EFFECT OF EXTRACTION METHODS ON PROTEASE YIELD AND PROTEOLYTIC ACTIVITIES OF PROTEASE ENZYME FROM DIFFERENT MATURITY STAGES OF STARFRUIT *(Averrhoa carambola)*

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Effect of Extraction Methods on Protease Yield and Proteolytic Activities of Protease Enzyme from Different Maturity Stages of Starfruit *(Averrhoa carambola)*

A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of B.Tech in Food Technology

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Approval Letter

This ***dissertation*** entitled ***Effect of Extraction Methods on Protease Yield and Proteolytic Activities of Protease Enzyme From Different Maturity Stages of Starfruit (Averrhoa carambola)*** presented by **Niraj Adhikari** has been accepted as the partial fulfillment of the requirement for the **B.Tech degree in Food Technology.**

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Abstract

The main objective of this work was to compare the effect of extraction methods (ammonium sulphate, acetone and acetone TCA precipitation) on protease yield from starfruit of different maturity stages (unripe, semiripe and ripe). Proteolytic activity (PA) and protein were measured spectrophotommetrically. Optimum hydrolysis condition: temperature and pH of protease for maximum proteolytic activity was determined by response surface methodology. Protease were incubated for 12 h in different buffers in the pH range of 3.5 to 8.5 before the determination of proteolytic activity. In order to determine the effect of temperature, the protease was incubated at different temperatures in the range of 40 to 90°C. Storage stability of the proteases were also determined for one week storage period at <4°C.

Higher yield and proteolytic activity were observed for protease using ammonium sulfate (40%) than acetone and acetone with TCA precipitations. As for maturity stages, PA and protein concentration yield from unripe stage were significantly ($p<0.05$) higher than those of semi ripe and ripe. Numerical optimization study revealed that the optimum temperature and pH for proteolytic activity were 65°C and 6.5 respectively. The optimized proteolytic activity was found to be 0.862±0.05 units/ml. Enzyme activity was significantly decreased during 7 days storage period at <4°C. This study suggested that ammonium sulfate precipitation is effective method for extraction of starfruit protease compared to acetone and acetone with TCA precipitations. In order to obtain higher protease activity, unripe starfruit will be better raw material for protease production.

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List of Abbreviations

Abbreviation	List of Abbreviations
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CCT	Central Campus of Technology
CDFT	Central Department of Food Technology
FAMA	Malaysian Federal Agricultural Marketing Authority
TCA	Tri-Chloro Acetic acid
MOA	Ministry of Agriculture
LSD	Least Significant Difference
PA	Proteolytic Activity
RFTQCO	Regional Food Technology and Quality Control Office
RSM	Response Surface Methodology

Part I

Introduction

1.1 General introduction

Starfruit, also known as carambola, is the fruit of *Averrhoa carambola*, categorized under oxalidaceae family a species of tree native to tropical Southeast Asia (Gepts, 2008). The sweet, mild acidic, sugary and juicy fruit with a fresh character and distinctive flavor is frequently eaten as fresh juices or used for flavour (Abdullah *et al.*, 2007).This star-shaped fruit has a waxy skin with several smooth brown seeds, and its flesh and skin are juicy and crisp (Paull and Duarte, 2012).

During the maturation process the starfruit skin transforms from dark green to orange during the maturation process, with the dark green colour representing immature starfruit and the orange color representing extremely mature starfruit (Mokji, 2009).Starfruit ripeness can be categorized into seven categories, namely index 1 to index 7. Index 1 represents the most immature starfruit (un-ripe), while index 7 represents the most mature starfruit (ripe) (Amirulah, 2012) .

Proteases are proteolytic (protein-digesting) enzymes that breakdown proteins (Sumantha *et al.*, 2006). They are classified based on their origins, (whether animal, plant, or microbial), catalytic action and nature of catalytic site (Taylor, 2013). Some plants which have promising levels of enzymes, desert date *(Balanite aegyptiaca)* (Beka *et al*., 2014), ginger *(Zingiber offcinale)* rhizomes (Gagaoua *et al.*, 2015), cardoon *(Cynara cardunculu)* (Silva and Malcata, 2005), tamarilla, *(Solanum betaceum)* (Li *et al.*, 2018) steam of pineapple (bromelian) (Mazumdar and Majumder, 2003), *Gliricidia sepium* (da Silva *et al.*, 2020), wild thistle*(Cynara humilis)* (Esteves *et al.*, 2003), lettuce *(Lactuca sativa)* (Lo Piero *et al.*, 2002), neem tree *(Azadirachta indica)* (Gupta and Chaphalkar, 2015) papaya *(Carica papaya)* (Maskey and Shrestha, 2020), *Moringa oleifera* (Banik *et al.*, 2018) etc. Proteases are routinely used in cheese making, baking and meat tenderization (Ward, 2011).

According to Kim *et al.* (2010) kiwi juice, (Shrestha, 2019) papaya is homogenized with buffer followed by centrifugation, and the other were the supernatant was precipitated by saturated ammonium sulfate followed by dialysis for extraction.

Protease are generally purified by salt precipitation and chromatography (Azarkan *et al.*, 2003), extraction by homogenizing in Tris-HCl buffer followed by purification in ammonium sulfate (Ismail and Kharoe, 2013), three-phase partitioning (TPP) (Dennison and Lovrien, 1997) extraction using phosphate buffer and subsequent purification with acetone precipitation (Simpson and Beynon, 2010), and extraction using phosphate buffer and subsequent purification with acetone with TCA precipitation (Méchin *et al.*, 2007).

In ammonium sulfate precipitation, precipitation proceeds when high concentrations of small, highly charged ions such as ammonium sulfate are added, these groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation (Dagley, 1955).

Acetone prevents dispersion of proteins among water-based solvents causing protein aggregation and precipitation. A mixture of TCA and sodium deoxycholate enhances the precipitation of very small amounts of proteins as deoxycholate binds to the hydrophobic parts of proteins (Boschetti and Righetti, 2013).

1.3 Objectives

1.3.1 General objective

The general objective of this study was to find the effects of extraction methods on protease yield and protease activity of protease extracted from different maturity stages of starfruit *(Averrohea Carambola).*

1.3.2 Specific objectives

The specific objectives of the study were as follows:

1. To extract enzyme from starfruit of different maturity stages (unripe, semi-ripe and ripe) using different precipitation methods as, ammonium sulfate, acetone and acetone with TCA precipitation.
2. To determine the proteolytic activity and protein content of extracted enzymes.
3. To optimize the pH and temperature of extracted enzyme.
4. To study the storage stability of the extracted enzyme.

1.4 Significance of the study

Starfruit is evergreen plant (Pingping *et al.*, 2017) as, flowering continues throughout the year and fruit is available most of the year (Dreyer and Marie, 1955). So, this research could be useful in determining an alternative method for the utilization of starfruit as a source of protease enzyme, which could be used in a variety of food processing industries as, proteases are commonly employed in the bakery sector. These enzymes are used to speed up the mixing process, reduce dough consistency and uniformity, control gluten strength in bread, and improve texture and flavor (Melim *et al.*, 2013).

Meat tenderization by protease enzyme tenderness can depend on the cut of the meat. One popular tenderizer is papain, which comes from the papaya tree. Papain works by breaking down meat proteins using a chemical process called hydrolysis. Thus resulting smaller meat proteins have a softer texture (Gerelt *et al.*, 2000), plant protease as milk clotting (Shah *et al.*, 2014) protease in brewing (Gomaa, 2018) and many more. As a result, its underutilization can be reduced.

1.2 Statement of problem

Under normal temperature and humidity conditions, starfruit have a limited shelf life (Karim and Wai, 1999). Since they are perishable by nature and have a high moisture content, they quickly lose their freshness, become vulnerable to mold and bacteria, and eventually decay, rendering them useless as human food (Yahaya and Mardiyya, 2019). Fruit utilization can be enhanced by food processing (Dongyu, 2017). Enzyme extraction is another method that can be useful in various food industries such as meat tenderization, milk clotting, baking industries etc. (Tucker and Woods, 1995).

It is widely available throughout the year in Nepal, especially in the Terai region. It is one of the most underappreciated fruits. It is neither processed nor widely consumed. As a result, enzyme extraction can be the one of the alternative choice used in the food industry.

1.5 Limitation of study

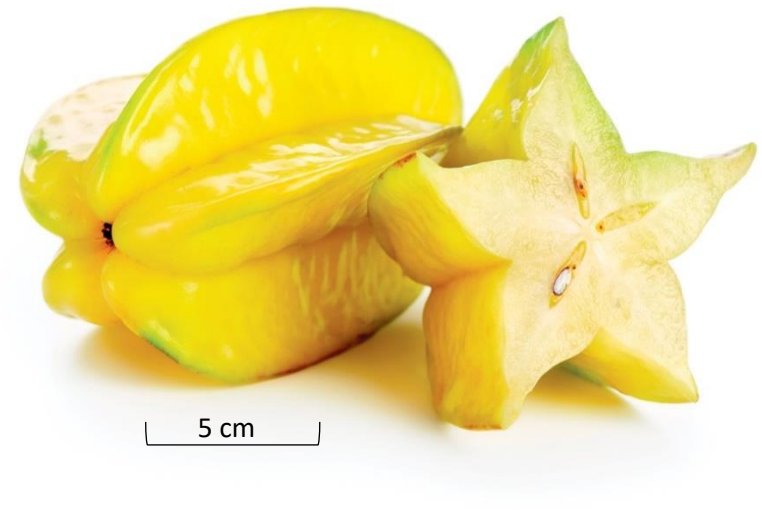
a. Purification of enzyme was not carried out due to lack of instrument in laboratory.
b. Variety was not distinguished.

Part II

Literature review

2.1 Introduction to starfruit

The, star fruit also known as the carambola, is a member of the Oxalidiaceae family, with the genus *Averrhoa Carambola*. The word Carambola is derived from Sanskrit word *karmaranga* meaning “food appetizer” (Monalisa *et al.*, 2014). Starfruit is a tropical fruit and is native to south east Asia i.e. Philippines, Indonesia, Malaysia, Vietnam, Nepal, India, Bangladesh, Sri Lanka, and Mauritius (Dasgupta *et al.*, 2013). The shape of cross-sectioned slices is that of a star is shown in Fig. 2.1.



Source: Hines (2013)

Fig. 2.1 Starfruit cross-section

The oxalidaceae family contains seven genera and over 200 species, most of which are found in tropical and subtropical regions of the world. Bilimbi *(Averrhoa bilimbi* L.*)* and carambola *(Averrhoa carambola* L.*)* are the two species in the genus *Averrhoa* (Hayes, 1960). In Brazil, Southeast Asia, South Asia, the South Pacific, Micronesia, portions of East Asia, the United States, and the Caribbean, the fruit is commonly consumed (Gepts, 2008).The tree is grown in tropical areas throughout the world (Blench, 2009). Starfruit is known by a variety of names in different parts of the world, including *balimbing* in Southeast Asia, *ma fen* in China , *kamaranga* in India, *kabra* and carambola in Spanish-speaking

countries (Young, 1987). In Nepal, it is called by different names- *aamrakh* (in *tharu* community), *kabra* and *charpate* in eastern region of Nepal (Chaudhary, 2019).

The small sour (or tart) type and the larger sweet type are the two main types of carambola. The sour varieties contain more oxalic acid than the sweet varieties. In recent years, a number of cultivars have been produced. The sweet types "*Arkin*" (Florida), "*Yang Tao*" (Taiwan), "*Ma fueng*" (Thailand), "*Maha*" (Malaysia), and "*Demak*" (Indonesia) are the most popular commercial cultivars, while the sour types "Golden Star", "Newcomb", "Star King", and "*Thaye*r" are the most common commercial cultivars (all from Florida). If left to ripen, some sour varieties, such as "Golden Star," may become sweet (Crane, 2001).

The carambola tree has a short trunk and several branches, reaching a height of 30 feet (9.1 m). It has 6–10 inch (15–25 cm) long deciduous leaves with 5–11 ovate leaflets that are medium green in color. Lilac flowers with purple streaks are about 0.25 inch (6.4 mm) wide and have a lilac hue (Warrier, 1993).

2.2 Taxonomical hierarchy and nomenclature of starfruit

Taxonomic hierarchy is the process of arranging various organisms into successive levels of the biological classification either in a decreasing or an increasing order from kingdom to species and vice versa (Moore, 1974). The taxonomical hierarchy is shown in the Table 2.1

Table 2.1 Taxonomical hierarchy of starfruit

Taxonomical hierarchy of starfruit	
Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyte
Sub phylum	Angiosperm
Class	Dicotyledonae
Order	Geraniales
Family	Oxalidaceae
Genus	*Averrhoa*
Species	*Averrhoa carambola*

Source: Guala (2017)

Over 800 species of herbs, shrubs, and trees, mostly of neotropical origin, make up the Oxalidaceae family, also known as the "wood sorrel." The genus name Averrhoa is named after the famous Andalusian philosopher *Averrhoea,* who lived in the twelfth century (Selin, 2008)

2.3 Botanical description

2.3.1 Botanical parts

The starfruit plant is a small, slow-growing evergreen tree with a short trunk or a shrub. The branches are drooping and the wood is white and turns reddish (Kapoor, 2018). It has a bushy shape with many branches producing a broad, rounded crown. The fruits have thin, waxy skin that is orange-yellow colored. The juicy fruits are yellow inside when ripe and have a crisp texture and when cut in cross-section are star-shaped. The fruits have an oxalic acid odor, which varies between plants from strong to mild, the taste also varies from very sour to mildly sweetish. Each fruit may have up to 6–12.5 mm long seeds, which are flat, thin, and brown. Some cultivated forms produce fruits with no seeds (Warrier, 1993).

The carambola shrub grows up to 12 m in height in cultivation, but in the wild, it grows much higher. The trunk is short and crooked. It branches near the base and has an irregular dense crown. The bark, greyish-brown to dark grey, is smooth. The leaves of the tree are small, measuring between 15-25 cm in length. They are arranged alternately and grow along a horizontal plane. When young, seven to nine leaflets are arranged together and they seem sensitive to touch or external stimuli. Carambola flowers are produced in a tuft measuring two to three cm long and are pentamerous. The starfruit is a large, fleshy berry of a rich amber color when ripe. When cutting cross sectionally, it is shaped like a star, hence its common name. It is a very juicy fruit with a sweet-sour taste (Yaacob and Subhadrabandhu, 1995). The botanical description is shown in the Table 2.2.

