVE PEPTIDES FROM FERMENTED SOYMILK

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CERTIFICATE

This is to certify that the project entitled "**Bioactive peptides from fermented soymilk**" which is being submitted by **Megha Bhatnagar** and **Neha Sharma** in partial fulfillment for the award of degree of B.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

Designation:

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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. The main focus of this project was to assess the performance of lactic acid cultures in regard of ability to produce bioactive peptides from fermented soymilk. For this, two lactic cultures namely *Lactobacillus rhamnosus* and *Lactobacillus rosaiae* were assessed for their performance in soymilk as fermentation media. Soymilk is considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for lactose-intolerant population and also considered cholesterol free product for cardiovascular disorders. Fermented soymilk is a good source of bioactive peptides such as anti-ACE, antioxidative, anti-cancer and immunomodulatory. Soymilk is an excellent source of protein and dietary fiber. The peptide fraction from soymilk fermented by *Lactobacillus rosaiae* showed the higher radical scavenging activity (36.88%) as estimated by ABTS method. No antimicrobial activity of the peptide fraction was observed against *E.coli*, *Shigella sp.* and *Cronobacter sp.*

CONTENTS

Introduction

Bioactive peptides are proteins which are synthesized in the cell in the form of large prepropeptides, are then cleaved and modified into active products. They generally consist of be 3 and 20 amino acids which are encrypted within the primary structure of a dietary protein. It is now well established that physiologically active peptides are produced from several food proteins during gastrointestinal digestion and fermentation of food material with the help of lactic acid bacteria.

Bioactive peptides are protein fragments which show a positive impact on the functions and conditions of living beings. These are the compounds produced by almost all species of life. However, they are made in limited quantities in nature. Bioactive peptides have been defined as peptides with hormone- or drug like activity which can helps to modulate physiological function through binding interactions to specific receptors on target cells further leading to induction of physiological responses. According to bioactive peptides functional properties, they may be classified as antimicrobial, antithrombotic, antihypertensive, opioid, immune modulatory, mineral binding and anti oxidative. These peptides play an important role for human health.

Bioactive peptides are released from dietary proteins by either gastrointestinal digestion or by the help of processing of foods. The bioactive peptides produced by soybeans consist of manifold and unique health benefits. Like in the prevention of age-related chronic disorders, such as cardiovascular disease, cancer, obesity, and decreased immune function. Evidence also suggests that these peptides can also get absorbed by the gastrointestinal system, thus exerting their action on specific target organs.

Recent research has shown that soymilk proteins can yield bioactive peptides having opioid activity, mineral binding, cytomodulatory, antihypertensive, immunostimulating, antimicrobial and antioxidative activities in the human body. Bioactive peptides are encrypted in soymilk proteins and can only be released by enzymatic hydrolysis *in vivo* by the means of gastrointestinal digestion, food processing or by using microbial enzymes in fermented products. Upon consumption, peptides with potent physiological activities may be liberated from Soymilk protein by the action of proteolytic enzymes in the gut and thus influence the major body's systems including endocrine, nervous, digestive, cardiovascular and immune systems.

Bioactive peptides have been isolated from many sources such as milk, soy proteins, gelatine, fish proteins; and maize , but maize, but soymilk appear to be the most important sources of bioactive peptides identified thus far. Numerous health advantages of soymilk protein derived bioactive pepides have been claimed for commercial interests in the environment of health sustaining-functional foods defined bioactive peptides as substances that can affect the biological processes of the body functions with beneficial effects.

Lactobacillus strains are the most important starter cultures used in traditional fermented soymilk manufacturing. Their application mainly stems from two important properties: rapid utilization of milk sugar leading to fast acidification of soymilk as growth medium, and highly developed proteolytic system capable of supplying essential amino acids required by a fast growing organism. A number of small and oligo- peptides with different physiological functions has been released from milk proteins through microbial proteolysis and has been well recognized and assessed.

In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released in three ways : (a) through hydrolysis by digestive enzymes, (b) through hydrolysis by proteolytic microorganisms and (c) through the action of proteolytic enzymes derived from microorganisms or plant.

At present vital research is being undertaken on the health effects of bioactive 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 in fermented soymilk, whey and ripened cheese. Varied of these peptides have been commercialized in the form of fermented soymilks. Bioactive peptides have the potential to be used in the formulation of health-enhancing nutraceuticals, or as potent drugs with well limited pharmacological effects.

Literature Review

Definition

Accordingly to a widely shared definition, a bioactive dietary substance is “a food component that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health.

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body function or conditions and may ultimately influence health of person. Upon oral administration, bioactive peptides, may affect the major body systems. The beneficial health effects may be classified as antimicrobial, anti oxidative, antithrombotic, and antihypertensive. The activity of these bifunctional peptides is based on their inherent amino acid composition and there sequence. The size of active sequences vary from 2- 20 amino acid residues.

Mechanisms of production of bioactive peptides

Milk-derived bioactive peptides, are generally food bioactive peptides, and are usually composed of 2-20 amino acids which become active only when they get released from the precursor protein when they are being encrypted.

