ISOLATION AND CHARACTERIZATION OF PLANT DERIVED BIOACTIVE PEPTIDES FOR FOOD APPLICATION

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By

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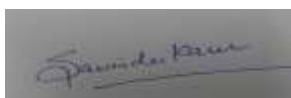
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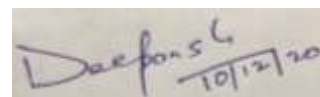
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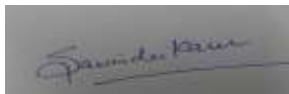**Dr. Sawinder Kaur**   **Dated:**

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This is to certify that the thesis entitled "**ISOLATION AND CHARACTERIZATION OF PLANT DERIVED BIOACTIVE PEPTIDES FOR FOOD APPLICATION**", is a bonafide work carried out by Ms. Poorva student of PhD (Food technology), Lovely Professional University, Phagwara, under my guidance towards the partial fulfillment of the degree in PhD (Food technology) of Lovely Professional University, Phagwara. This work has not been published or submitted elsewhere for the award of any degree.

(Dr. Sawinder Kaur)

ABSTRACT

Bioactive peptides are protein fragments which have a positive impact on the functions and conditions of living organisms. Apart from animal sources, plants are excellent source of bioactive peptides. In this study, pumpkin seeds, flaxseeds, quinoa seeds, chia seeds and peanuts has been used to isolate the bioactive peptides. *Lactobacillus plantarum* strain NCDC 374 was found to possess maximum proteolytic activity than other tested probiotic strains. Among all the tested substrates, flaxseed was found to possess best functional properties, thus, further studies were carried out with flaxseed. Optimization of fermentation condition to obtain maximum functional properties (Proteolytic activity, Antioxidant activity and ACE inhibition %) was investigated using response surface methodology. Optimal condition to produce the functional peptides were found to be 4.20% inoculum size with 126 hours of fermentation time. The fermented milk resulted in 67.38% inhibition in DPPH, 41.35% inhibition in ACE and 30.38 micro gram leucine/ml proteolytic activity. Molecular weight cut off membrane (Viva spin) were used to fractionate the peptides. 10kDa peptides showed optimal results for % DPPH inhibition, ACE inhibition, Antimicrobial activity and DPP-IV inhibition as compared to 5kDa. Further, effect of 10kDa peptide was checked on shelf-life enhancement of cheese and it was found to retain the growth of indigenous microorganisms. However, antagonistic behaviour was shown against pathogenic microorganisms.

Keywords: Flaxseed, Bioactive peptides, ACE inhibition, Antioxidant activity, Dipeptidyl peptidase-4 (DPP-4) inhibitors, Shelf-life, Cheese

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CHAPTER 1

INTRODUCTION

Bioactive peptides (or cryptides) have been defined as short-chained amino acids capable of imparting health benefits to the human body (Kehinde and Sharma, 2018). They impart numerous biofunctionalitites including; immunomodulatory, antidiabetic, antimicrobial, antihypertensive and antioxidative amongst several others. They are obtainable from diverse natural sources of remarkable protein contents such as food materials, venoms of scorpions and snakes, feathers, horns, cottonseeds and wool (Pennington et al., 2017; Juichi et al., 2018). Food sources possessing sufficient protein content are potential base materials for isolation of bioactive peptides. Although milk and milk products are greatly studied as source of bioactive peptides, many bioactive peptides are also found in other animal and plant sources like egg, fish, oyster, cereal (rice, wheat, soybean, buckwheat, barley, and corn), soybean, and radish seeds (Yoshikawa et al., 2003).

The disintegration of protein molecules with a successive liberation of peptides takes place during conventional food processing operations such as cooking, fermentation and germination; however, in scientific studies, the typical methodology for isolation of peptides especially from food sources has been by hydrolysis by one or more enzymes, food fermentation, in silico techniques or synthesis by chemical synthesis (Hou et al., 2017). However, probiotic fermentation is a functional technique involving the proteolytic breakdown of food proteins by probiotics such as *Lactobacillus plantarum, Lactobacillus acidophilus, Aspergillus oryzae* and *Bacillus subtilis* (Mechmeche et al., 2019, Kehinde and Sharma, 2018). Dairy foods have also been used in recent studies for the isolation of bioactive peptides. Plant substrate rich in protein are the good choice for substrate for production of bioactive peptides.

Cancer is a non-communicable disease of global reputation; one of the leading causes of death in the twenty-first century and observed to be more prevalent in developed and industrialized countries relative to less developed ones. Traditional approaches to cancer management such as radiotherapy and surgery have shown remarkable, underlying adverse effects. Chemotherapy, a drug-based treatment formulated for the destruction of malignant cells possesses the disadvantages of also harming healthy tissues. The medical com- plexities, high treatment costs and deleterious effects of the aforementioned therapies creates the need for a less harmful and affordable treatment for the ever-increasing population of cancer patients globally. Peptides with anticancer biofunctionalities and with desirous attributes such as specificity of action, efficacious solubility and tumor penetration and overall safety avails them of the befitting profile and optimal pharmacological choice for cancer treatment. The structural configuration of peptides has been long discovered to play a significant role in their functionalities, particularly those associated with destruction of patho genic and/or cancerous cells (Sharma et al., 2020) .

Furthermore, Diabetes, a metabolic syndrome of global importance has been on a progressive rise in recent years. Several pharmacological approaches have been made, which have proved effective, but with underlying side effects. Bioactive hydrolysates (BHs) and peptides (BPs) from food sources, however, have shown the relative advantage of imparting less adverse effects. Furthermore, BHs and BPs from food have been discovered to impart their antidiabetic potentials through one or more mechanisms such as inhibition of digestive enzymes, inhibition of the antigenic enzyme – Dipeptyl peptidase IV (DPP-IV), decrease in blood glucose levels and increase in insulin uptake. Several plants and animal sources have been used as protein sources for the isolation of antidiabetic hydrolysates and peptides through different mechanisms and analytical techniques (Kehinde and Sharma, 2020).

Additionally, Cardiovascular disease (CVD) is the single leading cause of death for both males and females in the world. Therefore, it is necessary to increased focus on improving diet and lifestyle as a strategy for CVD risk reduction. High blood pressure is one of the major independent risk factors for CVD. Angiotensin I-converting enzyme (ACE) plays a crucial role in blood pressure regulation which promotes the conversion of angiotensin I to the angiotensin II. Inhibition of these processes by bioactive component of food such as bioactive peptides can be a good alternative of synthetic drugs. Recently, several bioactive peptides have been identified from soybean having ACE-Inhibitory activities (Tsai et al.,, 2006). Oxidative stress and increased production of ROS (reactive oxygen species) with endogenous antioxidant mechanisms is another factor for the progression of several CVD (Pihlanto, 2013). Several bioactive peptides are known which are able to scavenge free radicals in the body and act as antioxidants. Antioxidative peptides from foods are considered to be safe and healthy ingredients with low molecular weight, low cost, high activity and easy absorption. They have some advantages in comparison to enzymatic antioxidants; that is, with simpler structure they have more stability in different situation and without any hazardous immunoreactions and they present nutritional and functional properties beside their antioxidant activity (Xie et al.,, 2008). Bioactive peptides derived from food proteins are able to inhibit the growth of pathogenic microorganism. Bioactive peptides are also less likely to accumulate in body tissues or to confer serious side effects because nature has provided the mechanism for their metabolism and utilization or excretion (Singh and Vij, 2015)

Bioactive peptides and protein hydrolysates hold great promise as valuable functional ingredients in healthy diets to fight the global epidemic of non-communicable diseases. During recent years, the isolation, identification and characterization of bioactive peptides have become emerging research subjects. The classical, empirical approach to production of bioactive protein hydrolysates and peptides involves first identifying a suitable protein

source, and then releasing peptide fragments with bioactivity through hydrolysis of peptide bonds, usually by the proteolytic action of enzymes sourced endogenously (autolysis) or exogenously (commercial enzyme preparations), or via fermentation (by addition of starter cultures). The resulting crude protein hydrolysate may undergo fractionation processes to yield an enriched bioactive peptide preparation or additional purification steps to isolate single peptides. Following the identification of the sequence of the isolated peptides, bioactivity is validated by testing chemically synthesized pure peptides (Udenigwe et al., 2014). The plant derived bioactive peptides are released from major protein sources such as flaxseed, soya, quinoa, pumpkin seeds and chia seeds by either gastrointestinal digestion or by the fermentation of milk prepared by using above mentioned substrates. It is also important to discover new peptides with health benefits in hydrolysates and fermented foods. Therefore, there has been a mounting research interest in the therapeutic potential of plant protein hydrolysates and their subsequent incorporation in functional foods and 'Food for Specified Health Uses' (FOSHU) related products where their biological activities may assist in the promotion of good health or in the control and prevention of diseases or extending the shelf-life of food products. Looking into the biofunctionality of bioactive peptides of fermented plant base milk, the present study was designed with following objectives;

1. To screen the proteolytic activity of different probiotic lactic acid bacterial strains.

2. To extract, analyze and compare the peptides derived from different substrates.

3. To investigate and identify the bioactive potential of the peptides for bioactivities.

4. To study the potential application of bioactive peptides in food industry.

CHAPTER 2

Review Literature

Bioactive peptides are fragments of proteins, having specific amino acid configuration and possessing desirable, positive effects on health and/or bodily functioning(Sánchez and Vázquez 2017). They are commonly classified on the basis of their functionalities as antimicrobial, antidiabetic, antihypertensive, immunomodulatory, anticancer and antioxidative (Ovando et al., 2018)

The study of bioactive peptides dates back to 1902 when Starling and Bayliss discovered a substance secreted from the intestinal lining with the potential of stimulating the secretion of digestive enzymes by the pancreas (Bayliss, 1902). The substance was named secretin and was found to be a peptide with its amino acid sequence determined. Several other peptides were discovered using a similar mechanism of purification and sequencing and the novel scientific concept of research was birthed (Schrader et al., 2014). In 1950, bioactive peptides from a food source was first discovered by Mellender who found that phosphorylated casein peptides stimulated the bone calcification of rechetic infants without Vitamin D (Berfenstam et al., 1955). Over the years, a considerable amount of research has been made on the isolation of bioactive peptides from various food sources.

Living things and products obtained from them; both at the microbial (fungi, bacteria) and complex categories are the most abundant sources of peptides (Daliri et al., 2017). Bioactive peptides have also been isolated from diverse food materials such as milk (Abd El-Fattah et al., 2017; Amorim et al., 2019), egg (Jahandideh et al., 2018; Du et al., 2019) soybeans (Lammi et al., 2018; Chatterjee et al., 2018), meat (Sayd et al., 2018; Zhang et al., 2019), rice and fish amongst several others. The breakdown of protein with a subsequent release of peptides occurs during food processing especially those involving operations like fermentation, germination and cooking but low efficacy, sensitivity towards gastrointestinal

enzyme and low intestinal permeability are the main challenges in the isolation and functionality of bioactive peptides. Nevertheless, the generation of potent peptides by enzymatic hydrolysis, chemical synthesis, microbial fermentation, chemical hydrolysis (acid or alkali) can benefit from a range of *in silico* techniques (FitzGerald et al., 2020; Hou et al., 2017).

2.1 Isolation of Bioactive peptides

Foods with remarkable protein content are potential raw sources of choice for bioactive peptide syntheses.

As per reported literature, common methods used for the production of bioactive peptides from foods or other protein-rich sources include enzymatic proteolysis, fermentation with optimally selected probiotics (Lee and Hur 2017) or directly by the chemical syntheses approach. In rare cases such, bioactive peptides have been isolated from uncommon plant parts and mushroom extracts (Geng et al., 2017; Prateep et al., 2017). From the reported literature, most common methods used in the production of antihypertensive peptides are:

2.1.1 Enzymatic hydrolysis

Enzymatic hydrolysis is the method used to produce bioactive hydrolysates via the addition of various protein hydrolysing enzymes like alcalase, achymotrypsin, bromelain, cryotin Flavourzyme, neutrase, orientase, papain, pepsin, pancreatin, pronase, protamex, protease N, protease A, thermolysin, trypsin, and validase (Halim et al., 2016). Gastrointestinal enzymes such as pepsin and trypsin have been used for the production of bioactive peptides. This method includes subjecting proteins to enzymes at optimum conditions including temperature and pH. (Morais et al., 2014). The controlled conditions are maintained using a thermostated instrument and by addition a solution of high pH and low pH condition. (Cheison and Kulozik 2017). The lentil protein concentrates have been treated with four different proteases (alcalase, savinase, protamex, and corolase 7089) at different hydrolysis times and their

angiotensin I-converting enzyme (ACE) inhibitory and antioxidant activities were evaluated. After a treatment of 2 h with savinase resulting hydrolysates showed the maximum ACE-inhibitory with IC_{50} value of 0.18 mg/ml and antioxidant activity (1.22 μmol of Trolox equivalent /mg of protein) (Garcia-Mora et al., 2014). Grass carp skin pieces were hydrolyzed with various enzymes such as alcalase (60°C and pH 8.0), proteinase K (37°C and pH 8.0), collagenase (37°C and pH 7.5), trypsin (after adjustment of the pH to 8.0) resulting in liberation of bioactive peptides with antioxidant as well as angiotensin-converting enzyme-inhibitory activity properties with usage of alcalase, collagenase and proteinase K (Yi et al., 2017).

Alcalase and papain enzymes were used to hydrolyse black-bone silky fowl. Peptides LER and GAG have been found with ACE inhibitory activity of 45.62± 2.40 μM; 253.07±6.66 μM respectively (Gu et al., 2012).

2.1.2 Fermentation

Fermentation entails the secretion of enzymes into their extracellular medium by the fermenting microbes (bacteria and yeast species) during their growth. Thus, inoculating protein-rich food materials with these types of microorganisms lead to lysis of protein hence, peptide production (Aluko 2015). The bioactive peptides, especially those are present in dairy sources released during lactic acid fermentation and during gastrointestinal digestion by enzymes and have been characterized for their usage as antihypertensive peptides (Sánchez and Vázquez 2017). To enhance their production, treatment of fermented peptides can be done by enzymes (Jakubczyk et al., 2013). Mostly commercial fermented milks with functional properties are based on *Lactobacillus helveticus* (Griffiths and Tellez 2013) Thus, with the adoption of one or more lactic acid bacteria, functional dairy products can be developed which support a healthy heart by maintaining blood pressure and heart rate (Beltrán-Barrientos et al., 2016).

2.1.3 Genetic Engineering

Recombinant DNA techniques have also been identified for the production of bioactive peptides. Microbiological genetic engineering technique has been proven more efficient as compare to enzymatic method in terms of yield and lower cost of production of antihypertensive peptides (Lv et al., 2003). However, this method has some shortcomings such as undesirable expression which can be dreadful for the host but this problem can be resolved by the separation of specific peptides from other peptides by the use of certain enzymes. Antihypertensive peptides were expressed with sequences SLVYPFPGPI, NIPPLTQTPV and DKIHPF in *Escherichia coli* (Losacco et al., 2007).

Antihypertensive peptides were obtained from ACE inhibitory peptides which were isolated from tuna frame protein. After consumption of recombinant or transformed *Lb. plantarum* NC8 (RLP) to SHR, there was decrease in SBP (P < 0.01), as well as decrease in levels of endothelin and Ang II (angiotensin) in plasma, heart, and kidney (Yang et al., 2015). VLVPV peptide have been isolated by the recombinant expression in modified transplastomic *Chlamydomonas reinhardtii*. It is cleaved by hydrolysing enzymes like chymotrypsin, pepsin and trypsin, and finally followed by high performance liquid chromatography. After 6 hours of the oral administration of the dose (peptide) of 30 mg/Kg of body weight to spontaneous hypertensive rats, systolic blood pressure reaching to normal levels (Ochoa-Méndez et al., 2016).

2.2 Sources of bioactive peptides

2.2.1 Animal sources

2.2.1.1 Dairy sources

Milk is often defined as the "ultimate" food because it provides all the nutrients which required to fulfil the nutritional needs of new born babies and ensures their development and

growth during the initial stages of life (Mills et al., 2011). Milk proteins are highly nourishing, possessing substantial biological value, and perform various functions in the body which promotes health. The identification of milk derived bioactive peptides was first reported by Mellander in 1950 (Berfenstam et al., 1955). After that discovery, numerous reports showed the presence of bioactive peptides in milk and its products show physiological effects on human health (Nagpal et al., 2011; Bhat et al., 2015).

Milk basically consists of two types of protein namely: casein (αs1, αs2, β, κ) representing about 80% of the total milk protein, and the whey protein (20%) comprising of; beta-lacto globulin, alpha-lacto globulin, immunoglobulin and bovine serum albumin (Mohanty et al., 2016). Bioactive peptides derived from milk are known to be versatile in health functionalities such as antimicrobial, antidiabetic, antithrombotic, antioxidant, and cholesterol-lowering (Korhonen and Pihlanto 2006).

Yusuf et al., (2018) investigated the antidiabetic potential of milk protein and its hydrolysate *in vivo* using diabetic rats. Their study proved that oral administration of milk proteins (MP) or milk protein hydrolysates (MPH) by the rats significantly reduced the blood glucose level, total lipids of blood plasma, including; low density lipoproteins (LDL), very low-density lipoproteins (VLDL), triglycerides and total cholesterol, in rat plasma, thus confirming that MP and MPH could be used as anti-diabetic agents.

Lacroix and Li-Chan (2013) researched on the inhibition of the enzymes DPP-IV and α-glucosidase by pepsin-treated whey proteins. Hydrolysates of whey protein isolates produced by digestion with peptic enzyme were explored for their functionalities as α-glucosidase and DPP-IV inhibitors. Their results revealed that α-lactalbumin hydrolysate, of all hydrolysates produced, displayed the greatest potential with an IC_{50} value of 0.036 mg/mL. They also discovered that only α-lactalbumin, WPI and β-lactoglobulin, and hydrolysates showed some inhibitory strength in opposition to α-glucosidase.

Nongonierma and Fitzgerald (2014) evaluated the DPP-IV inhibition potential of 12 different bioactive peptides derived from milk proteins. Only 8 peptides namely GL, AL, VA, WV, FL, HL, SL, and IP were found to possess inhibitory potentials. Results obtained showed that the WV peptide had the only non-competitive, highest potential with an IC_{50} value of 65.0; its inverse VW, however, displayed no inhibition. With the in-silico approach, Tulipano et al., (2011) were able to show that the tripeptide Ile-Pro-Ala obtained from whey proteins possesses the DPP-IV inhibition potential.

Zhou et al., (2016) checked the destructive functionality of the bovine milk peptide - ACFP against ovarian cancer cells and found it to be effective in apoptosis promotion and for viability inhibition of the cells.

Sah et al., (2014) conducted a study focused on evaluating the effect of probiotic strains on anticancer activity. Three lactic acid bacteria strains namely; *Lactobacillus casei* ATCC 393, *Lactobaciilus paracasei* and *Lactobacillus acidophilus* ATCC 4356 were used for proteolysis and the liberated peptides displayed strong antimutagenicity 26.32%.

Sah et al., (2016) examined the effect of refrigerated storage on the antiproliferation of HT-29 cells of synbiotic yoghurt formulated with pine apple peel powder. A significant inhibition against the cells was observed during storage, in comparison to the control (not containing pine apple peel powder) preparation thus, indicating its anticancer functionality.

Khalesi et al., (2017) mentioned the potential of camel milk to control the cancer cells somewhat higher than bovine milk. Rafiq et al., (2018) evaluated the anticancer activity of WSP extracted from cheddar cheeses obtained from buffalo and cow during their ripening process. An experimental study was conducted on colon cancer model (HT-29) cells with different concentrations ranging from 100-500 µg/ml of WSP of cow milk Cheddar cheeses (CCC) and buffalo milk cheddar cheeses (BCC) at different ripening stages. The best combination achieved was 500 µg/ml after 120 and 150 ripening days. It was proven that

BCC showed the higher inhibition on lung cancer cells than CCC. BCC with concentration of 500 μg/ml after the 150th ripening day decreased the cell count upto 18.12%. Medeiros et al., (2018) performed anti the crude protein extract (CPE) obtained from goat milk whey with ammonium sulphate precipitation used for protein fractionation. Subsequently, SDS-PAGE and a two-dimensional electrophoresis was performed to characterize the proteins. The protein extracts with concentrations of 0.05 and 0.1 μg/ml were observed effective for the reduction of C6 glioma cells in rat by 70%. This study also reported that CPE can be used as an antioxidant and bacteriostatic agent.

Chen et al., (2014) reported the production of ACE peptides, VPP and IPP, by fermentation of milk with *Lactococcus* lactis strains. These peptides were isolated by ultra-performance liquid chromatography with quadrupole-time-of-flight mass spectrometry. By oral dose of these peptides significantly reduced the systolic, diastolic, and mean blood pressure of SHR by 15 to 18 mm.

Elkhtab et al., (2017) identified three antihypertensive peptides from kombucha milk fermented by *Lactobacillus casei* and followed by purification with high performance liquid chromatography. These peptides sequenced as VAPFPEVFGK, LVYPFPGPLH, and FVAPEPFVFGKEK showed the angiotensin I-converting enzyme inhibition activity with IC_{50} values of 0.03, 0.03 and 0.75μM respectively.

Georgalaki et al., (2017) examined the milk of various animals such as cow, goat and sheep which was fermented with *Lb. delbrueckii* spp. and *S. thermophilus*. Fermented extracts were followed by HPLC and further all fractions subjected to the mass spectrometry. It has been reported that identification of Angiotensin I-converting enzyme inhibitory peptide differs with type of milk and type of strain. Angiotensin I-converting enzyme inhibitory activity % of goat milk fermented milk (*S. thermophilus*) was higher as compared to other milks.

Ibrahim et al., (2017) identified three most active antihypertensive peptides PEQSLACQCL (whey βlactoglobulin), QSLVYPFTGPI(βcasein) and ARHPHPHLSFM(κ-casein)) from milk proteins of goat after the digestion of gastric pepsin. After the fractionation of hydrolysates of proteins by size exclusion chromatography, peptides were isolated by the process of reverse phase high performance of liquid chromatography from fraction F4. The whey and casein derived peptide exhibit the IC_{50} values of 4.45 µM/l and 4.27 µM/l respectively.

In one of the recent studies observed by Cui et al., (2020) prepared and evaluated the macromolecular peptides from the milk after gastrointestinal digestion. Feeding of digestive milk to the spontaneous hypertensive rats leads to the reduction in blood pressure by value of 60 mmHg from the original blood pressure. Effect of macromolecular peptide was found to similar the effect of captopril (ACE inhibitor).