Table 2.2 Different botanical parts of starfruit

Botanical parts	Description
Tree	Carambola trees grow to be 5-12 m tall. It has a large number of branches, resulting in a large number of water shoots. The younger plant has a pyramidal shape, while the older plant is round.
Leaves	The leaf is oval in shape and small. The leaf's upper surface is smooth and yellowish-green in color. The color of the lower surface is dark green. The leaf measures 2-4 cm in width and 2-9 cm in length.
Flower	The purplish flower is tiny and has a purplish color. It has five petals, five sepals, and five stamens. Underneath the style is the ovary. Flowers bloom all over the trunk, roots, and twigs
Seed	There are normally only 10-12 seeds per fruit, although there are occasions when there are none. The seeds are edible and measure 1/4 to 1/2 inches (0.6-1.3 cm) long, small, light brown, and are encased in a gelatinous aril. After being removed from the fruit, seeds quickly lose viability
Fruits	The fruits are oval with 5.6 angles/ribs on each side. It will form a star shape when cross-cut. When the fruit is young, it is green, but when it ripens, it turns yellow or orange. Smooth, juicy, crispy flesh with a sweet yet sour flavor.

Source: Othman and Omar (2004)

2.3.2 Crop requirement

The starfruit is a tropical and subtropical fruit that can be produced up to 1,200 m in elevation (4,000 feet). It prefers full sun, but needs high humidity and at least 1,800 mm of annual rainfall (70 in). It doesn't have a specific soil type, but it does well in loam and needs good drainage. During the dry seasons, moderate irrigation helps it develop. During the dry seasons, moderate irrigation helps it develop (Othman and Omar, 2004).

2.3.3 Reproduction pattern

Averrhoa carambola is an angiosperm (Wu *et al.*, 2020), which undergo reproduction via alteration of generations. Starfruit tree are dioecious, meaning that a male and female star fruit tree is needed to create a new tree. Its pollination is not air born, but instead it is pollinated by insects. The two main insects that are responsible for the pollination of starfruit are honey bees and sting-less bees (Hilu *et al.*, 2003).

2.3.4 Maturity index of starfruit

In the starfruit maturity process, the starfruit skin changes from dark green color into orange color where dark green color is for immature starfruit while the orange color is for very mature starfruit (Amirulah *et al.*, 2010). Based on the Malaysian Federal Agricultural Marketing Authority (FAMA), the starfruit maturity can be classified into 7 levels of maturity, namely index 1 to index 7 as presented in Table 2.3

Table 2.3 Maturity index of starfruit according to FAMA

Index	Color	Maturity
Index 1	Dark green	Immature
Index 2	Green with little yellow	Mature
Index 3	More green than yellow	Mature
Index 4	Yellow-Green	Almost ripe
Index 5	Yellow with a little green	Ripe
Index 6	Yellow	Ripe
Index 7	Orange	Overripe

Source: Omar (2013)

2.3.5 Grading and sizes

Maturity at harvest is a critical factor that determines storage life and final fruit quality standard. Immature fruits are more subject to shriveling and mechanical damage, and are of inferior quality when ripe. Overripe fruits are likely to become soft and have mealy insipid

flavour soon after harvest. Any fruit picked either too early or too late in its season is more susceptible to physiological disorders and has a shorter storage life than fruit picked at the proper maturity (Kader, 1997). Therefore, grading is an integral part of the starfruit produce industry (Abdullah and Fathinul-Syahir, 2005). Grading of starfruit is shown in Table 2.4.

Table 2.4 Grading of starfruit according to the Malaysian Federal Agricultural Marketing Authority (FAMA).

Grade	Specifications	Range of flexibility (Maximum)
Premium	Fruits are collected from the same cultivar, fresh and clean. Uniform size and maturity index and free from damages	Maturity ≤ 3% Freshness ≤ 5% Damages ≤ 3% Abnormality ≤ 3% Size uniformity ≤ 5%
1	Fruits are collected from the same cultivar, fresh and clean. Uniform size and maturity index and slight or free from damages.	Maturity ≤ 5% Freshness ≤ 5% Damages ≤ 5% Abnormality ≤ 3% Size uniformity ≤ 10%
2	Fruits are collected from the same cultivar, fresh and clean. Uniform size and maturity index and slight or free from damages	Maturity ≤ 10% Freshness ≤ 10% Damages ≤ 10% Abnormality ≤ 10% Size uniformity ≤ 10%

Source: Ahmad *et al.* (2010)

2.3.6 Harvesting and yield

Starfruit are available in India in September and October, as well as December and January (Dasgupta and Chakraborty, 2013). They are produced all year in Malaya. Fruit is available throughout the year in Florida, but the main crop matures from late summer to early winter. Some trees produced a lot of fruit in November and December, as well as in March and April. It's possible that there will be three crops. Much of the seasonal variation is due to weather conditions. When the fruits are fully ripe, they naturally fall to the ground. They should be hand-picked while pale-green with a hint of yellow for marketing and shipping. Trees that have received proper horticultural care have produced 100 to 250, and even 300 pounds (45-113-136 kg) of fruit (Manda *et al.*, 2012). According to Ministry of agriculture, Nepal the yield of summer (tropical) fruit is 9.18% (MoA, 2013).

2.4 Varieties of starfruit

Starfruit, a commercial cultivar in Malaysia, is widely marketed and exported mainly to Europe in all states. Four states in Malaysia are cultivating star fruit, namely *Salangor*, the *Negeri Sembilan, Pahang and Johor* (Rahman *et al.*, 2007). The Department of Agriculture, Malaysia, is listed with nineteen varieties of starfruit. Of the 19 varieties, however, the best commercial clones, *'Belimbing Besi'* (B10) and *'Belimbing Madu'* are only two popular ones (B17) (Zabedah *et al.*, 2008). In addition to Malaysia, Starfruit plants are grown in the United States for their fruit.Taiwan has its own Starfruit plant accession collection, for example, *'Mih Tao,' 'Dah Pon*' and *'Tean Ma'* as well as *'Fwang Tung'* in Thailand (Gowrishankar *et al.*, 2018). Fig. 2.2 resembles the varieties of starfruit.

Fig. a
'Arkins'

Fig. b
'B-2'

Fig. c
'B-10'

Fig. d
'B-17'

Fig. e
'Dah Pon'

Fig. f
'Demak'

Fig. g
'Fwang Tung'

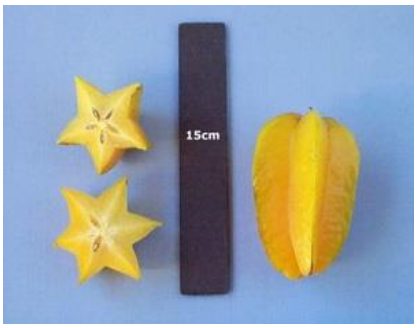


Fig. h
'Golden Star'

Fig.i
'Hew #1'

Fig. j
'Kajang'

Fig. k
'Kary'

Fig. l
'Sri Kambangan'

Source: Crane (2001)

Fig. 2.2 Varieties of Starfruit

2.5 Chemical composition of starfruit

The fruit of the starfruit is distinguished by its distinctive star shape and rich golden color. The composition of the fruit during ripening varies greatly (Narain *et al.*, 2001). Chemical composition is shown in Table 2.5.

Table 2.5 Chemical composition of starfruit

S.N	Parameters	Young	Half ripe	Ripe
1	Soluble protein (% fresh weight)	0.65	0.83	0.85
2	Reducing sugar (% fresh weight)	0.33	1.15	1.32
3	Total sugar (% fresh weight)	1.12	1.50	2.25
4	Ascorbic acid (% fresh weight)	9.5	13.5	18.0
5	Amino acid (% fresh weight)	0.12	0.16	0.17
6	Oxalic acid (% fresh weight)	0.63	0.85	1.64
7	Pectin (% fresh weight)	1.74	1.95	5.11
8	Chlorophyll (% mg/g weight)	4.05	13.60	2.10

Source: Patil *et al.* (2010)

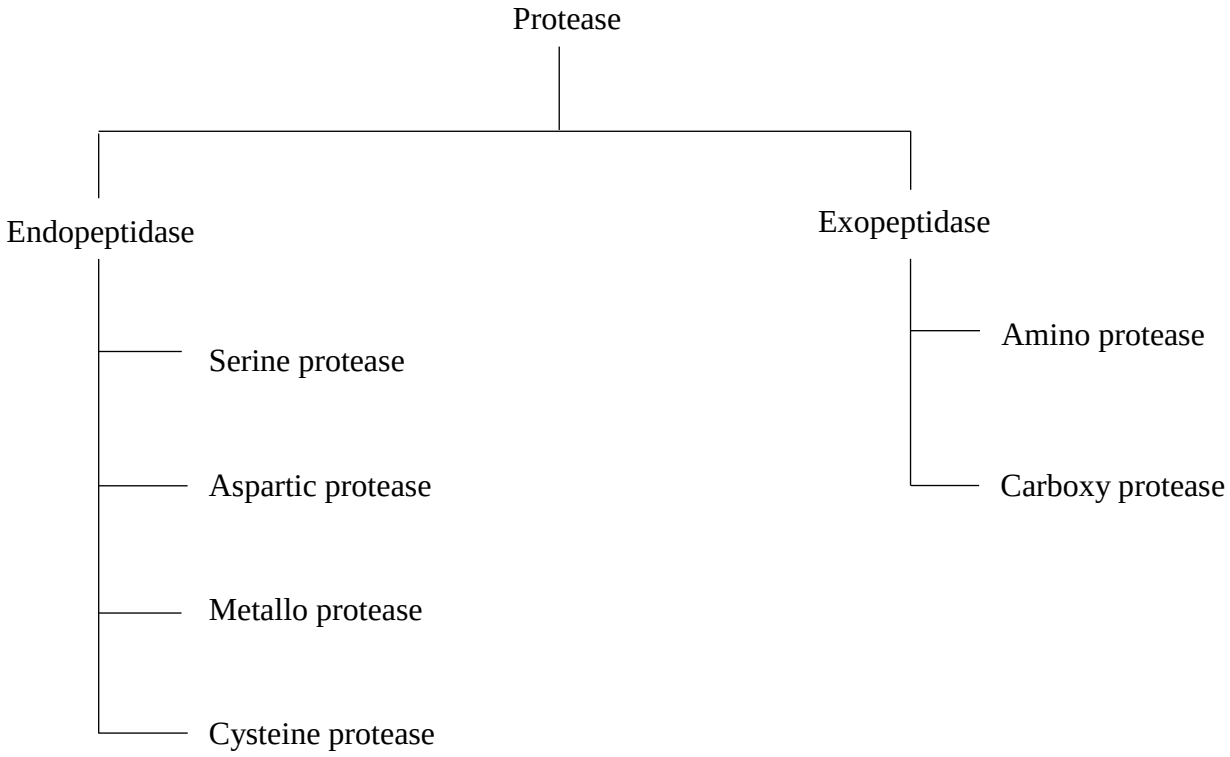
2.6 Plant protease

Proteases are the enzymes hydrolyzing molecules of proteins into peptides and amino acids. Proteases are the most commercially important enzymes because of their numerous uses in food and other industries, the interest in plant proteases has grown rapidly over the last few decades. There is still little, but quick, growth of the number of industrially used enzymes of plant origin. Since, plants need proteases over their entire life cycle. In most plant physiology and growth, plant proteases play an important role in signaling molecular production and as regulators of important cellular processes such as cell division and metabolism (Shah and Mir, 2019).

2.6.1 Classification of plant protease

Proteases can be classified into many ways. They are classified as exoproteases or endoproteases depending on where they act. Endoproteases cleave internal peptide bonds, whereas exoproteases cleave N- or C-terminal peptide bonds (Palma *et al.*, 2002). Based on their ability to cleave the N-terminal and C-terminal peptide bonds, exoproteases are further divided into aminopeptidases and carboxypeptidases. Endoproteases are classified according

to their catalytic mechanism, which is the active site of the enzyme (Rawlings and Barrett, 2010). The main catalytic types are aspartate, serine, cysteine, and metallo proteases (Bruno *et al.*, 2010). Fig. 2.3 shows the classification of protease.



Source. Sharma *et al.* (2019)

Fig. 2.3 Classification of protease

2.6.1.1 Aspartic protease

Aspartic proteases are commonly known as acidic proteases (Rao *et al.*, 1998). They are peptidases with a wide range of functions and specificities. Animals, plants, fungi, and viruses all have them. Aspartic proteases have been linked to a variety of physiological functions, including mammalian digestion of nutrients, pathogen defense, yeast virulence, breast cancer, mastitis, pollen-pistil interactions, parasite hemoglobin degradation, and HIV protein maturation (Claverie-MartÌn and Vega-Hernàndez, 2007). Aspartic proteases are endopeptidases with two aspartic residues in their active site, which are required for catalytic activity They are most active at acidic pH (3-4) and exhibit specificity against aromatic or bulky amino acid residues on both side of their peptide bond (Yegin *et al.*, 2011).

2.6.1.2 Serine protease

Serine proteases (or serine endopeptidases) are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the (enzyme's) active site (Rawat *et al.*, 2021). They are found in almost all plant parts, but are most abundant in fruits, and are found in a wide range of taxonomic groups, from trees and crops to legumes and herbs (Rawlings and Neil, 1994).

2.6.1.3 Cysteine protease

Cysteine proteases, also known as thiol proteases, are protein-degrading hydrolase enzymes.(Rawat *et al.*, 2021). Cysteine proteases are involved in both anabolic and catabolic processes in plants, as well as signaling pathways and protein maturation, degradation, and rebuilding in response to various external stimuli (Grudkowska and Zagdańska, 2004).

2.6.2 Production of plant protease

Proteases from plant beginning to plant death are required by plants for different physiological and developmental processes. They can be extracted from natural sources or prepared by tissue culture practice (González-Rábade *et al.*, 2011).

2.6.2.1 Production from natural process

Plant proteases can be extracted from natural sources by aqueous maceration of various plant organs such as flowers, fruits, seeds, roots, and leaves. Depending on the degree of purification, the resultant raw extract may be further purified to obtain partially purified enzymes and pure enzymes. Ammonium sulfate precipitation is effective in producing significant amounts of active proteases (Shah *et al.*, 2014). Some plants which have promising levels of enzymes, *Balanite aegyptiaca* (Beka *et al.*, 2014), *Zingiber offcinale* rhizomes (Gagaoua *et al.*, 2015*), Cynara cardunculus* (Silva and Malcata, 2005), tamarilla, (Li *et al.*, 2018), *Allium Cepa* L. (Ndidi *et al*., 2012), *Pissum sativum* L. (Basha and Beevers, 1975), *Zea mays* L.*(*Bassuony *et al.*, 2008*)* reported to have been extracted and purified.

