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Research Article

# Isolation and Characterization of Chitinolytic Bacteria for Chitinase Production from the African Catfish, *Clarias gariepinus* (Burchell, 1822)

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## Abstract

Chitinases are hydrolytic enzymes that break down the glycosidic bonds in chitin. Chitin is a component of the cell walls of fungi and exoskeletal elements of some animals (including worms and arthropods), therefore, chitinases are generally found in organisms that either needs to reshape their own chitin or dissolve and digest the chitin of fungi or animals. The importance of chitinase in industries cannot be overemphasized as it has been applied in agriculture, as a biopesticide for control of plant fungi infections, in medicine, as indicators of fungi infection and in waste management, for biodegradation of fish waste. African catfish (*Clarias gariepinus*) which plays host to bacteria is very readily available and easy to cultivate thus providing a cheap means of obtaining chitinolytic bacteria for the production of chitinase in commercial quantity. Bacteria populations isolated from the skin and gut of catfish were screened on colloidal-chitin agar medium. Chitinase production was determined by zones of hydrolysis produced after 96 h of incubation at 37°C. The result of this investigation revealed thirty-six pure bacterial isolates from the skin and gut of catfish. Gram staining test revealed, twenty five Gram positive bacteria while eleven were Gram negative. After four days of incubation, twenty-six bacteria isolates obtained from the gut and skin of catfish were selected as chitinase producing bacteria based on the clear zones of hydrolysis produced. The bacterial isolates obtained will be very useful for the production of chitinase which can be employed for the biocontrol of fungal pathogens and harmful insects. This study presents a first time report of Chitinase producing *Bacillus cereus* from the gut of catfish (*Clarias gariepinus*).

Key words: Chitinases, chitinolytic bacteria, African catfish

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Chitinases are enzymes that degrade chitin (Zarei *et al*., 2012). They contribute to nitrogen and carbon generation of the ecosystem (Jholapara *et al*., 2013). Chitinases have received special attention due to their role in the bio control of fungal pathogens and harmful insects (Mathivanan *et al*., 1998). A variety of pathogenic microorganisms contain chitin coats which provide protection against external factors. Chitinases have been employed to breakdown these protective coats and weaken the defense system of several pathogenic microorganisms and insects (Hamid *et al*., 2013). Chitinase, the best studied enzymes which hydrolyze chitin have broad spectrum of distribution in nature including bacteria, fungi, plants, insects and protozoa, human, animal and yeast (Saranya and Thayumanavan, 2013). Chitinolytic microorganisms inhabit a wide range of environments. Kopecny *et al*. (1996) Found chitinolytic bacteria in the feaces of wild herbivores (e.g., bison-*Bibos bonasus*, llama-*Llama vicugnapaca* and elk-*Elaphurus davidianus*) and domestic herbivores (e.g., sheep and cow). They were also found in the rumen fluid of cows, which are unable to produce enzymes for digesting chitin and thus offer a living environment for chitinolytic bacteria in exchange for help in digesting the compound (Brzezinska *et al*., 2014). Chitinolytic bacteria have also been found in human faeces (Simunek *et al*., 2002; Vernazza *et al*., 2005). It is generally accepted that commensal intestinal microflora has a major impact on gastrointestinal function and thereby on human health. The presence of bacterial chitinases in human colon can play an important role as part of defence mechanisms against fungal invasion (Vicencio *et al*., 2008). The roles of Chitinases in these organisms are diverse. In bacteria, chitinases are usually involved in mineralization of chitin nutrition and parasitism (Saranya and Thayumanavan, 2013). In general, the African catfish is omnivorous feeding on insects, plankton, snail etc. (most of which contain chitin) (Dadebo *et al*., 2014). However, species may also be cannibalistic as the larger ones may feed on much smaller ones (Adewumi and Olaleye, 2011). Fagbenro *et al*. (2001) reported that the digestive enzymes present in the gut of the fish species closely related to its feeding habit. According to Moreau (1988) the presence of appropriate enzymes determines the ability of an organism to digest a given food item. Abass *et al*. (2004) reported that only eight percent (8%) of *Clarias gariepinus* species were infected with aphanomyces fungus. This result was however, lower than other fish species (striped and thin lip gray mullets) examined, thus confirming the presence of chitinases as a result of its feed pattern. Fish is generally regarded as highly nutritious and a very rich source of protein (Egwui, 1986). However, studies have shown that the skin and gut of catfish habours a variety of microorganism which may be potentially pathogenic, causing spoilage and therefore causing serious economic losses not only due to mortality but also in the cause of treatment (Emikpe *et al*., 2011). The microflora of the alimentary tracts of freshwater fish contains species of Aeromonas and representative of family Enterobacteriaceae. Acinetobacter, Bacillus, flavobacterium are derived based on the diet whereas the microflora of the intestines is reported to consist predominantly of fermentative bacteria including Aeromonas and Enterobacteriaceae representatives (Davies, 1997). The intestinal flora of catfish, however may change with age, nutritional status, environmental conditions and the complexity of the fish digestive system (Ringo *et al*., 2006). The African catfish, *Clarias gariepinus* is of great economic importance in fisheries in most African countries where it represents a priceless source of protein for most African countries (Egwui, 1986). This study was therefore carried out to isolate, characterize and screen for chitinase producing bacteria from the gut and skin of the African catfish.

