"PRODUCTION OF PROTEASE ENZYME BY *Aspergillus niger* FROM AGRO-WASTES"

Dissertation submitted by

ABISHA. P (REG.NO: VM20FPT002)

In partial fulfilment of the degree of

MASTER OF VOCATIONAL STUDIES

In

FOOD PROCESSING TECHNOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

Affiliated to Mahatma Gandhi university, kottayam

Under the guidance of

Dr. Prabhakumari C

Dy. Principal Scientist

Undertaken at

DEPARTMENT OF BIOTECHNOLOGY

CEPCI- CASHEW EXPORT PROMOTION COUNCIL OF INDIA, KOLLAM

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DECLARATION

I, ABISHA. P (VM20FPT002) hereby declare that this dissertation entitled **"PRODUCTION OF PROTEASE ENZYME BY *Aspergillus niger* FROM AGRO-WASTES"** is a bonafide record of research work done by me during the course of research and that the dissertation has not previously formed the basis for the award to me for any degree, diploma, fellowship or other title of any other university or society.

Place: ERNAKULAM **ABISHA. P**

Date:

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First and foremost, we would like to thank God almighty for giving us the strength, grace, mercy and provision to undertake this project and to complete it satisfactorily. Without his blessings, this achievement would not have been possible.

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ABSTRACT

Solid state fermentation holds tremendous potentials for the production for the production of the enzyme protease by *Aspergillus niger*. Different agro wastes like pineapple peel, mango peel, orange peel, banana peel, sweet potato peel and sugarcane fibre waste are collected from local market. They are dried well and grinded to a powder mixture. It is used as the substrate. The agro wastes are cheap raw materials for protease production. *Aspergillus niger* was identified to be the best producer of protease. When *Aspergillus niger* was incubated for 5 days at 30°C, it showed high yield of protease in the substrate.

Keywords: Protease, *Aspergillus niger*, Solid state fermentation, Agro- wastes

INTRODUCTION

CHAPTER 1
INTRODUCTION

Proteases are multifunctional enzymes that catalyze the hydrolysis of proteins to polypeptides and oligopeptides to amino acids. These enzymes accounting for nearly 60% of the whole enzyme market and have been used in a wide variety of applications including the production of pharmaceuticals, detergents, fertilizers or textiles and in processes in leather, food and biotechnology industries (Ramakrishna et al., 2010; Yin et al., 2013; Abraham et al., 2014). They can be isolated from plants, animals and microorganisms. Of these sources, the microorganisms show great potential for protease production due to their broad biochemical diversity and their susceptibility to genetic manipulation. It has been estimated that microbial proteases represent approximately 40% of the total worldwide enzyme sales (Rao et al., 1998).

Several species of filamentous fungi have been exploited in industrial processes for the production of metabolites and industrial enzymes. *Aspergillus niger* has a long tradition of safe use in the production of enzymes and organic acids. Many of these products have listed as a "Generally Recognized as Safe (GRAS)" by the US Food and Drug Administration (Schuster et al., 2002). *A. niger* is one of the most important sources of fungal proteases. According Pel et al. (2007) genome sequencing shows that *A. niger* has 198 proteins involved in proteolytic degradation process.

Proteolytic enzymes can be produced by submerged and solid state fermentation. For the growth of fungi, solid state fermentation is most appropriate method because the solid substrates resemble the natural habitat of the fungi and improving their growth and the secretion of a wide range of extracellular enzymes. Some characteristics make solid state fermentation more attractive than submerged fermentation: simplicity, low cost, high yields and concentrations of the enzymes and the use of inexpensive and widely available agricultural wastes as substrates (Chutmanop et al.,2008). This process arouses most economical interest in regions such as Brazil, which has abundant biomass and agro-industrial wastes with low cost, such as the residues from the processing of soybeans, wheat, cottonseed and oranges, that reached a world production of approximately 1.1 billion tons in 2013 (FAO, 2014). The use of these wastes as substrates for the development of biotechnological processes such as the enzymes production by solid state fermentation is a promising example of obtaining biomolecules with high added value from low cost substrates.

1.1 Protease from agro-wastes

Waste is defined by (Oresanya, 1998) as any unwanted material intentionally thrown away for disposal. However, certain wastes may eventually become resources valuable to others once they are removed from the waste stream (Wiebe, 2003). Waste products arise from our ways of life and they are generated at every stage of process of production and development. Solid waste is used to describe non-liquid waste material arising from domestic, trade, commercial and public services. One of the most critical problems of developing countries is improper management of vast amount of wastes generated by various anthropogenic activities. More challenging is the unsafe disposal of these wastes into the ambient environment. Water bodies especially freshwater reservoirs are the most affected. This has often rendered these natural resources unsuitable for both primary and/or secondary usage (Fakayode, 2005).

