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Enhanced Production of Salinity-Induced Proteases from *Aspergillus flavus* and *Aspergillus niger*

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Abstract

Proteases are important industrial enzymes that account for about 60% of the total enzyme market globally due to their large application in food, feed, textile and pharmaceutical industries. The effect of salt stress on protease production was evaluated on *Aspergillus flavus* and *Aspergillus niger*. The enzyme production was enhanced by stepwise optimization of the culture parameters, notably, carbon source, nitrogen source, pH, and temperature of the submerged fermentation process while using a minimal salt media and casein as substrate for the protease activity. The fungi species were found to be good producers of both acid and alkaline proteases under 4% salt stress condition. The optimum culture conditions for alkaline protease production by *Aspergillus flavus* were sucrose 4%, peptone 1%, pH 8 at 40˚C with maximum enzymatic activities of 8.85 mM/min/mg protein, 5.22 mM/min/mg protein, 3.75 mM/min/mg protein, and 1.64 mM/min/mg protein, respectively. Lactose 4%, peptone 1%, pH 6 at 50˚C were the optimum culture conditions for acid protease production by *Aspergillus flavus* with maximum enzymatic activities of 4.59 mM/min/mg protein, 2.06 mM/min/mg protein, 1.24 mM/min/mg protein, and 1.23 mM/min/mg protein, respectively. For *Aspergillus niger*, the optimum culture conditions for alkaline protease production were corn starch 4%, yeast extract 1%, pH 6 at 40˚C with maximum enzymatic activities of 5.99 mM/min/mg protein, 3.85 mM/min/mg protein, 6.18 mM/min/mg protein, and 3.72 mM/min/mg protein, respectively. While lactose 4%, yeast extract 1%, pH 6 at 50˚C were the best culture conditions for acid protease production by *Aspergillus niger* with maximum enzymatic activities of 4.81 mM/min/mg protein, 0.93 mM/min/mg protein, 5.71 mM/min/mg protein, and 3.34 mM/min/mg protein, respectively.

Keywords

Protease, Salt-Stress, Fermentation, Enzymes, Optimization

1. Introduction

Microbial proteases are degradative enzymes, which catalyse the total hydrolysis of proteins [1]. Proteases are the most important industrial enzyme accounting for about 60% of the total enzyme market in the world and approximately 40% of the total worldwide enzyme sale [2]. They are very essential in the production of detergents for protein stain removal [3] [4]. In dairy industry, proteases are used to coagulate milk protein forming curds, which are used for cheese preparation. In food industry, proteases are essential for improving the functional, nutritional and flavour properties in proteins especially in baking where they are used to degrade proteins in flour for biscuits, crackers and cookies. In pharmaceutical industry also, proteases give a wide application such as in treatment of clotting disorder [5] and in the production of digestive and certain medical treatments of inflammation and virulent wounds [6].

According to Oyeleke *et al.* (2010), extracellular proteases can be isolated from various sources including plants, animals and microbes via fermentation process [7]. Although, most commercial proteases originated from bacteria belonging to the genus *Bacillus*, fungi exhibit a wider variety of proteases than bacteria. Fungi are the preferable source of proteases owing to the limited space required for their cultivation, biochemical diversity and their ready susceptibility to genetic manipulation [8]. In addition, fungi are normally GRAS (generally regarded as safe) strains and they produce extracellular enzymes, which can be recovered easily from the fermentation broth [9].

The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients [1]. The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases [10]. It can affect growth of the microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces. Another important environmental factor is the incubation temperature, which is important to the production of proteases by microorganisms. Higher temperature is known to affect the metabolic activities of microorganisms producing proteolytic enzymes [11]. However, some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature [1] [10]. In the production of proteases, it has been shown to be inducible and was affected by the nature of the carbon source and nitrogen source used in fermentation. Therefore, the choice of an appropriate carbon source and nitrogen source is also of great importance.

This study was carried out to determine the optimum pH, the optimum temperature, the best carbon source, and the best nitrogen source for extracellular

protease production from *Aspergillus flavus* and *Aspergillus niger* under 4% salt stress.

