A DISSERTATION REPORT

Effects of Gluten hydrolysates on Plant growth

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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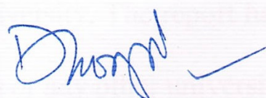
JULY, 2014

CERTIFICATE

This is to certify that the thesis entitled “Effect of Gluten hydrolysates in Plant growth ’ submitted by Ms. Jyotika in partial fulfilment of the requirements for the award of degree of Masters of Science in Bio-Technology to Thapar University, Patiala, is a record of students’s own work carried out by her under my supervision. The report has not been submitted for award of any other degree or certificate in this or any other University or Institute.



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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in dissertation entitled "**Effects of Gluten Hydrolysates in Plant growth** " in partial fulfillment of the requirements for award of degree of Master of Science, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of one year from January, 2014-July, 2014, under the supervision of **Dr. Dinesh Goyal**, Professor & Head, Department of Biotechnology, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Date: 18 -July -14

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Place: Patiala

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Acknowledgement

There are many individuals that merit recognition for their assistance and encouragement to me while working toward this degree.

I owe my deepest gratitude to my guide Dr. Dinesh Goyal, Professor & Head, Department of Biotechnology, Thapar university, Patiala for his excellent guidance, perennial encouragement and support during the course of my work. As my supervisor, his insight, observations and suggestions helped me to establish the overall direction of the research and to achieve the objectives of the work.

I am also thankful to Mr Nadeem, Ms. Prerna, Ms. Mahiti, Ms. Rajinder and all other lab mates and staff members of STEP (Science and Technology Entrepreneurship Park) for their constant assistance and co-operation.

My sincere thanks are due to all faculty members of Department of Biotechnology for their suggestions and constructive criticism. I would like to extend a heartfelt thanks to all my friends sheenu didi, Shilpi, Manmeet, Purnima, Ravleen, Manjeet, Geetika for their affectionate company, cooperation and moral support. I will always remain very grateful to them for their endless love and support.

The words are not sufficient to express my love and gratitude to my parents and friends for their blessings and moral support. I am in dearth of words in expressing my warm feelings to my family for their encouragement and affection.

Last but not the least, I wish to acknowledge all those, whose names have not figured here, but who helped me in any form during the period of my research work.

Date: 18-July-14

Place: Patiala

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Symbols

g	Gram
g/L	Gram per liter
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
µg	Microgram
µL	Microliter
°C	Celcius

Abstract

Gluten is a protein composite of gliadin and glutenin found in corn and related grains which is conjoined with the starch in the endosperm is produced as a by product of starch industry. Various chemical and enzymatic methods were applied to hydrolyse gluten and to characterize resulting hyrdrolysate by high performance thin layer chromatography. Hydrolysis was confirmed by estimating tyrosine amino acid. It was observed that using 1N HCL and 1M NaOH for hydrolysis of gluten concentration of tyrosine, methionine, and arginine was more whereas as tyrosine, glutamic acid and methionine was more when gluten was hydrolysed by 4N HCL and 4M NaOH. *Bacillus* N 15 was inoculated in nutrient broth and grown for overnight at 37°C and in cell free supernatant protease activity was checked against gluten. Supernatant of over night grown culture containing extracellular protease enzyme was used directly to hydrolyse gluten (0.5 gm) used as a substrate and incubated at 37°C for 4.30hr. In enzymatic hydrolysate the concentration of arginine, histidine, cysteine and glutamic acid was more. Hydrolysates were checked for their effect on moong and lentil seed germination and growth of transplanted rice in earthern pots. Gluten hydrolysates showed positive effect on plant growth, however more work is required to be done.

Introduction

Gluten is a protein composite of glutenin and gliadin, found in wheat and related grains, including barley and rye. Gluten, protein composite is cojoined with starch in the endosperm of various grass related grains. The prolamin and glutelin from wheat (gliadin, which is soluble in 70% alcohol, and glutenin, which is only soluble in dilute acids or alkalis) comprises of 80% of the protein contained in wheat.

The fruit of most flowering plants have endosperms with stored protein to nourish embryonic plants during germination. True gluten, with gliadin and glutenin, is limited to certain members of the grass family. The stored proteins of maize and rice are sometime called glutens, but their proteins differ from gluten.

There are two types of gluten mainly wheat gluten, corn gluten. Wheat gluten is obtained by washing wheat dough with water until all starch granules have been removed leaving the sticky insoluble gluten as an elastic mass. Wheat gluten is an alternative to tofu (soybean based food) sometimes used as meat substitutes. Wheat gluten is also used both as a protein source and binding ingredient in pet foods. If gluten is extracted and gently dried in hot air at moderate temperature it maintains its characteristic and named as vital gluten. Wheat gluten is very effective in binding meat chunks or trimming together to form restructured steaks or chops. The most common usage of gluten in western countries has been traditionally continued in baking goods. It also provide structural enhancement in cereals and snacks.

Corn gluten is obtained from corn kernels through wet milling . In wet milling process , corn is "steeped" and the kernel components are separated into corn bran, starch, corn gluten meal (protein), germ, and soluble components.A further definition might include the gene synthesis of gluten proteins in the developing grain – the *Gli-1* and *Gli-2* loci coding for the gliadin proteins and the *Glu-1* and *Glu-3* loci coding for the glutenin polypeptides. A biological definition might include the origins of the gluten-protein complex as being derived from 'storage proteins of wheat grain'.

Gluten is formed as a storage proteins according to their function for the wheat grain. The grain also contains the residue of many metabolic proteins (mainly water-soluble) that have been

needed by the developing grain, together with the proteins providing those putative mechanisms that must carry life on into the next generation of the wheat plant when the germination process commences.

In these respects, the storage protein of the mature wheat may not differ much from those of other grains. Corn gluten hydrolysate increases the crop yield,crop quality.Corn gluten hydrolysate is a non selective pre emergence or pre plant incorporated herbicide that inhibits the root development, decreases the weed growth. Christians(1993) investigated the weed control efficacy of corn gluten hydrolysate in several weed species including creeping grass(Agrotus palustris) , crab grass(Digitaria sp).Corn gluten hydrolysates are

The present strategy is to focus on hydrolysis procedures and to study its hydrolysed products and its explore its uses other than the food industry . The hydrolysed gluten can be used as Plant growth promoter.

Gluten a by product of wheat starch or corn starch industry, is a typically water insoluble protein. Gliadins are polymorphic polypeptides with a molecular mass between 30,000 to 80,000 kDa and Glutenins are multichained polypeptides with a molecular mass between 80,000kDa to several million.

Corn gluten is processed from corn kernels which have three main parts, the seed coat or pericarp,the starchy endosperm,and the embryo,commonly called germ . Outer hull is known as pericarp, for protection of seed. Endosperm is the main energy reserve having 80% of total weight of the kernel.It is about 90% starch and 7%gluten.

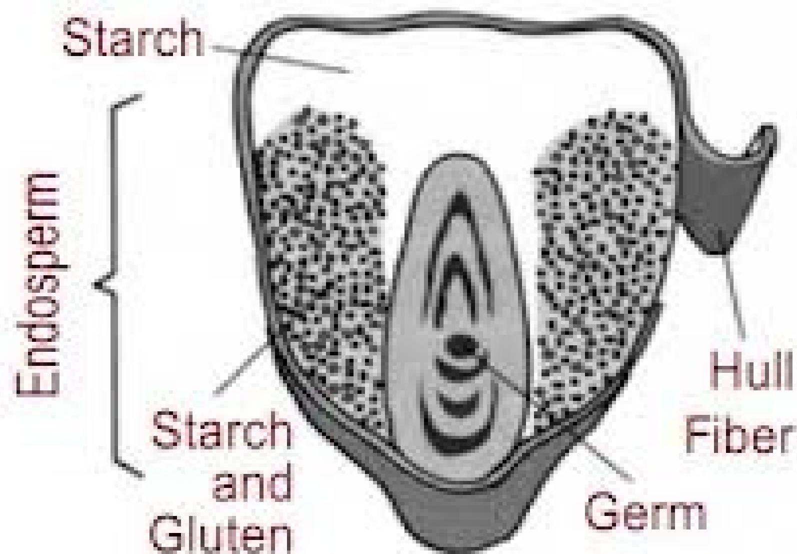

Fig1.Corn kernel.

Wheat gluten is processed from wheat kernels having bran,germ,endosperm.Bran is the outermost part of kernel. Endoperm is made up of starch and proteins and a germ which is a source of oils.

