Original Research Article

Optimization Studies on Extracellular Protease Production by *Aspergillus niger* and*Aspergillus terreus* Using Skim Milk Casein as Substrate

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ABSTRACT

Aim: Proteases are proteolytic enzymes that have a wide range of applications in the industrial sectors. This study investigated the production and optimization of protease by protease-producing fungi, (*Aspergillus niger* and *A. terreus)* using skim milk casein as substrate.

Materials and Methods:*Aspergillus terreus* and *A. niger* obtained from the Department of Plant Science and Biotechnology, University of Jos, Nigeria were screened for extracellular protease on skim milk agar medium. Optimization studies across different parameters~~;~~:~~T~~temperatures (30-80^{o}C), pH (3-10), s~~S~~ubstrate Concentration (0.25-2 %), and i~~I~~ncubation period (6 days) using s~~S~~ubmergedf~~F~~ermentation were done for maximum protease activity.

Results: The maximum temperature of protease activity was ~~at~~ 50^{o}C (116.5 IU/ml) for *Aspergillus niger* and ~~at~~ 40^{o}C (121.86 IU/ml) for *Aspergillus terreus.* The Optimum pH for protease activity was ~~at~~ pH 9 (106.85 IU/ml) for *Aspergillus niger* and ~~at~~ pH 8 (107.90 IU/ml) for *Aspergillus terreus.* Optimum protease activity (29.36 IU/ml) at an incubation period of 48 hours was recorded for *Aspergillus niger* and 72 hours (182.2IU/ml) for *Aspergillus terreus.* At 2% substrate concentration a maximum activity of 87.21 IU/ml and 76.5 IU/ml was recorded for *Aspergillus niger* and *A. terreus*,respectively.

Conclusion:The test fungi *Aspergillus niger* and *Aspergillus terreus*exhibited maximum hydrolytic potential for protease production which has a vast application in different industries.

Keywords: Hydrolytic, Enzyme,*Aspergillus niger*, *Aspergillus terreus*, Protease assay, Skim milk

1. INTRODUCTION

Enzymes are macromolecular biological catalysts, most of which are proteins in nature, although a few are catalytic RNA molecules. Enzymes catalyze or accelerate chemical reactions. At the start of a chemical reaction, substrates are converted by enzymes into products [1]. ~~Over the~~More than hundreds of enzymes areused industrially, more than half of whichare from fungi and yeast, and over a third are from bacteria, while the rest are divided between animal (8%) and plants (4%) sources. Enzymes are widely spread virtually in all plants, animals, and microorganisms. Microbial enzymes are preferred to plants and animal because of the following reasons~~;~~: low cost of production, predictable and controllable enzyme contents, and also because unlike those of plant and animal tissues contain~~s~~ less harmful materials [1]. Fungal enzymes are more suited for industrial applications as fungi are easily cultu~~red~~vated and are fast growing. Fungal proteases are used in the food, diary, detergent, leather, and pharmaceutical industries. The~~re~~y are used for bioremediation and production of therapeutic peptides. Furthermore, fungal proteases are more diverse and exhibit wider substrate specificity [2].

Proteases are a large and complex group of enzymes that catalyze the hydrolysis of protein molecules into peptides and amino acids [3, 4]. They are also called proteolytic enzymes or proteinases. Proteases are classified into three groups, that ~~is~~are, neutral, acidic, and alkaline proteases, based on their acid-base nature. Fungi are the main source of acid proteases [5]. Proteases form a large group of enzymes belonging to the class of hydrolases. Serine protease (EC 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartate proteases (EC 3.4.23), and metallo-protease (EC 3.4.24) constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market [1,6,7]. Traditionally the proteinases have been regarded as degradative enzymes which are capable of cleaving protein foods. They liberate small peptides and amino acids needed by the body. Proteolytic enzymes have the ability to carry out selective modification of proteins by limited cleavage, such as activation of zymogenic forms of enzymes, blood clotting add considerable interest to already important enzymes. These are also used in all crucial biological processes such as metabolism, enzyme modification, complement system, apoptosis pathways, prophenol oxidase activating cascade. Furthermore, a study of proteolytic enzymes is valu~~ed~~able because of their use in various forms of medical therapies important as reagents in laboratory and industrial processes [8]. Proteases play an important role in many processes in

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human's body such as fertilization, digestion, growth, maturation, aging, and even death of the organism. They regulate many physiological processes by controlling the activation of the synthesis and degradation of proteins~~)~~.