## MATERIALS AND METHODS

**Collection of samples:** A total of three *C. gariepinus* samples of average weight were collected from the reservoir of Entrepreneurial Development Studies Centre of Covenant University in September, 2014. They were transported live in plastic bags to the Microbiology Research Laboratory of Covenant University, Canaanland, Ota, Ogun State.

**Preparation of catfish samples:** Each of the catfish sample was thoroughly washed, slaughtered and the blood allowed to drain. The fish were then labelled as catfish 1, 2 and 3, respectively. The gut and intestines were extracted and blended and serial dilutions carried out according to the method described by Olayemi *et al*. (2012) whereby one gram of sample was added to 9 mL of sterile distilled water. Serial dilutions ($10^{-1}$–$10^{-4}$) of the homogenized samples were made.

**Isolation of bacteria population from the gut of catfish:** One gram of the gut of each catfish samples was collected and coded as $G_1$, $G_2$ and $G_3$ and serial dilution of $10^{-1}$-$10^{-4}$ was carried using sterile distilled water. A 0.1 mL of $G_1$ $10^{-2}$, $G_1$ $10^{-4}$ $G_2$ $10^{-2}$ $G_2$ $10^{-4}$, $G_3$ $10^{-2}$ and $G_3$ $10^{-4}$ of each catfish sample was inoculated on nutrient agar using the pour plate method and incubated at 37°C for 48 h.

**Isolation of bacteria from skin of catfish:** A sterile swab stick was used to collect a skin swab of catfish 1, 2 and 3, respectively. Serial dilutions $10^{-1}$ to $10^{-4}$ were carried out

using sterile distilled water. A 0.1 mL of the samples ($S_1$ $10^{-2}$, $S_1$ $10^{-4}$, $S_2$ $10^{-2}$, $S_2$ $10^{-4}$, $S_3$ $10^{-2}$ and $S_3$ $10^{-4}$) were incubated at 37°C for 48 h.

**Selection of pure cultures:** After 48 h of incubation, samples were further sub cultured in duplicates with each colony from each plate labelled as $A_1$, $A_2$, $B_1$, $B_2$, $C_1$, $C_2$, $D_1$, $D_2$, $E_1$, $E_2$, $F_1$ and $F_2$, respectively. Three series of sub culturing was carried out to obtain pure bacterial isolates.

**Identification of bacterial isolates:** The Bacterial isolates were subjected to various biochemical test, Gram staining, motility test, sugar fermentation test, starch hydrolysis, hydrogen sulphide production, utilization of urease, catalase test, oxidase test, Methyl Red Vogues Proskauer (MRVP) test, utilization of citrate and indole production. The obtained results were compared with the Bergey's manual of systematic microbiology in order to identify the organisms.

**Isolation of dna from chitinolytic bacteria:** The DNA isolation was carried out according to the protocol described in Trans Easy pure Genomic DNA kit purchased from Trans gene biotech (China).

