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Production of Cellulase and Protease from Microorganisms Isolated from Gut of *Archachatina marginata* (Giant African Snail)

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Abstract Cellulase and protease producing microorganisms were isolated from the gut of *Archachatina marginata* (Giant African snail). The microorganisms isolated were *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus casseliflavus*, *Streptococcus faecalis*, *Aspergillus flavus*, *Rhizopus* sp, *Fusarium* sp. and *Aspergillus niger*. When the isolates were tested on skimmed milk agar (1%) and Carboxymethyl Cellulase, *B. subtilis* and *A. niger* were the best producing microorganisms for both cellulase and protease enzyme. For cellulase production, *B. subtilis* revealed its highest biomass yield after 24 hours of incubation with activity of 0.62mg/ml/sec^{-4} at 80℃ and pH of 5. *Aspergillus niger* showed its highest biomass yield after 5 days with an activity of 0.74mg/ml/sec^{-4} and an optimum temperature of 60℃ and the optimum pH was 5. For protease production, *B. subtilis* was grown for 30 hours; its highest biomass yield was after 18 hours with an enzyme activity of 2.95µg/ml/sec^{-4} and an optimum temperature of 60℃, while *A. niger* was grown for 7 days and showed highest yield after 6 days with enzymatic activity of 3.14µg/ml/sec^{-}4, and an optimum temperature of 50℃. The optimum pH was at 7 and 8 for *B. subtilis* and *A. niger* respectively. The results from this study suggest that *B. subtilis* and *A. niger* can be harnessed for the production of cellulase and protease.

Keywords *Archachatina marginata*, Cellulase, Protease, Microorganisms, Enzymatic Activity

1. Introduction

Cellulose is a fibrous, insoluble, crystalline polysaccharide. It is a major polysaccharide constituent of plant cell walls, composed of repeating D-glucose units linked by α-1,4-glucosidic bonds (Jagtap and Rao, 2005) and being the most abundant carbohydrate polymer on earth (Guo *et al*., 2008). Cellulose is used as a food source by a wide variety of organisms including fungi, bacteria, plants and protists, as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, mollusks and nematodes (Watanabe and Tokuda, 2001; Davison and Blaxter, 2005). These organisms possess cellulases and the complete enzymatic system of them include three different types, thatis, exo-α-1,4-glucanases (EC 3.2.1.91), endo-α-1,4-glucanases (EC 3.2.1.4), and α-1,4-glucosidase (EC 3.2.1.21) (Wilson and Irwin, 1999). Protease (serine protease) {EC 3.4.21}, cystein (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 (Wellingta *et al*., 2003). Protease is an enzyme that breaks the peptide bonds of proteins (Mitchell *et al*., 2007). Protease breaks down peptide bonds to produce amino acids and other smaller peptides. It can be isolated from a variety of sources such as plants, animals and microbiota (fungi and bacteria). Its application is very broad and has been used in many fields for years, and is mainly used in food and detergent industries (Yandri *et al*., 2008). They find application in a number of biotechnological processes, viz. in food processing and pharmaceuticals, leather industry, detergent industry, etc. (Nascimento and Martins, 2004; Beg and Gupta, 2003).

Two third (2/3) of the industrial produced proteases are from microbial sources (Ellaiah and Adinarayana, 2002). A variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes are known to produce these enzymes (Madan *et al*., 2002; Devi *et al*., 2008). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Devi *et al*., 2008). Among the various proteases, bacterial protease is the most significant, compared with animal and fungal protease (Wellingta *et al*., 2004), and among bacteria species.

Snails contain a lot of organisms in its gut, and a variety of enzymes can be extracted and produced from it. Snails are

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cheap and can be easily obtained, especially during the rainy season. The dependence of snails on microbial activity within their gut would explain their extraordinary efficiency in plant fibre digestion (60-80%) (Charrier *et al*., 2006). In snails, the enzyme producing symbiotic protozoan present in its intestine (Charrier *et al*., 2006) hydrolyze cellulose to glucose in synergistic collaborative relationship with indigenously produced enzymes (Chevalier *et al*., 2003).

Cellulase and protease can be isolated, extracted and produced locally, thereby saving the cost of importation into the country. These enzymes are especially useful in biofuel industry, fruit juice clarity in fruit juice industry, paper and pulp industry, and biological detergents. Cellulase and protease are important commercial enzymes and the best way of producing these enzymes is via microbial fermentation which is very economical and convenient. This is because of their usefulness in pharmaceutical, paper, detergent industries and many others.

