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EVALUATION OF ENZYME-PRODUCING POTENTIALS OF BACTERIAL SPECIES ISOLATED FROM GOAT DUNG-CONTAMINATED SOIL AT AWKA SOUTH LOCAL GOVERNMENT, ANAMBRA STATE, NIGERIA

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Abstract

The increase use of synthetic enzymes in different industries has resulted in toxicity and decrease in specificity. This menace has necessitated for an alternative source of enzymes which could be friendly to both man and environment. This study was carried out to evaluate enzyme-producing potentials of bacterial species isolated from goat dung-contaminated soil at Awka South Local Government Area, Anambra State, Nigeria. Forty goat (40) droppings were collected from four different communities that were investigated (Umuawulu, Nibo, Mbaukwu, and Nise) at the LGA. The presence of bacteria in the goat dung-contaminated soil was evaluated using standard microbiological technique. The bacterial isolates were identified using cultural, morphological, and biochemical features. The digestive enzymes (amylases, lipases, and cellulases)-producing potentials of the bacterial isolates were investigated using standard microbiological techniques. The result revealed that all the bacterial isolates produced lipases, cellulases, and amylases, but the quantity of the enzymes varied as revealed in the enzyme activity index (EAI)S. The highest amylase was produced by *Klebsiella* species (75%) while the highest lipase was produced by *Bacillus* species (60%) and the highest cellulase was also produced by *Klebsiella* species (84.20%). Statistically, there was a significant ($P < 0.05$) difference in the quantity of enzymes produced by the bacterial isolates. This study has shown that bacterial species isolated from goat dung sites are capable of producing digestive enzymes, which are highly essential in different industries and biotechnology.

Key Words: Bacteria, Enzymes, Goat droppings, Goat barn

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1.0 Introduction

Microorganisms in the soil have been known to contribute immensely in nutrient recycling such as saprophytic roles of certain fungal and bacterial species (Lennon et al., 2020). In the soil, there are complex organic materials that cannot be optimized directly by plants such as polysaccharides, lipids etc. (Chouhan et al., 2020). These compounds are broken down by enzymes produced by microorganisms in the soil, and the ability of an organism to produce a specific enzyme depends on its physiology and environmental factors (Dash et al., 2015).

Dungs that are produced by domestic animals such as goat, sheep, cow, and chicken contain organic compounds, which interact with soil microbes, thereby influencing their metabolic processes (Lodha, 2018). The ability of a bacterium to survive in a high organic matter-enriched soil indicates high level of tolerance due to certain traits in their genetic composition (Lodha, 2018). Similarly, bacterial species that lack such adaptive traits are eliminated in the environment as described in the theory of evolution by Charles Darwin (Ilesanmi et al., 2020).

Research has shown that bacterial species that are capable of adapting in an environment produce metabolites that aid the organisms to thrive more in that habitat (Ehon et al., 2016). Microbial enzymes are beneficial metabolites that are produced by microorganisms such as bacteria and fungi in the environment (Ehon et al., 2016). These enzymes are highly specific in nature, as a given enzyme can only act on a specific substrate. Enzymes that are produced by bacteria in the soil play vital role in nutrient utilization and recycling (Alhamdani and Alkabbi, 2016). Complex organic compounds can only be optimized when there is an enzymatic degradation and the absorbable nutrients are made available for the growth of plants and soil microbes (Reddy et al., 2016).

Several researchers have evaluated enzyme-producing potentials of bacteria in animal dungs such as Ilesanmi et al. (2020), Ameri et al. (2015), Demissie et al. (2024) but little information has been documented on evaluation of enzyme-producing potentials of bacterial species isolated from goat dung-contaminated soil at Awka South Local Government Area. Hence, the aim of this study is to evaluate enzyme-producing potentials of bacterial species isolated from goat dung-contaminated soil at Awka South Local Government Area, Anambra State.

2.0 Materials and Methods

Isolation of Test Organisms from the Samples

The prepared and diluted samples (goat droppings) were aseptically grown on Nutrient agar (NA), MacConkey agar (MA), Centrimide Agar (CA), Sabouraud Dextrose agar (SDA), and Mannitol Salt Agar (MSA) (BIOTECH) which were prepared following the instruction of the manufacturer and procedures described in Cheesbrough (2010) and Frank and Robert (2015). The swab sticks used on the body of the goat were aseptically streaked in a sterile poured NA, MA, CA, MSA, and SDA. Plates (90 mm X 15 mm) as described by Frank and Robert (2015). Then, blood agar was used for the collection of air microbes (bioaerosol) using sedimentation techniques as described by Reddy *et al*. (2016). The cultured plates were carefully placed inside the bacteriological incubator (ST X B128) in an inverted position, and incubated at 35±2^{o}C for 24 h.