These are the different mechanisms which can be used to release the encrypted bioactive peptides from the precursor proteins:

(a) enzymatic hydrolysis by the help of digestive enzymes
(b) food processing and
(c) proteolysis by enzymes derived from microorganisms or by the plants

(a) Enzymatic hydrolysis:

The break of abeyant bioactive peptides from milk proteins frequently occurs during assimilation by pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy and aminopeptidases), bearing alive peptide bits in the gastrointestinal amplitude of the milk-consuming alone (Schlimme and Meisel, 1995). The physiological furnishings of bioactive peptides depend on their adeptness to ability their ambition sites intact, which may absorb assimilation through the abdominal epithelium above-mentioned to biking to the borderline organs (Vermeirssen et al., 2004).

Many of the accepted bioactive peptides accept been produced in vitro application gastrointestinal enzymes, usually pepsin and trypsin. ACE-inhibitory peptides and CPPs, for example, are a lot of frequently produced by trypsin.

Other digestive enzymes and altered agitator combinations of proteinases—including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as able-bodied as enzymes from bacterial and fungal sources—have aswell been activated to accomplish bioactive peptides from assorted proteins.

b) Food Processing:

The structural and chemical changes that action during the processing of food proteins may aftereffect in the absolution of bioactive peptides. In particular, heat and/or alkali treatment analysis can accomplish added inter-and intra molecular covalent bonds that are aggressive to hydrolysis. Such processing altitude as well advance the racemic about-face of L-amino acids to D-isomers and consequently, advance to indigestible peptide bonds. The abeyant accumulation of indigestible peptide sequences during aliment processing is noteworthy, because this may advance both accumulation and assimilation of bioactive peptides that do not action by itself in the forerunner protein.

Such bioactive peptides can be generated during accomplish of several milk articles and may appropriately be ingested as food components. For example, partially anatomize milk proteins for hypoallergenic baby formulae and for analytic applications in diet abide alone of peptides and accommodate bioactive peptides. Cheese contains phosphopeptides as accustomed capacity and accessory proteolysis during cheese ripening leads to accumulation of assorted ACE inhibitory peptides.

c) Microbial fermentation

Many industrially utilised dairy starter cultures are proteolytic in nature to some extent. Bioactive peptides can thus, be generated by the proteolytic activities of the strains of starter

And non-starter bacteria e.g .*Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilus is* used in the manufacturing of many fermented dairy products. The proteolytic system of lactic acid bacteria (LAB) is very well characterised. This system consists of a cell wall-bound proteinase and amount of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases. Extracellular proteinases could cause abasement of casein into oligopeptides. The longer chain oligopeptides can be a source of bioactive peptides which when further degraded by intracellular peptidases of lysed-lactic acid bacteria.

The most effective way to increase the concentration of bioactive peptides in fermented dairy products is to use highly proteolytic strains of LAB for fermentation or co-fermentation. The choice of the strains will also influences the release of effective bioactive peptides. The strain should not be too proteolytic otherwise the product may get destroyed and must have the right specificity to give good concentrations of active peptides. The concentration of ACE-inhibitory peptides seems to rely on a balance between their formation and further breakdown into inactive peptides and amino acids which totally depends on storage time and conditions.

Various bioactive peptides including ACE-inhibitory or antihypertensive peptides, immunomodulatory, antioxidative, antimutagenic peptides have been released from soymilk proteins through microbial proteolysis (Gobbetti *et al*., 2004; Korhonen and Pihlanto, 2001; Korhonen and Pihlanto, 2004, Matar *et al.*, 2003). The best accepted ACE-inhibitory peptides are, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), that have been identified in soymilk fermented with strains of *Lb. helveticus* and *Saccharomyces cerevisiae.* In addition to live microorganisms, proteolytic enzymes abandoned from LAB have been successfully employed to release bioactive peptides from soymilk proteins.

Properties of lactic acid bacteria

Lactic acid bacteria have been used to ferment or culture foods for at least 4000 years. They are used in particular in fermented milk products from all over the world. Lactic acid bacteria are therefore excellent ambassadors for an often maligned microbial world. They are not only of major economic significance, but are also of value in maintaining and promoting human health.

The lactic acid bacteria are defined as Gram –positive cocci or rods with a low-GC. These are acid-tolerant in nature and are generally non-spore forming bacteria which are associated by their common metabolic and physiological characteristics. They are able to grow both in the presence and absence of oxygen. Furthermore, LAB can be found in spoiling plants and lactic products produce lactic acid as the major metabolic end product as a result of carbohydrate fermentation. Several strains of LAB produce proteinaceous, bacteriocins that create an additional hurdle for spoilage and pathogenic microorganisms.

