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## Screening of soil microorganisms for amylase production

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### ABSTRACT

Soil microbes were isolated to determine their potential to produce amylase. The microbes were isolated and identified from soils of bakery waste, local flourmill waste, using pour plate method. The organism identified includes *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger*, *Aspergillus fumigatus*, *Escherichia coli*, *Micrococcus sp.*, *Staphylococcus aureus* and *Mucor*. The organisms were screened for amylase production and only *B. licheniformis*; *B. subtilis*, *A. niger* and *A. fumigatus* showed positive result to screening. Amylase activity was assayed using 3, 5-Dinitrosalicylic acid (DNSA) method. All strains yielded high amount of amylase at optimum temperature and pH. Optimum temperature of amylase produced by *B. subtilis*, *B. licheniformis*, *A. niger*, and *A. fumigatus* was at 70°C, 70°C, 70°C, 40°C respectively with amylase activity ranging from 0.00083 – 0.00457 mg/ml/sec. The optimum pH for all the isolate was pH 7 with amylase activity ranging from 0.000471 – 0.00457 mg/ml/sec. The data obtained from optimization of amylase activity assay condition was subjected to Pearson Correlation Statistical analysis which showed that the organisms (*B. subtilis*, *B. licheniformis*, *A. niger*, *A. fumigatus*) have significant growth with moderate temperature but decrease with increase in temperature. Generally, as the incubation time increases enzyme activity increase also increase in pH decreases the enzyme activity except for *B. licheniformis*. The result obtained reveals that these isolates are good producers of amylase and could be exploited for production of amylase in food, brewery, textile and detergent industry.

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### KEYWORDS

Soil microbes;  
Amylase;  
Activity;  
Optimum;  
Assayed.

### INTRODUCTION

Many microorganisms that live in the soil play indispensable role in maintaining life of this planet degrading or chemically modifying molecules. Considerable human interest in soil organism stems from their ability to synthesize a variety of useful chemicals. In-

dustrial microbiology concern itself with the isolation and description of microorganisms from natural environment such as soil and water<sup>[1]</sup>.

Amylase is group of important enzyme which is mainly employed in starch processing industries for hydrolysis of polysaccharides like starch into simple sugar<sup>[2]</sup>. These enzymes originate from different sources

such as plants, animals and microorganism. Enzymes from microbial source generally meet industrial demand, due to their high yield and thermostability. Microbial enzyme presents a wide spectrum of characteristics that make them useful for specific applications. These includes: they are of natural origin and non-toxic, have great specificity of action and hence and can bring about reactions not easily carried out, they work best under mild condition of moderate temperature and near neutral pH, thus not requiring drastic conditions of high temperature and pH, high acidity and the likes which necessitate special expensive equipment. These characteristics and special advantage offered by microbial enzyme made many industries interested in adapting enzymatic methods to the requirement of their processes<sup>[3]</sup>.

Due to the wide range of application of amylase enzyme in various sectors such as confectionaries, baking, paper, textile, detergent and many pharmaceuticals; many researchers have studied amylase production with variety of substrate and microorganisms like bacteria, yeast and fungi. Due to the ever increasing demand for this enzyme, people are still trying to increase the productivity of amylase by variety of approach like selection of high enzyme producing strains, process optimization, usage of cheap substrate e.t.c.<sup>[1]</sup>. Therefore the aim of this study is to screen microorganisms with the potential to produce amylase from soils of bakery waste, local mill waste located in Minna, Niger State.

## MATERIALS AND METHODS

### Sample collection

Ten grams of soil were collected in sterile polythene bags from the top soil of bakery waste of Federal University of Technology Minna Bakery, local flour mill waste site in Bosso Minna and were taken to the microbiology laboratory at Federal University of Technology, Minna for analysis.

### Microbiological analysis

Isolation of both bacteria and fungi was done by serial dilution of soil sample. One gram (1g) of each of the soil sample was weighed and dispensed into 9ml of sterile distilled water as described by<sup>[4,5]</sup>.

Serial dilution was carried out up to  $10^{-6}$  diluents from which 1ml was taken and pour plated into nutrient agar and sabouraud dextrose agar for bacteria and fungi respectively. The agar plates were incubated at  $37^{\circ}\text{C}$  for 24-48hrs (bacteria) at room temperature for 3-5days (Fungi)<sup>[4,5]</sup>.