In order to minimize the waste, one has to convert the waste to value- added products. Agro-industrial waste contains fat, crude fiber, carbohydrates and protein and can be converted to useful agricultural and industrial products by microorganisms with the supplementation of nitrogen sources (Yang, 2002). Thus providing an alternative avenue and value addition to these otherwise under or non-utilized residues. They are considered the best substrates for the enzyme production in solid state fermentation (Ellaiah et al., 2002).

1.2 Applications of protease enzyme in food industry

Food products are very complex and contain diverse matrices presenting different compositions that include diverse kinds of constituents. All components are interacting among them, resulting in the specific features of the final food. Among these constituents, peptides and proteins contained in the food medium take an outstanding position (Lacou and others 2015). Proteins play crucial roles in those aspects involved in the bio- and techno functional features and/or nutritional value of foods (Lacou and others 2015; Wouters and others 2016). The hydrolysis of these proteins affects the food matrix properties, which may produce positive effects such as modifications of sensory quality (such as texture or flavor), enhanced digestibility, decreased allergenicity, or liberation of bioactive peptides (Henzel and others 2003; Chen 2008; Lacou and others 2015). Changes of chemical processes by enzymatic hydrolysis processes is an attractive option as enzymatic treatments fully maintain the chemical species existing in the food sample. The acid protein hydrolysis onto the food will affect most constituents of the food matrix and/or will generate compounds that may be harmful for human wellbeing (Castro and Mart´ın Hernandez 1994.

Protease can be utilized to tenderize meat products and increase its flavor. It finds a wide utilization in the development of meat quality. Enzymatic hydrolysis of meat products will cause the protein to undergo internal cross-linking reactions, and produce special chemical groups to change the internal structure of the protein. The chemical properties of the protein in the meat product are changed, thereby changing its functional characteristics such as water solubility, hydration and emulsification, thereby improving the quality of the meat product.

The quality of meat depends to a large extent on its texture. As one of the important evaluation indexes of meat product quality, tenderness has become an important factor for consumers to evaluate meat product quality and palatability.

Meat is rich in fibrin, these proteins will make the structure of meat more closely linked, resulting in reduced tenderness of meat. Through the action of protease, the fiber units in the meat can be broken, so that the myofibrils are lysed and the meat becomes loose, so as to achieve the purpose of improving the tenderness of the meat.

In protein hydrolysis, the position and mode of action of different enzymes on proteins are different, so the tenderization effect and time are also greatly different. In addition, the protein itself is different, which further affects the time and effect of the enzyme.

How to turn rough meat into crunchy and tender brown-red meat? Protease is one of the first choices. Studies have shown that fig protease extracted from figs and bromelain obtained from pineapple are very effective in tenderizing meat. However, crude papain is most widely used in the meat industry for reasons of safety and cheapness. It has been reported that by using papain as an activator to tenderize beef, the color, taste and flavor of beef have been improved to a certain extent. It has also been reported that the use of bromelain combined with some inorganic salts to treat mutton produces ham intestines with tender meat, good elasticity and unique flavor.

After heat treatment, the meat product can emit its own meat flavor, and the cured meat product will produce a unique flavor of bacon during processing. Especially the fermented meat products are popular among consumers for their unique bacon flavor. However, it takes a long time to make bacon meat products, and its production process is difficult to control under natural conditions.

The use of protease can make meat products produce free amino acids and other precursor substances or intermediate products that affect the flavor of meat products, which is beneficial to accelerate the production of flavors of meat products, and has a certain effect on improving the flavor of meat products.

Meat products usually produce a large amount of by-products or leftovers. Proteases can convert waste protein into protein concentrates for human consumption or as feed.

As a highly efficient and healthy enzyme preparation, protease at Creative Enzymes has been widely used in the meat processing industry. Relevant technologies for meat tenderization, taste improvement, and flavor improvement of meat products using protease preparations have been continuously updated, and have made great contributions to the processing of meat products. With the rapid development of enzyme technology, the application potential of protease will be further developed, and it will also make greater contributions to the development of the meat processing industry and even the food processing industry. Fiona Bingly (2020)

1.3 RELEVANCE OF THE STUDY

Proteases are very beneficial enzymes having different applications in food industry. Hence it is more important to produce protease cost efficiently. In this study cheap raw materials such as agro wastes are used as substrate.

Usually agro wastes are discarded and it is completely a lost. Hence this study aims to utilize the agro waste as raw materials and minimize the wastage of these agro wastes.

1.4 OBJECTIVES

Proteases are universal enzyme product by plants, animals and micro organisms which play a dominant role in protein metabolism. Protease catalyses the hydrolysis of peptide bond in protein and give rise to amino acids.

a) Production of protease under solid state fermentation using agro wastes by *Aspergillus niger*
b) Utilization of agro-wastes for protease production
c) Effect of substrate in the production of protease and its activity

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

2.1 Proteases

2.1.1 O.P. Ward (2011) states that proteases are ubiquitous in biosystems where they have diverse roles in the biochemical, physiological, and regulatory aspects of cells and organisms. Proteases represent the largest segment of the industrial enzyme market where they are used in detergents, in food processing, in leather and fabric upgrading, as catalysts in organic synthesis, and as therapeutics. Microbial protease overproducing strains have been developed by conventional screening, mutation/selection strategies and genetic engineering, and wholly new enzymes, with altered specificity or stability have been designed through techniques such as site-directed mutagenesis and directed evolution. Complete sequencing of the genomes of key *Bacillus* and *Aspergillus* workhorse extracellular enzyme producers and other species of interest has contributed to enhanced production yields of indigenous proteases as well as to production of heterologous proteases.