The drop in pH that occurs as lactic acid is produced by the bacteria which is beneficial in the preservation of food. The lowered pH inhibits the growth of many other food spoilage microorganisms . Abundant growth of the lactic acid bacteria, and so production of lactic acid, is likewise hindered by the low pH. The low pH environment prolongs the shelf life of foods (e.g., pickles, yogurt, cheese) from contamination by bacteria that are common in the kitchen or bacteria that are able to grow at refrigeration temperatures. The drop in the oxygen level during lactic acid fermentation is also an inhibitory factor for potential food pathogens. Lactic acid bacteria produce antibacterial compounds also known as bacteriocins. Bacteriocins act by punching holes through the membrane that surrounds the bacteria. Thus, bacteriocins activity is usually lethal to the bacteria.

Moreover, it seems that lactic acid and other metabolic activity products contribute to the organoleptic and textural profile of a food item. Due to their ubiquitous presence in fermented foods and their contribution to the intestinal microflora of human mucosal surfaces, the industrial importance of the LAB is more manifested by their generally recognized as Safe(GRAS) status.

Sources of Bioactive peptides

Soybean:

Soybeans, is one of the most abundant plant sources of dietary protein, consists 36% to 56% of protein. They also contain 35% carbohydrates, including soluble di- and oligosaccharides, 18% to 22% fat, including polyunsaturated fatty acids, phytosterols, phospholipids, minerals, B vitamins, and fiber. Recent studies accepted that 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 peptides and can be utilized as a health enhancing ingredient in functional foods.

Soybean, is an important source of food proteins has received increasing interest from public due to its reported health benefits. The bloom allowances are attributed to its components, including isoflavones, saponins, proteins, and peptides. Soymilk also contains soy proteins that consist of higher quality of amino acid balance compared to animal proteins (Connes *et af.*, 2003).

The amount of isoflavones in soy products varies with the type of soybean, the condition and area of cultivation, and processing. Soy foods, especially soymilk, are considered a good substitution for dairy products for individuals who have milk intolerance. Milk intolerance including cow's milk protein allergy (CMPA), cow's milk protein intolerance (CMPI), and lactose intolerance is prevalent in the world, especially in children. CMPA can affect from 2 to 6% of children. Lunasin, Bowman-Birk inhibitor, lectin and 3-conglycinin are some of the biologically active peptides and protein found in soybean.

It is an excellent source of dietary peptides, have many activities like antihypertensive, anti cholesterol, and antioxidant activities, and also prevent cancer. The processing of soy protein into peptides in the GI tract increases their healthful effects by exposing active groups within the amino acid chain. These can be effective in many ways like from the prevention of age-related chronic disorders, such as cardiovascular disease, cancer, obesity, and decreased immune function. Despite the potential anti-nutritive effects, a large amount of research papers reported health benefits of soy foods in the area of disease prevention. The other possible mechanism of soy foods to prevent cancer is similar to the mechanism of blood vessel protection. Formation of new blood vessels is the key step for the growth of human cancers and facilitation of tumor cell metastases. Soy isoflavone is the major protective components, since it can restore the structure of blood vessels and inhibit cell transformation and tumor cell proliferation.

Soybean is a useful source of inhibitor of the angiotensin-converting enzyme (Ahn et al.,2000; Shin et al., 2001). Inhibitors of this enzyme are now widely used as antihypertensive agents, causing a fall in blood pressure comparable to that produced by thiazides, and calcium antagonists (Pool et al., 1989). Many peptides isolated by the hydrolysis of food proteins shows inhibitory activity against the enzyme and reduce blood pressure after oral administration . Daily usage of food with such peptides might be effective in maintaining blood pressure at the normal level.

Benefits of Soy Milk

- Soymilk is an excellent source of protein and dietary fiber.
- It consists of little saturated fat and no cholesterol and lactose.
- It is a good source of iron
- Soy protein may reduce the risk of certain cancers, including breast cancer, prostate and lung cancer.
- It conatin isoflavones which deal with many health issues, most important being the prevention of various cancers, heart disease, osteoporosis, antioxidant etc.
- As it doesn’t contain galactose, soy milk can safely replace breast milk in children with galactosemia.
- It lacks casein and is a good source of lecithin and vitamin E.
- It is safe for people with lactose intolerance, or milk allergy.
- It also helps in fighting the symptoms of menopause and in promoting eye health or anti-catract.
- It can conveniently be used as a weaning formula instead of cow’s milk for infants.
- It also shows anti-diabetic and anti-obesity property.

Fish:

The muscles, skeleton, skin and internal organs of fish can be used as a source of biologically active peptides. Enzymatic hydrolysis of these materials can provide biologically active peptides with different physicochemical properties. These peptides can demonstrate antihypertensive , anticoagulant, immunomodulative, antioxidant, and other pharmacological properties.