2.2.1.2 Egg

Egg has its protein in the egg white and yolk. The proteins obtained from hen eggs, predominantly comprise of ovotransferrin (Giansanti et al., 2012), ovalbumin, ovomucin, ovomucoid (Kovacs-Nolan et al., 2000), lysozyme, avidin, cystatin, ovoinhibitor (Kovacs-Nolan et al., 2005), lipoprotein and glycoprotein (Omana et al., 2010)**.** Egg is also known to be a functional food rich in protein of high biological value (Surai and Sparks 2001) from which several bioactive peptides can be derived. These proteins and peptides have been discovered to impart their functionality either in the raw form or when hydrolysed *in vivo* or *in vitro* (Hartmann and Meisel 2007). Egg peptides are known to possess anticancer (Watanabe et al., 1998) antimicrobial (Memarpoor-Yazdi et al., 2012), antioxidant (Carrillo et al., 2016), antihypertensive (Majumder and Wu 2011), antidiabetic (Yu et al., 2011) and bone growth promotion (Eckert et al., 2013). In a research conducted by Yu et al., (2011) the

peptides RVPSLM and TPSPR were isolated from egg white hydrolysate via enzymatic hydrolysis with alcalase and found to be effective (IC_{50} values of 23.07 and 40.02µmol/L) in inhibiting the enzyme α-glucosidase and thus of functional antidiabetic activity. Other peptides isolated in the course of the study include TPSPR, DLQGK, AGLAPY, RVPSL, DHPFLF, HAEIN and QIGLF and examined for α-amylase inhibition but none was found to show any inhibition (IC_{50} >150g/mol)

Using egg yolk by-product as the protein source, Zambrowicz et al., (2015), isolated multifunctional novel peptides of antioxidative and antidiabetic potentials. They found that the peptides: RASDPLLSV, RNDDLNYIQ and LAPSLPGKPKPD showed significant DPP-IV inhibition with IC_{50} values ranging from 361.5 to 426.25µmol/L. LAPSLPGKPKPD in particular was found to have an additional α-glucosidase inhibitory potential with IC_{50} value of 1065.6µmol/L

Nongonierma and Fitzgerald (2014b) developed an *in-silico* model to estimate the DPP-IV inhibition capability of 72 dietary proteins from various sources. Their calculations showed that chicken egg ovomucoid has a relatively lower potency index (PI) of chicken egg 0.13×10^{-6} $\mu M^{-1}g^{-1}$ in comparison with 17.89×10^{-6} μM^{-1} g^{-1} from bovine κ-CN. Chicken egg ovotransferrin was calculated to have a PI less than 5.00×10^{-6} μM^{-1} g^{-1}

With enzymatic hydrolysis (278P) of egg Ovotransferrin Moon et al., (2017) checked antihypertensive and anticancer potential of hydrolysates. The IC_{50} values of the promod 278P hydrolysate of ovotransferrin against 5 different cancer cells was MCF-7 (10.05±1.55), HeLa (3.45±0.94), HepG2(4.43±1.87), HT-29 (4.92±0.63), and LoVo (10.43±3.91) mg/ml with no activity shown by ovotransferrin.

Fan et al., (2019) identified antihypertensive peptides from egg white proteins after the gastrointestinal digestion by thermolysin. Twenty-seven peptides were identified through de

novo sequencing but among all these, highest ACE inhibitory was shown by pentapeptide and hexapeptide and the reason for this was the presence of hydrophobic N terminus and hydrophobic tetrapeptide C terminus. Feeding of thermolysin digested egg white hydrolysate at a dose of 1000mg/ kg of body weight, showed the reduction of SBP, DBP of SHRs from the values of 171.7, 139.4 mm Hg to the 112.3, 83 mm Hg respectively.

In a research conducted by Khueychai et al., (2018), a dipeptide, YV was isolated from ostrich egg white (ovalbumin). For the isolation of peptide, ovalbumin was subjected to anion exchange chromatography followed by enzymatic hydrolysis by the alkaline for 2-10 hours. After 8 hours of hydrolysis, hydrolysate was subjected to the reversed phase high performance liquid chromatography to get the purified peptide. This novel dipeptide was found to possess strong ACE inhibition activity with an IC_{50} value of 63.97µg/mL.

Using defatted egg yolk, Yousr and Howell (2015) isolated a peptide (SDNRNQGY) possessing similar ACE inhibitory activity as shown by captopril. They hydrolyzed the egg yolk by using enzymes; pancreatin and pepsin and then followed by ultrafiltration. Fractionation of peptide was done by gel filtration and subjected to the liquid chromatography –mass spectrometry analysis to identify the amino acid sequence. Fraction 2 exhibit the lower activity with IC_{50} of 5.44 mg/mL while fraction 56 exhibit the higher activity 69.2 % with IC_{50} value of 3.35 mg/mL.

2.2.1.3 Marine

Marine foods are rich sources of proteins and bioactive peptides with cosmeceutical, pharmaceutical and nutraceutical functionalities (Venkatesan et al., 2017). Such peptides have been discovered to exhibit versatile bioactivities such as antihypertensive, antioxidant, antioxidative, antimicrobial, antidiabetic and neuroprotective effects (Cheung et al., 2015). A slight number of fish protein hydrolysates have manifested the activity of stimulating glucose uptake *in vivo* and are applicable to the management of hyperglycaemia in addition to

methodical therapy. These hydrolysates can alleviate glucose tolerance either by inciting glucose uptake or by increasing insulin response in target cells (Cheung et al., 2015). Collagen, for example, is an abundant protein that can be isolated from common fish by-products such as skin (Venkatesan et al., 2017) which has been used extensively in the manufacture of antidiabetic pharmaceuticals (Lauritano and Ianora 2016).

Wang et al., (2015) in a research study compared the enzymatically isolated hydrolysates for DPP-IV from the skin gelatin of Tilapia and Halibut fishes. The halibut skin gelatin hydrolysates include SPGSSGPQGFTG, GPVGPAGNPGANGLN and PPGPTGPRGQPNIGF with IC_{50} values of 101.6, 81.3 and 146.7 respectively. The Tilapia skin gelatin hydrolysates include IPGDPGPPGPPGP, LPGERGRPGAPGP and GPKGDRGLPGPPGRDGM with IC_{50} values of 65.4, 76.8 and 89.6 respectively. As shown in their results, it was found that the warm-blooded fish gelatin skin (Tilapia) has a higher antidiabetic potential.

In another study conducted by Nasri et al., (2015) to investigate the antidiabetic capabilities of hdrolysates and the raw, undigested goby fish muscle proteins on hyperglycaemic rats fed with fat-high-fructose diet (HFFD), they found that these hydrolysates are very effective in controlling diabetes by decreasing the blood glucose levels, α-amylase activity and hepatic glycogen levels.

Several peptides of various functionalities have been synthesized using tuna cooking juice as the protein source. Such peptides have been found to possess antioxidative (Hsu et al., 2009), anticancer (Huang et al., 2014) and DPP-IV inhibition (Huang et al., 2012) potentials. Huang et al., 2012 conducted a study to determine the potential of peptides enzymatically isolated from tuna cooking juice (5.44% protein content) in inhibiting DPP-IV. The enzymes orientase (OR) and Protease XXIII (PR) were used and three peptides which showed dose-dependent DPP-IV inhibition were isolated. Three peptides PACGGFYISGRPG (1304.6 Da)

CAYQWGRPVNRIR (1690.8 Da) and PGVGGPMGPIGPCYQ (1412.7 Da) were isolated and found to possess DPP-IV inhibitory potential with IC_{50} values of 96.4 M, 78.0 and 116.1, respectively. Following their enzymatic isolation, simulated gastrointestinal digestion (SGID) was also carried out to check its effect on the DPP-IV inhibition functionality of the peptides. Their results showed that the functionality was secured or even enhanced following the SGID treatment.

Pan et al., (2016) extracted a novel anticancer hexapeptide Phe-Ile-Met-Gly-Pro-Tyr (FIMGPY) from Skate (*Roja porosa*) through the hydrolysis of its protein cartilage, with chromatographic and ultrafiltration purification techniques, with its anticancer strength measured with HeLa cells. The hexapeptide showed a strong antiproliferative tendency against the cells, and induced apoptosis by caspase-3 activation and up regulation of Bax/Bcl-2 ratio, thus suggesting the nutraceutical and food usage of the bioactive peptide for cancer therapy. Nurdiani et al., (2017) used protein fraction from flathead by-product to obtain bioactive peptides. Enzymatic hydrolysis by protease was performed to get low MW peptides. Presence of low MW (<3 kDa) peptides inhibited the growth of HT-29 colon cancer cells upto 91.04%. In another study conducted by Wang et al., (2012) an antimicrobial peptide Temporin-1CEa (FVD-LKKIAN-IINSIF-NH2) obtained from Skin of Chinese brown frog (*Rana chensinensis*) showed anticancer property against breast cancer cell line (MDA-MB-231 and MCF-7). RP-HPLC was used for purification of peptide from crude hydrolysate. Their results concluded that cytotoxic activity of peptides might be mediated by direct membrane-destruction and intracellular calcium–related mechanisms.

In a study conducted by Huang et al., (2017)**,** isolated 2 novel tetrapeptides SCH-P10 (Asp-Tyr-Val-Pro) and SCH-P9 (Leu-Pro-Gly-Pro) from *Sinonovacula constricta,* using enzymatic *(*trypsin (EC 3.4.21.4), pepsin (EC 3.4.23.1), papain (EC 3.4.22.2) and alcalase (EC 3.4.21.62) hydrolysis. The anticancer potentials of these peptides were checked against

two cancer cells: DU-145 and PC-3, usually proliferating from the prostate. Their results revealed that both peptides aided apoptosis in both cancer cells and could be employable for prostate cancer therapy. Quah et al., 2017 performed a comparative study of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), papain (EC 3.4.22.2) and alcalase (EC 3.4.21.62) hydrolysates of sponge *Xestospongia testudinaria* against cervical cell lines (HeLa) and found the papain hydrolysate is the most active against HeLA cell lines.

Wang and Zhang (2017) extracted and hydrolysed the whole protein of *Spirulina platensis* by using three gastrointestinal-derived endopeptidases (chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4) and pepsin (EC 3.4.23.1)). The hydrolysates were segmented into four fractions (Tr1-Tr4) using gel filtration chromatography, and the Tr2 fraction showed the strongest anti-proliferative potential against three tested cancer cells (SGC-7901, MCF-7 and HepG-2), with IC_{50} values of 48.25, 36.42 and less than 1.25µg ml^{-1}, respectively. In addition, the octapeptide - HVLSRAPR was identified from the Tr1 fraction and was found to exhibit a strong inhibition against HT-29 cancer cells with 99.88 µg ml^{-1} as its IC_{50} value.

In a study reported by Lee et al., (2012), the antihypertensive peptide sequenced as WYPAAP was isolated from by-products of duck skin after its proteolysis with 9 proteolytic enzyme preparations. These enzymes were alcalase, collagenase, flavourzyme, neutrase, papain, pepsin, protamex, trypsin, and α-chymotrypsin. This hexapeptide was obtained after purification process by using fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC). This identified peptide had an IC_{50} value of 137 µM. The mean systolic blood pressure (SBP) of SHR was quickly reduced by injecting this peptide at a dose of 1 mg/kg of body weight as compare to treatment of captopril. In a research study Cao et al., (2010) identified and evaluated the potential of antihypertensive activity of *Acetes chinensis* through vivo study. *Acetes chinensis* (shrimp marine) was hydrolysed with pepsin. Hydrolysate was fractionated with the gel filtration and the resulted

fraction (1320Da to 311Da) was further fractionated by reverse phase high performance liquid chromatography. Out of 15 fractions, fraction 9 showed the 92.7% ACE inhibitory activity and LHP peptide was identified with values of IC_{50} was 1.6μM. In addition, they have been that this tripeptide corresponds to structure of antihypertensive peptide because it contains proline at C-terminal and leucine at the N-terminal. After the oral administration of this peptide (at a dose of 6mg/kg of body weight) to the SHRs, SBP was reduced by 31±3.3mmHg at 4 hours and 36±2.8 mmHg at 6 hours. Antihypertensive peptides, GMNNLTP (728 Da) and LEQ (369 Da) were identified after hydrolysis of cultured biomass of the marine microalga (*Nannochloropsis oculate*) with pepsin followed by the purification method (Sephadex gel filtration).The observed IC_{50} value of these two purified peptides were 123 and 173μM respectively and production of nitric oxide was also increased (Samarakoon et al., 2013). Peptides with the amino sequence TFPHGP and HWTTQR were isolated from the seaweed pipefish muscle protein. The seaweed pipefish muscle protein was hydrolysed with various enzymes like papain, alcalase, neutrase, pronase, pepsin and trypsin, but amongst all of them alcalase showed the highest ACE-I inhibitory activity in fraction 3(II) and fraction 3(III) (0.62 mg /ml and 1.44 mg/ml respectively). After the fractionation done by reverse-phase high performance liquid chromatography (Wijesekara et al., 2011). After the enzymatic hydrolysation of tuna frame protein with various enzymes (alcalase, neutrase, pepsin, papain, a-chymotrypsin and trypsin.), ACE inhibitor which comprised of 21 amino acid residues (GDLGKTTTVSNWSPPKYKDTP) has been identified. While with the pepsin degradation, this peptide expressed the maximum IC_{50} value, was 11.28 μM. After the 6 hours of the oral administration of this peptide to the rats showed the reduction in systolic blood pressure(21 ± 2 mm Hg) at dose of 10 mg/kg of body weight and this antihypertensive activity was similar to that of captopril which is a commercial antihypertensive drug (Lee et al., 2010).

2.2.1.4 Meat and its by-product

Meat and its by-products such as; blood, collagen, mechanically recovered meats and trimmings are typically recognised as being composed of high protein content and accordingly, they are potential proteolytic substrates subjectable to processing for the isolation of bioactive peptides (Garcia-Mora et al., 2014).

2.2.2. Plant sources

2.2.2.1 Cereals and pseudocereals

Proteins from cereal-food sources contribute the larger fraction of the globally consumed dietary protein, especially for developing countries where wheat and rice are staple sources of plant protein (Van der Spiegel et al., 2013; Shewry and Halford 2002). The protein content in cereals ranges from 6-15% with a larger fraction located in the storage proteins (Shewry and Halford 2002). The prominent storage protein(s) vary with the cereal type, but general examples include glutelins (wheat, barley and rice), prolamins (maize), globulins (oats), thionin (rye and oat) and germins (wheat and barley) (Cunsolo et al., 2012).

Psedocereals are non-grassy, plant-based foods with similar composition and functionalities with cereals. However, they differ in their number of seed leaves (cotyledons) with two seed leaves (dicotyledons) as opposed to true cereals with one (monocotyledons) (Alvarez-Jubete, Arendt and Gallagher, 2010). Common examples include quinoa, buckwheat and amaranth.

Cereals and pseudocereals are potential plant sources of peptides with health promoting benefits (Malaguti et al., 2014). Lunasin, a bioactive peptide with a 43 amino acid peptide chain length was initially isolated from soyabean and later from cereals such as; rye, barley, wheat and rice and amaranth, a pseudocereal (Jeong et al., 2007; Jang et al., 2011). This peptide was discovered by Hernandez-Ledesma et al., (2016) to have antioxidative protection effects on DNA and the potential of terminating proliferative cell multiplication of cancer cells.

2.2.2.2 Rice

Rice is a staple food in most of the Asian countries. Its proteins have high biological value than other cereals proteins (Yang et al., 2017). In a study aimed at production of ACE inhibitory activity, broken rice was utilized as functional ingredient. Rice Protein was isolated from defatted broken rice flour and then hydrolysed with pepsin (0.1g pepsin/g protein). After that, fractionation was done by two times gel filtration liquid chromatography followed by reversed phase high performance liquid chromatography. Fractions (F5-IV) and (F5-V) was observed as most active fractions and amino acid sequence of these fractions were determined by the liquid chromatography-mass spectrometry. Similar peptides with sequence FNVPSRYGIY, PWHNPRQGGF and SPFWNINA were obtained in both fractions and binding energy of these peptides to ACE was -8.01, -5.03 and -5.67 Kcal/mol respectively (Pinciroli et al., 2019).

Bran of rice is a major by-product of rice milling industry. Boonla et al., (2015) conducted a study to evaluate the antihypertensive activity of the rice bran. Defatted rice (Jasmine rice) bran was hydrolysed with enzyme(protease) for 4 hours. Rice bran protein hydrolysate was freeze dried and subjected to the ultrafiltration. To observe the effect of rice bran protein hydrolysate, groups (2K-1C groups) and same operated control groups were made. Their findings showed that ACE activity level in plasma of 2K-1C was decreased after consumption of rice bran hydrolysate.

Shobako et al., (2017) isolated two antihypertensive peptides from the rice bran after the thermolysin digestion. This hydrolysate was subjected to the fractionation process (reversed phase high performance liquid chromatography). Amino acid sequence (LRA and YY) of peptides were determined by the 492-protein sequencer and then purified peptides were quantified by the liquid chromatography- mass spectrometry. The peptides LRA and YY exhibit the IC_{50} value of 62.0±1.8μM and 16.5±0.6μM respectively. To examine the ACE

inhibitory activity of the peptides, spontaneously hypertension rats were taken. Their findings showed that after 4 hours of the feeding of the LRA (0.25 mg/Kg of bodyweight) and YY (0.5 mg/Kg of bodyweight) to SHRs, reduced the blood pressure of rats.

Hatanaka et al., (2015) conducted a study on the evaluation of the antidiabetic functionality of peptides from two rice products – rice bran and sake lees enzymatically digested with a commercially available protease – Denazyme AP derived from *Aspergillus oryzae.* Their findings revealed that the hydrolysate produced from the bran had higher DPP-IV inhibition with an IC_{50} value of 1.28±0.18 mg/ml in comparison with the hydrolysate from sake lees which displayed a relatively lower inhibition bioactivity with an IC_{50} value of 27.55 ± 5.76 mg/ml.

Li (2014) derived a pentapeptide (EQRPR) from rice bran and showed antiproliferative properties against human cancer cell models (MCF-7 and MDA-MB-231). The pentapeptide, through a caspase-dependent route, was discovered to remarkably retard the proliferation of human breast cancer cells by initiating apoptosis.

Kannan et al., (2010) conducted an examination to evaluate the anticancer strength of the peptide (Glu-Gln-Arg-Pro-Arg) derived from rice bran, enzymatically digested with alcalase. Liver (HepG2) and human colon (Caco-2) cell lines were used and their findings revealed that peptides with molecular weight of <5 and 5-10 kDa had an 80% repression against the Caco-2 cancer cells and the <5 kDa segments howing a 50 % inhibition against HepG2 cell growth.

2.2.2.3 Wheat

Wheat is a worldwide staple food and its germ (inner layer of wheat grain) is quite rich in nutrients including proteins and essential amino acids (Brouns et al., 2012). Iwaniak et al., (2014) reported wheat germ hydrolysate as an effective antihypertensive agent and the main

reason of showing antihypertensive effect by wheat germ hydrolysate was the presence of tripeptide IVY. Effect of IVY was examined on mice and it was observed that after the 8 min of the injection of 5 mg/kg IVY, arterial pressure was reduced by 19.2 mm Hg. This reduction in pressure was observed due to the action of aminopeptidase, present in plasma, on IVY and formation of effective ACE inhibitor (VY). Karami et al., (2019) conducted a study on the release of antihypertensive peptides from crude wheat germ protein hydrolysate by proteinase K hydrolysis. Fractionation of hydrolysate was done with reversed phase high performance liquid chromatography and amino acid sequence was identified by LTQ-Orbitrap XL mass spectrometer. Identified peptides VALTGDNGHSDHVVHF, VDSLLTAAK, MDATALHYENQK, IGGIGTVPVGR and SGGSYADELVSTAK were found to exhibit good antihypertensive activity with IC_{50} values of 189.3 µg/ml, 159.7µg/ml, 303.6µg/ml, 125.7µg/ml and 128.2 µg/ml respectively.

Wheat gluten was also experimented for the isolation of antihypertensive peptides. A protease (alcalase) isolated from *Pseudomonas aeruginosa* was used for protein lysis. Two peptides sequenced as SAGGYIW and APATPSFW containing tryptophan at their carboxyl end and checked for antihypertensive activity. These peptides were found to exhibit the half maximum inhibitory concentration with values of 0.02 mg/mL, 0.036 mg/mL respectively (Zhang et al., 2020).

2.2.2.4. Brewers spent grain

Brewer's spent grain, which represents about 85% of the overall by-products occupies the largest fraction of by-products obtained during beer-brewing (Mussatto 2014; Xiros and Christakopoulos 2012). It is usually obtained from barley which is the raw material commonly used for beer production (Lynch et al., 2016). Connolly et al., (2017) isolated DPP-IV inhibitory peptides from protein-enriched brewer's spent grain isolate hydrolyzed using alcalase enzyme. Following the enzymatic digestion which occurred for a 240 minutes'

time period, the IC_{50} value for DPP-IV inhibition was determined to be 3.57 ± 0.19 mg mL^{-1}. After each sequential treatment of ultrafiltration fractionation, simulated gastrointestinal digestion and RP-HPLC, the IC_{50} value for DPP-IV inhibition was checked. Their results showed that ultrafiltration had no significant effect on the inhibition potential. Further exposure of the alcalase hydrolysate to simulated gastrointestinal digestion effected an increase in the DPP-IV inhibition activity. They reported that following the fractionation of the hydrolysates using (RP-HPLC), the 28th fraction displayed the highest DPP-IV inhibition. The novel peptides ILLPGAQDGL and ILDL with DPP-IV inhibition IC_{50} values of 145.5 and 1121.1 μm were discovered within the fraction.

Lin et al., (2012) in a study involving the optimization of the enzymatic hydrolysis of proteins extracted from brewer's spent grain by alcalase also investigated the *in vitro* inhibition of the hydrolysate obtained against α-glucosidase enzyme. Their findings showed that at a concentration of 4.0 mg/mL, α-glucosidase inhibition of 21.42% was achieved. Their study also showed that purification by ultrafiltration had a relatively higher inhibition in comparison to purification without ultrafiltration. The overall fraction of the protein hydrolysate with molecular weight not up to 5kDa was discovered to have an inhibition of 56.41%.

Wei et al., (2019) evaluated the prolamin based distilled spent grain to isolate the ACE inhibitory hydrolysates. Dried flour of the distilled spent grain was used for making the protein isolate (DDSG-PI) by using three different enzymes (alcalase, flavourzyme and neutrase). All 3 hydrolysates were exposed to check the ACE inhibitory activity. Among these, maximum activity (79.05% at 3 h treatment) was shown by alcalase hydrolysates followed by neutrase hydrolysate (67.58% at 4 h) and flavourzyme hydrolysate (42.84% at 5 h). As maximum activity was shown by alcalase hydrolysates, therefore, it was further fractionated by ultrafiltration in four fractions on the basis of their molecular weight (>5 kDa,

3–5 kDa, 1–3 kDa, and <1 kDa). Fraction with molecular weight <1kDa showed the highest ACE inhibitory activity with IC_{50} value of 6.750 µg/ml. Further fraction (<1kDa) was fractionated again into fraction (1-8) with reversed phase high performance liquid Chromatography and results showed that fraction 6 exhibited the 88.99% ACE inhibition activity. Six antihypertensive peptides (AVQ, YPQ, NQL, AYLQ, VLPVLS, and VLPSLN) identified by quadrupole time-of-flight mass spectrometry from the fraction 6. Peptides AVQ, YPQ, AYLQ, VLPVLS, NQL and VLPSLN exhibited the ACE inhibition with IC_{50} value of 181.0 µM, 220.0 µM, 228.6 µM, 248.1 µM, 704.6 µM, and 1246 µM respectively. Among all, VLPVLS was found to present in highest concentration (16.96 mg/g).

