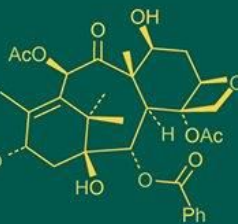
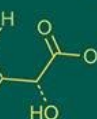


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## Studies related to the production of poultry enzymes protease from *Aspergillus niger* using different substrates

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### Abstract

Diet given to the poultry mainly consists of plant based ingredients which has certain anti nutritional factors such as phytin, non starch polysaccharides (NSP), proteinacious anti nutrients. To overcome these anti nutritional factors exogenous enzymes are added to the poultry feed. Our focus is to develop protease for poultry from fungal source. The objective of this project is to develop cheap commercial protease by solid state fermentation (SSF) using *Aspergillus niger* and an inexpensive and readily available agro-industrial substrates such as wheat bran and rice bran etc. Earlier studies using acid fungal protease proved to be effective in improving the bird weight (BW) gain and feed conversion ratio (FCR).

In the present study, we found wheat bran is most suitable for SSF using *A. Niger* and we optimized various parameters such as pH, temperature, moisture content, effect of mineral salt solution etc. for efficient protease production. During the course of study we analyzed thryrosine standard curve and casein hydrolysis is used to measure protease activity *in vitro*. In conclusion we isolated protease suitable for formulation in poultry application.

In summary there is a potential for protease supplementation in poultry diet, however interaction with other supplemented enzymes, dietary proteins and digestive architecture of the chicken required further investigation.

**Keywords:** Solid state fermentation, protease, *Aspergillus niger*, agro-industrial wastes, wheat bran, casein hydrolysis

### 1. Introduction

The competition between human and livestock population for the existing animal and plant resources has been of great concern to the poultry nutritionists. Today the poultry industry has already challenged by the high price of the feed ingredients. Moreover in the next century there will be a shortage of conventional feed stuffs for use in poultry production. Efforts are on to look forward for the new feed resources and to evaluate them for their inclusion in the poultry ration. However many of the feed stuffs are characterized by the presence of certain incriminating factors. The incorporation of feed stuffs containing incriminating factors may adversely affect the performance of poultry. The nutritional strategy involving the use of feed enzymes offer immense potential to overcome the problems

The use of enzymes in feed mixtures of growing poultry particularly for young chicks has been the subject of much research activity in the recent decades. The interest in the feed enzymes is a reflection in changing the attitude of the society and the economic climate of the feed industry.

### The objectives of this study were

1. To develop cheap commercial food grade protease by solid state fermentation (SSF) using *Aspergillus niger*.
2. To study the production of extracellular protease enzyme in solid substrate Fermentation using agro-industrial waste as the substrate for *Aspergillus niger*
3. To study the effects of substrate concentration to the extracellular protease Production
4. To evaluates the hydrolytic activities of the protease.

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5. To optimize cultivation conditions for protease production under SSF.
6. To confirm the use of the protease as a poultry diet.

### 1.1 Poultry enzymes

Feeding enzymes to poultry is one of the major nutritional advances in the last fifty years. It is the culmination of something that nutritionists realized for a long time but until 1980's it remained beyond their reach. Indeed, the theory of feed enzymes is simple. Plants contain some compounds that either the animal cannot digest or which hinder its digestive system, often because the animal cannot produce the necessary enzyme to degrade them. Nutritionists can help the animal by identifying these indigestible compounds and feeding a suitable enzyme. These enzymes come from microorganisms that are carefully selected for the task and grown under controlled conditions.

The poultry industry readily accepts enzymes as a standard dietary component, especially in wheat and barley-based rations. But still many questions are partially answered. For example, how do enzymes work? Do growth rates reflect differences in the potency of different enzyme preparations? What is the link between gut viscosity, enzyme action and growth rates? And are enzymes necessary in all poultry rations? This review aims to supply some background information about enzyme and its usage in poultry nutrition and also help to answer some of commonly asked questions regarding enzymes <sup>[1]</sup>.

### 1.2 Enzymes in Poultry Nutrition

The use of enzymes in animal feed is of great importance. Consistent increase in the price of feed ingredients has been a major constraint in most of the developing countries. As a consequence cheaper and nonconventional feed ingredients have to be used which contain higher percentage of Non-Starch Polysaccharides (soluble and insoluble/crude fibre) along with starch? Non Starch Polysaccharides (NSPs) are polymeric carbohydrates which differ in composition and structure from starch <sup>[2]</sup> and possess chemical cross linking among them therefore, are not well digested by poultry <sup>[3, 4]</sup>. A part of these NSPs is water-soluble which is notorious for forming a gel like viscous consistency in the intestinal tract <sup>[5]</sup> thus by reducing gut performance. Predominantly water soluble and viscous arabinoxylans, which belong to pentosan group, are assumed to be the factor responsible. These pentosans also greatly increase the water intake by the birds, which lead to unmanageable litter problems caused by wet and sticky droppings. This deteriorates the hygienic conditions and carcass quality <sup>[6]</sup>. On the other hand, B-glucans adversely affect all nutrients, especially protein and starch utilization and are known to give rise highly viscous conditions in the small intestine of the chicks <sup>[7]</sup>. Poultry do not produce enzymes for the hydrolysis of Non-Starch Polysaccharide present in the cell wall of the grains and they remain un-hydrolysed. This results in low feed efficiency. Research work has suggested that the negative effects of NSPs can be overcome by dietary modifications including supplementation of diets with suitable exogenous enzyme preparations (8). Enzymes break down the NSPs, decrease intestinal viscosity and eventually improve the digestibility of nutrients by improving gut performance.

### 1.3 Types of Enzyme Available for Poultry

Some of the enzymes that have been used over the past several years or have potential for use in the feed industry

include cellulase (B-glucanases), xylanases and associated enzymes, phytases, proteases, lipases, and galactosidases. Enzymes in the feed industry have mostly been used for poultry to neutralize the effects of the viscous, non-starch polysaccharides in cereals such as barley, wheat, rye, and triticale. These anti-nutritive carbohydrates are undesirable, as they reduce digestion and absorption of all nutrients in the diet, especially fat and protein. Recently, considerable interest has been shown in the use of phytase as a feed additive, as it not only increases the availability of phosphate in plants but also reduces environmental pollution. Several other enzyme products are currently being evaluated in the feed industry, including protease to enhance protein digestion, lipases to enhance lipid digestion, B-galactosidases to neutralize certain anti-nutritive factors in non-cereal feedstuffs, and amylase to assist in the digestion of starch in early-weaned animals.

### 1.4 Benefits of Enzymes

Benefits of using feed enzymes to poultry diets include; reduction in digesta viscosity, enhanced digestion and absorption of nutrients especially fat and protein, improved Apparent Metabolizable Energy (AME) value of the diet, increased feed intake, weight gain, and feed-grain ratio, reduced beak impaction and vent plugging, decreased size of gastrointestinal tract, altered population of microorganisms in gastrointestinal tract, reduced water intake, reduced water content of excreta, reduced production of ammonia from excreta, reduced output of excreta, including reduced N and P <sup>[9]</sup>.

#### 1.4.1 Reduction in Digesta Viscosity:

Enzymes added to poultry diets; especially diets containing cereals rich in NSP such as wheat, barley, and rye, reduced viscosity in the diet and digesta. Morgan *et al.*, (1995) and Muramatsu *et al.*, (1992) found that that enzyme supplementation of wheat based diets significantly reduced foregut digesta viscosity of birds. The reduction in foregut digesta viscosity was achieved primarily by reducing the molecular weight through hydrolysis of xylan backbone by endo-xylanase into smaller compounds and thus reduction in viscous effects of the feed because foregut digesta viscosity is directly proportional to the molecular weight <sup>[10]</sup> of wheat arabinoxylans. As a result of endo-xylanase and B-glucanase supplementation, the long backbones of the arabinoxylans and B-glucans are cleaved into shorter fragments, thereby reducing their viscosity <sup>[11]</sup>.

