STUDY ON DEGRADATION KINETICS OF WHEAT AND BARLEY PROTEINS BY *Lactobacillus paracasei CD4*

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IN

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By

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CERTIFICATE

This is to certify that the entitled work **"Study on degradation kinetics of wheat and barley proteins by *Lactobacillus Paracasei CD4*"** presented in this dissertation was carried out at Department Of Biotechnology and Bioinformatics, **Jaypee University of Information Technology, Waknaghat (Solan) India,** by Ms. Kritika Sharma and is an original and legal record of my work carried out under my supervision from June 2018 to May 2019. During the project work she had learnt most of the techniques used during the work. Therefore, the above articulation made by the candidate is genuine to the finest of my knowledge.

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There were a part of individuals who were without a doubt exceptionally supportive, kind, agreeable and affable along the advancement of this project as well. I would like to express my acknowledgement to those concerned.

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BTDD

DECLARATION

I do hereby declare that this dissertation entitled **"Study on degradation kinetics of wheat and barley proteins by *Lactobacillus Paracasei CD4*"** submitted towards fulfillment for the award of degree of **Masters of Technology in Biotechnology under the Biotechnology and Bioinformatics Department of Jaypee University of Information Technology** is wholly based on the study and results carried out under the guidance of **Dr. Saurabh Bansal.** Also, till now this work has not been submitted anywhere for any additional degree or diploma. Therefore, the declaration made by the candidate is true and genuine.

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ABBREVIATIONS

CD	Celiac Disease
GFD	Gluten Free Diet
GDB	Gluten Degrading Bacteria
LAB	Lactic Acid Bacteria
MRS	De Man, Rogosa and Sharpe agar
tTg	Tissue Transglutaminase
℃	Degree Celsius
µl.	Microlitres
ml.	Millilitres
gm.	Grams
rpm	Revolution per minute
min	Minutes
SDS	Sodium Dodecyl Sulphonate
PAGE	Poly acrylamide gel Electrophoresis
APS	Ammonium Persulfate
Rp- HPLC	Rversed phase Liquid Chromatography

ABSTRACT

This contemporary analysis has a goal of figuring out the proteolytic activity of indigenous lactic cultures against dietary gluten found in various food products of wheat, barley to underline a peculiar approach for reducing the epidemiology of this disease. Celiac disease is an intense situation of gluten sensitivity and intolerance hence gluten is the main causative protein that damages the small intestine. Based on probiotic attributes of lactic cultures four lactic cultures; *Lactobacillus paracasei CD4, L. gastricus BTM7, L. plantarum K 90* and *L. rhamnosus GG* showed their perceptible gluten hydrolyzing characteristics with the plate method containing MRS agar, gluten protein and the lactic cultures. Furthermore, lactobacilli strains were also examined for appurtenant selection of efficient strains that exhibit high proteolytic and metabolic activity. This prerequisite knowledge was further used in qualitative and quantitative analysis of the target protein. Accordingly, the gluten complex gets denatured within higher and lower molecular weight fragmented protein i.e. Glutenin and Gliadin. For further development of the fermented food product with probiotic attribute, *Lactobacillus paracasei CD4* is used for preparing sourdough (khamir) followed by their proteolytic analysis of gluten protein through SDS-PAGE and RP-HPLC. The results acquired in the current study demonstrated the effective propensity of *Lactobacillus paracasei CD4* to breakdown the gluten into minimal amount. Hence the selective *Lactobacillus paracasei CD4* strain can be used to stimulate the production of fermented food product with minimal gluten content and enriched with probiotic values as a boon for gluten intolerant people to reduce the risk of immunogenic reactions to the negligible quantum. The developed fermented product would also be healthier to the normal individuals and as well as it strengthen their gut microbiota

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Gluten intolerance (celiac disease)

Celiac disease (CD) is a T cell mediated inflammatory immunogenic condition that typically targets the gastro intestinal tract largely the small intestine, the insolubility of gluten is the major constraint for developing this pathological disease. Severity of this disease increases with the consumption of the endospermic protein like gluten constituted in many packed and homemade food products to provide elasticity to the dough.

Irrespective of gender and age this disease's pervasiveness is comparatively higher than the earlier decades (0.7-2%) globally, i.e. out of 96 individuals every one person is affected. Different researchers have studied the issues of high prevalence and concluded that lack of awareness is the major and primary issue of increasing rate of the disorder approximately 70%-80% of cases misdiagnosed annually. Secondly, it could be genetically inherited from parents to off springs, affected individuals may carry some Human Leukocyte Antigen receptors DQ2 and DQ8 that are responsible for the immunogenic reactions and hence elevate the symptoms of duodenal inflammation, discomfort and villous atrophy, responsible for other opportunistic diseases like malabsorption, anemia and diarrhoea. (Helmerhorst Eva J. 2014). Researchers showed that intestinal inflammation is purely associated with the CD71 cells overexpressions and also with IgA (Losurdo G et al 2016).

Till today the treatment known and preferred by physicians at most and exclusively is the strict gluten free diet that is however very challenging since, these dietary proteins could not be easily digested by the host microbial enzymes and abundantly constituted in various dietary products of wheat, barley or rye. (Helmerhorst Eva J. 2014)

In future a probable curative and restorative measure for auto immune triggered reactions acquired by gluten protein could be the use of bacterial derived small peptides or enzymes that degrade gluten protein before reaching the GI tract (Bethune MT et al 2007, Cerf-Bensussan N et al 2012). Up to now, the microbial enzymes aiding hydrolyses of causative protein are extracted and utilized from innumerable biological sources like *Sphingomonas capsulata* and *Aspergillus niger* including barley species itself responsible to produce prolyl endopeptidases, EP-B2 respectively but these enzymatic oral supplementation exhibit some restrictions over usage as these enzymes do not hold stability and pH under

acidic condition of the human stomach and therefore require needful clinical trials and FDA approval (Tack GJ et al 2013, Lahdeaho ML et al 2014). Alternatively, many studies have demonstrated that probiotics adhere the property of altering gluten protein into digestible peptides and also increases the physicochemical reaction, introduction of probiotics containing these live or attenuated beneficial bacteria helps in reducing the distressing of small intestine and gut by consuming food containing gluten (Berger M et al 2015). In addition to this many researches has been detailed that probiotics specifically microbes of gastrointestinal tract (GI) have many other health advantages irrespective of gluten indigestion issues.(Petschow B et al 2013, Serban DE et al 2013).

1.2 Causative agent: The GLUTEN

The protein allergenicity is predominantly induced by wheat and barley flour that is rich in gluten and are also known as endosperm proteins comprises of prolines and glutamines. It is a complex of water soluble glutenins and ethanol soluble gliadins, commonly β gliadins and γ gliadins with having molecular weights (MWs) ranging from 42-47 KDa (Kim N. 2017) while glutenin unit have classified into two subunits of high-molecular-weight (HMW) proteins with MW of 67– 88 KDa and low-molecular-weight (LMW) proteins MW around 32–35 KDa. (Kucek et al 2015)

1.3 Probiotic Microbes

These probiotic microbes are non- pathogenic usually and may assimilate many positive health benefits other than preventing the risk of CD such as strengthening the gut immune system if ingested in decent amount via means of oral consumption or through injections. *Bifidobacterium spp, Streptococcus spp, staphylococcus spp and lactobacillus* too are some of these examples; many dairy products like yoghurt are abundant with *Lactobacilli spp.* so it's easy to isolate these cultures/ beneficiary microbes from variety of Himalayan regions (Sharma K et.al, 2017).