A study conducted by Connolly et al., (2014) involving the evaluation of in vitro dipeptidyl peptidase IV (DPP-IV) and as well as angiotensin converting enzyme inhibitory properties of protein hydrolysate derived from pale brewer spent grain. Hydrolysis of protein enriched isolate of brewer spent grain was done with eleven commercial enzymes (alcalase, corolase PP, corolase L10, flavourzyme, prolyve, protex 6, protamex, promod, promod 144MG, promod 439, promod 24P and trypsin 250). Their finding revealed that ACE inhibition was increased with the increase in the concentration of the hydrolysate. ACE inhibition % of the brewer spent grain protein isolate 30.69 ± 4.58, 17.46 ± 4.76 and 0.00 ± 3.76% inhibition at 1.00 and 0.50 and 0.25 mg/ml of the hydrolysate concentration. Among all, brewer spent grain hydrolysed with Prolyve enzyme was found to exhibit the highest angiotensinogen converting enzyme inhibitory activity (89.25 ± 2.46% at 1 mg/ml).

2.2.2.5 Amaranth

Amaranth is a pseudocereal which can serve as cereal as well as green leafy vegetable. It contains bioactive components and can be used as an ingredient to express its functional properties (Bhattarai 2018). Ontiveros et al., (2020) examined the Amarnath hydrolyzate enriched cookies to verify their antihypertensive properties. The obtained results showed that

feeding of amaranth hydrolysate enriched cookies (1.2 g/kg of body weight) to the SHRs reduced the blood pressure of rats (p > 0.5) compared to the feeding of control cookies (p < 0.5).

Ramírez-Torres et al., (2017) analyzed the effect of amaranth derived protein hydrolysate after the hydrolysis with alcalase. Before hydrolysis of Amaranth protein extract, response surface optimization was done to optimize the degree of enzymatic hydrolysis. Optimized value of degree hydrolysis was 74.77% which exhibited 93.53% ACE inhibitory activity. To conduct the *in vivo* study in spontaneously hypertensive rats, hypertensive rats were supplemented with water (1.5 ml), captopril (25 mg/kg of body weight) and amaranth hydrolysate (1.2 g/kg of body weight) separately. Their findings revealed that mice that are supplemented with amaranth hydrolysate showed reduction in systolic blood pressure after the 4 hours and captopril supplemented rats showed reduction in systolic blood pressure after 3 hours compared to the water supplemented rats (p<0.5).

Suárez et al., (2019) examined the potential of inhibitory action of antihypertensive peptides derived from amaranth. After the preparation of Amaranth protein isolate (API) and amaranth protein hydrolysate (AH), emulsion (80:20) was prepared with protein mixture 1:1 of API and AH with or without 2% of VIKP peptide and sunflower oil. To conduct the *in vivo* study, 8 groups of spontaneously hypertensive rats (Negative control group, Captopril group, Aliskiren group, API group, AH group, VIKP group, Emulsion group, Emulsion + VIKP group) were made. Among all the groups, most significant reduction in systolic blood pressure was obtained in case of Emulsion group and Emulsion + VIKP group by the value of 42±2 and 35±2mmHg respectively from the original values.

Velarde-Salcedo et al., (2013) in a study regarding the potential of peptides obtained from Amaranthus protein hydrolysis to inhibit DPP-IV *in vitro* discovered that peptides contained in all protein fractions obtained from amaranth possess the potential to inhibit the DPP-IV

enzyme. In their study, albumins, globulins (7S and 11S), and glutelins were enzymatically digested with trypsin. Peptides released were then examined for their potential to inhibit DPP-IV. Their results showed that the glutelins fraction displayed the strongest inhibition potential and the weakest by the 11S globulins proteins.

2.2.2.6 Quinoa

Quinoa contains high biological value protein and albumin is the major fraction of quinoa protein. In a study conducted by Zheng et al., (2019), potential of quinoa protein (albumin) was evaluated. Albumin contained quinoa bran was sequentially hydrolyzed with alcalase and trypsin. The degree of hydrolysis of quinoa bran was 24.26±3.61% with 62.38±5.64% ACE inhibition. Fractionation of protein hydrolysate was done by gel chromatography, five fractions (A-E) were obtained and among the five fractions, fraction D showed the highest ACE inhibitory activity i.e. 79.78 ± 4.17%. Fraction D was further fractionated by reversed phase high performance liquid chromatography into 8 fractions(D1-D8). Among these, D7 exhibited the highest ACE inhibitory activity (78.08 ± 4.07) %. D7 fraction subjected to liquid chromatography-mass spectrometry analysis and obtained peptide (RGQVIYVL (946.6 Da)) exhibited the angiotensinogen converting enzyme inhibitory activity with an IC_{50} value of 38.16μM. Molecular docking of this peptide revealed the strong binding power of the peptides with active site of ACE enzyme. Feeding of this peptide to SHRs with different doses (100-150 mg/kg of body weight) showed the significantly reduction in SBP as well as DBP of rats.

Moreno-limón and González-luna (2018) investigated the antihypertensive potential of the protein hydrolysate derived from the Quinoa grains. Protein isolate from defatted quinoa seeds was hydrolyzed with alcalase and flavourzyme. After hydrolysis, fractions (10-15 kDa) were determined by the process of sodium dodecyl sulfate polyacrylamide gel electrophoresis which is difficult for shorter peptides (1-5 kDa). So, followed by the gel filtration

chromatography. Their findings revealed that alcalase hydrolysis leads to the identification of peptides with molecular weight (4.25 kDa, 2.71 kDa and 1.11 kDa) have high potential of ACE inhibition.

Ujiroghenea et al., (2019) conducted a study for the evaluation of ACE inhibition potential of germination-based protein derived from the quinoa yogurt beverage. Firstly, sprouted quinoa milk was inoculated with *Lactobacillus casei* SY13 and *Lactobacillus casei*. After 8 hours of fermentation followed by the enzymatic hydrolysis, protein hydrolysate of quinoa yogurt beverage (QYB) was isolated. Their results showed that inhibitory activity of *Lactobacillus casei* inoculated QYB was varied with germination hours (44.47–81.31%, 65.22–85.12%, and 47.37–87.18% at 0 h, 24 h and 72 h respectively). 19 fractions(F1-F19) from quinoa yogurt beverage protein hydrolysate were obtained. *Lactobacillus casei* inoculated hydrolysate showed the highest ACE inhibitory activity in fraction F14 (170.26± 37.28 mg/mL). After reverse phase high performance liquid chromatography and mass spectrometry analysis, five peptides LGGIWHL, VAHPVF, IRAMPVAV, ALFPTHR and LAHMIVAGA were identified in the fraction 14.

Vilcacundo et al., (2017) conducted a study on the release of antidiabetic peptides from Quinoa by a simulated gastrointestinal digestion, *in vitro*. The antidiabetic activities of the isolated peptides were evaluated on their inhibition of α-amylase, α-glucosidase and DPP-IV enzymes following the gastrointestinal digestion. Fractionation of the peptides by ultrafiltration occurred and the inhibitory potential of the fractioned peptides were also evaluated. Results were represented as D0- digestion starting point, GD120- gastric digest obtained after incubating with pepsin for 120min, ID60 and ID120 representing digests after 60min incubation with pepsin and 120min incubation with pancreatin respectively. Their results showed that fractionated peptide with molecular weight less than 5kDa had the highest α-glucosidase inhibition with IC$_{50}$ value of 1.45±0.12a and ID120 had the lowest inhibition

(1.81 ± 0.03b). ID120 showed the strongest inhibition for α-amylase with an IC_{50} value of 0.19 ± 0.02 and ID60 peptide wit molecular weight less than 5kDa had the weakest inhibition of 1.09 ± 0.04 IC_{50} value. Of all peptides isolated, only the D0 peptide showed no inhibition for DPP-IV. The ID60 peptide showed the strongest inhibition with IC_{50} value of 0.23 ± 0.01 and the GD120 value showed the weakest inhibition of 2.52 ± 0.06 IC_{50} value.

Protein isolated from quinoa was enzymatically hydrolyzed with two different enzymatic preparations viz papain and its microbial derivative by (Nongonierma et al., 2014). The DPP-IV inhibition potential of the hydrolysates from both preparations were examined with the protein isolate as control. They reported that the protein isolate had higher inhibition while both enzymatic preparations had similar inhibition capacities with similar IC_{50} values of $0.88 ± 0.05$ mg mL^{-1}.

2.2.2.7 Legumes

Legumes belongs to the family *Fabaceae* and they are consumed throughout the world due to their high protein content, fiber, minerals and other nutritional properties (Ouraji et al., 2020). They include common beans, peanuts, chickpeas, lentils, fava beans, velvet bean, and pigeon pea etc (Luna-Vital and De Mejía 2018).

Pigeon pea (legume) contained 20-28% protein and utilized as food in Nigeria. A study conducted on evaluation of potential of ACE inhibition of pigeon pea hydrolysate. Pigeon pea isolate was hydrolyzed with the pepsin and followed by pancreatin. 34.02 g of hydrophobic amino acid was determined from 100g of pepsin-pancreatin-hydrolysed pea protein. Angiotensin converting enzyme inhibition potential of pepsin-pancreatin-hydrolysed pea protein was 61.82% and pancreatin-hydrolysed pea protein exhibited 14.28% renin inhibition. After the oral administration of pepsin-pancreatin-hydrolysed pea protein and pancreatin-hydrolysed pea protein to spontaneously hypertensive rats (SHRs), systolic blood pressure

was reduced by the values of -33.0 mmHg (4 hour) and -34.6 mmHg (6 hour) respectively (Olagunju et al., 2018).

In a research conducted by Bhaskar et al., (2019), antihypertensive peptides TVGMTAKF and QLLLQQ were isolated from horse gram flour hydrolysate via hydrolysis with alcalase. Hydrolysate was subjected to the ultrafiltration, gel chromatography, high performance liquid chromatography followed by nano liquid chromatography-mass spectrometry. TVGMTAKF and QLLLQQ peptides showed an effective ACE inhibition with an IC_{50} values of 30.3±2.3 μM and 75.0±4.2 μM respectively. In addition, their results revealed that these two peptides retained their inhibition nature after the gastrointestinal digest with the enzymes (chymotrypsin, pepsin and trypsin).

Chel-Guerrero et al., (2017) examined the hypotensive and antihypertensive properties of the velvet bean *in vitro* and *in vivo* models. Protein extract from this legume was hydrolysed differently with alcalase, flavourzyme and alcalase-flavourzyme. Obtained degree of hydrolysis (at a 120 min of hydrolysis) of flavourzyme, alcalase and alcalase-flavourzyme were 39.39%, 24.14% and 33.14% and exhibited IC_{50} 0.630 mg/ml, 0.589 mg/ml and 0.993 mg/ml respectively. As shown in their results, at 120 min of injection of alcalase hydrolysate into animal models (hypertensive rats) at a dose of 5mg/kg of body weight, reduction was 27.29% in systolic blood pressure and 29.37% in diastolic blood pressure was obtained.

2.2.2.8 Soybean

Soybean is rich in proteins and the essential amino acids with less fat and cholesterol as compared to animal proteins (Chatterjee et al., 2018). Research study conducted by (Puchalska et al., 2014), antihypertensive peptides were extracted from the soybean based infant formulas. Soybean based infant formulas extract was subjected to ultrafiltration. Among all the fractions (< 3 kDa, 3–5 kDa and 5–10 kDa), fraction (<5kDa) showed highest ACE inhibition and RPSYT peptide was synthesized from the fractions.

Soy protein which is a good nitrogen source was fermented with *Lactobacillus casei spp. psuedoplantarum*. After the purification with the reverse phase high performance liquid chromatography, fraction F2 and F3 were isolated. These fractions exhibit the antihypertensive activity with 17±0.63 µg/ml and 30±0.13 µg/ml respectively. Further LIVTQ and LIVT peptides were synthesized with the use of fluorenylmethyloxycarbonyl solid phase peptide synthesis and these peptides showed inhibition against ACE enzyme having an IC_{50} value of 0.087 and 0.110 µM respectively (Vallabha and Tiku 2014).

In a research study, antihypertensive potential of *lactobacillus casei* fermented soybean milk was examined. Two *Lactobacillus* strains (CICC 20280 and CICC 23184) were selected as they showed the high ACE inhibition activity with the value of IC_{50} 1.13 and 0.89 mg/ml, respectively. In their results, it was found that peptide content was high in case of CICC 23184 (5.17 ± 0.22 mg/ml) fermented soybean milk while CICC 20280 fermented soybean milk showed 3.97 ± 0.67 mg/ml. But GABA content of CICC 20280 fermented soybean milk (21.71 ± 0.36mg/mL) was higher than CICC 23184 fermented milk (1.57 ± 0.21 mg/ml). After the fourth day of feeding of CICC 23184 fermented soybean milk to the spontaneously hypertensive rats obtained reduction values of SBP and DBP was 91.7 ± 5.9 and 74.2 ± 9.1 mmHg respectively while reduction obtained values in SBP and HBP of CICC 20280 fermented soybean milk were 122.4 ± 8.6 and 111.8 ± 6.4 mmHg respectively (Bao and Chi 2016).

Nishibori et al., (2017) conducted a study on evaluation of the biological activity (ACE inhibition) of soy pulp (okara) extract. Okara derived oligopeptides were separated by reversed phase – high performance liquid chromatography. Their findings revealed that ACE inhibition activity of fermented soybean milk was concentration dependent. It was found that the little increase in concentration reduces enzyme activity. Their findings revealed that effect of 7µl of extract is similar to the captopril at 5nM.

In a study conducted by Yang et al., (2012) the comparison of the antidiabetic potential of unfermented cooked soybeans and meju fermented by traditional and standardized methods was conducted on diabetic rats. Their results showed that the fermented products in overall had better insulinotrpic potential through improved hepatic insulin sensitivity by the activation of insulin signaling and also by the stimulation of glucose-stimulated insulin. Ademiluyi et al., (2014) also investigated the antidiabetic effects of fermented soybean diet *in vivo* and *in vitro*. The *in vivo* results on streptozotocin (STZ)-induced diabetic rats exhibited a reversal in the blood glucose level to normal. The *in vitro* studies reflected an α-glucosidase and an α-amylase inhibition by the soybean extract.

González-Montoya et al., (2018) researched on germinated soybean, hydrolysed with composite pepsin/pancreatin to isolate peptide fractions. After the germination for six days, protein concentrate from the germinated soybean was digested with the composite enzymes and using ultrafiltration, the hydrolysates were fractionated into < 5, 5–10, and >10kDa fractions respectively. They were found effective against HCT-116, HT-29 and Caco-2 cancer cell lines and reduced inflammation induced by microphagous lipopolysaccharides with the 5-10 kDa fractions found most effective relative to others.

2.2.2.9 Fruits and vegetables

Presence of low level of proteins (0.5-3.9%) make them a limited substrate for the production of bioactive peptides. Their concomitant proteins are commonly localized in their seeds, and resultantly bacterial disintegration in the large intestine and resistive to metabolic digestion in the small intestine (Kehinde 2018). For these reasons, only few studies have been done on fruits and vegetables for the production of bioactive peptides.

Dia and Krishnan (2016) using bitter gourd (*Momordica charantia*) isolated a novel anticancer peptide (BG-4) possessing a high trypsin inhibitory activity (about 8.6 times higher than control preparation).This characteristic was associated to the possibility of its

cytotoxic attribute against HT-29 and HCT-116. Another mechanism related to these results was the apoptosis induction as shown by the added percentage of HT-29 and HCT-116 colon cancer cells undergoing apoptosis from 8.5% (untreated) to 31.9% (BG-4 treated, 125 µg/ml for 16 h) and 5.4% (untreated) to 24.8% (BG-4 treated, 125 µg/ml for 16 h) respectively. Bloom (2018) conducted a study to evaluate the mechanism and potential of the BG4 peptide to induce cytotoxicity to cancer cells of the ovary (COV318 and A27801AP). Results obtained from their study showed that at a concentration of 250 µg/ml, treatment with the peptide reduced the viable cell count by 19.8 and 65.1 % for the COV318 and A27801AP cell lines respectively. Induction of apoptosis was discovered as the mechanism of action especially with the A27801AP cell line which showed an increased apoptosis from 7.1 to 23.9 % due to treatment.

Wang et al., (2018) however, conducted a research study on the enzymatic isolation of antidiabetic peptides from hydrolyzed proteins of the walnut fruit - *Juglans mandshurica* Maxim. The protein isolates were further fractionated by ultrafiltration with 10 and 3KDa membranes respectively. The antidiabetic potentials of the hydrolysed fractions were determined on the basis of their α-glucosidase inhibition *in vivo* using streptozotocin-induced diabetic mice. Their results showed that hydrolysate peptide fractions within the categories of lower (<3kDa) and medium (3-10 kDa) showed higher inhibition of the digestive enzyme with inhibition values of inhibition rates of 46.6±11.23% and 61.73±1.93% at concentration of 100mg/mL. The higher weight fractions (>10 kDa) showed lower inhibition of 56.22±1.55% at a concentration of 60 mg/mL. In addition, insulin secretion was found to increase at a rate of 23.71% and glycogen levels, liver glucokinase, and fasting blood glucose levels showed a decrease by 76.19, 69.54, 64.82% respectively.

Ren et al., (2016) in a research study identified and characterized two novel oligopeptides with α-glucosidase inhibition potentials from an unconventional plant source: the seed protein

of hemp (*Cannabis sativa* L.). Subsequent to the defatting and dehydration of the hemp seed, the protein was extracted and hydrolysed enzymatically with 6 proteases namely flavourzyme, protamex, neutrase, trypsin, papain and alcalase. The alcalase treatment showed the highest degree of hydrolysis and α-glucosidase inhibition and was further fractionated for the isolation of peptides. The dipeptide LR (287.2 Da) and the pentapeptide PLMLP (568.4 Da) were isolated.

Admassu et al., (2018) examined the α-amylase inhibition potential of protein hydrolysate from commercially dried laver (seaweed). In their study, the α-amylase inhibition of hydrolysates obtained from four different enzymatic treatments using neutrase, pepsin, alcalase and trypsin were examined. The pepsin hydrolysate was found to possess the strongest inhibition of 1.86 mg/mL as the evaluated IC_{50} value. Ultrafiltrative fractionation of this hydrolysate then followed in the order of <5 kDa, 5-10 kDa and >10 kDa fractions, among which the <5 kDa fraction showed the strongest α-amylase inhibition *in vitro*, with an IC_{50} value of 1.18mg/mL. Using gel chromatography, the <5 kDa fraction was further fractionated into three fractions classified as F-I to F-III and the F-III fraction found to possess the α-amylase strongest inhibition potential with IC_{50} value of 0.87mg/mL.

Arise (2016) isolated the protein in water melon seed and examined the α-amylase inhibition potential of its enzymatically digested hydrolysates, *in vitro*. The enzymes trypsin, pepsin and alcalase were used for hydrolysis and their hydrolysates were found to inhibit α-amylase with IC_{50} inhibition values of 0.234, 0.165 and 0.149 mg mL–1 respectively.

Alcaide-Hidalgo et al., (2019), used virgin olive oil (unfiltered) to isolate ACE inhibitory and antihypertensive peptides. Antihypertensive effect of olive extract on the systolic blood pressure was examined after feeding the peptides to spontaneously hypertensive rats (SHR) at a concentration of 0.425 mg/kg of body weight. The olive oil extract reduced an average

blood pressure of 10 mmHg at 4 hours (P < 0.01) and of 20 mmHg at 6 hours from the initial values of 203.8 ± 1.8 mmHg.

Montone et al., (2018) evaluated the antioxidant activity as well as angiotensinogen converting enzyme inhibitory activity of cauliflower by products (stems and leaves). Firstly, cauliflower by product protein pellet hydrolysed with alcalase and then followed by RP-HPLC. Their findings showed that APYDPDWYYIR and SKGFTSPLF were isolated from fraction 2 and 11 respectively. APYDPDWYYIR and SKGFTSPLF exhibited the ACE inhibitory activity with EC_{50} (half maximal effective concentration) values of 2.59 and 15.26 µmol/l respectively.

Peptides were identified from sweet potato protein by the method of enzymatic hydrolysis (alcalase, papain and pepsin). Hydrolysate which was prepared by alcalase, selected for fractionation and purification process. Peptides VSAIW, AIWGA, FVIKP, VVMPSTF and FHDPMLR) showed the good angiotensinogen converting enzyme inhibitory activity which was 90.73%, 62.34%, 51.45%, 46.74% and 39.89% respectively (Nazir and Mu 2019).

2.3 Pharmacological Application and New Perspectives of Bioactive Peptides

Nowadays, drugs derived from natural products have become recent trend; however, very limited compounds have reached the market. Among these, peptides derived from animal sources have shown their potential to inhibit the growth of tumors and many of them are under clinical trials. A peptide named as *Cemadotin* derived from sea slug and *Aplidine*, a potent apoptosis inducer depsipeptide isolated from tunicate *Aplidin albicans*, are under phase II clinical trials (Suarez-Jimenez et al., 2012). In addition, Schally and Comaru-Schally (1987) introduce LHRH (luteinizing hormone- releasing hormone) agonists as a therapy for prostate cancer. Cetrorelix was the first LHRH antagonist given marketing approval and, thus, became the first LHRH antagonist available clinically.

2.4 Conclusion and future prospects

Food-borne bioactive peptides have been proven to possess several health benefits varying from antipathogenic, antioxidative, anticancer, immunomodulatory, and antihpertensive amongst several others. Their relative availability and affordability, along with natural characteristic makes them suitable alternatives for consideration. More so, food by-products have been proven to be potential sources of bioactive peptides; a novel route for their efficacious valorization. Presently, several BHs and BPs have been isolated from dairy, meat, cereals and legumes, nonetheless, more investigation should be done on their isolation from exceptional sources like fruits and leafy vegetables. Though unconventional and with protein in petite quantities, BHs and BPs isolated from these sources can prove to be of extraordinary potential in the treatment of various diseases. Furthermore, there is need for more research with the purpose of concentrating and fortifying food-derived BHs and BPs to be as or even more effective than other pharmaceutical approaches while retaining their desired characteristic of minimal side effects. Also, nutraceutical applications of bioactive peptides can be explored.

CHAPTER 3

Materials and Methods

Locale of study

Most of the research work has been carried out in Department of food technology, Lovely professional University, Phagwara, Punjab. Partial work related to application has been performed in Amity Institute of Microbial Technology, Amity University Rajasthan, Jaipur, India.

3.1 Materials

Pumpkin seed (*Cucurbita maxima*), flaxseed (Brown color variety), Quinoa seeds (*Chenopodium quinoa*), Chia seeds (*Salvia hispanica*) and peanut (Bold Kernel) were used as substrate for bioactive preparation of bioactive peptide. Protein content of all seeds was determined by Lowry's method. Protein contents in pumpkin seed, flaxseed, quinoa seeds, chia seeds and peanut were 19.73g, 18.76g, 17.8g, 19g and 26g per 100 gm respectively.

