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CHARACTERIZATION OF CHITINASE 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. The role of Chitinases in the treatment and prevention of various diseases have been reported. They have been implicated in the human health care for the treatment of fungal infections, in Asthma and in the control of mosquito which causes the deadly malaria disease accounting for about 70% of infant mortality in Africa. Chitinase was obtained from chitinolytic bacteria inhabiting the skin and gut of the African Catfish (*Clarias gariepinus*). Bacterial population Isolated from catfish was screened on colloidal-chitin agar medium. The ability to produce Chitinase was determined by zones of hydrolysis produced after 96h of incubation at 37^{o}C. Isolation of chitinase was carried out with colloidal chitin as substrate in sodium phosphate buffer. Optimum conditions were therefore ascertained at a temperature of 50^{0}C and a substrate concentration of 0.15g for chitinase produced by bacteria *spp* (isolate code 17 and 36) while pH 5.5 was obtained for isolate code 36 and pH 6.0 for isolate code 17. The Michaelis – Mentens constant (Km) which is also known as the dissociation constant is the substrate concentration at half maximum velocity. Calculated from the Lineweaver-Burk plot, the apparent Km values for the hydrolysis of chitin by chitinolytic bacterial isolate code 36 and isolate code 17 were approximately 0.09mg/ml and 0.007mg/ml respectively. Isolation of DNA and PCR amplification carried out identified the bacteria as a member of the genus *Bacillus*. This study established that species of *Bacillus* inhabiting the gut and skin of the African catfish can be used for Chitinase production in appreciable quantity.

Keywords: Chitinases, *Clarias gariepinus*, Chitin, PCR, Bacillus sp.

I. INTRODUCTION

Chitinases are hydrolytic enzymes that break down the glycoside bonds in chitin (Zarei *et al.*, 2012). Chitin is a β-1, 4-linked polymer of *N*-acetylglucosamine and it is one of the most abundant biopolymers in nature (Trung and Bao, 2015). Chitin makes up the exoskeleton of arthropods, including crustaceans, insects, molluscs, nematodes and worms (Tjoelker *et al*., 2000). Therefore, crustacean, shrimps and fish scale waste is ideal as raw material for chitin production (Trung and Bao, 2015). Most microorganisms including many species of bacteria have been reported to have chitinase producing ability (Xia *et al.*, 2005). Medically, it has been reported that globally 300million people suffer and die yearly from fungal disease with an estimated number of 150 persons per hour (Global plague, 2013). Considering the vast importance of chitinase as biopesticides and indicators of fungi infections in humans, there is a need for locally produced chitinase. Biotechnological uses of Chitinases at the commercial scale are not maximally utilized due to their high cost (Matsumoto, 2006). Nigeria spends N200bn annually on importation of industrial enzymes (Peter-Albert *et al.*, 2014). The African catfish *Clarias gariepinus* is a sharp tooth fish of the family clariidae and they are air breathing fishes (George and Otubusin, 2007). The gut of the catfish which harbors a number of chitinolytic bacteria is a waste abundantly available because of the vast growing industry of local cultivation of catfish. Prameela *et al*. (2010) reported that about 130 million tons of fish waste is generated each year in the world. Chitinases, which hydrolyze chitin, are largely found in nature, for example, in the stomachs of fish (Gutowska *et al*., 2004; Molinaria *et al*., 2007). Due to the cannibalistic nature of *C. gariepinus* as reported by Adewunmi and Olaleye (2011), it suggests that these species may catabolize chitin not just to penetrate prey exoskeletons but also to derive nutrients from the chitin itself. Matsumoto (2006) reported that Chitinases are present in chitin-containing microorganisms, bacteria and plants with a diversity of roles. Chitinases are associated with chitin eating habit (feeding on crustaceans or insects) and have been detected in the stomachs of Atlantic cod, Japanese sea bass and trout (Okutani and Kimata, 1964; Danulat and Kausch, 1984; Danulat, 1986). Therefore, strains of microorganisms for production of chitinase with appreciable activity will be very useful in developing novel biological control strategies. This study is therefore an approach to decrease the high cost of importation of chitinase for agricultural and medical uses. It will also serve as a means of utilizing the abundant waste from catfish.

