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# CHARACTERIZATION OF CELLULASE PRODUCED BY MICROORGANISMS ISOLATED FROM SOIL

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**Abstract**

*The study was undertaken to characterize cellulase produced by microorganisms isolated from the soil. Cellulolytic bacteria and fungi were isolated from soil samples collected from ten different locations within Ilorin, Kwara State, Nigeria. Organisms were grown in a minimal medium containing carboxymethylcellulose for the production of cellulolytic enzymes. Endoglucanase activity of culture filtrates which represents the crude enzyme was determined using DNS reagent. Bacillus subtilis was identified using molecular technique; and fungal isolates by macroscopic and microscopic methods. Bacillus subtilis, Fusarium oxysporium, Aspergillus niger and Penicillium italicum showed relatively high cellulase activity with diameter of clearance zone of 10, 8, 9, and 10mm respectively, and were used in the fermentation process. The endoglucanase activity of the crude enzymes ranged from 1.64 to 1.74IUml$^{-1}$. Optimum activity was obtained at temperature range of 30 to 70$^{0}$C, pH between 3 and 9 and substrate concentrations of 1 to 5%. This study showed that highly cellulolytic fungi and bacteria that can be used in lignocellulosic ethanol production are available in the environment. A further study might discover a novel organism that could be modified genetically for improved cellulose activity.*

**Key Words:** *Endoglucanase activity, cellulolytic microorganisms, fermentation, carboxymethylcellulose*

## Introduction

Cellulose is the most abundant biomass on the earth (Venkata *et al.*, 2013). Plant biomass contains cellulose as the major component. Cellulose has attracted worldwide attention as a renewable resource that can be converted into biobased products and bioenergy

(Xing-hua *et al.,* 2009). Celluloses are observed as the most important renewable resource for bioconversion. It is of economic interest to develop an effective method to hydrolyze the cellulosic biomass (Saraswati *et al.,* 2012). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently (Sonia *et al.,* 2013).

Microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro* (Sonia *et al*., 2013).

Efficient cellulase activities are observed in fungi but there is increasing interest in cellulose production by bacteria, because bacteria have higher growth rate as compared to fungi, and have good potential to be used in cellulase production (Sonia *et al.,* 2013). Beyond free microbial cellulases, there are opportunities for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. And also as the final product of cellulose degradation by cellulase enzyme is glucose, a soluble sugar, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research, biodegradation and bioremediation.

Enzymatic hydrolysis of palm oil effluent solid using mixture of locally isolated fungi *Aspergillus niger* EB5 and *Trichoderma sp*.EB6 was reported by Wong *et al*. (2008). They also observed the effect of substrate pretreatment, different ratio of cellulase mixture and incubation, pH on the enzymatic hydrolysis of palm oil effluent solids. Cellulase enzyme production was studied by Charitha and Sunil (2010) using fungal strain *Aspergillus niger* against the lignocellulosic bio wastes like sawdust, paper cellulose at varying environmental parameters. Sharada (2012) studied production of cellulase using *Trichoderma reesei* and *Aspergillus niger* with black gram husk and green gram husk and reported maximum cellulase production using green gram husk as substrate in solid state fermentation by *Aspergillus niger*.

Cellulases, in particular EGIII and CBH I, are commonly used in detergents for cleaning textiles. Clarkson *et al*., (2000) showed that EG III variants, in particular from *T. reesei*, are suitable for the use in detergents. *T. viride* and *T. harzianum* are also industrially utilized natural sources of cellulases as *A. niger* (Kottwitz and Schambil, 2005). Cellulase preparations, mainly from species of *Humicola* (*H. insolens* and *H. grisea var. thermoidea*) that are active under mild alkaline conditions and at elevated temperatures, are commonly added in washing powders (Mitchinson and Wendt, 2001) and in detergents (Uhlig, 1998).

The aims of the study were:

(i)To isolate and screen cellulolytic bacteria and fungi from the soil; (ii)produce cellulase from potential isolates by submerge fermentation process; (iii)partially purify culture filtrates for improved cellulase activity; (iv)assay for activity of the partially purified cellulases; (v) To optimize

parameters for culture filtrates; and (vi) identify cellulolytic bacteria and fungi.

