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Characterization and Lipolytic Activity of Bacteria Isolates from Freshwater Clam (*Mercenaria Mercenaria*) in Bayelsa State, Nigeria

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ABSTRACT

Lipases form an important group of relevant enzymes which have applications in various fields including; food, pharmaceutical, detergent, textile and cosmetic industries. Lipases can be produced from diverse sources including microorganisms. This study evaluated the potential of bacteria isolates from fresh-water clam *Mercenaria Mercenaria* to produce lipolytic enzymes. Ten samples of Clam (*Mercenaria Mercenaria*) were screened for the presence of lipase producing bacteria using classical culture methods. Eleven bacteria species were obtained, of which six (*Actinomyces* sp., *E. coli*, *Bacillus* sp., *Pseudomonas* sp., *Clostridium* sp. and *Klebsiella* sp.) produced lipases that had lipolytic activity in breaking down olive oil used in media supplementation. The best culture media and conditions for optimal production of lipases was studied and it was shown that supplementation of growth media with 2% dextrose at neutral pH gave the greatest yield of lipases when lipase producing isolates were grown in shake flasks. Measurement of biomass by culture and turbidimetric methods indicates that the highest cell mass was recorded by *Pseudomonas* sp at 7.8 x 10^5 CFU/ml, closely followed by *Actinomyces* sp. and *Bacillus* sp., at 6.2 x 10^5 CFU/ml and 5.3 x 10^5 respectively. The produced lipases were partially purified by precipitating with ammonium sulphate followed by dialysis. The total protein content of produced lipases was evaluated by the Lowry's method, showing that estimated protein content followed the same trend as cell biomass with the highest recorded by *Pseudomonas* sp. at 1.53mg/ml, followed by *Actinomyces* sp. and *Bacillus* sp. at 1.47mg/ml and 1.32mg/ml respectively. The results obtained in this study shows that isolates obtained from freshwater clam can produce potent lipases which can be employed for industrial, food and other diverse uses.

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Introduction

Lipases are a class of hydrolytic enzymes which catalyse the breakdown/hydrolysis of triacylglycerol to glycerol and fatty acids [1]. Lipases are produced by a wide variety of organisms including bacteria, fungi, plants and animals. However, microbial lipases, especially from bacteria, are more useful than their plant and animal derivatives because of their stability [2]. Furthermore, bacterial lipases are preferable because of their ease of production through fermentation and the possibility of improving yield through modification of producer organisms and the optimization of growth conditions [3]. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications [4]. Bacterial lipases are used extensively in the food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butterfat and cream [3]. Currently, bacterial lipases are of great demand because of potential industrial applications [5]. The common lipase-producing bacterial strains are *Pseudomonas aeruginosa*, *P. fluorescens*. *Bacillus coagulans*, *B. cereus*, *Staphylococcus aureus*, *S. hyicus*, *Burkholderia glumae*, *B. cepacia*, etc. [6]. Lipase producing microorganisms have been isolated from a variety of sources including; petrol spilled soil Turkish pastirma agro-industrial wastes, dairy plants etc [7-10].

Mercenaria mercenaria commonly known as freshwater clams are invertebrates belonging to the phylum mollusks [11]. They are a popular delicacy especially for communities in the riverine areas of the Niger Delta Nigeria. *Mercenaria mercenaria* has a large diversity of microbial flora associated with it. However, the presence of some bacteria found in clam can at times be dependent on the microorganisms present in the water through contamination. This is because clams are filter feeders and when they dwell in areas with high microbial contamination, they concentrate both minerals and microorganisms present in the water body. Thus, the bacteria flora of the clam depicts the level of bacterial contamination of the water environment [12]. The presence of a wide bacteria population in clams can be of public health significance if the clam is colonized by pathogenic microorganisms or it can be of no effect. On the positive aspect, previous reports have indicated that the biological diversity of marine and estuarine environment may provide a wide array of enzyme secreting microbes which can be harnessed for the production of commercially relevant enzymes for industrial applications. These enzymes from microbial sources are attractive for industrial applications because of the diverse enzymatic properties and substrate specificity [4].

The present study focused on identifying bacteria which can yield the enzyme lipase from *M. mercenaria* harvested from freshwater environment, the selection of optimal growth conditions for lipase production and an attempt at partial purification and quantitation of produced lipases. This will be beneficial in the continual search for isolates with greater potential for enzyme synthesis with minimal growth requirement.