II. MATERIALS AND METHODOLOGY

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 Sample

Each of the catfish sample was thoroughly washed, slaughtered and the blood allowed to drain. The fish were then labeled 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 gramme of sample was added to nine (9) milliliter of sterile distilled water. Serial dilutions ($10^{-1} – 10^{-4}$) of the homogenized samples were made.

Isolation of Bacterial Population from the Gut of Catfish

One gramme 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} to 10^{-4} was carried using sterile distilled water. 0.1ml of G_1 10^{-2}, G_1 10^{-4} G_2 10^{-2} G_2 10-4, G_3 10^{-2}, G_3 10-4 of each catfish sample was inoculated on nutrient agar using the pour plate method and incubated at 37°C for 48h.

Isolation of Bacteria from the 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. 0.1ml of the samples (S_1 10^{-2}, S_1 10^{-4}, S_2 10^{-2}, S_2 10^{-4}, S_3 10^{-2}, S_3 10^{-4}) were incubated at 37°C for 48h.

Selection of Pure Cultures

After 48h of incubation, samples were further sub cultured in duplicates with each colony from each plate. Three series of sub culturing was carried out to obtain pure bacterial isolates.

Preparation of Colloidal Chitin

Colloidal Chitin was prepared according to the method of Bleatley (2012) whereby twenty grammes of chitin powder was treated with 150ml of 12M concentrated HCL in a 1000ml beaker. The HCL was added slowly with continuous stirring for 5mins, followed by stirring for 1min at an interval of every 5mins for 1h in a chemical fume cupboard at room temperature. The chitin-HCL mixture was then passed through eight layers of muslin cloth. The clear filtrate (100ml) obtained was treated with 2L of ice cold distilled water to allow precipitation of colloidal chitin. This was incubated overnight at 4°C and passed through two layers of filter paper. Tap water (3L) was passed through the colloidal chitin until the pH had risen to 7.0. This was then pressed between two layers of filter paper to remove additional moisture and autoclaved in a 100ml beaker wrapped with two layers of aluminum foil.

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). The composition of the colloidal chitin agar medium is as follows;

- NaH_2PO_4 6g
- KHP_2O_4 3g
- NH_4Cl 1g
- NaCl 0.5g
- Yeast extract 0.05g
- Colloidal chitin (w/v) 1.0%
- Agar 15g
- Distilled water 1000ml

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.

Chitinase Production

The bacterial isolates showing the highest zones of hydrolysis were selected. The Colony were inoculated into separate 25ml nutrient broth medium and incubated at 30°C until O.D was 1 at 600nm. It was then used to inoculate 100ml of basal medium (pH 6.5) in a ratio of 5% (v/v). Cultures were incubated at 30°C in a shaker at 150rpm for eight days.

Composition of Basal Medium

Chitin powder	2.5g
Yeast extracts	0.25g
$(NH_4)_2SO_4$	0.1g
$MgSO_4.7H_20$	0.05g
$Cacl_2.2H_20$	0.0 25g
Nacl	0.5g
KBr	0.05g
$MnCL_2.4H_20$	0.0005g
$ZnSO_4.7H_20$	0.0005g
$FeSO_4.7H_20$	0.01g

Chitinase Assay

Chitinase activity was determined according to a modified method of Vincy *et al.* (2014) using colloidal chitin as substrate. The enzyme solution (0.5ml) was added to 1ml substrate solution which contained 0.5% suspension of colloidal chitin prepared in 50mM Sodium phosphate buffer (pH 6.0) and incubated at 50°C for 1h. The reaction was terminated by adding 3ml of DNSA reagent and heated in a boiling water bath for 15mins. Absorbance was measured at 660nm. One unit of chitinase activity was defined as the amount of

enzyme, which produced 1 mole of N-actetylglucosamine in 1ml of reaction mixture under the standard assay condition.