2. Materials and Methods

2.1. Chemicals Used

Hydrated magnesium sulfate ($MgSO_4 \cdot 7H_2O$), hydrated iron (II) sulfate ($Fe_2SO_4 \cdot 7H_20$), sodium chloride (NaCl), ethanol, dipotassium monohydrogen phosphate (K_2HPO_4), and monopotassium dihydrogen phosphate (KH_2PO_4) were purchased from BDH chemicals (Philadelphia, USA.). Trichloracetic acid (TCA), sodium acetate (CH_3COONa), sodium carbonate (Na_2CO_3), Folin-Ciolcalteau's reagent, bovine serum albumin (BSA), and tyrosine standard were purchased from Sigma-Aldrich chemical company (St. Louis, MO, USA.). Potato dextrose agar (PDA), corn starch, maltose, lactose, sucrose, yeast extract, peptone, and urea were of analytical standard and were obtained from the Laboratory of the Department of Biochemistry, Federal University of Technology, Akure (FUTA), Ondo State, Nigeria.

2.2. Test Organisms

Aspergillus flavus and *Aspergillus niger* used for this study were collected from culture bank in Microbiology Department, FUTA. The pure stocks were grown on PDA for 72 hours (h) and were repeatedly sub-cultured to get pure cultures of microorganisms for this study. The pure cultures were stored on agar slant in a refrigerator for 72 h and were subcultured after every 72 h.

2.3. Fermentation

Submerged fermentation was carried out in 250 ml Erlenmeyer flask and contained the following: Carbon source 4%, nitrogen source 1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, $Fe_2SO_4 \cdot 7H_2O$ 0.001%, NaCl 4%, pH 6. These were autoclaved at 121˚C for 20 mins at a pressure of 15 psi. After cooling, inoculation of *Aspergillus flavus* and *Aspergillus niger* were done in aseptic conditions. The Erlenmeyer flasks were agitated at 150 rpm in an RSB-12 Remi Lab Water Bath Shaker (Maharashtra, India) for 72 h to promote submerged growth of the fungi, and by extension, production of the enzyme of interest. After fermentation, the culture broths were centrifuged at 5000 rpm for 20 mins at 4˚C in a TGL-16M Tabletop High-Capacity Refrigerated Centrifuge (Shanghai, China). The supernatant was used as crude enzyme extract. Meanwhile, the fermentation parameter under study in each case was varied while other fermentation parameters were kept constant.

2.4. Determination of Protein Concentration

The protein concentration of each crude enzyme sample was determined spectrophotometrically by the method of Bradford [12] using BSA as a standard.

2.5. Assay of Protease Activity

Protease activity was assayed by the modified method of Anson [13] using casein as substrate. One unit of enzyme activity was defined as the amount of enzyme liberating one μmole of tyrosine/ml/min under the defined conditions.

2.6. Effect of Carbon Sources on Protease Production

The effect of various carbon sources on the production of the extracellular protease from *Aspergillus flavus* and *Aspergillus niger* was determined by varying the carbon source used. Corn starch was substituted with sucrose, lactose, and maltose in the normal assay medium while other conditions were maintained during the experiment.

2.7. Effect of Nitrogen Sources on Protease Production

Organic nitrogen sources—yeast extract, peptone, and urea—were used as the nitrogen source in the minimal salt media to determine the best nitrogen source for the production of extracellular protease from *Aspergillus flavus* and *Aspergillus niger*. Other conditions were maintained during the experiment.

2.8. Effect of pH on Protease Production

The pH of the minimal salt media was varied from pH 5 to pH 9 to determine the optimum pH for protease production from *Aspergillus flavus* and *Aspergillus niger*. The pH of the medium was adjusted by using either 50 mM K_2HPO_4 or 50 mM KH_2PO_4. Other conditions were kept constant.

2.9. Effect of Temperature on Protease Production

The effect of temperature on the production of extracellular protease from *Aspergillus flavus* and *Aspergillus niger* was determined by incubating the fermentation flasks at 40˚C, 50˚C, 60˚C, 70˚C, and 80˚C. Other conditions were kept constant.

2.10. Statistical Analysis

Microsoft Excel 2016 was used to analyse the data obtained from this study.

