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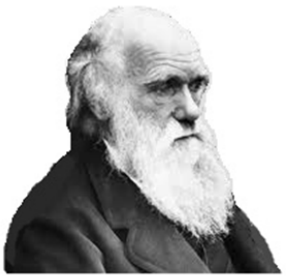

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Production of amylase by the intestinal microflora of cultured freshwater fishes (*Oreochromis niloticus* and *Clarias gariepinus*) rared localy in Calabar, south Nigeria

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ABSTRACT

This study focused on determining the amylase-producing ability of the intestinal microbes in cultured fresh water fishes – *Oreochromis niloticus* and *Clarias gariepinus*. The bacterial isolates were identified on the basis of standard cultural, morphological and biochemical characteristics. The amylase production ability of the bacterial isolates was determined using starch agar. The mean viable count of the intestinal microbes ranged from 1.2×10^5 CFU/ml to 7.1×10^5 CFU/ml for tilapias (*Oreochromis niloticus*) and from 2.0×10^4 CFU/ml to 8.9×10^4 CFU/ml for catfishes (*Clarias gariepinus*). *Staphylococcus* and *Micrococcus* were predominant for both tilapias and catfishes. Out of 24 isolates, 21 were amylase producers. These included the following bacteria genera: *Bacillus*, *Micrococcus*, and *Staphylococcus.* These results strongly suggest that intestinal microbes play a pivotal role in the digestion of starch in cultured freshwater fishes and should be explored for industrial amylase production.

***Keywords*:** Amylase, Intestinal Microflora, Cultured Fresh Water Fishes, *Oreochromis niloticus*, *Clarias gariepinus*

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1. INTRODUCTION

Amylase enzyme is one of the most important enzymes used in modern biotechnology (Rao *et al.*, 2017). It is an enzyme that hydrolyzes starch. Amylases can be derived from plants (like cassava, maize, barley etc.), animals (like man, rat, certain species of spiders etc.), and microorganisms (like bacteria, fungi, and actinomycetes). (Sanwo *et al*., 1992; Teotia *et al*., 2001; Pandey *et al*., 1999; Berlin, 2004; Erickson, 1992; Ben *et al.*, 1999). However, microbial amylases have been extensively used in recent biotechnology because of having greater thermal stability, capable of producing different sugar profile, and having a long history of safe use (Reddy *et al*., 2003; Rao *et al.*, 2007). Amylase was first discovered and utilized in the industry by the Japanese scientist Jokichi Takamine in the year 1894 at Peoria, Illinois (USA). In that very year and at the same place, the amylase produced was used as a pharmaceutical aid for the treatment of digestive disorders (Rao *et al*., 2007). There are three types of amylases, namely: alpha amylase (endo-1,4-α-D-glucohydrolase), beta amylase (β-1,4-glucan maltohydrolase), and glucoamylase (amyloglucosidase) (Rao *et al*., 2007). Being ubiquitous in nature, microbes could likewise be found in the intestinal tract of fresh water fishes. A great number of such microbes are heterotrophic bacteria including aerobes and anaerobes. Although many ecological studies on the intestinal microflora of fishes have been conducted, only recently have reports concerning their functions appeared (Sugita *et al.*, 1997). Researches show that intestinal bacteria can produce certain bioactive substances like: biotin, Vitamin B_{12}, and antibacterial substances beneficial to the host (fish). These facts strongly suggest a symbiotic relationship between fish and intestinal microflora (Sugita *et al.*, 1992; Sugita *et al*., 1991; Westerdahl *et al.*, 1991). Starch is the substrate for amylase production. A substrate is a "reacting molecule" that is acted upon by its specific enzyme to yield a product or products (Prescott *et al*., 2005). Each of the three types of amylases has a unique way of acting on starch. For instance, alpha amylase (being an endoamylase) cleaves or breaks the α-1,4-glucosidic linkages in starch internally to give glucose, maltose, or dextrins (Rao *et al*., 2007). Beta amylase, as an exoamylase, cleaves the glycolytic bonds removing two glucose units at a time thus producing maltose (Abe *et al*., 1988; Gupta *et al*., 2003). Glucoamylase, on the other hand, cleaves both the α-1,4- and α-1,6-glucosidic linkages to yield glucose, maltose, and limit dextrins (Rao *et al*., 2007). Microbial amylases have various industrial applications. They can be exploited for high fructose corn preparation, for the production of alcohol and brewing, for paper coating, for the preparation of detergents amongst all others (Abe *et al*., 1988; Bailey and Ollis, 1986). Industries requiring microbial amylases include: clinical, medicinal, analytical chemistry, textile, food, and distilling industries (Rao *et al*., 2007). Since microbial amylases have an extensive application in the industry thus this study was aimed at isolating and determining the intestinal microflora in cultured freshwater fishes rared locally and determining the amylase-producing ability of the intestinal isolates [24-35].