The protein content of the dialysate was determined by the method of lowry *et al*. (1951) (Appendix II). Chitinase activity was determined as described earlier.

Ammonium Sulphate Precipitation

Solid Ammonium sulphate (Analytical grade) was added to the crude enzyme preparation to 90% saturation. The suspension was kept 4^0C for 24hrs. The mixture was centrifuged at 4000rpm for 15min. the supernatant was discarded. The precipitate was re-dissolved in 0.05M phosphate buffer. The enzyme was dialyzed against four changes of the same buffer. Dialysis was performed using acetylated cellophane tubing as described by Whitaker *et al*., 1963 (Appendix I). The protein content of the dialysate was determined by the method of lowry *et al*. (1951)(Appendix II). Chitinase activity was determined as described earlier.

Characterization of chitinase

The effect of a number of factors on the activity of the partially purified enzyme was examined.

Effect of Temperature

To determine the optimum temperature for chitinase production, the partially purified enzyme was subjected to various temperature; 40°C, 45°C, 50°C, 55°C, 60°C and 65°C for 1hr. Chitinase activity was determined as previously described.

Effect of pH

The effect of pH on chitinase activity was determined by varying the pH of the substrate from pH 4.5 to pH 8. The substrate consisted of 0.15g of colloidal chitin dissolved in 50mM sodium phosphate buffer of varying pH. Incubation was at 50°C for 1h. Chitinase activity was determined as previously described.

Effect of Substrate Concentration

The effect of various substrate concentration was determined by using various concentrations of colloidal chitin in phosphate buffer; 0.05%, 0.10%, 0.15%, 0.2%, and 0.25%. The reaction mixture contained 1ml of substrate and 0.5ml of enzyme incubated at 50^OC for 2hr. Chitinase activity was determined as previously described.

Effect of Time of Heating

The effect of time of heating on the stability of the enzyme was determined. Samples of crude enzyme was heated at 70°C and 90°C for different periods of time (0, 5, 10, 15, 20, 25, 30min) respectively. The reaction mixture consisted of 1ml of the substrate and 0.5ml of the enzyme solution. Chitinase activity was determined as previously described.

Kinetic Determination

Initial reaction rates of chitinase were determined at different substrate concentrations ranging from 0.05% to 0.25% of chitin. The kinetic constant (Km) was estimated following the method of Line-weaver and Burk (1934) for chitinase from isolate code 17 and isolate code 36.

III. RESULTS

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 (40mm) (Table 1).

Chitinase Production

Isolate code 36 (from the gut of catfish) produced appreciable chitinase activity when compared with chitinase activity of isolate code 17 (from the skin) of the catfish.

TABLE 1: Diameter of Zones of Hydrolysis of Chitinolytic Bacteria

S/N	ISOLATE CODES	DIAMETER OF ZONES OF HYDROLYSIS
1	S_1A	14mm
2	S_1B	25mm
3	S_1C	15mm
4	S_1D	15mm
5	S_1E	32mm
6	S_1F	---------
7	S_1G	25mm
8	S_3A	24mm
9	S_3B	---------
10	S_3C	20mm
11	S_3D	22mm
12	S_3E	--------

13	S_3F	25mm
14	S_3G	--------
15	S_3H	24mm
16	S_3I	--------
17	S_3J	40mm
18	S_2A	------
19	S_3K	------
20	S_3L	------
21	S_3M	13mm
22	G_1A	12mm
23	G_1B	28mm
24	G_1C	24mm
25	G_1D	25mm
26	G_1E	33mm
27	G_1F	------
28	G_1G	25mm
29	G_1H	28mm
30	G_1I	27mm
31	G_1J	26mm
32	G_1K	20mm
33	G_1L	27mm
34	G_1M	30mm
35	G_1N	--------
36	G_1O	40mm