A peptide fraction with a molecular weight of approximately 10kDa isolated frm the protein hydrolyzate of cod skeletons demonstrated high antioxidative activity. Antiskeletons of yellow fin sole, *Limanda aspera*, tuna and the hoki, *Johnius bellengerii.*

Physiological effects of bioactive peptides:

Benefits of Soymilk to Human Health:

Soymilk has been suggested as an alternative to replace cow's milk which contain lactose.Soymilk is an alternative for this population as soymilk is rich in protein and does not contain lactose. Additionally, soymilk contains calcium, zinc, magnesium, iron and essential amino acids which is an excellent nutritional value for human diet (Sacks *et al.*, 2006). The cholesterol-lowering effect of soymilk has been recognized by most studies. Past studies reported that amino acid of proteins in soymilk contributes to hypocholesterolaemic effects. Past studies have shown that these isoflavonoids in soymilk reduced the risks of cardiovascular diseases due to their ability to reduce cholesterol . Due to their estrogen mimetic properties, soybean phytoestrogens have been found able to eliminate symptoms of menopause (Murkies *eta!.*, 1995).

Effect on Coronary Heart Disease:

Coronary Heart Disease (CHD) is a serious heart disease and a major cause of death in most developed countries. The high fat and sugar content of the diet particularly in the United States and many other variable factors, such as smoking and drinking alcoholic beverages, contribute to an individual’s risk for CHD. Low-density lipoproteins (LDL) play key roles in the atherosclerotic process. LDL is oxidized by free radicals along the walls of blood vessels and forms a glue-like material which builds up and eventually may block the vessel.

High-density lipoproteins (HDL) have the protective effect against LDL oxidation and eliminate the cholesterol along the blood vessel wall (Malik and others 2004). Soy foods contain a high quantity of soy isoflavone components that have effects on reducing the risk for CHD because of their antioxidant (Anderson and others 1998; Tikkanen and others 1998) and anti-inflammatory effects.

Furthermore, during the atherosclerotic process, blood vessels could be damaged by the abnormal spasm or "clamping down" which occurs when the atherosclerotic plaque is exposed to stress. Soy proteins and soy isoflavones may reduce the blood clot formation (Anderson and others 2001) and restore damaged blood vessels to protect blood vessels because of their anti-inflammatory effects (Honore and others 1997). Since soy proteins and their isoflavones may correct the balance of blood lipid levels, protect blood vessels, and have antioxidant and anti-inflammatory effects, in 1999 the U.S. Food and Drug Administration (FDA) approved a health claim that eating soy protein as part of a low saturated fat and cholesterol diet may decrease an individual's risk for CHD (FDA 1999). That same year, the U.S. FDA published that 25 g of soy protein per day provided an appropriate intake to reduce risk for CHD (FDA 1999).

Effect on Cancer :

Currently, more than 13 million people in the United States have had some type of cancer (American Cancer Society 2012). The main types of cancer are lung cancer, colon cancer, breast cancer, prostate cancer, and skin cancer. The high rate of cancer is often because of unhealthy lifestyles, such as smoking, high-calorie diets, drinking alcohol, lack of physical activities, etc.

Results from animal and in vitro studies have suggested that soy foods have a positive effect against cancer initiation due to its high isoflavone content. The one 8 possible mechanism of soy's effects against cancer is that the structure of isoflavones is similar to estrogen's structure, thus having a weak estrogenic effect. Consequently, the association of soy foods with cancer risk was first researched for breast cancer and possible mechanism of soy's effects against cancer is that the structure of isoflavones is similar to estrogen's structure, thus having a weak estrogenic effect. Consequently, the association of soy foods with cancer risk was first researched for breast cancer and prostate cancer prevention. The other possible mechanism of soy foods to prevent cancer is similar to the mechanism of blood vessel protection. Formation of new blood vessels is the key step for the growth of human cancers and facilitation of tumor cell metastases.

Soy isoflavone is the major protective components, since it can restore the structure of blood vessels (Honore and others 1997) and inhibit cell transformation and tumor cell proliferation (Anderson and others 2001). A prospective cohort study provided strong evidence that soy food consumption may protect against colorectal cancer in women (Yang and others 2009). In addition, in a large case control study, researchers demonstrated that soy food consumption may reduce the risk of colorectal cancer in men. Soy food consumption has a positive effect on lung cancer as well. A meta-analysis demonstrated that soy isoflavones were associated with a 27% risk reduction in lung cancer (Yang and others 2011).

Effects on immune system

Diet is known to play a very important role in the body's defense mechanism. Research concerning the role of functional peptides on the immune system is quite recent but seems to be promising. The two main activities being studied are the immunomodulatory (stimulation of immune system) as well as antimicrobial (inhibition of microorganisms) effects of bioactive peptides. Immunomodulating peptides have been found to stimulate the proliferation of human lymphocytes, the phagocytic activities of macrophages and also antibody synthesis. Also, it has been suggested that immunomodulatory milk peptides may alleviate allergic reactions in humans and enhance mucosal immunity in the gastrointestinal tract.

In this way immunomodulatory peptides may regulate the development of the immune system in newborn infants. Furthermore, it has been suggested that immunopeptides formed during milk fermentation may contribute to the antitumor effects of fermented milk (Matar *et al*., 2003).