### Identification and characterization of microbial isolate

Pure isolate of bacteria were characterized and identified according to the method of Chessbrough<sup>[6]</sup>. Isolates were identified based on Gram staining colonial morphology and biochemical test while that of fungi was based on colour of aerial and substrate hyphae.

### Screening for amylase producing bacteria and fungi

Identified isolate were screened for amylolytic activity by streaking individual isolate on 1% starch nutrient agar and 1% starch sabouraud dextrose agar medium. The agar plate was incubated at  $37^{\circ}\text{C}$  for 24-48hr and that of the fungi was incubated at room temperature for 3-5days. Culture plate was flooded with lugol iodine to identify zone of clearing around culture. The diameter of zone of clearing formed represents amylolytic activity of the isolated strain as described by<sup>[7]</sup>.

### Enzyme production

Amylase production was carried out by submerge fermentation technique using production media containing  $\text{KH}_2\text{PO}_4$  1.5g/l,  $\text{NH}_4\text{NO}_3$  10g/l, KCl 0.5g/l,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1g/l,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01g/l, soluble starch 20g/l, for fungal amylase production and bacteriological peptone 6g/l, KCl 0.5g/l,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5g/l, soluble starch 1g/l for bacterial amylase production. The medium was heated to homogenize 30-40ml was distributed into 100ml flask and then sterilized by autoclaving. The medium was inoculated with 0.5ml of fungal spore. The fungal spore was taking 10ml of sterile distilled water to it and scraping it with wire loop to loosen the spore. For bacteria, a loop full of the propagated selected strain was inoculated into the medium. The medium was then placed in a shaker incubator operated at 200 rev/min for 72hours<sup>[1]</sup>.

### Extraction of enzyme

For fungi, the whole content of enzyme production

## FULL PAPER

medium containing the growing fungi was poured through a funnel fitted with Watt man number 1 filter paper, the filtrate contains the crude enzyme. For bacteria, the bacteria culture was poured into centrifuge tubes and spin for 20min at 5000 rpm, the supernatant was then decanted which represent the crude enzyme.

### Amylase enzyme assay

Amylase activities was assayed as described by Bertrand *et al.*, (2004); by pipetting 1 ml of the culture extract “enzyme” into test tubes and 1ml of 1% soluble starch in citrate phosphate buffer having a pH 6.5. The reducing sugar liberated was estimated by 3, 5-Dinitro salicylic acid (DNSA) method<sup>[7]</sup>. The reaction mixture was incubated in water bath at 40°C for 30min. A blank consisting of 1ml of soluble starch in citrate phosphate buffer (pH 6.5) was also incubated in a water bath at the same temperature and time with the other test tubes. The reaction was terminated by adding 2ml of DNSA reagent in each test tube and then immersing the tube in boiling water bath for 5min after which they were allowed to cool and 5ml of distilled water was added. The absorbance for all the test tubes was measured at 540nm with spectrophotometer. Enzyme activity was defined as the amount of soluble starch hydrolyzed by 1ml of enzyme extract in 1minute.

### Optimization of amylase activity assay condition

The influence of pH, time of incubation and temperature was measured to optimize enzyme assay condition.

### Effect of incubation time

The effect of incubation time on the enzyme production was studied by checking the enzyme activity at 24, 48, 72 and 96 hours.

### Effect of temperature on enzyme activity

Effect of temperature was determined by assaying activity of this enzyme at different temperature range 50°C, 60°C, 70°C, 80°C, 90°C upon incubation for 30minutes.

### Effect of pH on enzyme activity

Optimum pH for enzyme activity was determined by running the assay activity between pH ranges of 5.0, 6.0, 7.0, 8.0, and 9.0. The pH was varied by adding

0.1N Hydrochloric acid and 0.1N Sodium Hydroxide.

The enzyme activity for each was determined using the Dinitrosalicylic acid method as described by<sup>[7]</sup>.

## RESULTS

The effect of incubation period on growth of *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger* and *Aspergillus fumigatus* is shown in Figure 1 and 2. There was increase in cell growth as the incubation time increase, maximum cell growth 0.2nm and 0.85nm was obtained at 72 and 96 hours of incubation time.