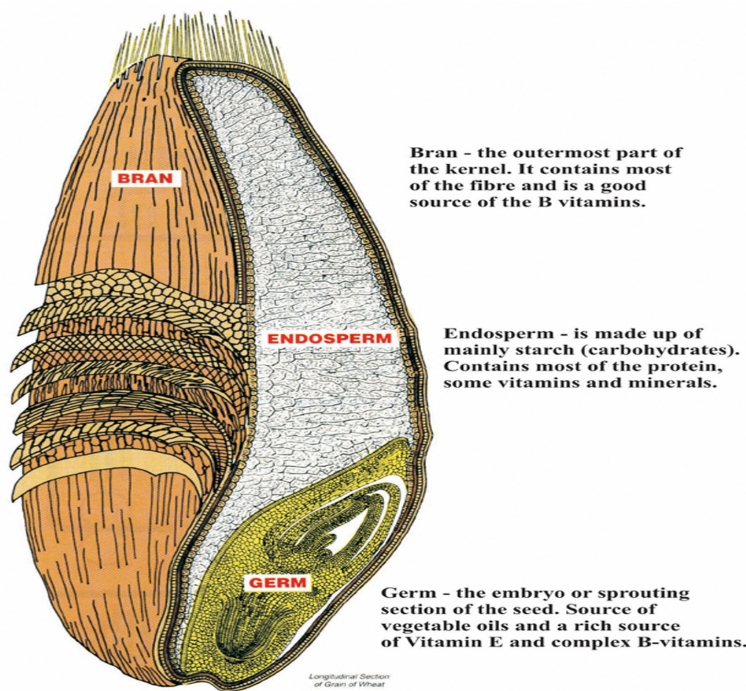

Fig2.Wheat kernel.

The insolubility of proteins sets limits for their utilization in formulated food systems. Gluten whether obtained as a by-product of cornstarch industry or wheat starch industry, attempts has been done to increase its utilization in industries. The insolubility is basically due to non polar amino acids (Krull et al.,1966) which are present in large quantities such as proline, leucine which tends to be involved in hydrophobic bonding and presence of glutamine in gluten plays an important role in stabilizing the protein structure and in promoting the association of gliadin and glutenin molecules through hydrogen bonding.

Gluten forms when glutenin cross links with gliadin which gives viscosity(thickness) and extensibility to the mixture. If this mixture left with yeats , it gets swells due to production of carbon dioxide bubbles which gets entrapped by this gluten network causes dough to rise. In food industry when a baker mixes flour with water at the start of bread making process, these protein hydrates to form gluten ,viscoelastic matrix holding the starch of bread making process,these protein hydrates to form gluten,viscoelastic matrix holding the starch granules that constitute bulk of flour.

Hydration of flour give dough is not just a simple mixing process but much mechanical work is needed because carbon dioxide bubbles are produced from fermentation process.Yeast ferment sugar liberated from starch by flour's natural complement of amylase enzymes. Gluten turn into sheets which entrap carbon dioxide,results in expansion of dough.

To enhance its elasticity affects the texture of baked goods.Gluten having the property of elasticity because of presence of sulphur containing amino acids which is responsible for cross linking.

Gluten was prepared from flour almost 300 years ago by an Italian named Beccari, who chad done a simple water washing experiment with wheat flour (Bailey, 1941). This discovery, which can be easily reproduced in the kitchen, has become the basis of a major cereal industry, utilizing millions of tones of wheat annually in North America, Europe and Australia. The present commercial process is basically an efficient repetition of Beccari's experiment.

Bakers had known the value of the gluten component of dough for decades. Although the enrichment of bakers' flour with gluten has been common practice in bread manufacture since the second half of 20th century, the purification of starch from wheat flour had been practised for a much longer time. For example, as far back as 1840 in Hull, England, Isaac Reckitt was washing starch from wheat flour. The procedure involved making a mash of the wheat after soaking in water for many days, and when soft enough, putting the mash through sieves, the wheat starch passing through leaving the bran in the sieves. The gluten was afterwards separated from the starch by washing.

The process begins in Wellington, New Zealand in the 1930s where a method was discovered for extracting gluten from flour using fermentation, water washing and addition of salt (Wrigley, 2000). Various tries has been done with the gluten enriching process produced bread of increased volume and improved texture. 'Procera' bread was patented and the same process was used. In 1932 the same process was introduced to Britain.

There were methods used to improve the texture of crumb , loaf volume which includes new type of drying process that de-vitalised the functional properties of the gluten.This process was firstly used in Austria(Dubois,1996).

The vital dry gluten was sold commercially as an additive for a number of breads, but it was not used as a blend with wheat flour. In 1955 gluten was added additionally in low protein flours.

Table1.Amino acid compostion of commercial vital gluten(Day and Batey(1995)

Amino acid	Content	Amino acid	Content
Alanine	3.0	Lysine	2.2
Arginine	4.3	Methionine	2.1
Aspartic acid	4.8	Phenylalanine	7.3
Cystine	2.6	Proline	14.6
Glutamic acid	39.0	Serine	5.6
Glycine	4.6	Threonine	3.1
Histidine	2.7	Tyrosine	4.3
Isoleucine	4.4	Valine	4.6
Leucine	8.4		

Content values expressed as g amino acid/100g protein

Glutamic acid and aspartic acid are predominantly in the amidated form, with about 90%existing as glutamine and asparagine, respectively.

Starting of the dried - gluten industry

Starch reduced was used for the Procera method as in this process starch was used to thrown away. However, this was the biggest problem, namely, the disposal of the starchy effluent produced by the protein-enriching step of washing out excess starch. This problem were the basis of development of an industrial - scale process to provide the ‘protein enriched’ ingredient for the Procera method in dried form , essentially as dehydrated gluten , at the same time permitting the recovery of the starch that was washed out , so it can be used in foods.

Advancement of Vital Dry gluten in North America

The industrial need of America was only for wheat starch not for gluten (Dubois, 1996).In 1920s heat dried gluten was found in the manufacture of low carbohydrate bread for diabetic.Later on gum gluten was used for the production of monosodium glutamate by acid hydrolysis. Until this stage gluten functional properties were changed by harsh drying process. But in 1950s another process was used in Australia (Dubois,1996), based on the ring or flash drying process. This modified gluten retained its functional properties. With in year this modified gluten was commercially sold in market. However, at the time, there was little success in attempts to blend the dry gluten with wheat flour at the mill to enhance protein quality.

Present industry of Gluten

Current practices of Gluten manufacture

The principle of gluten washing still remains similar to that of Beccari hundreds of years ago. Most commercial operations now use variations of either the Batter process or the Martin Process.

The Martin Process

In this method, a wheat flour dough is washed with water while it passes through a tumbling cylindrical agitator.This work increases the protein content of dough and starch comes out. This process helps in moving the dough through cylinder and through the small holes starch comes out and protein remains inside the cylinder.

The Batter process

In this process, a thick suspension or batter of flour is stirred slowly in a tank for several hours,during which time the starch seperates from the protein. The mixture is then passed through a fine sieve , which allows the starch granules to pass through, while the curds of gluten are retained on the screen. This gluten is washed with water to remove further starch in a similar manner to the Martin process. The gluten is then dried.

The Martin Process is a continuous process, while the nature of Batter Process makes it more suited to batch process.

Other Procedures

Most commercial operations use one or the other of the above methods with modifications, but there have been many other processes developed for the commercial production of gluten. While most have not made it the past the laboratory curiosity stage, others , for example the Alfa Laval Raisio process, have been applied in full scale production facilities. The basis of these conventional industrial centrifuges, which may involve either conventional industrial centrifuges or hydro cyclones to separate the starch from the protein. Many use hydro cyclones as the principle of cleaning the starch,and , in some cases , for the actual separation of starch and gluten .

Drying gluten

The functional properties of gluten are yery susceptible to heat when we heat when wet, and the relatively low temperatures destroy the cohesive, viscoelastic properties, which make it unique among food proteins. Attempts to dry gluten while retaining these properties were unsuccessful until the application of the ring drier to gluten in the first half of 20th century. This process has been the basis of gluten drying since then .The principle is simple –wet gluten ,whose moisture content is around 70% is mixed with sufficient dry gluten to reduce the moisture to about 20% .This lowered moisture material is comminuted and subjected to flash drying in a ring drier. A portion of dried gluten is removed and packaged while the rest is returned to the drying cycle to reduce the moisture content of more wet gluten .This procedure was still very sensitive to heat , but with careful control of the temperature , a vital wheat gluten is produced.

An alternative way of drying to prepare a vital gluten is to disperse the gluten in aqueous or acetic acid and then spray dried this suspension. The resulting product retains the visco elastic

properties of gluten, and it may be used for the most of same purposes as a vital gluten. The cost of this drying procedure, together with environmental concerns, limit its application except for special reasons.

Another dry gluten product is known as 'Devital gluten' .More severe temperatures are intentionally used in drying , often by drum drying .As a result, this material has lost the characterstic cohesive , visco elastic properties of native gluten, but it retains the insolubility and water binding capacity of vital gluten.
Devital gluten is commonly used where the cohesiveness of vital gluten can actually be a disadvantage.

Uses of Vital gluten

The most common use of gluten is used in baking goods. However, with an increasing awareness of gluten's unique structural and functional properties has come an expanding diversity of applications. The uses of gluten worldwide vary from country to country as shown in Table2.

Table 2. Usage of gluten in different regions (as percentage of total usage for gluten)(Maningat et al.,1994).