3. Results

3.1. Effect of Carbon Sources on Protease Production

Sucrose gave the highest enzymatic activity, 8.85 mM/min/mg protein, as carbon source for the production of extracellular alkaline protease from *Aspergillus flavus*. But corn starch with an enzymatic activity of 5.99 mM/min/mg protein served as the best carbon source for extracellular alkaline protease production from *Aspergillus niger* (**Figure 1**). However, lactose gave the highest enzymatic activities, 4.59 mM/min/mg protein and 4.81 mM/min/mg protein, when used as carbon source for the production of extracellular acid protease from *Aspergillus flavus* and *Aspergillus niger*, respectively (**Figure 2**).

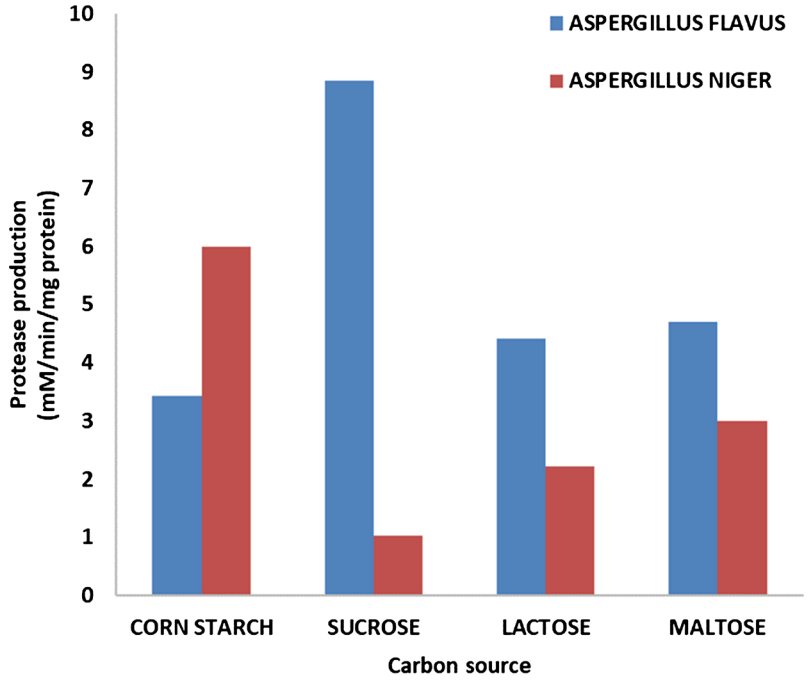

Figure 1. Carbon source variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 8.

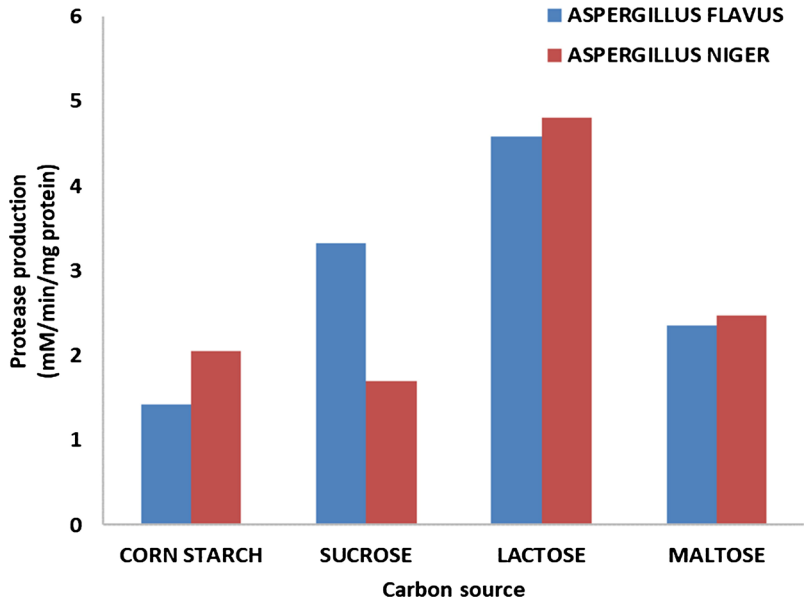

Figure 2. Carbon source variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 4.8.