The antimicrobial properties of milk have been widely acknowledged for many years. The antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins, such as lactoferrin, lactoperoxidase and lysozyme.. More recently, other whey proteins such as α-lactalbumin and ß-lactoglobulin have also been considered as potential precursors of bactericidal fragments. These peptides have been found to be active against a broad range of pathogenic organisms. Depending on the target microorganism, inhibitory concentrations of peptides vary.

Effects on nervous system

Recent studies have provided evidence that peptides exist in dairy products which play an active role in the nervous system; these are known as opioid peptides. The first major opioid peptides discovered wereß-casomorphins, fragments of ß-casein (Smacchi and Gobbbetii, 1998). Once absorbed into blood, these peptides can travel to the brain and various other organs and elicit pharmacological properties similar to opium or morphine. This may be the reason why human neonates generally become calm and sleepy after drinking milk. In contrast to the casomorphins, some peptides produced by the breakdown of κ-casein function as opioid antagonists, that is, they can inhibit the effect of morphine like substances.

Future perspectives for bioactive peptides

Fermented dairy products and other foods containing bioactive peptides would appear to have the potential to give specific health benefits to consumers. While there is a need for further basic research to clarify why these peptides have physiological effects, commercial products containing bioactive peptides are now commercially available. Food and pharmaceutical companies are actively working on how to exploit bioactive peptides in both human nutrition and in health promotion.

Bioactive peptide have the potential to be used in the formulation of functional foods, cosmetics and as potent drugs having well defined pharmacological effects. With the rise of consumer concerns about the deleterious effects of chemical preservatives and the increasing preference for natural components, milk derived bioactive substances may have high value in food preservation and nutraceuticals.

Application of enrichment protocols such as membrane processing and chromatographic isolation may also be an area of future interest in the extraction of potent biofunctional peptides from fermented dairy products and their subsequent utilization as functional food ingredients.

Molecular studies are required to study the mechanisms by which the bioactive peptides exert their activities. Ultimately this research may be helpful in understanding, preventing and treating life-style related diseases such as cardiovascular disease, cancers, osteoporosis, stress and obesity.

Materials and Methods

1) **Chemicals**

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

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) *Lactobacillus rosaiae* (isolated from curd)

3) **Maintenance of lactic culture**

The lactic cultures (*Lactobacillus rhamnosus* and *Lactobacillus rosaiae)* were maintained by inoculating each culture individually in MRS broth (de Man, Rogosa and Sharpe**)** and were incubated at 37°C for 24 hrs.

4) **Propagation of cultures**

An aliquot of inoculums of lactic cultures (*Lactobacillus rhamnosus* and *Lactobacillus rosaiae)* 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.

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

↓

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

↓

Slurry sieved through muslin cloth

↓

Boil the soy milk and analyzed.

6) Preparation of fermented soymilk

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

The flask were inoculated with different lactic cultures and were incubated for 24hrs at 37 ˚C.

Fermentation was observed next day.

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 lyophilized to obtain powder form.

Analysis

1) pH:- pH of fermented soymilk was estimated with the help of pH meter.

2) Determination of moisture content in soymilk:- About 20mls of the soymilk sample was weighed in empty petri-dish and was then dried in a hot air oven for 24 hours and it was cooled in a desiccator and weight .

Percentage of moisture content = $(W1-W0) - (W2-W0)/ (W1-W0) \times 100$

Where:

W0 = Weight of empty moisture dish

W1 = Weight of moisture dish and sample

W2 = Weight of dessicated sample

(W1 – W0) – (W2 – W0) = Weight loss

(W1 – W0) = Weight of sample

3) Determination of ash content in soymilk : A 2 ml of milk sample was taken in empty clean crucible and placed in a muffle furnace and ashed at 500°C - 600°C for 3 hours. The crucible was removed from the muffle furnace and cooled in a desiccator, and weighed.

Percentage of ash content = $(W2-W0)/ (W1-W0) \times 100$

Where:

W0 = Weight of empty crucible

W1 = Weight of sample and crucible

W2 = Weight of ash and crucible.

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

Standard – BSA(1mg/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 added and vortexed it and kept at 37˚C for 15 min.

0.5 ml of folin phenol reagent(1N) was added to each testtube and incubated it for 30 min in dark and absorbance was taken at 750nm.

5) Milk Fat Content Measurement by a Simple UV Spectrophotometric method :

Soymilk (30 μL) was added to 3 mL of absolute ethanol at −20°C.

All vials were capped and stored for 1 h at −20°C.

Samples were centrifuged at 13,000 rpm for 15min and allowed to reach room temperature. Sample absorbance is taken at 208 nm.

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 : distilled water (1:1)

Standard- BSA (1mg/ml)

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 1mg of sample in 1ml of distilled water.

5 ml of lowry solution was added and vortexed it and kept at 37^{o}C for 15 mins.