## Materials and Methods

### *Collection of Sample*

Soil samples were collected from different areas such as garden, car park and wood furnishing region within and outside the University of Ilorin campus. Sterile spatula was used to collect over 5g of each soil into a sterile container and labeled appropriately.

### *Sterilization of Materials*

Glass materials such as conical flasks, test tube, McCartney bottles, measuring cylinders used were washed with detergent and thoroughly rinsed with water. They were allowed to dry and sterilized in the hot air oven at $171^0$C for 1hrbefore use. Wireloop was sterilized with naked flame and the petridishes and syringes used were also sterile.

### *Isolation of Cellulolytic Organisms*

***Bacteria:*** Carboxymethylcellulose After autoclaving at 121°C and $2kg/m^2$pressure, the isolation was done by the pour plate method.. Cellulolytic activity of the isolates was detected after flooding the plates with 10ml of 1%congo red for 15min and washed with 10ml of 1M NaCl for another 15min. Cellulase activity was indicated by clear zone around bacterial colony. Diameter of zone of clearance was measured across along with the diameter of the organism. A difference in the diameter represented the zone of clearance.

***Fungi:*** This was done on Czapek-Dox medium and was screened for their ability to produce cellulases complex following the method of Teather and Wood (1982).

### *Preparation of Standard Inoculum*

***Bacteria:*** For preparation of standard inoculum, the isolate that showed a high zone of hydrolysis >5 was cultured in 20 ml inoculum medium. Microbial counts were determined using spectrophotometer. . The composition of production medium was same as of inoculums medium except the concentration of carboxymethyl cellulose which was 1% instead of 0.5%.

***Fungi:*** The isolated fungal cultures were used to know their potential for cellulase production and activities.

***Assay for cellulase activities*** Carboxyl methyl cellulase activity was determined using the method of the International Union of Pure and Applied Chemistry (Ghose, 1987).

### *Optimization of Conditions for the Activity of Crude Enzyme Filtrates*

The optimum parameters were determined for cellulase activity. The crude enzyme was optimized using different parameters including temperature, pH, and substrate concentrations.

### *Partial Purification of Enzyme Filtrates with Ammonium Sulphate*

Endoglucanase activity was determined following the DNSA method recommended by International Union of Pure and Applied Chemistry (Ghose, 1987).

### *Identification of Organisms*

This was done using colonial characteristics and other morphological tests and then viewed under the microscope (Barrow and Feltham, 1993).

## Results and Discussion

Microorganisms were isolated from ten different locations (Table 1). A total of 8 cellulolytic microorganisms were

obtained comprising of 3 bacterial and 5 fungal isolates. Bacteria and fungi capable of producing cellulase exist in soil samples obtained from the car-park, flower bed and wood processing floor. Sample site G had the highest bacterial counts of $3.6x10^4$ while sample site H and J had the least bacterial counts of $1.1x10^4$. Also, sample site J had the highest fungal counts of $3.0x10^3$ while sample site H had the lowest fungal counts of $1.2x10^3$.The difference in microbial counts across the different soil samples might be attributed to the nutrient composition of such soils. Similar result was reported by Stephen *et al*. (2013).

Table 1: The microbial counts of soil samples obtained from different sites

| Sample site | Bacterial counts cfu/ml ($x10^4$) | Fungal counts cfu/ml ($x10^3$ ) |
|---|---|---|
| A | 1.7 | 1.8 |
| B | 2.9 | 1.8 |
| C | 2.4 | 2.5 |
| D | 2.2 | 2.3 |
| E | 2.8 | 2.0 |
| F | 1.4 | 2.2 |
| G | 3.6 | 1.6 |
| H | 1.1 | 1.2 |
| I | 1.4 | 1.3 |
| J | 1.1 | 3.0 |

Key

A – Car park opposite block 3 B- Flower bed of block4
C- Flower bed of block 6 D- Sawmill Geri Alimi Ilorin point 1
E- Saw-mill Geri Alimi Ilorin point 2 F- Sawmill Geri Alimi Ilorin point 3
G- Saw-mill Geri Alimi Ilorin point 4 H- Sawmill Tanke point 1
I- Sawmill Tanke point 2 J- University of Ilorin complex

Three of the bacterial isolates showed varying degrees of cellulolytic activities as shown in Table 2 with highest cellulolytic activity of >5 occurring in *Bacillus subtilis*.