#### 1.4.2 Improvement in Nutrient Digestibility

Protease enzymes improve the digestibility and availability of proteins, optimising diet costs by allowing lower quality protein alternatives to be used in the diet. This helps save on feed costs, as grain and oilseed meal prices continue to fluctuate. Researchers have also been digging into the additional benefits of the protease enzyme and its link to gut health and bacteria balance in poultry and swine.

Protease enzymes are an important factor in protein digestion as they hydrolyse the less digestible proteins in animal feeds and break them down into more usable peptides. Improving dietary protein digestibility with a quality protease can reduce feed cost by allowing the use of lower crude protein feedstuffs with lesser quality amino acids, effectively lowering protein and digestible amino acids levels required from the feedstuffs up to

10%. Enzymes have been shown to improve performance and nutrient digestibility when added to poultry diets containing cereals, such as barley <sup>[12]</sup>, maize, oats, rye, wheat, and to those containing pulses, such as lupins <sup>[13]</sup>. The effect of enzyme supplementation on Dry Matter Digestibilities (DMD) in pigs and poultry depends on the type of diet and the type of animal: increases in DMD range from 0.9 to 17% in poultry.

#### 1.4.3 Impact on Environment

Enzymes have been approved for use in poultry feed because they are natural products of fermentation and therefore pose no threat to the animal or the consumer. Enzymes not only will enable livestock and poultry producers to economically use new feedstuffs, but will also prove to be environmentally friendly, as they reduce the pollution associated with animal production. As well as contributing to improved poultry production, feed enzymes can have a positive impact on the environment. Birds are omnivorous animals and therefore, are notable to digest non-starch carbohydrates, as those present in soluble and insoluble fibre. Thus, many vegetable ingredients usually used in poultry diets present inferior digestibility values, when administered to birds in comparison to animals with superior fermentative capacity, as swine. The improvement of birds' digestive capacity through the use of supplementary enzymes, is presented as a serious alternative to, not only improve animal performance, but also as a mean to reduce excreta amount, which decreases the contaminant potential of the production environment and therefore, becomes an alternative to be considered to attenuate the effects of growth promoters withdrawal (Vieira, 2003).

#### 1.5 Factors affecting the benefits of enzyme

The degree of improvement obtained by adding enzymes to the diet depends on many factors <sup>[14]</sup>, including the type and amount of cereal in the diet; the level of anti-nutritive factor in the cereal, which can vary within a given cereal (for example, low-versus high-B-glucan barley); the spectrum and concentration of enzymes used; the type of animal (poultry tend to be more responsive to enzyme treatment than pigs); and the age of the animal (young animals tend to respond better to enzymes than older animals); type of gut micro flora present and the physiology of the bird. Older birds, because of the enhanced fermentation capacity of the micro flora in their intestines, have a greater capacity to deal with negative viscosity effects <sup>[15]</sup>.

#### 1.6 Role of protease enzymes in poultry feed

Protease is a protein digesting enzyme that breaks down storage proteins binding starch within feed ingredients. This makes the energy from protein bound starch available to the bird to be used for productive purposes. Proteases are also effective in releasing protein anti-nutrients found in ingredients like soybean meal.

This function of proteases makes proteins more available. Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. These include: cell division, regulating protein turnover, activation of zymogene performs, blood clotting, lysis of blood clot, processing and transport of secretory proteins across membrane, nutrition, regulation of gene expression and in pathogenic factors. Proteases differ in their specific activity, substrate specificity, pH and temperature optima and

stability, active site, and catalytic mechanisms. All these features contributed in diversifying their classification and practical applications in industries involving protein hydrolysis.

## 2. Review of Literature

### 2.1 Introduction

This part included all the collected and well organized information related to the study from variety of literature being reviewed from journals and other related sources.

### 2.2 Industrial enzymes

Smythe (1950) defined enzymes as organic catalysts which are produced by living cells and they are responsible in catalyzing the chemical reactions of process life of the cell. There are many different kinds of enzymes and their existence is actually undetermined since only few known enzymes had been discovered. Some people proposed that all proteins are enzymes.

Global Industry Analyst, Inc. (GIA) forecasting the global market for industrial enzymes by 2015 is approximately to be US\$3.74 billion. This is due to new developed enzyme technologies that enhance the cost efficiencies and productivity, customers' favour in substituting petrochemical products with organic products and lastly because of the high demand from textile, detergent, cosmetic and pharmaceuticals manufacturers (Global Industry Analysts, 2011). The common enzymes are lipases, carbohydrases, proteases and many more.

One of the largest product segments in global industrial enzyme market is proteases (Rao *et al.*, 1998; Global Industry Analysts, 2011). Proteases monopolized around 60% of the world's industrial enzyme market as they are found to be used in many biotechnological processes and industrial applications (Rao *et al.*, 1998; El Enshasy *et al.*, 2008) such as in baking industry for gluten development, dairy industry as milk-clotting agents (Sumantha *et al.*, 2006) and pharmaceutical industries (Sambamurthy and Kar, 2006) and this is clearly seen in figure.

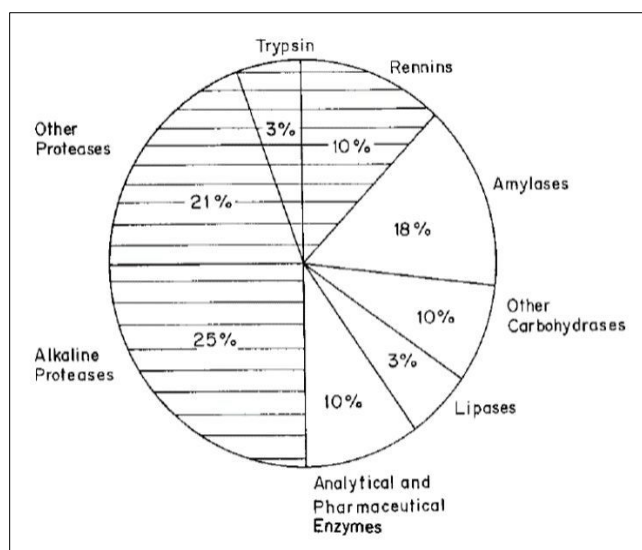


Fig 1: Distribution of enzymes sale .Source: Rao *et al.*, 1998.

The Figur describes the sale distribution of enzymes. The shaded area represents the sale of proteases enzyme and it shows that half of the sale is monopolized by protease. This figure really proved that proteases are important enzyme

used in the world. Since proteases were found to have such numerous applications in the industries, they were commercially produced nowadays.

## 2.3 Protease

Proteases, also known as proteolytic enzymes or proteinases, belong to a group of enzymes whose catalytically function to hydrolyze or breakdown the peptide bonds of proteins and they can either be limited proteolysis which break specific peptide bonds or unlimited proteolysis which break down a complete polypeptide chain to amino chain residues (Salahuddin and Khan, 2008). These enzymes can be found from various sources such as plants, animals and microorganisms (Rao *et al.*, 1998; Ikram-UI-Haq *et al.*, 2006).

### 2.3.1 Classification of Proteases

The microbial proteases differ widely not only in their functions but also in properties, based on which they can be classified into various groups. Protease can be classified according to three major criteria which is the type of reaction catalyst, chemical nature of the catalytic site and evolutionary relationship with reference to structure. Rao *et al.* (1998) studied further about microbial proteases. There are two major fungi that responsible in the production of protease which is filamentous fungi and yeast and can be further classified to acidic protease, alkaline protease, serine protease and metalloprotease (filamentous fungi)/other protease (yeast) [20, 21].