2.6.2.2 In vitro production

The plant cells are totipotent and can produce in vitro and in vivo the same chemical compounds. Between the two processes, the yield of enzymes differs (González-Rábade *et*

al., 2011). Different plant organs produce proteases with different activities (Pérez *et al.*, 2013). Proteases have been produced using a variety of in vitro techniques, including callus and cell suspension cultures. Examples include, proteases produced from *Mirabilis jalapa* culture (Tamer and Mavituna, 1997), cell suspension culture of *Centaurea calcitrapa* (Raposo and Domingos, 2008), and callus culture of *Silybum marianum* (Cimino *et al.*, 2006) and *Cynara cardunculus* (Oliveira *et al.*, 2010).

The advantages of in vitro techniques are numerous. Plant proteases produced in vitro yield higher enzyme yields and require fewer extraction procedures than those extracted from natural sources. Furthermore, these techniques mitigate the effects of climate change and pollution, seasonal variations, as well as the diversity of enzymes produced by different parts of the plant (González-Rábade *et al.*, 2011)

2.6.3 Starfruit protease

Protease yields from unripe starfruit are higher than those from ripe stage, which is consistent with protein concentrations obtained using various purification methods (Ismail and Zainuddin, 2015). Analysis from the freeze dried protease indicated that protease from ripe noni fruits *(Morinda citrifolia* L*)* had higher protein concentration and specific activity than protease from unripe noni fruits (Ismail and Razak, 2014). Protein concentration is higher for proteases extracted with 40% ammonium sulfate at both ripening stages (ripe and unripe) for bilimbi *(Averrhoa bilimbi* L.) (Ismail and Kharoe, 2013).

2.7 Extraction of protease

Proteases are the most commercially important enzymes because of their multiple applications in food and other industries. In recent decades, interest in plant proteases has been increased rapidly. The number of industrially employed enzymes of plant origin is still small but growing fast (Cotabarren *et al.*, 2020). Plants are an important source of proteases as plants require proteases throughout their life cycle. These are present in all kinds of plant tissues and, thus, can be extracted from their natural sources or can be prepared using in vitro techniques. Plant proteases can be extracted from natural sources by aqueous maceration of various plant organs. The crude extract thus obtained may be further purified to obtain a pure enzyme using various protein precipitation methods (Shah and Mir, 2019).

2.8 Protein precipitation

Protein Precipitation is the process in which protein is separated from any extra impurities that may be combined with it. It is a crucial aspect of downstream processing and can be done with a variety of different approaches. While there are a variety of precipitation procedures, the two most common are Salt Induced Precipitation ("Salting Out") using ammonium sulfate, acetone and precipitation with trichloroacetic acid (Biosciences, 2010).

2.8.1 Precipitation methods

2.8.1.1 Ammonium sulfate precipitation

One of the most frequent methods for protein purification from a solution is ammonium sulfate precipitation. Proteins create hydrogen bonds with water molecules in solution by exposing their polar and ionic groups. When large amounts of tiny, highly charged ions, such as ammonium sulfate, are introduced, these ions compete with the proteins for water molecule binding. This causes precipitation by removing the water molecules from the protein and lowering its solubility (Morgan *et al.*, 2021). Different saturation level can be used for precipitation (Burgess and Richard, 2009).

In ammonium sulfate precipitation, hundred milliliter crude extract is mixed with ammonium sulfate to a concentration of 30% to 60% (w/v) followed by 4 h incubation at 4°C to precipitate the protease. This is followed by centrifugation at 10,000×g for 10 min using a centrifuge. The precipitate is collected and then dissolved in 0.02 M Tris-HCI buffer, pH 7.5 and dialyzed at 4°C for 12 h (Wang *et al.*, 2008).

2.8.1.2 Acetone precipitation

Proteins are insoluble in acetone (particularly at low temperatures) whilst many small molecules which could interfere with downstream protein work are soluble. By precipitating proteins in this solvent it can remove buffer contaminant and concentrate protein into a pellet which can be re-dissolved by other solvents (IGEM, 2011).

Cold acetone (-20°C) is slowly added into the crude extract and the mixture was gently agitated to allow precipitation. This was followed by centrifugation using a centrifuge at 10,000×g for 15 min. The precipitate was then dissolved in phosphate buffer (50 mM), pH 7.2 and dialyzed (He *et al.*, 2008).

2.8.1.3 Acetone-TCA precipitation

TCA and acetone are often employed to precipitate proteins during 2-D electrophoresis sample preparation, and the combination is more successful than either TCA or acetone alone (Niu *et al.*, 2018).

Cold acetone (-20°C) is slowly added into the crude extract with the immediate addition of 10% TCA and the mixture was gently agitated to allow precipitation for 12hr. This was followed by centrifugation using a centrifuge at 10,000×g for 15 min. The precipitate was then dissolved in phosphate buffer (50 mM), pH 7.2. After that, it was collected and stored at 4°c until further use (Lenga, 1988).

2.8.1.4 TCA precipitation

TCA (Trichloroacetic acid) is a very effective protein precipitant. TCA is added to the extract at a final concentration of 10–20%, and the proteins are allowed to precipitate for 30 min on ice. In this method tissue can also be homogenized directly in a 10–20 % TCA solution. Proteolysis and other protein changes are limited using this method. The pellet should next be centrifuged and washed with acetone or ethanol to eliminate any remaining TCA (Link and LaBaer, 2011).

2.9 Uses of protease

Enzyme preparations from plant extracts have been used in industrial processes for a long time, even before much was known about the nature and properties of the enzymes. The great majority of commercial enzymes have been obtained mainly from microbial sources but plant enzymes are becoming increasingly important, with applications in industrial processes, biotechnology and pharmacology. Proteases like papain, bromelain and ficin are employed in different industrial processes and medicine (Uhlig, 1998). Some of these papain-like proteases are currently used in the food industry for cheese, brewing and beverage industries for the preparation of highly soluble and flavored protein hydrolysates (papain-like proteases), as a food complement to soften meats and dehydrated egg (Bailey and Light, 1989) and for the production of emulsifiers, among other uses (Pardo *et al.*, 2000).

Uses in other industries include culture medium formulation (Headon & Walsh, 1994), isolation of genetic material (Genelhu and Zanini, 1998) and the use of keratinases in the leather industry for dehairing and bating of hides to substitute toxic chemicals (Foroughi *et*

al., 2006). Also, they are used in the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems. Proteases also have an important application in the pharmaceutical industry. Plant extracts with a high content of proteolytic enzymes have been used in traditional medicine for a long time. They have been used for the treatment of cancer (Batkin *et al.*, 1988), as antitumorals (Otsuki *et al.*, 2010), for digestion disorders (Kelly, 1975), and swelling and immune-modulation problems (Otsuki *et al.*, 2010). A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation and metastasis due to its capacity of modifying cell surface structures by peptide cleavage. Salas *et al.* (2008) reviewed the pharmacological activity of plant cysteine proteases, emphasizing their role in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

In the dairy industry, some enzymes are required for the production of cheeses, yogurt and other dairy products, while others are used in a more specialized fashion to improve texture or flavor. Some functions of protease are:

1. Accelerates ripening and flavor development and increases the intensity of cheese-flavor.
2. Increases emulsifying properties.
3. Increases water absorption capacity.
4. Easily controllable protein hydrolysis at elevated temperatures and production of enzyme modified cheese (EMC) (Bathmanathan *et al.*, 2019).

Part III

Materials and methods

3.1 Materials

3.1.1 Star fruit

Starfruit of different maturity stages were procured from the local cultivator of central Nepal, Chitwan. It was stored at refrigeration temperature (<10°C) until used.

3.2 Methods

3.2.1 Extraction of starfruit juice

The starfruit of as required maturity stages were cut and the seeds were removed before being ground in a juice extractor (Model: Havel's max grind 14000). The juice was then filtered through a layer of muslin cloth and stored at <4°C.

3.2.2 Precipitation of starfruit juice

Three different partial purification methods were used to purify the crude extract comprising acetone, ammonium sulfate, and acetone with TCA precipitation.

3.2.2.1 Acetone precipitation

30 ml of cold acetone (-20 °C) was slowly added into the crude extract in the ratio of 3:1 and the mixture was gently agitated to allow precipitation for 12 h. This was followed by centrifugation using a centrifuge (Model: D3024R DLAB, UK) at 10,000×g for 15 min. Removal of supernatant and collection of precipitate and dissolve in phosphate buffer 50mM of pH 7.2 (He *et al.*, 2008). Fig. 3.1 shows flowchart for acetone precipitation.

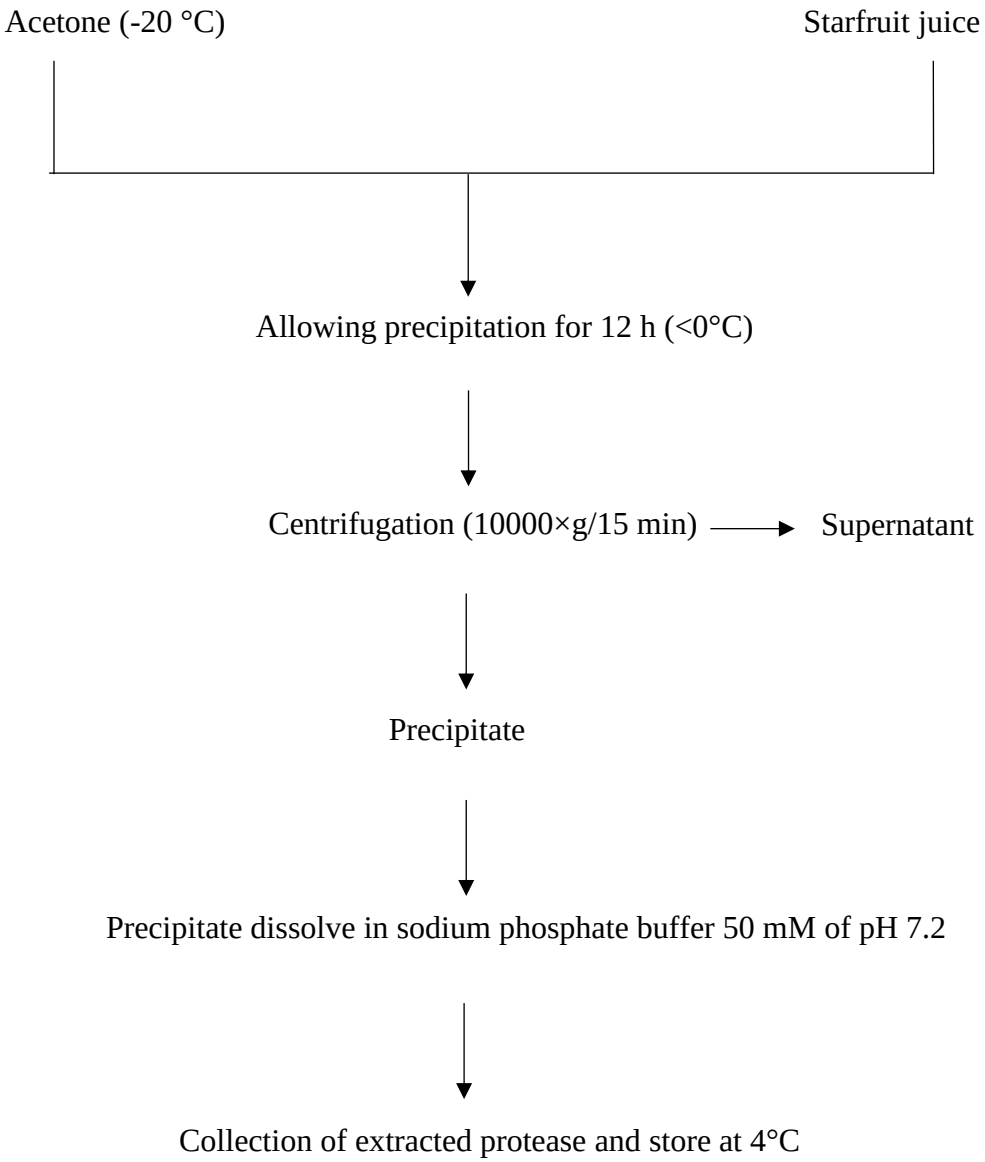


Fig. 3.1 Flow diagram for acetone precipitation.

3.2.2.2 Ammonium sulfate precipitation

Ammonium sulfate to a concentration of 40% or 50% or 60% saturation (w/v) followed by 12 h incubation at 4°C to precipitate the protease. This was followed by centrifugation at 10,000×g for 10 min using a centrifuge (Model D3024R DLAB, UK). The precipitate was then dissolved in 50 mM phosphate buffer pH 7.2. After that, it was collected and stored at 4°C until further use (Wang *et al.*, 2008). Fig. 3.2 shows flowchart for ammonium sulfate precipitation.

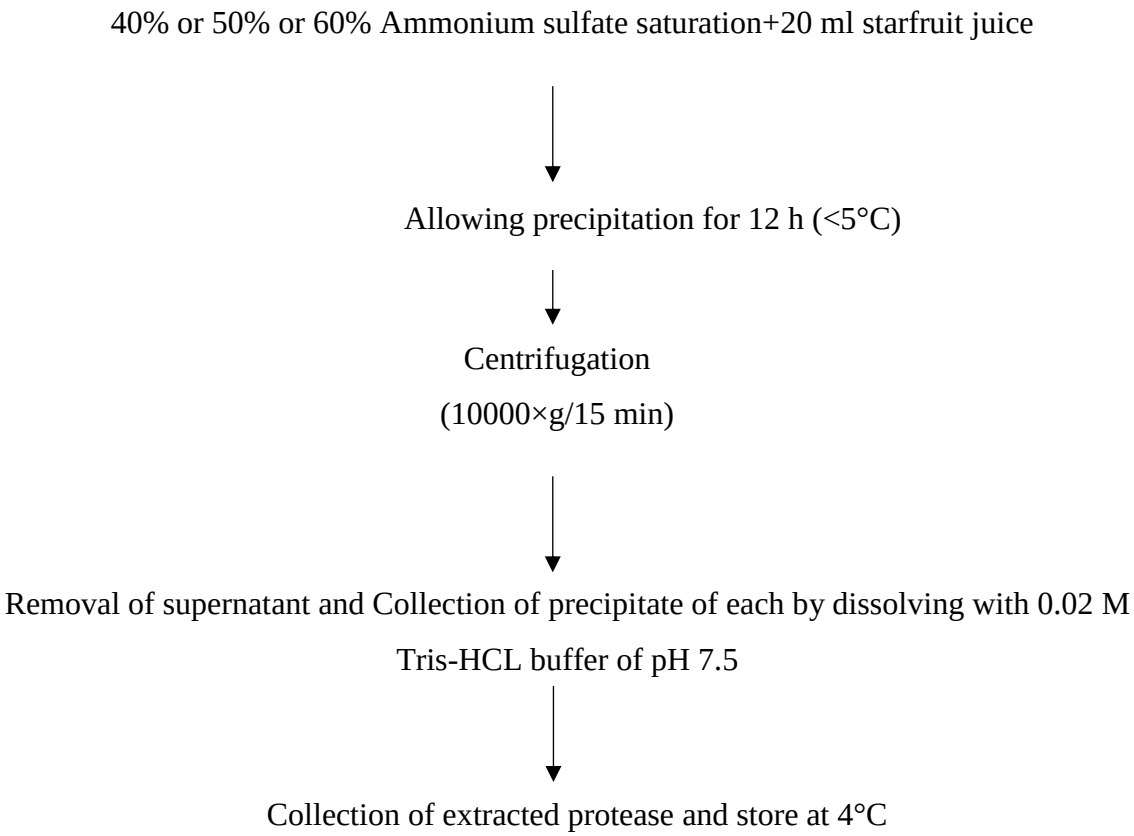


Fig. 3.2 Flow diagram for ammonium sulfate precipitation (Wang *et al.*, 2008).