Table 2: Cellulase activities of bacterial isolates

| Sample site | Isolate code | Isolate name | Diameter of zone of clearance (mm) | Diameter of colony (mm) | Zone of clearance (mm) |
|---|---|---|---|---|---|
| A | B1 | *Pseudomonas aeruginosa* | 23 | 21 | 2 |
| | B2 | *Bacillus subtilis* | 29 | 19 | 10* |
| C | B3 | *Bacillus thuringiensis* | 6 | 2 | 4 |

Key

A –Car park opposite block 3
C-Flower bed of block 6
* - Isolate with zone of clearance >5
> - greater than.

Five of the fungal isolates showed varying degree of cellulolytic activities with highest activity >5 occurring in *Fusarium oxysporum*, *Aspergillus niger*, and *Penicillium italicum* (Table 3).

Table 3: Cellulase activities of fungal isolates

| Sample site | Isolate code | Isolate name | Diameter of zone of clearance (mm) | Diameter of colony (mm) | Zone of clearance (mm) |
|---|---|---|---|---|---|
| B | F1 | *Fusarium oxysporum* | 43 | 35 | 8* |
| | F2 | *Aspergillus fumigatus* | 11 | 9 | 2 |
| C | F3 | *Penicillium notatum* | 25 | 21 | 4 |
| E | F4 | *Aspergillus niger* | 33 | 24 | 9* |
| G | F5 | *Penicillium italicum* | 30 | 20 | 10* |

Key
B-Flower bed of block4
C-Flower bed of block 6
E- Sawmill Geri Alimi Ilorin point 2
G- Sawill Geri Alimi Ilorin point 4
* - Isolates with zone of clearance >5
> - greater than.

The crude enzymes obtained from these isolates which were assayed for endoglucanase activities, with the cellulase activity ranged from 1.64-1.74IUml$^{-1}$ as shown in (Figure 1). Microorganisms with high cellulolytic activities included *Bacillus subtilis, Fusarium oxysporum, Aspergillus niger* and *Penicillium italicum.* The highest activity was seen in *Penicillium italicum* with 1.74IUml$^{-1}$, followed by *Aspergillus niger* with 1.68IUml$^{-1}$, then *Bacillus subtilis* with 1.67IUml$^{-1}$ and the least activity was observed in *Fusarium oxysporum* with 1.64IUml$^{-1}$ (Figure 1). Bacterial isolates were abundant than fungal isolates. However, fungi produced a considerable zone of clearance that was greater than 5 during cellulolytic screening with the use of the congo red test, which was indicative of a stronger cellulolytic potential compared to bacteria. A large number of the cellulolytic bacteria and fungi were found in samples originating from car park, flower bed and wood processing floor which was in consistent with the high content of decomposing wood and leaves that provided more abundant cellulosic substrates in this area. Microorganisms with high cellulolytic activity included; *Bacillus subtilis, Fusarium oxysporum, Aspergillus niger* and *Penicillium italicum.* The highest activity was seen in *Penicillium italicum* with 1.74IUml$^{-1}$, followed by *Aspergillus niger* with 1.68IUml$^{-1}$, then *Bacillus subtilis* with 1.67IUml$^{-1}$ and the least activity was observed in *Fusarium oxysporum* with 1.64IUml$^{-1}$ (Figure 1). *Fusarium oxysporum* showed its optimum activity at temperature of 60$^{0}$C with 1.75IUml$^{-1}$, pH 4.0 with 1.68IUml$^{-1}$ and substrate concentration of 3% with 1.69IUml$^{-1}$.

Cellulases of *Fusarium solani* on carboxymethylcellulose produced optimum activity at temperature of $40^0$C with 0.28g/L and pH 6.0 with 0.065g/L as reported by Lofti *et al.* (2011).