- a) **Serine Proteases:** Serine proteases are characterized by the presence of serine group at their active site. They are abundant and widespread among viruses, bacteria and eukaryotes, suggesting that they are vital to organisms. Serine proteases are inactivated by organic phosphate esters which acylate the active serine residue. Serine proteases are generally active at neutral and alkaline pH, with an optimum between 7 and 11 [22].
- b) **Cysteine/Thiol Proteases:** Cysteine proteases occur in both prokaryotes and eukaryotes. They have cysteine at their active site and have pH optima in the range of 6 to

8. They are activated by reducing agents such as hydrogen cyanide and inhibited by oxidizing agents. They are susceptible to sulfhydryl (-SH) agent such as p-CMB but are unaffected by DFP and metal-chelating agents [23]. The activation by reducing agents is due to regeneration of SH group.

- c) **Aspartic Proteases:** These proteases are widely distributed in animal, yeast and mould cells but rarely found in bacteria. They are commonly known as acidic proteases having aspartic acid residues at their active sites. They show specificity towards aromatic or bulky amino acids residues on both sides of the peptide bond and have pH optima between 3-4. The aspartic proteases are inhibited by pepstatin [24]. Microbial aspartic proteases are further divided into two groups, (i) Pepsin like proteases and (ii) rennin-like proteases produced by moulds and yeasts.
- d) **Metallo-proteases:** Metallo-proteases are the most diverse of the catalytic types of proteases [25]. They are characterized by the requirement for a divalent ion for their activity. These enzymes are sensitive to chelating agents but insensitive to sulfhydryl agents and phosphate esters have metal ion involved in the catalytic mechanism. Neutral and alkaline proteases from many microbial sources belong to this group of enzymes.

Mostly they were produced by fungi, bacteria and viruses (microbial proteases) (Rao *et al.*, 1998; Ikram-UI-Haq *et al.*, 2006) because of the inability of plants and animals proteases to satisfy the world demands (Rao *et al.*, 1998; Kumar *et al.*, 2008). Adinarayana and Ellaiah (2002) also reported that two third of industrial proteases are microbial proteases. This is due to their broad biochemical diversity and susceptibility to genetic manipulation (Sandhya *et al.*, 2005) and also because of the characteristics of microbial proteases that can satisfy the need in biotechnological application (Kumar *et al.*, 2008).

Below table shows the potential producers of proteases according to its types (Rao *et al.* 1998).

**Table 1: Fungal protease producers**

| Fungi             | Type of proteases  | Fungi species                           |
|-------------------|--------------------|-----------------------------------------|
| Filamentous fungi | Acid proteases     | a) Mucor                                |
|                   |                    | b) Rhizopus                             |
|                   |                    | c) Aspergillus                          |
|                   | Alkaline proteases | a) Aspergillus                          |
|                   |                    | b) Acremonium                           |
|                   |                    | c) Fusarium                             |
|                   | Serine proteases   | a) Tritirachium                         |
|                   | Metalloproteases   | a) Aspergillus                          |
| Yeast             | Acid proteases     | a) Saccharomycopsis                     |
|                   |                    | b) Saccharomyces                        |
|                   |                    | c) Candida albicans, Candida tropicalis |
|                   |                    | d) Yarrowia lipolytica                  |
|                   | Alkaline proteases | a) Yarrowia lipolytica                  |
|                   | Serine proteases   | a) Kluyveromyces                        |
|                   |                    | b) Saccharomyces cerevisiae             |
|                   | Other proteases    | a) Saccharomyces cerevisiae             |

Almost all industries have long used bacterial proteases but the preparation for obtaining enzymes that free from microbes is quite costly (Andrade *et al.*, 2002). Due to this issue, fungal proteases have increase in their industrial demand since they offer several advantages compared to

bacterial proteases. Sandhya *et al.* (2005) and Murthy and Naidu (2010) stated that fungi were safe in producing enzymes because they were known as GRAS (Generally Recognized As Safe) strains and referring to Food and Drug Administration (2001).

Proteases from *Aspergillus niger* and *Aspergillus oryzae* origins were declared to be GRAS, meaning that they are safe to be consumed by human. In addition, fungal protease was produced extracellularly that make ease to be recovered from the fermentation broth (Sandhya *et al.*, 2005; Murthy and Naidu, 2010). Moreover, the generated mycelium from the fermentation system can be easily tossed by simple filtration (Andrade *et al.*, 2002; Murthy and Naidu, 2010) and fungi can also easily grown on inexpensive substrates (Murthy and Naidu, 2010; Benazir *et al.*, 2011). Besides, these microorganisms are also capable in consuming organic materials in wastes for their both carbon and energy sources in order to grow (Mahmood *et al.*, 1998). Proteases can be produced either intracellularly or

extracellularly. Mostly microbial proteases were produced extracellularly which means the proteases abundantly found within the production medium. Andrade *et al.* (2002) said that extracellular enzymes capable to digest insoluble nutrient materials such as cellulose, protein and starch, and the nutrients from digested products are then transported into the cell to be used as for growth.

#### 2.4 *Aspergillus niger*

*Aspergillus niger* is one of the known fungi that have the potential in producing extracellular protease enzymes due to its cosmopolitan and ubiquitous nature (Benazir *et al.*, 2011). The scientific classification of *Aspergillus niger* is described by below

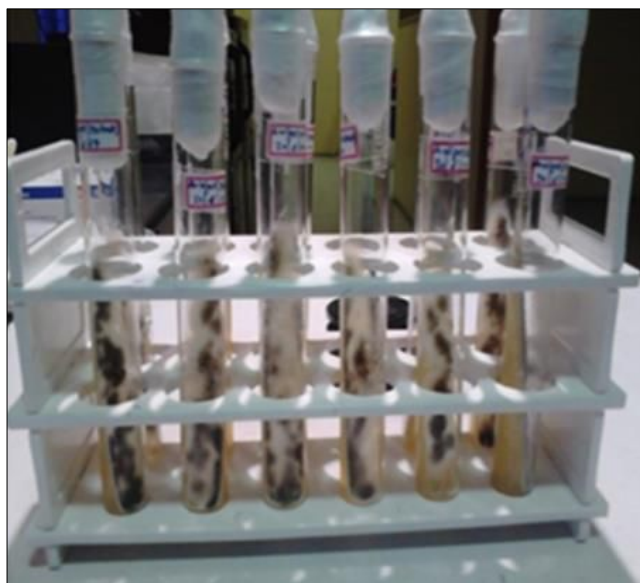


**Fig 2:** Culture slants of fungus A.niger

#### Taxonomy of *Aspergillus niger*

Kingdom: Fungi  
Phylum: Ascomycota  
Class: Eurotiomycetes  
Order: Eurotiales  
Family: Trichocomaceae  
Genus: *Aspergillus*  
Species: *Aspergillus niger*

*Aspergillus niger* is a filamentous fungus that gives an important role in biotechnology. *Aspergillus niger* does have spores and reproduces asexually meaning that it can produce its offspring individually. Purwanto *et al.* (2009) discovered

the morphology of *Aspergillus niger* using Scanning Electron Microscope (SEM) while Hoffmann (2010) identified the SEM of the asexual reproduction of *Aspergillus niger*.