The study is aimed at isolating microorganism present in gut of snails (*Archachatina marginata*) for their potential to produce cellulase and protease and to determine the optimum temperature and pH for the enzyme activities.

2. Materials and Methods

Collection of Samples

The snails (5 in number) were obtained from Kure Ultra Modern market in Minna, Niger State, Nigeria. They were placed in a clean, covered bowl, containing sand, some vegetables and water and kept safe in the microbiology laboratory of Federal university of Technology, Minna, Niger State, Nigeria prior to its use.

Media

Media used were Sabouraud Dextrose Agar for isolation of fungi and Nutrient agar for isolation of bacteria. The media were prepared according to manufacturer's instruction and sterilized in autoclave at 121℃ for 15 minutes.

Isolation of the Microorganisms from the gut of *Archachatina marginata*

The snails were washed with clean water and the outer shell cleaned thoroughly with ethanol for surface sterilization. The shells were aseptically broken to remove its flesh and then dissected to reveal its gut. A portion of the gut was streaked onto nutrient agar plates, using inoculating loop and incubated for 18-24hours at 37℃ for bacterial growth and streaked in Sabouraud dextrose agar (SDA) plates and incubated for 25℃ for 3-5days for fungal growth. Individual colonies observed were sub-cultured on Nutrient agar and Sabouraud dextrose agar plates for another 18-24 hours and 3-5 days respectively and finally grown on agar slants to preserve the pure cultures.

Identification of Microorganisms

The bacterial isolates were characterized based on colonial morphology, cultural characteristics and biochemical tests as described by Oyeleke and Manga (2008). The isolates were identified by comparing their characteristics with those of known taxa using the Bergey's manual of determinative bacteriology (Holts *et al*., 1994).

A small portion of the mycelia growth was carefully picked with the aid of a pair of sterile inoculating needles and placed in a drop of lactophenol cotton blue on a microscope slide and covered with a cover slip. The slide was examined under the microscope, first with (x10) and then with (x40) objective lens for morphological examination as described by Oyeleke and Manga, (2008). The isolates were identified by comparing their characteristics with those of known taxa using the schemes of Domsch and Gams (1970).

Screening for Protease Enzyme

One gram (1g) of powdered milk was included into Nutrient Agar and Sabouraud dextrose agar respectively and the bacteria and fungi isolates were inoculated unto the plates, for 24 hours at 37℃, and 5 days at room temperature (28±2℃) respectively. The bacteria plates were observed for a zone of clearance after 24hours and 5days for fungi plates.

Screening for Cellulase Activity

A loopful of grown culture of isolated colonies was inoculated on Nutrient broth containing 1% Carboxymethyl Cellulose (CMC) for bacteria in conical flasks, and Potato dextrose broth also containing 1% Carboxymethyl Cellulose (CMC) for fungi. They were incubated at 37℃ for 24hours for bacteria isolates and for 5days at room temperature (28±2℃) for fungi isolates and allowed to grow. After incubation, the cellulolity activity was measured using spectrophotometer by dinitrosalicylic acid (DNSA) method (Bertrand *et al*., 2004). This was done by centrifuging the culture broth at 8, 000 revolutions per minute (rpm) for five (5) and fifteen (15) minutes for bacteria and fungi respectively. It was then filtered using Whatman's filter paper No. 1 and then one ml (1 ml) of the culture filtrate, one ml (1 ml) 1% CMC in 0.05M citrate buffer, pH 4.8 incubated for 30minutes at 37℃ then reacting with DNS reagent (to stop the growth reaction) and boiled for 5 minutes. It was then read with the spectrophotometer at 540 nM.

Cellulase Enzyme Production and Assay

The bacteria species was grown for production of cellulase in a minimum salt medium, (500ml) containing 0.05g $MgSO_4$, 0.5g $(NH_4)_2SO_4$, 1g KH_2PO_4, 3.5g K_2HPO_4, 0.25g Sodium citrate, supplemented with carboxymethyl cellulose (CMC) as carbon source in distilled water (it is sterilized at 121℃ for 15 minutes, allowed to cool before use) . The cultures were grown at 37℃ for 30 hours. Culture broth was sampled at a six (6) hour interval during growth to enzyme activity in relation to biomass yield by measuring at an absorbance of 540 nM using, spectrophotometer. Cellulase activity was assayed by the determination of reducing sugar released from carboxymethyl cellulose (CMC). 0.5 ml of culture supernatant fluid was incubated with 0.5 ml 1% CMC in 0.05M citrate buffer, pH 4.8 at 40℃ for 30mins. The reducing sugar product was assayed by the dinitrosalicylic acid (DNSA) method (Bertrand *et al*., 2004), using glucose as the sugar standard.