3.2.2.3 Acetone with Tri-Chloro Acetic Acid (TCA) precipitation

Cold acetone (-20 °C) was slowly added into the crude extract in the ratio of 3:1 with the immediate addition of 10% TCA and the mixture was gently agitated to allow precipitation for 12 h. This was followed by centrifugation using a centrifuge (Model D3024R DLAB, UK) at 10,000×g for 15 min. The precipitate was then dissolved in 50 mM phosphate buffer pH 7.2. After that, it was collected and stored at 4°C until further use (Lenga, 1988). Fig. 3.3 shows flowchart for acetone with TCA precipitation.

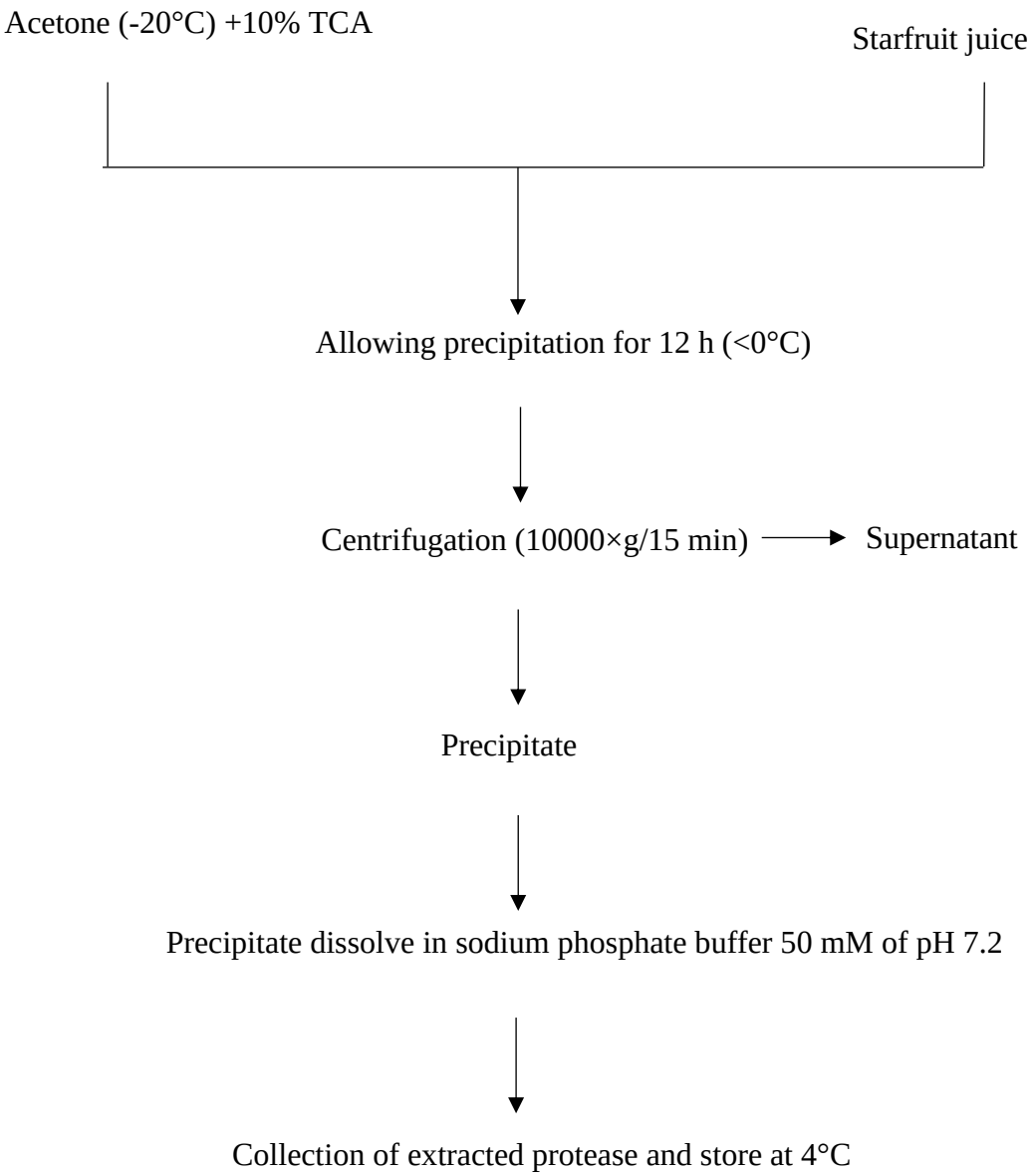


Fig. 3.3 Flow diagram for acetone with TCA precipitation (Lenga, 1988).

3.2.3 Proteolytic activity determination

Protease activity was determined using a protocol given by (Cupp-Enyard, 2008). It may be used as a standardized procedure to determine the activity of proteases. In this assay, casein acts as a substrate. When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin and Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue coloured chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the

protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve, the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

One protease unit was defined as the amount of casein hydrolysed to produce colour equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 7.5 and 37°C (colour by Folin's reagent) and was calculated by the following standard formula.

$$\text{Protease activity (units/mL)} = \frac{(\boldsymbol{mol\ Tyrosine})\times \boldsymbol{V_T}}{\boldsymbol{V_E}\times \boldsymbol{t}\times \boldsymbol{V_C}}$$

Where V_T is the total assay volume in ml, V_E is the volume of the enzyme used ml, t is the reaction time in min, and V_C is the volume used in the colorimetric reaction in ml.

3.2.4 Protein assay

Protein content of the starfruit juice was determined using the procedure given by (Bradford, 1976). It is a sensitive colorimetric protein assay based on binding of the dye Coomassie Brilliant Blue G-250 to the protein to be assayed. It is used to estimate protein concentration. For this, 1 ml of enzyme extract was mixed with 5 ml of alkaline copper solution in a test tube. Alkaline copper solution was prepared by mixing 50 ml of reagent A (2% sodium carbonate in 0.1 N NaOH) and 1 ml of reagent B (0.5% copper sulphate in 1% sodium potassium tartarate). The contents of the tubes were allowed to stand for 10 min and 0.5 ml of diluted Folin's reagent (1:1 mixture of Folin's reagent and 0.1 N NaOH) was added rapidly with immediate mixing and the tube was incubated at room temperature in dark for 30 min. The contents of the tube were read in a spectrophotometer at 595 nm and compared with a calibration or calibration curve; 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard solution (200 µg/ml of Bovine Serum Albumin (BSA)) was pipetted out in labeled test tubes. A tube with 1 ml of distilled water serves as blank. The volume in each test tube was made up to 1 ml with distilled water. 5 ml of alkaline copper solution was added to all the test tubes including the blank. The contents of the tubes were mixed by shaking the tubes and were allowed to stand for 10 min. 0.5 ml of diluted Folin's reagent was added rapidly with immediate mixing and the tubes were incubated at room temperature in dark for 30 min. The absorbance of all the tubes were measured in a spectrophotometer at 595 nm and a standard

curve was plotted by taking concentration of BSA along X-axis and absorbance at 595 nm along Y-axis.

3.2.5 Experimental design

The experimental design, data analysis and model building were performed using "Design Expert" software (Version 13, Stat-Ease Inc., USA). The optimization of starfruit enzyme was prepared with variations in: (a) pH and (b) temperature, as shown in Table 3.1. The independent variables and their levels were selected on the basis of literature and preliminary experiments. Starfruit proteases shows activity between pH range of 6-8 (Normah and Asmah, 2016) and thermal stability between 40-80 (Ismail and Zainuddin, 2015). In experiment pH were adjusted by using different buffers (sodium phosphate at pH levels of acidic region and Tris–HCl at pH levels of alkaline region) (Corzo *et al.*, 2012). A two-factor central composite design was employed. The response variable was Proteolytic Activity (PA).

Table 3.1 Different constraints for optimization of starfruit protease enzyme

Name	Goal	Range
Temperature of enzyme	In the range	40-90°C
pH of enzyme	In the range	3.5-9.5
Proteolytic activity (PA)	Maximum	

3.2.5.1 Analysis of data

The independent process variables were correlated using a second order quadratic model. Multiple regression analysis with Design Expert® software was used to determine the second order polynomial coefficient for each term of the equation. The statistical significance of the terms was investigated using analysis of variance for the response after the data was fitted to the selected model. R^2 (coefficient of determination- the amount of variation around the mean explained by the model), adjusted R^2 (a measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model), predicted R^2 (a measure of good the model predicts a model value) and Fisher's F test were used to test the adequacy of the model. Coefficient of determination R^2 is a measure

of degree of fit as it is the ratio of explained variation to the total variation. A better empirical model fits the actual data when R^2 approaches unity. The smaller the value of R^2, the less relevance the dependent variables in the model have in explaining the behavior variation. Then the effect of predictors on the response was interpreted using the models.

3.2.6 Storage stability

To determine the storage stability of protease enzyme, enzyme solution was stored at below 10°c and the activity was measured for 7 days by standard protease determination method.

3.2.7 Statistical analysis

Data was statistically processed by IBM SPSS statistics version 26. Means of the data was separated whether they are significant or not by using Tukey's HSD method at 5% level of significance.

Part IV

Results and discussion

In this research work, the effect of precipitation methods (Ammonium sulfate, acetone and acetone with TCA precipitation) on different maturity stages of starfruit (unripe, semi-ripe, and ripe) were observed. Based on the yield of proteolytic activity shown by different precipitation methods on different maturity stages, higher yielded maturity stage with its precipitation method is selected. The impact of pH and temperature on enzyme were analyzed by response surface methodology. Additionally, storage stability of extracted protease was also identified.

4.1 Selection of best precipitation methods and maturity stage

4.1.1 Selection of best saturation % level for ammonium sulfate precipitation

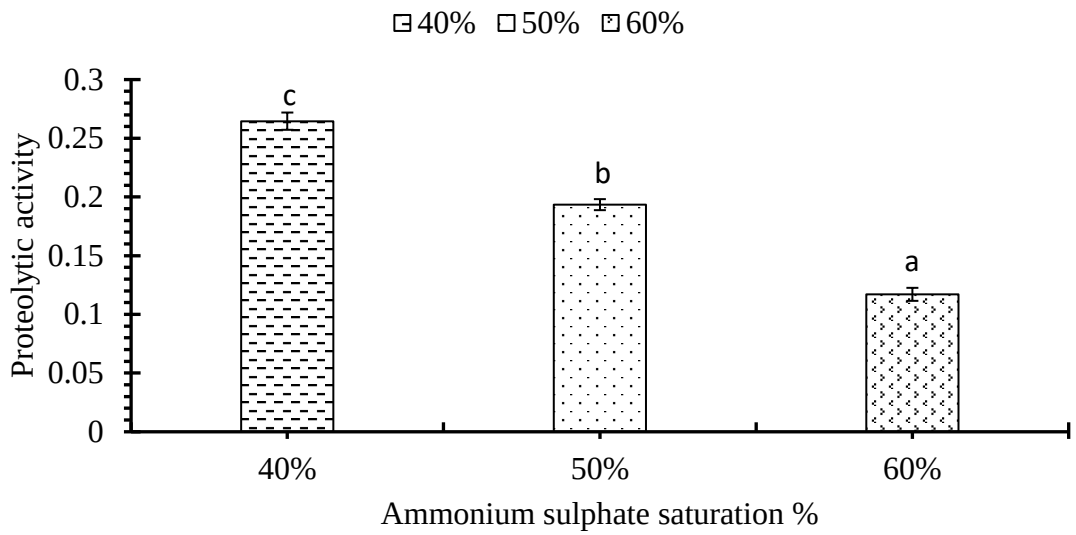


Fig. 4.1 Ammonium sulfate saturation

Proteolytic activity of starfruit protease for different saturation level (40, 50 and 60%) were found to be 0.264567 ± 0.00734^{c}, 0.193467 ± 0.00469^{b} and 0.117067 ± 0.00554^{a} respectively shown on Fig. 4.1.

Ammonium sulfate with 40% saturation was found to be more effective for precipitation than other saturation level.

Banik *et al.* (2018), Ismail *et al.* (2015), Beltagy and Adawy (2004) preferred 40% saturation level for protease extraction.