In our laboratory, the potency of these lactic cultures have already been studied and reported under in vitro gut simulated conditions and therefore, these cultures are complying to combat the accelerating trend of celiac disease in country's population consequently, the

objective highlights the study of degradation kinetics of wheat and barley proteins by *Lactobacillus paracasei CD4.*

1.4 Fermented sourdough

Sourdough prepared from lactic culture is a conventional process extensively used to regulate the organoleptic properties of baked foods such as bread, dosa, bhatura rich in probiotics. The mixture of probiotics has additionally been observed effective for protein (gliadin) degradation. With the aim of predigestion of gliadin that is the insoluble protein and foremost causative agent, this corresponding study deals with the production of smaller peptides that are being absorbed within the small intestine with subsequent digestion in the stomach and hence have a low allergenic reaction.

1.5 Nutrition value and cost

Regular consumption of fermented food certainly provide dietary and nutritional benefits over non fermented food products, fermented food have longer shelf life and can be preserved for longer time, super easy to digest, make food more nutritious and edible in case of lactose and gluten intolerant individuals. With the purpose of fermentation process for food products the utmost vital consequence is the enrichment of food with fundamental and vital bioactive compounds like “essential amino acids, vitamins and minerals” also, which is particularly crucial in those areas of the world where malnutrition is prevalent and rising health issue.

Coming, over the cost of the consumption of fermented food product over gluten free diet or other medications, so economically GFD is going to be more expensive and tiring routine to be followed concerning the taste and budget. The reason of expensive diet is only the hindrance to prepare food without the assimilation of gluten protein which is very complicated.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Background of Celiac disease and gluten toxicity

Celiac disease (CD) is one of the prevalent T cell mediated autoimmune inflammatory condition that might occur in the individuals who are genetically predisposed and based on many cohort studies it could be genetically inherited from first degree relatives to the next generation (Lundin et al. 2015). CD is nowadays very common food allergy transpired that gluten consumption is the primary reason to infect or inflamate the GI tract majorly the duodenum part of small intestine where absorption of nutrients from ingested food takes place but due to villous atrophy the nutrient absorption get dysfunctional. Although the symptoms of this disease are not very distinctive and peculiar and similar like indigestion problem, irritable bowel syndrome problem related to improper nutrient absorption, nausea, neurological disorders likewise these symptoms may appear with the frequent consumption and inhalation of gluten rich cereals such as wheat, rye, barley. To date, the prevalence study is based upon serological screening i.e. typically by detecting the titer of antitransglutaminase antibodies due to relatively high specificity and sensitivity towards antigliadin, anti tissue transglutaminase enzymes (Lundin et al. 2015). Definite diagnosis of histopathological changes with gastroduodenoscopy such as"villous atrophy, inflammation of the intestine, irritable bowel syndrome also" should be carried out accurately as the diagnosis based on clinical symptoms seems difficult since the heterogenecity of the disease is very high and risk of adhering the disease varies with the age groups also . (José Ibiapina S.N. et al 2004).

2.2 Gluten properties

Wheat is one of the staple food crops with annual harvesting rate of around 600 million tonnes in various regions of India along with the huge variety, other than maize and rice which is thoroughly being consumed and utilized in preparing different bakery products or food stuffs. The acceleration in the production and harvesting of wheat crop is because of the distinctive property that is viscoelastic characteristic in wheat dough for its processing to make bread, pasta, or bakery food stuffs and gives the chewy texture to the food products (Shewry Peter R. et al. 2002). The gluten is a complex protein formed with cross linking of

glutenin and gliadin constitutes high amount of glutamine and proline amino acids that are not easily be hydrolyzed in the gut of normal individual even, which is found in the endosperm of the dry grain and hence known as storage protein (Berger M et al. 2015).

2.3 Stages of celiac disease

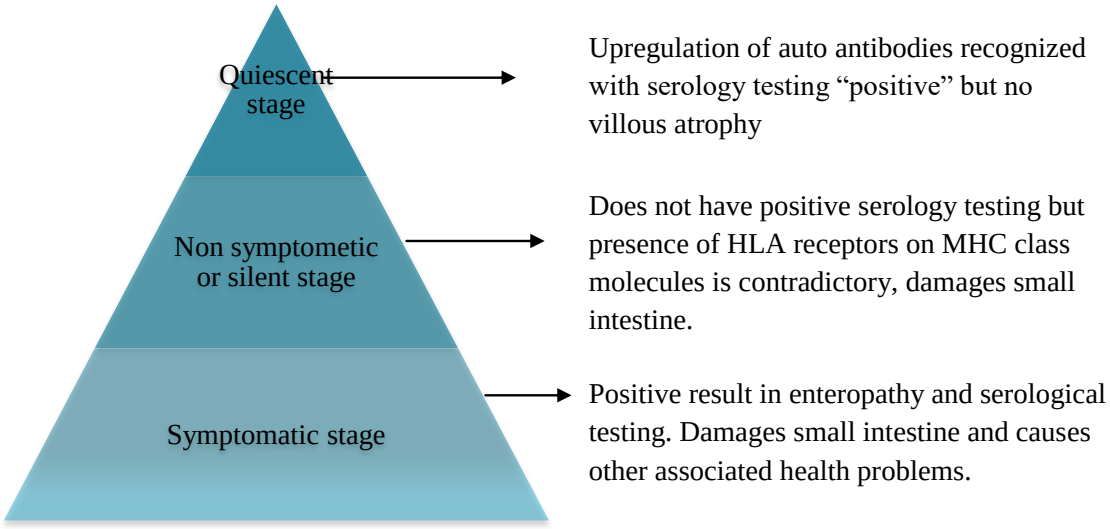

Figure 2.3: Pictorial representation of stages of CD

(Parzanese I et al. 2017)

2.4 Risk of other opportunistic autoimmune disorders

In many recent researches it has been detailed and explained the association between celiac disease and other autoimmune disorders like type 1 diabetes mellitus, neurological disorders, autoimmune thyroid disorder and many more. There have been almost hundred opportunistic diseases either autoimmune or not that are directly associated with celiac disease (Colin P et al. 1994). Over a survey, it is inferred that individuals suffering from gluten intolerance and celiac disease are more prone to have other autoimmune disorders with an incident rate of approximately 5% more than the normal or healthy people and hence reciprocate in a vice versa manner if the individual is suffering from any other disease like diabetes, indigestion etc often concurrently suffer from celiac disease issues (Lundin et al. 2015).

The incidents related to celiac disease and T1DM in association with each other is very frequent than the general healthy population. The ratio of prevalence may vary with respect to the age of the patient, genetic factor and also the environment factors such as diet therefore considered under multifactorial disorder (Smyth D J. et al. 2008). People are at more risk of developing neurological disorders also who endure CD because of the immunogenic antibodies generated that affect the neural part especially the neurons (Cats E A et al. 2011).