The demand for novel natural products with improved qualitiesto enhance the existence of men has led researchers to conduct a series of researches over the years. Among these natural products are thebiological enzymes. ~~Of~~Among all ~~the~~ enzymes, proteases have a renewed interest due to their role in the cellular metabolic processes and their importance in the industrial, pharmaceutical, and medical fields. ~~Its~~Their application is very broad andtheyha~~s~~ve been used in many fields for years~~, and is~~ mainly ~~used~~ in food and detergent industries [9]. Proteases are classified into three groups, that is, neutral, acidic, and alkaline proteases based on their acid-base nature.Fungi are the main source of acid proteases [5]. Extracellular proteases have high commercial value and multiple applications in various industrial sectors, such as the detergent, food, pharmaceutical, leather, and diagnostics industries, waste management, and silver recovery. Currently, the largest share of the enzyme market has been held by detergent proteases, being active and stable at alkaline pH [10]. Fungi of the genera *Aspergillus, Penicillium,* and *Rhizopus* are especially useful for producing proteases. Fungal enzymes are more suitable for industrial application as fungi are easily cultured and fast growing.Also, fungal proteases are more diverse and exhibit wider substrate specificity [2]. There is an increased demand for newer and novel natural products with improved qualities to enhance man's existence in life. Among these natural products are the biological enzymes. This study aims to investigate the extracellular proteases production of *Aspergillus niger* and *A. terreus* using skim milk casein as a substrate.

Comment [G2]: What is the meaning of this?

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2. MATERIALS AND METHODS

2.1 Collection and Isolation of Protease Producing Fungi

The test fungi (*Aspergillus niger* and *Aspergillus terreus*) were obtained from preserved samples ~~in~~at the Department of Plant Science and Biotechnology, University of Jos. The samples were inoculated on starch agar and kept in an incubator at 25±2°C for 5 days. Macroscopic and microscopic examinations were carried out to confirm the identities of thetest fungi. Identification of species was done using a fungal atlas and the identification schemes of [11,12]. Pure fungal colonies of *Aspergillus niger* and *A. terreus* were stored at 4°C until ~~required for~~ used [13].

2.2 Screening of the Test Fungi for Proteolytic Activity Using Plate Assay

Skim milk agar plates were prepared using selective medium containing [g/l: KH_2 PO_4 (0.7) NH_4NO_3 (5), KCl, 0.2; $MgSO_4.H_2O$, 0.2; $FeSO_4.H_2O$, 0.5; distilled H_2O, 1000L; Agar, 10; 1% skim milk casein] and inoculated with 5mm mycelia discs from the edge of actively growing 4-day old cultures of the test fungi. After 5 days of incubation on skim milk agar at 25±2^oC, the plates were observed for growth.

2.3 Effects of Incubation Period on Fungal Growth and Crude Protease Production Using Submerged Fermentation (SmF)

Theeffects of incubation period on crude protease production were determined using modified method of Asha *et al.* [14]. Submerged Fermentation ~~(SmF)~~ was performed by inoculating disc of pure cultures of actively growing *Aspergillus niger* and *A. terreus* into the modified protease production medium composed of [g/l: KH_2PO_4, 1.5; NaCl, 0.5; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2; skim milk substrate, 10g) and then incubated for 6 days at room temperature (27±2^oC) without agitation [14]. Aliquots were withdrawn at 24 hours' intervals centrifuged at 8000rmp and supernatant of crude enzyme was used for protease assay. The protease activity was assayed according to the method of Cupp Enyard [15] by using skim milk casein as substrate. The test tubes, containing the reaction mixtures, composed of 1ml ~~of the~~ crude enzyme, 1ml ~~of~~ phosphate buffer~~;~~(pH 7.5), and 1 ml of 1% skim milk casein, and blank containing 1 ml of the enzyme solution only, were incubated in ~~a~~ water bath, at temperatures of 37^oC for 30mins to allow for an enzymatic reaction. The reaction was terminated by the addition of 5ml of Trichloroacetic acid (TCA). The tubes were then allowed to stand for 15 min. at room temperature. Precipitate proteins from both test tubes were then filtered by centrifugation at 8000rpm for 10 minutes and 2 ml of filtrates mixed with 5 ml of $NaCo_3$ was then added to both test tubes turning the solution cloudy followed by the addition of 1ml of Folin ciocalteus phenol reagent which reacted primarily with free tyrosine liberated in the mixture. The resultant solution was mixed properly by swirling and incubated at 37^oC for 30 minutes for the development of blue color. 2 ml of the solution were then placed into suitable cuvettes and absorbance was measured at 650nm against a reagent blank using tyrosine standard. One protease unit was defined as the amount of enzyme that released 1μg of tyrosine per ml, per minute, under standard conditions [5].