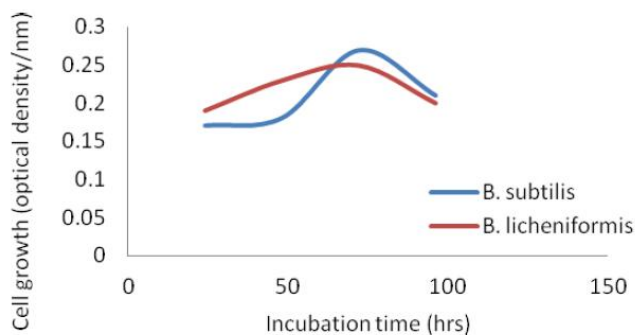


Figure 1

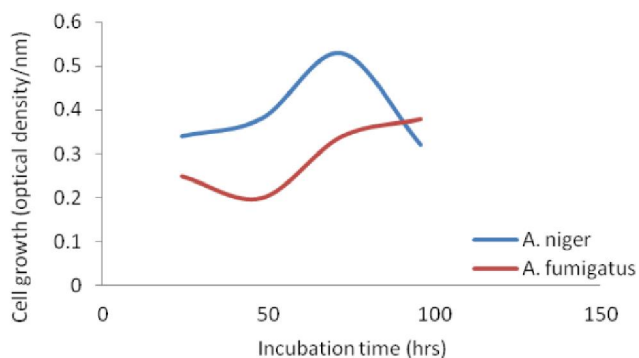


Figure 2

The effect of Incubation period on amylase activity of microbial isolates is shown in Figure 3 and 4. There was increase in amylase activity as the incubation time increases. Maximum amylase activity of 0.00035mg/ml and 0.00059mg/ml at 72hours of incubation time was obtained for bacterial isolate. Fungal isolate had maximum activity of 0.00457mg/ml and 0.000591mg/ml at 72hours and 92hours.

The effect of temperature on amylase activity of microbial isolate is shown in Figure 5 and 6. As the temperature increase, the amylase activity also increases with optimum amylase activity obtained at 70°C except for *A. fumigatus* which is at 40°C.