According to Ikram grow under varying environmental conditions such as fermentation time, pH, temperature and utilizing various sources of substrate as nutrients. Several fungi have been reported to produce proteases such as *Aspergillus niger* (Paranthaman *et al.*, 2009), *Aspergillus oryzae* (Murthy and Naidu, 2010) *Aspergillus fischeri* (Saravanakumar *et al.*-Haq *et al.*, 2006) and *Mucor circinelloides* (Andrade12, *Aspergillus oryzae al.*, 2010), *Penicillium et al.*, 2002).

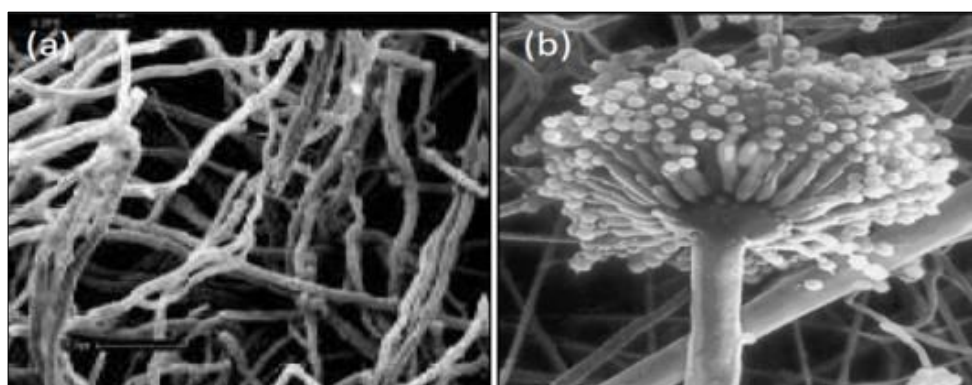


**Fig 3:** (a) Filamentous structure of *Aspergillus niger* (b) Asexual reproduction of *Aspergillus niger*

## 2.5 Production of proteases

Microbial proteases have been commercially produced by fermentation process either in solid-state fermentation (SSF) or submerged fermentation (SmF). In large scale fermentation, SSF is not often used due to severe engineering problems and it was suggested to apply the submerged fermentation process as it possesses few advantages (Gregori *et al.*, 2007). Submerged fermentation was said to provide more uniform and reproducible biomass and also requires uncomplicated downstream processing. Some researcher also discovered other method that was capable in producing high yield of protease enzymes. Samarntarn *et al.* (1999) used genetically engineered *Aspergillus oryzae* U1521 that enabled to produce five times more proteases yield than its parental strain since it contains multiple copies of protease gene while Dunne *et al.* (1997) had successfully forced *Stenotrophomonas maltophilia* W18 which was isolated from the rhizosphere of sugar beet to secrete extracellular protease activity for the purpose of biological control of *Pythium ultimum*. Unfortunately, the application of genetic engineered technique was still lacking among the researchers and most of them focused more in using the common fermentation technique either in submerged or solid state forms. With regard to proteases synthesis in the microorganism, Saravanakumar *et al.* (2010) discovered that medium composition and some physical factors such as the pH, fermentation period, temperature etc have influenced the extracellular protease production. El Enshasy *et al.* (2008) decided that the improvement of yields of proteases and the ptimization of the fermentation medium and production conditions need to be considered as to develop economically feasible technology. That was why many parameters were studied in former researches for examples, the pH, temperature, incubation time, different carbon and nitrogen sources, substrate concentrations and inoculums sizes. Better fermentation conditions will enhance higher production of proteases.

All commercially important alkaline proteases are produced by microorganisms. The enzymatic yield obtained from fermentation, cost of their production, and downstream processing cost determines the final cost of the enzyme. To develop a viable industrial process, lowering production cost, and increasing enzyme productivity are very important. Selection of best fermentation technique and optimization of culture conditions contribute much in achieving enzyme productivity (Barrios-Gonzalez *et al.*, 2005) [26]. Currently, enzyme production by microorganisms can be achieved using submerged fermentation or solid state fermentation. Submerged fermentation (SmF) submerged fermentation is defined as the cultivation of microorganisms in liquid nutrient broth. The growth of the microorganism at a large scale involve use of closed large vessel (up to 100 cubic meters in volume) containing a rich nutrient broth and enough amount of oxygen (Enshasy *et al.*, 2008) [27]. Fermentation parameters such as medium composition, pH, and aeration are important variables that affect the production of enzyme in SmF (Maurer, 2004) [28].

SmF has its own advantages and drawbacks. Serious pollution problems, low product concentration and high production cost are the major draw backs associated with the use of SmF. However, presence of high water content that is favourable for most bacterial growth, homogeneity of the fermentation system, ease in process parameter

measurement and presence of well developed industrial equipment are some of the advantages of submerged fermentation (Holker & Lenz, 2005) [29]. At present, more than 90% of the commercial microbial enzymes, including alkaline proteases, are produced using submerged fermentation (Underkofler *et al.*, 1958; Aguilar *et al.*, 2008) [30].

**Solid state Fermentation** Solid state fermentation is defined as the growth of microorganisms on moist solid substrates in the absence of free flowing water. The microorganism obtains water, carbon, nitrogen, minerals, and other nutrients from the solid substrate. The substrate also provides anchorage for the micro organism, thus stimulate the growth condition occurring in nature (Pérez-Guerra *et al.*, 2003) [31]. Control of fermentation parameters such as pH, moisture content of the solid substrate, and temperature during fermentation are important variables that affect enzyme yield in SSF (Pandey *et al.*, 1999) [32]. Recently the production of industrial enzymes using solid state fermentation has gaining more attention. This is because, compared to SmF, SSF has the following advantages (Holker & Lenz, 2005) [33].

First, cheap agricultural residues can be used as substrates, thus greatly reducing the cost of enzyme production.

Secondly, solid state fermentation requires easier operation. This includes requirement for a smaller space, simple aeration system, and less pollution. In addition, lower energy requirement for operation, possibility to use semi-sterilized condition, and reduced risk of contaminations are also important features of SSF that make the operation system simple (Pérez-Guerra *et al.*, 2003) [34].

Thirdly, solid state fermentation significantly minimizes catabolic repression in production of hydrolytic enzymes in nutrient rich solid state medium. This is because of limited exposure of nutrients to microorganism, and decline in mass transfer that favor slower but constant microbial growth rate. In addition, the presence of active regulatory mechanisms in SSF is also believed to play important role in inducing high volumetric product biosynthesis (Barrios-Gonzalez *et al.*, 2005) [35].

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g. the blood clotting cascade, the complement system and the invertebrate prophenol-oxidase activating cascade). However, as with the increase of the industrial demand, the market of protease enzyme has widened and alternate methods of producing protease is being researched.

Based on previously done researches, the protease enzyme can be produced by using or from microorganisms identified as potential in producing abundant amount of protease enzymes in need for industrial purposes. These microorganisms or microbes are such like bacteria, fungi and other microorganism classes. Cutting down from the list, under fungi there are many species of fungi which are capable in producing protease enzyme, in which the well-known types are under the *Aspergillus Niger*

The bacteria under the *Aspergillus* sp. are well known for producing extracellular proteases in use as an industrial enzyme such as *Aspergillus niger* and *Aspergillus oryzae*. The production method of protease production differs based on the capacity of the enzyme wanted or needed. For upscale or large scale production as for industrial purposes,

open tray system should be used for more efficiency and larger yield. For a small scale production or lab scale production for testing and research purposes, it is suitable to only use Erlenmeyer flasks shaken in appropriate incubators.

### 2.5.1 Extracellular Protease

Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Whereas, extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products<sup>[36, 37]</sup>. In addition, extracellular protease which is a hydrolytic enzyme can be classified further into two categories which are exo-peptidases that cleave off amino acids from ends of protein chain, and endo-peptidases that cleave peptide bonds within the protein<sup>[38]</sup>. Microbial proteases are subdivided into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme. In production of extracellular protease, protease enzymes are most active in temperature ranges from 40-55 °C.