3.2. Effect of Nitrogen Sources on Protease Production

Peptone was the best nitrogen source to produce extracellular alkaline protease and acid protease from *Aspergillus flavus* with enzymatic activities of 5.22 mM/min/mg protein and 2.06 mM/min/mg protein, respectively (Figure 3 and Figure 4). However, yeast extract gave the highest enzymatic activities of 3.85 mM/min/mg protein and 0.93 mM/min/mg protein for the production of ex-

tracellular alkaline protease and acid protease from *Aspergillus niger* respectively (Figure 3 and Figure 4).

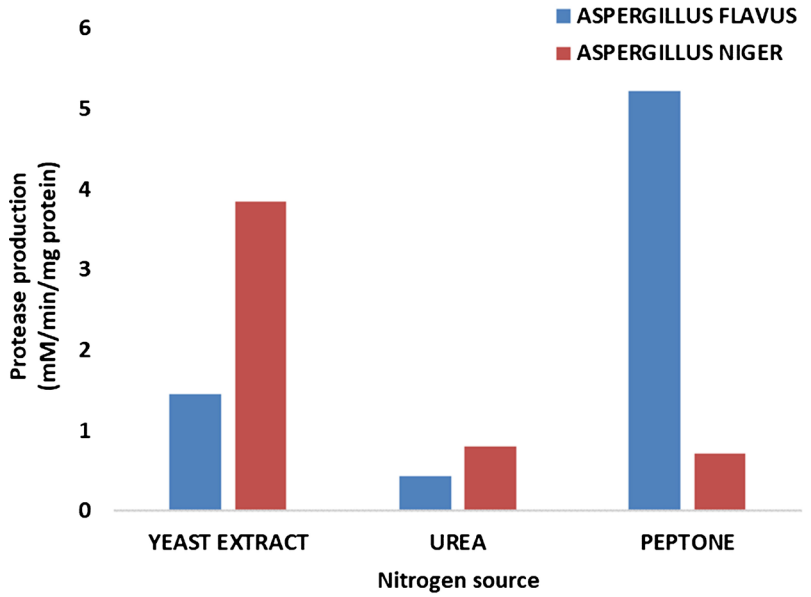

Figure 3. Nitrogen source variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 8.

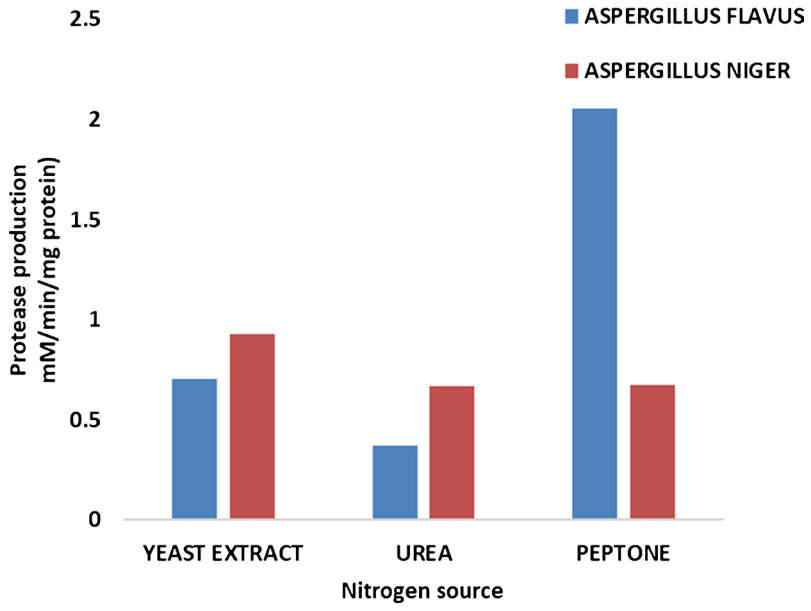

Figure 4. Nitrogen source variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 4.8.