2. MATERIALS AND METHODS

2. 1. Media and Reagents

Criterion dehydrated culture medium (Nutrient Agar, Simmons Citrate Agar, Starch Agar, MR VP broth, and Trypticase Soy broth) from Hardy Diagnostics, USA. All media were prepared according to manufacturer's instructions.

Analytical chemicals from Titan Biotech Ltd, India were used to prepare the following reagents: Gram staining reagents (crystal violet, lugol's iodine, 70% ethanol, and neutral red), kovac's reagent, methyl red indicator, 40% potassium hydroxide, and 5% alpha-naphthol in alcohol. Other reagents included: indole reagents and oxidase test strips (Hardy Diagnostics, USA), analytical grade alcohol, and hydrogen peroxide from Sigma Aldrich, USA.

2. 2. Sample Collection

The fish samples – tilapias (*Oreochromis niloticus*) and catfishes (*Clarias gariepinus*) – were obtained from Cross River Basin Authority (CRBA) and University of Calabar (UNICAL) farm all located within Calabar. Figures 1 and 2 below show the pictures the two fish genera respectively.

Fig. 1. *Oreochromis niloticus* (Linnaeus, 1758) rared locally in Calabar, Cross River State, Nigeria

Fig. 2. *Clarias gariepinus* (Burchell, 1822) rared locally in Calabar, Cross River State, Nigeria

The fishes were transported in sterile plastic containers to the laboratory (within two hours after purchase) for analysis. The fishes were dissected aseptically. The intestines were pulled out and immersed in 9 ml sterile water in universal containers. Ten-fold serial dilutions were carried out for each sample. One ml from 10^{-2} and 10^{-3} dilutions (for catfishes) and from 10^{-3} and 10^{-4} dilutions (for tilapias) were plated out in duplicates on Nutrient Agar culture media using pour plating technique. Plates were incubated for 24 hours at room temperature.

2. 3. Characterisation and identification of bacterial isolates

Pure isolates were extracted after repeated sub-culturing for characterization on the basis of their gram's morphology and biochemical reactions (Cheesbrough, 2006). The bacterial isolates were then identified by comparing their characteristics with those of known taxonomy using the schemes of Cowan and Steel (1993)

2. 4. Amylase Production

Each isolate was inoculated onto freshly prepared starch agar and incubated for 24 hours at 37 ºC. After which the plates were flooded with iodine reagent and observation was made for zones of clearance. The zones of clearance indicate starch hydrolysis by the enzyme, amylase, produced by the bacterial isolates.

3. RESULTS

The results from the analysis of the four intestines from four cultured freshwater fishes obtained from two pond sites, Cross River Basin Authority (CRBA) and University of Calabar (UNICAL) farm, are presented as follows:

Table 1 shows the mean viable count of the intestinal microbes isolated from the four cultured freshwater fishes. Enumeration was done at varying dilutions; 10^{-3} and 10^{-4} dilutions (for tilapias) and 10^{-2} and 10^{-3} dilutions (for catfishes). The mean viable count of the intestinal isolates for tilapias (*Oreochromis niloticus*) ranges from 1.2×10^5 CFU/ml to 7.1×10^5 CFU/ml. For catfishes (*Clarias gariepinus*), it ranges from 2.0×10^4 CFU/ml to 8.9×10^4 CFU/ml.

Table 2 shows the morphological and biochemical characteristics of the intestinal isolates. Four bacteria genera were isolated, namely: *Micrococcus*, *Staphylococcus, Bacillus*, and *Pseudomonas.* Isolates W_1 to Y_3 were obtained from a pair of tilapias whilst isolates A_1 to E_3 were obtained from a pair of catfishes. The biochemical characterization of the isolates was based on Cowen and Steel's identification manual by Barrow and Feltham, (1993).

