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ISOLATION OF CELLULOLYTIC AND METHANOGENIC BACTERIA FROM THE MID AND HIND GUT OF TERMITES (ISOPTERA)

BY

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ABSTRACT:
The aim of this study is to isolate cellulolytic and methanogenic bacteria from the mid and hind gut of termites. Ten (10) termites were randomly sampled from their nest along Kalgo Birnin Kebbi road in Kebbi State. They were pretreated with 70% ethanol, then dissected using surgical blades and forceps, both the mid and hind gut were aseptically transferred into sterile petri dishes and were crushed. An aliquot of each sample of both mid and hind gut were serially diluted. Colonies were identified at generic level using their phenotypic characteristics. The results of viable plate count were 18.0 x 10^{-4} and 17.0 x10^{-4} for hind and mid gut respectively. Totally twenty six (26) bacteria were isolated out of which 14 were cellulolytic with an average halo zone of 2.5cm while 12 were non cellulolytic, also out of the 26 isolates nine (9) were methanogens while 17 were non methane producing. From the result obtained it is quite indicative that gut of termites habour bacteria that may be economically viable, with great environmental sanitization potentials. Most research on cellulose utilization and methane production has been performed on laboratory scale. Therefore there is the need to extend such studies to full scale field applications.

Keywords: **Termites, Cellulolytic, Methanogenic, Aliquot, Phenotype**

1.0 INTRODUCTION

Termites are found in soil, in nest called termiterium where they exhibit a microbial symbiotic relationship (Geo and Gupta 2011). Termites belong to the class Isoptera (equal - wing), they feed mostly on dead material, soil and animal dung's (Denovo *et al*., 2001). Termite is delicacy in diet of some human cultures and is used in many traditional medicines. Some insect such as termite, wood feeding, beetles, and leaf cutting ants, white grubs and snails uses cellulosic substrates as their main food source and are highly efficient at degrading cellulose to glucose as energy source.

Sustainable resources, which are in need of human beings, are derived from plant biomass; interest in bio energy has been sharply increasing in the recent years due to the necessity of sustainable economics and clean environment (lee *et al.,* 2008). Cellulose and hemicelluloses are the most abundant biomass on earth and therefore, have the greater potential to resolve both the energetic and environmental demands of bio energy (Saito *et al.,* 2003). Cellulolytic raw materials can be converted to methane by hydrolysis and fermentation (Denovo *et al.,* 2001). Therefore, the ability to isolating ideal biofuel bacteria with ability to degrade different cellulosic materials with high yield has an important role in developing biofuel production system (lee *et al.,* 2008). Cellulolytic organisms are ubiquitous in nature, mostly rod shape, obligate anaerobic gram negative bacteria (Saito *et al.,* 2003). Both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases and hemicellulases enzymes capable of degraded cellulose and hemicellulose. Bacteria have some advantages over fungi; cellulolytic bacteria have been isolated from a wide variety of environments such as decaying plant material originating from agricultural wastes, faeces of ruminants, soil, gastrointestinal tract of insects, and snail (Doi, 2008). A number of fungi and bacteria had been reported to be capable of utilizing cellulose as a carbon source (Murashima *et al*., 2002). Cellulases are produced by both fungi and bacteria such as from the genus *Aspergillus, Rhizopus* and *Trichoderma* (Murashima *et al*., 2002; Saito *et al*., 2003 and Fahrurrozi *et al*., 2010) and the genus *Bacillus (*Lee *et al*., 2008). Cellulases from both bacterial and fungal sources appear to function in a similar manner when acting on crystalline cellulosic substrates.

Methanogens are cocci or bacilli, they are anaerobic organisms. Some methanogens are called hydrogenotrophic used carbon dioxide as source of carbon and hydrogen as a reducing agent. Methanogens are microorganism that produced methane as a metabolic by product; they are responsible for mash gas in the digestive tract of ruminants, animal dungs, cellulose, hemicelluloses, lignin and human faecal material, Methanogen play vital ecological role in anaerobic environments of removing excess hydrogen and fermentation products that have been produced by other forms of anaerobic respiration (Gao and Gupta, 20011).

Increases in gas price have become the driving force for developing alternative energy sources especially fuel, bio ethanol for automobile using the abundant plants biomass (Gupta *et al.,* 2011). Activities such as plant cultivation and earth burning is believed to be the cause of increase methane level in the atmosphere, to add to these are the source of methane producing bacteria from the body surface of insects such as; Termites, snail, white bug (Dillon and Dillon

2004). The availability, sustainability in production and low starting value of this biomass on earth draw the attention of this research work.