Materials and Methods

Sample Collection and Bacteria Isolation

10 samples of clam (*Mercenaria mercenaria*) were collected from the lower Nun River in Bayelsa State, Niger Delta region of Nigeria in sterile disposable sample bags and immediately transported to the laboratory for further analysis. The shells of the clams were removed with a sharp sterile knife and the meats transferred into a stomacher bag. 10grams of clam meat was homogenized with 90ml of sterile peptone water using a stomacher. This was serially diluted and cultured by the pour plate method on nutrient agar incubated at 37°C for 24 h. Resulting representative colonies were sub-cultured twice onto nutrient agar to obtain pure cultures.

Phenotypic characterization of isolates

The morphological and cultural characteristics of colonies growing on agar plates were noted. The physiological and biochemical reactions of test isolates including Gram staining, cell morphology, catalase, indole, citrate reactions, motility test, glucose, lactose, gas and hydrogen sulphide production were evaluated to aid in the identification of the bacteria according to the Bergey's Manual of Determinative Bacteriology [13].

Screening of Isolates for Lipolytic Activity

All isolates obtained were screened for their ability to breakdown lipids (lipolytic activity) by culturing on phenol red agar supplemented with 1.0% (v/v) olive oil by the streak plate method and incubated at 37 °C for 48 h. The ability of the isolates to break down lipids (lipolytic activity) was detected by the occurrence of an opaque zone around the colonies.

Production of crude enzyme

Isolates showing lipolytic activity were sub-cultured onto nutrient broth supplemented with 1% olive oil and 2% dextrose at neutral pH in a rotary shaker for 24hours at 37 °C in duplicates. After overnight incubation, the cell biomass was evaluated by the spread plate and turbidimetric method using an inoculum withdrawn from the shaker.

Partial Purification and Quantification of Produced Lipases

The fermentation broth was centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant was collected, filtered and utilized as crude enzyme for assay. The supernatant obtained was purified further by the addition of 50% ammonium sulphate. This was allowed to stand before being subjected to dialysis using a dialysis membrane to obtain partially purified lipases. Partial quantitation of the protein content was undertaken according to the method of Lowry et al. (1951) [14].

Results and Discussion

Eleven (11) bacterial isolates were obtained from harvested freshwater clams based on their colonial morphology and biochemical characteristics. The organisms isolated were a mixture of both normal flora, pathogenic and lipase producing organisms and this is expected in seafood harvested from water bodies that are used for both domestic and commercial purposes [15]. The isolates were designated L1-L11 as shown in Table 1. below.

Table 1: Bacterial isolates from fresh clam

Isolate	Gram reaction	Mor-phology	Catalase test	Indole test	Citrate test	Motility test	Glu test	Lac test	H_2s	Gas	Suspected organisms
L1	-	Rod	+	-	+	+	+	+	-	+	*Enterobacter* sp.
L2	-	Rod	+	-	+	+	+	+	+	+	*Proteus* sp.
L3	-	Rod	+	+	-	+	+	+	-	+	*Escherichia coli*
L4	+	Rod	+	-	+	+	+	-	-	+	*Pseudomonas* sp.
L5	+	Cocci	+	-	-	-	+	+	-	+	*Staphylococcus* sp.
L6	-	Rod	+	+	+	+	+	+	-	+	*Citrobacter* sp.
L7	-	Rod	+	-	-	+	+	+	-	+	*Salmonella* sp.
L8	+	Rod	+	-	+	+	+	-	-	+	*Bacillus* sp.
L9	-	Rod			+	+	+	+	-	+	*Klebsiella* sp.
L10	+	Rod	-	-	+	+	+		+	+	*Actinomyces*
L11	+	Rod	-	+	-	+	+	-	+	+	*Clostridium botulinum*

Of the 11 isolates, upon culture on phenol red agar supplemented with 1% olive oil, six isolates: *E. coli*, *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp., *Clostridium* sp., and *Actinomyces* sp. showed ability to produce lipases by the formation of halos around the colonies as shown in Table 2. below. This result agrees with a previous study by Bharathi et al., 2019 which obtained similar lipase producing organisms. In this study, the highest halo intensity was recorded by *Bacillus* sp., followed by *Pseudomonas* sp. and *Actinomyces* sp. whereas *E. coli*, *Klebsiella* sp., and *Clostridium* sp. gave moderate-intensity halos [7].

Table 2: Lipolytic Activity of Isolated Bacterial species

ISOLATE	LIPID DEGRADATION (lipase production)
Escherichia coli	+
Bacillus species	+
Proteus species	-
Klebsiella species	+
Citrobacter species	-
Pseudomonas species	+
Staphylococcus species	-
Salmonella species	-
Enterobacter species	-
Clostridium species	+
Actinomyces species	+

The choice of olive oil as a lipase inducer was selected because of the report of Bornscheuer (2002) which pointed out that amongst the natural oils, olive oil has proven to be the best substrate for the induction of lipase production. Several studies confirm a high production of lipase in culture media in the presence of olive oil at a concentration of 0.1% to 3% which corroborate to the results obtained in the present work. The work of Vishnupriya et al. (2010) and Essakiraj et al. (2010) also demonstrated the ability of olive oil as the best source of carbon for bacterial lipase production when compared to other oils. Dandavate et al. (2009) evaluated the effect of castor, olive, corn and peanut oils on lipase production by bacteria of the genus *Burkholderia* and the best production was observed in the cultivation of olive oil [16-20].

