STUDIES IN PRODUCTION AND APPLICATION OF PLANT PROTEASE

Dissertation Report-1

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CERTIFICATE

This is to certify that Srishti Mehta has personally completed M.Sc. Dissertation Report-1 entitled '**Studies in production and application of plant protease'** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of dissertation report has ever been submitted for any other purpose at any university.

The project report is appropriate for the submission and partial fulfillment of the conditions for the evaluation leading to the award of Master of Food Science and Technology.

Signature of Supervisior

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DECLARATION

I hereby declare that the work presented in the dissertation entitled **'Studies in production and application of plant protease'** is my own original work. The work has been carried out by me at School of Agriculture, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Dr. Yogesh Gat**, Assistant Professor (Food Technology) of School of Agriculture, Lovely Professional University, Phagwara, Punjab, India for the award of the degree of Master of Science in Food Technology.

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I certify that the above statement made by the student is correct to the best of my knowledge and belief.

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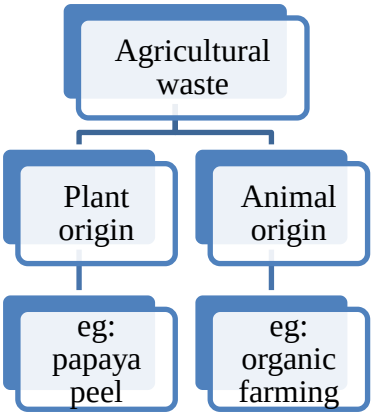
CONTENTS

INTRODUCTION CHAPTER-1

The emphasis was on to extract the plant protease or called proteinase from agro waste as agro-waste being enriched in various essential nutrients so plant protease can easily be extracted out of it using various different techniques. Different techniques used produce different yield of plant protease from various sources.

1.1 Agricultural waste

Agro-industrial waste is divided into two parts, named- agricultural waste and industrial waste. In which agricultural waste is further divided into two sub parts that are: field residues and process residues. Field residues are generally comprise of stem, stalks, leaves and seed pods whereas process residues are comprise of husks, seeds, roots, molasses and bagasse. Agricultural waste is also entitled as “agro-waste”, “green waste”, “organic waste” and “biodegradable waste”. Waste which is comprised of garden waste, like- flower cuttings, hedge trimmings, grass, domestic and commercial food waste. This is largely high in nitrogen content. 50-60% of valuable solid stuff can be procured from agricultural waste (Sadh et al, 2018).



1.2 Plant proteases

Proteolysis i.e. protein catabolism or breakdown is the housekeeping function performed by an enzyme called protease, also called proteinase or peptidase. All existing living forms and microorganisms such as fungi, yeast and bacteria they contain an enzyme protease. Due to its wide area of action and application in waste management, detergents, silver recovery and leather made it as an important enzyme for Industries (Babu et al, 2005). Enzymes are biocatalyst which

helps in increasing the rate of chemical reaction and they commercially used in food, chemical, detergents and pharmaceuticals industries (kumar et al, 1999). Bacteria such as *Bacillus species* and many enzymes reported to produce alkaline protease (Sharmila et al, 2014). Among animal, bacterial and fungal protease, bacterial protease is more efficient (Sharmila et al, 2014).Metallo and Serine protease are the main classes of bacteria protease. Serine and metallo are present in *Lysobacterenzymogene, E.coli,B.amyloliquefaciens, Bacillus subtilis*, *Pseudomonas sp.*, (Sharmila et al, 2014)

Plants are also the good source of protease enzymes. They are present in latex of plants and also in cell wall .Plant protease such as papain, ficin, bromelain were traditionally used for many purposes that is fig tree latex used for cheese making and milk clotting. Fig tree latex and pine juice is used for its antihelmentic property (Siota et al., 2010).Fruit and vegetables processed for the manufacturing of canned food and juice results in large amount of waste which can be used as feed and fertilizer (Gil and Moupoey, 2017) Proteolytic enzymes have the ability to modify proteins through limited or extensive cleavage , releasing free amino acids, peptides or polypeptides with physicochemical properties different from the original protein (Aguilar *et al.*, 2008). Proteolytic enzymes from proteolytic system of starters, proteases endogenous to food or added enzymes (eg: rennet) differ in their specificity and therefore, in their capacity to release bioactive sequences (Korhonun and Pihlanto 2006; Sabione et al., 2016).