Comment [G5]: Too long so that one cannot understand it. I suggest the authors separate the text in several different sentences.

Comment [G6]: Is this the right verb?

2.4 OPTIMIZATION STUDIES ON THE ENZYME USING DIFFERENT

PARAMETERS

2.4.1 Effects of Temperatures of incubation on Protease Production

The effects of different incubation temperatures on enzyme activity of *Aspergillus niger* and *A. terreus* were studied using modified method of Asha *et al.* [14]. The optimum temperature for protease activity was determined by performing the enzyme reaction at incubation temperatures:~~between~~ 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C ~~and the~~. Standard protease assay method was performed, using the phosphate buffer at ~~a~~ pH ~~of~~ 7.5, to analyze the results.

2.4.2 Effects of pH on Protease Activity

The effects of different pH on the protease activity of *Aspergillus niger* and *A. terreus* were determined using 1% skim milk casein as substrates The phosphate buffer ~~pH 7.5 was alternated to a~~ vary~~inged~~ pH of 3, 4, 5, 6, 7, 8, 9 and 10, using 2M Hydrochloric acid (HCl) and 1M of Sodium hydroxide (NaOH). The pH was measured using a pH meter and then the standard protease assay was performed. The valueswere monitored and recorded in triplicates Asha *et al.* [14].

> **Comment [G7]:** Which values – of the pH or of the ...

2.4.3 Effects of Substrate Concentration on Protease Activity

Standard protease assay at ~~a~~ pH ~~of~~ 7.5 (phosphate buffer) was performed to determine the effects of substrate concentration on protease activity. Varying concentrations (0.25%, 0.5%, 1.0%, 1.5%, and 2.0%) of skim milk casein (substrate) were used for the assay. The values were monitored and recorded in triplicates Asha *et al.* [14].

> **Comment [G8]:** Which values?

3. RESULTS

Identification of test fungi (*Aspergillus niger* and *Aspergillus terreus*) using their distinct characteristics was confirmed. The screening for proteolytic activity, done using plate assay, showed that *Aspergillus niger* and *A. terreus* initiated growth on the skim milk media used. It was observed that by the end of the incubation period the test fungi have covered the entire plates, indicating that were both proteolytic. The details of the structure are shown in ~~Plates 1 and 2~~, respectively.

> **Comment [G9]:** This should be Fig.1 and Fig.2

The effects of the incubation period of 6 days on fungal growth and crude protease production are shown in Figure ~~1~~3. Both test fungi had growth with progression from the 1st day. *Aspergillus niger* had the highest ~~peak of~~ enzyme activity on day 2 (48 hours) liberating 29.36 IU/ml. *Aspergillus terreus* had ~~thehighesta~~peak of enzyme activity on day 3 (72 hours) of incubation, liberating 182.21 IU/ml.

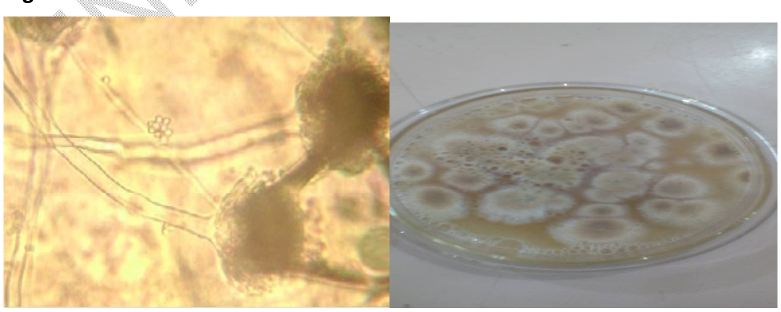
> **Comment [G10]:** One cannot say 'highest peak' here. Use either maximum or peak value
>
> Actually, the maximum value, if one follows the curve is not exactly there.