Table 3 shows the result of the intestinal isolates capable of hydrolyzing starch. All the other isolates, excluding the genera *Pseudomonas*, are amylase producers.

Table 4 shows the varying distribution of amylase producers in their respective genera. *Micrococcus* occurs more frequently than *Bacillus* and *Staphylococcus*.

Table 5 shows the distribution of amylolytic intestinal microbes in cultured freshwater fishes. It was observed that tilapias have higher percentage of intestinal microbes capable of producing amylase than catfishes.

Table 1. Enumeration of intestinal microbes in tilapias and catfishes

SAMPLE	MEAN VIABLE COUNTS (CFU/ml) OF ISOLATES IN RESPECTIVE DILUTIONS		
	10^{-2}	10^{-3}	10^{-4}
Tilapia I	NG	1.3×10^5	7.1×10^5
Tilapia II	NG	1.6×10^5	1.2×10^5
Catfish I	2.0×10^4	8.9×10^4	NG
Catfish II	2.0×10^4	6.2×10^4	NG

Key: NG = No Growth

Table 2. Characterization of intestinal isolates from cultured freshwater fishes

Isolates	Morphology	Gram Stain	Catalase	Oxidase	Citrate	MR	VP	Indole	Coagulase	Glucose	Sucrose	Mannitol	Lactose	Probable Organism
W_1	cocci	+	+	+	+	+	-	-	-	A	AG	NR	NR	*Micrococcus*
W_2	cocci	+	+	+	+	+	-	-	-	AG	AG	A	NR	*Micrococcus*
W_3	cocci	+	+	+	+	+	-	-	-	NR	NR	NR	NR	*Micrococcus*
X_1	cocci	+	+	+	+	-	-	-	-	NR	NR	NR	NR	*Micrococcus*
X_2	cocci	+	+	+	-	-	-	-	-	NR	NR	NR	NR	*Micrococcus*
X_3	cocci	+	+	+	+	-	-	-	-	A	NR	NR	NR	*Micrococcus*
Z_1	cocci	+	+	-	+	+	+	-	+	AG	A	A	A	*Staphylococcus*
Z_2	cocci	+	+	-	+	+	+	-	-	AG	AG	AG	AG	*Staphylococcus*
Z_3	cocci	+	+	-	+	+	+	-	-	AG	AG	AG	NR	*Staphylococcus*
Y_1	cocci	+	+	-	+	+	+	-	-	A	NR	NR	NR	*Staphylococcus*
Y_2	cocci	+	+	+	+	+	-	-	-	A	NR	NR	NR	*Micrococcus*
Y_3	cocci	+	+	-	+	+	+	-	-	NR	NR	NR	AG	*Staphylococcus*
A_1	rod	+	+	-	+	-	-	+	-	AG	A	AG	NR	*Bacillus*

A_2	rod	+	+	-	+	+	-	-	-	NR	NR	NR	NR	*Bacillus*
A_3	rod	+	+	+	+	-	+	-	-	AG	AG	AG	AG	*Bacillus*
B_1	rod	-	+	+	-	-	+	-	-	A	NR	NR	NR	*Pseudomonas*
B_2	rod	-	+	+	+	-	+	-	-	A	NR	NR	NR	*Pseudomonas*
B_3	rod	+	+	+	+	-	+	-	-	A	NR	NR	NR	*Bacillus*
D_1	cocci	+	+	+	+	+	-	-	+	A	AG	AG	AG	*Micrococcus*
D_2	cocci	+	+	+	-	-	-	-	-	NR	AG	NR	NR	*Micrococcus*
D_3	rod	-	+	+	+	+	+	-	+	AG	A	AG	A	*Pseudomonas*
E_1	cocci	+	+	+	+	+	-	-	-	A	NR	NR	NR	*Micrococcus*
E_2	cocci	+	+	+	+	+	-	-	-	A	A	NR	NR	*Micrococcus*
E_3	cocci	+	+	-	+	+	+	-	-	A	A	AG	A	*Micrococcus*

Keys: + = Reactive, A = Acid MR = Methyl Red - = Unreactive, AG = Acid and GasVP = Voges Proskauer NR = No Reaction