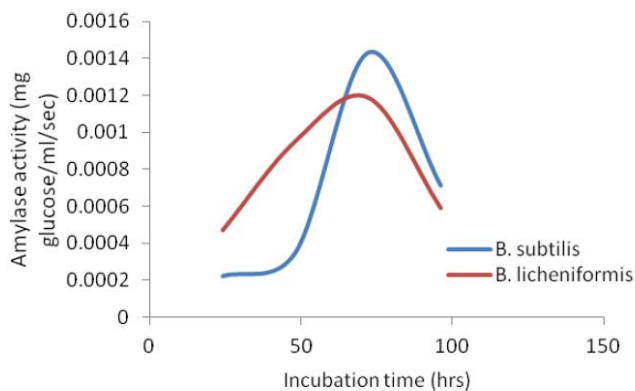


Figure 3

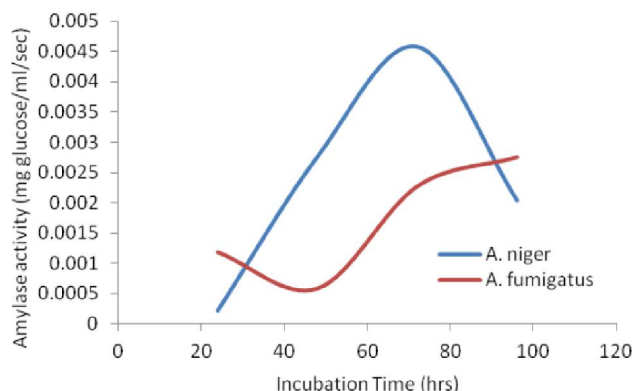


Figure 4

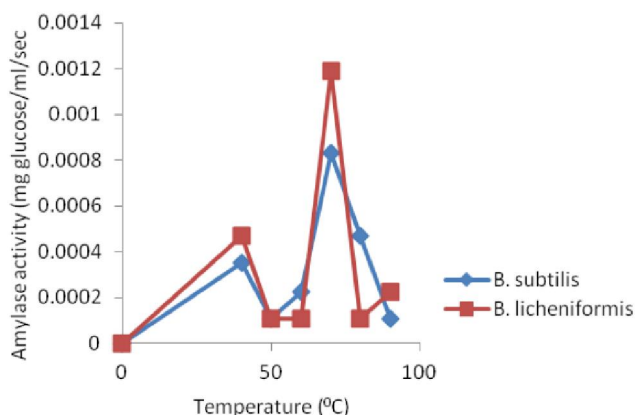


Figure 5

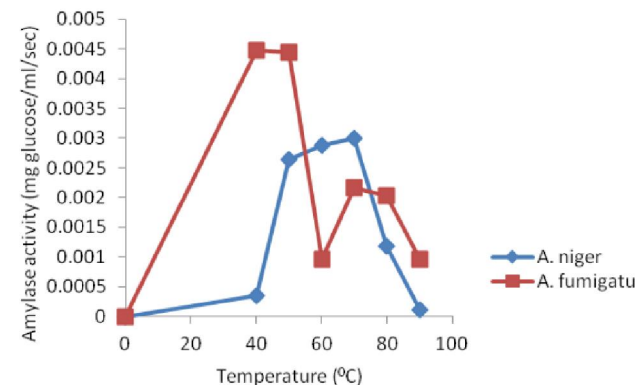


Figure 6

Figure 7 and 8 shows the effect of pH on the activity of amylase produced by microbial isolate with optimum amylase activity obtained at pH 7 for all the isolates.

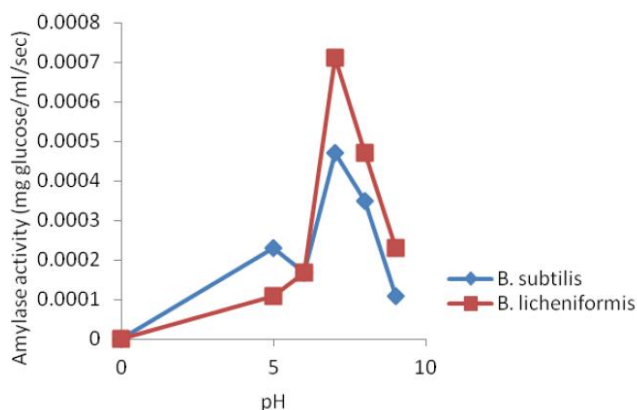


Figure 7

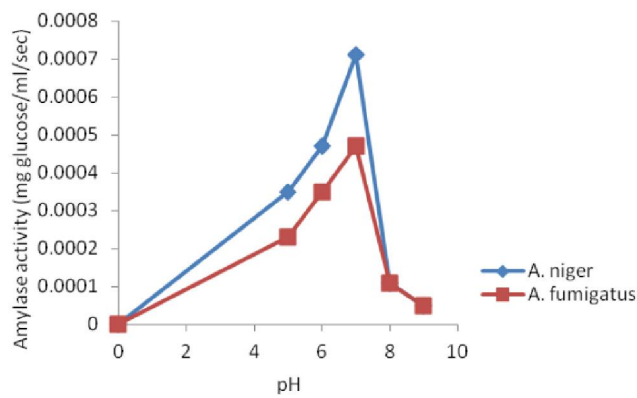


Figure 8

The Pearson correlating matrix for pH, temperature and time on enzyme activity is shown in TABLE 1 below:

The relationship between the enzyme activity and temperature is negative for all the isolates except *B. subtilis*.

The relationship between the pH and the enzyme activity is negative for all the isolates except *B. licheniformis*.

The relationship between the incubation time and the enzyme activity is positive for all the isolates.

TABLE 1 : Pearson correlating matrix for pH, temperature, and time on enzyme activity.

Organism	Temperature	Time	pH
<i>Bacillus subtilis</i>	0.095	0.607	-0.066
<i>Bacillus licheniformis</i>	-0.407	0.231	0.343
<i>Aspergillus niger</i>	-0.222	0.521	-0.563
<i>Aspergillusfumigatus</i>	-0.789	0.833	-0.552



## FULL PAPER

## DISCUSSION OF RESULT

Following biochemical test (bacteria), cultural and morphological characteristic (fungi), the isolates found include *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus aureus*, *Micrococcus* sp., *Escherichia coli*, while the fungi species are *Aspergillus niger*, *Aspergillus fumigatus*, *Mucor* sp., *Bacillus* sp. was found to be the predominate genus isolated.

This finding is in agreement with<sup>[3]</sup> and<sup>[8]</sup> who reported that *Bacillus* was the predominant genus found in hospital waste. *Bacillus licheniformis* was isolated from Bakery waste (FUT Minna Bakery). This finding was in agreement with<sup>[9]</sup> who isolate *Bacillus licheniformis* from bakery waste. It was also similar to<sup>[10]</sup> who isolated *Bacillus licheniformis* from soil sample in Kartoum state.