The fungi species were placed in a minimal salt medium:

$FeSO_4$ 0.001g; $NaNO_3$, 1.5g; Na_2HPO_4, 0.5g; $MgSO_4$, 0.05; and KCl; 0.25g; Carboxymethyl cellulose (CMC); 5g in 500ml capacity distilled water (it was sterilized at 121℃ for 15 minutes and allowed to cool before use). The culture was grown for 7days at room temperature (28±2℃). The culture broth was sampled every 24 hours during growth to determine cell density/biomass growth by measuring at an absorbance of 540 nM, using spectrophotometer. Cellulase assay was done according to the method of Mandels (2005). Half milliliter of 1% CMC in 0.1M citrate buffer (pH 4.8) was placed in a test tube and 0.5 ml of culture filtrate was added. The reaction mixture was incubated at 50℃ for 30 min and the reaction terminated by adding 1.5 ml 3,5- dinitrosalisylic acid (DNSA) reagent. The tubes were heated at 100℃ in a boiling water bath for 5 minutes and then cooled at room temperature (28±2℃). The absorbance was read at 540 nM. Enzyme activity is expressed as mg/ml glucose released per min-1ml of culture filtrate as enzyme solution.

Culture filtrate was obtained by filtration through Whatman No. 1 filter paper and the culture filtrate served as the enzyme solution (Singh, 2003; El-Naghy *et al*., 1991).

Protease Enzyme production and Assay

The bacteria isolate was grown in culture medium containing 2.5g glucose, 3.75g peptone, 2.5g $MgSO_4$, 2.5g KH_2PO_4, 0.05g $FeSO_4$, 3g Casein in 500ml distilled water in a conical flask (it is sterilized at 121℃ for 15 minutes, allowed to cool before use) and incubated for 24hours. The fungal isolates were grown in KH_2PO_4, 0.5g; $MgSO_4{\cdot}7H_2O$, 2.5g; KCl, 2.5g; $FeSO_4{\cdot}7H_2O$, 0.05; glucose, 2.5g; peptone, 3.75g; casein, 5g in 500ml distilled water in a conical flask (it is sterilized at 121℃ for 15 minutes, allowed to cool before use) for protease production. The culture broth for bacteria is sampled every six (6) hours, during growth and that of fungi every 24 hours during growth to determine the enzyme activity in relation to biomass growth for protease enzyme. The protease activity was assayed by the method of Lovrien *et al*., (1985). Three ml (3 ml) of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-HCl buffer, pH 8.0 and 0.1 ml of each enzyme was incubated at 40℃. After 30 min, the reaction was stopped by adding 3 ml of cold 10% trichloroacetic acid. After 1 h, each of the culture filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate and absorbance of the supernatants was read with spectrophotometer at 540 nm. The amount of amino acids released was calculated from a standard curve plotted against a range of known concentrations of tyrosine. One unit of enzyme (μ/ml/min) was defined as the amount of enzyme that liberated 1.5 g tyrosine per ml per minute under assay conditions (Bertrand *et al*., 2004).

Effect of pH on the Production of Cellulase

The optimum pH was determined by incubating 1 ml appropriately crude enzyme, 1ml 1% CMC in 1 ml citrate buffer, pH 4.8 (Sami *et al*., 2008) different pH (4 – 9) for 30mins at 50℃. Reducing sugars thus released were estimated by the dinitrosalicyclic acid reagent method (Bertrand *et al*., 2004).

Effect of Temperature on the Production of Cellulase

The optimum temperature of CMCase was determined by incubating 1 ml appropriately diluted enzyme with 1 ml 1% CMC in citrate buffer pH 4.8 (Sami *et al*., 2008) at different temperature range (30 – 80℃) for 30mins. Reducing sugars were estimated by the dinitrosalicyclic acid reagent method (Bertrand *et al*., 2004).

Effect of pH on the Production of Protease

The effect of pH on protease production was carried out by growing both fungi and bacteria species at different pH range (4-9). Protease activity was measured using spectrophotometer.

Effect of Temperature on the Production of Protease

The optimum temperature of protease produced was studied by incubating 1 ml crude enzyme, 1 ml 1% CMC in citrate buffer, pH 5 growing both fungi and bacteria species at various temperature range (40-90℃). Protease activity was measured using spectrophotometer.