Purification of the Isolates: The plates that showed discrete colonies were selected after 24 h and aseptically streaked each colony on sterile plates (90 mm X 15 mm) containing NA (BIOTECH) prepared according to manufacturer's description. The streaked plates were placed in a bacteriological incubator in an inverted position and incubated at 35±2^{o}C for 24 h as described in Cheesbrough (2006) and Goldman and Green (2009).

Characterization of the Pure Isolates

The pure isolates were characterized using cultural, morphological, and biochemical characteristics as described in the study published by Iheukwumere *et al*. (2018).

Cultural and morphological characteristics of the isolates: The cultural description (size, appearance, edge, elevation, colour, surface) of the isolates was carried out as described in Goldman and Green (2009). The Gram staining technique which revealed Gram reaction, cell morphology, and cell arrangement was carried out using the procedure described by Cheesbrough (2010), Goldman and Green (2009) and Frank and Robert (2015). The presence or absence of capsule was ascertained as described by Goldman and Green (2009).

Gram staining technique :A thin smear was made in a cleaned grease free microscopic slide (75mm×25mm), air dried and heat fixed. The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with cleaned water. Gram iodine solution (0.01%) was then applied and allowed for 60 seconds. This was rinsed with cleaned water. This was followed by decolourizing the slide content with 95% w/v ethyl alcohol for 10seconds and then rinsed with cleaned water. The smear was then counter stained with safranin solution (0.025%) for 60 seconds, rinsed with cleaned water, blot drained and air dried. The stained smear was covered with a drop of immersion oil and observed under a compound binocular light microscope using x40 and x 100 objective lenses.

Capsule Staining Technique: A thin smear was also made in a cleaned grease free microscopic slide (75mm×25mm), air dried and heat fixed. Crystal violet (1%) was applied and allowed for 2 minutes. The crystal violet was gently washed off with 20% copper sulphate solution. The slide was blotted and dried, and observed under compound binocular light microscope using oil immersion lens (X100).

Motility test: A semi-solid medium prepared by mixing 5.0g of bacteriological agar (BIOTECH) with 2.0 g of nutrient broth (BIOTECH) in 1 litre of distilled water was used. The solution was dissolved and sterilized using autoclaving technique after dispensing 10ml portion in different test tubes. The test tubes were allowed to set in vertical positions and the test organisms were inoculated by performing a single stab down the centre of the test tube to half the depth of the medium using sterile stabbing needle. The test tubes were incubated in a vertical position.

Biochemical characteristics of the isolates: The ability of the isolates to produce catalase, tryptophanase, oxidase, acetoin, grow in 6.55% NaCl and to utilize sugars, sugar alcohols and other substances (ribose, sorbitol, arabinose, sacharose, glucose trehalose, lactose, starch, inulin, salicin, hiparate) were evaluated using the methods described by Cheesbrough (2010), Goldman and Green (2009) and Frank and Robert (2015).

Indole test: Indole is a nitrogen containing compound formed when the amino acid tryptophan is hydrolyzed by bacteria that have the enzyme tryptophanase. This is detected by using KOVAC’s reagent. For this test, isolates were cultured in peptone water in 500.0 ml of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The isolates were inoculated on the medium and incubated at 37 ± 2^0C for 48 h. Five drops of KOVAC's reagent were carefully introduced into the top of 24 h old pure culture. The presence of indole was revealed by the formation of red layer colouration on the top of the broth culture.

Sugar Fermentation test : This test was carried out to evaluate the ability of the isolates to metabolize sugars and sugar alcohol (glucose, xylose, ducitol, maltose, arabinose, inositol, mucate and lactose) with the resultant production of acid and gas or either. One litre of 1% (w/v) peptone water was added to 3 ml of 0.2% (w/v) bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham's tubes. The medium was then sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes. The medium was then inoculated with the appropriate isolates and the cultures incubated at 37 ± 2^0C for 48 h and were examined for the production of acid and gas. A change in colour from purple to yellow indicated acid production while gas production was assessed by the presence of bubbles in the inverted Durham's tubes.