*Aspergillus niger* showed its optimum activity at temperature of $50^0$C with 1.69IUml$^{-1}$, pH 3.0 with 1.69IUml$^{-1}$ and substrate concentration of 1% with 1.73IUml$^{-1}$. Cellulases of *Aspergillus niger* produced optimum activity at $30^0$C with 7.9U/ml and pH range of 5.0 with 7.9u/ml and substrate concentration of 1% with 7.46U/ml (Jaafaru and Fagade, 2010). Optimum activity was also reported by Saliu and Sani (2012) at temperature of $50^0$C, pH 5.0 and substrate concentration of 10% with 0.34IUml$^{-1}$.

*Penicillium italicum* showed its optimum activity at a temperature of $70^0$C with 1.76IUml$^{-1}$, pH 5.0 with 1.75IUml$^{-1}$, and substrate concentration of 1% with 1.77IUml$^{-1}$. Saliu and Sani(2012) reported optimum activity of *Penicillium decumbens* at temperature of $50^0$C, pH 5.0 and substrate concentration of 10% with 0.24IUml$^{-1}$.

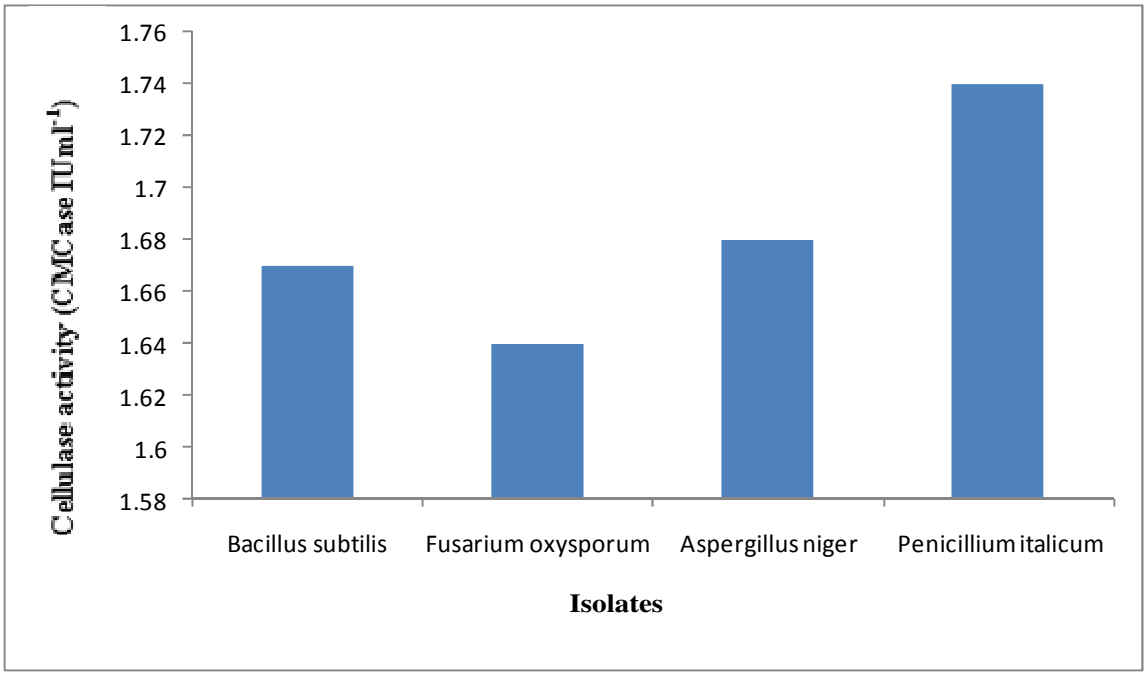


Figure 1: Endoglucanase activity of the crude enzyme from different microorganisms

The optimization of the crude enzyme at different temperature is depicted in Figure 2, the enzyme activity of *Bacillus subtilis*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium italicum* ranged from 1.65 to 1.75IUml$^{-1}$, 1.62 to 1.75IUml$^{-1}$, 1.67 to 1.69IUml$^{-1}$ and 1.69 to 1.76 IUml$^{-1}$ respectively. The crude enzymes produced by these microorganisms were optimized at temperature range 30-$70^0$C.