2.1.2 M.R. Kanost et al.,, (2005) reported that a protease cleaves a peptide bond, called the scissile bond, between two amino acid residues named P1 and P1′. Residues on the N-terminal side of the scissile bond are numbered in the C to N direction, whereas residues on the C-terminal side of the scissile bond (the “prime” side) are numbered in the N to C direction. The substrate specificity of most endopeptidases is highly dependent on the nature of the side chain of the P1 residue, but the sequence of other residues near the scissile bond can also affect binding of the substrate to the active site and thus influence substrate specificity.

2.1.3 Kirti Rani et al.,, (2012) states that the proteases are enzymes with highly specialized proteolytic functions. They are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. They not only have several physiological functions and roles in the living beings but are also of great importance in various industries as well thus providing a lot of economic benefits.

2.1.4 Sumit kumar dubey (2017) reported that Proteases (EC 3.4) belong to hydrolases class of enzyme that performs proteolytic action to hydrolyze the peptide bonds of a polypeptide chain. Proteases are classified into four group’s viz., Serine proteases, Cysteine proteases, Aspartic

proteases and Metallo-peptidases. Proteases are produced by animals, plants, fungi, bacteria and viruses and have a potential application in food processing, pharmaceuticals and detergent industries. Activities of the poultry animals and shedding of their high protein content feathers and nails make the soil rich in protein degrading microbial flora.

2.2 Protease production under solid state fermentation

2.2.1 Paranthaman et al.,, (2009) reported that the production of enzymes by bioprocesses is a good value added to agro industry residues. A comparative study was carried out on the production of protease using different varieties of rice brokens from rice mill waste as substrates in solid state fermentation by *Aspergillus niger.* Among the all tested varieties of rice broken PONNI produced the highest activity as 67.7 U/g while ADT-66 produced lowest protease as 44.7 U/g under solid state fermentation conditions. The optimized conditions for producing maximum yield of protease were incubation at 35ºC, 96 h and pH 7.0.

2.2.2 Mais E Ahmed states that the best condition for protease production in the moisture is 1:30 and incubation at 40˚C for 8day at PH 8. Inoculation with 2.2×104 cell/ml of fungal media containing, different nitrogen sources the peptone the bast. Protease was precipitated from production media by 70% saturation of ammonium sulphate with 3.5 U/mg protein specific activities, the final purification in DEAE-cellulose 9.8 fold of purification and 0.188 yield.

2.2.3 RJS de Castro et al.,, (2075) reported that the substrate formulation using different agro-industrial wastes (wheat bran, soybean meal, cottonseed meal and orange peel) as a tool to purpose a versatile system for protease production by *Aspergillus niger* LBA 02 under solid state fermentation. The results showed interesting synergistic and antagonistic effects between the agro-industrial wastes during the protease production. The highest protease activity was found using the medium containing wheat bran (0.50) and soybean meal (0.50), reaching 262.78 U g−1 after 48 h fermentation.

2.2.4 Sonia Couri et al.,, states that a mixture containing polygalacturonase, cellulase, xylanase and protease enzymes was produced using *Aspergillus niger* 3T5B8 on different agro-industrial residues by solid-state fermentation and tested for vegetable oil extraction. The enzymic activities were evaluated using second-order empirical models from experimental data as a function of fermentation time and cellobiose concentration in the fermentation medium. The use of wheat bran as substrate without cellobiose addition and 42 h of fermentation were the

most favourable conditions for producing the mixture of hydrolytic enzymes (polygalacturonase 30.75 U/ml, xylanase 30.62 U/ml, protease 5.27 U/ml).

2.2.5 Hamid Mukhtar et al.,, (2009) reported that thirteen *A. niger* strains capable of producing proteases were isolated from the soil. Of all the isolates, Aspergillus niger IHG9 was found to be the best producer of protease. Different agricultural by-products were evaluated as fermentation substrate and maximum enzyme biosynthesis (5.2U/g) was obtained when sunflower meal was used as a substrate. Optimum temperature, pH and fermentation period for the production of protease were 30°C (7.2 U/g), 4.5(7.0 U/g) and 72 hrs (7.2 U/g), respectively. The growth and production of protease by Aspergillus niger IHG9 was also studied by varying the carbon and nitrogen sources.