Hydrogen sulphide production: This was performed using triple sugar iron (TSI) agar. The TSI agar was prepared according to the manufacturer's instruction. This was sterilized using autoclave and allowed to cool to 45˚C. The isolates were aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37 ± 2^0C for 24-48 h. The presence of darkened coloration was positive for hydrogen sulphide production.

Methyl red test : The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37 ± 2^0C for 48 h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution was added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

Voges-proskauer test : The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37 ± 2^0C for 48 h. After incubation, 1.0 ml of 40% potassium hydroxide (KOH) containing 0.3% Creatine and 3 ml of 5% solution of αnaphthol was added to the absolute alcohol. Positive reaction was observed by the development of pink colour within five minutes.

Citrate utilization test: The Simmon's Citrate Agar was prepare according to the manufacturer's direction and the isolates were inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37 ± 2^0C for 48 h. Positive test was indicated by the appearance of growth with blue colour, while negative test showed no growth and the original green colour was retained.

Catalase test: This was carried out as described by Cheesbrough (2010). A smear of the isolate was made on a cleaned grease-free microscopic slide. Then a drop of 30% hydrogen peroxide (H_2O_2) was added on the smear. Prompt effervescence indicated catalase production.

Oxidase test: Here, two drops of freshly prepared oxidase reagent was introduced to whatman No 1 filter paper placed in Petri dish, and a smear of the test isolates were made on the spot using a sterile stick. Production of blue-black colouration was checked within 15 seconds.

Coagulase test: The test was carried out as described by Cheesbrough (2010). A smear of the isolate was made on a clean grease-free microscopic slide that contained a drop of physiological saline, a drop of human plasma was added, and gently mixed, after 10 seconds, presence of clump indicated positive result.

Screening the Bacterial Isolates for Enzymes-producing Potential

Screening for amylase activity: This was carried out using Modified Basal Medium (MM), consisting of 1 g glucose, 2.5 g yeast extract, 16 g bacto agar, 1000 ml distilled water with the addition of 1% soluble starch. Bacterial isolates were streaked on selective amylase media and incubated for 24 h. After incubation, the plate was flooded using lugol's iodine solution as an indicator of starch and was allowed to stand for 15 min. The clear zone was formed around the bacterial colonies that revealed amylase activity. The clear area and index diameter were measured and calculated appropriately (Abd-Elhahlem et al., 2015).

Screening for cellulase activity: The cellulose activity was checked using MM media with the addition of 1% Caroxymethyl-Cellulose (CMC) as a substrate. After incubating for 48 h, the media were flooded using 0.1% Congo red solution, and were allowed to stand for 15 – 20 min and then rinsed using 1 M NaCl solution for 15 – 20 min. Cellulase activity was revealed by the formation of yellow colour around the colonies. The yellow colonies diameter and the diameter of the colonies were read using meter rule and pair of divider (Abdel-Aziz et al., 2021).

Screening for lipase activity: The lipase activity was detected using a medium containing 10 g peptone, 0.1 g $ACl_2.2H_2O$, 5 g NaCl, 10 ml tween 80.16 g bactor agar and 1000 ml of distilled water. The formation of white precipitate around the colonies indicated lipase activity. White precipitation is the disposition of crystal from calcium salts formed by acids from microorganisms (Mazzuotelli et al., 2013). The formular index value was calculated thus: Diameter of white precipitation- Diameter of the colony/ Diamater of the colony (Chouhan et al., 2020).

Statistical Analysis: The data obtained in this study were presented in Tables. One way Analysis of variance was used to determine the significance of the sample sources and distribution of the isolates in the sampled communities at 95% confidence level. Pairwise comparison was used to carry out student "t" test (Iheukwumere et al., 2018).

3.0 Results

Characterization of the Bacterial Isolates

The cultural and morphological characteristics of the bacterial isolates are presented in Table 1. The result revealed the selected bacterial isolates exhibited different colours on different selective media. All the bacterial isolates were circular and medium except isolate CA12 that showed large colonies while isolates CA7, CA3, and CA9 had regular margin and smooth surface. Also, all the isolates had rod shape and motile except isolate CA12, that was non-motile while endospores were only found in CA16 and CA9, which were all Gram positive unlike non spore formers which were Gram negative. The biochemical characteristics revealed that all the isolates produced catalase while indole and oxidase were produced by CA16 and CA9, respectively. Meanwhile, none of the isolates produced coagulase. However, all the isolates utilized glucose and maltose as their carbon and energy sources but lactose and sugar alcohols were only utilized by 50% of the isolates (Table 2).