3.3. Effect of pH on Protease Production

An optimum yield of extracellular alkaline protease from *Aspergillus flavus*—at 3.75 mM/min/mg protein—was achieved when the production pH was 8 (Figure 5) while pH 6 gave the highest yield, 1.24 mM/min/mg protein, of extracellular acid protease from *Aspergillus flavus* (Figure 6). For *Aspergillus niger*, the optimum

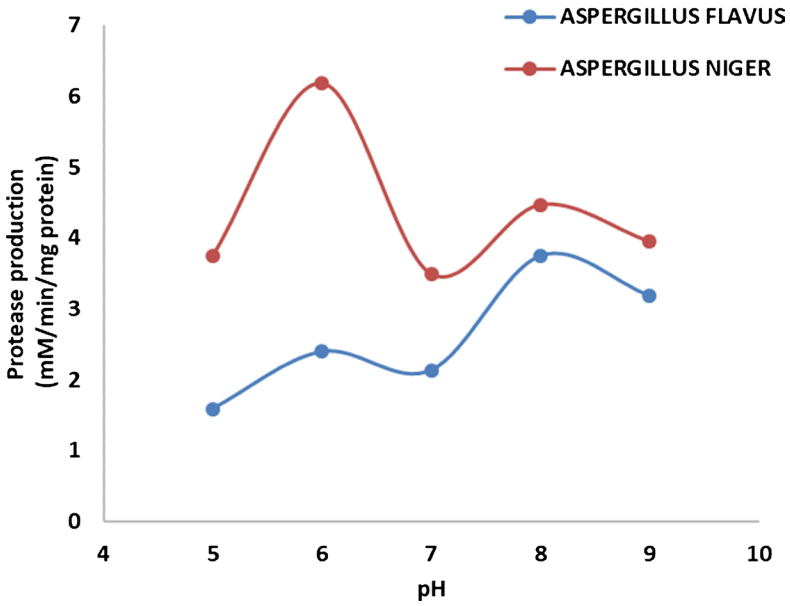

Figure 5. pH variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 8.

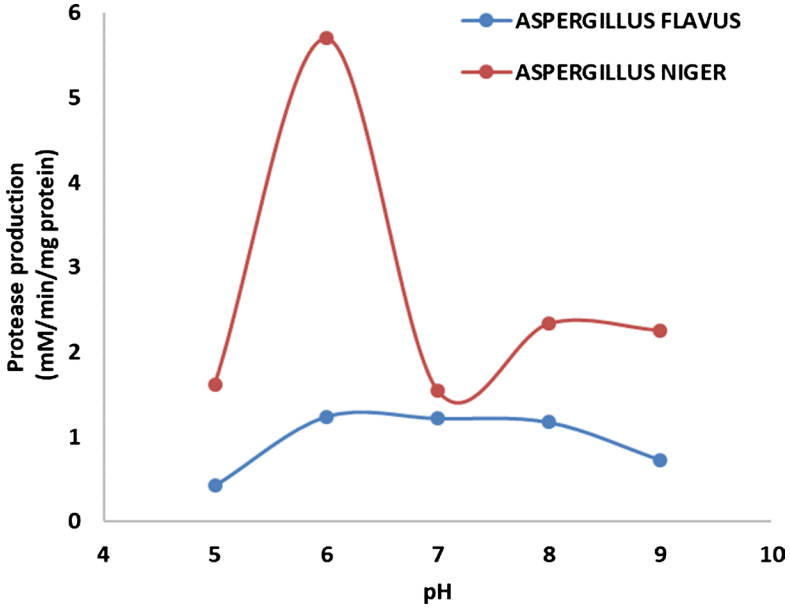

Figure 6. pH variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 4.8.

pH for the production of extracellular alkaline protease and acid protease was pH 6 with optimum yields of 6.18 mM/min/mg protein and 5.71 mM/min/mg protein, respectively (Figure 5 and Figure 6).

3.4. Effect of Temperature on Protease Production

The production of extracellular alkaline protease from *Aspergillus flavus* and *Aspergillus niger* was optimized at 40˚C with enzymatic activities of 1.64 mM/min/mg protein and 3.72 mM/min/mg protein respectively (Figure 7). However, the

production of extracellular acid protease from *Aspergillus flavus* and *Aspergillus niger* was optimized at 50˚C with 1.23 mM/min/mg protein and 3.34 mM/min/mg protein as the enzymatic activities respectively (Figure 8).