**PCR amplification:** The RAPID PCR assays were performed in a 20 µL reaction mixture using a PCR master mix (Solis Biodyne, Estonia), containing IX PCR buffer, 2.5 mM magnesium chloride, 200 µM each of dNTP, 2 U Taq DNA polymerase. Additionally, 50 pmol of S30 primer (5'-GTGATCGCAG-3'), 10-200 ng of DNA and sterile water was used to make up the reaction mixture. The PCR was carried out in an Eppendorf thermal cycler nexus series (Eppendorf, Germany) with the following amplification conditions, an initial denaturation step at 96°C for 5 min, followed by 40 consecutive cycles of 95°C for 15 sec, 35.5°C for 15 sec, 72°C for 2 min and a final extension at 72°C for 10 min. The RAPD-PCR products were separated on 1.5% agarose gel electrophoresis. The agarose gel was prepared by boiling 1.5 g of agarose powder in 100 mL of 0.5X Tris Acetate EDTA (TAE) buffer. After boiling, the solution was allowed to cool and 50 µL of 1 mg $mL^{-1}$ ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 min and the comb was removed. Ten microliters of the DNA samples were loaded into the wells after mixing with 2 µL of bromophenol blue. A 100 bp DNA ladder (Solis Biodyne, Estonia) was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at constant voltage of 80°C for about 1 h 30 min.

**Preparation of colloidal-chitin agar medium:** The colloidal chitin agar medium used for screening of chitinolytic bacteria species was prepared according to the method described by Saima *et al.* (2013).

**Screening and selection of chitinolytic bacteria:** Screening of chitinolytic bacteria was carried out according to the method of Saima *et al.* (2013). The pure isolates obtained were inoculated into the colloidal chitin agar medium and incubated at 37°C for four days. Chitinolytic bacteria were selected based on clear zones of hydrolysis produced after incubation for four days.

## RESULTS

**Identification of bacterial isolates:** The result of this investigation revealed thirty-six pure bacterial isolates from the skin and gut of catfish (*Clarias gariepinus*) (Table 1). Gram

Table 1: Identification of isolates from the gut and skin of catfish

| Isolate code | Origin | Identification |
|---|---|---|
| $S_1A$ | Skin | *Bacillus* spp. |
| $S_1B$ | Skin | *E. coli* |
| $S_1C$ | Skin | *Bacillus* spp. |
| $S_1D$ | Skin | *Bacillus* spp. |
| $S_1E$ | Skin | *E. coli* |
| $S_1F$ | Skin | *Staphylococcus* spp. |
| $S_1G$ | Skin | *Bacillus* spp. |
| $S_3A$ | Skin | *Aeromonas* spp. |
| $S_3B$ | Skin | *Staphylococcus* spp. |
| $S_3C$ | Skin | *Bacillus* spp. |
| $S_3D$ | Skin | *Aeromonas* spp. |
| $S_3E$ | Skin | *Bacillus* spp. |
| $S_3F$ | Skin | *Aeromonas* spp. |
| $S_3G$ | Skin | *Bacillus* spp. |
| $S_3H$ | Skin | *Bacillus* spp. |
| $S_3I$ | Skin | *Staphylococcus* spp. |
| $S_3J$ | Skin | *Bacillus* spp. |
| $S_2A$ | Skin | *Bacillus* spp. |
| $S_3K$ | Skin | *Vibro* spp. |
| $S_3L$ | Skin | *Staphylococcus* spp. |
| $S_3M$ | Gut | *Bacillus* spp. |
| $G_1A$ | Gut | *Bacillus* spp. |
| $G_1B$ | Gut | *Bacillus* spp. |
| $G_1C$ | Gut | *Bacillus* spp. |
| $G_1D$ | Gut | *Aeromonas* spp. |
| $G_1E$ | Gut | *Aeromonas* spp. |
| $G_1F$ | Gut | *Bacillus* spp. |
| $G_1G$ | Gut | *Staphylococcus* spp. |
| $G_1H$ | Gut | *Bacillus* spp. |
| $G_1I$ | Gut | *Bacillus* spp. |
| $G_1J$ | Gut | *Bacillus* spp. |
| $G_1K$ | Gut | *Bacillus* spp. |
| $G_1L$ | Gut | *Bacillus* spp. |
| $G_1M$ | Gut | *Staphylococcus* spp. |
| $G_1N$ | Gut | *Bacillus* spp. |
| $G_1O$ | Gut | *Bacillus* spp. |