The growth of these microorganisms on a culture medium containing cellulose as unique carbon source (carboxymethylcellulase) indicated that the organisms had the potential to

degrade cellulose. This finding concurs with previous reports that showed small variations in carbon sources, pH, macro and micro nutrients, temperature and time of incubation induced differences in microorganism growth (Arifoglu and Ogel, 2000; Ogel *et al.,* 2001; Grigorevski de Limaa *et al.,* 2005; Ahamed and Vermette, 2008).

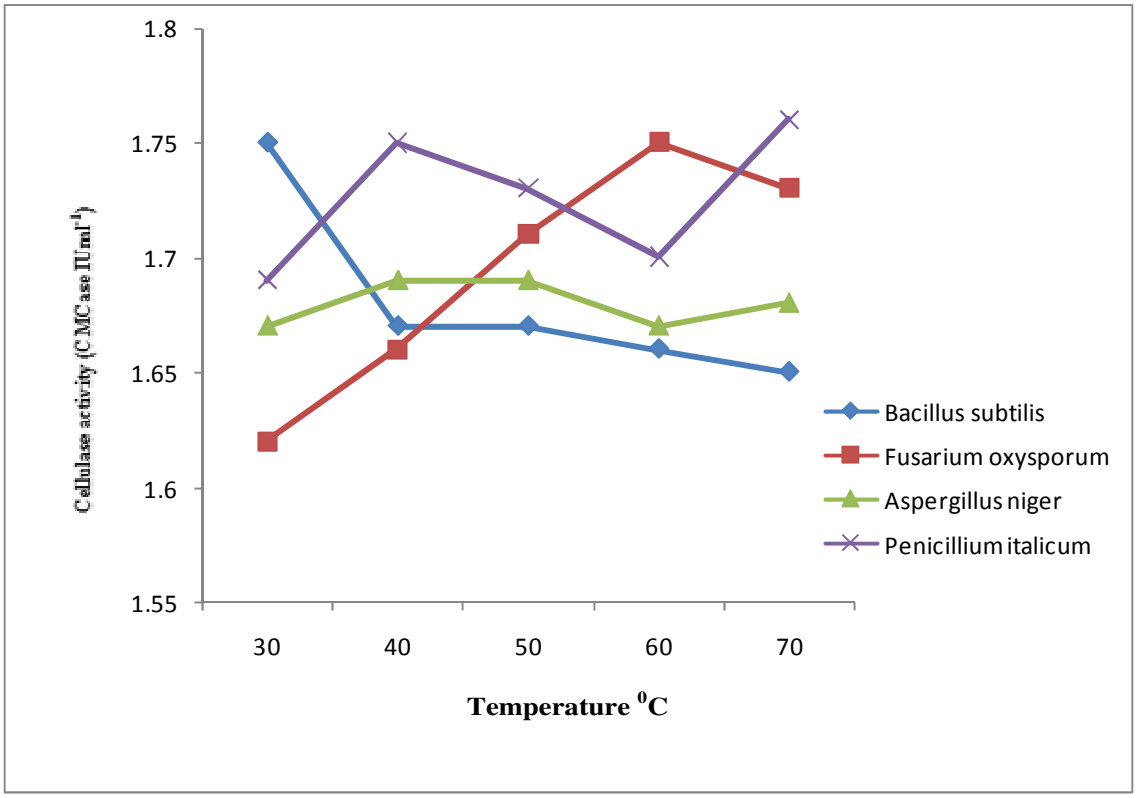


Figure 2: The optimization of temperature on endoglucanase activity of the crude enzyme produced by different microorganisms

The optimization of the crude enzyme at different pH is shown in Figure 3, the enzyme activity of *Bacillus subtilis*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium italicum* ranged from 1.59 to 1.70IUml$^{-1}$, 1.60 to 1.68IUml$^{-1}$, 1.62 to 1.69IUml$^{-1}$ and 1.65 to 1.75IUml$^{-1}$ in that order. The crude enzyme produced by these microorganisms were optimized at pH value 3.0-7.0 for fungi and 5.0-9.0 for bacteria respectively.