2.4.1 Risk of Neuropathy and Stroke

According to many population based studies it has been inferred that CD adhere bidirectional association with many other immune mediated disorders and preventive measures must be taken at the early stage of this CD to reduce the risk and prevalence of other opportunistic disorders hence proper screening could be beneficial in the patients of neuropathy or neurological disorders (Thawani S et al. 2015).

Stroke is the second most common reason of human deaths globally and this rate of prominence get hike because of inflammatory immune mediated diseases like very common CD. In current studies it was analyzed that the percentage of affected people are increasing very rapidly and approximately touches to 1-2% mostly in the western regions of the hemisphere (Ludvigsson JF. et al. 2012).

2.4.2 GFD AND T1DM

Gluten free diet (GFD) is generally recommended as a preventive measure for CD population but, in this modern era normal and healthy people started using gluten free food stuffs in the daily routine but adhering a gluten free diet is also not healthy because GFD only contains carbs and starch in very high amount and hence the glycemic index of gluten free food is very high and may cause many health issues such as increasing blood glucose level of the body and may cause insulin dependent diabetes mellitus (Ouaka-Kchaou A et al. 2008)

2.5 Concurrent and associated auto immune disorders with CD

These diseased conditions occur concurrently with celiac disease the main reason that have been concluded till today is tissue damage and that is relatively causing and damaging the other tissues hence chances of developing multi diseases at later stage of CD is undoubtedly very high.

Table 2.5: Depicting the prevalence of other diseases

Associated disorders	Prevalence of disease in CD patients
Anemia / Malabsorption	15-50%
Type 1 diabetes mellitus	10%
Neurological disorders (Peripheral neuropathy)	~12%
Liver dysfunctioning	~10%
Condition of Infertility	8%

(World Gastroenterology Organization)

2.5.1 Worldwide statistics:

According to the different studies reviewed for the celiac disease, prevalence of this disease rumored globally is very high and arising consequently that affects the living standard as well as quality of life a person cohere:

Table 2.5.1 Estimated global epidemiology

Population type	Percentage of prevalence
Healthy population	0.23-1.4%
Population at risk (western countries)	1-12%
European and North American regions	~1%
Asian region	0.2-1%

(Ashtari S et.al. 2014)

The prevalence of CD amongst first degree relatives in Asian pacific region is comparatively lower than the Western countries and hence shows a great nonuniformity because of various numbers of responsible factors that includes:

- Gluten intake in daily diet.

  The latest research on gluten intake showed that people suffering from gluten intolerance or celiac disease have to avoid gluten from their diet as ingesting a gluten around 50 mg daily can cause villous atrophy and hence, individuals therefore are more prone in developing this health issue. FDA has approved 20 ppm of gluten in a gluten free labeled product (GRAS).

- HLA- DQ2/DQ8 (Receptors) abundance within the population.

  According to the research HLA typing in CD patients is around 96% which is higher as compared to normal individuals which lies around 40% and those individuals who serve as first degree relatives for this disease the frequency starts rising from 40% i.e. more the closer relative more would be the HLA frequency and hence higher will be the prevalence of the CD **(**Cecillo LA et.al, 2015**).**

2.6 Prevalence in North India:

In a questionnaire-based survey in Chandigarh (northern part of India) where around 4300- 4400 school children were included approximately age ranges from 3–17 years, and based on the over cohort community based study this widespread prevalence of the CD though increasingly proliferating and escalating. In India around 6-7 million of total population is expecting to have this CD and although CD is not a new disease which is gathering more attention rather, lack of knowledge was the main reason (Gupta R et.al 2009). CD cases have majorly reported from the northern part of the country India where wheat is mainly consumed in daily routine this includes states like Chandigarh, Punjab, Haryana, Rajastan, UP, Delhi, some portion of Madhya Pradesh collectively known as CELIAC BELT (Catassi. C et.al 2014).

2.6.1 Prevalence of CD in type 1 diabetic population of North India:

Year 2010, population study conducted to identify the prevalence of CD in type 1 diabetic population which was nearly 11.1% on an average including all ages and genders and this prevalence percentage may change by some external as well as internal factors such as, genetic factors (Bahdada et al. 2010**).**

According to the American Diabetes Association, there might be a genetic link between CD and type 1diabetes:

1. **Genetic predisposition:** Human leukocyte antigens (HLA GENES) HLA DQ2/ HLA DQ8.
2. **Biomarkers:** Certain biomarkers in the human blood that are receptive to celiac disease antigen may also increase the risk of developing type 1 diabetes.

Both these above said conditions associated with inflammatory constituent which provokes the immune system to damage the organs or the tissues of the body such as intestinal damage and damage occur in the beta pancreatic cells that causes CD and Diabetes.

2.7 Tissue Transglutaminase; The receptive enzyme

The ingestion of gluten rich dietary food instigate the immunogenic reaction because of the secretion of Immunoglobulin class A (IgA) and hence these IgA target transglutaminase that are relatively in high concentration than normal individual and act as substrate found and generated in small intestine therefore the enzyme act as biomarker and is used as diagnostic aid nowadays for CD (Rief S. et al. 2003). Tissue transglutaminase (tTg) counts under a wide group of transglutaminase enzyme that form the peptide bonds (isopeptide linkages) with the cross linking between tTg and gluten. Transglutaminase is a pervasive enzyme counting around 680- 686 amino acids, it is an intracellular enzyme but also be localized in extracellular matrix and over the cell surfaces as well act as multifunctional enzyme or protein (Di Sabatino A et al. 2012).

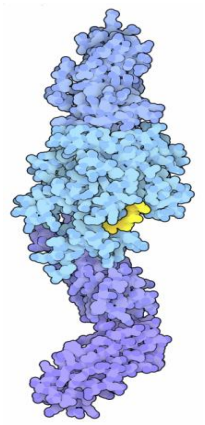

Figure 2.7: Gluten molecule (Yellow) binds to the active site of tTG (Blue)

(Costanzo L.D., et.al, 2017) [PDB-101] WHO and World Gastroenterology Organisation

2.8 tTG- Gluten complex, prerequisite for disease development

Various human transglutaminase enzyme form a cascade that have homologous gene sequences and structures but encoded by different genes that changes their functionality with specificity of substrate (Di Sabatino A et al. 2012).

tTG is a protein binder enzyme binds with the gluten protein with peptide bonds giving rise to enzyme-protein complex (tTG- Gluten) whenever the gluten is consumed by the body. These complex molecules then detected by the B cells of the body producing humoral responses via specific antibodies generation and as a result of the following imbibing of gluten-enzyme complex, tTG then subsequently removes the ammonia group from glutamine (amino acid present in gluten with ammonia group) (Rief S et al. 2003).

