Production of Protease and Amylase from *Bacillus subtilis* and *Aspergillus niger* Using *Parkia biglobossa* (Africa Locust Beans) as Substrate in Solid State Fermentation

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Abstract  *Bacillus subtilis* and *Aspergillus niger* were utilized for the production of amylase and protease enzymes in this study. *Parkia biglobossa* (Africa Locust Beans) shell was used as substrate by both organisms for the production of amylase and protease enzyme. The optimum temperature for the activity of amylase and protease enzymes produced by *Bacillus subtilis* was 50°C, while 30°C and 40°C was recorded for amylase and protease enzyme produced by *Aspergillus niger* with an activity of 1.1 mg/ml/sec, 0.8 mg/ml/sec for amylase and protease enzyme by *Bacillus subtilis* and 0.87 mg/ml/sec, 0.77 mg/ml/sec for amylase and protease produced by *Aspergillus niger* respectively. Optimum pH was attained at pH 9 for amylase and protease enzyme produced by *Bacillus subtilis* with an activity of 1.2 mg/ml/sec and 0.83 mg/ml/sec respectively. The optimum pH for the activity of *Aspergillus niger* was recorded at pH 5 and pH 6 for amylase and protease with an activity of 0.87 mg/ml/sec and 0.74 mg/ml/sec respectively. The result showed that both organisms utilized *Parkia biglobossa* to produce extracellular amylase and protease, but the activity of amylase enzyme produced by both organisms was greater than the activity of protease enzyme and enzymes produced by *Bacillus subtilis* showed superior activity which can be useful industrially.

Keywords  *Bacillus subtilis*, *Aspergillus niger*, Amylase, Protease Enzymes, *Parkia biglobossa*

1. Introduction

Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it (Oyeleke and Oduwole, 2009).

Microbial enzymes are preferred to those from both plant and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan et al., 2003). These naturally occurring enzymes are quite often not readily available in sufficient quantities for food applications or industrial use. However, by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth, commercial quantities can be obtained. This technique, well known for more than 3,000 years, is called fermentation. Some of the typical applications include enzyme use in the production of sweeteners, chocolate syrups, bakery products, alcoholic beverages, precooked cereals, infant foods, fish meal, cheese and dairy products, egg products, fruit juice, soft drinks, vegetable oil and puree, candy, spice and flavour extracts, and liquid coffee, as well as for dough conditioning, chill proofing of beer, flavour development, and meat tenderizing. Enzymes also play a significant role in non-food applications. Industrial enzymes are used in laundry and dishwashing detergents, stonewashing jeans, pulp and paper manufacture, leather dehairing and tanning, de-sizing of textiles, deinking of paper, and degreasing of hides (Enzyme Technical Association, 2001).

The West African locust bean (dawadawa) belongs to the family leguminosa. The English common name is baobab, probably derived from the Arabic bu hibab, which means “fruit with several seeds” (Kurebgaseka, 2005). Readily available agricultural waste such as the shell of *Parkia biglobossa* which presently constitutes part of the menace to solid waste management may be used as substrate for growth by microorganism in solid state fermentation.

Agro-industrial residues are generally considered the best substrates for the solid state fermentation processes, and use of solid state fermentation 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 sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull,
The medium was prepared by weighing the following medium composition in grams per litre; Bacteriological peptone-6g, MgSO₄·7H₂O-0.5g, KCL-0.5g, substrate-1.0g. The above medium composition were dissolved in 1000ml of distilled water after which 100ml of the medium was measured into a conical flask (250ml capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121°C for 15 minutes after which they were removed and allowed to cool before the organism was inoculated.

**Preparation of the Medium Used for Amylase Production by A. niger**

The medium was prepared by weighing the following medium composition in grams per litre; KH₂PO₄-1.4g, NH₄NO₃-10g, KCL-0.5g, MgSO₄·7H₂O-0.1g, FeSO₄·7H₂O-0.01g, Substrate-20g. The above medium composition were dissolved in 1000ml of distilled water after which 100ml of the medium was measured into conical flask (250ml capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121°C for 15 minutes after which they were removed and allowed to cool before the organism was inoculated.