Uses	North America	Europeon Union	Australia	Japan
Baking	83	17	54	63
Flour fortification	1	66	9	14
Pet food	12	13	13	8
Meats	1	-	9	5
Breakfast cereals	1	-	12	2
Noodles	-	-	-	-
Sausages	-	-	-	-
Other	2	4	3	8

Non food applications include aquaculture feed, biodegradable plastics ,films ,coatings, adhesives, inks, cosmetics and pharmaceuticals.

Gluten has been modified in various ways to produce a wide range of food ingredients, providing water binding and emulsifying properties , nutritional advantages for sports drinks and medical supplements, dairy supplements.

Uses of gluten

1- In baking goods
2- In non baking goods

Table 3. Use of gluten in bakery foods (Maningat et al.,1994)

Bakery products	Use level(% gluten content on a flour basis)
Pizza crust	1-2
Vienna bread	2-3
Frozen doughs	2-5
Raisin bread	3-4
Wheat bread with bran	3-4
Salad rolls	4
Multi grain bread	4-5
High protein bread, bagels	5-6
Whole meal fiber increased bread	5-7
Whole white bread from flaked wheat	10
High-fibre, reduced-calorie bread	8-12
Bread with low slice weight	30

Gluten in non-bakery food products

The desired property of gluten in its ability to bind fat and water while at the same time increasing the protein content makes gluten attractive to various type of application. A major use of gluten is the replacement of meat in vegetarian foods. Gluten's viscoelastic properties can be used in preparing synthetic cheese with the characteristic texture and quality of cheese. If gluten used alone or in combination with soy protein, has been used to replace approximately 30% of the more expensive sodium caseinate used in imitation-cheese products.

Table 4. Non-bakery foods and animal feed applications of gluten. (Maningat et al.,1994).

Non bakery products and animal feeds	% Gluten content, product basis
Sea food analogues	1.3
Crab food analogues	2.1
Artificial caviar	1-30
Frankfurters	3.2
Sausage analogue	8
Meat like sausages	16.7
Meat like balls and hamburger	10.6
Restructured beef steaks	3.6
Imitation cheese	5.8-6.3
Synthetic cheese	14.2
Meringue	15.1
High- protein snack	1-50
High – protein pasta	1.6
Flour tortillas	1-4
Extruded wheat gluten products	20-23
Light colored seasoning liquid	>25
Pet foods	3-28.2
Aquaculture diet	5-10
Calfmilkreplacer	15-30

Other uses of gluten

Changing in properties of gluten makes it suitable for food use also makes it ideal for use in certain types of industrial products. Table 5 lists possible applications of gluten in non-food uses. The list includes some products that are already commercialized, as well as ones tried by researchers to demonstrate the potential of gluten in non-food uses.

Gluten is having adhesive property, which makes it useful in bandages and adhesives tapes. Its reactivity makes it useful for binding heavy metals in industrial processes, removing ink from waste paper, or solidifying waste oils. Some peptides from gluten can be used in cosmetics, lotion. Gluten is hydrophobic and insolubility properties permit slow release encapsulation of flavours, colours, medicines. Gluten can be used into building materials, such as light weight, frost resistant concrete.

Currently used procedures for Modification of gluten

Gluten like many other protein ingredients has multifunctional properties.
However while a range of functionalities allows an ingredient to be used in a range of applications, not all of the functionalities inherent in gluten or engineered into modified ingredients are needed in a single application. For an ingredient to be the ingredient of choice for a particular application, the functionality of the ingredient has to be matched with attributes required for the ingredient in the target application. While the inherent functionality of a gluten gives an indication of its potential functionality of the ingredient in an application, it should be realized that when an ingredient is added to a food , the expressed functionality of the ingredient may be modified due to changes in the molecular environment of the ingredient(e.g., pH,presence of salts, sugars, salts), the interaction of the ingredient with other components in the formulated food and the processing treatment applied in the manufacture of the final product. It is essential for an ingredient supplier to be aware of how and the extent to which his ingredient can be modified by conventional chemical, physical and enzymatic means, as well as the effects of newer processing technologies(e.g., ultrasonic, high pressure) on the properties of the ingredient.

Chemical alterations

The main modification applied to gluten is solubilisation. Gluten is soluble, or at least dispersible, in a variety of solvents including urea solutions, lactic acid, soaps, and detergents, acetic acid, hydrochloric acid, sodium hydroxide, 70% ethanol, and 2-chloroethanol. Many of these are incompatible with the food products, but for non-food purposes, there are few limitations other than cost, safety and environmental concerns. Deamidation method was used for the solubilisation for gluten. This may either achieved by acid or alkali. Approximately 90% of the glutamic acid is in the form of glutamine. Removal of amide group of these residues to form the corresponding carboxylic acid changes the potential ionic charge on the protein, thus increasing its solubility above a certain pH. In acidic deamidation, there is also a degree of peptide hydrolysis to form lower molecular weight polypeptides, which are also usually more soluble than larger ones.

Acidic hydrolysis (Fadil , Babiker and Fujisawa.,1996)

To 5g of gluten was added 200ml of 0.05N HCl,and then the mixture was incubated at 120°C for 60 min. The treated mixture was centrifuged to remove a small amount of unhydrolysed protein, and then the supernatant was dialyzed(3000-4000,molecular weight cut off) against distilled water or 0.1M phosphate buffer (pH7.0).The former was freeze dried, and the latter was used for transglutaminase treatment. The yield of acid hydolysate was more than 80% after dialysis.

Transglutaminase Treatment

The acid treated gluten, which dialyzed against 0.1M phosphate buffer (pH7.0) (10mg/ml) was reacted with TGase (0.5mg/ml).The enzyme was incubated at 55°C for 60min. The enzyme was inactivated by N-ethylmaleimide (0.1ml) (Kato et al.,1991b). The treated samples were dialyzed against distilled water and then freeze-dried.

Measurement of solubility

Freeze dried samples of acid hydrolysate with or without TGase treatment were used for the determination of solubility at various pHs: pH 2-3, 0.05M citrate buffer; pH 6-8 ,0.05M phosphate buffer; pH9-11, 0.05Mcarbonate buffer; and pH12, 0.05M NaOH slightly adjusted with 0.05 M HCl. Samples were dissolved in buffer and shaken with a vortex mixer for 10 s, and the turbidity was measured at 500nm.

Others methods of hydrolysis(Gallus and Jennings(1970)

Hydrolysis 1- A suspension of gluten (50g) in water (200ml) was extracted with two portions each ether and n butanol. An equal volume of concentrated hydrochloric acid was added and the mixture left for 19 days at room temperature. The dark brown hydrolysate was neutralized to about pH 3 with 10N KOH and extracted with ether and n butanol as before. The hydrolysate was adjusted to pH 8.5 with 10NKOH and re extracted as before with ether and n butanol.

Hydrolysis 2.-gluten (50g) was suspended in 6N HCl (200ml) and left, with occasional mixing, for 64hr at room temperature. The hydrolysate was extracted twice with ether and three times with n-butanol, adjusted to pH 10 with 10N KOH (in an ice water bath), then re extracted with ether and n- butanol.

Hydrolysis 3- Gluten was suspended in 2N KOH (200ml) for 64hr at room temperature. It was necessary to add 400ml distilled water to the hydrolysate. The hydrolysate was adjusted to pH 2 with HCl and re extracted with ether and n-butanol.

Hydrolysis 4- Gluten was macerated with n butanol in a mortar and pestle. The residue was washed with water and suspended in 6N HCl for 10 days at room temperature, with occasional mixing. The hydrolysate was neutralized to pH 3 with 10N KOH (in an ice water bath) and treated subsequently as for hydrolysis1.

Enzymatic hydrolysis :- (Verma and McCalla.,1966)

Gluten samples were prepared from ether extracted and enzymes used in this process included the fermex MT, crystalline papain, Enzymatic digestion was carried out either in Erlenmeyer

flasks or in cellophane tubing. Weighed amounts of the enzyme to be used were added to specific volumes of the dispersed gluten which had been brought to the reaction temperature in a thermostatically controlled water bath. In some experiments, dialysis was carried on simultaneously with the digestion; in all such experiments, cellophane tubing was used. A bag made from the tubing was filled, the enzyme added, and the bag tied to a rotary dialyzer and immersed in a vessel containing a large volume of dispersing medium at the reaction temperature.

Since the enzymes could not be inactivated by adding chemicals or changing pH without seriously altering the solubility properties of the protein and its degradation products, no inactivation was attempted. Subsequent procedures were carried out as promptly as possible after the end of the reaction period and, where necessary to hold the samples, the reaction products were kept in a cold room at 3 °C. Aliquots of some digests were subject to precipitation by bringing them to half saturation with magnesium sulphate. The precipitate was separated by centrifugation at 9,500 X g for 20 min.

Sodium salicylate (8%) with or without added bisulphate, has been used by many workers as a dispersing agent for gluten. Aluminium lactate has been frequently used . Since the pH value of these agents are roughly equivalent to the optimum for the action of three of the four enzymes to be tested , it was decided to make preliminary trials using each enzyme with gluten dispersed in aluminum lactate(0.02M in respect to aluminum),sodium salicylate (8%), and 8% sodium salicylate to which 50mg sodium bisulphate per 100 cc. had been added.