4.1.2 Precipitation methods and maturity stage (unripe)

Three different precipitation methods were used namely, Ammonium sulfate (40%), Acetone, and Acetone with TCA precipitation for protein precipitation. Proteolytic activity and protein assay were determined spectrophotommetrically. Fig. 4.2 shows the effects of precipitation method on maturity stage (unripe) proteolytic activity and protein assay

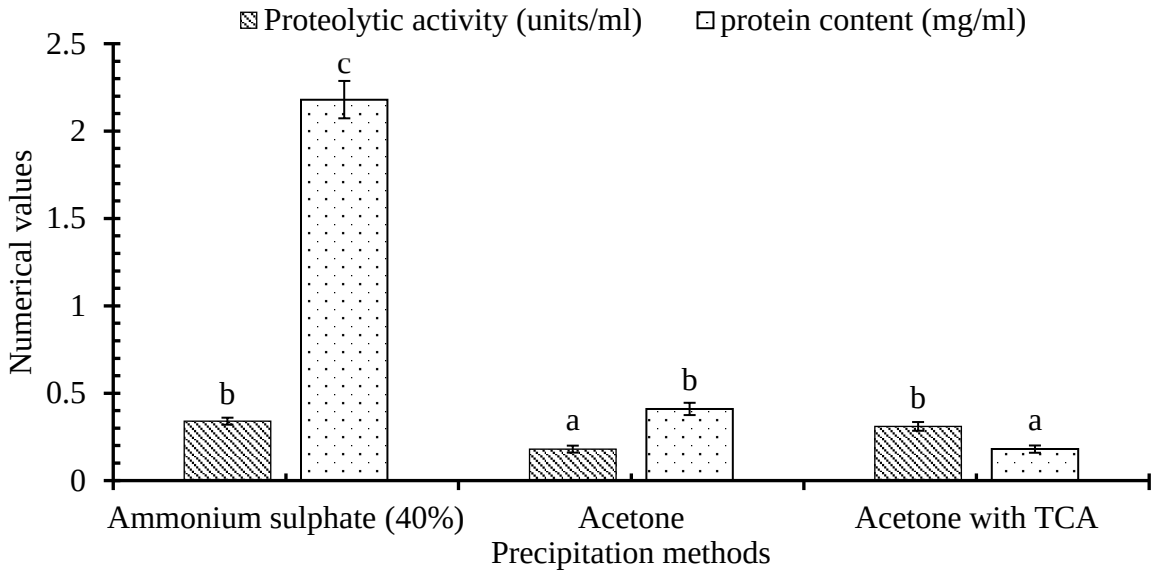


Fig. 4.2 Effects of precipitation method on maturity stage (unripe) proteolytic activity and protein

Note: Values on top of the bars bearing similar superscript were not significantly different at 5% level of significance. Vertical error bars represents ± standard deviation of PA and protein assay respectively.

The mean value of proteolytic activity was found to be significantly higher ($p<0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone precipitation. Whereas, the mean value of protein assay was found to be highest for ammonium sulfate (40% saturation), followed by acetone and TCA-acetone precipitation. (Ismail and Zainuddin, 2015) found similar result of higher yield in unripe starfruit than other maturity stages. (Ismail and Kharoe, 2013) also recommend purification using 40%

ammonium sulfate precipitation to partially purify proteases from *Averrhoa bilimbi* especially from the unripe stage.

4.1.3 Precipitation methods and maturity stage (semi-ripe)

The mean value of proteolytic activity was found to be significantly higher ($p<0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone precipitation. Whereas, the mean value of protein assay was found to be highest for ammonium sulfate (40% saturation), followed by acetone and TCA-acetone precipitation. Fig. 4.3 shows effects of precipitation method on maturity stage (semi-ripe) proteolytic activity and protein assay.

In Chaurasiya *et al.* (2013) studies shows bromelain derived from partially ripe fruits yielded more protein than bromelain extracted from completely ripe fruits. Bromelain activity reduces as ripening continues, according to their findings.

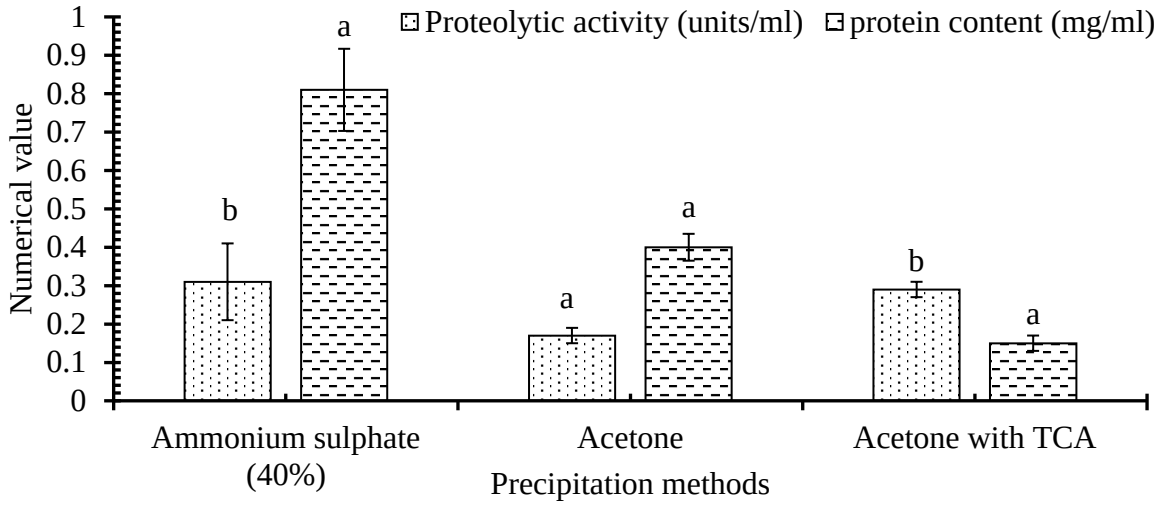


Fig. 4.3 Effects of precipitation method on maturity stage (semi-ripe) proteolytic activity and protein assay

Note: Values on top of the bars bearing similar superscript were not significantly different at 5% level of significance. Vertical error bars represents ± standard deviation of PA and protein assay respectively.

4.1.4 Precipitation methods and maturity stage (ripe)

The mean value of proteolytic activity was found to be significantly higher ($p<0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone

precipitation. Fig 4.4 shows the effects of precipitation method on maturity stage (ripe) proteolytic activity and protein assay.

In studies of (Vallés *et al.*, 2007) the crude extract was obtained from ripe fruits, and its proteolytic activity was determined.

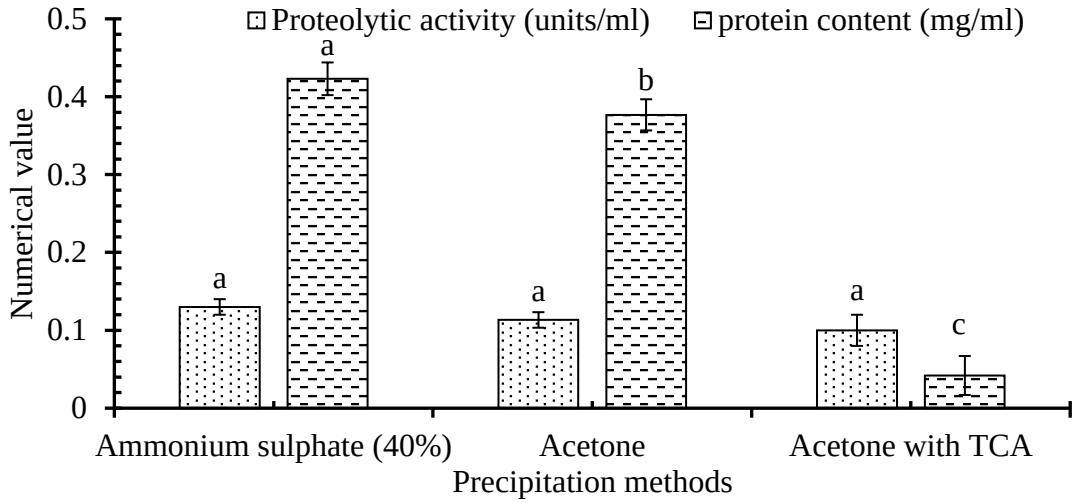


Fig. 4.4 Effects of precipitation method on maturity stage (ripe) proteolytic activity and protein assay

Note: Values on top of the bars bearing similar superscript were not significantly different at 5% level of significance. Vertical error bars represents ± standard deviation of PA and protein assay respectively.

4.2 Proteolytic activity and maturity stages

The results for effect of maturity stage on PA were calculated using equation deduced from standard curve (Appendix E). Fig.4.5 shows the effect of maturity stage on PA.

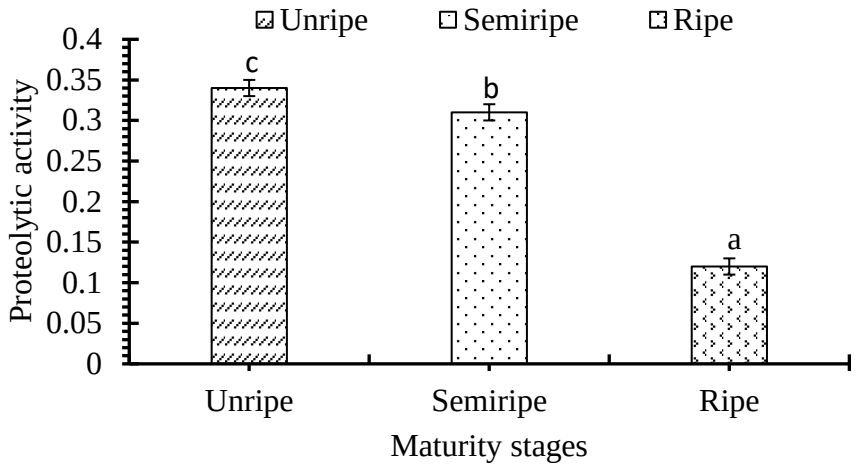


Fig. 4.5 Effect of maturity stage on ammonium sulfate 40% extracted PA

Note: Values on top of the bars bearing similar superscript were not significantly different at 5% level of significance. Vertical error bars represents ± standard deviation of PA and protein assay respectively.

Statistical analysis at 5% level of significance shows that all there stages are significantly different with each other. Highest value for unripe (0.34±0.01) and lowest for ripe (0.12±0.01). This may occur because during ripening there is decrease in amino acid profile of tropical or climacteric fruits (Brady, 1987).

4.3 Optimization of pH and temperature

On the basis of high proteolytic activity, selection of best precipitation method (ammonium sulfate (40%) and maturity stage (unripe) is identified and optimization study was done in accordance of temperature and pH.

4.3.1 Numerical optimization for protease activity (PA)

4.3.1.1 Effects of pH and temperature on PA

The parameters range were conducted with the help of a design expert, and proteolytic activity was identified (Table 4.3).Table 4.4 and 4.5 show the coefficients of model and other statistical attributes of proteolytic activity. The proteolytic activity of starfruit protease were ranged from 0.352 to 0.8632 units/ml. The coefficients of the model and other statistical attributes of PA are shown in Tables 4.2. The Model F-value of 19.71 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B^2 is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 1.22 implies the Lack of Fit is not significant relative to the pure error. There is a 41.05% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good. So, it can be used to fit model. The Predicted R^2 of 0.7027 is in reasonable agreement with the Adjusted R^2 of 0.8863; i.e. the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. So, ratio of 9.854 indicates an adequate signal. This model can be used to navigate the design space. Considering all the above criteria, the model (Equation 4.1 and 4.2) was selected for representing the variation of pH and temperature for PA and further analysis.

Eq. 4.1 Coded equation for Proteolytic activity = $0.8136+0.0161\times A-0.0358\times B-0.027\times AB-0.0655\times A^2-0.3490\times B^2$

Eq. 4.2 Actual equation for Proteolytic activity = $-2.21972+0.224557\times A+0.071506\times B-0.000054\times A\times B-0.016388\times A^2-0.000558\times B^2$

Where, A and B are pH and temperature (°C) respectively.

In the quadratic equation 4.2, for the starfruit protease, PA had significant ($P>0.05$) positive effect of pH (A) but non-significant ($P>0.05$) negative effect of temperature (B) at 95% level of confidence.

Table 4.1 Responses by variables of enzymes from *starfruit (Averrhoa carambola)*

Std	Run	Space type	Factor 1 A: pH	Factor 2 B: Temperature	Response 1 Proteolytic activity (units/ml)
1	1	Factorial	4.5	40	0.3822
2	2	Factorial	8.5	40	0.4003
6	3	Axial	8.5	65	0.822
9	4	Centre	6.5	65	0.8523
13	5	Centre	6.5	65	0.8116
5	6	Axial	4.5	65	0.763
10	7	Centre	6.5	65	0.8623
8	8	Axial	6.5	90	0.4364
4	9	Factorial	8.5	90	0.3682
12	10	Centre	6.5	65	0.7216
11	11	Centre	6.5	65	0.7313
3	12	Factorial	4.5	90	0.3527
7	13	Axil	6.5	40	0.5817

Table 4.2 Analysis of variance (ANOVA) for proteolytic activity

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	0.4730	5	0.0946	19.71	0.0005	**Significant**
A-pH	0.0016	1	0.0016	0.3234	0.5874	
B-Temperature	0.0077	1	0.0077	1.61	0.2457	
AB	0.0000	1	0.0000	0.0060	0.9406	
A^2	0.0119	1	0.0119	2.47	0.1598	
B^2	0.3364	1	0.3364	70.09	<0.0001	
Residual	0.0336	7	0.0048			
Lack of fit	0.0161	3	0.0054	1.22	0.4105	**Non-significant**
Pure error	0.0175	4	0.0044			
Cor. total	0.5066	12				

*Significant at p<0.05, DF: Degree of freedom

Table 4.3 Model summary statistics for proteolytic activity

Source	Sequential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2
Linear	0.9119	0.0071	-0.1782	-1.0651
2FI	0.9823	0.0052	-0.3089	-3.8358
Quadratic	10.0001	0.4105	0.863	**Suggested**
Cubic	0.6316	0.1812	0.8627	**Aliased**

* Case(s) with leverage of 1.0000: PRESS statistic not defined.

4.3.1.2 Solution with expected response

The selection of optimized temperature and pH was take on the basis of desirability and optimum protease activity, done by central composite response surface method, setting the constraints above (Table 4.3). Fig.4.6. shows solution with expected response.