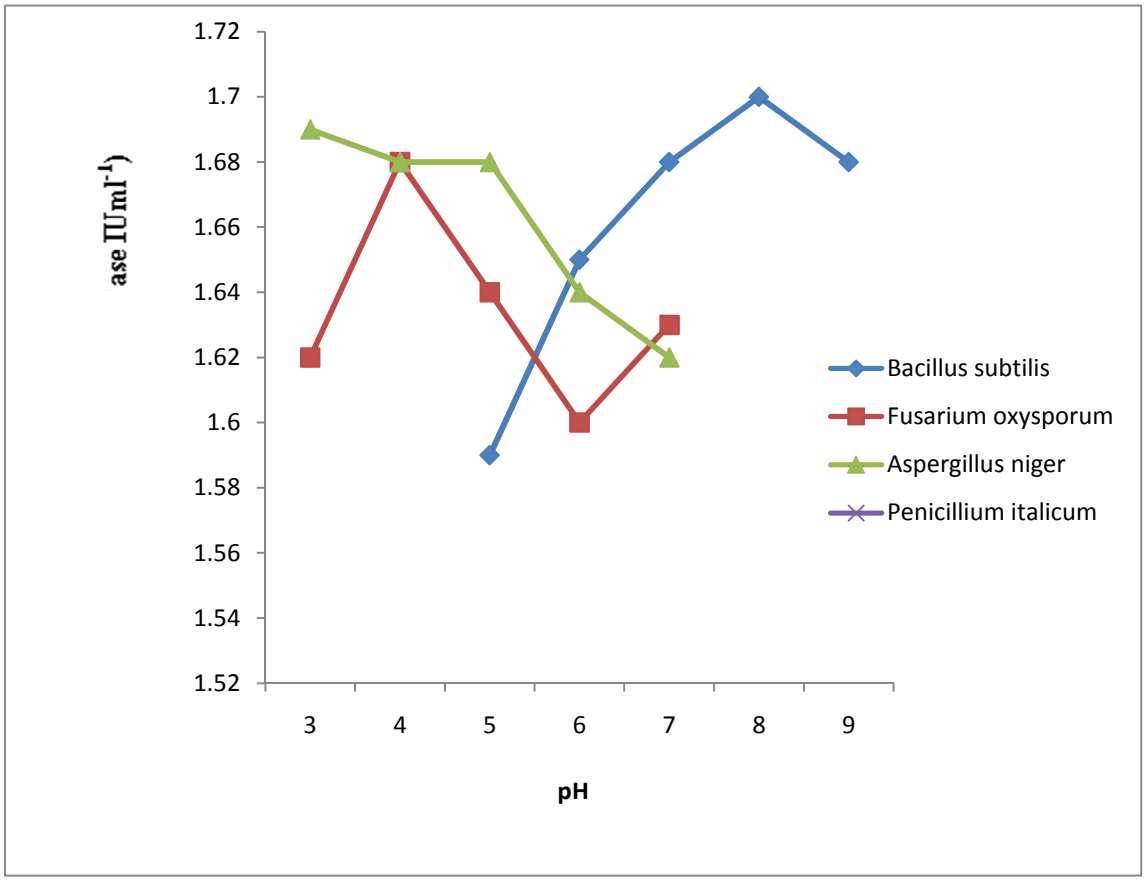


Figure 3: The optimization of pH on endoglucanase activity of the crude enzyme produced by different microorganisms

The optimization of the crude enzyme at different substrate concentrations is depicted in Figure 4, the enzyme activity of *Bacillus subtilis*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium italicum* ranged from 1.59 to 1.68IUml$^{-1}$, 1.56 to 1.69IUml$^{-1}$, 1.62 to 1.73IUml$^{-1}$ and 1.66 to 1.77IUml$^{-1}$ respectively. The crude enzyme produced by these microorganisms was optimized at substrate concentration of 1-5%.