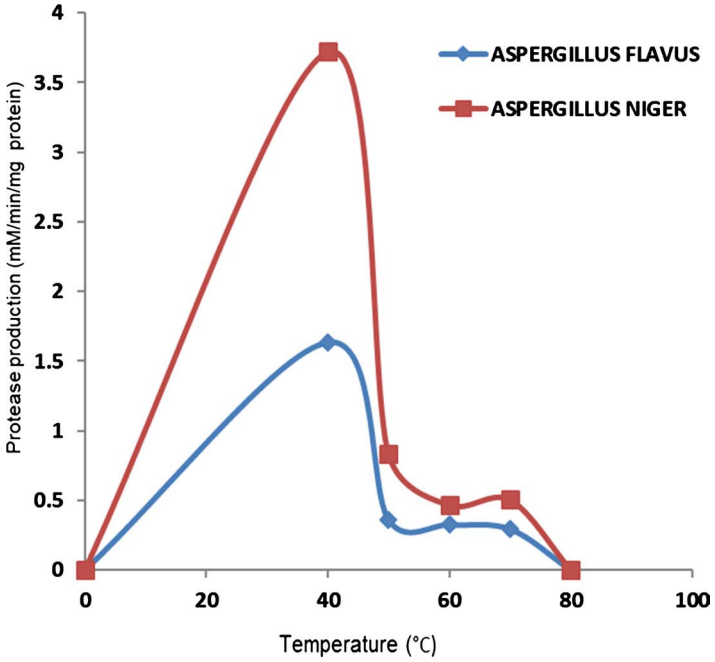

Figure 7. Temperature variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 8.

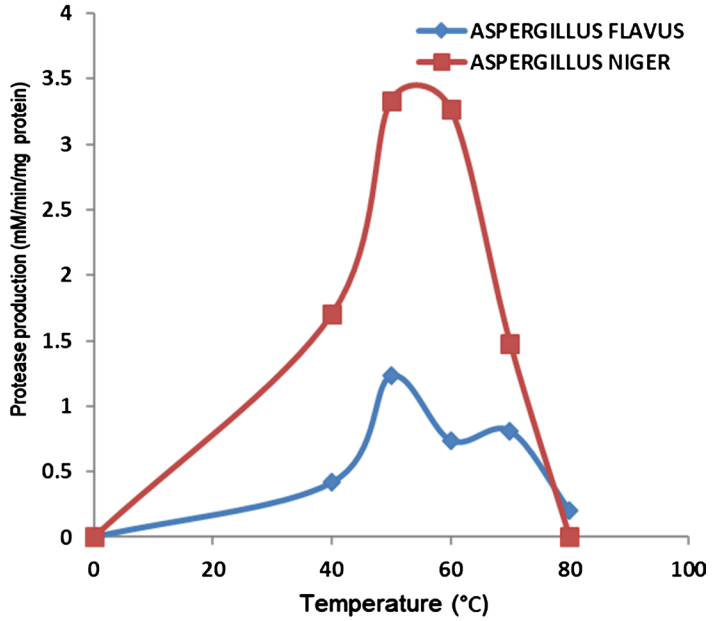

Figure 8. Temperature variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 4.8.

4. Discussion

During the preliminary stage of this study, the NaCl concentration in the minimal

salt media was varied at an interval of 2% from 0% to 6% for alkaline and acid protease production by *Aspergillus flavus* and *Aspergillus niger* (**Figure S1**). The result showed that maximum extracellular alkaline and acid proteases were produced when the *Aspergillus* spp. were subjected to 4% salt stress. The 4% salt stress was able to create an isotonic environment for the fungal species, which in turn supported cell growth and protease production.

It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the fermentation conditions. There are several reports showing that different carbon sources have different influences on extracellular protease production by different microorganisms [14]. Increased yields of alkaline proteases have been reported by other workers who used different sugars such as lactose, maltose, sucrose and fructose [15] [16], though not under salt stress. Our study corroborates these reports. Lactose was the best carbon source for the production of extracellular acid protease by *Aspergillus flavus* and *Aspergillus niger*; while sucrose and corn starch were the best carbon source for extracellular alkaline protease in *Aspergillus flavus* and *Aspergillus niger*, respectively.