2.2.6 Chee Kuan Ooi et al.,, (2021) states that the produced protease from SSF was slightly alkaline. The correlation between factors operating parameters (incubation temperature, inoculum size, moisture content) for enzyme production is analysed using statistical software, Minitab 16. A 23 full factorial experimental design was employed, and the enzyme produced was optimized by the method of desirability function. The optimal conditions for protease production of 3.7 U/mg were 35 °C of incubation temperature, 60% of initial moisture content, and 1.0 inoculum size. It is concluded that SSF protease was successfully produced from Aspergillus niger by utilizing shrimp waste as substrate.

2.2.7 Chamraj Gokul Madhumithah et al.,, reported that the wastes like potato, pumpkin, cauliflower, cabbage and brinjal procured from local market served as substrates for the solid state fermentation. Various parameters like pH, temperature and incubation time were optimized. Among the various substrates examined, it was inferred that cauliflower and cabbage yielded the maximum enzyme activity 1.082 U g of substrate and 0.886 U g of substrate after 96 hours, respectively.

2.2.8 Joel Gnanadoss et al.,, (2010) states that different types of oil cakes which were used for the isolation of protease producing organism. The isolated fungal strains were labeled as LCJ 21- 29. After incubation, the inoculated fungal strains (LCJ 21- 29) present in the basal medium showed different size and colour of fungal colonies. Generally, protease produced from organisms is constitutive or partially inducible in nature and strongly influenced by medium components such as carbon / nitrogen ratio and presence of easily metabolizable sugars, such as starch etc.

2.2.9 Javaid iqbal qazi et al.,, (2008) reported that The *A. niger* isolate and its mutant strains appeared capable of producing protease enzyme in solid state fermentation. Among 5 carbon sources, wheat bran along with soybean proved most satisfactory one for the enzyme yield.

An incubation period of 48 hours and pH 3.00 were found optimum for the protease yield

The maximum enzyme synthesis at 30° C and at pH 3.0.

2.2.10 The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling (Singh et al., 2009).

2.2.11 Major impediments to the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physicochemical characteristics and low production cost have been focus of much research (Kabli, 2007).

2.2.12 Solid state fermentation (SSF) was chosen for the present research because it has been reported to be of more grated productivity than that of submerged fermentation (Ghildyal et al., 1985; Hesseltine, 1972).

2.2.13 Economically, SSF offers many advantages including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with submerged fermentation (Paranthaman et al., 2009).

2.2.14 Sri Venu Madhav et al.,, (2019) states that solid state fermentation of *A. niger* on wheat bran produces maximum enzyme activity (1.210 O. D @ 660 nm corresponding to 179.86 Units/ml) which is a very high activity as compared to protease activity isolated from bacterial source. Efforts are being made to formulate protease from *A. niger* for the purpose of poultry feed supplement.

2.2.15 Nafisa S. A. Ahmed et al.,, (2017) reported that the optimum conditions for protease enzyme production were: 3.0% of spores' inoculum (16.80U/ml), pH 7.0 (18.19U/ml), 5.0% of NH4NO3 as a nitrogen source (13.28U/ml), 3.0% of fructose as a carbon source (11.38U/ml) during 96hrs incubation period. These results indicate the potentiality of this fungus as a source for protease production. In addition, the proteases produced from the treatment of local agro-waste could be used in a variety of industrial applications.

2.3 Applications of protease enzyme in food industry

2.3.1 Fiona Bingly (2020) states that the protease can be utilized to tenderize meat products and increase its flavor. It finds a wide utilization in the development of meat quality. Enzymatic hydrolysis of meat products will cause the protein to undergo internal cross-linking reactions, and produce special chemical groups to change the internal structure of the protein. The chemical properties of the protein in the meat product are changed, thereby changing its functional characteristics such as water solubility, hydration and emulsification, thereby improving the quality of the meat product.

2.3.2 Jermen Mamo et al.,, (2018) reported that the major application of acid proteases is for cheese production in dairy industry. The microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 and 40,000 [4]. The major role of acid proteases in cheese production is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-K-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which accounts for its exceptional performance in cheese production. The aspartic proteases produced by microbes such as Mucor miehei, B. subtilis, and Endothia parasitica, which is GRAS (genetically regarded as safe), are gradually replacing chymosin in cheese making.

2.3.3 Jermen mamo et al.,, (2018) states that the treatment of nine immunogenic epitopes of the 26-mer and 33-mer gliadin fragments by prolyl endopeptidase from Aspergillus niger (AN-PEP) successfully degraded all the nine epitopes in the stomach pH range at considerable lesser dosage than the digestive enzyme supplements. The digestive enzyme supplements showed comparable proteolytic activities with near neutral pH optima and modest gluten detoxification properties as determined by ELISA.

2.3.4 Jermen mamo et al.,, (2018) reported that the addition of protease enzyme during beer fermentation also has a significant benefit beyond haze removal. The expression of aspartyl protease in recombinant yeast used for fuel ethanol production improves the capability of the yeast to metabolize soluble proteins and leads to significant increase in ethanol production. Furthermore, the recombinant yeast strains exhibited advanced growth rate, viability, and lower

yields of by-products such as glycerol and pyruvic acid [39]. Likewise, the use of the multicomponent protease enzyme (Flavourzyme) with brewer's yeast strain Weihenstephan 34/70 for beer fermentation showed a significant increase in nitrogen availability during the course of beer fermentation.