The deaminated glutamine is then bound to HLA DQ2 and HLA DQ8 receptors present on APCs "Antigen Presenting Cells" surface and thus, the T cells especially Th1 type cells will recognize gluten as foreign or external antigen, this prophecy will activate the cytokynines and thus generate auto antibodies that damages the intestine (Caputo I et 2008). Moreover, it was analyzed that deamination of tTG is a responsible reason of the pathogenesis occurrence hence named as celiac disease. (Sabatino A et.al 2012)

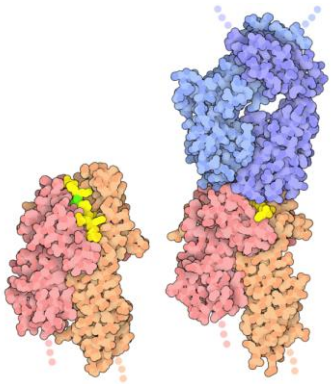

Figure 2.8: Deaminated gluten (yellow), tTG (green) form complex. HLA DQ2 and DQ8 molecules (orange and pink), T-cell receptor (blue) recognizes the whole formed complex.

(Costanzo L.D., et.al, 2017) [PDB-101] WHO and World Gastroenterology Organization

2.9 Transpired purposeful therapeutic and dietary approaches

The deamidation of gluten manipulates the digestible property of gluten and hence evokes stimulatory response furthermore, this disease can be treated with massive therapeutic and dietary approaches that includes initially, a gluten free diet "gluten

should be less than hundred ppm" constituting maize, or carbohydrates rich food although to control the gluten concentration but in case of severe conditions proper medications like anti transglutaminase immunoglobulin introduction to prevent the gluten deamination and thus reducing the prevalence of CD in the respective patients. Although recovery of damaged duodenal and mucosal layer of small intestine takes months of recovery period and that's why immediate recovery of epithelial cells is not so possible that might establish the other digestive issues.

2.10 "Probable Enzymatic therapies" Mode of Action

To either suppress or eliminate the risk of adhering CD there are majorly two ways to treat gluten toxicants:

1. One is the digestion of immunogenic gluten protein by intra-luminal enzymatic therapies.
2. Another is, transforming targeted immunogenic larger peptides of gluten into relatively smaller and non immunogenic peptides using bacterial strains directly.

2.10.1 Target areas for therapeutic approaches:

- **Suppressing the receptive epitope sites**

Using specific HLA blocking substrates or biomolecules to inhibit the binding of deaminated gluten peptide and thereby, triggers the generation of auto-antibodies by cytokines hence forms a barrier for the activation of T cell to respond and multiply rapidly (McCarville JL et al 2015).

- **Intestinal permeability**

Gluten is a long peptide chain of glutamines and prolines remnants also known as prolamins that are generally and mostly indigestible by common gut proteases. These indigestible molecules get into the lamina propria by transcytosis. Futhurmore, these peptides derived from gluten get modified, activates the APCs and hence induce

cytokines proliferation that tends to tissue damage (Gordon S.R. et al. 2012). In comparison between healthy and the affected individual the intestinal permeability has found to be more permeable in CD patients than others therefore, in future the researchers could think of the aspect of decreasing the permeability of lamina propria at initial stage and otherwise targeting the intestinal permeability so to not induce the immune system of the body (McCarville JL et al 2015).

- **Use of synthetic binders and therapeutic enzymes:**

At present time, there are many advanced approaches to deal with the gluten intolerance problem. One of the upcoming approaches is use of polymeric binders that inhibits the generation of immunogenic peptides from gluten. One of the limitations could be, this polymeric binder can act successfully over a minimum concentration of gluten. Use of endopeptidases is the approach that helps in digesting the gluten and thus many such enzymes have been isolated from different origins like microbes that include bacteria, fungi. Chemically derived oral enzymatic therapies have also proceeded the clinical trials some of them are Vercimon, Elafin, Larazotide acetate, NexVax2 (Makharia GK , 2014).

Table 2.10.1: Recent Enzyme and vaccine therapies

ENZYMES	SOURCE	MODE OF ACTION	DRAWBACKS	REFERENCE
Prolyl oligopeptidase	Bacteria	Cut after a proline residue in peptides	Unstable and non-functional under acidic conditions of the stomach	Shan et al 2002, 2004

ENZYMES	SOURCE	MODE OF ACTION	DRAWBACKS	REFERENCE
DPPIV (dipeptidyl peptidase)	Fungi	Cut after a proline residue in peptides	Sensitive to low pH and no endoprotease activity	König et al, 2017
Prolyl endoprotease	Aspergillus niger	Cut those epitopes into smaller non immunogenic peptides	Require high dose and provide only 50% degradation	Janssen et al, 2015; König et al, 2017

(Makharia GK, 2014)

Table 2.10.2: Vaccines against gluten immunogenic response

THERAPY	TREATMENT CLASS	MODE OF ACTION
Larazotide acetate	Tight junction protein binder	Decrease the permeability of tight junctions within the intestine.
BL- 7010	Polymer binds with gluten	Scavenges the gliadin and prevents immunogenic peptides generation.
Nex Vax 2	Vaccine	To reduce the allergic response.

THERAPY	TREATMENT CLASS	MODE OF ACTION
Necator americanus	Modulate immune response	Inhibits the activation of Tcells.
Lactobacilli spp. And Bifidobacterium	Probiotics	Strengthen the gut microbes and reduces inflammatory immunogenic response.

(Veeraraghavan G et.al 2015)

- **Limiting the Gluten consumption**

One way of reducing the gluten exposure is strictly adapting the gluten free diet for lifetime and follow up a proper and healthy diet without ingesting gluten which is tedious somehow. The other way is inheriting the habit of using ancient wheat in daily diet and this has been observed over few decades back the reason is, nowadays the wheat cultivars that is being commercialized is hexaploid resulted from hybridization process between two different varieties of wheat i.e. diploid and a tetraploid. From many studies it has been observed that ancient wheat like *Triticum monococcum*; a tetraploid wheat cultivar) tends to have a shorter sequence of these 33-mer gliadin and glutenin sequences and thus having relatively lesser proline titer which inferred low incidences of celiac disease. The major reason of consumers attraction towards the hybrid variety of wheat due to chewy texture and elasticity that aids in processing the dough easily without much efforts and give good texture and other organoleptic properties to bread or bakery cookies etc. (Makharia GK , 2014).

- **Role of Probiotics and gut microbiota:**

In many recent reviews the reason of CD occurrence has been explained briefly with the evidence of loose or increased permeability of intestine as well as dysbiosis of gut microbiota that wholly controls the body functions. This dysfunctioning of gut microbes leads to health issues not only associated with the gut but every cell functioning is directly or indirectly regulated by gut microbes hence play a very vital role. This

dysfunctioning and alteration in gut microbiota commonly due to allergens such as gluten in case of CELIAC (gluten intolerant) patients.