Chromatographic Procedures

The extracts were concentrated in a rotary evaporator and chromatographed on Whatman No 1 paper with the different solvent systems.Thechromatograms were examined after applying staining reagents.

A- nigrosine, to detect polypeptides and proteins (Gallus and Jennings 1968);

B- ninhydrin, to detect amino acids and peptides (Stepka 1957; Mabry and Todd 1963);

Other procedures of enzymatic hydrolysis:

1**-By Yvanne Rabe ,Lynzee Reyes(2012)** – Enzymatic hydrolysis was performed with proteases.To a small lad of gluten 10 ml of distilled water was added and mixed with 10 ml of protease enzyme and then 10ml of phosphate buffer was added having pH7.5. Then it was left for overnight at room temperature.

2- By **N.A Hardt, A.J.van der Goot,R.M.Boom(2013)** – The hydrolysis reactions was carried out in water bath, having hydrolysis temperature 50°C .Before hydrolysis, gluten was allowed to stand for 5min at the reaction temperature .The enzyme was mixed with the water before adding it to the substrate. Enzyme used was protease from *Aspergillus oryzae*.

3-By **Xiangzhen Kong, Huiming Zhou, Haifeng Qian(2006)-** Wheat gluten was hydrolysed with six different enzymes, under the conditions available given in the table based on optimum hydrolysis conditions.

Table6 –Conditions for the hydrolysis of wheat gluten with different proteases.

Reaction conditions	Proteases				
	Neutrase	Alcalase	Pepsin	Pancreatin	Protamex
pH	7.0	8.5	2.0	8.5	6.5
T(°C)	50	60	37	37	50

Wheat gluten was dissolved as a 5% solution; the suspension was adjusted with appropriate temperature and pH, depending upon used enzyme incubated for 30 min with continuously stirring. For pepsin, mixture was incubated @37°C for 24 hrs and pH was not changed till that procedure. However, for other enzymes pH was not same. It was continuously changing.

Today: Automated, Micro waved, and more

Clearly, there is room for improvement when one considers all exceptions for acid and base hydrolysis. Solving the above problems requires high levels of operator skill and knowledge. More generally, the three problem areas of hydrolysis that need to be addressed are contamination, incomplete hydrolysis, and degradation. Incomplete hydrolysis is a major

concern with proteins that have groups of aliphatic amino acids such as valine, isoleucine and alanine that have bulky side chains. These bulky side chains sterically hinder hydrolysis. Some often employ extended hydrolysis times to completely hydrolyze these bulky groups. Unfortunately, such prolonged treatment may also increase the degradation of labile amino acids. Another approach is to elevate the hydrolysis reaction temperature to 150-160°C. This elevated temperature can only be achieved in a sealed vessel that can tolerate several atmospheres of pressure.

Degradation of labile amino acids can be minimized by adding agents that serve as protectants and antioxidants.Thioglycolic acid, a readily oxidized compound, will scavenge any oxygen present during hydrolysis. Addition of this compound minimizes degradation of tyrosine, methionine, serine. There are some other automated procedures which can be used for hydrolysis of proteins which are summarized in a table7.

Table7- Features of Amino Acid Hydrolysis Systems(Micheal V.Pickering,Paul Newton.,1990)

Hydrolysis system	Type	Sample capacity	Automation	Temp(°C)
Pierce Reacti-ThermIII	Liquid	24	Manual	110-200
CEM MDS-81D	Vapor	40	Semiautomatic	100-180
ABI 420H	Vapor	3	Automatic	150-170

Materials and methods

1- Acidic and alkaline hydrolysis

To perform Acidic hydrolysis of gluten(Chiu H.Wu et al .,1976)

Requirements: Chemicals- 4NHCL,1NHCL,1MNaOH, Distilled water.

Procedure- 1- Weighed 0.5gm gluten.

2- Put it into round bottom flasks

3-To the flasks 20ml 4NHCl and 1NHCl was added.

4- Then it was placed on the hot plate @ 50°C for 4-5 hrs for complete hydrolysis

5-After that 10 ml distilled water was added and 1MNaOH was added.

6- The hydrolysed gluten was stored under 4°C for further analysis

To perform Alkaline hydrolysis of gluten(Chiu H.Wu et al .,1976)

Requirements:Chemicals- 4MNaOH,1MNaOH, 1NHCl, Distilled water

Procedure-1- Weighed 0.5gm gluten.

2- Put it into round bottom flasks

3-To the flasks 20ml 4MNaOH and 1MNaOH was added.

4- Then it was placed on the hot plate @ 50°C for 4-5 hrs for complete hydrolysis

5-After that 10 ml distilled water was added and 1NHCl was added.

6- The hydrolysed gluten was stored under 4°C for further analysis

Estimation of tyrosine and residual protein content in hydrolysates.

For tyrosine standard curve-

Requirements- Tyrosine standard (0.2mg/ml) , folin ciocalteau reagent.

Procedure-.1- Prepared 0.2mg/ ml of tyrosine as a stock solution.for standard curve

2- Different dilutions of tyrosine were prepared.

3-To this distilled water was added to make up the volume up to 2ml.

4- Test tubes were incubated at room temperature for 30min.

5- Then filter the solution , to the filterate 5ml of Na_2CO_3 .

6- To this 1ml of folin reagent was added and further incubated @37^0 C for 30min.

7- Recorded the absorbance @660nm

8- Like wise gluten hydrolysate were checked to perform tyrosine estimation.

For residual protein content

Standard curve of BSA

Requirements- SolutionA- 2% Na_2CO_3 in 0.1N NaOH

Solution B- 0.5% Copper sulphate in 1% Rochelle's salt.

SolutionC- Mix 50ml of reagent A with 1ml of reagent B just prior to use.

Solution D – Folin Cioclateau reagent

Standard BSA (0.2mg/ml)

Procedure- 1 The stock BSA solution was prepared and diluted for the standard curve

2 The lowry solution was prepared by mixing SolA+SolB+SolC with a ratio of (100:1:1)

3 The samples were vortexed and diluted with distilled water.

4 0.5 ml of sample was transferred to 10 ml test tube.

5 Test tubes were vortexed and incubated for 20min at room temperature in dark .

6 In between folin reagent was prepared and 0.1 ml of folin reagent was added in the test tubes.

7 The tubes were vortexed and incubated for 30min.

8 Absorbance was taken @750nm.

9 Gluten hydrolysates were taken to perform residual protein test.

2 Enzymatic hydrolysis

To perform enzymatic hydrolysis and check the protease activity of over night grown culture of *Bacillus* N15.

Requirements- Culture of *Bacillus* N15 was provided by Research Lab 3 Department of biotechnology of Thapar university,Patiala, Nutrient broth.

Procedure- 1 – Overnight grown culture of Bacillus N15 was taken.

2- From the culture supernatant was taken for enzyme source protease.

3- Different volume sof supernatant was taken in test tubes for different time intervals for action of protease.

4- Added 3ml of Phosphate buffer (PH 7.5) to each of the test tubes of different volume of supernatant.

5- Added 3ml of gluten hydrolysate in in each test tube.(gluten as a substrate for the action of proteases).

6- Incubated the samples at 35^0c for 30minutes, 60min, 90min, 120min,150min,180min,210min,240min.

7- Added 5ml of TCA (Tri- chloro acetic acid) after the completion of incubation time.(TCA is added to stop the ongoing reaction).

8- Filtered the solution (Filteration is done to remove turbidity which developed due to addition of TCA.

9- Taken1ml of filterate and add 2 ml of 20% Na_2CO_3.

10- Then added 1ml of folin reagent.

11- Incubated for 30 minutes at room temperature.

12- Added 6ml of distilled water.

13—Noted the absorbance at 660nm. .

14--Then graphs of Time Vs Concentration were plotted fir 1ml of supernatant, 2ml supernatant, 3ml supernatant.

3 Characterization of hydrolysates

To confirm the presence of particular amino acid

Xanthoproteic test- Aromatic amino acids, such as Phenyl alanine, tyrosine and tryptophan, respond to this test. In the presence of concentrated nitric acid, the aromatic phenyl ring is nitrated to give yellow colored nitro-derivatives. At alkaline pH, the color changes to orange due to the ionization of the phenolic group. Hydrolysed gluten were used as test sample of xanthoproteic test

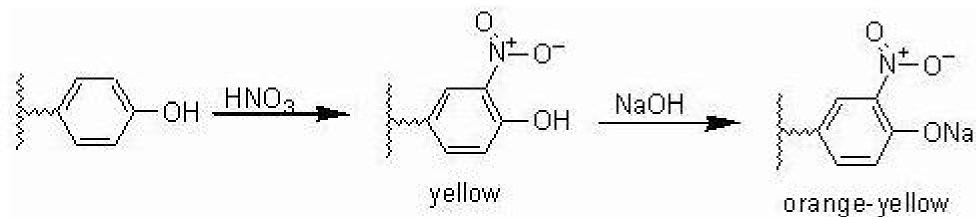

Fig.3- Chemical reaction of Xanthoproteic test.