3. Results

Screening of Bacterial Isolates for the Production of Cellulase

Five bacteria were isolated from the gut of the snail. Of the four (4) bacterial isolated and screened for cellulase enzyme production, *B. subtilis* showed the highest cellulase activity (2.2 mg/ml/sec^{-4}) while *S. aureus* showed the least (Table 1).

Table 1. Screening of bacterial isolates for cellulase enzyme using spectrophotometer

Microorganisms	Optical Density (540nM)	Enzyme Activity (mg/ml sec^{-4})
Bacillus subtilis	0.14	2.2
Streptococcus casseliflavus	0.11	1.7
Streptococcus faecalis	0.09	1.4
Staphylococcus aureus	0.01	0.2

Screening of Fungi Isolates for the Production of Cellulase

Four fungi were isolated from the gut of the snails. Of the four (4) fungi isolated and screened for cellulase enzyme production, *Aspergillus niger* showed the highest cellulaase activity (14.46 mg/ml/sec^{-4}) followed by *A. flavus* (14.18 mg/ml/sec^{-4}) while *Fusarium* sp. and *Rhizopus* sp. showed the least (Table 2).

Table 2. Screening of fungal isolates for cellulase enzyme

Microorganisms Activity	Optical Density (540nM)	Enzyme (mg/ml sec^{-4})
Fusarium sp.	0.33	9.33
Aspergillus flavus	0.50	14.18
Rhizopus sp.	0.33	9.33
Aspergillus niger	0.51	14.46

Screening for Protease Enzyme

Both bacteria and fungi were tested on skimmed milk agar (1%) of which *B. subtilis* and *Aspergillus niger* showed the highest zone of clearance out of all the microorganisms isolated. They were then chosen to be used for protease

enzyme assay.

Cellulase activity

The enzyme activity of *B. subtilis* was highest yield after 24 hours as shown in Figure 1, with an activity of 0.62mg/ml/sec^{-4}. *Aspergillus niger* showed its highest biomass yield after 5 days with an activity of 0.74mg/ml/sec-^{4}, as shown in Figure 2.