The advanced therapeutic and dietary approaches nowadays come into existence that can be approachable to combat this issue many of these are isolated from microbes such as bacteria, fungi that show proteolytic properties against the causative food allergen "gluten". Other aspects are isolating the active enzymes from beneficial gut and digestive system microbes such as *Lactobacilli species, Streptococcus species, E.coli* and much more (Losurdo G et. al 2016)

Ideally to combat this issue generally and most commonly people gets inclined towards "GFD" for rest of the life which is difficult where country food is wheat and gluten is used in the processing of baked foods. In recent times, people are very health conscious and nowadays more habitual towards organic products and probiotics hence, this new trend is giving a potential solution against the rising issue of CD (de Sousa Moraes LF et al. 2014). There are many probiotic products being commercialized in the market such as yoghurt having high content of *Lactobacilli*, these live microbes when ingested firstly, they help in digestion and secondly these bacteria strengthen the gut microflora by colonizing there in the small intestine and proliferates there only (Lorenzo Pisarello MJ et.al. 2015). Other examples of probiotics are fermented food like South Indian dishes are mostly having probiotics properties due to the incorporation of starter cultures used to ferment the dough that enhances the food texture and also the nutritional value of the food therefore, can be used as condiments in Indian Kitchens.

Currently, many lactic cultures have been registered for clinical trials that constitute the efficient degrading ability against the responsible peptides for instance: *L. plantarum* KKP 593/p and W37/54, *L. helveticus* Lh10, *L. rhamnosus* Lr23, *L. sakei* 750, *L. curvatus* 750, *L. coryniformis*spA, *W.cibariaEKO31, P.pentosaceusEKO23*and*1850* and *P.acidilactici* EKO26 are some of them but do not allow complete degradation (Stefanska et al. 2016). Consequently, this probiotic treatment could be served as "additional treatment to enhance the efficacy and potency of the therapeutic and medicinal treatment" (Veeraraghavan G et.al, 2015).

2.11 Socio-Economic efficiency and cost benefits

The cost of producing the fermented product would be less than their existing cost. Lactic cultures which we would be using to produce these fermented products are economically very feasible than adapting gluten free diet. A gluten free flour marketed with a cost of 160-200/ kg that's what not economically feasible.

India features a wealthy culture of generation of conventional matured nourishment method like consumption of fermented food whereby the individuals since ages have been subordinate on maturation of different delights via fermentation process, where nourishment arranged from distinctive conventional techniques that are accessible and represented by ethnic inclination, individuals religion and also the social and cultural ethos hence, easy to adapt this routine in daily diet routine.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Prerequisites

3.1 Apparatus required:

- 45 ml tubes for centrifugation, a temperature controlled centrifuge, distilled and milliQ water for SDS PAGE and Rp- HPLC analysis, 0.2 mm filters for HPLC, C8 Columns, Round bottom flasks, UV Spectrophotometer, SDS PAGE Assembly, Lyophillizer, Sonicator, vacuum pump, HPLC vials.

3.1.1 Reagents used:

1. Chemicals (0.02M phosphate buffer, NaCl, 70% Ethanol, 0.075N NaOH to extract gliadin, glutenin, albumin)
2. Bradford reagent for protein quantification with the BSA standard.
3. SDS PAGE reagents "Acrylamide, Bis-acrylamide, tris- HCl, SDS to linearize the protein, and a polymerizing agent TEMED in different concentrations to prepare stacking and resolving gel of 12% and 5% respectively".
4. Sample buffer constitutes "Glycerol to make sample denser, Bromophenol blue as a tracking dye, 2-β mercaptoethanol to disintegrate sulphur bonds, Tris Base and Glycine".
5. Acetonitrile (ACN) and Tri-Fluoro Acetic acid (TFA) (HPLC GRADE)

3.1.2 Wheat and barley flour:

Barley and wheat flour was procured from commercial stores to extract albumin, glutenin and gliadin from the mix of these two flours. Gliadin is the protein insoluble in water and thus can be easily dissolved in 60-70% of ethanol. Barley requires more water to knead it into dough hence mix of wheat and barley gave required texture of bread hence preferred.

3.1.3 Fermentation:

It is an anaerobic condition used to ferment the dough of wheat and barley into sourdough with the help of lactobacillus species named as "*Lactobacillus paracasei*

CD4 (*L4*)" to degrade the storage protein into smaller peptides that are not harmful and easily consumable by celiac prone patients.

3.2 Methodologies followed:

3.2.1 Revival of the culture

The preliminary results acquired in gluten degrading activity were assessed and indicated the potential of indigenous probiotic lactic cultures. The *L4* culture is then taken for further analysis of protein denaturation.

- Hence, first of all, *L4* culture suspension was prepared at normal room temperature (37°C) on shaking.
- After the desired growth the culture suspension is then centrifuged and the cells were pelleted down after discarding the supernatant.
- The pellets were then dissolved in calculated amount (50 ml) of distilled water used to knead dough.

3.2.2 Fermentation of the dough

When adding the lactic culture cells mixed in distilled water to knead dough, the cells at survival temperature starts degrading the long protein peptides into small fragments with the help of fermentation process this leads to easy digestion of fermented food with the help of lactic culture, this mode of action is commonly known as "pre-digestion of gluten" during the processing of food. Fermentation was observed with increase in loaf volume.

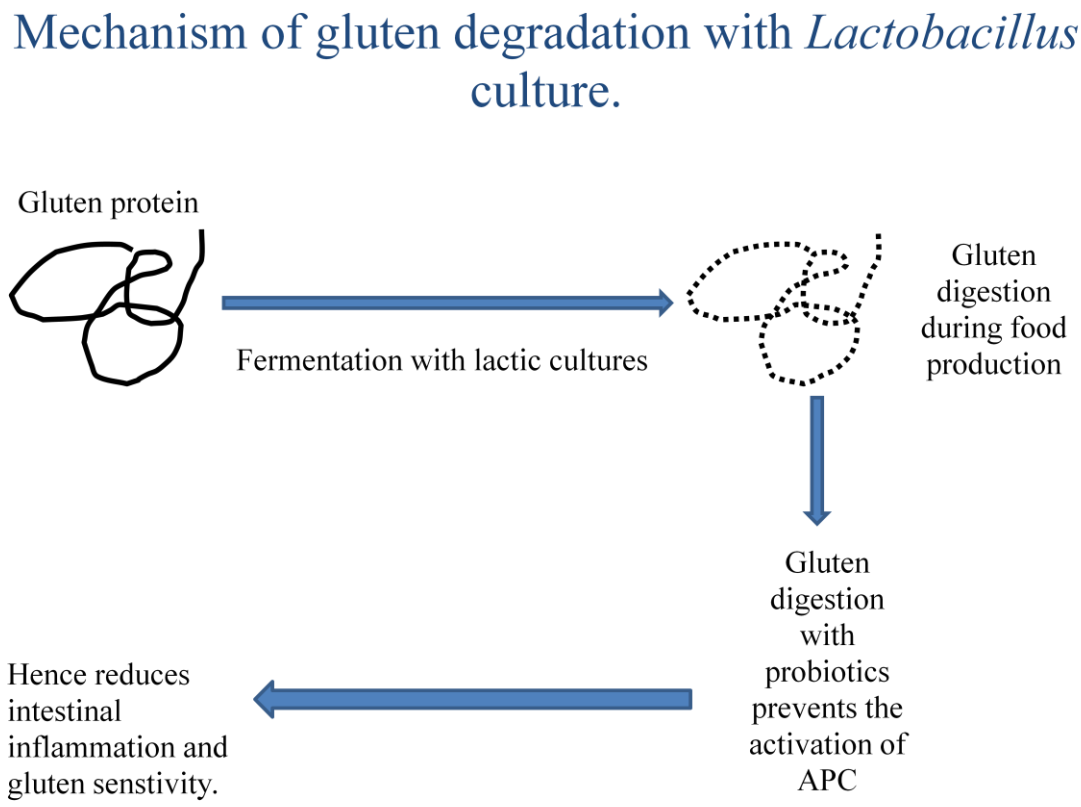

Figure3.2.2: Pictorial representation of mechanism of lactic culture used. (Deora NS et al. 2014)