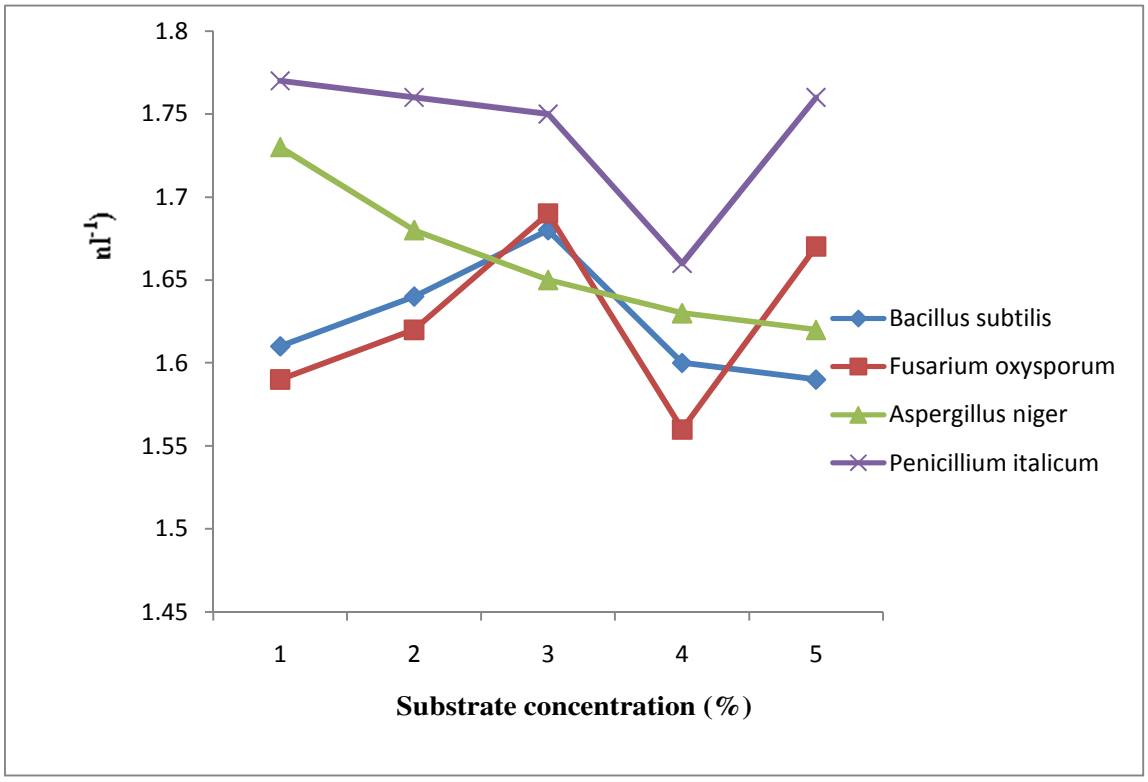


Figure 4: The optimization of substrate concentrations on endoglucanase activity of the crude enzyme produced by different microorganisms

The endoglucanase activity of the crude enzyme and partially purified enzyme is shown in Figure 5. Partial purification of the crude enzyme with ammonium sulphate increased the enzyme activity. The enzyme activity of *Baciilus subtilis, Fusarium oxysporum, Aspergillus niger* and *Penicillium italicum* increased from 1.67 to 1.70IUml$^{-1}$, 1.64 to 1.65IUml$^{-1}$, 1.68 to 1.69IUml$^{-1}$ and 1.74 to 1.83IUml$^{-1}$ respectively. This was also reported by Saliu and Sani (2012) with increase in the cellulase activity of *Aspergillus niger* on carboxymethylcellulose from 0.168 to 0.174IUml$^{-1}$ and *Penicillium decumbens* 0.038 to 0.220IUml$^{-1}$.