Table 3. Reaction of intestinal isolates on starch

ISOLATES	REACTION ON STARCH	MICROORGANISMS INVOLVED
W_1	+	*Micrococcus*
W_2	+	*Micrococcus*
W_3	+	*Micrococcus*
X_1	+	*Micrococcus*
X_2	+	*Micrococcus*
X_3	+	*Micrococcus*
Z_1	+	*Staphylococcus*
Z_2	+	*Staphylococcus*
Z_3	+	*Staphylococcus*
Y_1	+	*Staphylococcus*

Y_2	+	*Micrococcus*
Y_3	+	*Staphylococcus*
A_1	+	*Bacillus*
A_2	+	*Bacillus*
A_3	+	*Bacillus*
B_1	-	*Pseudomonas*
B_2	-	*Pseudomonas*
B_3	+	*Bacillus*
D_1	+	*Micrococcus*
D_2	+	*Micrococcus*
D_3	-	*Pseudomonas*
E_1	+	*Micrococcus*
E_2	+	*Micrococcus*
E_3	+	*Staphylococcus*

Table 4. Distribution of amylase producers in different genera

AMYLASE PRODUCERS	NUMBER	PERCENTAGE (%)
Bacillus	4	19.05
Micrococcus	11	53.38
Staphylococcus	6	28.57
Total	**21**	

Table 5. Distribution of amylase producers in cultured freshwater fishes

SAMPLES	NUMBER OF AMYLASE PRODUCERS	% DISTRIBUTION OF AMYLASE PRODUCERS
Tilapias	12	57.14
Catfishes	9	42.86

4. DISCUSSION

This research work on cultured fresh water fishes – tilapias and catfishes – revealed that their intestines were colonized by heterotrophic bacteria at varied levels. The mean viable count of the intestinal microbes ranged from 1.2×10^5 CFU/ml to 7.1×10^5 CFU/ml for tilapias (*Oreochromis niloticus*) and from 2.0×10^4 CFU/ml to 8.9×10^4 CFU/ml for catfishes (*Clarias gariepinus*) as Table 1 shows. A total of 24 isolates were classified on the basis of their morphological and biochemical reactions as Table 2 shows. The characterization revealed four generic groups, namely: *Micrococcus, Staphylococcus, Bacillus*, and *Pseudomonas.* Amongst these genera, *Micrococcus* and *Staphylococcus* were predominant in both fishes. This result contrasted the works of Sugita et al., (1997) were *Aeromonas* and *Bacteroidaceae* were predominant as well as the works of Naviner et al., (2006) were *Aeromonas hydrophila* was predominant. Out of the 24 isolates, 21 were amylase producers and 3 were non-amylase producers as Table 3 shows. 19.05% of these amylase producers were *Bacilli,* 53.28% were *Micrococci,* and 28.57% were *Staphylococci* as noted in Table 5. The non-amylase producing isolates were members of the genus *Pseudomonas*. Though the former results agree with the works of Sugita et al., (2008), the latter contrasts with their works as *Pseudomonas* was isolated as an amylase producer. Since *Pseudomonas* was recovered as a non-amylase producer in this research work, its presence in the intestines of cultured freshwater fishes may contribute to spoilage by the production of histamines in the fish tissues (Austin, 2002).

Amylase producing intestinal microbes could either be aerobic (as in *Bacillus* and *Micrococcus*) or facultative (as in *Staphylococcus*). As seen in Table 5, tilapias harbor a greater percentage of amylase producers than catfishes. Variances in the population of the intestinal microbes existing in cultured freshwater fishes may be due to: fish ecology, seasonal fluctuations in the water's temperature, developmental stages of the fish, and structure of the digestive tract (Austin, 2002; Izvekova et al., 2007; Naviner et al., 2006; Sugita et al., 1991). These factors are most likely to affect the amylase producing abilities of the intestinal microbes (Takeuchi, 1991).

5. CONCLUSION

Majority of heterotrophic bacteria colonizing the intestinal tract of cultured freshwater fishes are amylase producers. Although the pancreas and secretory cells in the intestinal walls of the fish can produce amylase, microbial amylases also play a key role in starch hydrolysis. This research work did not include quantitative assessment and purification of the amylase enzyme produced. However, it is vital for these to be done in order to determine the best amylase producers and the volume of the enzyme produced. Microbial amylases are safe for use in industries for the production of useful goods and services.

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