Kumara *et al.* (2012) have shown that organic nitrogen sources are beneficial in the production of microbial proteases. They reported that peptone and yeast extract are the ideal nitrogen sources for producing extracellular protease from microorganisms [17]. In this study, peptone was the best nitrogen source for *Aspergillus flavus* while yeast extract was the best nitrogen source for *Aspergillus niger* for producing alkaline extracellular protease and acid extracellular protease in the fungi *sp.*, respectively. This is in line with the findings of Kumara *et al.* (2012).

Protease production by microbial strains strongly depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production [18]. In this study, the production of extracellular alkaline and acid proteases from *Aspergillus flavus* and *Aspergillus niger* was optimized at a pH range of 6 - 8. At pH below or above this range, the protein structure of the protease could change, thus leading to an observable decline in the enzymatic activity.

Temperature is an important factor that has effects on enzymes. Paranthaman *et al.* (2009) reported that fermentation carried out at 35˚C was best suited for the production of fungal proteases [6]. Higher temperature has been reported to have some adverse effects on metabolic activities of microorganisms and also inhibit the growth of fungi [11]. In this study, the production of extracellular alkaline and acid proteases from *Aspergillus flavus* and *Aspergillus niger* was optimized at a temperature range of 40˚C - 50˚C. Above this temperature range, the metabolic activities of *Aspergillus flavus* and *Aspergillus niger* will be adversely affected and their cell growth will be inhibited. Furthermore, their enzymes will be denatured because at higher temperature, the weak hydrogen bonds within the enzyme structure will be broken. To the best of our knowledge, this study is

the first to optimize the culture conditions for the production of extracellular acid and alkaline proteases from *Aspergillus flavus* and *Aspergillus niger* under salt stress.

The continuous sourcing and production of industrially useful biocatalysts are invariably important for advancement in biotechnological processes. Taken together, the salt stress condition employed in this study favours an enhanced extracellular production of both acid and alkaline proteases from *Aspergillus flavus* and *Aspergillus niger* which could find considerable importance in industrial processes.

Authors' Contributions

All authors contributed to the development of methodology. MOO and OSB performed the experiments. All authors analyzed and interpreted the data. MOO wrote the manuscript. All authors read and approved the final manuscript and have agreed to be accountable for all aspects of the work.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

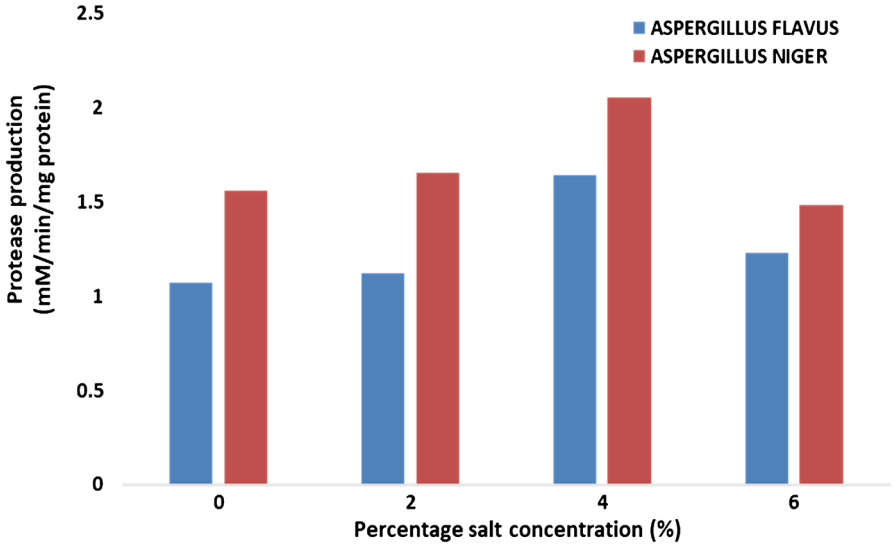

Figure S1. Percentage salt concentration variation for extracellular protease production from *Aspergillus flavus* and *Aspergillus niger* in a medium containing casein at pH 7.0.