Ninhydrin test-In the pH range of 4-8, all α- amino acids react with ninhydrin (triketohydrindene hydrate), a powerful oxidizing agent to give a purple colored product (diketohydrin) termed Rhuemann's purple. All primary amines and ammonia react similarly but without the liberation of carbon dioxide. The imino acids proline and hydroxyproline also react with ninhydrin, but they give a yellow colored complex instead

of a purple one. Besides amino acids, other complex structures such as peptides, peptones and proteins also react positively when subjected to the ninhydrin reaction.The hydrolysed gluten samples were used to test this.

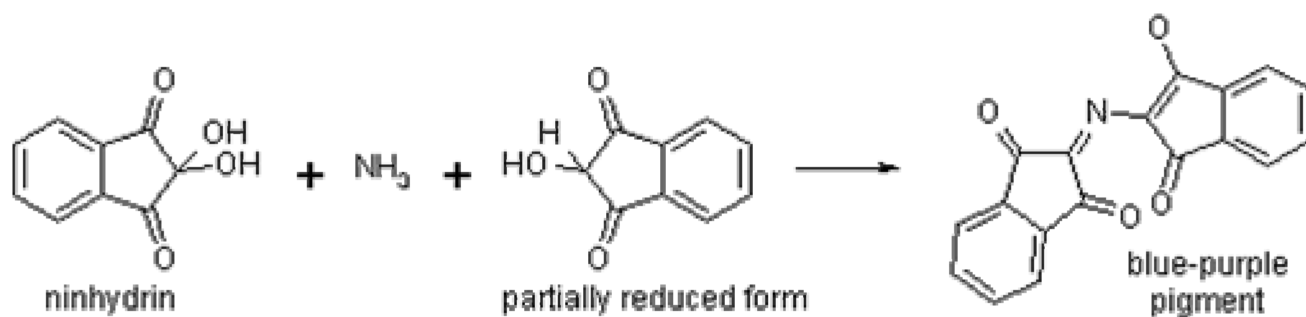

Fig.4- Chemical reaction of ninhydrin test

Seperation of amino acids of hydrolysed samples of gluten (Gallus, and Jennings.,1970).

Requirements-Solvent mixture (n-butanol:acetic acid:water)(4:1:5), TLC plates.

Procedure-1- 30 g silica gel g was dissolved in 60 ml of water in a beaker.

2-With the help of applicator silica gel g was poured onto the TLC plates.

3- Then TLC plates were dried in Oven.

4- In between solvent chamber was made by putting solvent in to the chamber so to get a saturation point.

5- With the help of capillary tube amino acids were spotted on the TLC plate and hydrolysed samples of gluten was also spotted.

6-Then TLC plate was put in to the TLC chamber to allow the separation of amino acids.

7- Then TLC plate was removed from the chamber and ninhydrin was sprayed .

8-TLC plate was dried in hot air oven for 10-15 min

9-Recorded the Rf value.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

Quantitative estimation of amino acids in Hydrolysed samples of gluten by HPTLC.

Reagents-Distilled water, solvent (butanol:acetic acid:water)(4:1:5)

Layer- Precoated HPTLC plates silica gel MERCK 60 F 254, 20X10cm.

Procedure- 1-Prepared samples were taken and with the help of CAMAG Automatic TLC Sampler III was apllied on to the HPTLC plate.

2- Along with the samples , standard of amino acids was also applied to the plate.

3- Total of 2µl of samples were apllied.

4- Then HPTLC plate was transferred to CAMAG Twin Trough Chamber 20X10cm

5- In the chamber prepared solvent mixture was added.

6- Then after the HPTLC plate was put into the CAMAG TLC scanner with Labdata system andCATS software.

7- Scanning was done by fluorescence at 460nm with mercury lamp, monochromator bandwidth 30nm.

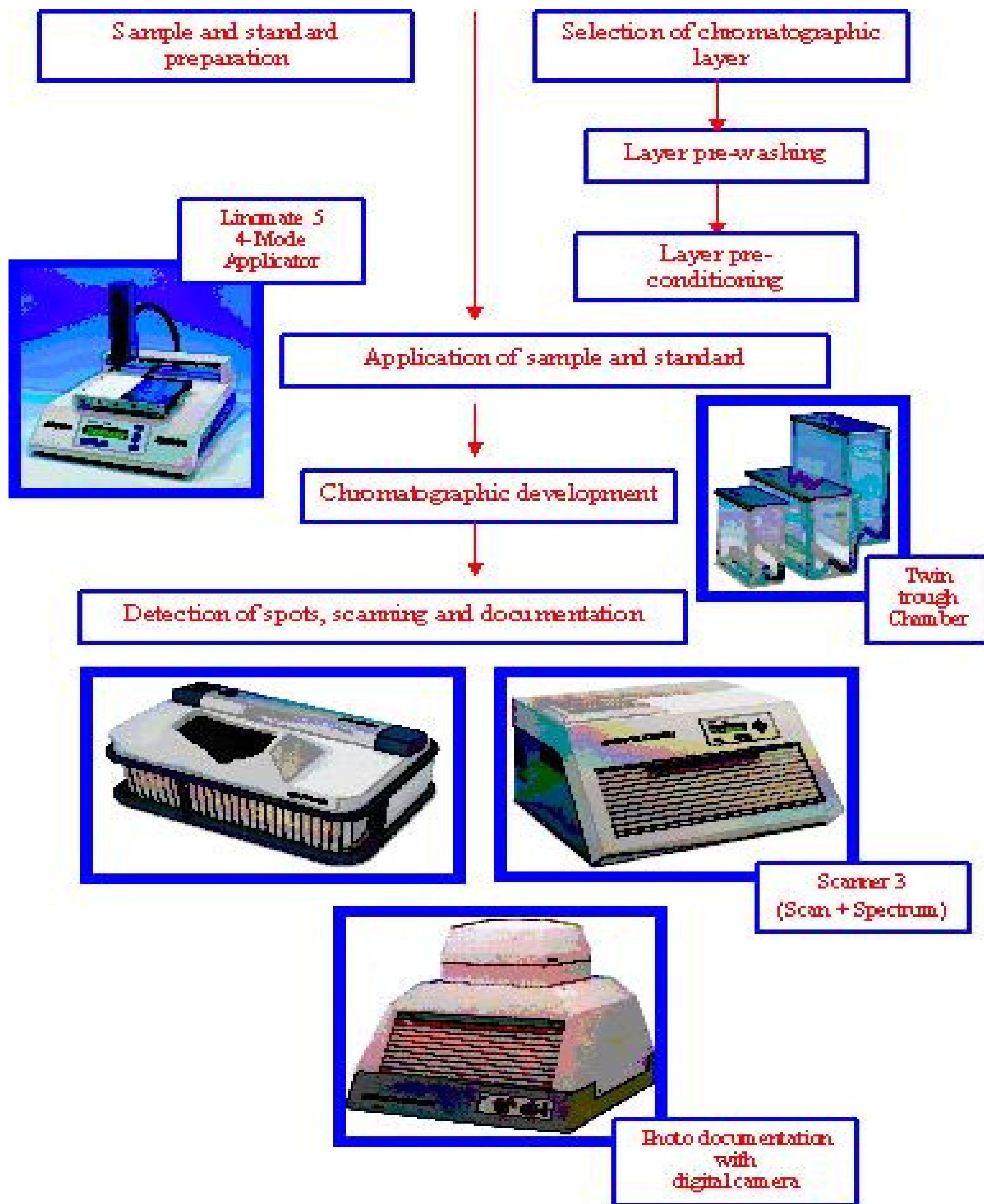

Fig.5-HPTLC methodology.

4 Effect of hydrolysates on plant growth.

To check the growth of seeds by applying nutrient solution and gluten hydrolysate

Requirements- Hoagland solution, enzymatic Hydrolysate (Autoclaved supernatant, filter sterilized supernatant),Moong beans, Black lentils.

Procedure- 1- Seeds of Moong beans and black lentils were taken at put into the Petri plates

2- To the seeds 1ml of different solutions were poured and stored at room temperature

3- Growth of seeds and percentage of seeds germination was recorded.

Calculations:

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds on plate}} \times 100$$

To check the effect of gluten hydrolysate on rice in pot culture.

Requirements- Acidic hydrolysate, Alkaline hydrolysate , Enzymatic hydrolysate, *Pusa 1121*.

Procedure – 1-2.5gm of gluten was taken and to this 100ml of 4NHCL was added and kept @50°c for 5-6 hrs

2- 2.5gm of gluten was taken and to this 100ml of 4MNaOH was added and kept @50°c for 5-6 hrs.

3- 2.5 gm of gluten was taken and to this 100 ml of filter sterilized supernatant was added and kept @37°C for 4.30 hrs.

4-First of all Paddy seeds were grown in pot and then after some growth was observed transferred into 12 other pots .

5- After transferring the Paddy to the pots acidic hydrolysate , alkaline hydrolysate, enzymatic hydrolysate was added and observed their effects on the paddy.