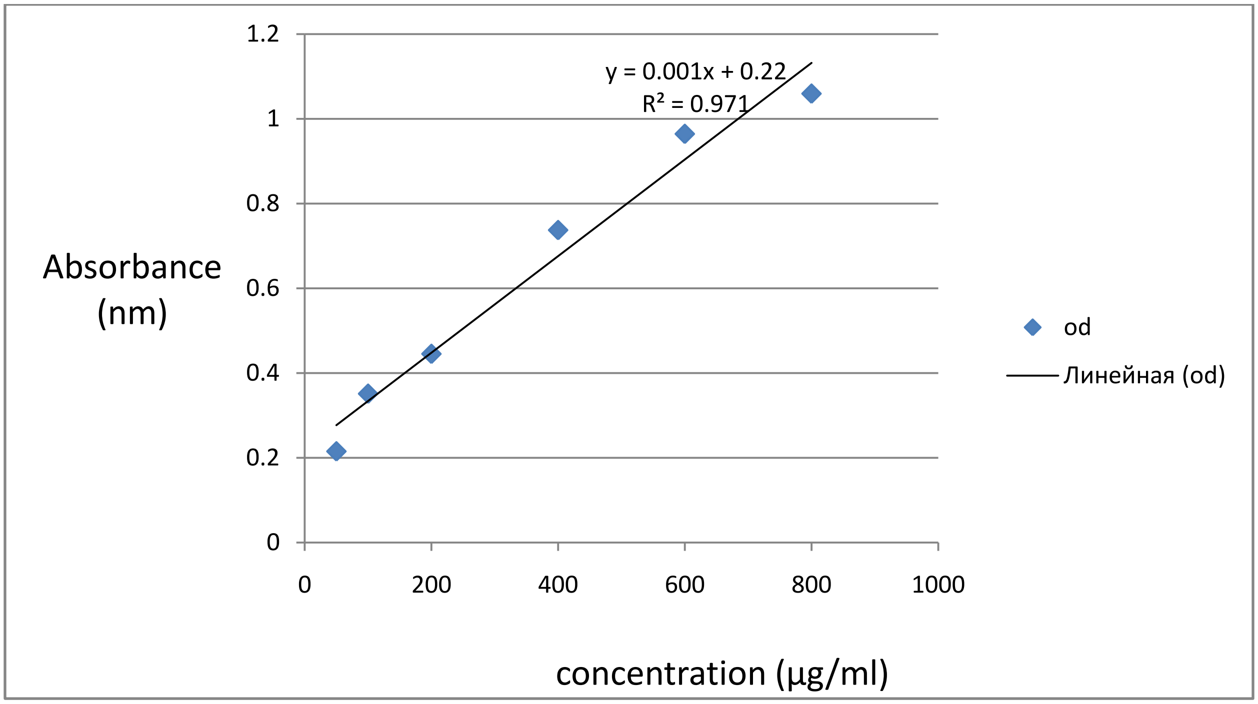
0.5 ml of folin phenol reagent (1N) was added and incubated it for 30 min in dark and absorbance was taken at 750 nm.

Sample measuring:- The sample solution was prepared as follows:

1 mg of lyophilized protein samples in 1 ml of distilled water, and dilutions were made.

5 ml of lowry solution were made and vortexed it and kept at 37^{o}C for 15 min.

0.5 ml of folin phenol regent (1N) was added and incubated it for 30min in dark and absorbance was taken at 750nm.



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 quantities and left to stand for 12-16 h at room temperature in dark until reaching a stable oxidative state.

The ABTS solution was diluted with 80% ethanol to an absorbance of 0.80 ± 0.05 at 734 nm.

100µL of sample was mixed with 2.9 ml of ABTS solution and the mixture was allowed to stand at room temperature for 30 min in dark condition.

The absorbance was determined at 734 nm.

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

Estimation of Anti microbial activity:

Anti microbial activity of bioactive peptides was estimated against *E. coli*, *Salmonella*, and *Cronobacter* strains with the help of Agar well method

Agar plates were made (1.3g of Nutrient Broth and 1.2 g of Agar Agar was dissolved in 100ml of distilled water, autoclaved at 121^{o}C , Each petri plate was filled with 20 ml of media in LAF)

- Plates were incubated at 37^{o}C to check contamination.
- Cultures were activated in nutrient broth and were incubated at 37^{o} C in shaking incubater.
- Spreading was done after 5 hrs with individual strains, left for 30min.
- Wells were made on the plates.
- Wells were inoculated with 50µL of bioactive peptide.

Estimation of Proteolytic activity:

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

The OPA reagent was prepared as follows:

7.620g di- Na tetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) were dissolved in 150 mL deionized water. The reagents were completely dissolved before continuing. 160mg o-phthaldialdehyde 97% (OPA) was dissolved in 4 ml ethanol.

The OPA solution was then transferred quantitatively to the above mentioned solution by rinsing with deionized water.176mg dithiothreitol 99 % (DTT) was added to the solution by rinsing with deionized water.

The solution was made up to 200 ml with deionized water.

Procedure:-

Standard:- Tyrosine 1 mg/ml

Tyrosine standard was made using dilutions 10, 20, 50, 100µL.