2.3.5 Jermen mamo et al.,, (2018) states that the acid protease enzyme is suitable for the degradation of the turbidity complex produced from proteins in fruit juices and alcoholic liquors [6]. Some fungal aspartic proteases have been used to hydrolyze proteins that cause turbidity in juices and wine. These are the protease BcAP8 from Botrytis cinerea and the aspergillopepsin I from Aspergillus saitoi (commercially marketed as Molsin F by Kikkoman Corp., Japan), both used in the winery as they successfully remove haze-forming proteins and hence reducing bentonite requirements [19]. The addition of AGP (a combination of Aspergillopepsin I (EC 3.4.23.18) and Aspergillopepsin II (EC 3.4.23.19)) into two clarified grape juices (Chardonnay and Sauvignon blanc juices) with heat treatment (at 750°C, 1 min) and without heat treatments prior to fermentation exhibited about 20% total protein reduction as compared to the control wine. However, it showed the best activity when the enzymes were combined with juice heating (≈90% total protein reduction). But the more heat-stable grape proteins (i.e., those do not contribute to wine hazing) were not affected by the treatments and hence account for the remaining 10% of proteins still found in the solution after the treatments. The major physicochemical parameters and sensorial characteristics of wines produced with AGP were comparable with the control.

2.3.6 Jermen mamo et al.,, (2018) reported that the clarification of blackcurrant juice with acid protease (Enzeco and Novozyme 89L) after precentrifugation and cold storage showed a significant reduction in haze development. The addition of Enzeco protease (conc. 0.025 g/L) and gallic acid (conc. 0.050 g/L) into blackcurrant juice and allowing it to react in the juice for 90 min at 50°C showed the lowest levels of haze formation after 28 days of storage at 20°C [43]. In other studies, the treatment of cherry juice with protease (Enzeco, enzyme preparations derived from Aspergillus spp.) resulted in a considerable reduction in immediate turbidity but had low clarification impact during the subsequent cold storage [44]. The combined treatment of wines with heat (90°C for 1 minute) and enzymes (Trenolin blank, a mixed pectolytic and proteolytic

enzyme solution and porcine pepsin) also reduced 40%–80% of the protein level in wines.

2.3.7 Jermen mamo et al.,, (2018) states that the application of BcAP8 (Botrytis aspartic protease) from the grape fungal pathogen Botrytis cinerea, into Australian Semillon and Sauvignon blanc juices, noticeably degraded chitinase, a major class of haze-forming proteins without heat denaturation. Therefore, BcAP8 could potentially benefit winemakers by removing haze-forming proteins under normal winemaking conditions [12]. The extracellular acid protease secreted by Saccharomyces cerevisiae PlR1 during alcoholic fermentation was also found to be active against grape proteins (molecular mass ≈ 25 kDa) at 38°C and pH 3.5.

2.3.8 Jermen mamo et al.,, (2018) reported that the wines treated with protease enzyme have been shown to have a higher amino acid contents than the non-enzyme-treated wines except for arginine and histidine. These results indicated that protease treatment could enhance the concentration of assimilable nitrogen, which was one of the important nutrients for yeast in wine fermentation.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

In present study, production of protease using agro waste under solid state fermentation by *Aspergillus niger*, following materials and methods are followed:

3.1 Microorganisms

The selection of suitable microbial strain as a source of enzyme is depend on the fact, that the microorganisms must have high yield of enzyme in shortest possible fermentation time. The microorganism used for the present study is a fungal strain *Aspergillus niger*. The Potato Dextrose Agar (PDA) media is used as growth medium. For its culture, spore of *Aspergillus niger* was provided by the institution, Cashew Export Promotion Council of India Laboratory (CEPC) Kollam.

3.2 Growth media

Growth media meant for the cultivation of production of a strain and subsequent production of either microbial culture or a biochemical product. Potato dextrose agar is a common microbiological growth media made from potato infusion and dextrose. Potato dextrose agar (PDA) is the most widely used medium for growing fungi and bacteria which attack living plants or decaying dead plant matter. Potato Dextrose Agar is composed of dehydrated potato infusion and dextrose that encourage luxuriant fungal growth. Agar is added as the solidifying agent. Many standard procedures use a specified amount of sterile tartaric acid (10%) to lower the pH of this medium from 3.5 to 0.1, inhibiting bacterial growth. Do not reheat the acidified medium, heating in the acid state will hydrolyze the agar.

3.3 Preparation of Potato Dextrose Agar

1. To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min.
2. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
3. Mix with Dextrose, Agar and Water and boil to dissolve.
4. Autoclave 15 min at 121°C.