**Preparation of Medium used for Protease Production by B. subtilis**

The media used for optimized production of protease enzyme consisted of substrate 1% (w/v), casein 0.5%, yeast extract 0.55, KH₂PO₄ 0.2%, Na₂CO₃ 1%, MgSO₄·7H₂O 0.2%, substrate and pH 8.0. The above medium composition were dissolved in 1000ml of distilled water after which 100ml of the medium was measured into a conical flask (250ml capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121°C for 15 minutes after which they were removed and allowed to cool before inoculating.

**Preparation of Medium used for Protease Production by A. niger**

The medium was prepared by weighing the following composition in grams per litre (g/L); peptone, 5; yeast extract 3; malt extract 2, substrate 2 and pH 8. The above medium composition were dissolved in 1000ml of distilled water after which 100ml of the medium was measured into a conical flask (250ml capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121°C for 15 minutes after which they were removed and allowed to cool before inoculating.

**Extraction of Amylase and Protease Enzyme from B. subtilis**

After incubation, the production medium was centrifuged at 6000rpm for 30 min to separate the cells. The supernatant was collected as it contained the crude enzyme and stored at 4°C till further use.

**Extraction of amyrase and protease enzyme from A. niger**

After the incubation period, extraction of the crude enzyme was done by centrifugation of the fermented media at 2000rpm (revolution per minute) for 5 minute. Supernatant
collected were then filtered off using whatman’s number 1 filter paper. The filtrate contained the crude amylase enzyme.

Protease Enzyme Assay
Casein solution of 2% (1ml) was incubated with 1ml of enzyme solution and 1ml of sodium phosphate buffer (pH 7) for 20 minutes at 40°C. The reaction was stopped using 10% Tricholoroacetic acid solution. After 20 minutes, the mixture was centrifuged at 10,000rpm for 5minutes. After centrifugation the supernatant was developed with Bradford reagent and read at 580nM

Amylase Enzyme Assay
Amylase activity was assayed as described by Bertrand et al. (2004) by taking 1ml of the crude enzyme into each of the test tubes and 1ml of 1% soluble starch in sodium phosphate buffer having a pH of 6.4.

Effect of Temperature on the Production of Amylase
The effect of temperature on amylase production was carried out using the following temperature values; 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C for both B. subtilis and A. niger after which an assay was carried out based on Dinitrosalicylic acid method (DNSA), (Bertrand et al., 2004).

Effect of pH on the Production of Amylase
The effect of pH on the amylase production was carried out using the following pH values of 6,7,8,9, and 10 for B. subtilis pH values and pH values 4,5,6,7and 8 was taken for A. niger while after which an assay was also carried out based on Dinitrosalicylic acid method (DNSA) (Bertrand et al., 2004).

Effect of temperature on protease production
The effect of temperature on amylase production was carried out using the following temperature values; 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for both B. subtilis and A. niger after which an assay was carried out (Bertrand et al., 2004).

Effect of pH on protease production
The effect of pH on the protease production was carried out using the following pH values; 6, 7, 8, 9, and 10 for the B. subtilis while pH values of 4, 5, 6, 7 and 8 was used for the A. niger after which an assay was also carried out.

Determination of reducing sugar
The reducing sugars liberated were estimated by the 3, 5 Dinitrosalicylic acid (DNSA) method. The reaction mixed was incubated in a water bath at 40°C for 15minutes and the reaction was terminated by adding 1ml of the prepared DNS reagent in the reaction tubes and then immersing the tubes in a boiling water bath (100°C) for 5 minutes after which they were allowed to cool under running tap water. The absorbance of the resulting coloured solution was measured using a Jenway colorimeter at 540mm against a blank prepared by substituting the hydrolyzed sample with 5ml of distilled water. The reducing sugar content was determined by making reference to the standard curve of known concentration for glucose (Bertrand et al., 2004).