1.3 Examples of plant protease

Enzyme	**Source**	**Application**
Bromelain	Pineapple	Baking
Ficin	Fig	Meat tenderisation
Papain	Papaya	Meat tenderisation and baking

1.3.1 Bromelain (a proteolytic enzyme), (Rabiu, 2018) is abundantly found in stem of the *Ananas cosmosus L. Merr* (Pineapple) that's why can also be known as "stem bromelain". In fruits also it is available in good amount. CAS number assigned to bromelain enzyme is 9001-00-7. Key suppliers for bromelain enzyme include- Sigma, MP Biomedicals, Pfaltz and Bauer, Beta Pharma, Enzyme Development Corporation and Biochem Europe (Siota and Villa, 2011).

1.3.2 *Ficin* Figs (*Ficus carica L*.) with respect to habit, growth forms and life forms are the broadly cultivated genus of woody plants of kingdom plantae. They are recognized mainly for their calcium content. It has been insisted that figs comprise of three times more calcium than diverse fruit varieties found in African and Asian countries.(Homaei et al, 2017)

Ficin (Ficain) is a plant thiol protease which can be drawn out from the latex of *Ficus carica Linnaues*. This enzyme is a member of C1 papain family.(Katsaros et al, 2009)

1.3.3 *Papain* Papaya (*Carica papaya Linnaeus*), in tropical and subtropical territory is dominant fruit crop. Antioxidants, proteins, polysaccharides, ascorbic acid, minerals (iron, calcium, potassium, etc.) and vitamin A (2020 IV), can be accounted in large quantity from papaya.(Lata et al, 2018). Papain (a proteolytic enzyme), is a plant endopeptidase that has been extracted out initially from the latex of *Carica papaya*. CAS number assigned to papain is 9001-73-4 (Siota and Vilaa, 2011)

PROBLEM BACKGROUND CHAPTER-2

In present scenario, as the consumers demand for healthy food at reasonable price. Consumers want a food to serve major benefits. Fermentation being one of the primitive methods for plant protease extraction as this method found to include high cost and large manpower. Because in this method, particular media for production of plant protease like- MRS media which is a selective and differential media but it's of high cost. Also a biofermenter also needed which was of high cost. It was the majorly used method and also extraction was done using acid, which found to leave certain residues in extracted plant protease thus declined its purification. Despite the fact that there are certain reports about the extraction and isolation of plant protease and its utilisation in food system. To the best of our knowledge very limited literature is available on extraction and isolation of plant proteases from agro-waste. As well there is extensive scope for the application of plant protease into different food system.

Thus, extraction of plant protease directly from agro-waste being new and beneficial method with all preserved quality parameters. As it is direct and easy method. High production can also be achieved because of nutrient composition of agro-waste. Health benefits also improvised because of natural substrate used for extraction.So, nutrients can be preserved and appropriate protein utilization or absorption by body can be easily achieved say for an example: application of naturally extracted plant protease in meat which causes hydrolysis of complex proteins into simpler form to increase biological value.

OBJECTIVES CHAPTER-3

1. Process parameter optimization for extraction of plant protease from agro-waste.
2. Process standardization for purification of extracted plant protease.
3. Application of isolated plant protease into suitable food

RESEARCH GAP CHAPTER-4

Despite the fact that there are certain reports about the extraction and isolation of plant protease and its utilisation in food system. To the best of our knowledge very limited literature is available on extraction and isolation of plant proteases from agro-waste. As well there is extensive scope for the application of plant protease into different food system. Hence present study is designed to focus on above mentioned aspects.