Results and Discussion

Acidic and alkaline hydrolysis of gluten

Gluten comprises of gliadin and glutenin proteins having different molecular weights. Hydrolysis of protein takes place by cleavage of peptide bonds by insertion of water molecules between the bonds. Amino acids are linked to each other in which carboxyl group of one amino acid is condensed to amide group of another amino acid. 0.5gm of gluten was separately treated with acid (1N HCl and 4N HCl) and alkali (1M NaOH and 4M NaOH) for 5hr at 50-60° C in a water bath. In acid treatment the resulting pH of hydrolysate was 2 and neutralized to 7 and in alkali treatment pH of hydrolysate was 13 and neutralized to 7. Treatment with concentrated hydrochloric acid and sodium hydroxide leads to vaporization of acid and alkali, when it comes in contact with gluten amide group of gliadin reacts with hydrochloric acid and NaOH which increases the solubility of gluten and resultant hydrolysates are the cocktail of amino acids.

Among different amino acids tyrosine concentration in hydrolysate was estimated using a standard curve of tyrosine (Fig. 1) and residual concentration of protein was estimated by Lowry method using standard curve of BSA (Fig. 2).

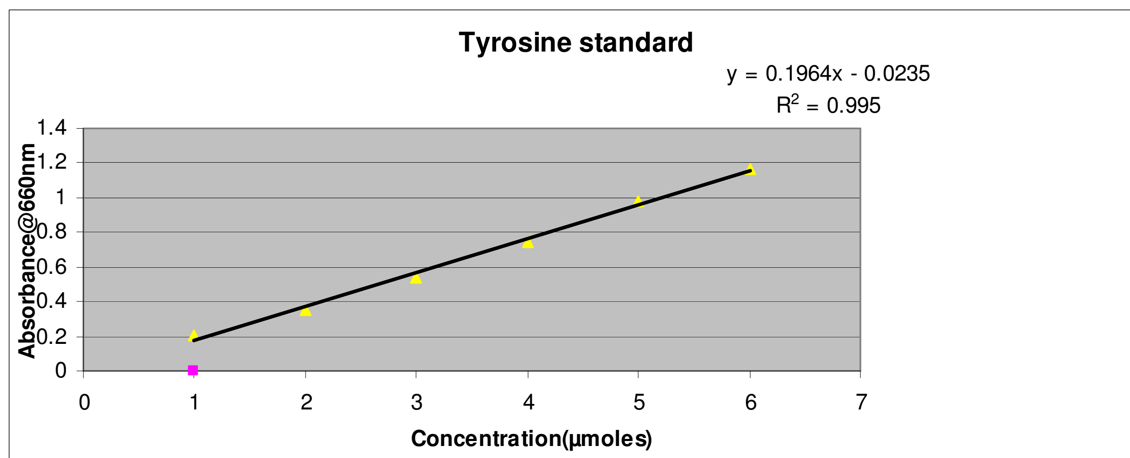

Fig. 3 Standard curve of tyrosine.

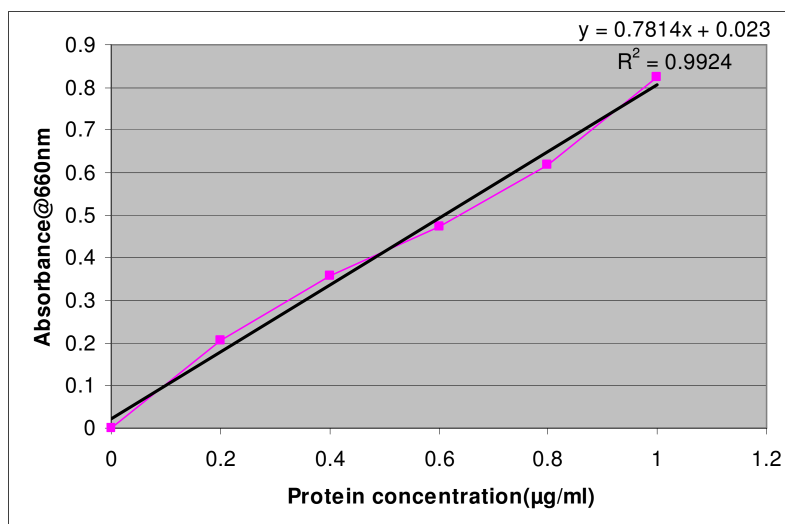

Fig. 4 Standard curve of BSA

Table 8. Estimation of protein and tyrosine concentration in hydrolysed gluten

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Acid/alkali hydrolysis	Absorbance at 660 nm for tyrosine	Concentration of tyrosine (µg/ml)	Absorbance at 750 nm for protein	Concentration of protein (µg/ml)
1M NaOH	0.820	0.789	0.822	0.957
4M NaOH	0.708	0.722	0.775	0.871
1N HCl	0.784	0.899	0.856	0.971
4N HCl	0.770	0.890	0.945	1.02

Acidic and alkaline hydrolysates of gluten were checked for tyrosine content and residual protein (Table 8). Tyrosine concentration was maximum in acidic hydrolysate of gluten as compared to alkali hydrolysis. With increase in concentration of acid or alkali there was no increase in tyrosine content indicating for hydrolysis of 0.5 g of gluten 1N HCl and 1M NaOH is sufficient. There was no tyrosine found in untreated gluten samples. Residual protein was nearly 0.8-1.0 µg/ml, which was determined only after hydrolysis of gluten.

Enzymatic hydrolysis

Bacillus N 15 was inoculated in nutrient broth and grown for overnight at 37°C and in cell free supernatant protease activity was checked against gluten. Supernatant of over night grown

culture containing extracellular protease enzyme was used directly to hydrolyse gluten (0.5 gm) used as a substrate and incubated at 37°C for 4.30hr. Proteases present in supernatant act on gluten resulting in its hydrolysis and producing tyrosine. Determination of amount of tyrosine produced among all other amino acids indicates extent of hydrolysis. Protease activity in the supernatant was also checked by taking different amount of supernatant (1, 2 and 3 ml) and estimating amount of tyrosine formed at different time intervals as a result of enzymatic hydrolysis of proteins present in gluten (Table 9; Fig.5). When protease digests the gluten, tyrosine is liberated along with other amino acids and peptides. Folin reacts with tyrosine to produce blue colored chromophore, which is detected by the spectrometer at 660 nm.

Table 9. Concentration of tyrosine formed by the action of protease in the supernatant of overnight grown culture of *Bacillus* N 15 at different time interval.

Time (hr)	Concentration of Tyrosine (µg/ml)		
	1 ml supernatant	2 ml supernatant	3 ml supernatant
0.5	0.335	0.98	1.57
1.0	0.470	0.99	1.61
1.5	0.552	1.15	1.62
2.0	0.636	1.15	2.20
2.5	0.740	1.24	2.34
3	0.930	2.60	2.10
3.5	1.789	2.76	2.00
4	2.56	2.20	1.79
4.5	2.84	2.00	1.65

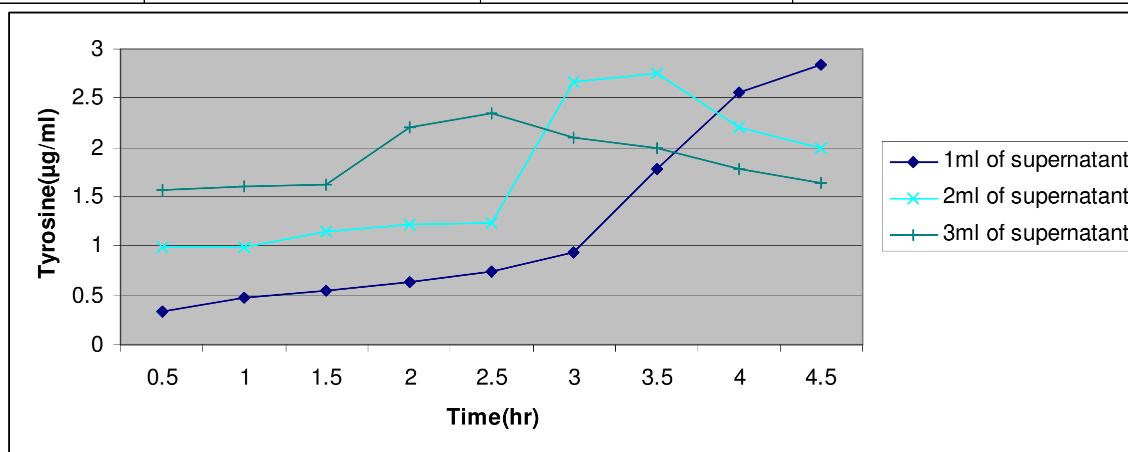


Fig. 5 Different volume of supernatant used for the hydrolysis of gluten.