3.2 Bacterial strains and Growth condition

Probiotic strains *Lactobacillus helveticus NCDC 292, Lactobacillus acidophilus NCDC15, Lactobacillus plantarum NCDC 374, Lactobacillus fermentum NCDC 141*, *Lactobacillus casei NCDC 297 were* procured from National Dairy Research Institute (Karnal). All LAB strains were maintained as frozen stock at -80°C in de Man Rogosa Sharpe (MRS) broth with 15% glycerol. Cultures were propagated twice in MRS broth and incubated at 37°C prior to use.

3.3 Proteolytic activity assay

Qualitative assay

Proteolytic activity of all probiotic strains was examined by the method given by Beganovic et al., 2013. Agar well diffusion method by using skim milk agar was used for the assay. Actively grown culture of probiotic strains was placed in the well of skim milk agar plate at the centre of plate and incubated at 37°C for 4h. After 4h incubation absence or presence of clear zone around the agar well inoculated with a particular strain was recorded.

3.4 Strain compatibility

Strains compatibility was evaluated by the plate diffusion assay (Pescuma et al., 2010). Briefly, overnight cultures grown in MRS were washed twice with saline solution and suspended at the initial volume. Plates were prepared by pouring 15 ml of MRS soft agar (MRS plus 0.7%, w/v, agar) containing 60 μl of the cell suspension on the agar. After overlay solidification, 5 mm diameter wells made with sterilized plastic straws were inoculated with 60μl of culture supernatants from the other strains. After incubation at 37°C for 16 h, appearance of inhibition zones were observed.

3.5 Sample Preparation

3.5.1 Flaxseed

To prepare the sample for bioactive peptide production flaxseeds were de mucilaged and deoiled. Demucilage of flaxseed was done as per the method given by Marambe et al., 2008. Briefly, 100 gm of seeds were stirred with 800 ml of 0.5M $NaHCO_3$ at 50°C for 1 hr and then seeds were recovered by using sieve. Seeds were rubbed and washed thoroughly with distilled water. Washing steps were repeated 2 more time. After washing, flaxseeds were dried at 45°C in an oven for 24 hr. After drying of seeds grinding was performed by using home style grinder and passed through a 100μm screen and subjected to deoiling by soxhlet method using n-hexane. After deoiling solvent was removed from the flour by keeping the

sample in hot air oven till complete removal of solvent. Dried flour of flaxseed was stored in air tight container.

3.5.2 Pumpkin seed, Quinoa seed, Peanut seed and Chia seed

Pumpkin seeds, Quinoa grains, Peanut seeds and Chia seeds were broken by shear impact in a laboratory-grade mortar and pestle and then deoiled with n-hexane. The deoiled pieces were further dried in a hot air oven for complete evaporation of the solvent and further milled to powder using a high-speed blender. Dried flour of all samples were stored in air tight container.

3.6 Preparation of fermentation medium

Proteins are the precursor of bioactive peptides. Therefore, protein content of milk should be high for the production of bioactive peptides. Thus, keeping this in view, protein content of milk prepared from different seeds were standardized. Four products, separately for different seeds were prepared A, B, C and D, keeping the quantity of all seeds same in all products, only added water was varied. From product A, 50 mL of milk was prepared, from B, 75 mL, from C, 100 mL and from D, 125 mL of milk prepared and their protein content was estimated by using Lowry's method by taking BSA (Bovine Serum Albumin) as standard.

3.7 Protein estimation by Lowry's method

Dilutions of different concentration of Bovine serum albumin (BSA) was prepared as ranged between 0.05 to 1 mg/mL by mixing stock BSA solution (1 mg/mL) with distilled water. Pipetted out 0.2 mL protein solution from these dilutions to different test tubes and added 2 mL of alkaline copper sulphate reagent (analytical reagent). The solutions were then mixed well and incubated at room temperature (25ºC) for 10 min. Then 0.2 mL of Folin-Ciocalteau

solution (reagent solutions) was added to each tube and incubate for 30 min. Zero the colorimeter with blank and the optical density at 660 nm was taken. The absorbance against protein concentration was plotted to get a standard calibration curve. The absorbance of unknown sample was checked and determined the concentration of the unknown sample using the standard curve.
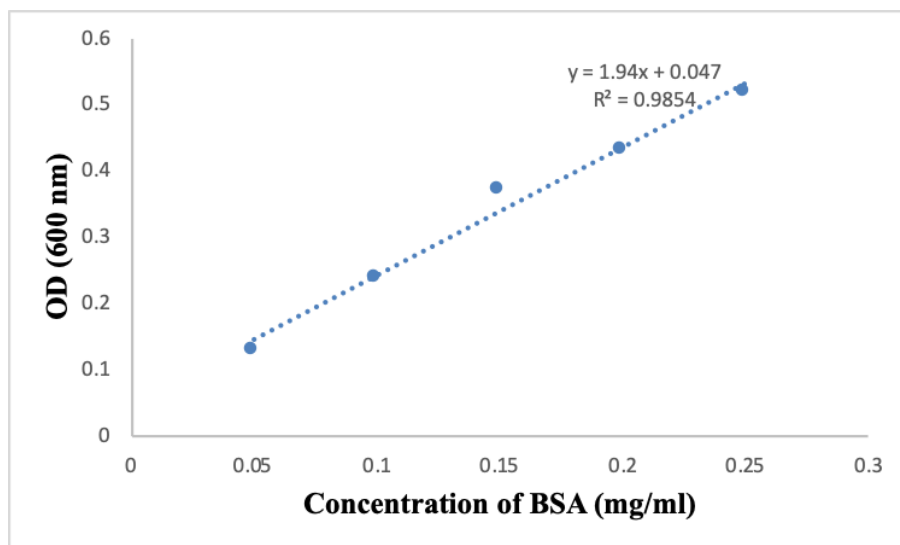
Figure 1: Standard curve of Bovine Serum Albumin

3.8 Fermentation condition

Inoculum for fermentation was prepared from the static fermentation of MRS-Cysteine broth till tertiary seed culture for 7 h. After that, whole cell culture fluid was centrifuged (10000 rpm for 10 min at 4ºC), washed with sterile saline solution (0.85% NaCl) and resuspended in original medium to its original volume and incubated at 37ºC.
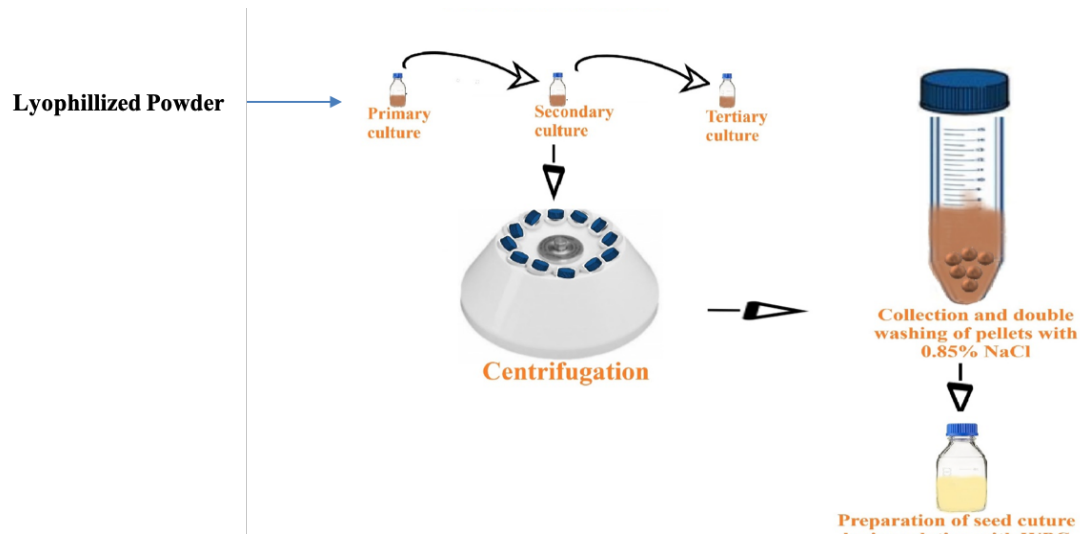
Figure 2: Preparation of seed Culture

3.9 Proteolysis assessment of fermented milk mediums

Proteolytic activity of probiotic bacteria in the fermented sample was determined by using the *o*-phthalaldehyde (OPA) test. The increase in optical density at 340 nm relative to the control was determined by using the spectrophotometer (Pescuma et al., 2010).

3.9.1 Principle

α – Amino group released by hydrolysis of protein react with *o*-phthalaldehyde and 2-mercaptoethanol to form an adduct that absorbs strongly at 340 nm. The absorptivity is similar for all α – amino groups.

3.9.2 Preparation of OPA solution

For the preparation of 50 ml OPA solution 2.5 ml of 20% (w/v) SDS (Sodium dodecyl sulphate), 25 ml of 100 millimolar/l sodium tetraborate, 40 mg of OPA dissolved in 1 ml of methanol, 100µl of 2-mercaptoethanol was mixed properly and final volume was made up with distilled water.

3.9.3 Standard curve of L-leucine

For the preparation of standard curve of L-leucine, a stock solution of leucine containing 0.02 g leucine in 10 ml was prepared. Different concentrations of L-leucine covering the range of 2 - 10μl of stock were prepared in addition to a blank that did not contain L-leucine. Each sample volume was made up to 50μl by using distilled water and mixed with 1.0 mL of OPA solution. The samples were incubated at room temperature (25ºC) for 5 minutes. The absorbance was determined at 340 nm using spectrophotometer.
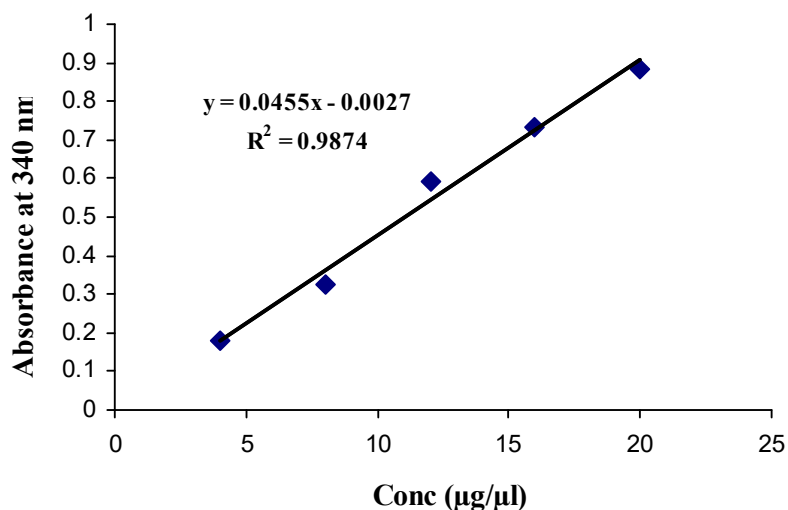
Figure 3: Standard curve of leucine

3.9.4 Preparation of sample for proteolysis assessment

For the preparation of sample for proteolysis assessment, fermented samples were incubated with 0.75mol/l trichloroacetic acid (1:3) at 4ºC for 30 minutes and centrifuged (5000 rpm, 10 min). Supernatant was used to check the proteolytic activity.

3.9.5 Determination of proteolytic activity

50μl of obtained supernatant of sample remove was mixed with 1 ml of OPA solution and incubated at room temperature for 5 min. Optical density was taken at 340 nm. Proteolytic activity was expressed as micro gram leucine released per ml by using the standard curve of L-leucine.

3.10 Antioxidant activity: Antioxidant activity during the study was checked by using two methods, DPPH inhibition and ABTS. DPPH inhibition was checked as per the method suggested by Abd El-Fattah et al.,, (2017).

DPPH radical scavenging activity (%) = $(A_0 - A_s)/A_0 \times 100$

- A_0- Absorbance of control at 517nm
- A_S - Absorbance of sample at 517nm

ABTS method was used for assessing antioxidative potential as suggested by Singh and Vij., 2017. In brief, an aliquot of 10 µl of sample was added to 990 µl ABTS solution (Diluted with phosphate buffer saline to adjust the absorbance at 734 nm to 0.7 ± 0.02) into 1 ml cuvette. The decrease in absorbance at 734 nm was recorded over the period of 10 min at 30 sec interval. The percentage of inhibition of ABTS was calculated by using the following formula.

ABTS activity (%) = $(A_0 - A_s)/A_0 \times 100$

- A_0- Absorbance of control at 734 nm
- A_S - Absorbance of sample at 734 nm

3.11 Selection of substrate

On the basis of better proteolytic activity and antioxidant activity one substrate was selected for further research. Out of all five substrate i.e. Flaxseed, Quinoa, Pumpkin and Peanut, better activity was shown by flaxseed. However, no growth was observed in Chia seeds due to gummy texture of Chia seeds. Hence, further work was carried with flaxseed.

3.12 Physico-chemical analysis of flaxseed milk

3.12.1 pH and Acidity

The pH of flaxseed milk was determined with pH meter (Orion, Thermo Scientific) Acidity was determined as per method performed by (Mudasir et al., 2018). Total solids of flaxseed milk were determined using Sharma et al., 2017.

3.12.2 Carbohydrate content

Carbohydrate content of flaxseed milk was determined as per the method given by Ilodibia et al., (2017). Forty-five mL of sample extracts was diluted to 450 mL with distilled water. One mL of each of the diluted extract was pipetted into different test tubes. Water and glucose were used as blank and standard respectively. To each of the test tubes, 5 mL of freshly prepared 0.10% Anthrone reagent was added, stoppered and mixed thoroughly by gently shaking followed by incubation in water bath at (30°C) for 12 min. The absorbance of the samples and standard were read from a spectrophotometer (Shimadzu corporation) at 630 nm against the blank. The green colour which shows the presence of glucose was stable for about 2 h. Total available carbohydrate as percentage glucose is calculated as shown below:

$$\text{Glucose (\%)} = \frac{25\ A_1}{X \times A_2}$$

X   =   Weight of sample (g)

A   =   Absorbance of diluted sample

A_2 =   Absorbance of diluted standard

3.13 Growth profile of probiotic strains in flaxseed medium

To examine the growth pattern of probiotic bacteria in flaxseed medium, static fermentation was carried out at 37°C. Seed culture was prepared as per the method suggested by Sharma et al., (2017) by fermenting the medium till tertiary culture. Microbial count in tertiary culture was 3.7×10^{10} CFU/mL. Subsequently, the whole tertiary cell culture fluid was centrifuged (10000 rpm for 10 min at 4ºC), washed with sterile saline solution (0.85% NaCl), resuspended in 50 ml flaxseed medium to its original volume of MRS broth. From the same medium different inoculum % i.e. 1 and 5% of was used to check the growth of probiotic microorganisms for 8 days (Sharma et al., 2017).

3.14 Optimization for peptide production

Optimization of conditions for maximum production of bioactivities of flaxseed bioactive peptides were carried out by Lactobacillus plantarum NCDC 374 at 37°C using RSM (Response Surface Methodology). Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Design Expert 9.0 (Stat-Ease, Inc. Minneapolis, MN, USA) statistical software with central composite design (CCD) was used to demonstrate the effect of factors (inoculum level, incubation time) on proteolysis, antimicrobial, antioxidant and ACE-inhibitory activities of fermented flaxseed milk. The parameters and responses were selected on the basis of literature available and low and high values were decided on the basis of preliminary studies.

3.15 Proteolysis assessment of fermented flaxseed milk medium

The proteolytic activity of *Lactobacillus plantarum* (NCDC 374) probiotic bacteria in the flaxseed medium was determined by using the o-phthalaldehyde (OPA) test. The increase in

optical density at 340 nm relative to the control was determined by using the spectrophotometer (Shimadzu Corporation, Japan) (Sharma et al., 2017). This test works on the principle that the α – amino group released by hydrolysis of protein reacts with o-phthalaldehyde and 2-mercaptoethanol to form an adduct that absorbs strongly at 340 nm. The absorptivity is similar for all α – amino groups (Pescuma et al., 2007).

3.16 Antimicrobial activity

Agar well diffusion method was used to evaluate the antibacterial activity of fermented milk against Bacillus cereus, Staphylococcus aureus and E. coli as per the method suggested by Sharma et al., (2016). For each design fermented milk was centrifuged at 10,000 rpm for 15 min and sterilized by using syringe filter of 0.22µm. Nutrient agar plates were seeded with vegetative cells of test bacteria (50 µl) and then 8.0 mm well was punched in the centre of plate by using a sterile cork borer. 100 µl of fermented sample was then added to the wells separately along with non-fermented sample as control. All the agar plates were incubated at 37°C for 24 h and zone of inhibition was measured using Hi-media zone reader scale.

3.17 Antioxidant activity

Antioxidant activity: Antioxidant activity of fermented milk was checked for antioxidant activity by using DPPH scavenging activity (Abd El-Fattah et al., 2017)

DPPH radical scavenging activity (%) = $(A_0 - A_s)/A_0 \times 100$

- A_0- Absorbance of control at 517nm

- A_S - Absorbance of sample at 517nm

ABTS [2, 29-Azinobis (3-ethylene benzothiazoline) 6-Sulphonicacid)] method was used

for assessing antioxidative potential as suggested by Singh and Vij., 2017.

In brief, an aliquot of 10 µl of sample was added to 990 µl ABTS solution (Diluted with phosphate buffer saline to adjust the absorbance at 734 nm to 0.7 ± 0.02) into 1 ml cuvette.

The decrease in absorbance at 734 nm was recorded over the period of 10 min at 30 sec interval. Trolox (6- hydroxy. 2, 5, 7, 8 – tetramethyl chroman-2-carboxylic acid) was used as standard and results were expressed as Trolox equivalent antioxidant capacity.

3.18 ACE-inhibitory potential

ACE-inhibitory activity of fermented flaxseed milk was analysed by the method of Cushman and Cheung (1971) with few modifications. In brief, 50 μL of sample was mixed with 50 μL of ACE (50 mU /mL), the mixture then pre-incubated for 10 min at 37^OC. Then 150 μL of 4.15 mm HHL solution was mixed and the mixture was incubated for 30 min at 37^OC. The reaction was terminated by the addition of 500 μL of 1 M HCl. The hippuric acid liberated by the ACE was extracted with 1.5 mL ethyl acetate followed by heat evaporation at 95°C for 10 min. The residue containing hippuric acid was dissolved in 1 ml of deionized water and the absorbance of the solution was measured at 228 nm. For blank all components except ACE was added and for control all components except sample was added. The extent of inhibition was calculated as follows:

ACE Inhibition (%) = [(Control-Sample)/ (Control-Blank)] x 100

3.19 Degree of hydrolysis (%)

Degree of hydrolysis was calculated by the O-phthaldialdehyde (OPA) method described by Nielsen et al., (2001).

3.19.1 Principle:

Degree of hydrolysis (DH) can be defined as the proportion of cleaved peptide bonds of a sample of protein. O-phthaldialdehyde (OPA) method is based on the reaction of amino groups and O-phthaldialdehyde (OPA) in the presence of dithiothreitol (DTT) forming a

coloured compound detectable at 340nm in a spectrophotometer. Serine is taken as the standard (Nielsen et al., 2001).

Materials: Spectrophotometer

3.19.2 Chemicals: 0.1% L-serine, di-sodium tetraboratedecahydrate, sodium dodecyl sulfate (SDS) or sodium lauryl sulfate, o-phthaldialdehyde 97% (OPA), ethanol, dithiothreitol 99% (DTT).

3.19.3 Procedure:

OPA reagent: 7.620 g di-sodium tetraboratedecahydrate, 200 mg sodium dodecyl sulfate (SDS) or sodium lauryl sulfate were completely dissolved in 150 mL deionized water. 160 mg of o-phthaldialdehyde 97 % (OPA) was dissolved in 4 mL of ethanol. Transfer the OPA solution to the above mentioned solution. 176 mg dithiothreitol 99% (DTT) should be added to the solution. Make the volume up to 200 mL with DDW.

- 3 ml of OPA reagent was added to every single test tube.

- 400μLDDW and 3 mL of OPA were taken as the control in both the cuvettes.

- 400μL L-serine and the same amount of sample were added to OPA containing test tubes and let them react exactly for 2 minutes.

- One cuvette containing water was replaced with reacted OPA and L-serine.

- The same cuvette was replaced with the reacted samples and OPA.

- Every spectrophotometric reading was taken at 340 nm.

Equation 1:   Serine NH2 $= \dfrac{OD\ sample - OD\ blank}{OD\ standard - OD\ blank} \times 0.9516 \times 0.01 \times \dfrac{100}{x \times P}$

Equation 2: $h = \dfrac{serine\ NH2 \quad \beta}{\alpha}$

Equation 3:   DH$= \dfrac{h}{h\ total} \times 100$

P = Protein %

x = amount of sample in grams

α = 0.97

β = 0.342

h = no of hydrolysed bonds

h total = 7.8

3.20 Fractionation and quantification of peptides

Fractionation and quantification of peptides was done as prescribed by Marambe et al., (2008). Peptides of fermented flaxseed milk were fractionated into 10 and 5 kDa peptides using molecular weight cut-off (MWCO) membranes (Sartorius India Pvt. Ltd, New Delhi, India) and stored at low temperature.

3.21 Biofunctionality of bioactive peptides

To check the biofunctionality antioxidant, antimicrobial and ACE inhibition activity was checked as per the above-mentioned procedure. Antidiabetic activity was examined on the basis of DPP-IV inhibition. DPP-IV-inhibitory activity was determined as per the method suggested by Zambrowicz et al., (2015) with few modifications. Briefly, DPP-IV was purchased from Sigma (D7052). The test sample (25 mL) was preincubated with an equal volume of the substrate Gly-Pro–p-nitroaniline (1.6 mM) at 37°C for 10 min. Afterwards, 50 mL of DPP-IV (0.01 U mL^{-1}, in 0.1 M Tris–HCl buffer, pH 8.0) was added and the mixture was incubated at 37 ^{O}C for 60 min. The reaction was stopped by the addition of 100 mL of 1 M sodium acetate buffer, pH 4.0. The released p-nitroaniline as a hydrolysis product was measured at wavelength of 405 nm. All samples were tested in 2 replications. The inhibition activity was expressed in terms of IC$_{50}$ values.

3.22 Hydrolytic assessment

Hydrolytic assessment was done as per method suggested by Sharma et al., (2016).

3.22.1 Principle

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification, and, because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular weight of proteins. SDS is an anionic detergent. Samples to be run on SDS-PAGE are firstly boiled for 5 minutes in sample buffer containing 2-mercaptoethanol and SDS. The 2-mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the SDS molecules. The sample buffer also contains an ionizable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. Once the samples are loaded a current is passed through the gel. The samples to be run are not in fact loaded directly into the main separating gel. When the main separating gel has been poured, a shorter stacking gel is poured on top of the separating gel. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. Typically, the separating gel used is a 15% polyacrylamide gel.

The relative molecular weight of a protein can be determined by comparing its mobility with those of a number of standard proteins of known molecular weight that are run on the same gel.