5.1 *Action of proteases of plant origin as milk-clotting enzymes in cheese manufacturing*

Shah et al., 2013. Milk coagulantin compounds are basic for generation of products like- cheese and plant proteases can be extracted from a few sources of plant and can be analyzed for milk coagulating capacity. These compounds can also be isolated through their by in vitro culture or regular origin. Extraction of milk thickening proteases through the plant parts is work serious thus plant's invitro is aother option to get coagulating proteins. The rough concentrates can be additionally filtrated to get partiallly refined or unadulterated pure protein on the level of its filtration.Proteases of plant origin were utilized as coagulant of milk in cheese making for many past years either as rough concentrates or inpurified forms. These coagulants are a other option to the calf rennet because of the availablility limitations and religious factors, more cost of rennet.. These catalysts are found in almost every kind of plant tissues and also can be extracted from their common origin or by in vitro method to guarantee a limitless flow of plant proteases (Shah et al., 2013).Almost every one of the chemicals utilized as coagulating agents belongs to proteases-aspartic, however proteins from different gatherings, for example, serine and cysteine proteases have additionally been accounted for and have the capacity to cluster milk in appropriate conditions. The proteolytic nature of most plant coagulanting agents has restricted their utilization in cheddar production because of lower yields of cheddar, unpleasant flavors, surface imperfections. The studies for advanced coagulating chemicals of milk from plants still proceeds because of the increasing worldwide demand for differentiated and great quality cheese production (Shah et al, 2013).

5.2 *Proteases from leaf extract of medicinally beneficial plants studied for their detection and analysis*

Chinnadurai et al, 2018. The parts of the plants, extracts from the plants and sometimes the entire plants are being used in the medicine for the treatment of the various disorders and the dieseases in the human. And mainily the plants with the higher activity of the protease are being used in many purposes to treat tumors, sweelings, coagulation of the blood, digestive problems and even for the immunological disorders from many past years. Here,that has been conducted in the medicinally non-important plants during the period in which all the studies are going on only on

the medicinally important plants, to identify the correlation between the activity of the proteases and the medicinal uses of that particular plant. As a result there has been no correlation was observed but the higher action of the proteases was observed in the leaf extracts of the *Pongamiapinnata* (Fabaceae), *Wrightiatinctoria* (Apocyanaceae) *Acalyphaindica* (Euphorbiaceae), *Adhatodavasica* (Acanthaceae) and *Curcuma longa* (Zingiberaceae) (Chinnadurai et al, 2018).

5.3 ***Meat Tenderization with Ginger Rhizome Protease***

LEE et al, 1986. Beef steaks and cut meat marinated with various levels of unrefined ginger concentrate were assessed for the tenderness and auxiliary changes. A huge (P<O.O5) upgrade of tenderness with expanding measures of ginger concentrate was seen at low levels of concentrate (0, 0.05, 0.1, and 0.2 ml/6.5 cm2 for the steaks; 0, 0.25, 0.5, and 1 ml/lOOg for the cut beef), wheras the change of tenderness at 0.2 ml/6.5 cm2 or 1 ml/lOOg was very low. Electron microscopy of the treated beef showed the particular degradation of thin fibers in the I-groups, bringing about broad fragmentation of myofibrils. so they concluded, ginger rhizome protease is avery good meat tenderizer. For both the loin and best round steaks, a relatively direct decrease of shear esteems with expanding measure of ginger concentrate was seen in between the 0 and 0.2 mL for every 6.5 cm surface region. A significantly a very little change in sensory scores was seen in between the 0.2 and 0.4 ml. These outcomes show that 0.2 ml of rough ginger concentrate per 6.5 cm surface region appears to be the ideal level to accomplish the biggest tenderization impact.

5.4 ***Plant proteases extraction from Zingiber officinale roscoe (Ginger rhizome) and Cucumis trigonus Roxb (Kachri) and its application in buffalo tenderization of meat***