From the above data it was observed that maximum concentration of tyrosine in hydrolysates was found after 4.5 hr of incubation using 1 ml of supernatant (Table 2; Fig. 5) revealing that

enzyme and substrate react with each other in 4.5 hr to completely hydrolyse gluten. But when 2ml of supernatant form over night grown culture was taken then this time interval decreases to 3.5 hr which occurs due to less availability of substrate for enzyme to act upon and when 3 ml of supernatant from over night grown culture was taken then time reduces to 2.5 hr, due to less availability of substrate for enzyme. Keeping the amount of substrate constant and increasing the concentration of enzyme by using more quantity of supernatant, the time required for hydrolysis decreases.

Characterization of hydrolysates

Xanthoproteic test

Xanthoproteic reaction is a qualititative test used to determine the amount of protein soluble in a solution, using concentrated nitric acid. The test gives a positive result in those proteins with amino acids having aromatic groups, especially in the presence of tyrosine test. The yellow colour produced is due to xanthoproteic acid, which is formed due to nitration of certain amino acids, most common examples being tyrosine and tryptophan. Gluten hydrolysate were used to detect the presence of aromatic amino acids. Yellow coloured compound in test tubes of hydrolysed gluten shows the presence of tyrosine and tryptophan (Fig. 6). Figure 6 represents that **1** no represents that Acidic Hydrolysate and **2** no represents alkali hydrolysate does not produce yellow colour compound therefore does not show the presence of tyrosine and tryptophan content. In **3** test tube 1 N acidic hydrolysate and in **4** no test tube, 1 M alkaline hydrolysate shows the light yellow colour complex which confirms the presence of tyrosine and tryptophan in 5 and 6 test tubes having enzymatic hydrolysates shows yellow colour complex which confirms the presence of aromatic amino acids.

Xanthoproteic acid test

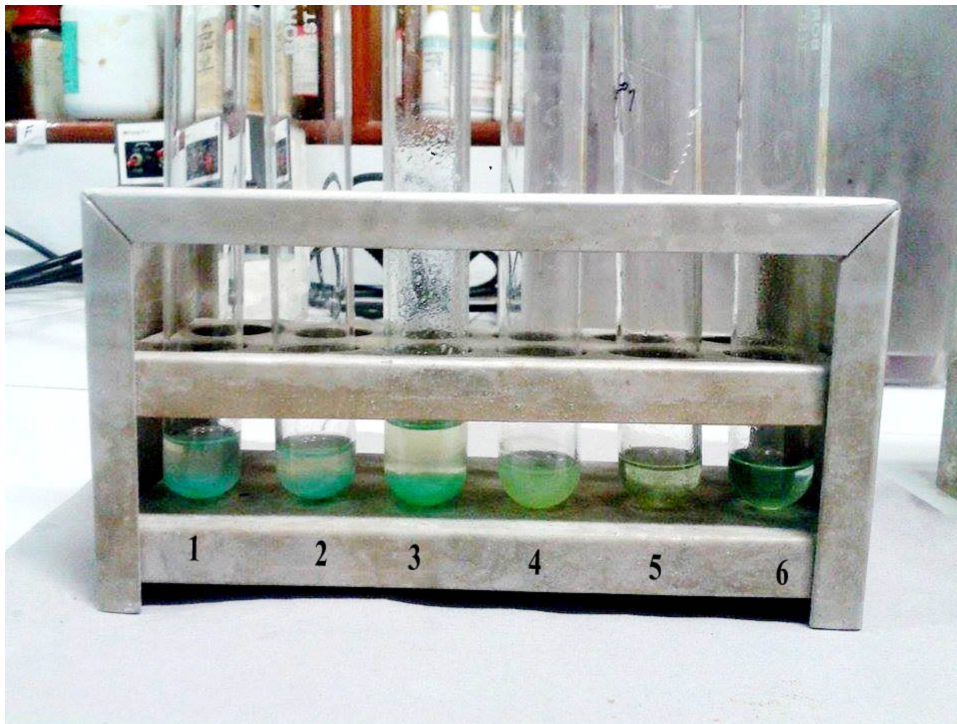


Fig. 6 Gluten hydrolysates produced coloured complex after reacting with xanthoproteic acid.

Ninhydrin test

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia, primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is produced. When ninhydrin was added to gluten hydrolysates purple colour formation takes place in all test tubes, which shows the presence of primary and secondary amines in the hydrolysates. Fig.7 shows that gluten hydrolysates having primary amines that's why deep purple colour was formed in all test tubes.

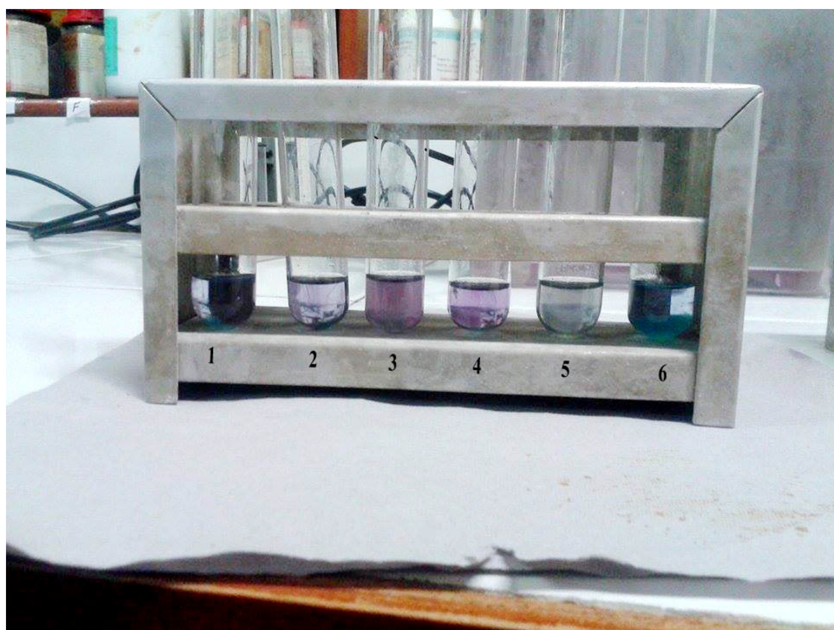


Fig. 7 Gluten hydrolysate after reacting with ninhydrin reagent shows purple coloured compound.

Separation and identification of amino acids through thin layer chromatography

Thin layer chromatography is a separation technique facilitated by the principle of polarity towards the two phases, stationary and mobile phase. Arginine moves slowest from the point of origin that's why it has lowest Rf value which means this amino acid has greatest affinity for stationary phase, on the other hand tyrosine had highest Rf value and moved farthest from the point of origin. This means that it has greatest affinity for mobile phase. Gluten hydrolysate obtained after acid, alkali and enzymatic hydrolysiss was loaded on TLC plates and separation of amino acids was performed using solvent (butanol: acetic acid: water) (4:1:5) (Fig.8). Standards of Glycine, Tyrosine, Glutamic acid, alanine, arginine were spotted on TLC plate along with these standards, hydrolysate of gluten were also spotted. The Rf values indicated the polarities of amino acids in samples of hydrolysed gluten (Table 10; Fig. 8). A lower Rf value denotes the highest polarity and high Rf value denotes the lowest polarity. n-butanol is the non polar solvent carries the non polar amino acids up to the chromatogram and keeps polar amino acids at the baseline. Due to the quantitative difference of n-butanol and acetic acid (4:1), n-butanol keeps polar amino acids at the baseline. But in the sample of hydrolysed gluten glycine, arginine and glutamic acid are present shown by the Rf values of hydrolsed samples

Table 10. Rf values of different amino acids.

Components	Distance traveled by solvent front (cm)	Distance traveled by component (cm)	Rf value
Glycine	11	14	0.78
Tyrosine	12	14	0.85
Glutamic acid	11	14	0.78
Alanine	11.5	14	0.82
Arginine	10	14	0.71
Acidic hydrolysate (4N)	12.5	14	0.89
Alkaline hydrolsate (4M)	12.5	14	0.89
Acidic hydrolysate (1N)	11.5	14	0.82
Alkaline hydrolysate (1M)	11	14	0.78
Enzymatic hydrolysate (4hr)	11	14	0.78
Enzymatic hydrolysate (4.30 hr)	11	14	0.78
Control (gluten)	1	14	0.071

Fig. 8 Using solvent (n butanol :acetic acid:water) spots of amino acids were seen on TLC plate.