3.4 Inoculation procedure

1. Take 4 test tubes
2. Prepare the potatoes dextrose agar medium using standard composition
3. Sterilize the conical flask containing medium and test tubes by autoclaving
4. At laminar airflow cabin (LAF), transfer the medium to test tubes (switch on the LAF with UV for 20 minutes and swap the LAF cabin with alcohol before using LAF)
5. Allow the media in the test tube to solidify
6. At LAF, after solidification inoculate this spore of *Aspergillus niger* into the medium
7. Plugged the test tubes and wrapped with paper

3.5 Solid state fermentation

Solid state fermentation was carried out for the present study, to study the effect of various incubation periods taken as parameters required for the optimum production of protease by *Aspergillus niger*. The incubation period is taken as a parameter with an interval pf 24 hours (24, 48, 72, 96, 120), a total of five days. Requirements and procedures for solid state fermentation are as follows:

3.5.1 Mineral salt media

Mineral salt media is used as nutrient solution for *Aspergillus niger* for its growth and protease production. Mineral salt medium is prepared by using the composition shown in table no.1 with a final volume of 50 mg/50 ml. mineral salt medium used as a nutritive media for A.niger on the substrates.

Table no: 1

Composition for mineral salt solution- macro nutrients

Si no:	Name of constituents	g/ml (g in 100 ml)
1.	Ammonium nitrate	1.65
2.	Potassium nitrate	1.90
3.	Magnesium sulphate	0.37
4.	Potassium dihydro phosphate	0.17
5.	Ammonium ferrous sulphate	0.005
6.	Calcium chloride	0.004
7.	Distilled water	100

Table no: 2

Micro nutrients

Si no:	Name of constituents	g/ml (g in 50ml)
1.	Boric acid	0.3125
2.	Manganese sulphate	1.115
3.	Zinc sulphate	0.43
4.	Sodium molybdate	0.0125
5.	Copper sulphate	0.01
6.	Distilled water	50

3.5.2 Preparation of spore suspension

1. Take 4 test tubes
2. Add 2 ml of distilled water to each test tube
3. Each test tube was plugged with cotton and autoclaved for 10 minutes
4. Allow them to cool at room temperature
5. At LAF, dissolve 2 full loop (before inoculation make the loop red hot in flame) of *Aspergillus niger* colony taken from the culture into the test tube to produce spore suspension. Repeat the step for rest of the 3 test tube.
6. Switch on the LAF with UV for 20 mins and swap the LAF cabin with alcohol before using LAF.

3.5.3 Substrate

The solid substrate taken for solid fermentation of the present study is agro industrial waste. The agro industrial wastes are generated during the industrial processing of agricultural products. Those derived from agricultural activity include materials such as pineapple peel, mango peel, banana peel, sugarcane fibre waste, orange peel and coconut oil cake are collected from local market. They are good sources for protease production.

Procedure for substrate preparation

- Pineapple peel, mango peel, banana peel, sugarcane fibre waste, orange peel and coconut oil cake are collected locally and they are dried using air dryer.
- The dried substances are grinded to powder form.

- From this mixture, 10 gram of sample was collected as substrate is taken in a Erlenmeyer flask of 250 ml.
- Plug the flask with cotton and autoclaved for 10 minutes and cooled at room temperature.
- At LAF, add spore suspension (2 ml distilled water + 2 full loops of *Aspergillus niger* colony) and 10 ml of mineral salt solution to the flask containing 10 gm of substrate.
- Flask is plugged with cotton and incubate it on five different incubation periods with an interval of 24 hours, (24, 48, 72, 96 and 120) with optimum temperature 30ºC.

3.5.4 Buffer

Phosphate buffer is used in the present day with a pH 7.0. buffers are used to control the pH.

Preparation of phosphate buffer

It is made by diluting a 39.0 ml, 0.2M solution of Monobasic Sodium Phosphate (2.78g in 100ml) and 61.0 ml of 0.2 solution of Dibasic Sodium Phosphate (5.36 in 100 ml), to a total of 200 ml.

3.6 Extraction of enzyme

The fermentation flask containing flasks were taken after completing its incubation period 120 hr for enzyme extraction by following procedure:

Procedure for enzyme extraction

- Take fermentation flask after completing its incubation period
- At LAF, add 10 ml of phosphate buffer (pH 7.0) into the fermentation flask.
- Place the flask on the shaker for shaking the mixture at 200 rpm for 30 minutes.
- Filter the mixture using filter paper.
- Transfer filtered solution into a centrifuge tube and centrifuge at 5000 rpm for 10 minutes
- Supernatant was used as enzyme source for the estimation of enzyme activity and pellet was discarded.

3.7 Enzyme assay

The enzyme activity was routinely assayed by measuring the protein liberated in the reaction mixture. Enzyme activity was expressed in units U.