3.3 PROTEIN EXTRACTION AND QUANTIFICATION

The extraction of different constituted proteins was done according to the procedure reported by Siddiqi et al.2016. The various steps involved were as follows:

1. From each dough of Wheat Barley mix 5gm was taken before and after fermentation at 0 hour and 12th hour.
2. That 5gm of fermented and non-fermented dough was incubated for 30 mins with 0.02 M phosphate buffer +0.9% NaCl (pH 6.8) then followed by centrifugation at 10,000 rpm for 15 mins to extract Albumin.
3. The gliadin protein was extracted with the 70% ethanol incubation for half an hour then followed by centrifugation for 15 mins.
4. 0.075 N NaOH was used to extract Glutenin from the two dough (fermented and non-fermented).

3.3.1 Lyophilization

Also known as “freeze drying process” used to dehydrate the biological samples at low temperature first crystallizes the liquid sample into solid state that is ice formation then, directly passing from solid to vapor state without transforming to liquid state commonly called as “Sublimation”.

- All the protein samples were then lyophilized after freezing them at -80°C under the vacuum conditions.

3.4 Protein quantification steps

The lyophilized protein samples were then mixed in a phosphate buffer or ethanol and then with Bradford analysis the protein is quantified using BSA standard curve.

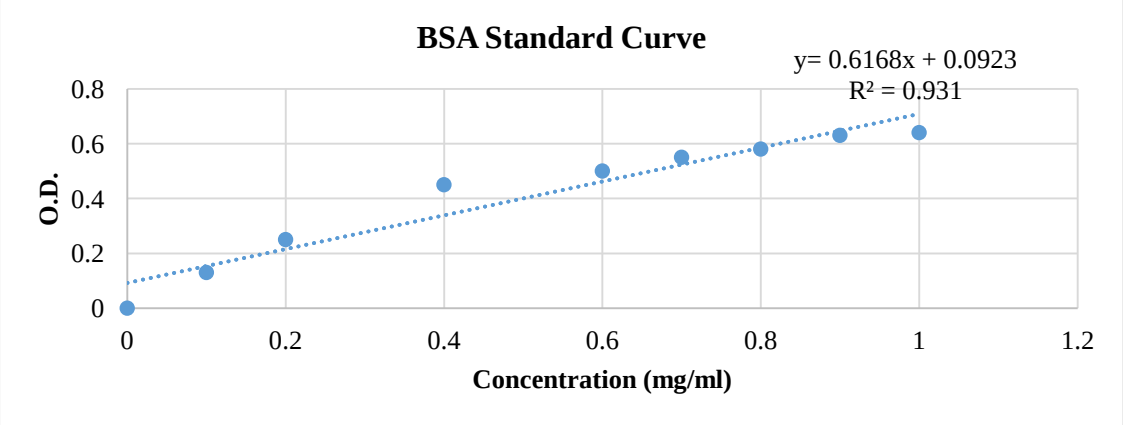

Figure 3.4: BSA Standard Curve for protein quantification

3.4.1 SDS PAGE ANALYSIS

- Stacking and the resolving gels were prepared to cast in between the gel plates and then left for solidification.
- Sample buffer was added into the extracted protein samples including the dye help in tracking the protein sample and secondly sample buffer constitutes beta

mercaptoethanol or DTT to break sulphur bonds of the protein and aid to linearize the protein.

- Running buffer is the another main constituent of SDS PAGE that helps in running the sample in electric field acts as a mobile phase.
- Stainer constitutes Coomassie blue (CB), Methanol and Glacial Acetic Acid (GAA). CB stains the protein band while GAA fixes the stained band whereas Methanol removes the extra stain from the gel.
- Destainer consisting GAA and Methanol which is used to remove excess of unbound dye to make the protein band easy to visualize.

Table 3.4.1: SDS PAGE reagents (Helmerhost Eva J et al. 2014)

Constituents	**12% gel (Resolving 10ml)**	**5% gel (Stacking 3ml)**
30 % Acrylamide and Bisacrylamide	4 ml	0.495 ml
Tris HCl	2.6 ml (1.5 M; pH 8.8)	0.375ml (1M; pH 6.8)
SDS (10%)	0.1 ml	0.03 ml
APS (10%)	0.1 ml	0.03 ml
TEMED	0.004 ml	0.003 ml
WATER	3.2 ml	2.1 ml

3.4.2 Degradation analysis by Rp-HPLC

The degradation of protein was analyzed using Reversed phase HPLC according to the procedure described and followed by Helmerhost et al. 2014

Following were the steps to proceed:

Mobile Phase Preparation:

1. Mobile phase A (milliQ water) and B (80% of Acetonitrile (ACN) and 100µl of Tri-Fluoroacetic Acid) were prepared (from literature).

HPLC Sample and Standard Preparation:

2. The gliadin samples of different time intervals were filtered with 0.2mm of filters.
3. The samples and standards were properly labeled and required adequate volume approx.1ml.

Purging

4. After placing the tubling's of pump within the respective pumps purging of pumps A and B was done for 5 mins.

Flow Rate

5. The isocratic flow was set and the flow rate of pumps was 1ml/min. after changing the pumps to remote mode.

Sample Loading

6. The test samples of extracted gliadin protein and standard were placed in the slots of Carlos Wheel accordingly.

Selection/ designing the protocol

7. The desired software operation was selected, and equilibration (washing) of the columns was done for at least first 60 mins.

8. Then, the blank control and standards and likewise the test samples were run at 219 to 230 nm.
9. Lastly, again the equilibration of column was done before shutting down the process of HPLC.
10. Finally, the samples were analyzed based on peak width and peak height with respect to protein standard.

3.5 Organoleptic characteristics of sourdough compared with control

Any food product is viable if it is competent and complied with the already available market product, this can be achieved by several examining parameters such as rheological properties and organoleptic properties in case of any food product.

The organoleptic characteristics of treated dough fermented with the lactic culture (wheat and barley) and non fermented dough as a control were studied over different parameters such as taste, softness, chewy nature etc. this was done with the baking of dough afterwards, taste and texture were analyzed by selecting a group of people to taste the same.

The sourdough prepared from fermentation develops different taste and texture with time and this is because of the acid production in the dough that rejuvenates the activity of many enzymes dealt with protein denaturation and gives health beneficiaries too, in consequence persuade the change in such characteristics.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Preliminary results for Screening of Lactic culture strains.

4.1.1 Plate assay

Plate assay was done to select the appropriate and efficient strain of lactic culture procured from many fermented and non fermented sources. The strain which makes clear zone, in the plates of MRS agar the respective strain was then selected.