1 2 3 4 5 6 7 8 9 10 11

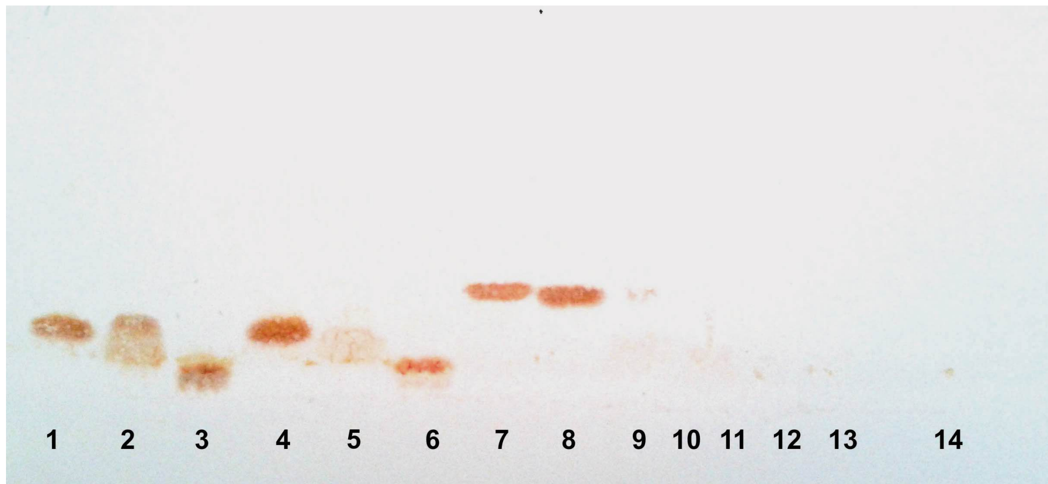


Fig. 9 Bands of amino acids observed under HPTLC scanner.

Fig. 10 Peaks of amino acids obtained after Scanning.

Quantitative estimation of amino acids in hydrolysed gluten by HPTLC

HPTLC is used to determine the concentration of amino acids present in the solution. There were 14 peaks were observed in TLC Scanner (Fig.10), having different peak areas. Peak area is related to concentration of amino acid. Out of 14 peaks, 8 peaks were of standard amino acids used and rest 6 peaks were of hydrolysed sample of gluten. Concentration of amino acids was detected in hydrolysates (Fig. 9). It was observed that using 1N HCL and 1M NaOH for hydrolysis of gluten concentration of tyrosine, methionine, and arginine was more whereas as tyrosine, glutamic acid and methionine was more when gluten was hydrolysed by 4N HCL and 4M NaOH. In enzymatic hydrolysate the concentration of arginine, histidine, cysteine and glutamic acid was more.

Effect of gluten hydrolysate on plant growth

Gluten hydrolysate are a cocktail of amino acids which can promote seed germination and growth of seedlings. Percentage of seed germination was observed after applying Hoagland solution alone and with the combination of enzymatic hydrolysate (Table 11). Water + enzymatic hydrolysate also showed high percentage of seed germination. Percentage of seed germination provides good estimate of potential field performance of seeds. Average growth of both Moong beans and Black lentil was observed maximum when enzymatic hydrolysate which was applied to seeds after filter sterilization.

Table 11. Percentage of seed germination and growth of seedling on petri plate containing soaked tissue paper treated with hydrolysate.

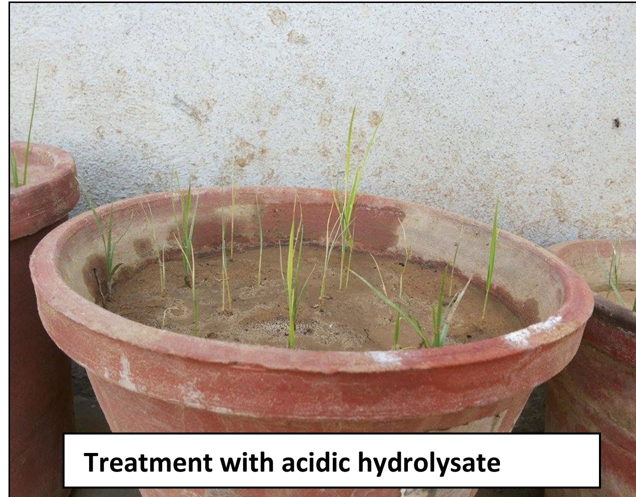
Treatment		Moong Beans		Black lentil	
		Average length of radical (cm)	% age of seed germination	Average length of radical (cm)	%age of seed germination
1	Water +enzymatic hydrolysate (filter sterilised)	1.62	70	0.66	80
2	Water+ enzymatic hydrolysate (autoclaved supernatant)	3	50	0.93	40
3	Water	2.8	90	1.96	90
4	Hoagland solution	0.5	80	0.3	70
5	Hoagland solution+ enzymatic hydrolysate (autoclaved supernatant)	0.5	40	0.46	30
6	Hoagland solution+ enzymatic hydrolysate (filter sterilized)	0.66	70	0.36	70

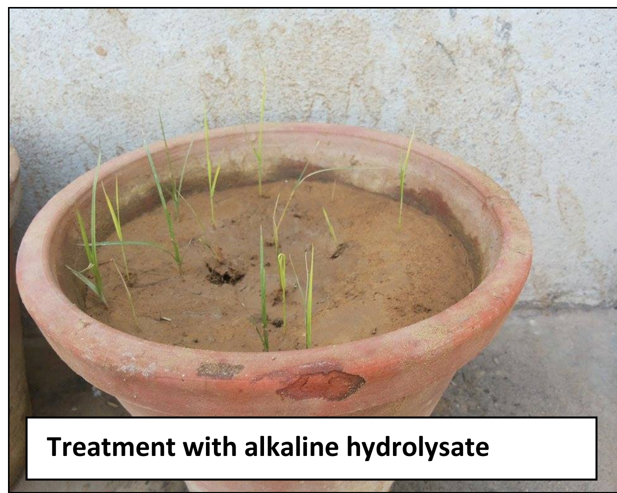
Effect of gluten hydrolysates on growth of transplanted rice seedling in earthern pots

Rice was grown and separately and transplanted in earthern pots and treated with different solution of hydrolysates and water. Good growth of rice seedling was observed on application of water and enzymatic hydrolysate (Table 12; Fig. 11). Acidic and alkaline hydrolysates had less effect on growth.. Gluten hydrolysate had positive effect on plant growth and are used as growth stimulators or as organic fertilizers. However these results needs to be reconfirmed and more work is required to be done in this direction since different hydrolysates are actually a mixture of amino acids and each one had accordingly different effect on seed germination and seedling growth.

Table 12. Average shoot height growth of rice seedlings after one week of transplantation.

Treatments	Average Shoot Height of rice seedlings on Pots.
Water	8.8±1.0
Acidic hydrolysate	5.10±.2.2
Alkaline hydrolysate	7.8±2.3
Enzymatic hydrolysate	8.4±2.2





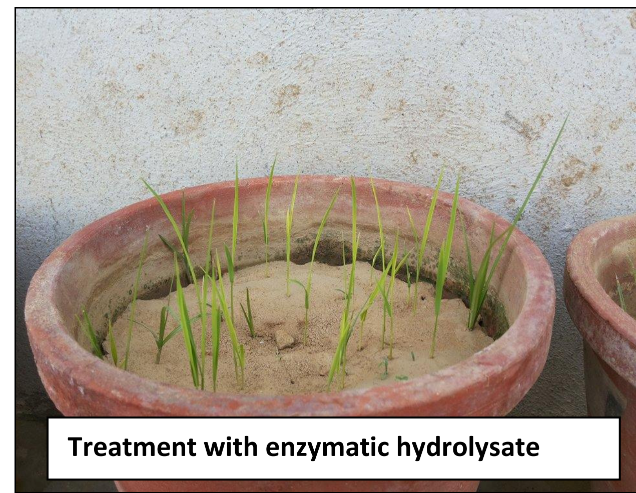


Fig. 11 Effect of gluten hydrolysates on growth of transplanted rice in earthern pots**.**

Conclusion

Gluten as a by product of corn starch and wheat starch industry having wide number of applications. After its hydrolysis its solubility increases and hydrolysis results in breaking of peptide bonds and formation of amino acid cocktail.This hydrolysis was performed either with acid or with alkaline or with enzyme. Resulting hydrolysates can be characterize to confirm the presence of amino acids. These amino acids plays an important role in plant growth.

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Compostion of nutrient broth per 100 gm.

Glucose	0.5%
Peptone	0.75%
Magnesium sulphate	0.5%
Potassium dihydrogen phosphate	0.5%
Iron sulphate heptahydrate	0.01%
Sodium carbonate	0.1%

Folin Lowry

Solution A – 2% sodium carbonate in 0.1N NaOH

Solution B – 0.5%copper sulphate in 1% sodium potassium tartrate

Solution C- Mix 50 ml of solution A with 1 ml of solution B just prior to use

Solution D- Folin ciocalteau reagent – 5ml of 2N Folin with 6 ml of distilled water.

Standards of amino acids

0.05M glycine- 0.058 g in 15 ml of HCL

0.05M gluatmic acid -0.113g in 15ml of HCL

0.05Mmethionine – 0.116g in 15 ml of HCL

0.05M arginine – 0.136 g in 15ml of HCL

Hoagland solution

Composition

1 Cobalt nitrate	236.1g
2 Potassium nitrate	101.1g
3 Potassium dihydrogen phosphate	136.1g
4 Magnesium sulphate heptahydrate	246.5g
5 Trace elements	

Boric acid	2.8g
Mangense chloride	1.8g
Zinc sulphate	0.1 g
Copper sulphate pentahydrate	0.025 g
6 Fe EDTA	
EDTA 2Na	0.104g
Ferrous sulphate	0.78g
Potassium hydroxide	0.561g