Requirements for enzyme assay:

- 2% casein dissolved in 100 ml of .01M phosphate buffer (pH 7.0)
- Enzyme extracts
- 0.4M sodium carbonate reagent
- Folin and ciocalteau reagent

Procedure for enzyme assay:

- Stock standard (Tyrosine- L)

  Tyrosine- L is used as stock standard (100 mg/100 ml distilled water) in enzyme assay. Optical density of stock standard was taken at 660 nm using UV spectrophotometer as following:

  a) Tyrosine -L
  b) Distilled water

Procedure:

- Supernatant is taken
- Take 2 test tubes and marked as T & C respectively.
- Add 1 ml of casein reagent to each test tubes
- Add 1 ml of enzyme extract to the tube marked T
- Incubate at 37° C for 10 minutes
- Then add 2 ml of TCA (0.4M) reagent in both tubes
- Add 1 ml of enzyme extract to the tube marked C
- Again incubated for 20 min, then filtered through What's man filter paper and filtrate is collected
- From the filtrate, collect 1 ml of sample
- Add 5 ml sodium carbonate and 1 ml of folin reagent to each test tubes
- Mix well, and incubate at room temperature for 30 mins, and allow them to cool at room temperature
- 1 ml of 2% phosphate buffer, 5 ml sodium carbonate and 1 ml of folin & colin reagent is taken as blank
- Absorbance of each tube is read at 660 nm

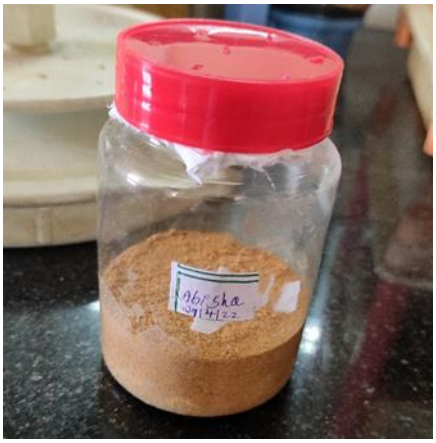
RESULT AND DISCUSSIONS

CHAPTER 4

RESULT AND DISCUSSION

4.1 In the present study, the fungal strain *Aspergillus niger* was used to produce protease using agro-wastes as solid substrates in solid state fermentation, the *Aspergillus niger* was cultured in potato dextrose agar (PDA). In solid state fermentation (SSF) with agro wastes as solid substrate, protease enzyme was produced under incubation period of five days (120 hrs) at an optimum pH of 7 and temperature 30ºC, that we provided. In the present study, Tyrosine (100mg/100ml) is used as stock solution in different concentration. Tyrosine is used for enzyme assay. Maximum optical density was recorded for the standard at 660 nm. The result obtained was, for test sample the absorbance value was 2.813 and for control sample the absorbance value was 2.791. The protease enzyme activity is calculated by using graphical method. In the graph, X axis is standard concentrations and Y axis is absorbance values. After plotting the graph, there forms a straight line. Then a dash line was drawn from the absorbance value of test sample that is 2.813 and it was drawn straight to the straight line. It was then drawn down to standard concentration. The point where the dash line touches is denoted as the protease activity of the sample.

Figure no: 1

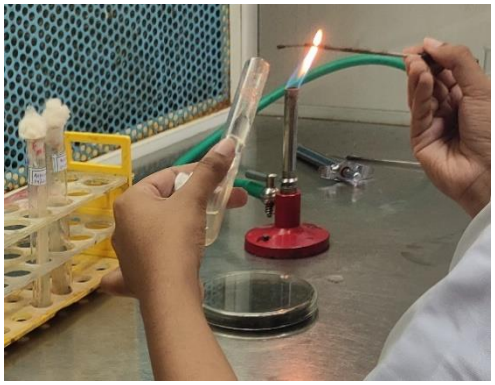


Substrate prepared by drying and grinding different agro wastes like pineapple peel, banana peel, mango peel, coconut oil cake, sweet potato peel and sugarcane fibre waste