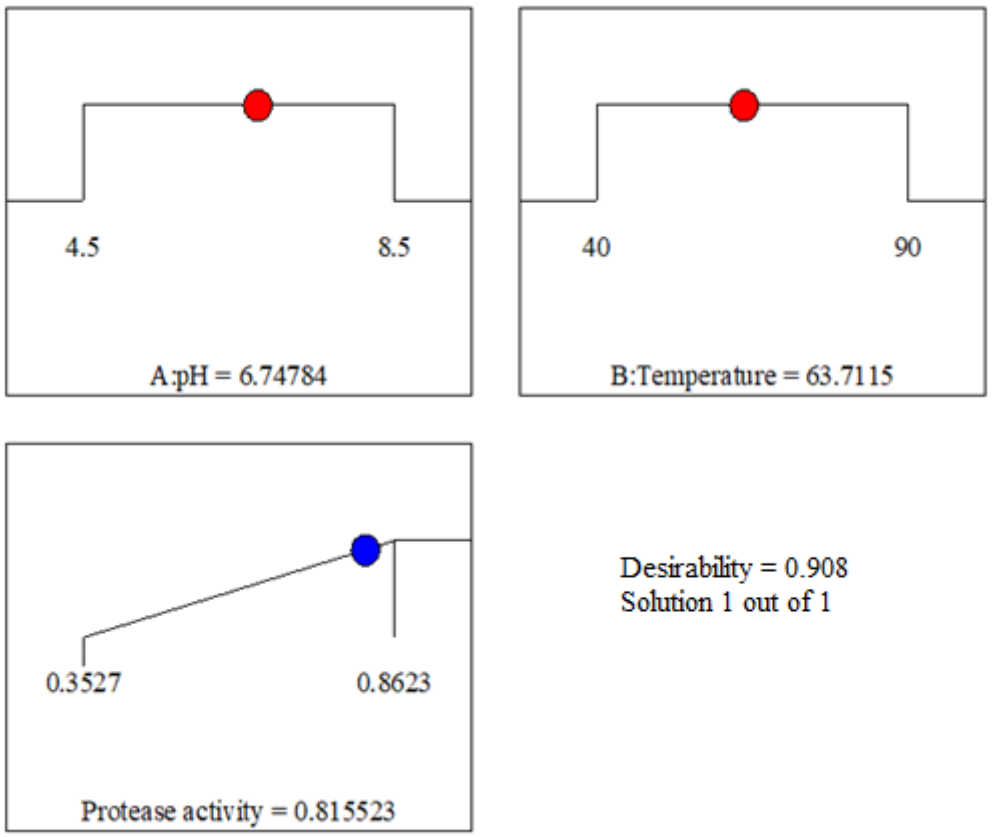


Fig. 4.6 Solution with expected response

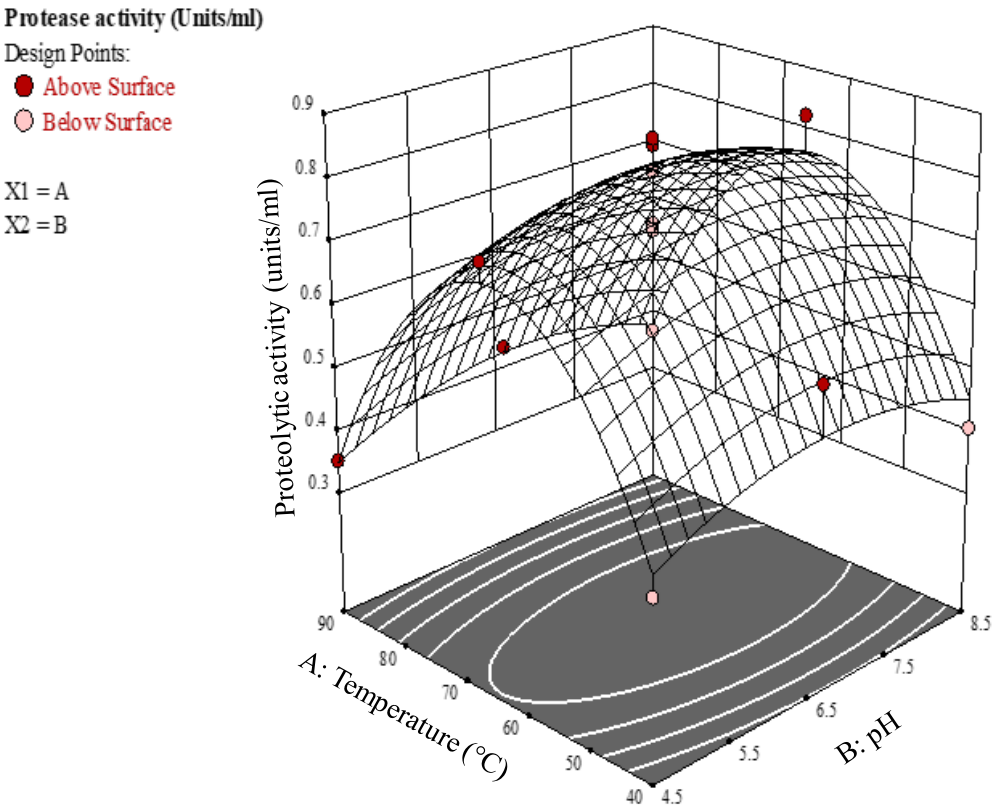


Fig. 4.7 Response surface plot of proteolytic activity of starfruit protease as a function of pH and temperature of enzyme

The combined increase of pH and temperature in enzyme shows non-linear fashion of proteolytic activity of starfruit protease as, it increased up to a maximum point and further increase led to decline in PA.

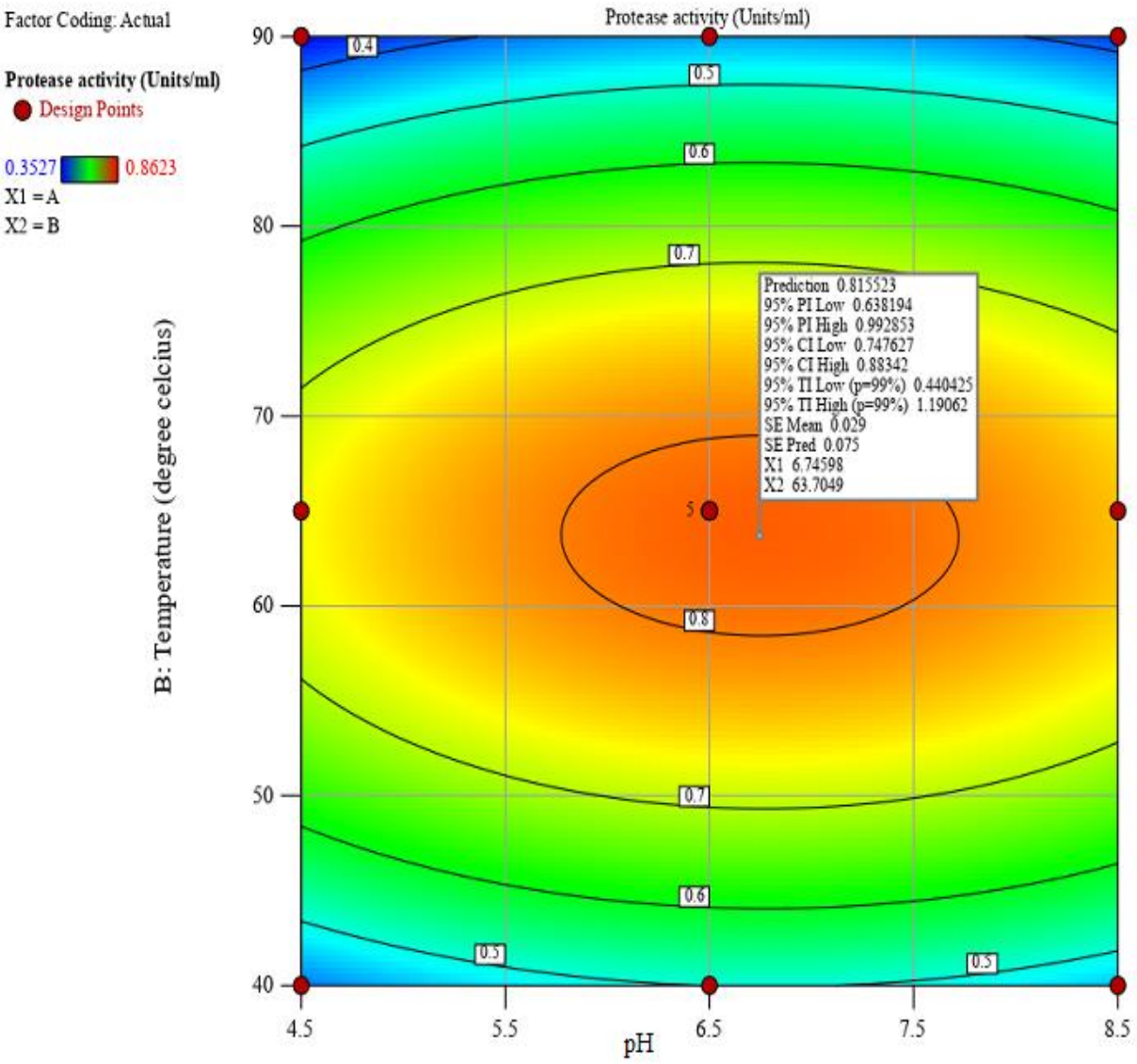


Fig. 4.8 Response surface plot (Counter plot) of proteolytic activity of starfruit protease as a function of pH and temperature of enzyme

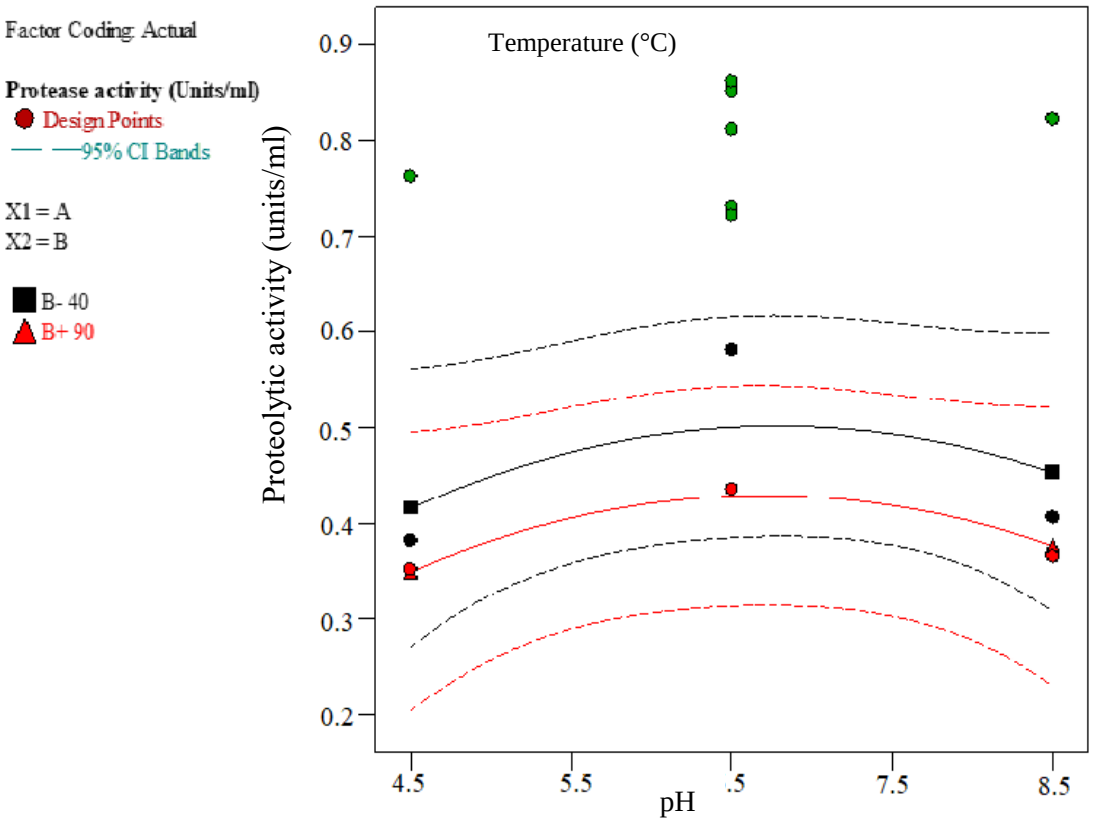


Fig. 4.9 Multiple interaction graph of PA of Starfruit protease for both factor A: pH and factor B: Temperature

4.3.2 Optimization of starfruit protease

A numerical response optimization technique was applied to determine the optimum combination of temperature and pH of starfruit protease enzyme for the Proteolytic activity. Different constraints for optimization of starfruit protease is shown in Table 4.4.

Table 4.4 Different constraints and predicted PA for optimization of starfruit protease

Name	Goal	Range
Temperature of enzyme	In the range	40-90
pH of enzyme	In the range	3.5-8.5
Proteolytic activity (PA)	Maximum	0.813

Under the assumptions by Design Expert (version 13), the optimum conditions (pH and temperature) for starfruit proteases activity were found to be 6.5 and 65ºC. The response predicted by the software for these optimum conditions reported proteolytic activity of 0.813 units/ml.

4.3.2.3 Verification of model

Within the scope of the variables studied in Central Composite Design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. Crude proteases were subjected to protease activity determination at enzyme concentration 1%, pH 6.5 and temperature 65°c.The results for protease activity were calculated using equation deduced from standard curve (Fig. C.1).The conditions and the results of the confirmatory experiments are presented in Table 4.8.

Table 4.5 Predicted and actual values of the responses at the optimized condition

Response	Condition		Predicted value	Mean observed value
	pH	Temperature		
(PA)	6.5	65 °C	0.813	0.862

The Predicted R^2 of 0.7027 is in reasonable agreement with the Adjusted R^2 of 0.8863; i.e. the difference is less than 0.2.

4.3.2.4 Model Validation

Model validation was identified on the basis of variables given by RSM. Fig. 4.10 shows the predicted PA vs Actual PA.

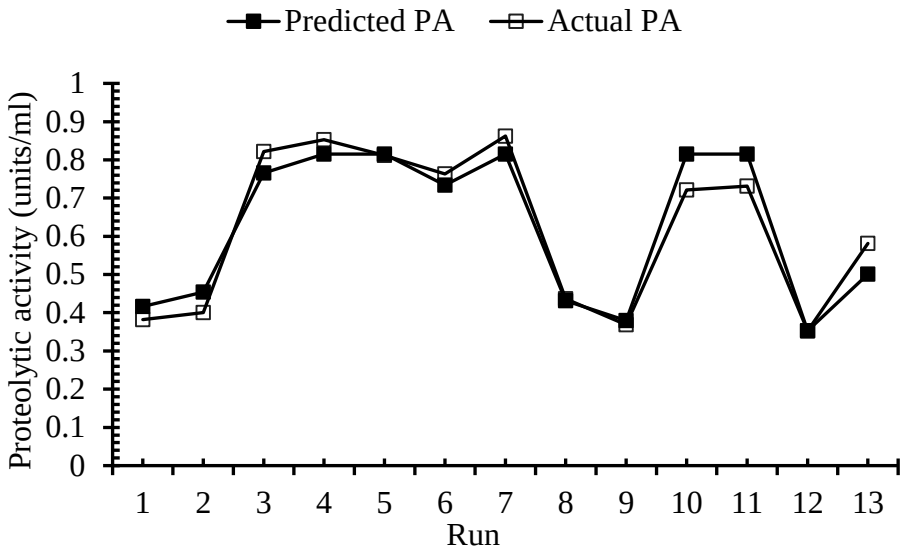


Fig. 4.10 Predicted PA vs Actual PA

From the predicted data and actual data, the varaibility (%) ranges from 0.02-13.88 % which shows (%) validity of range from 86.12-99.98 % (Appendix D).

4.4 Storage stability of starfruit protease

Starfruit proteases of Unripe maturity stage were incubated for one week at below 10°C and the proteolytic activity was determined which is shown in (Fig. 4.11).