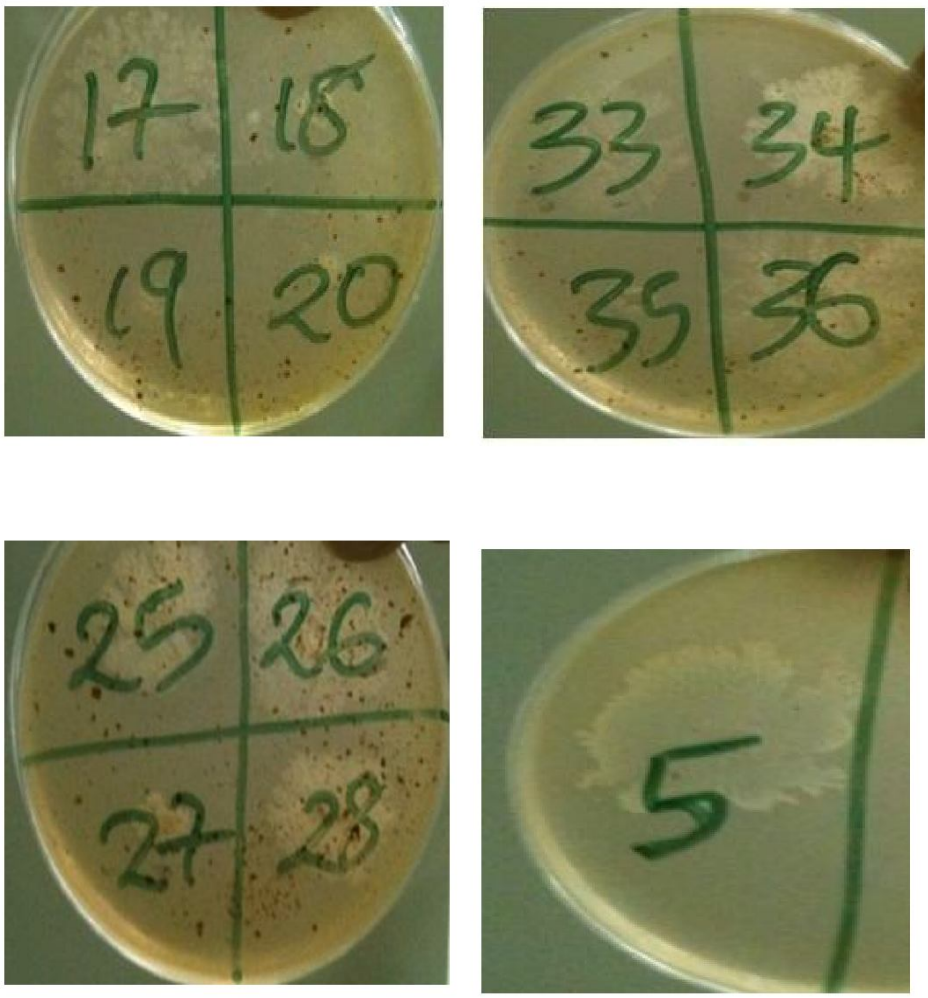

FIG 1: Bacterial isolates showing zones of hydrolysis on colloidal chitin agar medium

TABLE 2: Partial Purification of Chitinase obtained from Bacteria (Isolate code 17) from Catfish (*Clarias gariepinus*)

Protein Step	Total Activity (Units)	Protein (mg/ml)	Specific Activity (Units/mg protein)	Yield (%)	Purification Fold
Crude Extract	0.048	0.093	0.52	100	1
Ammonium Sulphate Precipitation	0.040	0.032	1.25	83.33	2.4

TABLE 3: Partial Purification of Chitinase obtained from Bacteria (Isolate code 36) from Catfish (*Clarias gariepinus*)

Protein Step	Total Activity (Units)	Protein (mg/ml)	Specific Activity (Units/mg protein)	Yield (%)	Purification Fold
Crude Extract	0. 028	0.090	0.31	100	1
Ammonium Sulphate Precipitation	0.023	0.020	1.15	82.14	3.7

IV. PROPERTIES OF CHITINASE

Effect of Temperature

The temperature of incubation affected the chitinase activity tremendously. The activity of the chitinase increased with increase in incubation temperature until an optimum was reached (Fig. 2; Fig. 3). Subsequent increase in temperature beyond the optimum temperature led to a reduction in the enzyme activity. The optimum temperature for the chitinase was 50^{o}C.