### 2.5.2 Production of Extracellular Protease

As the term extracellular applies, it is known as that to produce enzymes which function outside the cell in which it originates. Be it a fungi or bacteria, the source can be mesophylls obtained everywhere. Mesophilic bacteria and fungi are usually obtained from earth soil, given there is no temperature restraint to abide to in the experimental work. As acknowledged the *Aspergillus* species are well known for production of extracellular protease. Most fungi from the *Aspergillus* species are preferred to be used in production of extracellular enzymes. As the title of the research implies, the aim is to produce extracellular protease from *Aspergillus* species are favourable in terms of extracellular proteases. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments<sup>[39]</sup>.

Most certainly, in terms of protease, as well known for food and industrial purposes the *Aspergillus* sp. is a major producer for alkaline, extracellular proteases

### 2.5.3 Effect of pH on Protease Production

The pH is the measure of acidity or alkalinity of an aqueous solution and it has been proved strongly to affect the production of protease in the process of fermentation. It influences most of the enzymatic processes and the transport process of diverse of components across the cell membrane (Sandhya *et al.*, 2005; Murthy and Naidu, 2010). The pH is related to the amount of hydrogen (H<sup>+</sup>) concentration and this H<sup>+</sup> will give effect to the growth of the microorganisms. At neutral pH, it can act as the substrate that might be used by the cells in order to grow but in acidic and alkaline conditions, it can become inhibitor to the cell growth and this will affect the enzyme production by the cells. Natarajan and Rajendran (2009) also said that variety of pH values may also lead to the changes of protein structure meaning that maybe at certain pH the protease might be denatured once it is secreted to the outside of the cells or it can be said that the enzyme is already inactive and

not stable at that pH. Murthy and Naidu (2010) found out that the increase in pH can cause the decrease of protease activity. In addition, different microorganisms might have different kinds of metabolic process and due to that the enzyme activity might also be affected. Coral *et al.* (2002) reported that fungal proteases are active at neutral pH and alkaline pH around pH 7 to pH 11 but Chakrabarti *et al.* (2000) proved that *Aspergillus terreus* managed to produce protease between pH 5.5 and pH 9.5.

### 2.5.4 Effect of Substrate Concentration on Protease Production

All microorganisms need carbon sources in order to live as it is the food for them. According to Adinarayana *et al.* (2003), the carbon sources are the utmost nutritional factors that influenced the protease production. Logically, if the microorganisms are supplied with adequate amount of nutrients, they will grow effectively meaning that they got all the nutrients needed in order to grow and product production will be maximized. If the microorganisms grow in lack of nutrients, their growth may be retarded and this will probably affect the product formation within the fermentation process.

Other metabolites or maybe the main products itself can repressed the product formation and it said that commonly the product production will decrease if the substrate concentration increase (Escobar and Barnett, 1993). When the substrate concentration is too high, it may also prohibit the enzyme activity (Dekleva *et al.*, 1985). Andrade *et al.* (2002) showed that protease activity was at its maximum when 40 g/l of substrate was used.

### 2.5.5. Utilization of wastes as substrate

Fermentation process needs nutrient medium that must satisfy the elemental requirements for the cell biomass, product formation and energy. The nutrients consist of macronutrients such as carbon sources, nitrogen sources and micronutrients such as trace elements and vitamins. According to El Enshasy *et al.* (2008), 40% of the production cost of large scale industrial enzyme is based on the cost of substrate so, it is important to use cost-effective substrate in the production process.

Normally most research used alternative sources of substrate to substitute the carbon and nitrogen sources in the fermentation process. There are many relevant sources of substrate to be used in the fermentation and the utilization of these alternative sources is hopefully promising for cheaper substrate cost but with efficient enzyme production especially in large scale production.

### 2.5.6. Optimization of protease production

Nowadays, researchers are more focused in optimizing the process parameters so that high yield of protease can be achieved. Optimization is classically done by applying one-factor-at-a-time (OFAT) method where it is known to be very time-consuming method. There are several parameters are going to be optimized but in this method other parameters need to be constant while studying a parameter at a time.

Fermentation is really affected by various factors such as pH, temperature, agitation speed, type of carbon and nitrogen sources and many others. In optimization, the best value for every studied factor is to be determined for its highest production of protease. For example, the effect of

temperature to the fermentation process has been studied so that the exact temperature at which the protease yield is relatively high can be determined but by implementing OFAT method, each factor can only be studied alone since the other factors need to maintain constant. This method used to consume a long period of time if many parameters have to be studied. Furthermore, the effect of interaction between parameters cannot be observed in this way. The results obtained from OFAT can be further studied for the interaction between two or several factors.

The researchers study the different parameters and came out with different outcomes (maximum protease activity). From their studies they manage to identify at what range of parameters will actually result in high protease activity.

## 2.6. Tyrosine

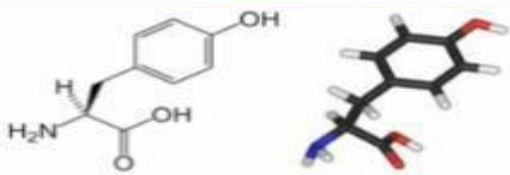
Tyrosine (from the Greek *tyros*, meaning *cheese*, as it was first discovered in cheese), 4-hydroxyphenylalanine, or 2-amino-3-(4-hydroxyphenyl)-propionic acid, is one of the 20 amino acids that are used by cells to synthesize proteins. It has a phenol side chain with a hydroxyl group. Upon the location of the hydroxyl group, there are three structural isomers of Tyr, namely para-Tyr (p-Tyr), meta-Tyr (m-Tyr) and ortho-Tyr (o-Tyr). Enzymatically, only the first isomer

(p-Tyr) is produced from L-Phe by the Phe-hydroxylase enzyme. The other two isoforms, m-Tyr and o-Tyr can be produced as a consequence of free radical attack on Phe in states with increased oxidative stress.

It plays a key role in signal transduction, since it can be *tagged* with a phosphate group (phosphorylated) by protein kinases to alter the functionality and activity of certain enzymes. (In its phosphorylated state, it is sometimes referred to as phosphotyrosine.) Tyrosine is also precursor to the thyroid hormones *thyroxine* and *triiodothyronine*, the pigment *melanin*, and the biologically-active catecholamines *dopamine*, *norepinephrine* and *epinephrine*. In *Papaver somniferum*, the opium poppy, it is used to produce morphine.

Tyrosine cannot be completely synthesized by animals, although it can be made by hydroxylation of phenylalanine if the latter is in abundant supply. It is produced by plants and most microorganisms from prephenate, an intermediate on the shikimate pathway. Prephenate is oxidatively decarboxylated with retention of the hydroxyl group to give p-hydroxyphenylpyruvate. This is transaminated using glutamate as the nitrogen source to give tyrosine and  $\alpha$ -ketoglutarate.