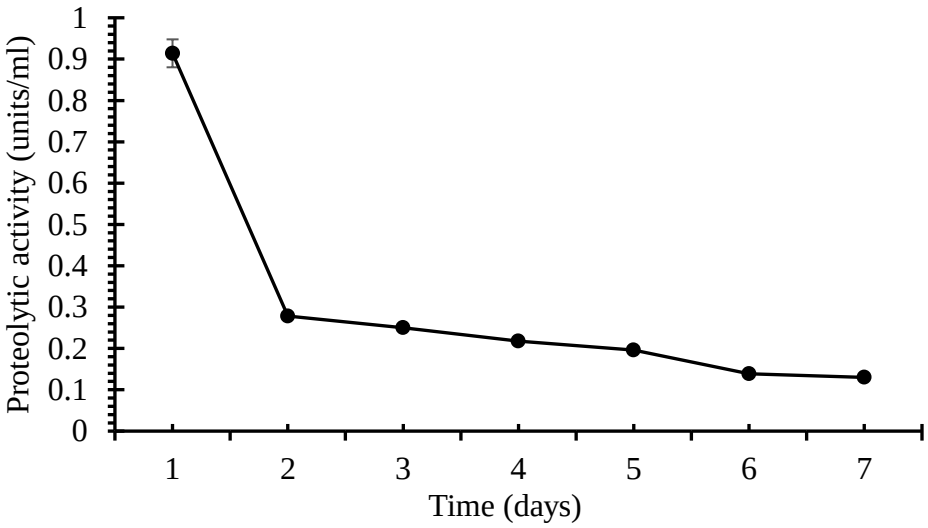


Fig. 4.11 Proteolytic activity of starfruit protease of ammonium sulfate during 7 days storage

The proteolytic activity of protease partially purified with 40% ammonium sulfate rapidly decreased during the first day of storage and slowly decreased thereafter until the storage stability is approximately 1/9 at day seven. When stored in more less temperature like <0°C its stability can be increased. Vallés *et al.* (2007) reported that when preserved for 180 days at (-20°C), the enzyme activity of the protease from ripe fruits of *Bromelia antiacantha* was 100%. If starfruit proteases were held at temperatures below 4°C, their relative activity would most likely be restored and the rate of degradation would be slower.

Part V

Conclusion and recommendations

5.1 Conclusion

On the basis of this research, the following conclusions can be drawn:

1. The effective precipitation method for starfruit protease was found to be ammonium sulfate (40%) with maximum proteolytic activity and protein content.
2. Among three maturity stages unripe stage have highest PA with protein content having values 0.862 units/ml and 2.18 mg/ml respectively.
3. Numerical optimization study revealed that the optimum condition for maximum protease activity for starfruit protease was pH 6.5 and temperature 65ºC.
4. The proteolytic activity of protease purified was significantly decreased during the 7 day of storage.

5.2 Recommendations

Based on the current study the following recommendations can be made:

1. Purification of starfruit protease can be carried out.
2. Use of stabilizer in protease can be carried out.
3. Its application can be studied.

Part VI

Summary

The main objective of this dissertation work was to perform comparative study on different precipitation methods and its effects on maturity stages of starfruit (unripe, semi ripe and ripe) proteases. Proteolytic Activity (PA) and protein assay were measured. Optimum condition of temperature and pH of protease enzyme for maximum proteolytic activity was determined by response surface methodology.

Protease from star fruit has been successfully extracted and partially purified by three purification or precipitation methods comprising 40% ammonium sulfate, acetone and acetone with TCA. Maturity stage (unripe) contains higher protein concentration compared to other stages (semi ripe and ripe). The yield for three precipitation method for proteolytic activity and protein content ranges from 0.01-0.33 units/ml and 0.15-2.17 mg/ml respectively.

Response surface methodology was used to investigate the impact of parameters such as pH and temperature for best precipitation and maturity stage on proteolytic activity. An empirical quadratic model was applied to experimental data on average enzyme activity, yielding an equation representing the best conditions. Data indicated that the extracted protease enzyme was stable at alkaline region with an optimal pH recorded at 6-8 and temperature 60-70°C. For starfruit protease the optimized temperature and pH were 65°C and 6.5 respectively. Higher protein concentration and PA was found in unripe stage. The proteolytic activity of protease purified was significantly decreased during the 7 day of storage and cold storage (<0°C) may increase its stability. This study suggested that unripe starfruit was a better source of protease than semi ripe and ripe. Therefore, starfruit may serve as another alternative source of plant protease.

Part VII

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Appendices

Appendix

Table A.1 Factors and point prediction from optimization by design expert

Factors

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	pH	6.50	4.50	8.50	0.0000	Actual
B	Temperature	65.00	40.00	90.00	0.0000	Actual

Point prediction

Two-sided Confidence = 95% Population = 99%

Analysis	Predicted Mean	Predicted Median	Std Dev	SE Mean	95% CI low for Mean	95% CI high for Mean	95% TI low for 99% Pop	95% TI high for 99% Pop
Protease activity	0.8136	0.8136	0.069278	0.0287661	0.745579	0.881621	0.438402	1.1888

Appendix B

B.1 Test of between-subjects (unripe)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	PA	.042[a]	2	.021	44.116	.000
	Protein	7.166[b]	2	3.583	808.251	.000
Intercept	PA	.683	1	.683	1430.326	.000
	Protein	7.636	1	7.636	1722.409	.000
Precipitation	PA	.042	2	.021	44.116	.000
	Protein	7.166	2	3.583	808.251	.000
Error	PA	.003	6	.000		
	Protein	.027	6	.004		
Total	PA	.728	9			
	Protein	14.829	9			
Corrected Total	PA	.045	8			
	Protein	7.193	8			

a. R Squared = .936 (Adjusted R Squared = .915)

b. R Squared = .996 (Adjusted R Squared = .995)

B.2 Test of between-subjects effects (semiripe)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	PA	.033[a]	2	.017	59.560	.000	.952
	Protein	.246[b]	2	.123	2.214	.190	.425
Intercept	PA	.573	1	.573	2061.160	.000	.997
	Protein	1.225	1	1.225	22.010	.003	.786
Precipitation	PA	.033	2	.017	59.560	.000	.952
	Protein	.246	2	.123	2.214	.190	.425
Error	PA	.002	6	.000			
	Protein	.334	6	.056			
Total	PA	.607	9				
	Protein	1.805	9				
Corrected Total	PA	.035	8				
	Protein	.580	8				

a. R Squared = .952 (Adjusted R Squared = .936)

b. R Squared = .425 (Adjusted R Squared = .233)

B.3 Test of between-subjects effects (ripe)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	PA	.002[a]	2	.001	2.771	.140	.480
	Protein	.281[b]	2	.141	62.835	.000	.954
Intercept	PA	.132	1	.132	339.457	.000	.983
	Protein	.704	1	.704	314.266	.000	.981
Precipitation	PA	.002	2	.001	2.771	.140	.480
	Protein	.281	2	.141	62.835	.000	.954
Error	PA	.002	6	.000			
	Protein	.013	6	.002			
Total	PA	.137	9				
	Protein	.999	9				
Corrected Total	PA	.004	8				
	Protein	.295	8				

a. R Squared = .480 (Adjusted R Squared = .307)

b. R Squared = 954 (Adjusted R squared = 939)

Appendix C

C.1 Post hoc test for proteolytic activity and protein (unripe)

PA

Tukey HSD[a,b]

		Subset	
Precipitation	N	1	2
Acetone	3	.1800	
Acetone with TCA	3		.3100
Ammonium sulfate (40%)	3		.3367
Sig.		1.000	.358

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .000.

a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = .05.

Protein

Tukey HSD[a,b]

		Subset		
Precipitation	N	1	2	3
Acetone with TCA	3	.1833		
Acetone	3		.4033	
Ammonium sulfate (40%)	3			2.1767
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .004.

a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = .05.

C.2 Post hoc test for proteolytic activity and protein (semiripe)

PA

Tukey HSD[a,b]

		Subset	
PRECIPITATION	N	1	2
Acetone	3	.1667	
Acetone with TCA	3		.2900
Ammonium sulfate (40%)	3		.3000
Sig.		1.000	.753

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .000.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

PROTEIN

Tukey HSD[a,b]

		Subset	
PRECIPITATION	N	1	
Acetone with TCA	3	.1500	
Acetone	3	.4067	
Ammonium sulfate (40%)	3	.5500	
Sig.			.175

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .056.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

C.3 Post hoc test for proteolytic activity and protein (ripe)

PA

Tukey HSD[a,b]

PRECIPITATION	N	Subset 1
Acetone with TCA	3	.1000
Acetone	3	.1267
Ammonium sulfate (40%)	3	.1367
Sig.		.136

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .000.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

PROTEIN

Tukey HSD[a,b]

PRECIPITATION	N	1	2	Subset 3
Acetone with TCA	3	.0423		
Acetone	3		.3300	
Ammonium sulfate (40%)	3			.4667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .002.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

C.4 Maturity stage and ammonium sulfate precipitation (40%)

PA

Tukey HSD[a,b]

		Subset		
Precipitation	N	1	2	3
Amm.sulfate 40%(ripe)	3	.1200		
Amm.sulfate 40% (semiripe)	3		.3100	
Amm.sulfate 40% (unripe)	3			.3400
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 1.00E-004.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

C.5 Ammonium sulfate saturation level (40%, 50% and 60%) and starfruit

PA

Tukey HSD[a,b]

		Subset		
Saturation	N	1	2	3
60%	3	.1171		
50%	3		.1935	
40%	3			.2646
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 5.341E-5.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

Appendix D

D.1 Model validation table

pH	Temperature	Actual PA	Actual Eq. PA	Variability (%)	(%) Validity
4.5	40	0.3822	0.41665	9.013	90.987
8.5	40	0.4003	0.454062	13.43	86.57
8.5	65	0.822	0.765486	6.87	93.13
6.5	65	0.8523	0.815033	4.37	95.63
6.5	65	0.8116	0.815033	0.42	99.58
4.5	65	0.763	0.733475	3.86	96.14
6.5	65	0.8623	0.815033	5.48	94.52
6.5	90	0.4364	0.431658	1.08	98.92
8.5	90	0.3682	0.379411	3.04	96.96
6.5	65	0.7216	0.815033	12.94	87.06
6.5	65	0.7313	0.815033	11.44	88.56
4.5	90	0.3527	0.352799	0.02	99.98
6.5	40	0.5817	0.500908	13.88	86.12

Appendix E

Calibration curve for protease activity (unripe) respectively

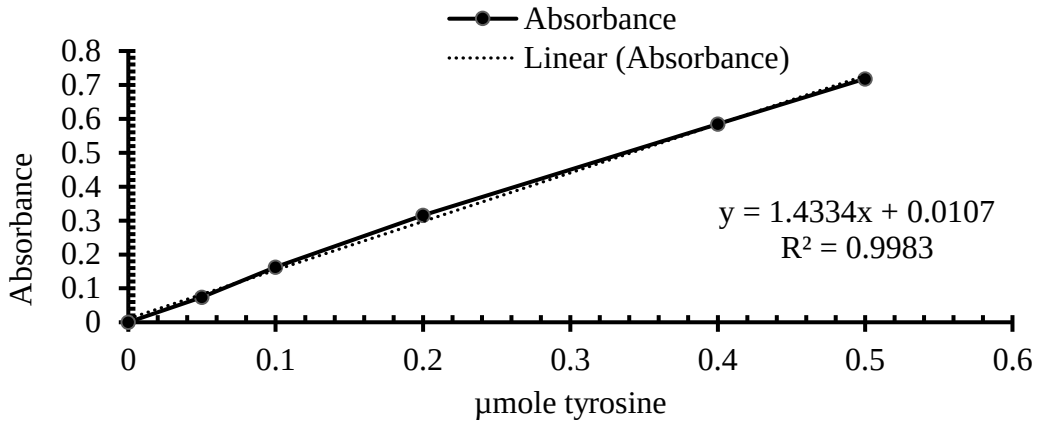

Fig.E.1 Standard curve of L-Tyrosine for protease activity (unripe)

Calibration curve for protease activity (semiripe)

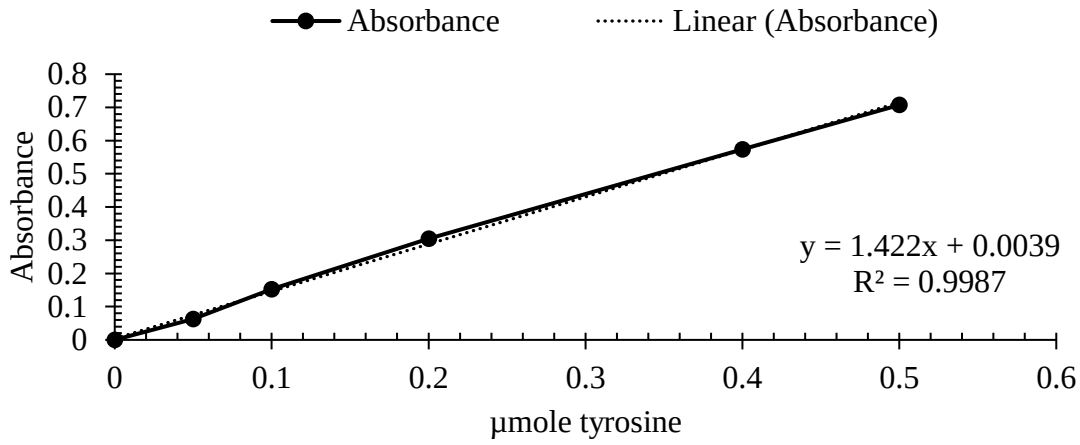

Fig.E.2 Standard curve of L-Tyrosine for protease activity (semiripe)

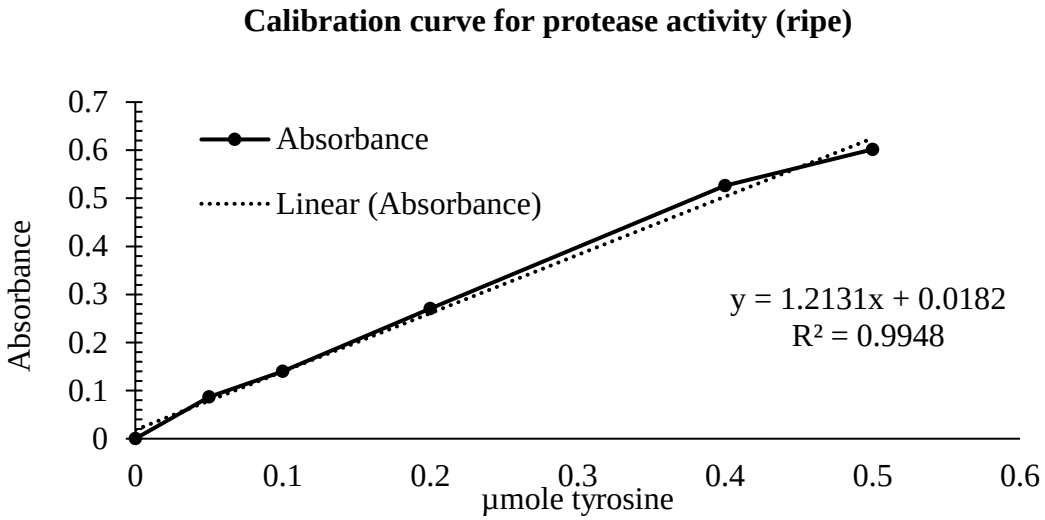

Fig.E.3 Standard curve of L-Tyrosine for protease activity (ripe)