Effect of PH

The pH of the reaction mixtures remarkably affected the activities of all enzymes produced from both bacteria isolates. The activities of the enzymes increased with increase in pH until an optimum pH was reached. Subsequent increase in pH led to a reduction in enzyme activity. The optimum pH was 5.5 and 6.0 for isolate code 36 and 17 respectively (Fig. 4; Fig. 5).

Effect of Substrate Concentration

The activity of the chitinase produced by the bacteria isolated from the skin of the catfish increased with increase in substrate concentration until an optimum substrate concentration was reached (Fig. 6, Fig. 7). The optimum substrate concentration for chitinase produced by bacteria isolated from skin and gut was 0.15mg.

KINETIC DETERMINATION

The apparent Km values for the hydrolysis of chitin by chitinolytic bacteria isolate code 36 and isolate code 17 calculated from the Lineweaver-Burk plot were approximately 0.09mg/ml and 0.007mg/ml respectively (Fig. 8; Fig. 9).

4.4.4 EFFECT OF TIME OF HEATING

The activity of chitinase with heating at 70^{o}C until an optimum was reached. Subsequent increase in time of heating led to a reduction in enzyme activity. However heating at 90^{o}C led to a continuous reduction in enzyme activity until the enzyme was completely denatured (Figs. 10, 11, 12, 13)

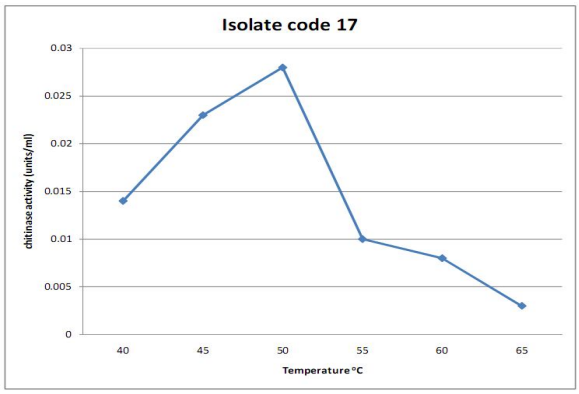

FIG 2: Effect of Temperature on chitinase obtained from bacterial specie (Isolate code 17)

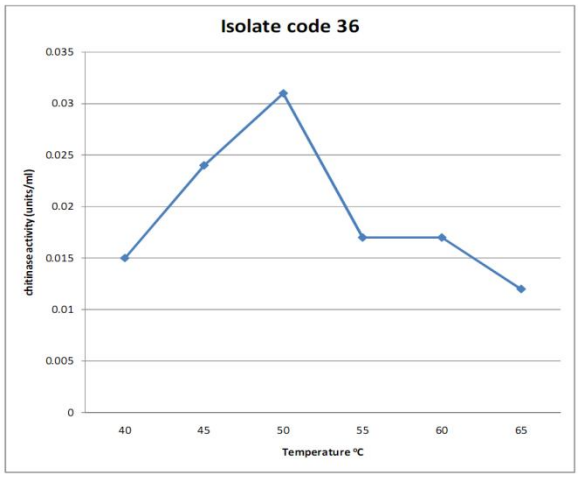

FIG 3: Effect of Temperature on chitinase produced by bacteria specie (Isolate code 36)