3.22.2 Materials

- Gel Apparatus
- Gel Assembly (Glass plates, gasket, spacer, holder etc.)
- MilliQ water

3.22.3 Reagents

- 29% Acrylamide/1.0% Bis-acrylamide
- 1.5M Tris base (pH 8.8)
- 0.5M Tris base (pH 6.8)
- 10% SDS
- 10% APS
- TEMED
- 4X Sample buffer
- 5X running buffer
- Coomassie Blue R-250
- Methanol
- Acetic acid

3.22.4 Reagents preparation for SDS PAGE

1. **30% Acrylamide/0.8% Bis-acrylamide**
   - 29.0 gm Acrylamide

- 1.0 gm Bis-acrylamide

- 100ml MilliQ

2. **Tris-HCl, pH 8.8**

- 22.72gm Tris base

- Dissolved in 60ml MilliQ

- Adjust pH to 8.8 with 5NHCl

- Dilute to 100ml

3. **Tris-HCl, pH 6.8**

- 15.12gm Tris base

- Dissolved in 60ml MilliQ

- Adjust pH to 6.8 with 5N HCl

- Dilute to 100ml

4. **10% SDS**

- 10gm SDS

- 100ml MiiliQ

5. **10% APS (Ammonium per sulfate)**

- 0.5gm APS

- 5ml MilliQ (Prepared fresh)

6. **Running/Electrophoresis Buffer (5X), pH 8.3**

- 15.1gm Tris-HCl

- 72gm glycine

- 5gm SDS

- 1000ml MilliQ (Dilute to 1X before use)

7. **Sample Buffer (4X)**

- 2.5ml Tris-HCl, pH 6.8.

- 4 ml glycerol.

- 0.8ml 10%SDS.

- 4 ml 2-mercaptoethanol.

- 2 mg Bromophenol blue. (Composition for 10 ml)

8. **Staining solution**

- 0.25gm Coomassie brilliant blue R-250

- 125ml Methanol

- 25ml Acetic acid

- 100ml MilliQ (Composition for 250 ml)

9. **Destaining solution**

- 45ml Methanol

- 10ml Acetic acid

- 45ml MilliQ

3.22.5 Procedure

- Assemble the glass plates, sandwich of the electrophoresis apparatus using two clean glass plates and two 0.75mm spacers. Lock the sandwich to the casting frame.

- Prepare the Separating Gel (15%)- for 10 ml

  ✓ Acrylamide/Bisacrylamide = 5.0ml

  ✓ Tris HCl pH 8.8   = 2.5ml

  ✓ 10% SDS   = 0.1ml

  ✓ MilliQ   = 2.3ml

  ✓ APS   = 0.1ml

  ✓ TEMED   = 10μl

Pour the separating gel to the sandwich along an edge of one of the spacers.

Slowly cover the top of the gel with around 1cm of water saturated butanol. Allow the gel to polymerize for 15 minutes.

- Pour off butanol and rinse completely with MilliQ.

- Prepare the Stacking Gel (4%)- for 5 ml

    - ✓ Acrylamide/Bisacrylamide = 0.85ml

    - ✓ Tris HCl pH 6.8   = 0.626ml

    - ✓ 10% SDS   = 50μl

    - ✓ MilliQ   = 3.422ml

    - ✓ APS   = 50μl

    - ✓ TEMED   = 5μl

Stacking gel solution was poured over separating gel. Insert a 0.75mm Teflon comb. Allow the gel to polymerize for 15minutes.

- Dilute an aliquot of protein sample with 4X sample buffer and incubate for 5 minutes at 100°C.

- After removing the Teflon comb the wells were filled with 1X running buffer. Using a 25 or 100-μl pipette with a flat tipped needle, samples were applied in the wells. Control wells were loaded with markers.

- Whole chamber was then filled with 1X running buffer.

- Power supply was connected and run at 25 mA constant current for 180 minutes, then the power supply was switched off and the gel was stained in staining solution for 2 hours on shaker and then destained overnight on shaker.

3.23 Applications of Bioactive peptides

Isolated bioactive peptides were checked for shelf-life enhancement of bioactive peptides.

3.23.1 Materials

Whey protein isolate was obtained from Mahaan Pvt. Ltd., and characterized according to the following composition data (dry-weight basis): 91.0% (wt/wt) protein, 0.97% (wt/wt) lipid, 1.03% (wt/wt) lactose, 2.0% (wt/wt) ash and 4.0% (wt/wt) moisture.

Semi-hard cheese was obtained from local market, being the samples stored at 4 °C until further use. The cheese physicochemical composition is: moisture 47.2% (w/w), fat 23.4% (w/w), protein 19.4% (w/w), total ash 3.58% (w/w), pH 4.8 and total acidity 1.40 (glactic acid/ 100 gcheese).

3.23.2 Preparation of coating solution

Coating solution was prepared as per the method given by Ramos et al., 2012 with slight modifications. Briefly, coating solution was prepared by slowly dissolving 10% WPI powder in deionized water. Glycerol (5%,) was used as a plasticizer. The resulting solution was magnetically stirred for approximately 2 h. Subsequently, solution was heated for 20 min in a water bath at $80 \pm 2°C$ under continuous agitation. The solution was cooled to 45°C over 1 h and 0.7% (wt/wt) guar gum was added at this temperature and stirred for approximately 20 min to ensure good dissolution. Guar gum is a natural food thickener aimed at increasing viscosity (Zúñiga et al., 2012). Then, 10% (wt/wt) olive oil was incorporated to reduce the water vapor permeability of the coating matrix base, to minimize the dehydration (Cerqueira et al., 2009), Tween 20 (0.2%, wt/wt) was incorporated as a surfactant to overcome the tendency for phase separation between polymer mixtures of WPI and polysaccharide gums (Syrbe et al., 1995), and as an emulsifier to assist in essential oil dissolution via the hydrophilic and hydrophobic parts of that molecule (Ojagh et al., 2010). Both compounds

were added under stirring for approximately 20 min at room temperature (25±2°C). The solution was homogenized at 19,000 rpm for 4 min using an UltraTurrax T25 homogenizer. The bioactive peptides of molecular weight 10kDa (0.25g/l) were mixed with coating solution. For comparison, Natamycin (0.25 g/L) mixed with coating solution and coating solution without any functional compound were used as control. Finally, the solutions were adjusted to pH 7.0 using 0.1 mol/L NaOH.

Incorporation of guar gum, olive oil, and Tween 20 in the edible coating matrix base at the aforementioned concentrations (i.e., 10% WPI with 5% glycerol, wt/wt) was decided from review literature.

3.23.3 Cheese Coating

Coating solution was adjusted to pH 7.0 (using 1 mol/L NaOH) to guarantee that the coatings were devoid of any significant antimicrobial activity associated with pH itself; hence, any antimicrobial activity observed would be caused by the antimicrobial compounds included in the formulation. Cheese was cut into the uniform size. The coatings were applied directly on the surface of fresh cheeses after manufacture (in the absence of any other type of protective coating added onto the cheese surface). Coatings were applied by dipping cheese samples for 2 min until all surfaces were covered, with the residual coating being allowed to drip off. Coating application was performed in an appropriate aseptic chamber. The cheeses were then left for 8 h at 12°C (85% relative humidity), in a temperature- and humidity-controlled room, turning them periodically (every 30 min or so) until the coating was essentially dry (based on visual inspection). Then, cheeses were stored for 30 d at 10°C and 85% relative humidity. The coated cheeses were compared with their uncoated (negative control) counterparts. Coated and un-coated samples were investigated for weight loss percent, pH, titrable acidity (TA), antioxidant activity and growth of bacteria and yeast at 7 days interval upto 28 days.

3.23.4 Weight loss percentage

Coated and uncoated samples were weighed at the beginning, just after coating and air-drying. Weight loss percentage was calculated by the method given by Sharma et al., 2017. Cheese pieces were weighed at 1st, 7th, 14th, 21th and 28th day of storage by using a digital balance (Sartorius, GC 1603 S-OCE). The results were expressed as percentage loss of the initial weight (Gol et al., 2013). Weight loss was calculated from the initial weight by using the formula:

$$\text{Weight loss (\%)} = [(Wi - Ws)/Wi]*100$$

Where Wi = initial weight

Ws = weight at sampling period

3.23.5 Determination of pH

Small pieces of cheese were cut and homogenized in a grinder and then filtered. The change in pH and titrable acidity (TA) of coated and un-coated samples were monitored on 1st, 7th, 14th, 21th and 28th day of storage period. Change in pH was monitored by using a digital pH meter (Thermo scientific, Orion 2 Star pH Benchtop).

3.23.6 Titratable acidity

The titrable acidity was determined by measuring the produced lactic acid as per method given by Horwitz, 2010. Briefly, 10 ml sample was titrated with 0.1 N NaOH to pH 8.2 in the presence of phenolphthalein as an indicator. The amount of NaOH used (milliliter) was multiplied by two, and titratable acidity was thus obtained in Soxhlet-Henkel degrees (ºSH)

while the volumetric productivity was expressed in g l^{-1}h^{-1}, calculated by multiplying the ºSH by 0.225 and dividing by the fermenting time.

3.23.7 Antioxidant activity

DPPH free radical scavenging assay was performed to check the antioxidant capacity of cheese samples. For this, 10 µl supernatant was mixed with 90 µl of distilled water and 3.9 ml of 25 mM DPPH methanolic solution. The mixture was then vortexed and incubated in dark at room temperature (25±2°C) for 30 min. After 30 min of incubation absorbance was taken at 515 nm against a blank of methanol. Results were expressed as percentage of inhibition of the DPPH radical according to the following equation:

$$\text{Inhibition of DPPH (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control is the absorbance of DPPH solution without extracts (Alothman et al., 2009)

3.23.8 Microbiological Analyses

Cheese samples were evaluated for the presence of different microorganisms via enumeration of viable cells at different time intervals i.e. 1st, 7th ,14th, 21th and 28th after application of coatings. Aseptically, 20-g sample was taken from cheese surface and diluted to 1:10 (wt/vol) in sterile 1% (wt/vol) sodium citrate and mixed properly. Subsequently, decimal dilutions were prepared with 0.1% (wt/vol) peptone water (Sigma). Different growth medium were used to check the presence of different microorganisms. *Lactococcus* spp. and *Lactobacillus* spp. were enumerated on M17 agar and Rogosa agar respectively, and incubated under anaerobic conditions at 37°C for 72 h. Plate count agar plates were used to check the growth of mesophilic aerobic bacteria and incubation was done at 30°C for 72 h under aerobic

condition. *Staphylococcus* spp. were enumerated on Baird-Parker agar. Pseudomonas spp. were enumerated on Pseudomonas agar base (pH 5). In both cases, incubation was done at 37°C for 48 h under aerobic condition. Yeasts and molds were determined on potato dextrose agar plates at 25°C. Surface plating technique was used for all microorganisms.

Statistical analysis

Analysis of variance test was carried out using commercial statistical package, SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA). All results of chemical analysis were recorded as mean ± SD of three replicates. Mean values were compared and significant differences were given using Duncan's LSD test (p≤0.05).

CHAPTER 4

Result and discussion

4.1 Objective 1: To screen the proteolytic activity of different probiotic lactic acid bacterial strains

Proteolytic activity of tested strains was evaluated as indicated by Beganović et al., (2013) and maximum proteolytic activity was shown by *Lactobacillus plantarum* NCDC 374 followed by *Lactobacillus fermentum.* Diameter of zone of proteolysis was 18±0.5 and 16±0.8 for *Lactobacillus plantarum* and *Lactobacillus fermentum* respectively as shown in **Figure 4**. However, no proteolytic effect was shown by *Lactobacillus helveticus* NCDC 292, *Lactobacillus acidophilus* NCDC 15 and *Lactobacillus casei* NCDC 297. Results for the same are given in **Figure 5**. Partovi et al., (2018) obtained analogous results regarding the proteolytic activity of *Lactobacillus plantarum* in an investigation regarding the technological attributes of 10 of its strains isolated from an Iranian traditional dairy product – the Siahmazgi cheese. Their results showed that all isolated strains showed significant proteolytic activity with the SC6 and SD6 strains manifesting the highest proteolytic potentials. The high proteolytic strength of the probiotic is ascribed to the enzymatic proteases it secretes (Khalid and Marth, 1990; Margono et al., 2014).
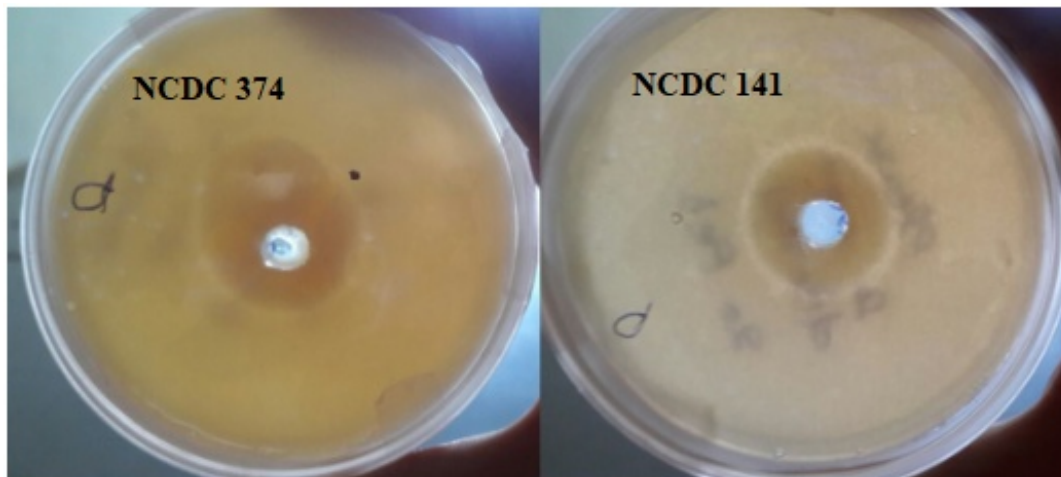
Figure 4: Proteolytic activity of NCDC 374 and NCDC 141.
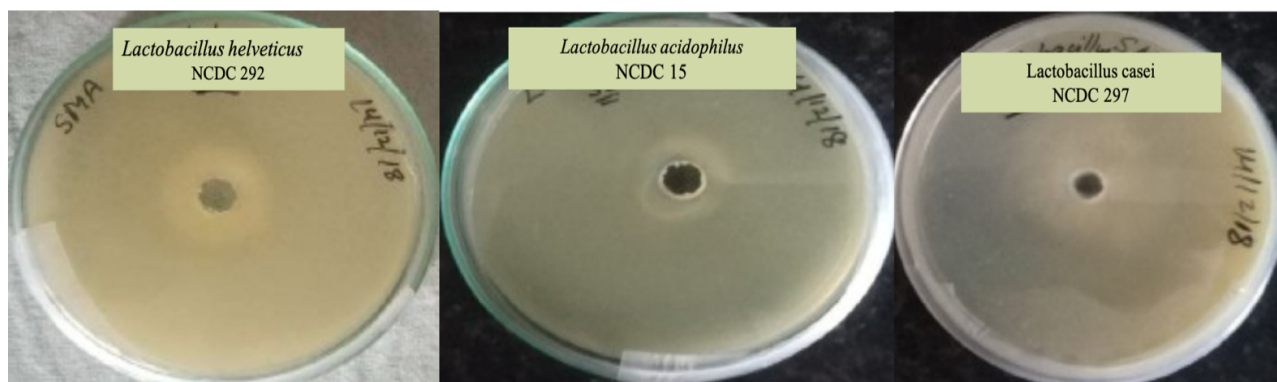
Figure 5: Proteolytic activity of NCDC 292, NCDC 15 and NCDC 297.

4.2 Strain compatibility

Strain compatibility was checked as suggested by Pescuma et al., (2010) and tested strains were found incompatible (**Figure 6**). A clear zone (indicating incompatibility) was observed by NCDC 141 (*Lactobacillus fermentum*) around NCDC 374 (*Lactobacillus plantarum*) growth. Consequently, the mixed culture was avoided for further studies. Similar results on compatibility were obtained by Kandola (2018) in a research study involving the compatibility evaluation of mixed probiotic culture in the production of synbiotic yogurt. *Lactobacillus acidophilus* was found to show complete inhibition to the yoghurt starter - *Lactobacillus delbrueckii* ssp.

The compatibility appraisal of mixed probiotic cultures is important for the assurance of peak viability and activity of probiotic microorganisms for optimum proteolytic activity. (Awaisheh, 2012). This is due to the likelihood of antagonistic interactions which may be of strong or weak inhibitions chiefly through the production of organic acids (Heller, 2003). Additionally, growth pattern of Lactobacillus plantarum on MRS agar plate is given in **Figure 7.**
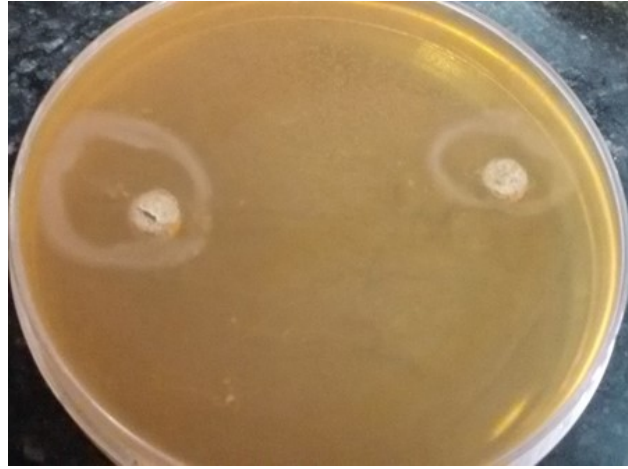
Figure 6: Strain compatibility of probiotic bacteria

Figure 7: Growth pattern of *Lactobacillus plantarum* NCDC 374 on MRS agar plate

4.3 Standardization and physico-chemical analysis of standardized milk

Milk was prepared by using all seeds i.e. Flaxseed, Quinoa, Pumpkin and Peanut as per the protocol mentioned above. Protein content in all samples was standardized for the production of bioactive peptides. Four lots of product (A, B, C and D) were prepared by keeping the

amount of all seeds separately and water in different rations (1:2, 1:3, 1:4, 1:5). The protein

contents of all lots were estimated. Protein content was high in product A followed by B, C

and D with protein content mentioned in Table 1,2,3,4.. The variation on protein content was

on the basis of dilution. Product D was selected on the basis of protein and viscosity of milk.

Albeit, protein content was high in A, B and C but viscosity was not suitable for probiotics

growth. Similar trend was followed by Singh et al.,, 2017 in which soybean milk was

prepared with a ratio of 1:5 to maintain the thickness of milk for probiotic growth. With

similar composition of seed and water (1:5) highest protein content was observed in peanut

milk (14.18 ± 0.09) followed by pumpkin seed milk (13.134 ± 0.24) and flaxseed (12.189 ±

0.58). However, lowest protein content was found in quinoa milk (10.25 ± 0.38). This is

basically due to the availability of high protein content in peanut seeds as compare to others.

A comparative analysis of all seeds are mentioned in Table 5.

Table 1 : Standardization of flaxseed milk for protein contents

Product	Flaxseed (gm)	Flaxseed milk (ml)	Ratio	Protein (%)
A	25	50	1:2	12.189 ± 0.58[a]
B	25	75	1:3	9.11 ± 0.75[b]
C	25	100	1:4	7.61 ± 0.27[c]
D	25	125	1:5	5.29 ± 0.6[d]

Table 2 : Standardization of Pumpkin milk for protein contents

Product	Pumpkin (gm)	Pumpkin milk (ml)	Ratio	Protein (%)
A	25	50	1:2	13.134 ± 0.24[a]
B	25	75	1:3	10.12 ± 0.18[b]
C	25	100	1:4	8.26 ± 0.54[c]
D	25	125	1:5	6.08 ± 0.26[d]

Table 3: Standardization of Quinoa milk for protein contents

Product	Quinoa (gm)	Quinoa milk (ml)	Ratio	Protein (%)
A	25	50	1:2	10.25 ± 0.38^a
B	25	75	1:3	8.26 ± 0.15^b
C	25	100	1:4	6.14 ± 0.84^c
D	25	125	1:5	4.38 ± 0.42^d

Table 4 : Standardization of Peanut milk for protein contents

Product	Peanut (gm)	Peanut milk (ml)	Ratio	Protein (%)
A	25	50	1:2	14.18 ± 0.09^a
B	25	75	1:3	11.09 ± 0.65^b
C	25	100	1:4	9.27 ± 0.77^c
D	25	125	1:5	7.06 ± 0.45^d

Table 5: Comparison of protein and Physicochemical analysis of different substrate milk (1:5)

	Flaxseed	Pumpkin	Peanut	Quinoa
pH	6.6 ± 0.04^a	6.6 ± 0.06^a	6.5 ± 0.09^a	6.3 ± 0.05^b
Total Solids	9.85 ± 0.25^a	8.95 ± 0.16^b	9.14 ± 0.09^c	9.42 ± 0.26^{ac}
Protein	5.29 ± 0.6^c	6.08 ± 0.26^b	7.06 ± 0.45^a	4.38 ± 0.42^d

Means within the same row (lowercase letters), for each sample, with the different letter are statistically differ from each other ($P > 0.05$).

4.4 Fermentation condition

Fermentation of all samples with probiotic strain *L. plantarum* NCDC 374 by using 5% inoculum size was done till pH reached below 4 as probiotic microorganisms are not able to survive owing to their acid sensitivity. At acidic condition, hydrogen ion concentration increases in the cytoplasm of microbes and energy consumption get increases due to maintenance of intracellular pH. Therefore, all energy of microorganisms get utilize in this

process hence, hamper enzymatic reactions and growth (Shabala et al.,, 2006). The loss of probiotic bacterial viability also reduces its functionality. During the fermentation it was inferred that pH of the fermentation medium decreased due to the production of organic acids. However, proteolytic activity and antioxidant activity was found to increase with fermentation in all samples. Proteolytic activity was determined by using standard curve of L-leucine and expressed as micro gram leucine released per ml due to the proteolytic action of probiotic microorganisms. During the initial hr of fermentation no proteolytic activity was found due to consumption of free amino acids present in the medium by the microorganisms. These results are in agreement with the Sharma et al., (2017) in which they ferment whey by a mixed culture of probiotic microorganisms. Results for change in pH, proteolytic activity and antioxidant activity are given in table 6,7,8,9.