Many bacteria including *Bacillus licheniformis* produces extracellular amylases during the fermentation of starch, as reported by<sup>[10]</sup>.

The fungal species identified, include *Aspergillus niger* which is frequently isolated. *Aspergillus* is capable of utilizing an enormous variety of substrates because of the large number of enzyme they produce<sup>[11]</sup>.

Primary screening was carried out on starch agar plate with *Bacillus licheniformis*, *Bacillus subtilis*, *Aspergillus niger*, *Aspergillus fumigatus* showing positive result. At lag phase, there was no growth as bacteria cells adapted to the culture medium for few hours. Exponential phase followed with increase in biomass

Effect of incubation period on cell growth shows that at lag phase, there was no growth. *Bacillus subtilis* had its maximum growth of 0.27nm at 72hours.

*Bacillus licheniformis* had maximum growth of 0.25nm at 72hours upon incubation in starch medium in shaker-incubator. This reflects that the bacteria metabolize the nutrient in the culture medium. The effect in incubation period on enzyme production period revealed that the highest enzyme production was at 72hours with maximum activity of 0.000143mg/ml.

*Bacillus licheniformis* had maximum activity of 0.00119mg/ml at 72hours. Activity was lost after this hour, this is said to be stationary phase where there is no cell death and the viable cell remain constant after 72 hours, viable cells began to decline, thus resulting to death phase. Similar reports where observed by<sup>[10]</sup> re-

garding a potential new isolate for the production of thermostable extracellular  $\alpha$ -amylase. Enzyme production was found to be concomitant with cell growth. Beyond 72hours there was decline in amylase activity. This may be due to the fact that after maximum production of enzyme at maximum incubation time. There was production of other by-products. These by-products inhibited the growth of organism hence enzyme formation<sup>[12,13]</sup>. Fungi had maximum growth at the 3<sup>rd</sup> and 4<sup>th</sup> day (96h) for *Aspergillus niger* and *Aspergillus fumigatus*. This agrees with report of<sup>[14]</sup>.

Optimum temperature of amylase produced by *Bacillus subtilis* was at 70°C 6and 70°C, 80°C, 40°C for *Bacillus licheniformis*, *Aspergillus niger*, and *Aspergillus fumigatus* respectively. The four isolate had amylase activity ranging from 0.00083 – 0.00487mg/ml/sec. This result agrees with<sup>[10]</sup> who stated that *Bacillus licheniformis* was found to be active in a broad temperature range of 50°C – 90°C and show a maximum activity at 70°C. Also similar to the report of<sup>[2,3,11]</sup> that during isomerization temperature is preferably maintained within the 20°C - 90°C and the best activity is obtained with 50°C - 75°C.

Optimum pH of amylase produced by *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger*, and *Aspergillus fumigatus* is uniform for all the four isolate in that all had their optimum activity at pH 7 which was then followed by a steady decrease in amylase activity. *Bacillus subtilis* had an amylase yield of 0.000471 mg/ml at pH 7; *Bacillus licheniformis* had an amylase yield of 0.000417mg/ml at pH 7. Considerable amount of amylase was produced by *Bacillus licheniformis* at pH 9 (0.00029mg/ml). This is in line with<sup>[15]</sup> also<sup>[10]</sup>. The enzyme stability trend, as reported in present study agrees with the behavior of amylase from *Bacillus* sp. Investigated by<sup>[16]</sup> in which a soluble starch medium was used. This result agrees with<sup>[3]</sup> who recorded optimum pH activities at 6.5 to 7.5 in the production of amylase, using *Bacillus subtilis*.

Statistically, in agreement with Pearson correlation, the organisms (*B. subtilis*, *B. licheniformis*, *A. niger*, *A. fumigatus*) showed significant growth with moderate temperature but decreases with increase in temperature. Generally, as incubation time increases; enzymes activity increases positively. Also, increase in pH decreases the enzyme activity except for *B. licheniformis*.

## CONCLUSION

From the results obtained in this research, it could be concluded that organism with potential to produce amylase can be isolated from bakery waste, dump site, flour mill waste, and waste dump site. This isolates could be exploited for the commercial production of the enzyme in industry like baking, brewery, detergent, and textile industry. Optimum temperature and pH for production of amylase was in the range of 40°C – 90°C, and pH 7. This shows the amylase produced by microbial isolate is thermostable.

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