3. Result

Effect of pH on the activity of amylase enzyme produced by B. subtilis
Figure 1 shows the effect of pH on the activity of enzyme produced by B. subtilis. The optimum pH for the activity of B. subtilis was recorded at pH 9 in this study with a concentration of 1.2mg/ml/sec.

Effect of pH on the activity of amylase enzyme produced by A. niger
Figure 2 shows the effect of pH on the activity of enzyme produced by A. niger. The optimum pH for this study was recorded at pH 5 with a concentration of 0.87mg/ml.
Effect of pH on the activity of protease enzyme produced by *B. subtilis*

Figure 3 shows the effect of pH on the activity of protease enzyme produced by *Bacillus subtilis*. The optimum pH was recorded at pH 9 with a concentration of 0.83mg/ml/sec.

Effect of pH on the activity of protease enzyme produced by *A. niger*

Figure 4 shows the effect of pH on the activity of protease enzyme produced by *Aspergillus niger*. The optimum pH was recorded at pH 6 with a concentration of 0.74mg/ml/sec.

For *A. niger*, optimum temperature for enzyme activity was recorded at 40°C with a concentration of 0.77mg/ml/sec.

Effect of temperature on the activity of amylase enzyme produced by *B. subtilis* and *A. niger*

Figure 5 shows the effect of temperature on the activity of enzyme produced by *Bacillus subtilis* and *A. niger*. The optimum temperature for the activity of *B. subtilis* was recorded at 50°C with a concentration of 1.1mg/ml/sec.

The optimum temperature for enzyme activity was recorded at 30°C with a concentration of 0.87mg/ml/sec for *A. niger*.

Effect of temperature on the activity of protease enzyme produced by *B. subtilis* and *A. niger*

Figure 6 shows the effect of temperature on the activity of protease enzyme produced by *Bacillus subtilis* and *A. niger*. The optimum temperature for enzyme activity was recorded at 50°C with a concentration of 0.80mg/ml/sec.

4. Discussion

The production of amylase and protease enzymes by *B. subtilis* and *A. niger* using *P. biglobossa* as substrate in solid state fermentation was carried out in shake flask. *Bacillus subtilis* showed highest protease enzyme activity at temperature range from 40-60°C. The optimum temperature was attained at 50°C with a concentration of 0.80mg/ml/sec. Protease enzyme activity decreases from 60°C, this may be due to the fact that at higher temperature enzymes are denatured. Gitishree *et al.* (2010) reported maximum protease production at 40°C. The effect of temperature on the activity of protease enzyme produced by *A. niger* revealed that there was a sudden increase in protease production from 30-40°C with a concentration of 0.43mg/ml/sec and 0.63mg/ml/sec. However there was sudden decrease in the activity of enzyme produced by *A. niger* when the temperature was increased above 50°C. This result agrees with Kalpana *et al.* (2008), that protease production decrease with increasing temperature from 35-45°C and that protease production ceases at higher temperature. Oyeleke *et al.* (2010), reported maximum protease yield for *A. flavus* and *A. fumigatus* at 30°C.

The optimum pH for the enzyme produced by *A. niger* for this study was recorded at pH 6 with pH range of 7-8 also supporting enzyme activity. Kalpana *et al.* (2008) reported maximum protease production at for *A. niger* at pH 7-9 and recorded maximum protease production at pH 8.5. In a similar findings, Oyeleke *et al.* (2010) reported the optimum pH for the activity of protease produced by *A. flavus* and *A. fumigatus* as pH 8 and pH 5 respectively. The optimum pH for protease production by *Bacillus subtilis* in this study was recorded at pH 9 with a concentration of 0.83mg/ml/sec. In a similar findings (Gitishree *et al.*, 2010), reported maximum protease production at pH 8.

Temperature and pH are the most important factor affecting enzyme activity. The effect of temperature and pH was studied for the production of amylase enzyme by *A.


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