3ml OPA reagents was added and mixed 5s.

The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer. Note the readings.

Sample measuring :- The sample solution was prepared as follows: 0.01 gm of lyophilized protein samples in 1 ml of distilled water.

And following dilutions of sample were made (10, 20, 50, 100 µL).

3ml OPA reagents was added and mixed for 5s.

The mixture stood for exactly 2 min before being read at 340nm in the spectrophotometer.

Tyrosine = (OD sample - OD blank) / (OD standard - OD blank) x 0.915 meqv x *S* x *D/ (P/V)*

Where S is sample volume in litre; D is dilution volume; P is protein content in the volume of the sample ; V is sample volume in assay.

SDS PAGE:

Materials:

Stacking gel composition: (5%)

Component	4ml	5ml	10ml	15ml
dh_2o	2.66	3.4	6.8	10.2
30% acrylamide	670µl	830 µl	1.66	2.49
1M Tris (pH=6.8)	500 µl	630 µl	1.26	1.89
10% SDS	40 µl	50 µl	100 µl	150 µl
10%APS	40 µl	50 µl	100 µl	150 µl
TEMED	4 µl	5 µl	10 µl	15 µl

Resolving gel composition: (12%)

Component	10ml	15ml	20ml	30ml
dh_2o	3.175	4.9	6.35	9.525
30% acrylamide	4	6	8	12
1.5 M Tris (pH= 8.8)	2.503	3.8	5.06	7.509
10% SDS	100 µl	150 µl	200 µl	300 µl
10%APS	100 µl	150 µl	200 µl	300 µl
TEMED	4 µl	6 µl	8 µl	12 µl

Protocol

1. Prepared polyacrylamide gel according to standard protocol.

2. Loaded samples and run gel @ 25 mA (2 gels run @ 50 mA) in 1x SDS Running Buffer.

3. Placed the gel in a plastic container. Covered with isopropanol fixing solution and shake at room temperature. For 0.75 mm-thick gels, shake 10 to 15 min; for 1.5 mmthick gels, shake 30 to 60 min.

4. Poured off fixing solution. Cover with Coomassie blue staining solution and shake at RT for 2 hr.

5. Poured off staining solution. Wash gel with 10% acetic acid to destain, shaking at RT ON.

Results

Analysis:

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 refers to the mineral content in the material, and is determined by burning a given quantity of material under prescribed conditions and measuring the residue. 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.

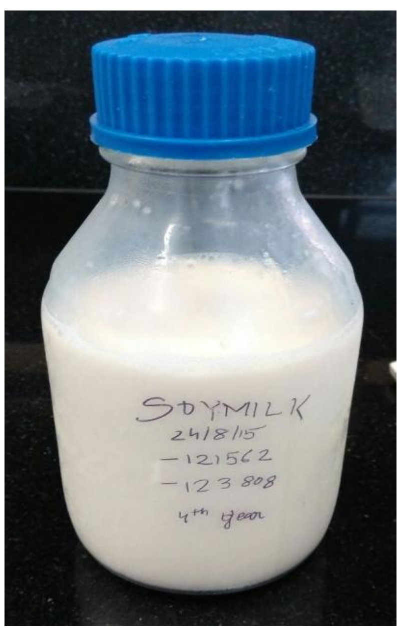


FIG 1: Soymilk

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 which were maintained at 37 C for 24hrs in the sterilized skimmed milk the tubes were checked for setting of the curd and hence curdling was observed.

FIG 2: Curdling of Soymilk

Protein content and Degree of Hydrolysis:

Production of amino acid and peptides from the degradation of milk protein by LAB enzymes and utilization of these amino acid are a central metabolic activity of LAB . Lactic acid bacteria depend on proteolytic system allowing degration of milk proteins for the growth.

Protein content in the fermented soymilk(*Lactobacillus rhamnosus*) was calculated as 1017µl/ml and for (*Lactobacillus rosaiae)* was 1302µl/ml.