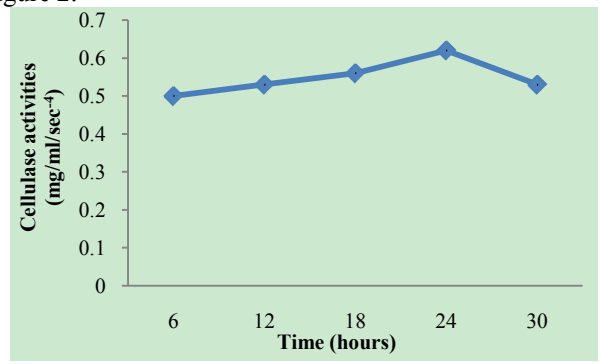

Figure 1. Cellulase activity of *B. subtilis*

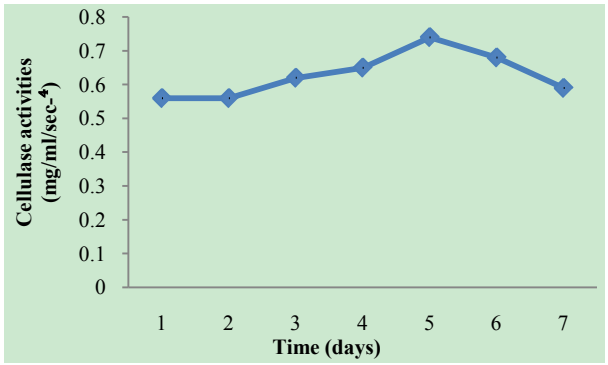

Figure 2. Cellulase activity of *A. niger*

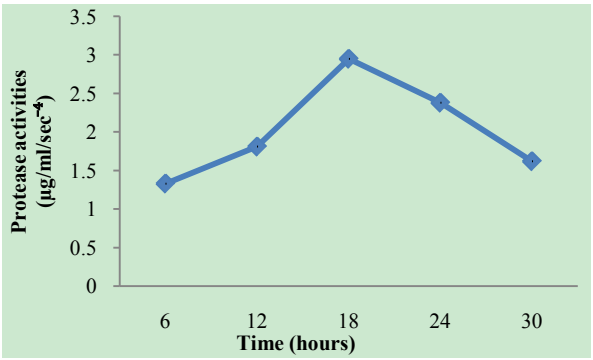

Figure 3. Protease activity of *B. subtilis*

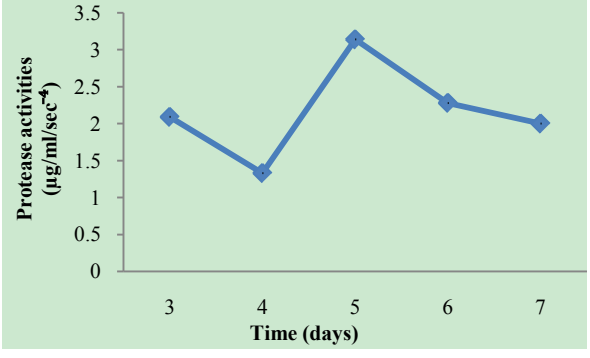

Figure 4. Protease activity of *A. niger*

Protease activity

The highest yield for protease enzyme was noticed for *Bacillus subtilis* and *A. niger* in Figure 3 and Figure 4 after 18 hours with enzyme activity of 2.95µg/ml/sec-^{4} and after 5 days with enzyme activity of 3.14µg/ml/sec-^{4} the production of protease by *B. subtilis* and *A. niger* decreased with the passage of time.

Effect of pH on cellulase production

The optimum pH of *B. subtilis* was at pH 5 with the activity of 2.85 mg/ml/sec-^{4}, pH below and above pH 5 yield low production of cellulase as shown in Figure 5 and pH 7 for *A. niger* with an activity of 0.75 mg/ml/sec-4 as shown in Figure 5.

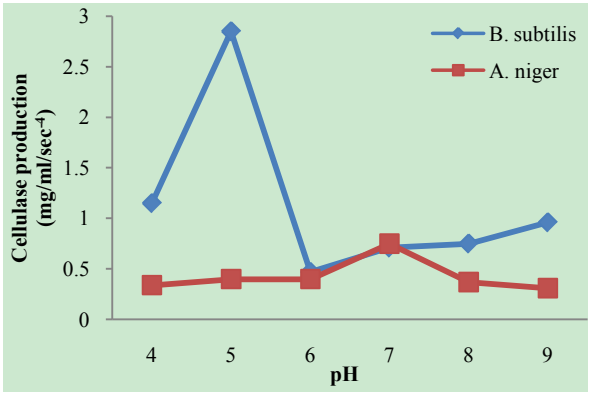

Figure 5. Effect of pH on cellulase production

Effect of temperature on cellulase production

The optimum temperature of *B. subtilis* was at 80℃ with activity of 0.81 mg/ml/sec^{-4} temperatures above and below 80℃ yielded low production of cellulase, as shown in Figure 6 and *A. niger*, was at 60℃ with an activity of 0.81 mg/ml/sec^{-4} as shown in Figure 6.

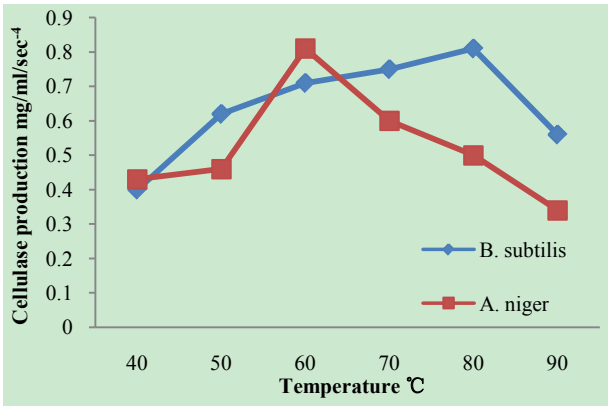

Figure 6. Effect of temperature on cellulase production

Effect of pH on protease production

The optimum pH of protease enzyme by both *B. subtilis* and *A. niger* was pH 7 and 8 respectively with an activity of 6.18µg/ml/sec-4 and 6.66×10^{-4}µg/ml/sec-4 respectively as shown in Figure 7. pH values below or above the optimum pH showed reduced enzymatic activities.

Effect of temperature on protease production

The optimum temperature for protease enzyme by *B. subtilis* was 60℃ with an activity of 4.19×10^{-4}µg/ml/sec^{-4} as

shown in Figure 8. Temperatures below or above 60℃ led to a decrease in protease enzyme yield. The optimum temperature for *A. niger* was 50℃ with an activity of 3.62 µg/ml/sec^{-4}, temperatures below or above 50℃ brought about a low productivity.