Naveena et al, 2004. Among the 166.4 million of the buffaloes from all over the world, the 161.4 million of the buffaloes are only from the Asian part of the world, and among which the half of the entire population is from the India. While the buffalo meat has the health benefits like the reduction of the blood cholesterol, on the other hand, due to the tough structure of it, it is not much preferable (Naveena et al, 2004). Thus, the study has been done to make the buffalo meat more acceptable, b maintaining of its tenderness and by increasing its overall qualities by reducing its toughness by using the plant proteolytic enzymes from the *CucumistrigonusRoxb* (Kacari) and *Zingberofficinake roscoe* (Ginger rhizome) and their action was studied by comparing with the papain which is a very most effective enzyme and as a result, it was observed

the very good increase in the quality parameter like the tenderness, juiciness, flavour, and even the overall acceptance has scored high. Among all these the one treated with the Ginger extract was with higher acceptance score. Thus, it was concluded that the Ginger and the Cucumins were the very good alternatives to the most efficient papain

5.5 SDS-PAGE extraction of protease

SDS-PAGE stands for Sodium Dodecyl Sulfate- Polyacrylamide Gel, is widely used for the evaluation and character analysis of protease deriving out of plant tissues. Mature cells of plants basically having big vacuoles whichever consist of high amounts of proteolytic enzymes, in addition phenolic compounds, pigments, and tannins. While separating by method of extraction, protease were coming out of plant tissues, the above-mentioned substances extracted are coming out of ruptured cells and might obstruct extremely with all the evaluation of protease using SDS-PAGE. The process of degradation of high molecular or sub atomic- mass proteins could present as quickly as the cells were ruptured in protease-rich tissues. Pulverizing the plant tissues within the presence of SDS doesn't at all times stop protein degradation (or destruction) therefore a few proteolytic enzymes are functional in aqueous solutions containing SDS. Inhibitors availability for a few exact proteases nothing of them had a universal inhibitory outcome upon all almost proteases (Wu and Wang, 1984).

We discovered that one proteolytic activeness within the arising anther of petunia was serious, which high-molecular-weight proteases were denatured in a couple of minutes afterwards homogenize tissues into solution of buffer. The procedures or operational plans described presently was established to stop proteolytic actions for the duration of homogenization and to favor the evaluation of proteins with high molecular weight by this method. Extraction of protease by homogenization of the tissue in slotion of buffer either with the (solution B) or without (solution C) of SDS led to a entire loss of high-molecular weight proteins

PROPOSED RESEARCH METHODOLGY CHAPTER-6

PROCUREMENT OF RAW MATERIAL

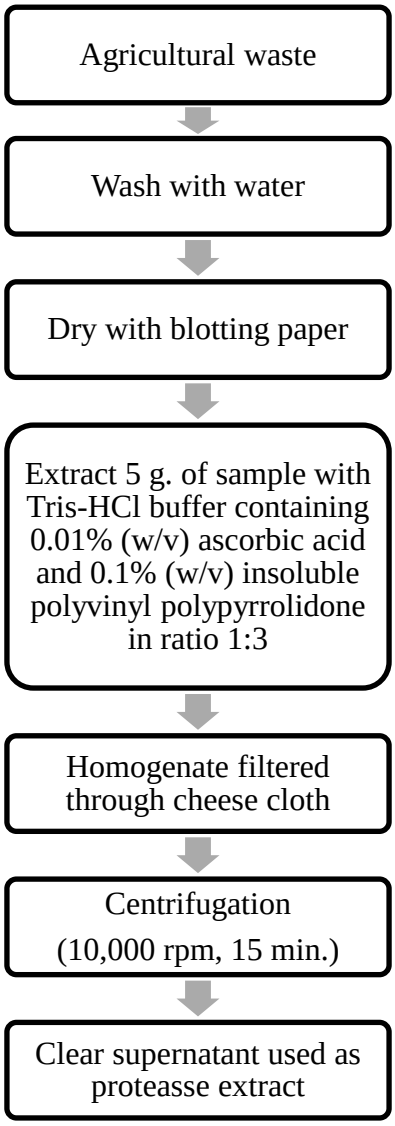
The current study in production and application of plant protease was planned to use agricultural waste which were bought from the local fruit market of Phagwara, Punjab and from agriculture field at Yamunanagar, Haryana. The agricultural waste collected was cut, dried, sieved and then grounded into the fine powder.