The resultant "*Lactobacillus paracasei CD4"* culture showed positive degradation characteristics against gluten. Hence, used further for detailed degradation kinetics.

Figure 4.1.1: Degrading activity was observed in *Lactobacillus paracasei CD4*

4.1.2 Sourdough observation

The dough were kneaded with and without culture, and left for few hours for fermentation.

After few hours the rise in loaf volume was observed in those that are having lactic cultures.

Figure 4.1.2: Rise in loaf volume of the flours prepared with lactic culture

4.2 Quantitative analysis

4.2.1 Estimating protein with Bradford test

After lyophillization method the extracted proteins were dissolved in buffer or in the same extraction reagents for estimating the protein content before and after the fermentation takes place. The Bradford reagent consists of Coomassie Blue dye that forms complex with the charged proteins like proline, glutamine etc. From the BSA Standard the protein content of glutenin, gliadin and albumin was determined.

Table 4.2.1: Dry weight (in grams) of the protein samples extracted from barley and mix of barley and wheat flours before and after fermentation.

PROTEIN/ FLOUR TYPE		BARLEY CONTROL	BARLEY + L4	WHEAT+ BARLEY CONTROL	WHEAT+ BARLEY +L4
ALBUMIN	0^{th} Hour	0.97	0.71	0.57	1.12
	12^{th} Hour	0.76	0.14	0.66	0.96
GLIADIN	0^{th} Hour	0.005	0.02	0.06	0.08
	12^{th} Hour	0.11	0.11	0.13	0.09
GLUTENIN	0^{th} Hour	0.60	0.15	0.20	0.14
	12^{th} Hour	0.42	0.008	0.09	0.32

4.2.2 SDS PAGE Analysis

- With the span of time the gliadin degradation was observed as the bands were found dissociated in those samples that are treated with lactic culture.
- In the image different bands of gliadin protein were observed and considered as "high molecular and low molecular weight" proteins or peptides.
- This shows that lactic culture used in this study adhere efficient and required amount of proteolytic activity against gliadin.
- The gliadin samples (Lane3 and 5) were hydrolyzed after fermentation as shown in the gel.

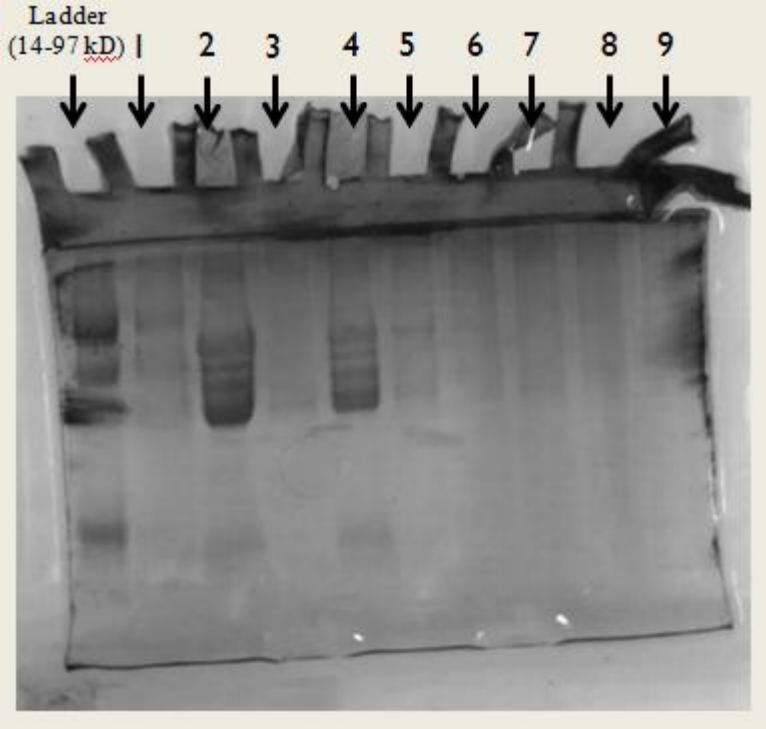

Figure 4.3.2: Gliadin degradation using lactic culture

Lane 1: Gliadin standard

Lane 2: 0^{th} hour control gliadin

Lane 3: 0^{th} hour L4 gliadin (Hydrolyzed gliadin of wheat and barley mix)

Lane 4: 12th hour control gliadin

Lane 5: 12th hour L4 gliadin (Hydrolyzed gliadin of wheat and barley mix)

Lane 6: 0th hour control glutenin

Lane 7: 0th hour L4 glutenin

Lane 8: 12th hour control glutenin

Lane 9: 12th hour L4 glutenin

4.2.3 Degradation kinetics with Reversed Phase HPLC

High Performance Liquid Chromatography is one of the column chromatography technique used to analyse even minute protein samples on the basis of the peak which is then calculated as the protein concentration. To validate the results a standard is must to analyze the whole process. Detector senses the presence of compound and therefore provides electronic signals to data acquisition based on the residence time of the compound.

From the following procedure it was observed that incase of 12th hour wheat and barley mix dough treated with the culture shows almost 50% degradation in gliadin protein analyzed and compared with the peak height of standard gliadin as well as the 0th hour untreated wheat and barley mix dough.