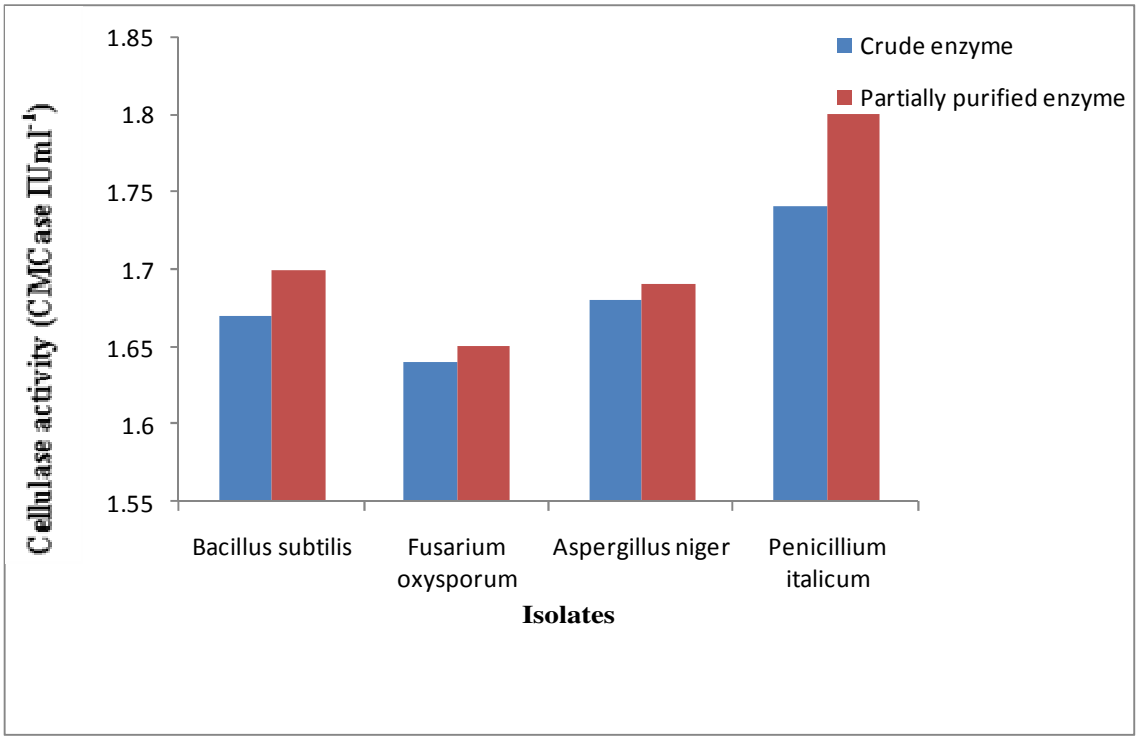


Figure 5: Endoglucanase activity of crude enzyme and partially purified enzyme of different microorganisms

The cultural, morphological and biochemical characteristics of the cellulolytic bacterial isolates are presented in Table 4.

Table 4: The cultural, morphological and biochemical characteristics of the bacterial isolates

| Isolate code | Cultural characteristics | Morphological characteristics | Gram stain | Spore stain | Motility | Glucose | Lactose | Sucrose | Mannitol | Catalase | Citrate | Oxidase | Indole | Coagulase | Vogesproskauer | Methyl red | Tentative identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B1 | Raised with an entire edge, yellowish cream in chains | Rod shaped | - | - | + | + | - | - | + | - | + | + | - | - | - | - | *Pseudomonas aeruginosa* |
| B2 | Flat, dull, whitish in colour and arranged in clusters | Rod shaped | + | + | + | + | - | - | + | + | - | - | - | - | - | + | *Bacillus subtilis* |
| B3 | Flat, smooth, creamy in colour and arranged in chains | Rod shaped | + | + | + | + | - | + | - | + | + | - | - | - | - | + | *Bacillus thuringiensis* |

Key - =negative reaction; += positive reaction

## Conclusion

The results of this study show that organisms with the potential to produce cellulase are present in the soil. The isolates showed a potential to convert cellulose into reducing sugars, which could be readily used in many applications such as animal foods and feed stock for production of valuable organic compounds. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and a possible food supplement or in pharmaceutical industry. However, further studies are recommended on the isolation of cellulase producing organisms from the soil so as to discover more organisms from the soil with the potential to produce the enzyme cellulase on a large scale, and the fermentation procedure be optimized for better yield. Also genetically modified organisms could also be used in the production of the enzyme cellulase.

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