The supplementation of media with 2% dextrose was informed by a previous study by Zouaoui and Bouziane, 2012 which showed that the addition of dextrose gave the highest yield of lipases in a shake flask. A neutral pH was maintained for the fermentation because previous work has reported maximum lipase production at pH at 6–7. Similarly, fermentation was undertaken at 37 °C as Gaur et al., 2008 has previously shown this temperature range to be optimal for lipase production. After fermentation in the shake flasks for 48 hours using different lipase producing isolates, inoculum was obtained from the flasks to measure call biomass through serial dilution and spread plate method. The results are shown in Table 3. Below[21-23].

Table 3: Total viable count (TVC) of lipase producing isolates

Isolate	Total viable count (cfu/ ml)
Actinomyces sp.	5.4×10^5
	6.2×10^5
E. coli	3.7×10^5
	4.43×10^5
Bacillus sp.	5.3×10^5
	3.8 x 105
Pseudomonas sp.	7.8×10^5
	5.5×10^5
Clostridium sp.	4.4×10^5
	3.7×10^5
Klebsiella sp.	4.0×10^5
	3.0×10^5

From the result shown above, the highest cell mass was recorded by *Pseudomonas* sp. This is closely followed by *Actinomyces* sp. and *Bacillus* sp., with the lowest recorded for *Klebsiella* sp. This tallies with previous reports that a greater proportion of bacterial lipases comes from Gram-negative bacteria with the most important being *Pseudomonas* which contains at least seven lipase producing species [24]. Furthermore, investigations by Ankit et al. (2011) on lipolytic microorganisms for industrial use, isolated species of lipolytic bacteria from the genera *Pseudomonas* and Bacillus from highly contaminated water samples of several rivers in the Bhopal region of India which produced potent lipases [15].

The cell-free supernatant was precipitated using ammonium sulphate, followed by dialysis. This was used to determine the total protein content according to the Lowry's method. Table 4. below shows the protein content of the lipases.

Table 4: Estimation of protein content

Bacterial strain	Estimated protein content (mg/ml)
Pseudomonas sp.	1.53
Actinomyces sp.	1.47
Bacillus sp.	1.32
Clostridium sp.	1.20
E. coli	1.10
Klebsiella sp.	1.02

Previous studies have successfully synthesized lipases from microorganisms especially bacteria, fungi and yeasts [25]. Esakkiraj et al. (2010) reported that, the potent lipolytic bacterium *Staphylococcus epidermidis* CMST Pi 1 was isolated from the gut of shrimp P. indicus. In this current study, results obtained from primary screening inferred that, out of the tested 11 bacterial strains, 6 strains showed lipase activity[19]. These bacterial strains that produced extracellular lipases includes; *Bacillus* spp, *E. coli*, *Klebsiella* spp, *Pseudomonas* spp, *Clostridium* spp, and *Actinomyces* spp. and the presence of extracellular lipolytic activity is consistent with previous reports for *Bacillus* sp. [26], *Acinetobacter* sp., *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp., Proteus sp., Staphylococcus sp [1]. The lipolytic strains isolated in this work were both Gram-negative and Gram - positive bacteria, which is in agreement with the research of Rousenau and Jaeger (2000) who reported some Gram positive lipolytic bacteria genera including; *Bacillus* sp., *Staphylococcus* sp., *Clostridium* sp., and Gram negative lipolytic bacteria in the genera *Pseudomonas*.

From the results shown in the table above, it can be inferred that enzyme production corelates with high biomass as suggested by Bharathi et al., (2019) [7,27]. This is seen through the graduation in the estimated protein content which corresponds with the biomass of the producer organisms. The isolates obtained from fresh water clams has shown to be potent lipase produces, highlighting their potential use as source of the commercially important enzyme.

Conclusion

Lipases have acquired much attention because of their ability to be utilized in a wide variety of reactions and the roles they play in different industrial applications such as food production, pharmaceuticals etc. The results obtained in this study show that freshwater clam *M. mercenaria* can be a potential source of lipase producing bacteria. This study identified six species of lipase producing bacteria and highlighted an optimized growth medium and conditions that favour the improved production of lipases by the isolates. It opens up potentially new source for the production of lipases in a cost-effective manner for diverse uses.

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