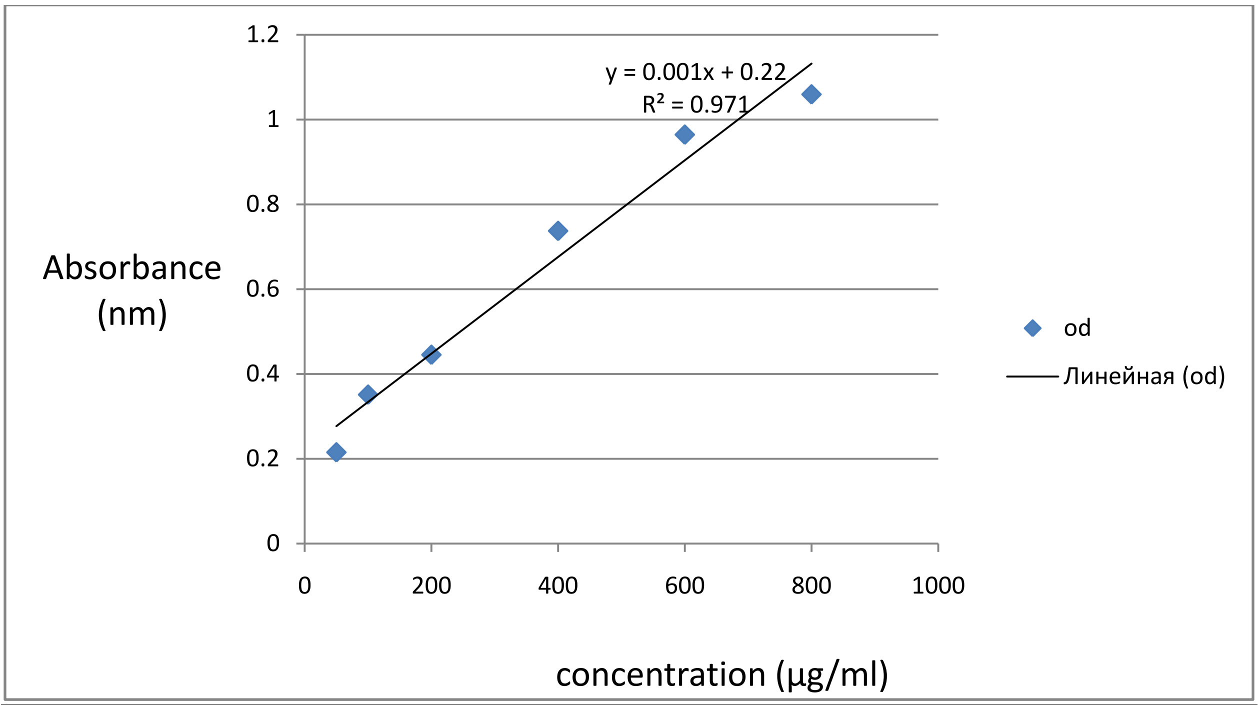


Fig 3. Total protein content

Anti microbial activity:

A method for quantitatively determining the effect of the substance by its inhibiting effect on the growth of susceptible microorganism. The most frequently used methods are based on agar well method. The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts . Similarly to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.

This activity was estimated by agar well assay method and the respective result of various lactic culture as given below.

Samples	*E.coli*	*Shigella sp.*	*Cronobacter sakazakii*
Lactobacillus rhamnosus	-	-	-
Lactobacillus rosaiae	-	-	-

Anti-microbial activity of *Lactobacillus rhamnosus* and *Lactobacillus rosaiae* was not seen against *E.coli, Shigella, Cronobacter strains.*

Anti-oxidative activity:

Oxidative metabolism is very crucial for the survival of human's cells. The risk of this activity leads to the production of free radicals which causes oxidative changes. Free radicals are linked with many pathological conditions such as diabetes, arthritis, atherosclerosis. Inhibition of the free radicals formed in the living body is one of the important way to protect body from above serious diseases.

The antioxidant potency of plant extract or food product has been measured by ABTS assay. ABTS is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods. In this assay, 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.

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

100µL of sample was mixed with 2.9 ml of ABTS solution and the mixture was allowed to stand at room temperature for 30 min in dark condition. The absorbance was determined at 734 nm. The scavenging effect was calculated with the formula given below:

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

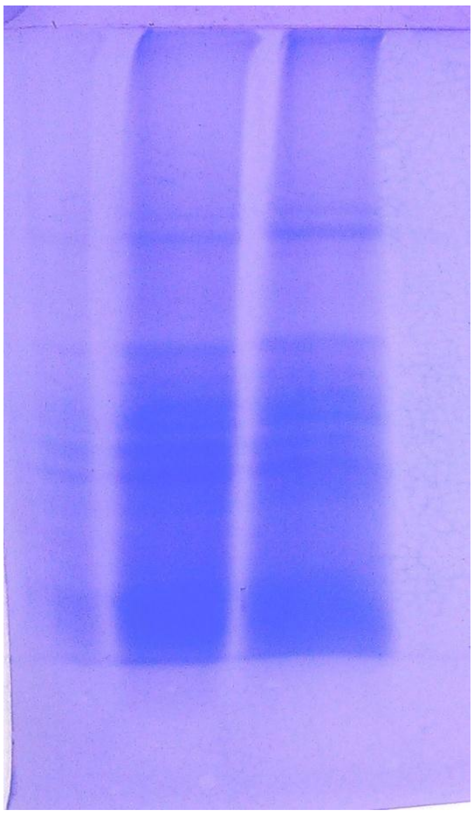
Absorbance of control was calculated as 0.293 and for *Lactobacillus rosaiae* and

Lactobacillus rhamnosus was calculated as o.111 and 0.078 respectively.

Culture	**Inhibition %**
Lactobacillus rhamnosus	**25.62%**
Lactobacillus rosaiae	**36.88%**

SDS PAGE:

In SDS PAGE bands was observed but they were not resolved properly.



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