Table 6: Change in pH, Proteolytic activity and % DPPH Inhibition in Flaxseed medium

Days	pH	Proteolytic activity	% DPPH Inhibition
0	6.64 ± 0.04^a	16.56 ± 0.1^f	12.61 ± 0.86^h
1	6.59 ± 0.01^a	11.65 ± 0.46^i	24.43 ± 1.59^g
2	5.98 ± 0.04^b	15.25 ± 0.18^h	29.85 ± 2.05^f
3	5.49 ± 0.07^c	15.76 ± 0.070^g	42.9 ± 1.27^e
4	5 ± 0.02^d	17.53 ± 0.1^e	52.35 ± 0.63^d
5	4.89 ± 0.02^e	19.39 ± 0.14^d	65.15 ± 0.77^c
6	3.78 ± 0.03^f	25.24 ± 0.09^b	67.81 ± 0.82^b
7	3.81 ± 0.02^f	27.71 ± 0.23^a	72.15 ± 0.35^a
8	3.84 ± 0.04^f	22.59 ± 0.11^c	71.2 ± 0.98^a

Mean value with different subscript in the same column differ significantly (Duncan's LSD test, P<0.05)

Table 7 : Change in pH, Proteolytic activity and % DPPH Inhibition in Quinoa medium

Days	pH	Proteolytic activity	% DPPH Inhibition
0	6.36 ± 0.02^a	18.67 ± 0.04^e	15.58 ± 0.21^g
1	6.27 ± 0.07^a	17.49 ± 0.06^g	16.25 ± 0.25^f
2	6.07 ± 0.04^b	15.23 ± 0.07^h	17.44 ± 0.28^e
3	5.84 ± 0.03^c	17.97 ± 0.06^f	23.23 ± 0.3^d
4	5.20 ± 0.02^d	19.18 ± 0.1^d	27.64 ± 0.14^c
5	4.75 ± 0.17^e	20.21 ± 0.09^c	28.39 ± 0.07^c
6	4.04 ± 0.03^f	20.9 ± 0.08^b	34.73 ± 0.06^b
7	3.73 ± 0.01^g	21.66 ± 0.11^a	36.81 ± 0.18^a
8	3.65 ± 0.04^g	21.62 ± 0.04^a	36.8 ± 0.09^a
9	3.62 ± 0.04^g	20.39 ± 0.08^c	36.50 ± 0.03^a

Mean value with different subscript in the same column differ significantly (Duncan's LSD test, P<0.05)

Table 8: Change in pH, Proteolytic activity and % DPPH Inhibition in Pumpkin medium

Days	pH	Proteolytic activity	% DPPH Inhibition
0	6.57 ± 0.04^a	11.99 ± 0.07^f	18.71 ± 0.04^h
1	6.37 ± 0.08^b	10.89 ± 0.12^g	20.18 ± 0.07^g
2	6.13 ± 0.05^c	9.14 ± 0.03^h	25.41 ± 0.05^f
3	5.78 ± 0.12^d	12.51 ± 0.11^e	28.91 ± 0.04^e
4	4.94 ± 0.04^e	15.41 ± 0.04^d	30.21 ± 0.09^d
5	4.04 ± 0.04^f	17.83 ± 0.07^c	33.36 ± 0.14^c
6	3.79 ± 0.07^g	18.1 ± 0.04^b	35.05 ± 0.09^b
7	3.41 ± 0.06^h	19.70 ± 0.10^a	40.41 ± 0.09^a
8	3.40 ± 0.06^h	19.75 ± 0.12^a	40.36 ± 0.15^a

Mean value with different subscript in the same column differ significantly (Duncan's LSD test, P<0.05)

Table 9: Change in pH, Proteolytic activity and % DPPH Inhibition in Peanut medium

Days	pH	Proteolytic activity	% DPPH Inhibition
0	6.5±0.02[a]	14.11±0.04[h]	16.46±0.11[h]
1	6.42±0.02[a]	14.28±0.02[g]	17.13±0.07[g]
2	6.28±0.07[b]	15.21±0.07[f]	19.47±0.09[f]
3	5.94±0.04[c]	15.43±0.08[e]	23.51±0.09[e]
4	5.22±0.06[d]	16.29±0.07[d]	25.30±0.26[d]
5	4.27±0.09[e]	16.81±0.09[c]	29.38±0.13[c]
6	3.7±0.11[f]	17.94±0.11[b]	35.17±0.07[b]
7	3.07±0.05[g]	18.48±0.07[a]	38.73±0.35[a]

Mean value with different subscript in the same column differ significantly (Duncan's LSD test, $P<0.05$)

4.5 Selection of substrate

On the basis of better proteolytic activity and antioxidant activity one substrate was selected for further research. Out of all five substrate i.e. Flaxseed, Quinoa, Pumpkin and Peanut, maximum activity was shown by flaxseed. However, no growth was observed in Chia seeds due to gummy texture of Chia seeds. Hence, further work was carried with flaxseed.

4.6 Physicochemical analysis of flaxseed milk (1:5)

Protein content was high in product A (12.189%), followed by B, C and D with protein content of 9.11±0.75, 7.61±0.5 and 5.29±0.8%, respectively. The variation of protein content was on the basis of dilution. Product D was selected on the basis of protein and viscosity of flaxseed milk. Albeit, protein content was high in A, B and C but viscosity was not suitable for probiotics growth as probiotic microorganisms are not able to tolerate high osmotic pressure which is arise due to highly viscous solution. In experimental work at our laboratory,

it was observed that growth of *L. plantarum* reduced in terms of cell biomass and number over the 20mPas viscosity. However, optimum viscosity for growth of microorganisms also varies with substrate and type of strain used. Similar trend was followed by Singh and Vij (2017) in which soybean milk was prepared with a ratio of 1:5 to maintain the thickness of milk for probiotic growth. Lactose content (2%) was added in flaxseed medium to initiate the growth of probiotic microorganism. The composition of selected flaxseed milk (Table 10) comprised 9.85±0.25% of total solid. The initial pH of flaxseed milk was 6.67±0.08 and titratable acidity was 0.102±0.006%. Carbohydrate content of flaxseed milk was found to be 6.02±0.28%. Composition of flaxseed milk actually depends on the ratio of seed to water taken. Flaxseed is considered as a good source of protein, consequently, protein content in flaxseed milk will also be sufficient to produce bioactive peptides (Singh and Vij, 2017).

Table 10: Physicochemical analysis of flaxseed milk (1:5)

S.No.	Parameters	Value
1.	Protein	5.29 ± 0.6%
2.	Total solids	9.85±0.25%
3.	Titratable acidity	0.102±0.006%
4.	pH	6.6±0.04
5.	Carbohydrate content	6.02±0.28 %

4.7 Growth profile of probiotic strains in flaxseed medium

Growth profile of probiotic bacteria (Figure 8) clearly indicates that the growth of probiotic strain *Lactobacillus plantarum* NCDC374 is quite slow in flaxseed medium. Exponential phase triggered from the 1st day of incubation in 5% inoculum size and from 2nd day in medium having 1% inoculum and remained till the 6th day in both the concentrations. Microbial cell numbers were significantly low in 1% (7.5×10^6 CFU/ml) inoculum size as compare to 5% (9.7×10^9 CFU/ml). Afterwards, the growth of probiotic bacteria entered the decline phase (Figure 8). It was also observed that pH of fermenting medium sharply decreased in exponential phase while gradual change was observed during decline phase. Fermentation was carried out for 8 days and pH decreases (lactic acid production) from 6.67±0.02 to 4.69±0.04 and 6.67±0.02 to 3.81±0.08 in medium having 1 and 5% inoculum size respectively, (Figure 2), and terminated thereafter due to the pH sensitivity of the probiotic. Probiotics have been found to have their cellular energy metabolism and integrity disrupted by pH drops in their immediate environment, a condition termed as acid stress, which would consequently disrupt the functionality of the probiotic. Similar trend was observed by Guo et al., (2017) in an evaluative study carried out to compare the acid-stress tolerance of two *L. plantarum* strains: *Lactobacillus plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 at cytoplasmic and membranous levels. Their findings showed that though the L. plantarum ZDY 2013 proved more tolerant to pH drop, noticeable amounts of deleterious alterations were affected by the pH change on their cellular organelles and energy metabolism.
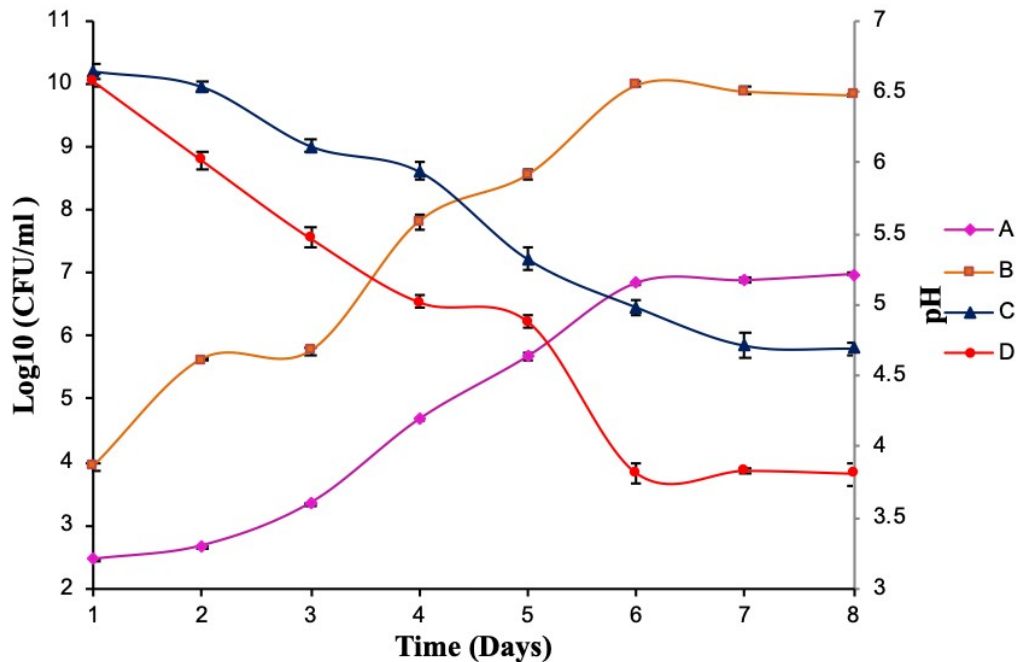
Figure 8: Growth profile and change in pH in flaxseed medium at different inoculum size

A – Inoculum size 1%; **B** – Inoculum size 5%; **C** – pH at Inoculum size 1%; **D** – pH at Inoculum size 5%

4.8 Optimization of peptide production

Screening of variables

A preliminary study was carried out to evaluate the level of independent variables. Based on

the preliminary study and literature independent variables Incubation time and inoculation

(%) were decided which are mentioned in Table 11.

Table 11: Optimization for peptide production

Parameters	-	Low	0	High	+
	-1.41	-1	0	1	1.41
Incubation Time	22	40	83	126	144
Inoculation %	1.5	2	3.25	4.5	5

Table 12: Summary of designs of experiment with responses results against factor

STD	Incubation Time	Inoculum %	Incubation Time (hr)	Inoculum %	DPPH % Inhibition	ACE % Inhibition	Proteolytic activity (µg/ml)
1	-1	-1	40	2	24.89	16.81	13.12
2	1	-1	126	2	51.78	32.84	22.17
3	-1	1	40	4.5	30.78	22.89	17.12
4	1	1	126	4.5	69.87	40.61	26.18
5	-1.41	0	22	3.25	22.78	18.74	10.58
6	1.41	0	144	3.25	68.27	41.64	32.85
7	0	-1.41	83	1.5	32.84	29.68	17.1
8	0	1.41	83	5	49.78	36.82	24.17
9	0	0	83	3.25	43.29	33.84	29.72
10	0	0	83	3.25	43.29	33.84	29.72
11	0	0	83	3.25	43.29	33.84	29.72
12	0	0	83	3.25	43.29	33.84	29.72
13	0	0	83	3.25	43.29	33.84	29.72

Optimization of fermentation condition

4.8.1 DPPH scavenging activity

The results for the DPPH scavenging activity are shown in Table 12 and Figure 9. ANOVA for the model of DPPH inhibition as fitted shows significance ($P<0.05$) and the lack of fit is non- significant ($P<0.05$) (Table 3). The response surface regression model on DPPH inhibition excellently fits with coefficient of determination ($R^2 = 0.999$). Incubation time has a significant positive linear effect ($p<0.0001$) depicting increase in DPPH inhibition with incubation time as well as positive quadratic effect ($p<0.001$) represent lower response at the centre value of incubation time. Similar trend was observed by Li et al., (2019) during the fermentation of apple juice by Lactobacillus plantarum where DPPH inhibition (%) was observed to increase from 24.95 to 43.95% with incubation time of 72 hr. Furthermore, inoculum (%) has a significant positive linear effect ($p<0.0001$) constituting increase in inhibition % of DPPH with inoculum % and a negative significant quadratic effect ($p<0.01$) represent higher response at the centre value of inoculum %. Reason for this trend can be associated with the metabolic reactions of probiotic microorganism causing a release of biochemical products of antioxidant potentials. Similar results were observed by Mantzourani et al., (2019) in a study aimed at preparing a fermented pomegranate juice by using the

probiotic - Lactobacillus plantarum ATCC 14917. During this study, it was reported that total phenolic content increased with a corresponding increase in antioxidant potential from 90 mg TE/100 mL to over 140 mg TE/100 mL during 2 weeks of storage. These readings were attributed to the possible offset of phenolic synthesis by enzymatic releases from lactic acid bacteria. Equation 1 describes the correlation between the incubation time and inoculum % on DPPH inhibition (%).

$$\% \text{ DPPH inhibition} = 43.29 + 16.28A + 5.99B + 1.345A^2 - 0.7618B^2 + 3.05AB \ldots\ldots\ldots$$

(equation 1)

Where, A= Incubation time and B= Inoculum %.

Table 13: Anova Table for DPPH

Source	Sum of squares	Degree of freedom	Mean squares	F value	p-value (prob F)
Model	2465.937	5	493.1875	1722.168	< 0.0001
A	2122.671	1	2122.671	7412.185	< 0.0001
B	287.2418	1	287.2418	1003.024	< 0.0001
A2	12.59622	1	12.59622	43.98492	0.0003
B2	4.037937	1	4.037937	14.10013	0.0071
AB	37.21	1	37.21	129.9341	< 0.0001
Residual	2.004631	7	0.286376		
Lack of fit	2.004631	3	0.66821		**Non-significant**
Pure error	0	4	0		
Total	2467.942	12			

R^2 =0.999; adjusted R^2 =0.9986
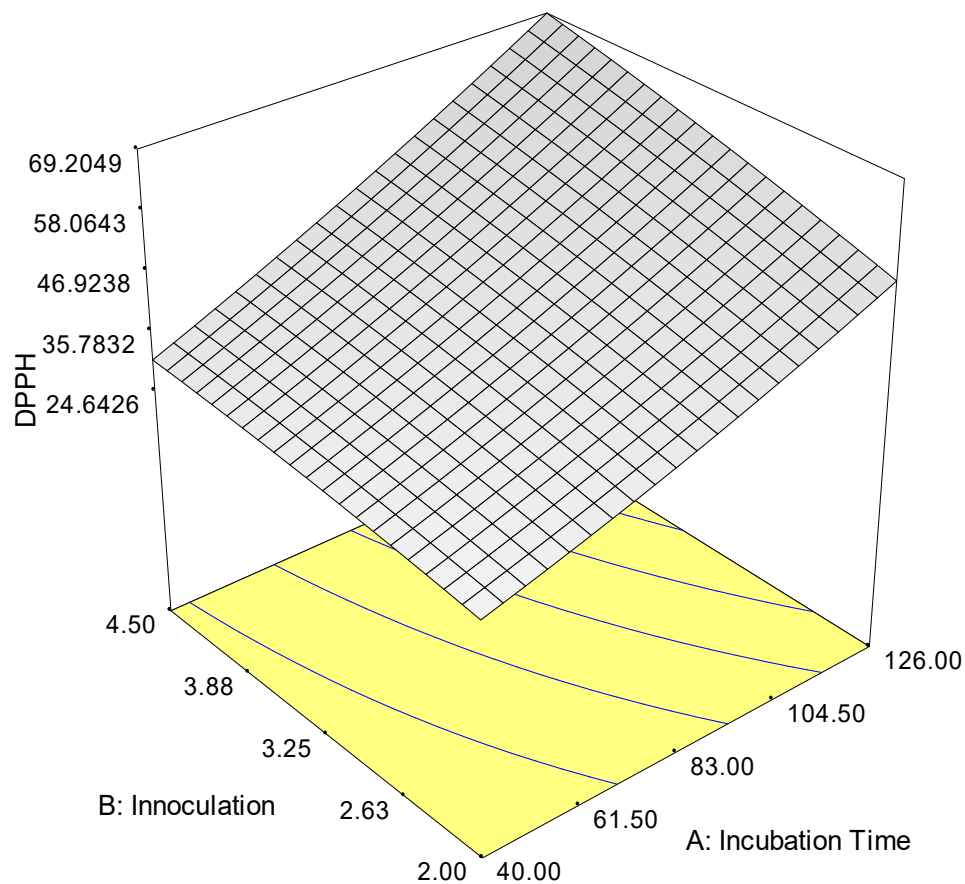
Figure 9: Response surface on 3 D plot for DPPH % Inhibition

4.8.2 Proteolytic activity

ANOVA results for the proteolytic activity are shown in Table 13 and Figure 10. Incubation time has a significant positive linear effect ($p<0.0001$) depicting increase in proteolytic activity with incubation time as well as significant negative quadratic effect ($p<0.05$) representing higher response at the centre value of incubation time. This trend can be associated with the utilization of free amino acids by the microorganisms and then act upon the protein for its metabolic requirements. Similar results were observed by Pescuma et al., (2010) while fermentation of whey by lactic acid bacteria. During their work, proteolytic

activity was observed to increase from 82.3 µg/ml to 626 µg/ml by *Lactobacillus delbrueckii* while 12 hr of incubation. Furthermore, inoculum (%) has a significant positive linear effect (p<0.0001) constituting increase in proteolytic activity with inoculum % and a negative significant quadratic effect (p<0.001) represent higher response at the centre value of inoculum %. These results are in accordance with the Sharma et al., (2017) in which whey was fermented by using a mix culture of *Lactobacillus plantarum* and *L. fermentum*. Equation 1 describes the correlation between the incubation time and inoculum % on proteolytic activity.

$$Proteolytic\ activity = 29.72 + 6.20A + 2.251B - 4.384A^2 - 4.924B^2 + 0.0025AB \ldots\ldots$$

.(*equation 2*)

Table 14: Anova table for proteolytic activity

Source	Sum of squares	Degree of freedom	Mean squares	F value	p-value (prob F)
Model	615.905	5	123.181	31.29371	0.0001
A	307.5762	1	307.5762	78.1387	< 0.0001
B	40.53821	1	40.53821	10.2986	0.0149
A2	133.7234	1	133.7234	33.97199	0.0006
B2	168.692	1	168.692	42.85562	0.0003
AB	2.5E-05	1	2.5E-05	6.35E-06	0.9981
Residual	27.554	7	3.936286		
Lack of fit	27.554	3	9.184667		**Non significant**
Pure error	0	4	0		
Total	643.459	12			

$R^2 = 0.957$; adjusted $R^2 = 0.9265$
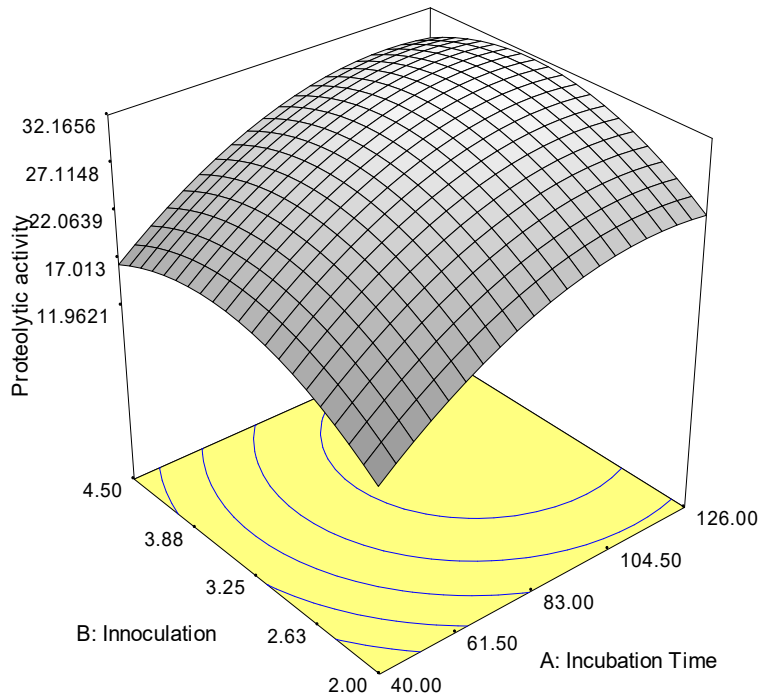
Figure 10: Response surface on 3 D plot for proteolytic activity

4.8.3 ACE Inhibition: ACE inhibition activity ranged from 16.81 to 41.64%. ANOVA for

inhibition is shown in Table (14) and Figure 11. Significant positive linear effect ($p < 0.0001$)

was shown by incubation time depicting increase in ACE inhibition with incubation time as

well as significant negative quadratic effect ($p < 0.05$) representing higher response at the

centre value of incubation time. Result similar to this study were shown by Ong and Shah

(2008) while ripening of cheese by *Lactobacillus acidophilus* and *Lactobacillus helvetics*.

ACE inhibiton was shown maximum on 12th week of storage at 12°C. Furthermore, inoculum

(%) has a significant positive linear effect ($p < 0.0001$) constituting increase in ACE inhibition

(%) with inoculum (%) and a negative significant quadratic effect ($p < 0.001$) showing higher

response at the centre value of inoculum %. Similar trend was shown by Pan and Guo (2010)

during the fermentation of sour milk by *Lactobacillus helveticus* and during their research 4% inoculum shows maximum ACE inhibitory activity (75.46%). Also maximum production of Peptides were observed at this condition.

$$ACE\ Inhibition\ (\%) - 33.84 + 8.266A + 2.993B - 2.683A^2 - 1.115B^2 + 0.4225AB.....($$

equation 3)

Table 15: Anova table for ACE

Source	Sum of squares	Degree of freedom	Mean squares	F value	p-value (prob F)
Model	673.7833	5	134.7567	36.90949	< 0.0001
A	546.7379	1	546.7379	149.7501	< 0.0001
B	71.68525	1	71.68525	19.6344	0.0030
A2	50.08111	1	50.08111	13.71708	0.0076
B2	9.250068	1	9.250068	2.533569	0.1555
AB	0.714025	1	0.714025	0.19557	0.6717
Residual	25.55702	7	3.651003		
Lack of fit	25.55702	3	8.519007		
Pure error	0	4	0		
Total	699.3403	12			

R^2 =0.963; adjusted R^2 =0.9373
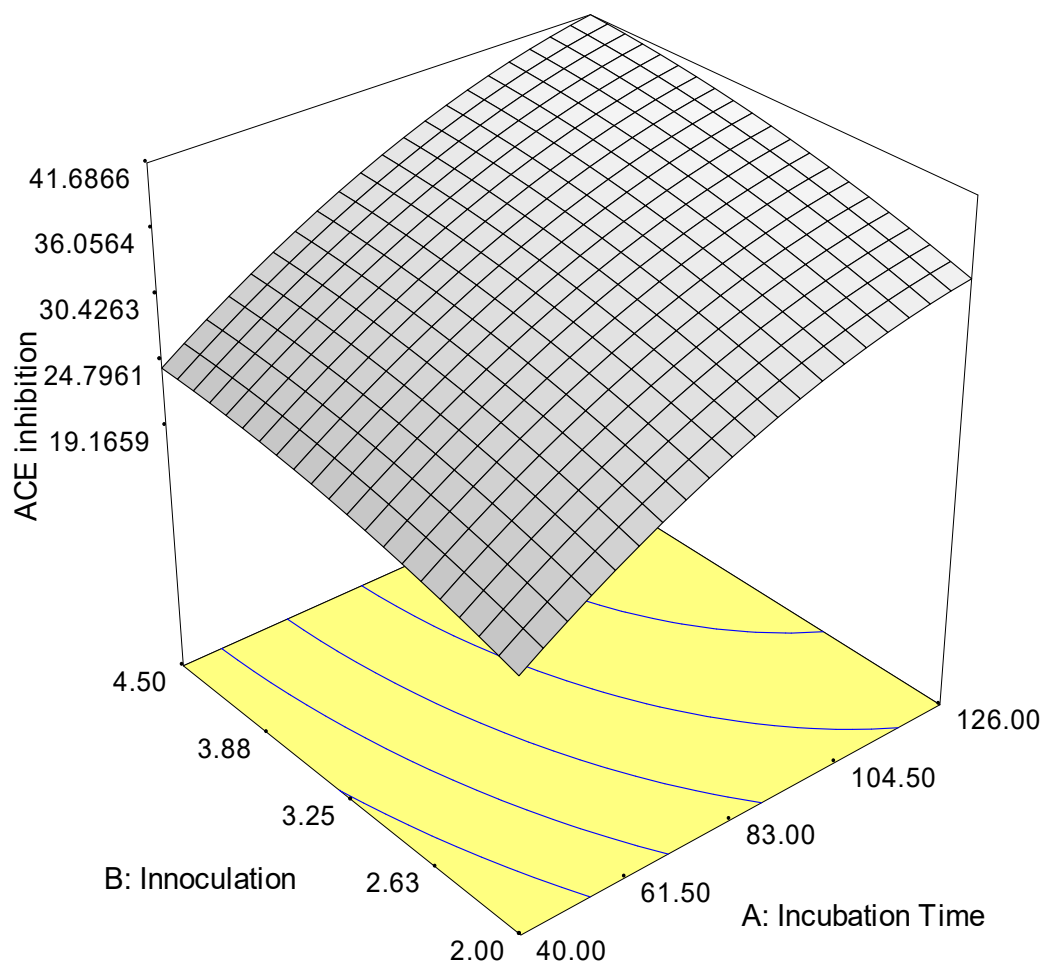
Figure 11: Response surface on 3 D plot for ACE Inhibition

4.9 Selection of Optimum condition

Optimization of conditions for the production of bioactive peptides with maximum proteolytic, antioxidant and ACE inhibition activity was done by design expert software version 7.0. The software setting used for the numerical optimization was that the both independent variables (incubation time and inoculum %) were kept within range and all responses (DPPH inhibition, ACE inhibition and proteolytic activity) were kept at maximum.