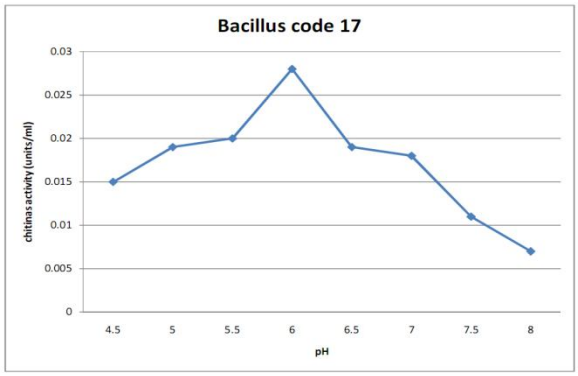

FIG 4: Effect of pH on chitinase obtained from bacteria specie (Isolate code 17)

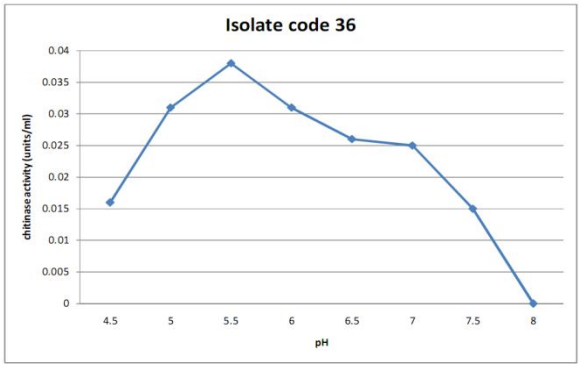

FIG 5: Effect of pH on chitinase obtained from bacteria specie (Isolate code 36)

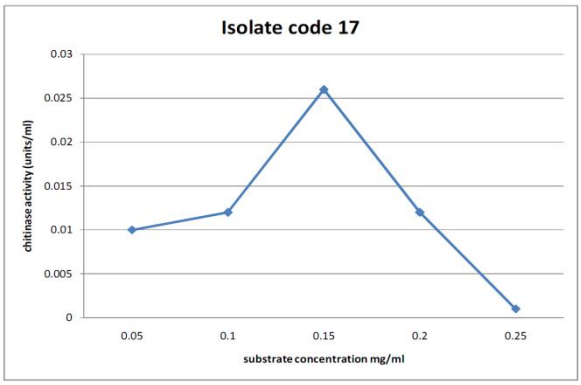

FIG 6: Effect of substrate concentration on chitinase obtained from bacteria specie (Isolate code 17)

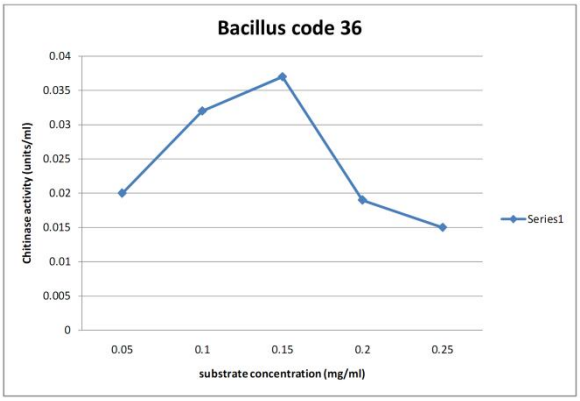

FIG 7: Effect of substrate concentration on chitinase obtained from bacteria specie (Isolate code 36)

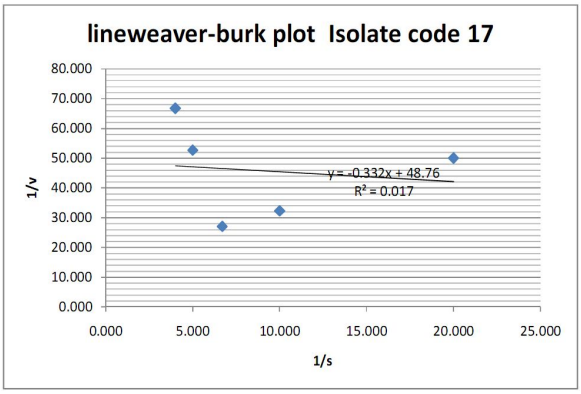

Fig 8: lineweaver-Burk plot for hydrolysis of chitin by partially purified chitinase from bacteria specie (Isolate code 17)