Table 1: Cultural and morphological characteristics of the bacterial isolates

Parameter	CA16	CA7	CA3	CA12	CA9
Appearance	Fuzzy white on NA	Creamy on CA	Red on MA	Pinkish On MA	Greyish On BA
Colony Size	Medium	Medium	Medium	Large	Medium
Margin of Colony	Irregular	Regular	Regular	Regular	Regular
Elevation	Flat	Convex	Convex	Convex	Flat
Surface of the Colony	Rough	Smooth	Smooth	Smooth	Smooth
Shape of the Colony	Circular	Circular	Circular	Circular	Circular
Gram Reaction	Positive	Negative	Negative	Negative	Positive
Morphology	Rods	Rod	Rod	Rod	Rods
Endospore	Present	Absent	Absent	Absent	Present
Position of Spore	Sub-terminal	-	-	-	Central
Motility	Yes	Yes	Yes	No	Yes
Bacterium	*Bacillus* species	*Pseudomonas species*	*Escherichia coli*	*Klebsiella species*	*Clostridium* species

NA= Nutrient agar; CA= Cetrimide agar; MA= MacConkey agar; BA= Blood aga

Table 2: Biochemical characteristics of the bacterial isolates

Parameter	CA16	CA7	CA3	Ca12	CA9
Catalase	+	+	+	+	+
Oxidase	–	–	–	_	+
Indole	+	–	_	_	–

Coagulase	–	–	_	_	–
Citrate	–	+	–	+	+/_
H_2S	–	+	+	+	–
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	–	+	+/–
Xylitol	+/–	+/–	–	_	+/–
Inositol	–	+	–	_	+/–
Ducitol	–	+	–	+	–
Trehalose	+	+	–	+	+
Possible Bacterium	*E. coli*	*Bacillus* species	*Clostridium* species	*Klebsiella species*	*Pseudomonas* species

Screening for Enzyme-producing Potential of the Bacterial Isolates

The screening for enzyme-producing potential of the bacterial isolates is presented in Table 3. The result revealed that all the bacterial isolates produced lipases, cellulases, and amylases but the quantity of enzymes varies as revealed in the enzyme activity index. Isolate CA12 produced the highest quantity of amylase (75%) followed by isolate CA7 (66.7%) while the least amylase was produced by isolate CA9 (50%). In other hand, isolate CA7 produced the highest lipase as shown in the enzyme activity index (60%) followed by isolate CA12 (50%) while the lipase was produced by isolates CA3 and CA9 (10%). Similarly, isolate CA12 produced the highest quantity of cellulase (84.2%) followed by isolate CA7 (83.33%) while the least cellulase was produced by isolate CA9 (50%).

Table 3: Screening for digestive-enzymes-producing potentials of the bacterial isolates

Isolate	Enzymatic activity index (%)		
	Amylase	Lipase	Cellulase
CA7	66.70	60.00	83.33
CA3	58.30	10.00	60.00
CA12	75.00	50.00	84.20
CA9	50.00	10.00	50.00
CA16	64.30	30.00	70.00

CA (Bacterial isolates)

4.0 Discussion and Conclusion

The use of synthetic enzymes in different industries has not been fully satisfactory due to high toxicity and low specificity. Biologically synthesized enzymes have been observed to exhibit high level of specificity and low toxicity. This study examined enzyme-producing potentials of bacterial species isolated from goat dung-contaminated soil at Awka South Local Government Area, Anambra State. The bacterial isolates in this present study were majorly Gram negative bacteria (*Klebsiella* species, *Escherichia coli, Pseudomonas* species) and Gram positive bacterium (*Bacillus* species). Similar result was reported by Demissie, et al. (2024) who screened for bacteria present in cow dung and identified *Bacillus* species, *Escherichia coli,* and *Micrococcus* species and Tomar et al. (2020), who screened bacteria present in cow dung and also identified *Bacillus* species and *Enterococcus* species.