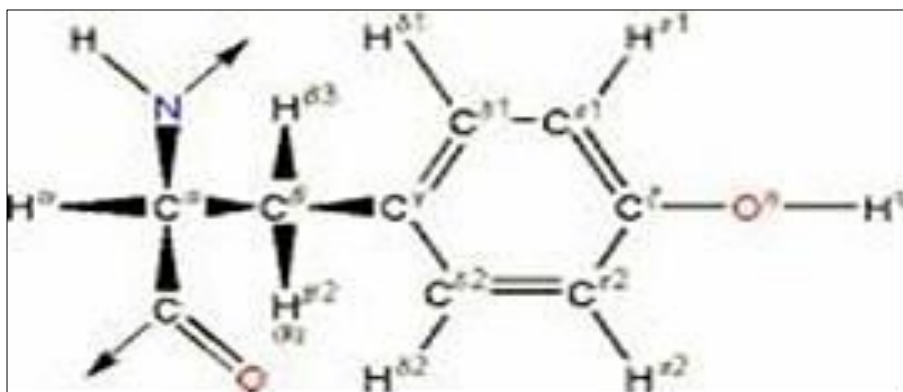
**Table 2:** Properties of tyrosine

| Tyrosine                                                                            |                                                |
|-------------------------------------------------------------------------------------|------------------------------------------------|
|  |                                                |
| Systematic name                                                                     | (S)-2-Amino-3-(4-hydroxyphenyl)-propanoic acid |
| Abbreviations                                                                       | Tyr<br>Y                                       |
| Chemical formula                                                                    | C <sub>9</sub> H <sub>9</sub> NO <sub>3</sub>  |
| Molecular mass                                                                      | 181.19 g mol <sup>-1</sup>                     |
| Melting point                                                                       | 343 °C                                         |
| Density                                                                             | 1.456 g cm <sup>-3</sup>                       |
| Isoelectric point                                                                   | 5.66                                           |
| pK <sub>a</sub>                                                                     | 2.24<br>9.04<br>10.10                          |
| CAS number                                                                          | [60-18-4]                                      |
| EINECS number                                                                       | 200-460-4                                      |

L-Tyrosine is sometimes recommended by practitioners as helpful for weight loss, clinical depression, and phenylketonuria; however, a study found that it had no impact on performance. L-tyrosine is a protein amino acid. It is classified as a conditionally essential amino acid.

Under most circumstances, the body can synthesize sufficient L-tyrosine, principally from L-phenylalanine, to meet its physiological demands. However, there are conditions where the body requires a dietary source of the amino acid for its physiological demands. For example, L-tyrosine is an essential amino acid for those with phenylketonuria. L-tyrosine is found in proteins of all life

forms. Dietary sources of L-tyrosine are principally derived from animal and vegetable proteins. Vegetables and juices contain small amounts of the free amino acid. The free amino acid is also found in fermented foods such as yogurt. L-tyrosine is also known as beta-(para-hydroxyphenyl) alanine, alpha-amino-para-hydroxyl-hydrocinnamic acid and (S)-alpha-amino-4-hydroxybenzenepropanoic acid. It is abbreviated as either Tyr or by its one-letter abbreviation Y. The molecular formula of L-tyrosine is C<sub>9</sub>H<sub>9</sub>NO<sub>3</sub>, and its molecular weight is 181.19 Dalton's. L-tyrosine is an aromatic amino acid with the following structural formula:

**L-Tyrosine (Tyr)****Fig 4:** L-Tyrosine structure**2.6.1 Tyrosine Absorbance**

Tyrosine, an essential amino acid, is also an aromatic amino acid and is derived from phenylalanine by hydroxylation in the para position. While tyrosine is hydrophobic, it is significantly more soluble than is phenylalanine. The phenolic hydroxyl of tyrosine is significantly more acidic than are the aliphatic hydroxyls of either serine or threonine, having a pKa of about 9.8 in polypeptides. As with all ionisable groups, the precise pKa will depend to a major degree upon the environment within the protein. Tyrosine's that are on the surface of a protein will generally have a lower pKa than those that are buried within a protein; ionization yielding the phenolate anion would be exceedingly unstable in the hydrophobic interior of a protein.

Tyrosine absorbs ultraviolet radiation and contributes to the absorbance spectra of proteins. The absorbance spectrum of tyrosine will be shown later; the extinction of tyrosine is only about 1/5 that of tryptophan at 280 nm, which is the primary contributor to the UV absorbance of proteins depending upon the number of residues of each in the protein.

**2.6.2 Tyrosine Standard Calibration Curve**

As a reference to protease enzyme activity, tyrosine standard curves are usually generated using an appropriate amount of tyrosine diluted in suitable fractions with solvents, favourably water. The suitably diluted samples will be read at a spectrophotometer at a wavelength measurement of 66

**3.1 Materials and Methods**

- 1) Conical flasks
- 2) Test tubes
- 3) Petri plates
- 4) Autoclave
- 5) Rotator shaker
- 6) Microscope
- 7) Incubator
- 8) U V spectrophotometer
- 9) Cooling centrifuge
- 10) Pipette
- 11) Laminar air flow chamber
- 12) Magnetic stirrer
- 13) pH meter

**3.1.1 Materials for culturing**

- 1) Potato dextrose agar (PDA)
- 2) Potato dextrose broth (PDB)
- 3) Fungal inoculum

**3.1.2 Materials for SSF (Solid State Fermentation)**

- 1) Minerals and salt solution
- 2) Different substrates
- 3) fungal inoculum

**3.1.3 Reagents required**

- 1) Tricarboxylic acid buffer (TCA) 10%
- 2) Casein solution 1% (w/v)
- 3) Phosphate buffer (pH 7)
- 4) Sodium carbonate (0.5M)
- 5) Folin-ciocalteau reagent
- 6) Tyrosine
- 7) Extractions (Tris buffer, Chilled water, Phosphate buffer)

**3.2 Preparation of materials for culturing:****1) Potato dextrose agar (PDA)****Table 3:** Preparation of Potato Dextrose Agar

| S. No | Compound               | Amount    |
|-------|------------------------|-----------|
| 1     | Filtrate potato liquid | 200ml     |
| 2     | Dextrose               | 2 grams   |
| 3     | Yeast extract          | 0.3 grams |
| 4     | Agar                   | 4 grams   |
| 5     | Distilled water        | 200ml     |

**2) Potato dextrose broth (PDB)****Table 4:** Preparation of Potato Dextrose Broth

| S. No | Compound               | Amount    |
|-------|------------------------|-----------|
| 1     | Filtrate potato liquid | 200ml     |
| 2     | Dextrose               | 2 grams   |
| 3     | Yeast extract          | 0.3 grams |
| 4     | Distilled water        | 200ml     |

**3.2.1 Preparation of fungal inoculums:** Fungal inoculums were prepared in 500 ml conical flasks containing 100 ml of sterilized potato dextrose broth and was aseptically inoculated with the *A.niger* from a fresh slant and allowed to grow at 35 °C for 48/72 hours in a incubator, after 48/72 hours of growth, the fungal culture was used as inoculums.

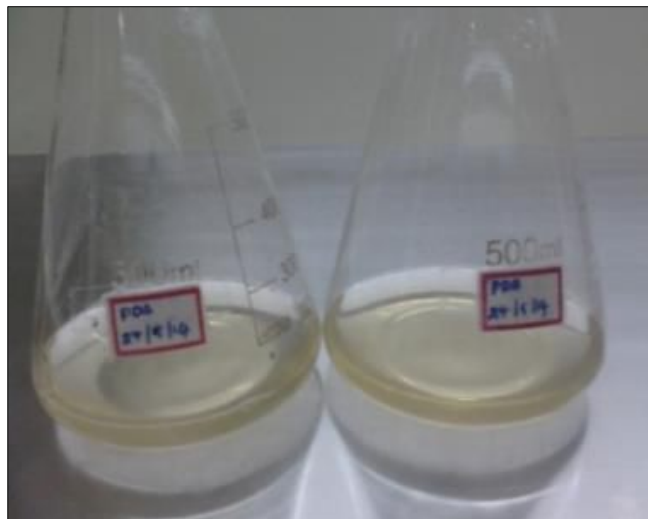


**Fig 5:** PDB culture broths for fungus before inoculation



**Fig 6:** PDB broths with fungus culture

### 3.3 Preparation of materials for SSF

#### 1) Minerals and salt solution

##### a) Diluents type 1: (D1)

**Table 5:** Preparation of Diluent (D1)

| S. No | Compound          | Grams  |
|-------|-------------------|--------|
| 1     | CaCO <sub>3</sub> | 0.5g/l |
| 2     | Peptone           | 1g/l   |
| 3     | Glucose           | 1g/l   |
| 4     | Yeast extract     | 1g/l   |
| 5     | Distilled water   | 1000ml |

#### Selection of best substrate for Protease production by SSF

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrates. However, some of the nutrients may be available in sub optimal concentrations, or even absent in the substrates. In such case, it would become

necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF process, thereby making them more easily accessible for microbial growth.