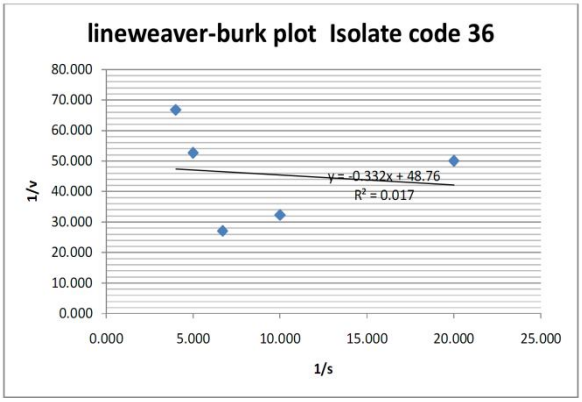

FIG 9: lineweaver-Burk plot for hydrolysis of chitin by partially purified chitinase from bacteria specie (Isolate code 36)

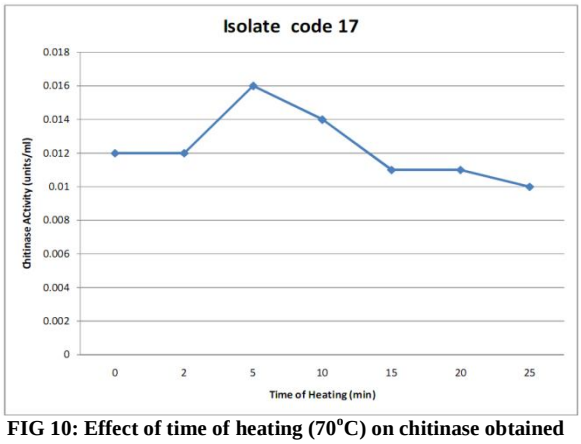

FIG 10: Effect of time of heating (70°C) on chitinase obtained from *bacillus sp* (sample code 17)

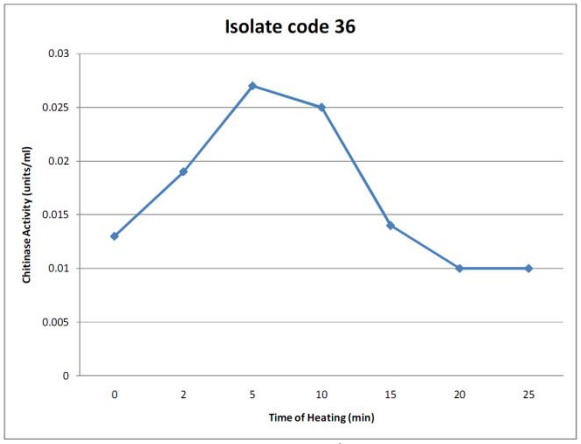

FIG 11: Effect of time of heating (70°C) on chitinase obtained from bacteria specie (Isolate code 36)

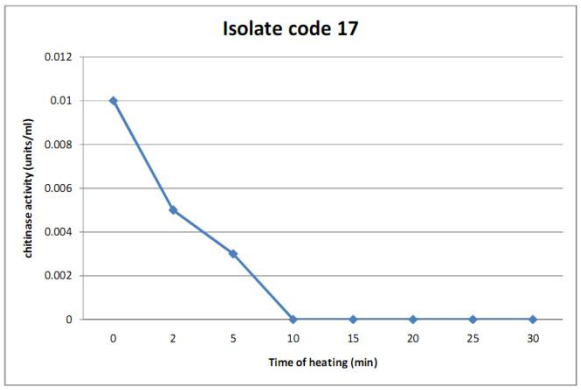

Fig 12: Effect of time of heating (90°C) on chitinase obtained from bacteria specie (Isolate code 17)