Table 2: Gram stain reactions

| Sample codes | Results |
|---|---|
| $S_1A$ | Gram positive short rods |
| $S_1B$ | Gram negative short rods |
| $S_1C$ | Gram positive short rods |
| $S_1D$ | Gram positive short rods |
| $S_1E$ | Gram negative short rods |
| $S_1F$ | Gram positive cocci |
| $S_1G$ | Gram positive short rods |
| $S_3A$ | Gram negative long rods |
| $S_3B$ | Gram positive cocci |
| $S_3C$ | Gram positive short rods |
| $S_3D$ | Gram negative short rods |
| $S_3E$ | Gram positive short rods |
| $S_3F$ | Gram negative short rods |
| $S_3G$ | Gram positive short rods |
| $S_3H$ | Gram positive short rods |
| $S_3I$ | Gram positive cocci |
| $S_3J$ | Gram positive long rods |
| $S_2A$ | Gram positive short rods |
| $S_3K$ | Gram negative short rods |
| $S_3L$ | Gram positive cocci |
| $S_3M$ | Gram positive short rods |
| $G_1A$ | Gram positive short rods |
| $G_1B$ | Gram positive short rods |
| $G_1C$ | Gram positive short rods |
| $G_1D$ | Gram negative long rods |
| $G_1E$ | Gram negative rods in pairs |
| $G_1F$ | Gram positive short rods |
| $G_1G$ | Gram positive tiny cocci |
| $G_1H$ | Gram positive short rods |
| $G_1I$ | Gram positive short rods |
| $G_1J$ | Gram positive short rods |
| $G_1K$ | Gram positive short rods |
| $G_1L$ | Gram positive short rods |
| $G_1M$ | Gram positive cocci |
| $G_1N$ | Gram positive short rods |
| $G_1O$ | Gram positive short rods |

staining test revealed twenty five Gram positive bacteria while eleven were Gram negative (Table 2). The isolates were identified based on biochemical characteristics of the isolates (Table 3 and 4).

**Growth of chitinolytic bacteria:** After four days of incubation, twenty-six bacteria isolates of the thirty-six bacterial isolates obtained from the gut and skin of catfish were selected as chitinase producing bacteria based on the clear zones of hydrolysis produced (Fig. 1). Isolate code 17 from skin and isolate code 36 from gut were selected for further studies based on larger zones of hydrolysis (40 mm) (Table 5).

**Identification of chitinolytic bacteria:** The isolates code 17 and 36 were identified as *Bacillus cereus* based on the 16S rRNA analysis.

Table 3: Sugar fermentation test

| Isolate codes | Glucose | Fructose | Galactose | Maltose | Sucrose | Lactose |
|---|---|---|---|---|---|---|
| $S_1A$ | + | + | - | A | A | A |
| $S_1B$ | + | A | G | + | A | + |
| $S_1C$ | + | + | - | + | A | + |
| $S_1D$ | + | + | + | + | A | A |
| $S_1E$ | _ | - | + | + | + | - |
| $S_1F$ | + | A | - | + | A | - |
| $S_1G$ | A | + | - | A | A | - |
| $S_3A$ | G | A | - | + | A | - |
| $S_3B$ | A | + | - | A | A | - |
| $S_3C$ | A | A | A | A | + | - |
| $S_3D$ | A | + | A | A | A | - |
| $S_3E$ | A | G | G | - | A | A |
| $S_3F$ | A | A | G | A | + | A |
| $S_3G$ | A | + | + | A | + | - |
| $S_3H$ | A | A | G | A | + | A |
| $S_3I$ | A | + | A | A | A | A |
| $S_3J$ | A | + | + | + | + | + |
| $S_2A$ | A | + | - | + | A | - |
| $S_3K$ | A | + | A | + | + | A |
| $S_3L$ | A | G | - | A | A | - |
| $S_3M$ | + | + | - | + | + | A |
| $G_1A$ | + | + | + | A | + | A |
| $G_1B$ | A | A | + | + | + | - |
| $G_1C$ | + | A | A | A | + | - |
| $G_1D$ | + | A | - | A | + | + |
| $G_1E$ | A | A | - | A | A | - |
| $G_1F$ | + | A | A | - | G | - |
| $G_1G$ | A | A | - | A | + | - |
| $G_1H$ | A | A | - | + | A | A |
| $G_1I$ | - | + | + | A | A | - |
| $G_1J$ | A | A | - | A | - | - |
| $G_1K$ | A | A | - | A | + | + |
| $G_1L$ | A | A | A | + | - | G |
| $G_1M$ | A | + | + | + | A | - |
| $G_1N$ | A | + | A | A | + | A |
| $G_1O$ | A | + | A | + | + | G |