The effects of incubation temperature on enzyme activity indicate that the organisms had enzyme activity at all ~~the~~ incubation temperatures but the optimum temperature for enzyme activity was 50°C (116.5 IU/ml) for *Aspergillus niger* and 40°C (121.86 IU/ml) for *Aspergillus terreus*. Enzyme activity then declined up to 49 IU/ml and 47.57 IU/ml at 80°C for *Aspergillus niger* and *A. terreus*, respectively. The details of the effects of incubation temperature on enzyme activity ~~is~~are shown in Figure ~~2~~4.

The results of the effects of varying the pH range from 3-10 on enzyme activity for both test fungi are presented in Figure ~~3~~5. The optimum protease activity for *Aspergillus niger* was recorded at pH 9 (106.85 IU/ml) and optimum protease activity for *Aspergillus terreus* was recorded at pH 8 (107.9 IU/ml). The decline on enzyme activity was observed at pH 10 with *Aspergillus niger* and *A terreus* recording 78 IU/ml and 90 IU/ml respectively.

~~The effects of~~Using different substrate concentration ~~of substrate(~~ of skim milk showed that protease activity ~~had a~~ gradual increase with an increase in ~~substrate~~ concentration (**Fig.4**). Enzyme activity was highest at 2% concentration for both *Aspergillusniger* and *A. terreus* yielding 87.21U/ml and 76.5 IU/ml, respectively.

~~Plate~~Figure1. (a) Culture of *Aspergillus niger*b) Microscopic Image of *Aspergillus niger*



Comment [G11]: Actually, the same values are observed at low pH as well. These values are not commented at all.

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~~Plate~~Figure 2:(a) Pure Culture of ***Aspergillus terreus***.(b) Microscopic image of ***A. terreus***

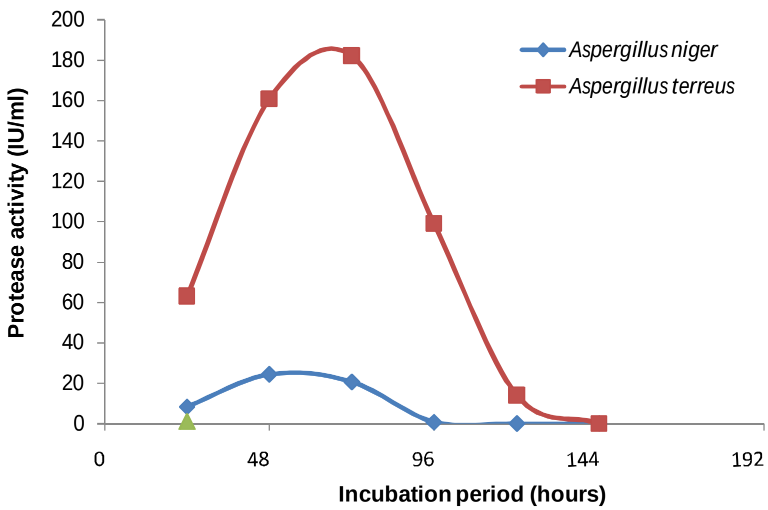

Figure 3.Effects of Incubation period on Protease Production.