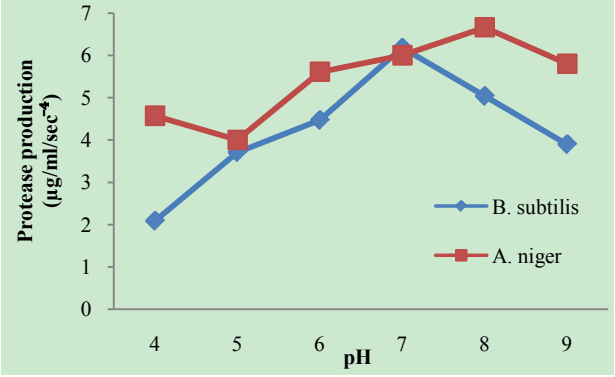

Figure 7. Effect of pH on Protease production

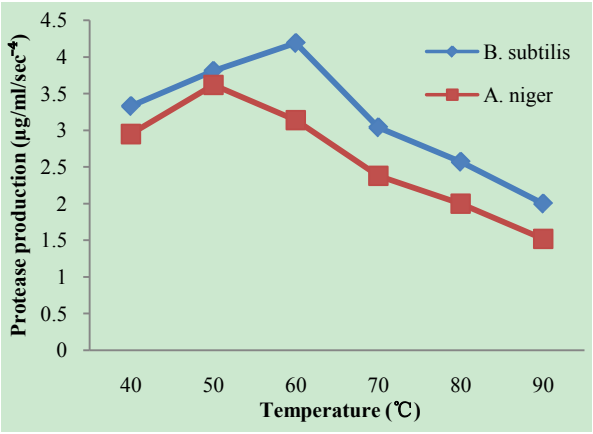

Figure 8. Effect of temperature on protease production

4. Discussion

For cellulase enzyme, *B. subtilis* was cultivated in mineral salt medium for 30 hours, at 37℃. After 24 hours, it showed its highest enzymatic activity to produce cellulase at 0.62mg/ml/sec^{-4}. These are in correlation to the findings of Mohamed *et al.* (2010) who recorded maximum cellulase productivity after 24 hours for *B. subtilis* KO strain. *Aspergillus niger* was also cultivated in mineral salt medium for 7 days at room temperature, and it showed its highest enzymatic activity after 5 days with an activity of 0.74×10^4 mg/ml/sec. These were slightly above findings by Umbrin *et al*. (2011) that recorded maximum cellulase productivity after 4 days in the solid state fermentation of *A.niger* by *Vigna mungo*. Different experiments, such as temperature and pH were carried out to optimize the culture conditions for the enzyme produced. *B. subtilis* had an optimum pH of 5, other *Bacillus* species, according to previous studies, by Ogura *et al.* (2006) had an optimum pH range of 5-6.5. The optimum temperature was at 80℃, however, this is in contrast with the findings of Umbrin *et al.* (2011) who recorded an optimum temperature of 45℃ for *B. subtilis* KO strain and *B. Amyloliquefaciens*. Ahmeh *et al*. (2004) and Kahan *et al*. (2006) recorded an optimum temperature of 40℃ for *Bacillus* strains DLG but Howard *et al.* (2003) recorded a temperature value of 65℃ as optimum for cellulase activities by *Bacillus* sp. The optimum pH for *A. niger* was 7 which is similar to the findings of Ogura *et al*. (2006), for other *Aspergillus* species with an optimum pH of 6-7. The optimum temperature was 60℃. This value is higher than that reported for *Streptomyces* sp. (Alam *et al*., 2004) with optimum temperature of 50℃, but lower than that reported for endoglucanase from *Aspergillus niger* (Howard *et al*, 2003) with optimum temperature of 70℃.

For protease enzyme, *B. subtilis* exhibited its highest enzymatic activity after 18 hours with an activity of 2.95×10^4 µg/ml/sec after cultivating in mineral salt medium for 30 hours. Although, this is different from findings by Wellingta *et al*.(2004) that had maximum enzymatic after 9 hours for *Bacillus* sp. *A. niger* had its highest enzymatic activity in 6 days, with an activity of 3.14×10^4 µg/ml/sec after cultivation in mineral salt medium for 7 days. *B. subtilis* had an optimum pH of 7, this is quite similar to the findings of Wellingta *et al.* (2004) that reported an optimum pH of 8 and optimum temperature of 60℃. *A. niger* recorded an optimum pH of 8, similar to findings by Oyeleke *et al.* (2010), and an optimum temperature of 50℃. Thus, *B. subtilis* and *A. niger* isolated from gut of *A. marginata* can potentially produce cellulase and protease and can be exploited for the commercial production of the enzymes in industries.

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