The ability of all the bacterial isolates in this study to produce digestive enzymes could be attributed to their high metabolic processes. *Bacillus* species showed highest enzyme production followed by *Klebsiella* species. The enzyme that was highly produced was amylase, which could be attributed to the vital functions it plays in breaking down complex carbohydrates into simpler sugars such as glucose. Similar observation was reported by several researchers (Pranay et al., 2019; Sreeleekshmi et al., 2019; John and Elangovan, 2013; Abd-Elhahlem et al., 2013; Mohammed et al*.,* 2011; Xie et al., 2014; Sahoo et al*.,* 2017; Dash et al., 2015) who screened for amylase-producing potentials in *Bacillus* species. Meanwhile, Oztat *et al.* (2024) specifically documented that *Bacillus thurigiensis* produced amylase when they evaluated amylase-producing potentials of bacterial isolates while Klinfoon et al. (2022) reported that *Bacillus* species, *Pseudomonas entomophla*, and *Pseudomonas putida* produced substantial quantity of amylase. In another study, *Klebsiella* species were reported as potential amylase producers by several researchers (Garba et al., 2021; Shiyon et al., 2020). Similarly, the ability of *Bacillus* species to produce lipase was reported in several studies (Chouchan et al., 2020; Muthumari et al., 2016; Reza et al., 2014) while the ability of *Klebsiella* species to produce lipase was also documented in several studies (Lin et al., 2012; Fashogbon et al., 2021; Alhandani and Alkabbi, 2016). Similarly, several researchers (Sadhu et al., 2013; Patagundi et al., 2014; Rasul et al., 2015; Ehon et al., 2016; Reddy et al., 2016; Abu-Gharbia et al., 2018; Niranjan et al., 2023; Demissie et al., 2024) had reported that *Bacillus* species produced cellulase as their metabolites during their exponential growth phase. The ability of *Klebsiella* species to produce cellulase was also reported in several studies (Chantarasiri, 2020; Abdel-Aziz et al., 2021; Korsa et al*.,* 2022). Statistically, there was no significant (P > 0.05) in the enzyme production by the bacterial isolates. These digestive enzymes synthesized by the bacterial isolates have a wide range of applications in biotechnology. For instance, amylase is optimized industrially for production of sugar syrups, which are used in pharmaceutical (as an excipient) and food industries (Sahoo *et al.,* 2017). In brewing industry, amylase is optimized in breaking down starches in sorghum and barley grains to release fermentable sugars and in textile industry, amylase is also used in the removal of starch from fabrics (Garba *et al*., 2020). Similarly, lipase is optimized in food industry for cheese and yoghurt production. In the production of biodiesel, lipase catalyses the transesterification of oils (Ameri et al., 2015). Also, lipase plays a vital role in bioremediation of organic pollutant and lipids (Ameri et al., 2015). Similarly, in bioethanol production, cellulase is optimized in breaking down cellulose in plant releasing fermentable sugars (Reddy et al., 2016). Cellulase is also essential in the production of paper as it breaks down cellulose in wood pulp and in brewing industries for clarification of wine and overall quality enhancement (Rasul et al., 2015). In the agriculture sector, cellulase, lipase, and amylase are essential in breaking complex organic matter to smaller ones that could add nutrient to the soil, thereby improving soil structure and texture (Chouhan et al., 2020; Demissie et al.,2024;). These digestive enzymes have been proved to play vital role in the production of biofertilizer due to the ability of the enzymes to break down complex substances. For instance, amylase in biofertilizer enables the production of carbon sources for soil microorganisms when starch is split into simple sugars. In biofertilizer, lipase facilitates the production of fatty acid and glycerol which are optimized by soil microorganisms for proliferation and general metabolic processes and cellulase also produces a carbon source for soil microorganism when cellulose is degraded (Niranjan et al., 2023). Enzyme-based biofertilizers have been proved to enhance sol structure, soil fertility, acceleration of crop yield due to availability of vital nutrients to beneficial soil microorganisms, and in the reduction of the effect of chemically synthesized fertilizer (Pranay et al., 2019).

Conclusion

This study has revealed that bacterial species are found in goat barns. These bacteria are capable of producing digestive enzymes such as amylase, lipase, and cellulase, which are frequently produced in various quantities. This shows that these bacteria could play significant roles in industries and biotechnology for production of essential products.

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Conflict of Interest

There was no conflict of interest among the authors.

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