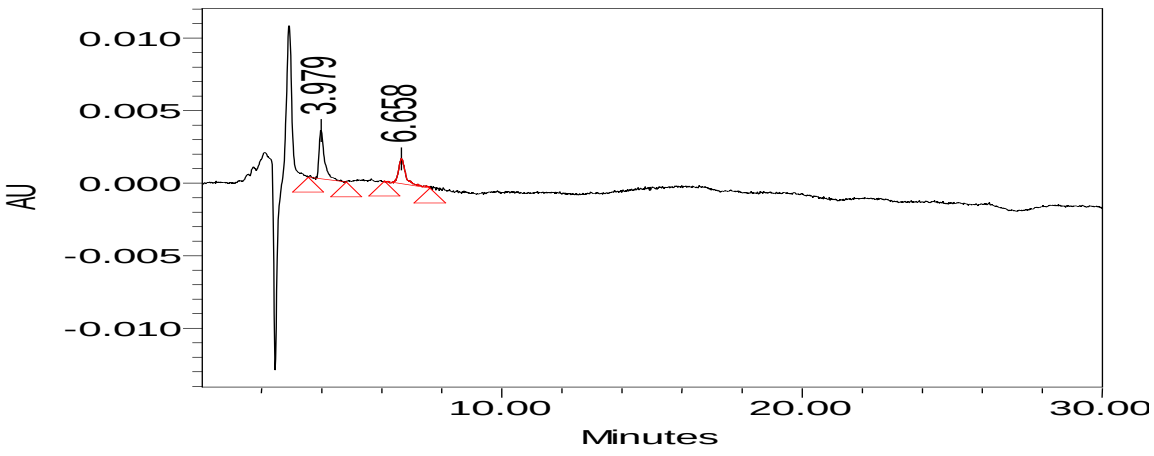

Figure 4.3.3.1: Gliadin standard

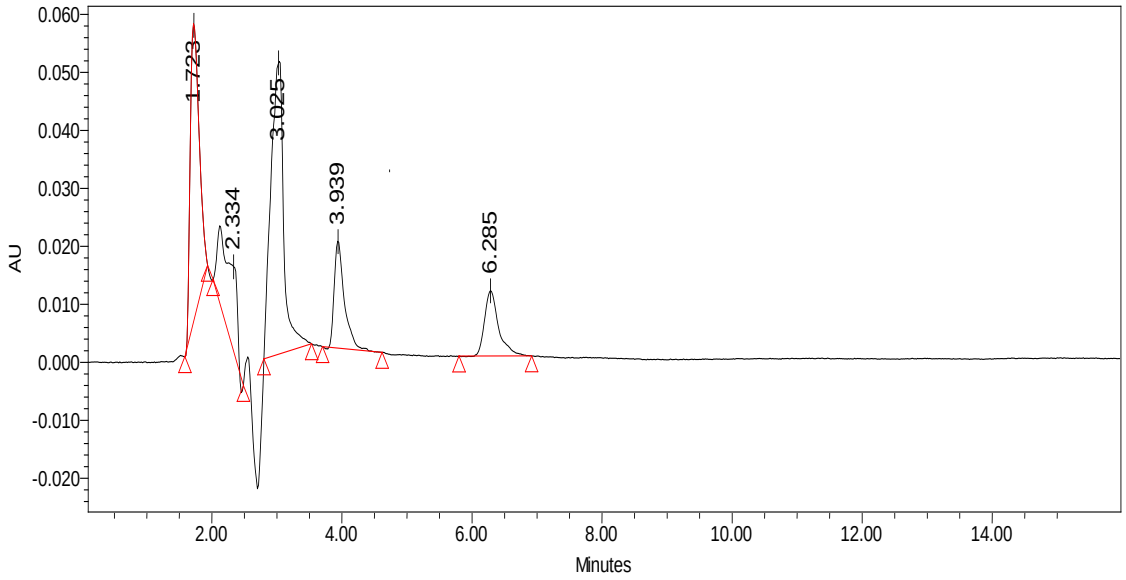

Figure 4.3.3.2: Wheat and Barley 0th hour L4

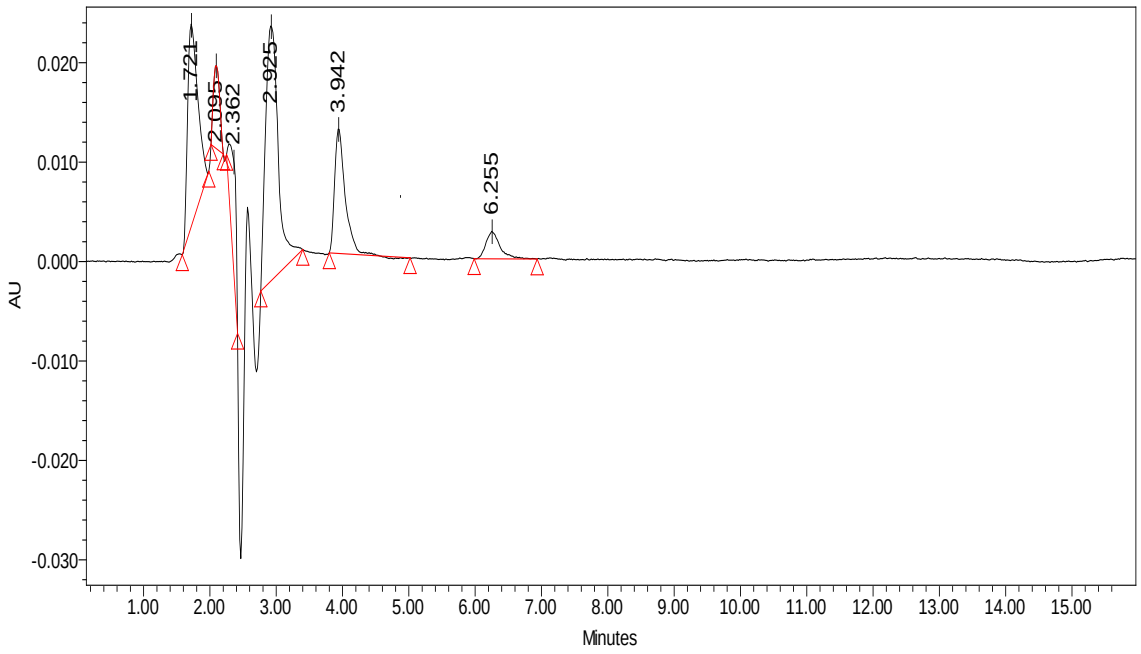

Figure 4.3.3.3: Wheat and Barley 12th hour L4

4.3 Organoleptic evaluation

For any new food product approval organoleptic evaluation should be done and that is required to be competent with the already commercialized product. Organoleptic evalution or study is based upon the sensory organs like the smell of the product, the flavor, the color of the product and many more.

Here, the organoleptic evaluation of fermented and non fermented dough was based on some of these above recited parameters like flavor, smell, chewy nature of the bread commonly known as “Bhatura”

Table 4.4: Organoleptic survey of the product

Culture/ Normal	Flavor (sourness)	Smell (normal)	Texture (soft)
Wheat and Barley+ L4	2	5	4
	0	3	5
	0	4	5
	1	3	4
	0	3	3
	2	5	5
	A bit sour in taste	Somehow Normal	Soft
Wheat and Barley(control)	0	4	3
	1	5	3
	0	4	5
	1	3	4
	0	4	3
	2	5	4
	Normal	Normal	Not exactly soft
	0	5	3
Barley control	1	5	2
	0	3	2
	0	4	1
	0	5	3
	1	5	1
	Normal taste	Normal	Not soft

CHAPTER 5

CONCLUSION

5. CONCLUSION

The results obtained from this study are significant and hence we can infer that the used lactic culture has observable degrading quality against gliadin that is the part of gluten protein. Moreover, this study has shown positive comparable response with respect to CD and gluten free diet. The respective study also enclosed with the suggestion that the fermented foods prepared from lactic culture can be used as condiments and as well as a probiotic substitutes.

In this era of modernization people are greatly aware of the products to be consumed that are healthy too. Nowadays, people are more inclined towards gluten free food because of the wrong perception that gluten free food is healthy which really does not. Moreover, due to readily high consumption the glucose level of the body may get disturbed. The high glycemic index foods such as carbohydrates and sugar rich products are the probable reasons of increased glucose levels in humans.

Leading a gluten free diet is not very easy for even a normal individual and affected ones with gluten. This epidemiology of disease can be controlled with many enzyme derived medicines and vaccinations. But most recent and cost effective method with nutritional value is the consumption of lactic culture treated food product that is having reduced amount of gliadin, which is the main causative agent of this disease. The reduced peptides of gliadin does not show allergic response and hence can be consumed by the diseased prone person as well as normal person to attain probiotic attributes of the food while digestion. Concluding with the statement that the results obtained in SDS PAGE and RP- HPLC showed breakdown of gliadin protein into smaller fragments that are having "*Lactobacillus Paracasei CD4*". Till now, the above used lactic culture for fermentation purpose showed positive therapeutic characteristics hence; in future the product developed from this method could be preferred by most of the affected or unaffected population globally.

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