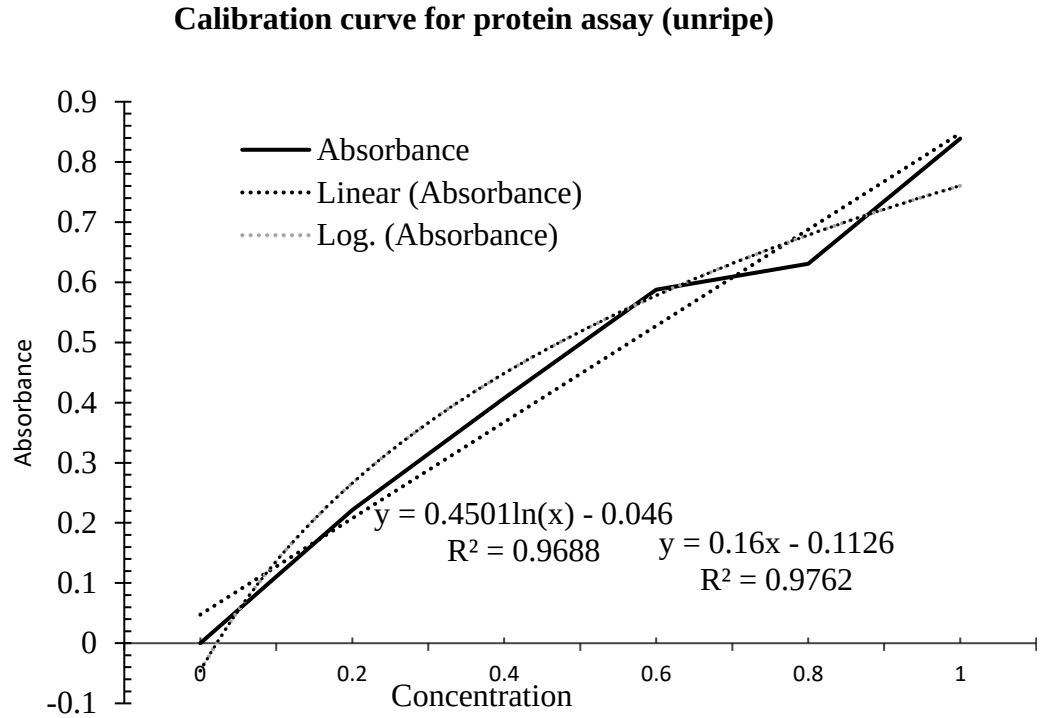

Fig. E4 Standard curve of protein concentration for protein determination (unripe)

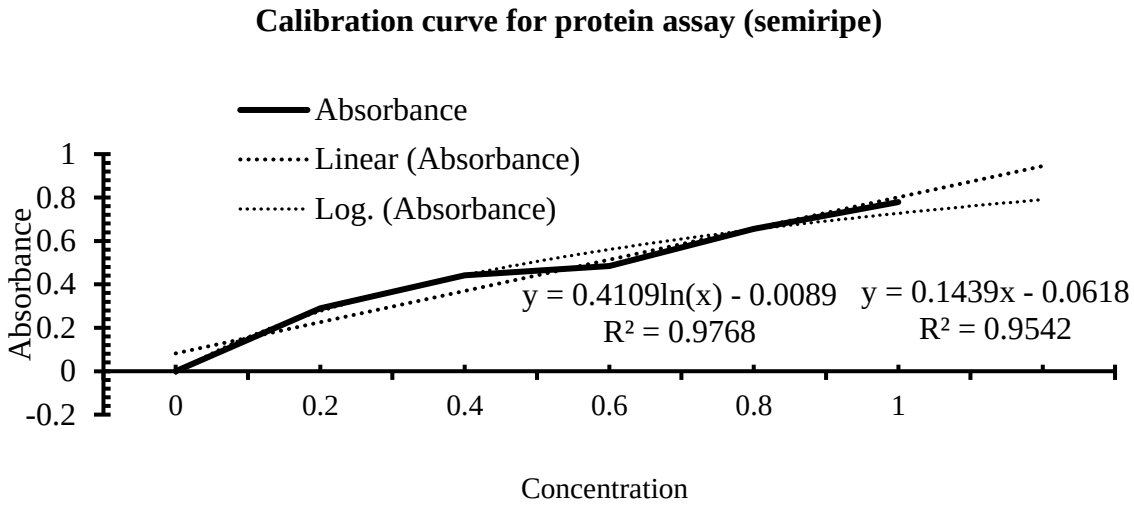

Fig. E5 Standard curve of protein concentration for protein determination (semiripe)

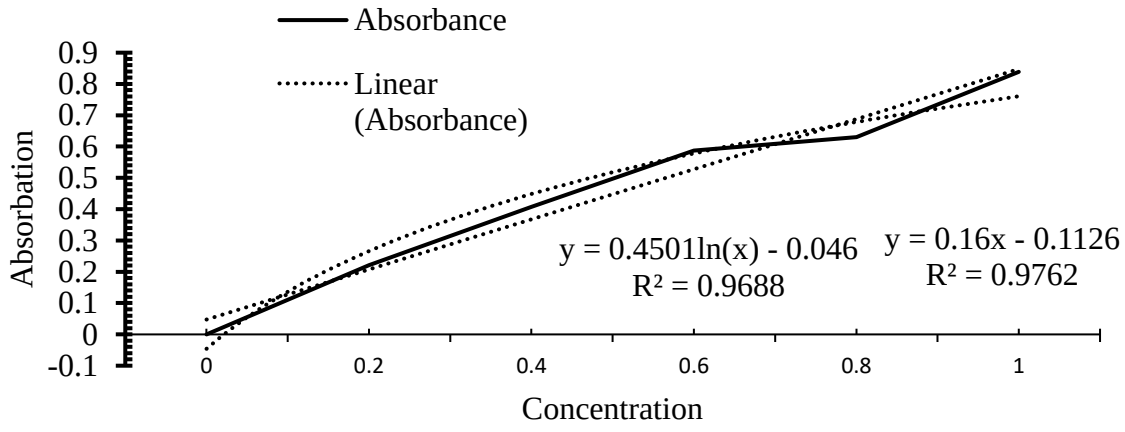

Fig. E6 Standard curve of protein concentration for protein determination (ripe)

Appendix F

F.1 Overlay plot for PA by pH and temperature

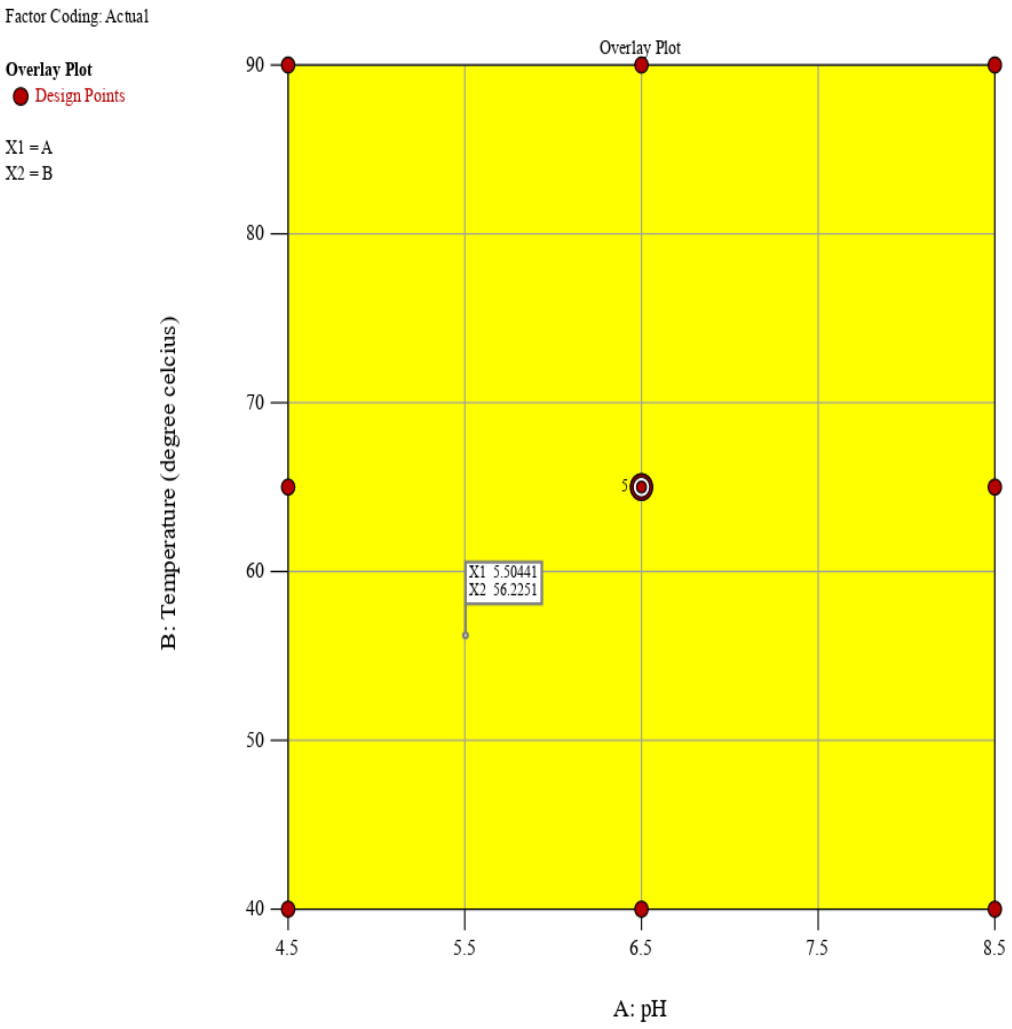

Fig.F.1 Overlay plot for PA by pH and temperature

Appendix G

G.1 (Prediction vs Actual) graph for PA

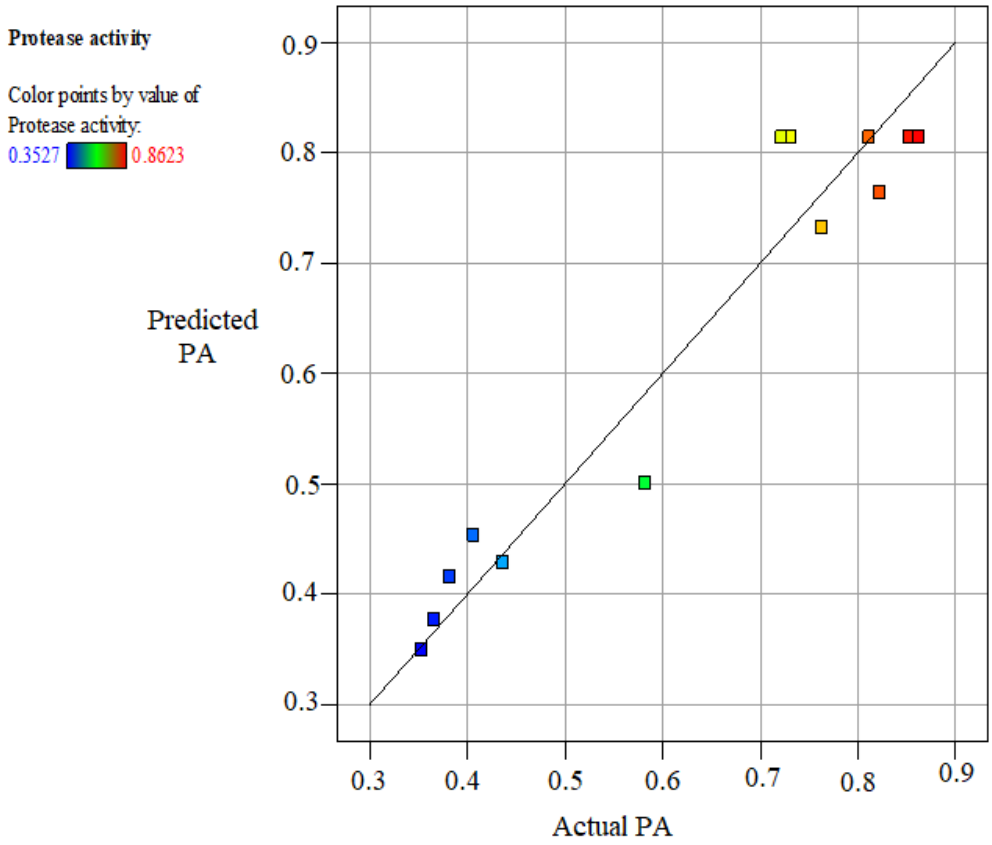

Fig F.1 (Prediction vs Actual) graph for PA

Instruments and chemical used in dissertation

Instruments name and model
Spectrophotometer (Carry 60, Version2.00)
Centrifuge (Model D3024R DIAB, UK)
pH meter (ESICO, Model1010)

Chemical names and manufacturers
Ammonium sulfate (OXFORD)
Tris-buffer (CDH)
Coos-Massine Brilliant Blue G-250 (OXFORD)
Acetone (SRL)
Ethanol (Changshu Hong sheng fine chemical ltd.)
Casein (HIMEDIA)
Tri-Chloro Aceticacid (TCA) (OXFORD)
Potassium dihydrogen phosphate (MERCK)
Tyrosine (LOBA.CH.PVT.LTD)
Bovine Serum Albumin (BSA) (HIMEDIA)
FC reagent (Fisher)

Note. All instrument and chemicals involved in dissertation are not listed

Color plates

Plate 1. Unripe

Plate 2. Semiripe

Plate 3. Ripe

Plate 4. Starfruit crosssection

Plate 5. Pellets after centrifuge

Plate 6. Performing proteolytic activity

Plate 7. Filtration through syringe filter

Plate 8. Performing centrifugation

Plate 9.Right to left (Asst. Prof. Bunty Maskey (Supervisor), Niraj Adhikari (myself), Kuber Osti (lab boy)).