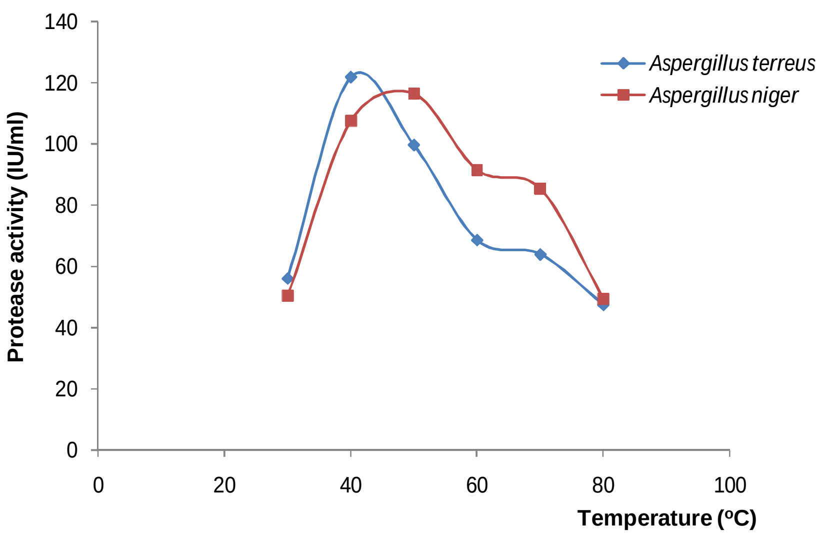

Figure 2: Effects of Different Temperature on Protease Production

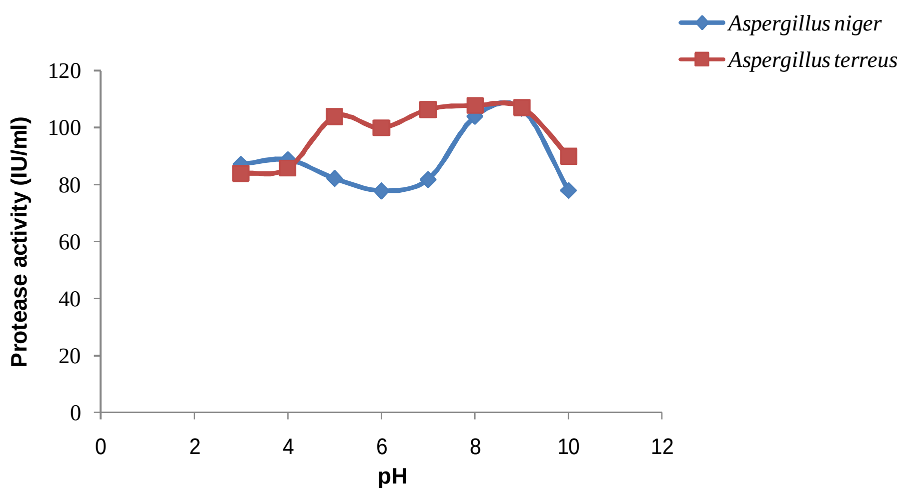

Figure 3: Effects of Different pH on Protease Production

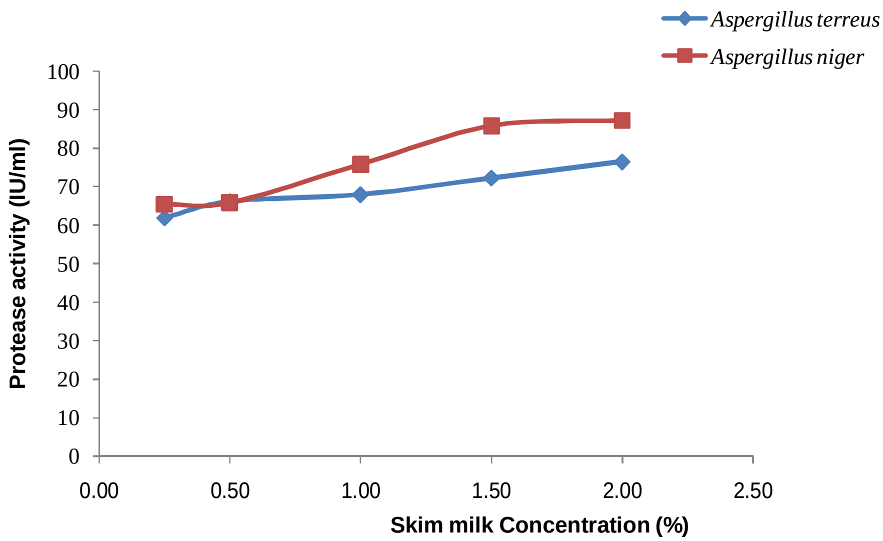

Figure 4: Effects of Different Substrate Concentration on Protease Production

4. DISCUSSION

The proteolytic activity of *Aspergillus niger* and *A. terreus* was studied using skim milk casein as asubstrate. Incubation period was an important parameter for enzyme production forthe investigatedfungi ~~such as~~*Aspergillus niger* and *A. terreus*. *Aspergillus niger*showed a maximum protease activity ~~by~~on the second day (48 hours) of its incubation after that there was a slight fall in protease activity. The maximum protease activity (Fig. 1) for *Aspergillus terreus* was observed ~~in~~on day 3 (72 hours) after which there was a decline which could be due to the exhaustion of nutrients as reported by [1,13,16]. The findings of this research work ~~is~~arein linewith the work of Chellapandi [17]. For both *Aspergillus niger* and *A. terreus* there was a gradual decrease in enzyme activity with increasing incubation period clearly suggesting the enzymes role as primary metabolite being produced in the log phase of the growth of the fungi for utilization of nutrients (proteins) present in the substrate. The later decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases [18].

Environmental conditions play an important role in the microbial growth and in suppression or initiation of the enzymes by specific compounds [19]. Temperature is a critical parameter that needs to be controlled and is usually varied from one organism to the another [20]. The metabolic activities of microorganisms are affected by high

Comment [G13]: The authors pretent slight but there is decrease to 0.

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Comment [G15]: It is not varied just like that. Here the sentence has to stress on the temperature effect on the specific parameters temperature resulting in adverse effects [1]. The optimal temperature for fungal proteases ranged between 35°C and 50°C with few exceptions [21]. For *Aspergillus niger* (Fig 2), there was a gradual increase in protease activity from 30°C ~~to 40°C and~~ up to ~~an optimum at~~ 50°C (116.5 IU/ml) and then decline from 60°C to 80°C~~,~~Similar result was ~~recorded~~reported by Siala*et al* [22]. For *Aspergillus terreus* (Fig 2), the results showed that there was a gradual increase