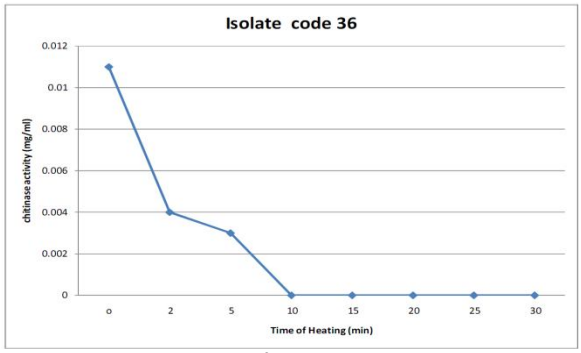

FIG 13: Effect of heat (90°C) on chitinase obtained from bacteria specie (Isolate code 36)

V. DISCUSSION

The result of this investigation revealed that bacterial isolate from the skin and gut of catfish (*Clarias gariepinus)* produced chitinases.. Fagbenro *et al.* (2000) 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; Xia *et al*., 2005).

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.* (1990). 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 96h of incubation. Priya *et al.* (2011) reported that chitinase production after 49h of incubation with maximum production between 72 - 96hrs of incubation. Kamil *et al.* (2007) also reported maximum chitinase production. The optimal temperature of the chitinase in this study was 50°C as supported by Zarei *et al. (*2012); Duskova *et al.* (2011). Priya *et al.* (2011) had earlier reported that the optimal temperature for chitinases ranges from 40 - 60°C depending on source. However, this report contrasts with earlier reports by Saima *et al.* (2013) that chitinase production by *Aeromonas spp* was maximum at 37°C.

Optimal pH was identified as 5.5 (isolate code 17) and 6.0 (isolate code 36). Previous reports of chitinase is most active between pH 5.0 and 6.0 (Haggag and Abdallh, 2012; Park *et al.*, 2000). For effect of substrate concentration, various concentration of colloidal chitin were tested as previous reports confirms that chitinase produced by bacteria hydrolyzed colloidal chitin more rapidly than crude chitin or chitin from fungal cell walls (Taechowisan, 2003). The optimal substrate concentration was 0.15mg/ml for chitinases produced by both isolate code 17 and isolate code 36. In this investigation, the effect of time of heating was investigated over two specific temperatures (70°C and 90°C) over a period of 30min. For chitinase produced by both bacterial isolates, heating at 70°C did not result in a loss in enzyme activity confirming earlier reports by Galante *et al.* (2011). They reported that chitinase were active for over 20mins of heating at 80°C. However, heating at 90°C has a tremendous effect on chitinase from both bacterial isolates. There was a continuous reduction in enzyme activity as heat was applied. After 10min of heating, enzyme activity had been completely lost in both cases. Wang *et al.*

(2002) reported that the chitinases studied lost more than fifty percent (> 50%) of their initial activities after only three minutes of heating at 100^{o}C. The Chitinase from *Isaria japourica* lost 0% of its enzyme activity at 50^{o}C (Kawachi *et al.*, 2001) while the chitinase from *Aspergillus fumigatus* lost 30% of its activity after 60min of heating at 55^{o}C. Galante *et al.* (2012) reported ChitMPI$_2$, a chitinase from *Moniliphthora Perniciosa* to have lost 10% of its initial activity after 20min of heating at 80^{o}C and were completely inactivated after 60min of heating. Chitinase has been described as one of the cell wall lytic enzymes which have differing physiological properties, molecular structure and molecular weights.

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 optimum condition for the chitinase produced has been determined as 0.15mg/ml (isolate 17 and isolate 36), 50^{o}C, pH 5.5 (isolate 36) and pH 6.0 (isolate 17) for substrate concentration, temperature and pH respectively.

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