It indicated that an incubation time of 126 hr with inoculum 4.20% will give the maximum value for proteolytic activity (30.38 μg leucine/ml), ACE inhibition (41.35%) and DPPH inhibition (67.38%) with overall desirability of 0.934.

Table 16: Analysis of variance (ANOVA) for quadratic model and lack of fit for proteolysis, antioxidant and ACE-Inhibitory activity as per CCD

Responses	Source	Sum of Squares	Degree of freedom (df)	Mean Square	F Value	p-value Prob (probb F)	
Proteolysis	Model	615.9	5	123.18	31.29	0.0001	Significant
	A	307.57	1	307.57	78.13	< 0.0001	
	B	40.53	1	40.53	10.29	0.0149	
	A2	133.72	1	133.72	33.97	0.0006	
	B2	168.69	1	168.69	42.85	0.0003	
	AB	2.50E-05	1	2.50E-05	6.35E-06	0.9981	
	Residual	27.55	7	3.93			
	Lack of fit	27.55	3	9.18			Non Significant
	Pure error	0	4	0			
	Total	643.45	12				
ACE Inhibition	Model	673.78	5	134.75	36.9	< 0.0001	Significant
	A	546.73	1	546.73	149.75	< 0.0001	
	B	71.68	1	71.68	19.63	0.003	
	A2	50.08	1	50.08	13.71	0.0076	
	B2	9.25	1	9.25	2.53	0.1555	
	AB	0.71	1	0.71	0.19	0.6717	
	Residual	25.55	7	3.65			
	Lack of fit	25.55	3	8.51			Non Significant
	Pure error	0	4	0			
	Total	699.34	12				
% DPPH Inhibition	Model	2465.93	5	493.1875	1722.168	< 0.0001	Significant
	A	2122.67	1	2122.671	7412.185	< 0.0001	
	B	287.24	1	287.2418	1003.024	< 0.0001	
	A2	12.59	1	12.59622	43.98492	0.0003	
	B2	4.0379	1	4.037937	14.10013	0.0071	
	AB	37.21	1	37.21	129.9341	< 0.0001	
	Residual	2.004631	7	0.286376			
	Lack of fit	2.004631	3	0.66821			Non Significant
	Pure error	0	4	0			
	Total	2467.942	12				

4.10 Verification of Optimized value

Investigation of fitness of model (Table 15) was done by carrying out the experimentation at the obtained optimum conditions (Inoculum size and incubation time) generated by the software i.e. Incubation time 126 hr with Inoculum % 4.20. Table 16 represents the experimental values which are within 93% of anticipated values. Therefore, suitability of model.

Table 17: Comparison between Predicted and Experimental value at Optimized condition (126 hr, 4.20% Inoculum size)

	DPPH % Inhibition	ACE Inhibition	Proteolytic activity
Predicted	67.38 ± 2.23	41.35 ± 2.38	30.38 ± 1.15
Experimental	62.13 ± 1.75	37.45 ± 0.73	34.34 ± 1.65

4.1 Fractionation and quantification of peptides

4.11.1 % degree of hydrolysis

Degree of hydrolysis is a measurement of total number of peptide bonds cleaved with respect to total peptide bonds present in sample. In this study, degree of hydrolysis of flaxseed protein by *Lactobacillus plantarum* was 58.96%.

4.11.2 Peptides concentration in fermented flaxseed milk

Highest peptides content was found in 10kDa fraction, 0.589±0.02 mg/ml, and this concentration was significantly (P< 0.05) higher than in comparison to 5kDa (0.514±0.02 mg/ml) while non-fractionated (NF) sample was having peptide content, 0.279±0.01 mg/ml. Because peptides were separated according to their molecular mass by MWCO, therefore they showed differences in peptide content (Puchalska et al.,, 2014). There results are in agreement with the data observed by Singh and Vij (2017).In this study they checked the potential of *Lactobacillus plantarum* to hydrolyse the soy protein.

4.11.3 Hydrolytic assessment

SDS PAGE was used to check the hydrolysis of flaxseed protein. Figure 12 shows the proteolysis effect of *Lactobacillus plantarum* on flaxseed protein in fermented sample as compare to non-fermented sample. It can be clearly observed from Figure 12 that the concentration of indigenous protein has been decreased and pepides of lower molecular weight has been released after the fermentation. Similar results were observed by Pescuma et

al., (2007) during the hydrolysis of whey protein by a mixed culture of three lactic acid

bacteria involving *Lactobacillus acidophilus, Streptococcus thermophilus and Lactobacillus delbrueckii*. A protein smear was observed in non-fermented sample due to the presence of

high molecular weight native proteins. Native proteins in flaxseed are reported to possess

molecular weight around 365 kDa. Also, some proteins having molecular weight 41, 48, 31,

23 and 20 kDa were also reported by Chung et al.,, 2005. Similar results of protein lysis after

fermentation were observed by Pescuma et al., (2007) during the hydrolysis of whey protein

by a mixed culture of three lactic acid bacteria involving *Lactobacillus acidophilus, Streptococcus thermophilus and Lactobacillus delbrueckii.*
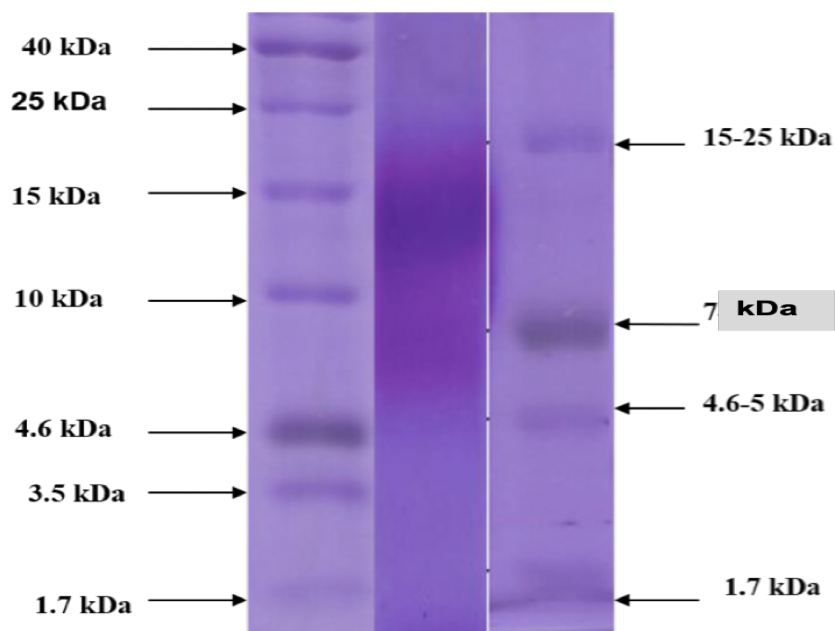
Figure 12: Hydrolysis of flaxseed proteins: Lane1: Pre-stained protein molecular marker, **Lane2**: Non-Fermented flaxseed sample**, Lane3**: Fermented flaxseed sample

4.12 Biofunctionality of flaxseed peptide

4.12.1 Antioxidant activity

ABTS and DPPH method was used to determine the antioxidant potential of peptides fraction of fermented flaxseed milk. During the experiment, 10kDa fraction of peptides showed highest ABTS (37.54±0.58%) and DPPH (73.74±0.27%) inhibition, followed by 5kDa, ABTS (28.84±0.64%) and DPPH (61.02±0.38%), respectively. Comparatively, lowest activity ABTS (19.78±0.26%) and DPPH (47.85±0.83%) was observed in unfractionated fermented flaxseed sample. Dudonne et al, (2009) reported that not only extract composition but also the conditions of the test used determine the antioxidant capacity of sample.. Different values for ABTS and DPPH represents the presence of different compounds. However, in case of peptides antioxidant activity depend upon the chemical structure and amino acids present in the peptides and the reason for low ABTS scavenging can be related with the presence of hydrophobic amino acids (Zhou et al.,, 2016). Higher activity in fermented sample is due to the presence of bioactive peptides which prevents the oxidation reactions and behave as antioxidants. Jeong et al., (2007) and Kim et al., (2007) reported the similar studies and support the fact that antioxidant property of peptides is highly influenced by its molecular mass and size. Also, Moure et al.,, (2006) supported the dependence of antioxidant activity on molecular weight distribution. Peptides with smaller size are known to be associated with higher antioxidant activity as compared to indigestive native large proteins. Similar trend was also observed by Sun et al.,, (2011) in which antioxidant activity of porcine haemoglobin hydrolysate or unfractionated sample was lower than its four

fractionated peptides named as PHH-I, PHH-II, PHH-III and PHH-IV with molecular weight > 10 kDa, 5–10 kDa, 3–5 kDa and < 3 kDa respectively.

4.12.2 Antimicrobial activity

Inhibiting the growth of pathogenic intestinal microflora is a proof for antimicrobial activity of flaxseed milk fermented by *Lactobacilli*. The inhibition of the growth of pathogens can be because of the production of antimicrobial peptides and other bioactive components. The agar well diffusion method was used to study antimicrobial activity of peptides (5kDa and 10kDa). The highest antimicrobial activity was shown by 10kDA against *Bacillus cereus* (17.0 ±1.15 mm) followed by *Staphylococcus aureus* (10.0±0.5 mm) (Figure 4). However, no activity was shown by 10 kDa against *E. coli* as given in Figure 13. Furthermore, 5kDa peptide showed a minimum zone of inhibition (9.0±1.0 mm) against *B. cereus*. Antibacterial activity of flaxseed peptide formed by *Lactobacillus fermentation* has not been reported yet, but Hassan et al., (2014) reported the antibacterial activity of flaxseed protein against *Entrococcus foecalis, Salmonella typhimurium and Escherichia coli*. Additionally, Mohanty et al., (2014) reported the bioactive potential of peptide produced by Lactobacillus from milk and milk products against *Escherichia coli* MTCC82, *Bacillus cereus* ATCC10702, *Salmonella enteritidis* 125109, *Salmonella typhi* MTCC3216, *Salmonella typhimurium* SB300, *Aeromonas hydrophila* ATCC7966 and *Staphylococcus aureus* MTCC96 and an effective zone of inhibition was shown by bioactive peptides against all the microorganisms.
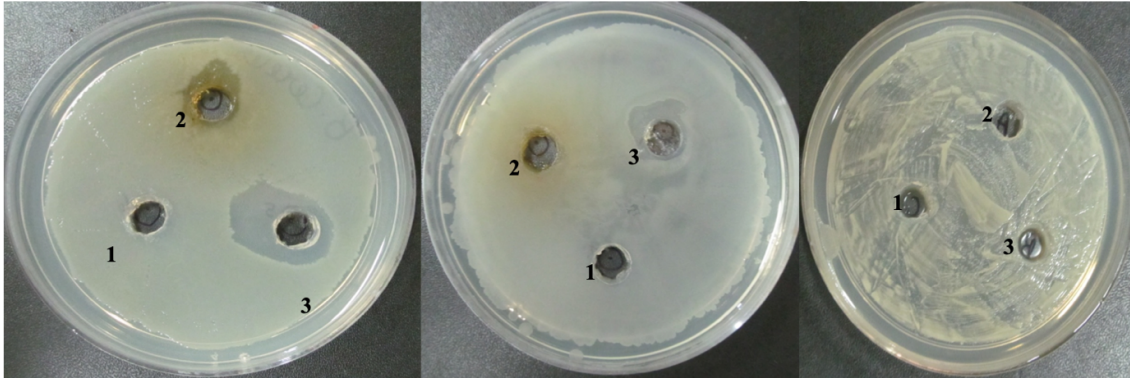
Figure 13: Antimicrobial Activity; A- *B. cereus*, B - *S. aureus*, C- *E.coli*, where, 1 – non fermented sample, 2- 5 kDa, 3 – 10 kDa

4.12.3 ACE-inhibitory activity

N-hippuryl-L-histidyl-L-leucine (HHL) was used to check the ACE-Inhibitory activity (*in-vitro*) of peptides fractions. Significant difference was observed in activity of 10 and 5kDa fraction of peptides, with values of 67.15±1.2% and 52.46±1% ACE inhibition, respectively. However, all the tested fractions showed higher activity as compare to non-fractionated sample (42.68±2.8%). Angiotensin-converting enzyme (ACE) is the intermediary between Angiotensin I and II enzymes with the target functionality of regulating blood pressure by proper maintenance of the volumetric quantity of bodily fluids. Angiotensin II, is the enzyme of interest for the hypertension syndrome since it is responsible for actuation of several metabolic reactions in the body's biochemistry in relation to rise in blood pressure such as; prompting the adrenal medulla to liberate catecholamines, decreasing the excretion of water and sodium in urine, aiding the hypertrophy of cardiac myocytes and vascularized muscular cells, and instigating the manufacture and distribution of aldosterone (Herman and Bhasir, 2019). Consequently, the inhibition of the biochemical lytic conversion process of Angiotensin-I to Angiotensin-II by the ACE enzyme would be remarkable for the management of hypertension; a procedure achievable by bioactive peptides. The proteolysis

of food proteins by probiotics into peptidic aromatic amino acids of bulky conformity and with profound hydrophobicity and those situated in positions of close proximity to the C-terminal have been established as the possible factors responsible for the inhibitory activities of peptides against enzymes (Girgih et al., 2014; Moayedi et al., 2018). These could be the rationale for the higher ACE inhibition by the 10 kDa relative to the 5 kDa fraction results obtained for this study. The results obtained from this study are in consonance with those obtained by Elkhtab et al., (2017) when they fermented milk with probiotic strains including; *Pediococcus acidilactici, Lactobacillus paracasei, Lactobacillus casei, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus helveticus,* and *Lactobacillus delbrueckii* ssp. *bulgaricus* for the proteolysis of inherent proteins and examined the antihypertensive functionality of the isolated peptides. Subsequent to ultrafiltration to 3 and 10 kDa fractions, the peptide fractions were analyzed on basis of their inhibition of ACE enzyme extracted in vivo from the lung tissue of rabbits and their results showed that the 10 kDa fraction had the superior inhibition.

4.12.4 DPP-IV inhibitory activity

The inhibition of digestive enzymes such as α -amylase, Dipeptyl peptidase IV (DPP-IV) and α -glucosidase by food and/or pharmaceutical agents such as bioactive peptides have been established as potential mechanism of action for the management of diabetes (Kehinde 2018). Fermentation of food materials have been recognised as a formidable procedure for improving their antidiabetic functionality (Sivamaruthi et al., 2018). Probiotic proteolysis of food proteins for this purpose is also gaining much research attention. The results obtained from this research shows that smaller peptide units on the basis of their molecular weight are more serviceable for the inhibition of the DPP-IV enzyme and consequently, the management of diabetes. IC_{50} value observed during the study was 0.58±0.02, 0.64±0.01 and 0.42±0.03

(mg/ml) for 5kDa, 10kDa and non-fractionated respectively. Results similar to those obtained in this research were acquired by Lu et al., (2019) for the isolation of DPP-IV inhibitory peptides from black tea. Peptides were fractionated into 1, 5, 10 and 50 kDa fractions respectively and had values of 72.7 ± 2.57, 75.7 ± 1.00, 73.8 ± 0.54, and 75.5 ± 0.47 % for DPP-IV inhibition respectively, with the 5 kDa being most effective. Satto et al.,, 2018 isolated bioactive peptides from Natto (soybean fermented with *B. subtilis*) and examined their antidiabetic potential on the premise of their inhibition of DPP-IV extracted from *Aspergillus oryzae*. The duopeptides LR and KL were evaluated and found to offer dose-dependent inhibition with IC_{50} values of 598.02 ± 18.35 and 41.40 ± 2.68 μg/ml, in concentrations of 85 and 50 μg/g natto respectively. A possible explanation for the DPP-IV inhibitory property of peptides is their ability to form hydrogen bonds at the enzyme's active site and block the entry of any species to it, thereby reducing its functionality.

4.13 Application of bioactive peptides for shelf life enhancement of cheese

4.13.1 Outer appearance of Cheese

Outer appearance of coated cheese was comparatively shiny as compared to uncoated sample. However, no variation was observed in appearance owing to incorporation of bioactive peptides and natamycin. Additionally, the functional coating applied on cheese surface dried in similar time interval (7 hr) as without functional agents. Besides, functional coating containing bioactive peptides showed similar adhesion like coating contained natamycin and without functional agents, to the cheese surface by observing visually. Similar results were observed by Ramos et al., 2012 while antimicrobial coating on cheese.

Outer appearance attributes of minimal and fully processed food materials are significantly important for their acceptability by consumers especially at the point of purchase. Accordingly, waxy mimetics are formulated with edible coatings of foods to offer them the

shiny appearance that will appeal to consumer's attraction. In studies, several natural ingredients have been employed for the shiny appeal such as: thyme essential oil for raisin by Youseftabar-Miri et al., (2020), *Opuntia ficus-indica* mucilage extract for kiwi fruits by Allegra et al., (2016), electrostatically sprayed alginate-based coating for strawberry by Peretto et al., (2017), and the application of *Aloe vera* coating on table grapes by Tripathi and Dubey (2004). Moreover, protein and protein derivatives have also shown that in addition to their metabolic functionalities, they can also be beneficial for sensory-related characteristics. Results similar to those obtained from this study showing a shiny appearance on the cheese tested subsequent to peptide coating has been reported in previous studies. Lysozyme and other prominent peptides such as nisin, usually secreted by organisms for defense have been found useful as sensory-enhancing edible coatings for food products (Costa et al., 2018). Ünalan et al., (2013) prepared lysozyme-based coatings for Kashar cheese and reported an inhibition of oxidation and consequently its undesirable rancid effects due to the controlled-release attribute of lysozyme of added antioxidants. Cui et al., (2016) used nisin with other ingredients on cheddar cheese and examined the resulting appearance changes after 15 days of storage at 4°C.

4.13.2 Physicochemical analysis of cheese

Physicochemical properties were performed by comparing cheeses coated with the edible coating solutions and uncoated samples. Coated and uncoated samples were checked for physicochemical properties at 1st, 7th, 14th, 21th and 28th of storage.