6.1 Experiment 1: To conduct proximate analysis of raw material.

TEST	METHOD
Moisture Content	AOAC 2005
Total Mineral Content	AOAC 2005
Fat Content	AOAC 2005
Protein content	AOAC 1990
Carbohydrate Content	Pathak et al., 2016

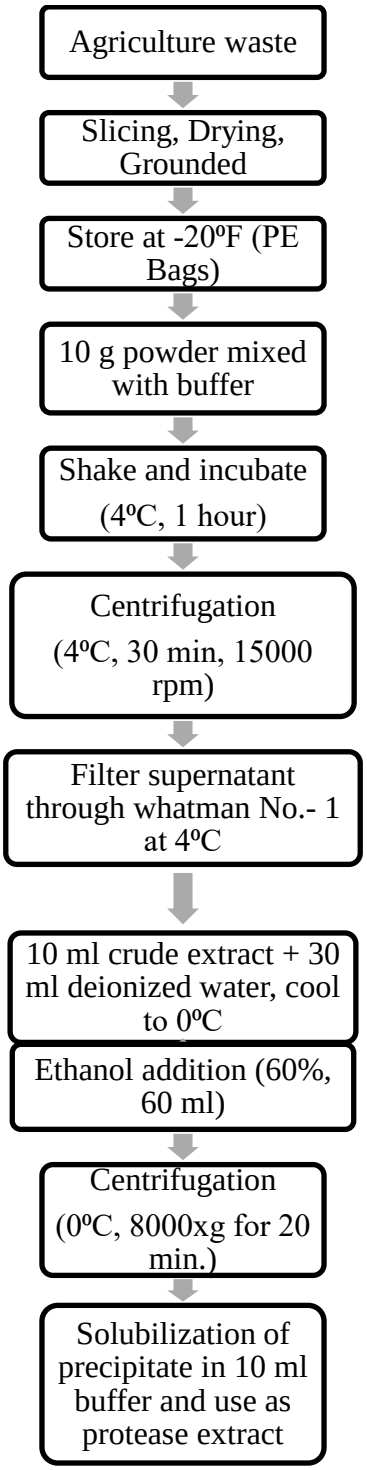
6.2 Experiment 2: To extract plant protease from agro-waste

6.2.1 Flow Sheet for extraction of proteases



Source: (Chinnadurai et al., 2018)

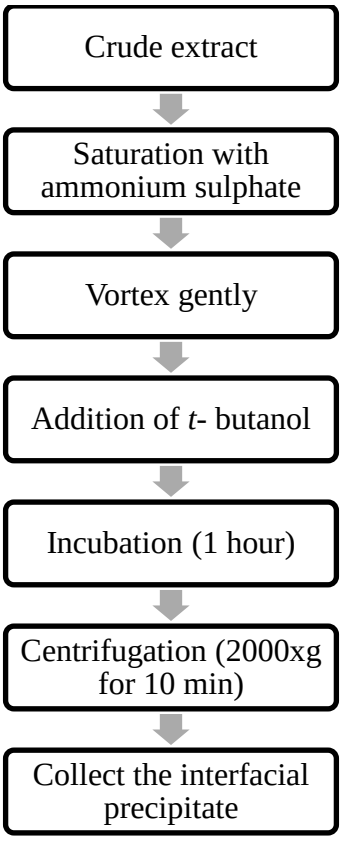
6.2.2 Flow Sheet for extraction of proteases



Source: (Chinnadurai et al., 2018)

6.3 Experiment 3 To do purification of extracted plant proteases.

6.3.1 Flow sheet for enzyme purification



Source: (Sharma and Gupta, 2001)

EXPECTED RESEARCH OUTCOMES CHAPTER-7

Process parameters will be optimized for the extraction of plant protease from agro-waste. In this study comparative analysis of different new techniques will be provided and also the best suited method i.e. which will provide the highest yield. Also, study of application of this naturally extracted plant protease will be carried out suitable food system. The effect of addition of plant protease on food product will be executed to improve consumer acceptability.

PROPOSED WORK WITH PLAN TIMELINE CHATER-8

Work Plan	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov
Review of literature	√	√	√								
Report Submission				√							
Extraction							√	√			
Purification								√			
Application									√	√	
Result compilation											√

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