Agro-industrial residues are generally considered the best substrates for the SSF process, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included wheat bran, rice bran, rice husk, soya meal, cotton meal, wheat bran plus skimmed milk, etc. Wheat bran however holds the key, and has most commonly been used, in various processes.



**Fig 7:** Fungal culture (seed) inoculating in to the substrate flask



**Fig 8:** Growth of *A.niger* on substrate (wheat bran)



**Fig 9:** Crude extract from Solid substrate fermentation

### 3.4 Reagents preparation

- 1) Preparation of Phosphate buffer (pH 7):** 10 ml of 1M K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> has to be prepared separately, from these preparations 6.15 ml of K<sub>2</sub>HPO<sub>4</sub> and 3.85ml of KH<sub>2</sub>PO<sub>4</sub> was taken in beaker and to this 90ml of distilled water was added which will gives the 100 ml of 0.1M pH 7 phosphate buffer .
- 2) Preparation of casein solution (1% w/v):** To the 100 ml of pH7 phosphate buffer 1gram of casein powder was added and mixed by gentle heating to dissolve the casein, which gives us the 100ml of casein solution.
- 3) Preparation of 10% TCA (tricarboxylic acid buffer):** 5 grams of TCA was added to 2.27ml of distilled water which gives the 100% TCA and this will comes up to 5ml of 100% TCA,by adding the 45ml of

distilled water to the 5ml of 100% TCA will gives the 50ml of 10% TCA.

- 4) **Preparation of 0.5M sodium carbonate ( $\text{Na}_2\text{CO}_3$ ):** 5.3 grams of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was dissolved in 100ml of distilled water, which gives the 100ml of 0.5M sodium carbonate ( $\text{Na}_2\text{CO}_3$ ).
- 5) **5) Preparation of 1.1mM Tyrosine:** 0.0497g of tyrosine was dissolved in 250 ml of distilled water and mixed up on magnetic stirrer by gentle heating, thus the final concentration was 1.1 mM and further diluted to different concentrations.

#### 4. Methodology

##### 4.1 Microorganism and inoculum development

###### 4.1.1 Fungi: *Aspergillus niger*

The fungal culture was obtained from the culture bank and the culture was revived by transferring to the potato dextrose agar slants which were placed at the 35 °C for 24 hours. The culture was maintained by transferring into the new slants and stored in the cool laboratory at 4 °C.

##### 4.2 Preparation of fungal inoculums:

Fungal inoculums were prepared in 500 ml conical flasks containing 100ml of sterilized potato dextrose broth and was aseptically inoculated with the *A.niger* from a fresh slant and allowed to grow at 35 °C for 48/72 hours in a incubator, after 48/72 hours of growth, the fungal culture was used as inoculums

##### 4.3 Fermentation technique

Solid state fermentation was carried out for the production of protease by *A.niger*. Different types of substrates such as the wheat bran, soya meal, rice bran, rice husk, cotton meal, wheat + soya mixture etc., were used, these different types of substrates were taken in different amounts and to this different amounts of different minerals and salt solution was added, and sterilized in an autoclave at 15 lbs pressure and

121 °C for about 15 minutes. After sterilization medium was cooled and different percentages (%) of microbial inoculums were inoculated to the substrates in the laminar air flow chamber in order to maintain the aseptic conditions, the substrates were mixed vigorously for uniform distribution in the medium and were incubated at 35 °C and room temperatures for different incubation periods.

##### 4.4 Extraction of enzyme

After incubation period of SSF, different amounts of water/buffers was added to flask and the flasks were rotated on rotary shaker for one hour at a speed of 200rpm for the extraction of protease from fermented substrate, after shaking, the contents of flaks were centrifuged at 4 °C and 10000rpm. After centrifuge the supernatant (crude enzyme) was stored at 4 °C for further analysis.

##### 4.5 Assay of protease

The activity of protease was assayed by the modified method of McDonald and Chen (1965). Two tubes were taken each with 5ml of casein and in one of the tube 1ml of diluted enzyme was added and incubated at 37 °C for 10 min after incubation 5ml of TCA was added in both the tubes and 1ml of enzyme was added in other tube and incubated both the tubes at 37 °C for 30 min, after incubation the contents were filtered through the whattman filtered paper. 2mL of filtrate was mixed with 5mL of alkaline reagent (sodium carbonate) and to this 1ml of 1:1 diluted folin and ciocalteau reagent was added; as a result, blue colure was produced. The optical density of the test mixture was read against blank at 660nm on UV/VIS Spectrophotometer.

##### 4.6 Standard Curve

Standard curve was done by pipetting the following reagents into suitable tubes (in milliliters):

**Table 6:** Preparation of tyrosine standard curve

| Serial order | Std soln (Tyrosine standard) 1.1mM | Distilled Water | Conc of tyrosine ( $\mu\text{M}$ ) | $\text{Na}_2\text{CO}_3$ (0.5M) | F-C phenol reagent | O.D Values @660nm |
|--------------|------------------------------------|-----------------|------------------------------------|---------------------------------|--------------------|-------------------|
| Std Blank    | -----                              | 2               | -----                              | 5                               | 1                  | 0.000             |
| Std 1        | 0.05                               | 1.95            | 27.5                               | 5                               | 1                  | 0.128             |
| Std 2        | 0.10                               | 1.90            | 55                                 | 5                               | 1                  | 0.252             |
| Std 3        | 0.20                               | 1.80            | 110                                | 5                               | 1                  | 0.482             |
| Std 4        | 0.30                               | 1.70            | 165.1                              | 5                               | 1                  | 0.725             |
| Std5         | 0.40                               | 1.60            | 220                                | 5                               | 1                  | 0.910             |
| Std6         | 0.50                               | 1.50            | 275                                | 5                               | 1                  | 1.117             |
| Std7         | 0.60                               | 1.40            | 330                                | 5                               | 1                  | 1.321             |
| Std8         | 0.70                               | 1.30            | 385                                | 5                               | 1                  | 1.507             |
| Std9         | 0.80                               | 1.20            | 440                                | 5                               | 1                  | 1.764             |
| Std10        | 0.90                               | 1.10            | 495.4                              | 5                               | 1                  | 1.854             |
| Std11        | 1                                  | 1               | 550                                | 5                               | 1                  | 2.102             |

Standard graph was drawn by placing O D values on X-axis and tyrosine concentration on Y-axis