in protease activity ~~from 30°C and~~ aup to ~~maximum at~~ 40°C (121.86 IU/ml) and then successive decrease ~~from 50°C to 80°C~~. The optimum temperature 40°C as observed in this study is in line with the previous works by other researchers [23, 24] ~~who reported a similar result for *Aspergillus terreus*~~. Fungal proteases are usually thermo labile and show reduced activities at high temperature. Higher temperature is found to have adverse effects on metabolic activities of microorganisms and cause inhibition to the growth of fungi. The enzyme is denatured by losing its catalytic properties at high temperatures due to stretching and breaking of weak hydrogen bonds within enzyme structure [25].

Secretion of enzyme by microbial strains is strongly influenced by the extracellular pH. Changes in pH can make and break inter- and intramolecular bonds leading to ~~and~~ changes in the structure of the enzyme and its effectiveness [1]. The transport of nutrients and enzymatic processes across the cell membrane are strongly influenced by the culture pH [14]. From the result obtained (Fig. 3) *Aspergillus niger* showed an optimum activity at pH 9.0. According to reports recorded by Devi [26] and Dubey *et al.* [27], *Aspergillus niger* produced proteases at varied pH in the ranges(4.4- 9.0). *Aspergillus terreus* ~~had~~optimum activity was observed at pH 8.0. Gopulkumar *et al.* [28] reported a pH optimum for *Aspergillus terreus*also at 8.0 ~~which agrees with the results of this research~~.

Substrate concentration critically affectstheenzyme activity. Enzyme production ~~was~~ influenced by nitrogen source;and skim milk (casein) has been reported to be a major nitrogen source for many microorganisms for maximum protease production [29]. From the result in Fig. 4, *Aspergillus niger* and *A. terreus* showed a maximum protease activity at 2.0% substrate concentration. According to studies by Kezia [29], it was reported that protease showed the highest activity at the highest concentration of the substrate.

5. CONCLUSION

Proteases have wide range of applications in different industries. The findings of this studies indicates the options that fungi isolates; *Aspergillus niger* and *Aspergillusterreus* produces proteases which can be used in diverse industries.

Comment [G16]: Terible sentence.

Comment [G17]: 1.6 % is the higest concentration!

Comment [G18]: The conclusion has to include all main findings and achievments f the study.

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