A+ve: Acid production A-ve: Acid not produced, G+ve: Gas production, G-ve: Gas not produced, +: Positive, -: Negative

## DISCUSSION

The result of this investigation revealed that bacterial isolate from the skin and gut of catfish (*Clarias gariepinus*) produced chitinases. Most of the bacteria isolated from the skin and gut were members of *Bacillus* sp., *Staphylococcus* species and *Escherichia coli*. Oladosu-Ajayi *et al.* (2011) reported the presence of *Bacillus* sp., *Staphylococcus* spp. and *E. coli* in all parts of the catfish evaluated. Fagbenro *et al.* (2001) established the presence of chitinolytic enzymes in the gut of several fish species. Previous researchers have reported chitinases from bacteria, fungi and insects (Tjoelker *et al.*, 2000; Xiao *et al.*, 2005). Matsumoto (2006) reported that microorganisms produce Chitinases in higher amounts than animals and plants and that the Chitinases are generally

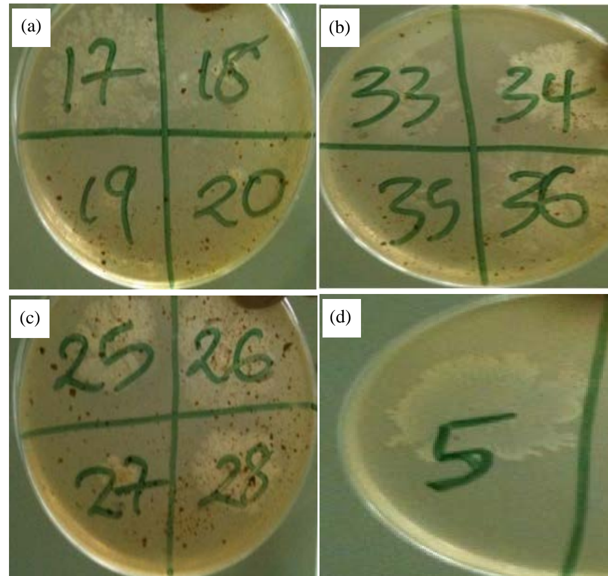


Fig. 1(a-d): Bacterial isolates (a) 17, 18, 19 and 20, (b) 33, 34, 35 and 36, (c) 25, 26, 27 and 28 and (d) 5 showing zone of hydrolysis on colloidal chitin agar medium

Table 4: Identification of bacterial isolates with biochemical tests

| Isolate codes | Oxidase | Citrate | Urease | MR | VP | Catalase | Indole | $H_2S$ | Starch | Motility |
|---|---|---|---|---|---|---|---|---|---|---|
| $S_1A$ | - | + | + | - | + | + | + | - | - | + |
| $S_1B$ | - | - | - | + | + | + | + | - | - | + |
| $S_1C$ | + | + | + | - | - | + | - | - | - | - |
| $S_1D$ | + | + | + | - | - | - | + | - | - | + |
| $S_1E$ | - | - | - | - | - | + | - | - | - | - |
| $S_1F$ | + | + | + | - | - | + | - | - | - | - |
| $S_1G$ | + | + | - | - | - | + | + | - | - | + |
| $S_3A$ | - | + | - | - | - | + | + | - | - | + |
| $S_3B$ | - | + | + | - | + | + | + | - | - | - |
| $S_3C$ | - | + | + | - | + | + | - | - | + | - |
| $S_3D$ | - | + | + | - | - | + | - | - | - | + |
| $S_3E$ | + | - | + | - | - | + | - | - | - | - |
| $S_3F$ | - | + | + | - | - | + | - | - | + | + |
| $S_3G$ | - | - | + | + | - | + | + | - | - | + |
| $S_3H$ | - | - | + | + | + | + | + | - | - | + |
| $S_3I$ | + | + | + | + | - | + | + | - | - | + |
| $S_3J$ | + | + | + | + | - | + | - | - | - | + |
| $S_2A$ | + | + | + | + | - | + | + | - | - | + |
| $S_3K$ | + | + | - | + | - | + | + | - | + | + |
| $S_3L$ | - | + | + | + | - | + | + | - | - | + |
| $S_3M$ | + | + | + | + | + | + | + | - | - | - |
| $G_1A$ | - | - | + | + | - | + | + | - | + | + |
| $G_1B$ | + | + | + | + | - | + | + | - | + | + |
| $G_1C$ | - | + | - | + | - | + | + | - | - | - |
| $G_1D$ | + | + | - | + | + | + | - | - | - | - |
| $G_1E$ | + | + | - | + | - | + | - | - | - | - |
| $G_1F$ | - | + | - | + | - | + | + | - | - | - |
| $G_1G$ | - | + | + | + | - | + | - | - | - | - |
| $G_1H$ | + | + | + | + | - | + | - | - | + | + |
| $G_1I$ | - | + | - | + | - | + | - | - | - | - |
| $G_1J$ | - | + | - | + | - | + | - | - | + | + |
| $G_1K$ | + | + | - | + | - | + | - | - | - | + |
| $G_1L$ | - | - | - | - | + | + | - | - | - | + |
| $G_1M$ | - | + | + | + | - | + | - | - | - | - |
| $G_1N$ | + | + | - | + | - | + | + | - | - | + |
| $G_1O$ | + | + | ND | + | - | + | + | - | - | - |