Figure no: 2

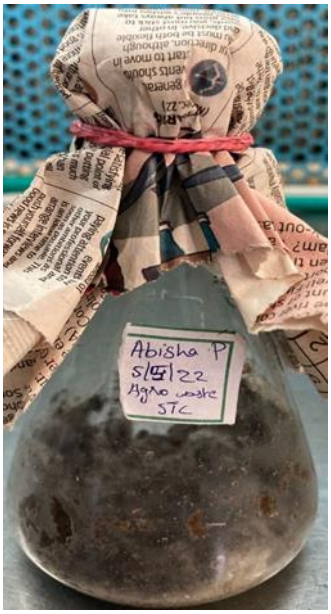
10 g of sample taken

Figure no: 3



Spore suspension

Figure no: 4



Sample after fermentation

Figure no: 5

Crude enzyme extract prepared by filtering the fermentation flask

Figure no: 6

Test and control sample

4.2 Table no: 3

Absorbance value of test and control

Test	2.813
Control	2.791

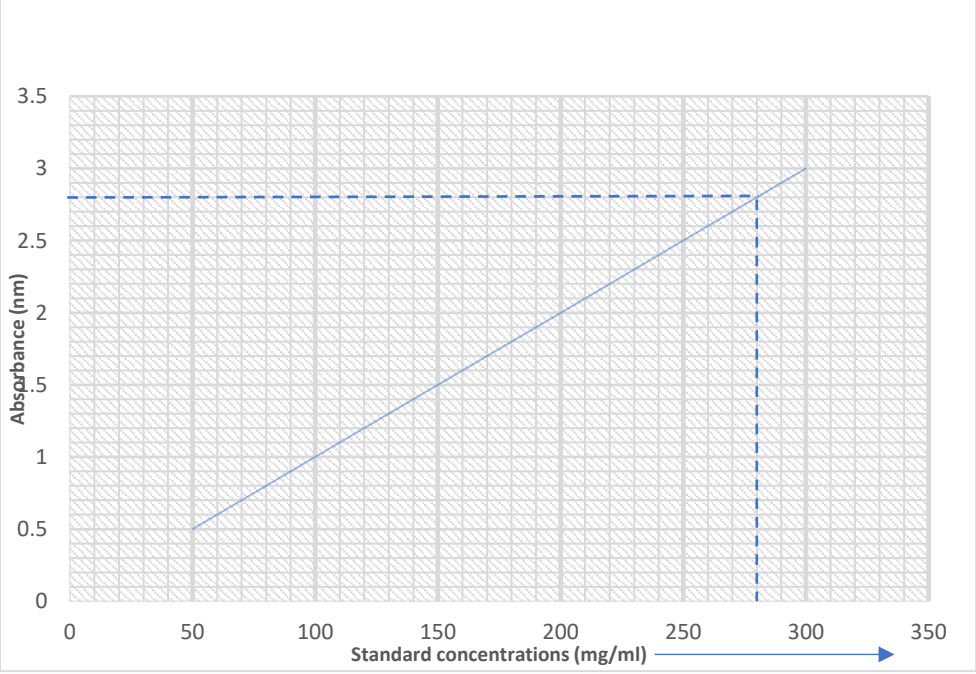
4.3Table no: 4

Standard concentrations of protease

50	100	150	200	250

4.4 Figure no. 7

Graphical representation for calculation of protease activity



The protease enzyme activity obtained is 280 µ/ml. Hence, the 10g of agro waste sample as substrate for solid state fermentation by *Aspergillus niger* gives 280 µ/ml protease activity.

CONCLUSION

CHAPTER 5

CONCLUSION

Proteases or proteolytic enzymes are found in all living organisms, e.g. bacteria, algae, plants and animals and in some of the viruses too. In this study protease was produced from a mixture of agro wastes such as pineapple peel, orange peel, mango peel, banana peel, sweet potato peel and sugarcane fibre waste. It is done by solid state fermentation by Aspergillus niger. In solid state fermentation (SSF) with agro wastes as solid substrate, protease enzyme was produced under incubation period of five days (120 hrs) at an optimum pH of 7 and temperature 30ºC, that we provided. In the present study, Tyrosine (100mg/100ml) is used as stock solution in different concentration. Tyrosine is used for enzyme assay. Maximum optical density was recorded for the standard at 660 nm. The result obtained was, for test sample the absorbance value was 2.813 and for control sample the absorbance value was 2.791. The protease enzyme activity is calculated by using graphical method. Hence, the 10g of agro waste sample as substrate for solid state fermentation by Aspergillus niger gives 280 µ/ml protease activity.

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CHAPTER 6

APPENDICES

APPENDICE I

Equipment

Sl.no.	Equipment
1.	Centrifuge ❖ A centrifuge is a laboratory device that is used for the separation of fluids, gas or liquid, based on density. ❖ Separation is achieved by spinning a vessel containing material at high speed; the centrifugal force pushes heavier materials to the outside of the vessel. ❖ There are various types of centrifuges, depending on the size and the sample capacity. ❖ A centrifuge works by using the principle of sedimentation: Under the influence of gravitational force (g-force), substances separate according to their density
2.	Spectrophotometer ❖ It is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. ❖ With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. ❖ Consists of three primary components: a light source, optics to deliver and collect the light, and a detector.

APPENDICE II

❖ Preparation of Potato Dextrose Agar

Composition	
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 L

❖ Composition for mineral salt solution- macro nutrients

Si no:	Name of constituents	g/ml (g in 100 ml)
1.	Ammonium nitrate	1.65
2.	Potassium nitrate	1.90
3.	Magnesium sulphate	0.37
4.	Potassium dihydro phosphate	0.17
5.	Ammonium ferrous sulphate	0.005
6.	Calcium chloride	0.004
7.	Distilled water	100

Micro nutrients

Si no:	Name of constituents	g/ml (g in 50ml)
1.	Boric acid	0.3125
2.	Manganese sulphate	1.115
3.	Zinc sulphate	0.43
4.	Sodium molybdate	0.0125
5.	Copper sulphate	0.01
6.	Distilled water	50