4.13.3 Moisture loss percentage

A striking objective of cheese storage is to fashion the parameters of external conditions for the proper and regulatory control of the cheese ripening cycle. Storage environment conditions especially relative humidity and temperature are to be maintained at specific

values to obtain optimal quality for the type of cheese desired since they firmly influence quality parameters such as weight and moisture losses, ripening rate, surface flora development and rind formation (Hay, 2017). More precisely, the storage parameters, with the inclusion of storage period have been reported to affect several physicochemical attributes such as titratable acidity and nitrogen profile (water soluble-nitrogen, trichloroacetic acid-soluble nitrogen, and phosphotungstic acid soluble nitrogen) (Andiç et al., 2010). In this study, the moisture loss of all coated samples (with bioactive peptides, natamycin or without functional agent) was comparatively lower than the uncoated samples. However, no significant ($P<0.05$) difference was observed in coating solution incorporated with bioactive peptides and natamysin. Moisture loss after 28 days was observed 14.4, 14.3, 14.6 and 23% for cheese coated with coating solution+ bioactive peptides, coating solution + natamycin, coating solution and uncoated sample. The uncoated samples had an unrestrained contact with the environment and in the course of attaining a humidity equilibrium loose moisture to it. However, the higher percentage of moisture loss was observed by Cerqueira et al (2009) during his study in which he applied glactomannan coating on cheese for 21 days. Difference in values depend upon the material used for making the coating solution. The slight differences in the results from the coating solutions could be due to pH differences. Relatively higher acidity will bring about a contraction effect on the cheese protein matrix curd, causing them to squeeze out moisture, an effect termed as syneresis (Perveen et al., 2011). Furthermore, the water vapor permeability (WVP) differences of the cheese coatings could sufficiently influence their moisture loss effects. The WVP is dependent upon the intermolecular distances of the molecular networks of the coatings used with the peptides being more molecularly compacted than natamycin (Mei et al., 2013)
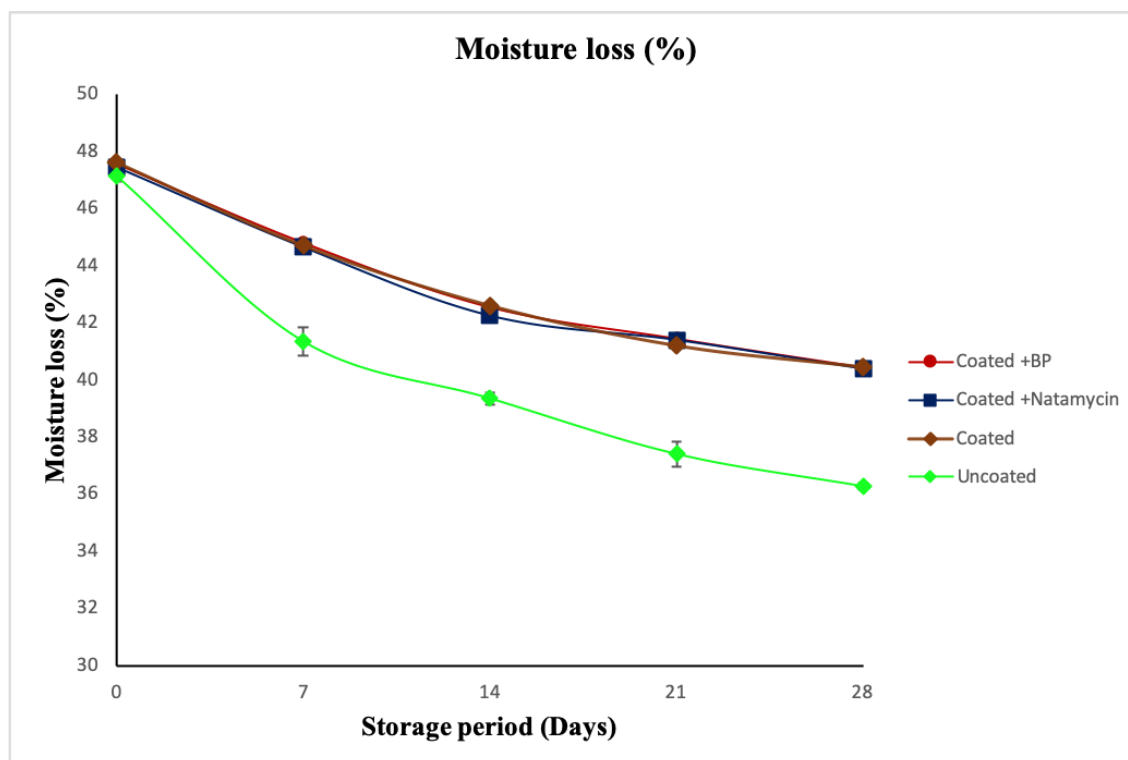
Figure 14: Moisture loss (%) in coated and uncoated sample

4.13.4 Change in pH and titratable acidity

The variation in pH as storage time elapsed is depicted in Figure 15; pH decreased for all samples, statistically significant differences ($P > 0.05$) was observed between coated and uncoated cheeses. Titratable acidity was found to increase with fermentation due to production of lactic acid owing to fermentation by lactic acid bacteria. Change in titratable acidity is given in Figure 16. Such a pH decrease may be due to the activity of indigenous lactic acid bacteria that metabolize lactose to lactate, thus leading to acid production. However, more alleviation was observed in cheese samples coated with coating material incorporated with bioactive peptides owing to retention of more moisture, thus more viability of indigeneous microflora. Nonetheless, the samples coated with coating material incorporated with natamycin showed minimum declination in pH due to limited growth of lactic acid bacteria. The addition of some additives to cheeses have been reported to affect their protein networks and cause significant pH reductions and increased solubilization of

calcium. Pastorino, Hansen and McMahon, 2003 injected glucono-δ-lactone solution into cheddar cheese blocks and observed a decrease in cheese weight, moisture content and pH from 5.3 to 4.7. The pH change was associated with contraction of the protein matrix of the cheese. In addition, both synthetic and microbially-derived peptides have mostly exhibited some functional properties including production of bioactive metabolites and antimicrobial effects at lower pH values. These effects have been attributed to the electrostatic interactions of charged amino acid residues in peptides that occur at such pH ranges. The interactions cause significant absorption and attachment to the surfaces of the substrates and make them more suited to cause metabolic changes. For example, several studies have shown that peptides impart their antimicrobial properties at lower pH ranges. Ureña et al.,, 2015 reported that the peptides Tet-124-G-DOPA-G and Tet-124 showed a better inhibition against E. coli at a pH of 4.75 better than at 6.9. Walker et al.,, 2005 also reported the increased production of desirable butyrate in the gut environment by anaerobic continuous cultures at a lower pH of 5.5 relative to 6.5.
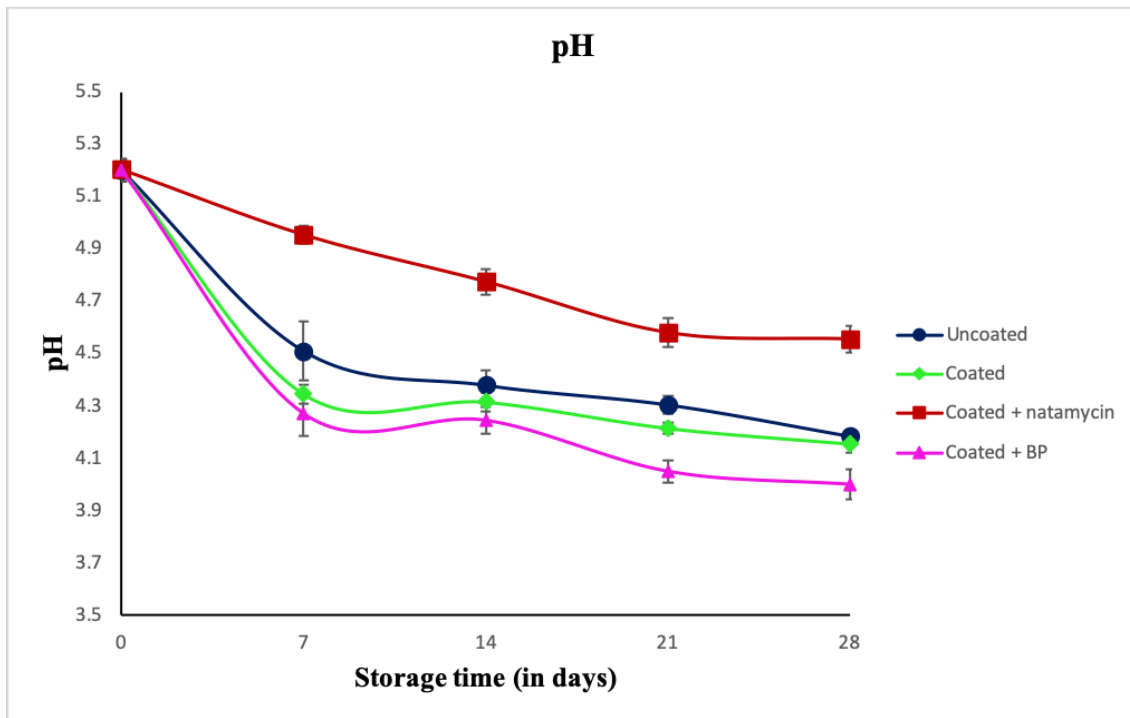
Figure 15: Change in pH in coated and uncoated sample
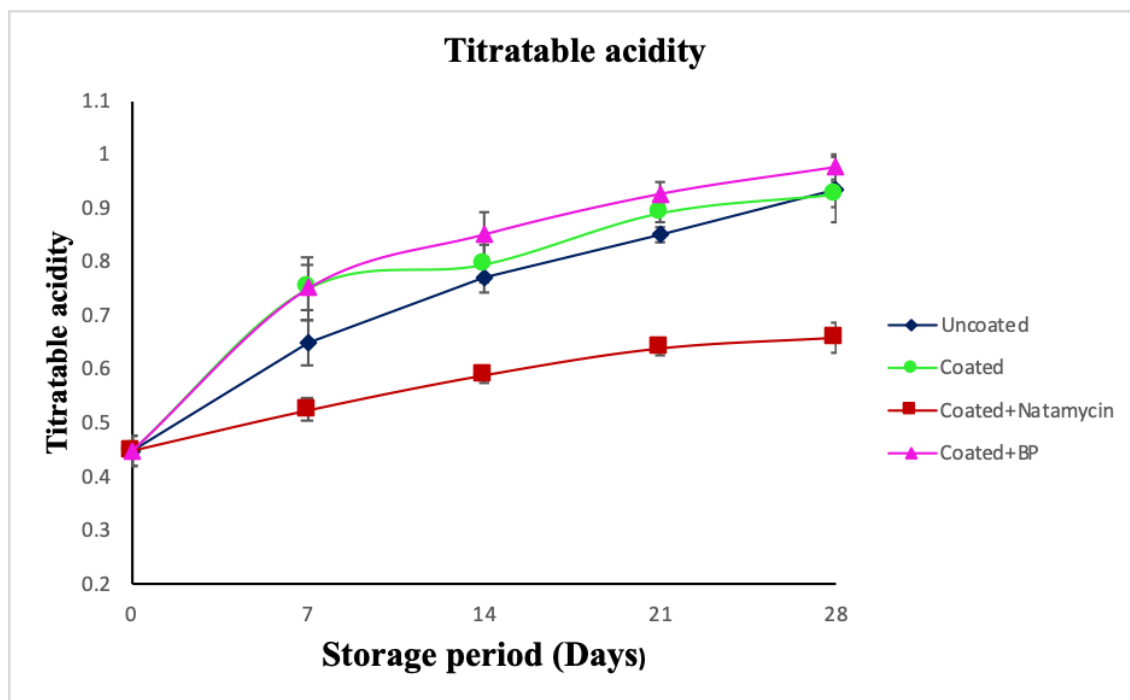
Figure 16: Change in titratable acidity in coated and uncoated sample

4.13.5 Antioxidant activity

The reduction in the concentration of DPPH was monitored by decrease in the absorbance at a characteristic wavelength when it encountered proton radical scavengers. Table depicts the DPPH radical scavenging activity (% inhibition) of water soluble extract (WSE) of the cheese samples. DPPH scavenging activity (%) was found to increase upto 7 days of storage in all the samples. DPPH (%) inhibition was 14.78 ± 0.89, 17.47 ± 0.75, 17.84 ± 1.14 and 44.34 ± 1.25 for uncoated, coated, coated+natamycin and coated+peptides respectively. Highest scavenging activity in coated+peptide sample can be related with the presence of bioactive peptides as bioactive peptides possess antioxidant potential. Besides, significant ($P<0.05$) decrease in scavenging activity was observed after 7 days in all samples. However, less variation was observed in bioactive peptides incorporated coated cheese samples due to less

loss in cheese samples. Dairy products have been longed used as raw materials for the isolation of bioactive peptides of antioxidant potentials. More specifically, cheeses have been investigated and found to be relatively higher with regards to the possession of bioactive peptides due to the processing, ingredients and profile of their inherent microbes. Factors such as ageing time, moisture content, milk source, production technology, and the selected strain of bacteria, yeasts and molds have been found to be of significant effects (Santiago-López et al.,, 2018). Such microorganisms proteolyze the milk proteins and release numerous amounts of bioactive peptides though the effects of these peptides have been found to reduce after reaching their peaks within some time periods. Chen et al.,, 2019 added *Lactobacillus plantarum* and *Lactobacillus casei* to cheddar chesses, exposed them to simulated gastrointestinal conditions and monitored their antioxidant potentials during aging based on their hydroxyl and DPPH radical scavenging activities. Their results showed a continuous increase up in these activities up to the 16th week before continuous decrease set in till the 36th week. Öztürk and Akin, 2017 studied the rate of increase of the antioxidant properties of water-soluble peptide extracts from Tulum cheeses made from goat milk during a 120-days ripening operation. Samples were examined at 0, 15, 30, 90, and 120 days with increase percentage inhibition of DPPH radicals found to be about 0, 2, 2 3, 1, and 1 % respectively to the nearest whole numbers with the maximal rate increases found on the 60th ripening day.

Table 18: **DPPH (%) inhibition in coated and uncoated samples**

Treatments	0 day	7th day	14th day	21th day	28th day
Uncoated	14.78±0.18[aA]	15.84±0.24[bA]	13.78±0.37[cA]	11.84±0.13[dA]	9.72±0.46[eA]
Coated	17.47±0.94[aB]	18.25±0.58[aB]	17.24±0.21[bB]	16.44±0.20[cB]	15.29±0.10[dB]

Coated+Natamycin	17.84±0.51^{aB}	18.29±0.65^{aB}	17.35±0.62^{aB}	16.23±0.54^{bB}	15.25±0.09^{cB}
Coated+Bioactive peptides	44.18±0.64^{aC}	48.34±0.34^{bC}	47.18±0.25^{cC}	43.24±0.98^{dC}	39.84±1.34^{dC}

Means within the same row (lowercase letters) and column (uppercase letters), for each microorganism, with the same letter, do not statistically differ from each other (Duncan's LSD test) ($P < 0.05$).

4.13.6 Microbiological profile

During the microbiological analysis of cheese samples as mentioned in (Table 19)presence of pathogenic microorganisms was negligible. However, *Lactococcus* spp, *Lactobacillu*s spp and mesophilic bacteria was ranged between 4 and 6 log(cfu/g) (Table 18). These results are similar as observed by Ramos et al.,, 2012 in which antimicrobial edible coating was applied on cheese. However, their microbial count was in range of 6-8 log CFU/ml. Presence of *Lactococcus* spp., *Lactobacillus* spp ensure the presence of starter culture in cheese. Moreover, a significant ($P < 0.05$) increase in the number of the above mentioned microorganisms occurred at the early stages of ripening (i.e., the first 20 d of storage), and a slight decrease was observed at later stages. These results are in accordance with Manolopoulou et al., (2003) in which they apply antimicrobial coating on feta cheese. According to obtained result, it was concluded that edible coating material maintain the growth of starter microflora throughout the storage period. Furthermore, log value for starter culture was significantly higher in coated samples except coated with natamycin. Thus, the presence of coating solution support the growth of beneficial microflora by retaining the moisture in cheese samples. Not only this but in cheese samples coated with coating solution incorporated with bioactive peptides, growth of starter culture was observed more than other. It may be related with the symbiotic relationship between starter culture and bioactive peptides.

Table 19: **Viable cell counts [log(cfu/g), means ± SD] of coated and uncoated cheese sample 28 d of storage at 10°C and 85% relative humidity**

Microorganism	Treatment	logCFU/g with storage time				
		0 day	7th day	14th day	21th day	28th day
Lactobacillus spp.	Uncoated	3.785±0.04^{aA}	4.182±0.01^{bA}	4.882±0.02^{cA}	4.083±0.07^{dA}	3.651±0.05^{eA}
	Coated	3.748±0.06^{aA}	4.487±0.03^{bB}	4.972±0.01^{cB}	4.893±0.03^{dB}	4.233±0.09^{eB}
	Coated+natamycin	3.737±0.05^{aA}	3.847±0.09^{bC}	3.941±0.02^{bC}	3.173±0.02^{cC}	2.847±0.04^{dC}
	Coated+ BP	3.741±0.07^{aA}	4.813±0.05^{bD}	4.948±0.03^{cD}	4.918±0.08^{cD}	4.883±0.02^{cD}
Lactococcus spp.	Uncoated	4.195±0.03^{aA}	4.881±0.08^{bA}	5.211±0.02^{cA}	4.872±0.02^{bA}	3.981±0.05^{dA}
	Coated	4.224±0.07^{aA}	4.974±0.04^{bA}	5.383±0.04^{cB}	5.354±0.04^{cB}	5.083±0.06^{dB}
	Coated+natamycin	4.247±0.01^{aA}	4.192±0.03^{bB}	4.227±0.09^{abC}	3.768±0.06^{cC}	3.587±0.03^{dC}
	Coated+ BP	4.213±0.05^{aA}	4.981±0.03^{bA}	5.748±0.05^{cD}	5.682±0.09^{cD}	5.132±0.05^{dD}
Mesophiles	Uncoated	5.433±0.08^{aA}	5.842±0.02^{bA}	5.177±0.03^{cA}	4.987±0.05^{dA}	4.213±0.04$_{eA}$
	Coated	5.562±0.03^{aA}	5.973±0.03^{bB}	6.342±0.07^{cB}	5.891±0.03^{dB}	5.273±0.02^{eB}
	Coated+natamycin	5.564±0.04^{aA}	5.825±0.02^{bC}	5.623±0.05^{aC}	4.927±0.02^{cA}	4.028±0.02^{dC}
	Coated+ BP	5.617±0.07^{aB}	6.023±0.06^{bD}	6.741±0.07^{cD}	6.231±0.07^{dC}	5.777±0.07^{dD}

Storage condition: 28 d of storage at 10°C and 85% relative humidity

Means within the same row (lowercase letters) and column (uppercase letters), for each microorganism, with the same letter, do not statistically differ from each other (Duncan's LSD test) ($P < 0.05$).

Cheese coated with coating solution incorporated with bioactive peptides did not show any growth of yeast and molds, which shows the efficacy of these coating against the growth of spoilage microflora. These results can be related with antimicrobial action of bioactive peptides as previously mentioned. Conversely, a limited growth of harmful and contaminant bacteria on cheese coated with coating solution compared with uncoated cheese samples can be related with controlled flow of gas especially, oxygen. Owing to this, oxygen become unavailable for aerobic bacteria, hence, no growth of aerobic bacteria. In addition to that,

water loss is also protected by coating therefore, a reduction in a_W which is a critical factor for bacterial growth. Moreover, as the coating included only natamycin, yeast and mould growth began after 21 d for yeast and mould. However, negligible growth (< 100 cFU/g) was observed for *Staphylococcus* and *Pseudomonas*.

Table 20: Viable cell counts [log(cfu/g), means ± SD] of spoilage microflora in coated and uncoated cheese sample

Pathogenic Microorganism	Treatment	logCFU/g with storage time				
		0 day	**7th day**	**14th day**	**21th day**	**28th day**
Staphylococus spp.	**Uncoated**	0.00±0.0aA	<2.00±0.0bA	2.8±0.06cA	3.1±0.03dA	3.5±0.07eA
	Coated	0.00±0.0aA	0.00±0.0aB	<2.00±0.0bB	2.3±0.01cB	2.9±0.02dB
	Coated+natamycin	0.00±0.0aA	0.00±0.0aB	<2.00±0.0bB	<2.00±0.0bC	<2.00±0.0bD
	Coated+ BP	0.00±0.0aA	0.00±0.0aB	<2.00±0.0bB	<2.00±0.0bC	2.3±0.03cC
Pseudomonas spp.	**Uncoated**	0.00±0.0aA	0.00±0.0aA	2.1±0.01bB	2.4±0.04cB	3.2±0.05dC
	Coated	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	<2.00±0.0bA	2.3±0.04cB
	Coated+natamycin	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	<2.00±0.0bA	<2.00±0.0bA
	Coated+ BP	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	<2.00±0.0bA	<2.00±0.0bA
Yeast and moulds	**Uncoated**	0.00±0.0aA	<2.00±0.0bB	2.5±0.6cB	3.8±0.08dC	4.5±0.07eD
	Coated	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	2.3±0.03cB	2.9±0.06dC
	Coated+natamycin	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	<2.00±0.0bA	2.6±0.04cB
	Coated+ BP	0.00±0.0aA	0.00±0.0aA	<2.00±0.0bA	<2.00±0.0bA	<2.00±0.0bA

Storage condition: 28 d of storage at 10°C and 85% relative humidity

Means within the same row (lowercase letters) and column (uppercase letters), for each microorganism, with the same letter, do not statistically differ from each other ($P > 0.05$).

CHAPTER 5

Summary and Conclusion

The present study was conducted to explore the potential of plant substrates Pumpkin seed, flaxseed, Quinoa seeds, Chia seeds and peanut for the production of functional bioactive peptides by fermentation method. So, the objectives of the proposed research work were: a) To screen the proteolytic activity of different probiotic lactic acid bacterial strains b) To extract, analyze and compare the peptides derived from different substrates c) To investigate and identify the bioactive potential of the peptides for bioactivities d)To study the potential application of bioactive peptides in food industry.

As first objective was to identify the potential proteolytic strain so different probiotic microorganisms *Lactobacillus helveticus* NCDC 292*, Lactobacillus acidophilus* NCDC15*, Lactobacillus plantarum* NCDC 374*, Lactobacillus fermentum* NCDC 141, *Lactobacillus casei* NCDC 297 were checked for their proteolytic activity by measuring the zone of proteolysis by using agar well diffusion method and among these *Lactobacillus plantarum* NCDC 374 showed maximum proteolytic activity with a zone of proteolysis of (18±0.5) mm followed by *Lactobacillus fermentum* NCDC 141 with a zone of proteolysis of (16±0.7) mm. However, no proteolytic behavior was observed among other tested strains *Lactobacillus helveticus* NCDC 292*, Lactobacillus acidophilus* NCDC15 and *Lactobacillus casei* NCDC 297. Therefore, *Lactobacillus plantarum* NCDC 374 *and Lactobacillus fermentum* NCDC 141 were selected on the basis of their proteolytic behavior. However, these two strains were found incompatible to each other upon checking their compatibility. Hence, *Lactobacillus plantarum* NCDC 374 was used for further study.

Second objective was to compare the functional potential of bioactive peptides isolated from different substrates. Parameters used for comparison were proteolytic activity and antioxidant

activity. In order to check these activities flaxseed, quinoa, pumpkin and peanut milk of ratio (1:5) was fermented with *Lactobacillus plantarum* NCDC 374 (5% inoculum size) and proteolytic activity and antioxidant activity was checked during fermentation. Among flaxseed, quinoa, pumpkin and peanut, maximum proteolytic activity (27.71 micro gram leucine/ml) and antioxidant activity (72.15% DPPH inhibition) was shown by flaxseed. However, no growth was observed in chia seeds due to its gummy texture of chia seeds. Hence, further work was carried out with flaxseed.

Third objective was to investigate and identify the bioactive potential of the peptides for bioactivities. Therefore, optimization of fermentation condition of flaxseed to obtain maximum functional properties (Proteolytic activity, Antioxidant activity and ACE inhibition %) was investigated using response surface methodology. Optimal condition to produce the functional peptides were found to be 4.20% inoculum size with 126 hours of fermentation time. The fermented milk resulted in 67.38% inhibition in DPPH, 41.35% inhibition in ACE and 30.38 micro gram leucine/ml proteolytic activity. Molecular weight cut off membrane (Viva spin) were used to fractionate the peptides. 10kDa peptides showed optimal results for % DPPH inhibition, ACE inhibition, Antimicrobial activity and DPP-IV inhibition as compared to 5kDa.

Further, objective was to check the application of 10 kDa bioactive peptides. Therefore, whey base edible coating incorporated with bioactive peptides, along with positive control coating+natamycin and negative control coating solution without any functional agent and uncoated samples were evaluated for shelf-life enhancement of cheese. Among all, whey coating incorporated with bioactive peptides extend the shelf-life of cheese upto 21 days without any growth of pathogenic microorganisms. Nevertheless, same coating material was found to maintain the growth of indigeneous microflora.

Consequently, it may be concluded that flaxseed proteins underwent an extensive degradation process during fermentation and generated a large number of bioactive peptides. Potential health effects of fermented milk mainly due to the presence of bioactive peptides produced by proteolytic action of *Lactobacillus plantarum* NCDC 374. Moreover, use of fermented flaxseed milk and their bioactive peptides as nutraceutical and functional food or inclusion in therapeutic diet for patients with diseases linked to oxidative stress and hypertension could be beneficial. However, the present study did not address the precise mechanisms governing these effects and further investigations aimed at understanding the mode of action of these peptides in in-vivo system are required. Also, peptides generated in this study can be used for nutraceutical preparation or can be added in food products to enhance their nutritional properties.

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