+: Positive reaction and -: Negative reaction, MR: Methyl red and VP: Voges-proskauer

Table 5: Diameter of zones of hydrolysis of chitinolytic bacteria

| Isolate codes | Diameter of zones of hydrolysis (mm) |
|---|---|
| $S_1A$ | 14 |
| $S_1B$ | 25 |
| $S_1C$ | 15 |
| $S_1D$ | 15 |
| $S_1E$ | 32 |
| $S_1F$ | - |
| $S_1G$ | 25 |
| $S_3A$ | 24 |
| $S_3B$ | - |
| $S_3C$ | 20 |
| $S_3D$ | 22 |
| $S_3E$ | - |
| $S_3F$ | |
| $S_3G$ | |
| $S_3H$ | 24 |
| $S_3I$ | - |
| $S_3J$ | 40 |
| $S_2A$ | - |
| $S_3K$ | - |
| $S_3L$ | - |
| $S_3M$ | 13 |
| $G_1A$ | 12 |
| $G_1B$ | 28 |
| $G_1C$ | 24 |
| $G_1D$ | 25 |
| $G_1E$ | 33 |
| $G_1F$ | - |
| $G_1G$ | 25 |
| $G_1H$ | 28 |
| $G_1I$ | 27 |
| $G_1J$ | 26 |
| $G_1K$ | 20 |
| $G_1L$ | 27 |
| $G_1M$ | 30 |
| $G_1N$ | - |
| $G_1O$ | 40 |

produced as inducible extracellular enzymes which are either of two types: endochitinases and exochitinases. Bacillus was amongst the bacterial chitinase producers. Others were Serratia, Chromobacterium, Klebsiella and Streptomyces. The PCR amplification and biochemical test identified the chitinolytic bacteria as members of the genus Bacillus. This was earlier established by Kamil *et al*. (2007). They reported that out of twenty chitinolytic bacteria isolated from rhizosphere soil, several species of Bacillus including *B. lincheniformis* and *B. thuringiensis* showed the highest chitinase activity. Similarly, six chitinases from *Bacillus circulans* WL-12 were reported by Watanabe *et al*. (1992) Members of the genus Bacillus are well known for their potential to secrete a number of degradative enzymes such as chitinases (Schallmey *et al*., 2004). This investigation revealed chitinase production after 96 h of incubation. Priya *et al*. (2011) reported that chitinase production after 49 h of incubation with maximum production between 72-96 h of incubation. Kamil *et al*. (2007) also reported maximum chitinase production.

## CONCLUSION

This research work has established the fact that the African cat fish (*Clarias gariepinus*) plays host to a number of chitinolytic bacteria from which chitinase can be produced for commercial purposes. The PCR amplification shows that two of the bacteria specie which produced the chitinase used in this study were *Bacillus cereus* after sequencing.

This research study recommends the following:

- Further purification processes may be carried out to bring about more yield of the enzyme
- The potential for the use of the chitinase in the treatment of fungi infections in humans may also be studied
- Chitinase produced from *B. cereus* from the gut of catfish can be tested on fungal pathogens to confirm its efficacy

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