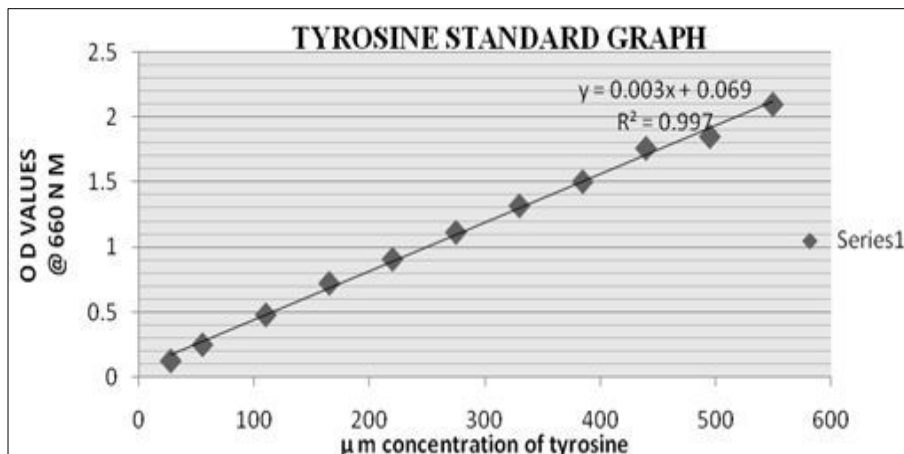


Fig 10: Tyrosine standard graphs

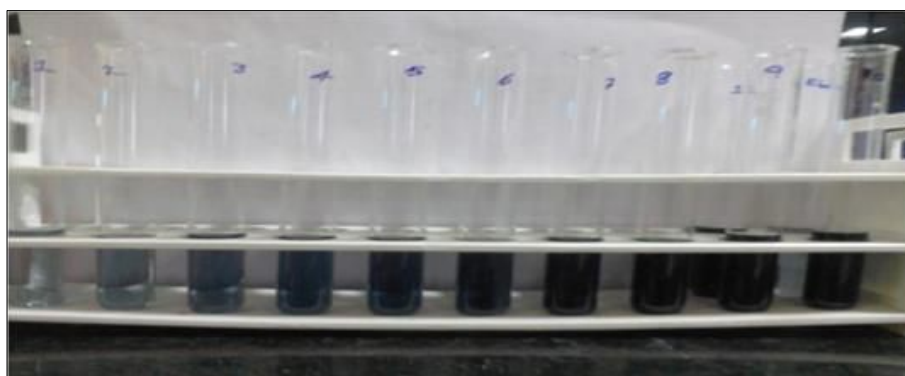


Fig 11: Tyrosine standard assay results showing concentrations gradient

#### 4.7 Calculations

##### Standard Curve

$\Delta A_{660nm} \text{ Standard} = A_{660nm} \text{ Standard} - A_{660nm} \text{ Standard Blank}$

Plot the  $\Delta A_{660nm}$  Standard vs.  $\mu\text{moles}$  of Tyrosine.

##### Sample Determination

$\Delta A_{660nm} \text{ Sample} = A_{660nm} \text{ Test} - A_{660nm} \text{ Sample Blank}$

Determine the  $\mu\text{Moles}$  of Tyrosine equivalents liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{Mole Tyrosine equivalents released}) (11)}{(1) (10) (2)}$$

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the Unit Definition

1 = Volume of enzyme (in milliliter) of enzyme used

2 = Volume (in milliliters) used in Colorimetric Determination

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{Mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{Mg protein/ml enzyme}}$$

#### 5. Results and Discussion

##### 1) Effect of substrate

Up to 40% of the total production cost of enzymes is due to the growth substrate. The real and beneficial production of enzyme is produced from the natural sources and industrial wastes. The production medium costs of about 30-40% of the total production cost of the enzyme at industrial level. The production of protease was done by replacing the nitrogen sources and common substrates with Rice bran, Wheat bran, Soya meal, Cotton meal, Ground nut cake. The maximum production was occurred when Wheat bran used as natural substrate (179.86 U/ml). The result indicates that the wheat bran can also be used as the cheap substrate for protease production.

Table 7: Effect of substrate on enzyme production

| Serial number | Type of substrate         | O D values @ 660 nm | Enzyme Units/ml |
|---------------|---------------------------|---------------------|-----------------|
| I             | Wheat bran (WB)           | 1.210               | 179.86          |
| II            | Wheat bran + Rice bran    | 0.185               | 27.5            |
| III           | Wheat bran + Wheat flour  | 0.566               | 84.13           |
| IV            | WB + WB + ground nut cake | 0.263               | 39.09           |
| V             | WB + casein               | 0.163               | 2.422           |
| VI            | WB + cotton               | 0.084               | 12.48           |

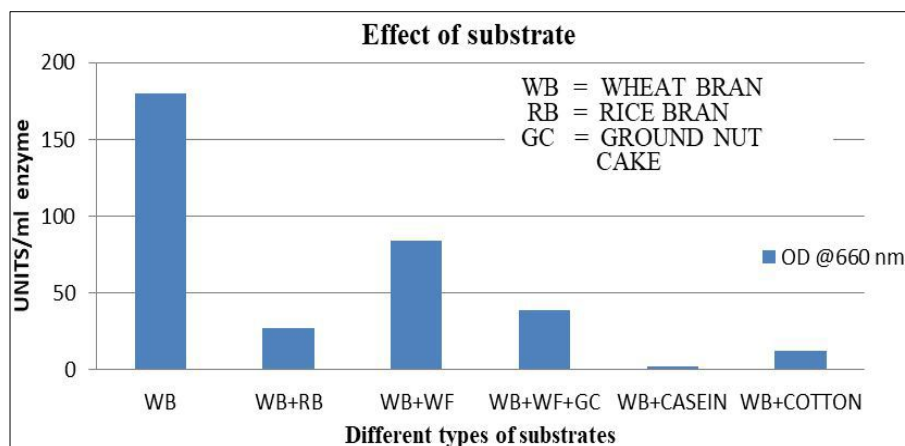


Fig 12: Effect of substrate on protease production by *Aspergillus niger*

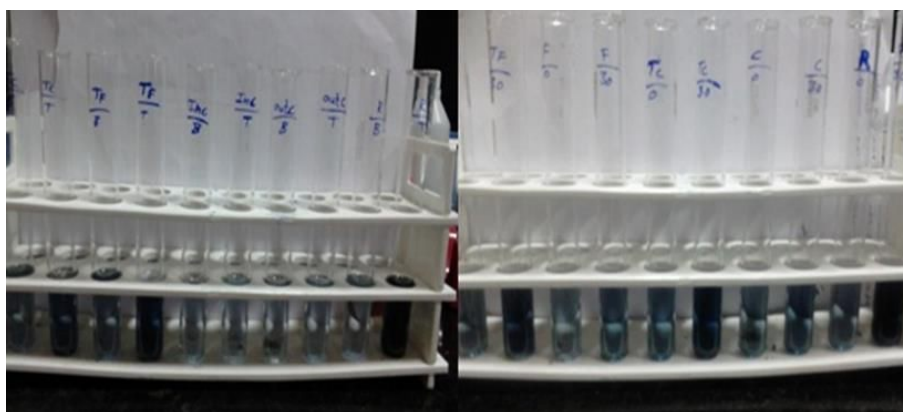


Fig 13: Protease assay of crude enzyme of SSF at different temperature

## 6. Conclusions

Based on the results of this work, the following conclusions can be drawn:

In this study an industrial strain of *Aspergillus niger* is used for protease production

This strain of *A. niger* is maintained by following methods such as sub culturing and glycerol stocks. Protease production is through solid state fermentation, growing *A. niger* on agro-industrial waste such as wheat bran etc. This study includes the optimization of different parameters such as substrates, pH, temperature, moisture%, Diluents etc. 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.

Therefore, the following activities for future research recommended:

- Searching for more potent protease producers from *Aspergillus* spp
- Testing the protease from *Aspergillus niger* as poultry feed and for other potential Industrial applications.
- Scale up studies.

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