AIM

To isolation cellulolytic and methanogenic bacteria from the mid and hind gut of Termite

OBJECTIVE

1 To enumerate number of isolates in colony forming unit per ml (cfu/ml)

2 To determine cellulose utilization

3 To formulate sugarcane bagasse agar medium

4 To quantify the volume of methanegas produced by isolates

2.0 MATERIALS AND METHOD

2.1 Sample collection

Ten (10) live Termites were randomly collected from their nest (Temiterium) along kalgo Birnin Kebbi road in Kebbi State Nigeria. In a sterilized sample bottle containing some soil particles of the nest and was transported to microbiology Laboratory of the Department of Science Laboratory of Waziri Umaru Federal Polytechnic Birnin Kebbi for further analyses.

2.2 Media preparation

2.2.1 Nutrient agar

11.8g of nutrient agar was weigh and dissolved in 500ml of distilled water, media was gently heated by autoclaving at 121^0c for 15minutes it was then allowed to cool for about 5^0c and then dispensed in to a sterilized Petri dishes and allowed to solidify gently. The sterilized Petri dishes with solidified nutrient agar were incubated for 24 hour at 37^0c to test for their sterility; plates that showed no growth of bacteria were used.

2.2.2 Sugar cane Bagasse agar (SCB)

9.6g of nutrient agar and 4.6g of crushed sugar cane bagasse fine powder were weighed and dissolved in 400ml of distilled water, sugar cane basal agar were heated over a hot plate to ensure proper dissolution of the media. Sugar cane bagasse agar was sterilized by autoclaving at $121^0{}_C$ for 15minutes and then allowed to cool for about 5^0c and incubated for 24hours at 37^0c.

2.3 Sample Treatment

Termites were placed in a sterilized stainless bowel; their surfaces were cleaned with 70 percent alcohol, and they were placed on a sterile white tiles, each of the sample were anatomically dissected using a sterilized surgical blade and forceps, sample were dissected into three

Anatomical portion namely, fore gut, mid gut, and hind gut. The mid and the hind gut were transferred into a sterilized Petri dishes and crushed with a bent glass rod.

2.4 ENUMERATION OF CELLOLULYTIC AND METHANOGENIC ISOLATES

The paste obtained was serially diluted to a factor $10^{-1,}$ 10^{-2} and 10^{-3} in other to reduce the microbial load. Sterilized test tube were arranged and labelled as sample M_1 - M_{10} for mid gut and sample H_I-H_{10} for hind gut, for each of the sample were labelled.

Three (3) set of test tube in triplicate with 9ml each were arranged, 1ml liquor from each of the paste of the crushed mid and hind gut was transferred into 9ml distilled water of factor 10^{-1}, 10^{-2} and 10^{-3} for both the mid and hind gut respectively. 1ml from factor $10^{-3}$for each of the sterilized test tube were transferred into a prepared nutrient agar and incubated for 48 hours at 37^0c colonies grew on the plates were counter and recorded.

2.5 Maintenance of cellulose degrading bacterial culture

The individual colonies that appeared during isolation studies were sub-culture on nutrient agar until pure culture were isolated. Each purified strains were maintained at 4^0C on two nutrient agar slants. One slant was stored as stock culture and other was used as working culture.

2.6 DETERMINATION OF CELLULOLYTIC ACTIVITY OF THE ISOLATE

Ability of the isolates to utilize cellulose as source of carbon was checked by inoculating each isolate into enriched media of sugar cane bagasse agar. Colony from nutrient agar plates were inoculated in a prepared sugar cane bagasse nutrient agar media (SCBNA), the plates were then flushed with 0.1percent methyl red and incubated for forty eight hours (48hr) at 37^0c, after the incubation period a cleared zone called the halo zone were observed. The colonies that showed halo zones were recorded as cellulose utilization bacteria positive while colonies with no halo zone were also recorded as cellulose utilization bacteria negative.

2.7 DETERMINATION OF GAS PRODUCTION ON WATER MELON PEEL

Colonies that are cellulase positive were tested for methane gas production. Water melon peel (Rind) was used as the cellulose (substrate); the water melon peel was blended using a blender and mixed with distilled water in the ration of 1: 2. The blended mixture was dispensed in a set up digesters, two (2) loopful of bacterial colonies were emulsified in a 2ml of distilled water to make an aliquot of the isolates and then transferred it into the different set up digester.

The cover of air tight container was pierced open with a giving set; super glue was used to seal the giving set to the container. The other end was connected to a measuring cylinder filled with water, the measuring cylinder was held within a clamp and a retort stand. The set up was left four weeks to check for gas production, gas from the digester set up cylinder they displacement of water by methane was observed and recorded as positive for gas production while cylinder that were still fill with water no displacement of water are recorded as negative.

3.0 Results and Discussion

3.1 ENUMERATION OF COLONIES IN CFU/ML

Table 1.0 and 1.1 shows the result of isolates colony count in cfu/ml for both the mid and hind gut of termites, results reveals that the hind gut of termites had 18 cfu / ml which is higher when

compared with the mid gut with 17 cfu/ ml, this indicate that the hind gut habour more bacterial than the mid gut. This result is not agreement with that of Begnell, (2006) in their study on isolation and screening of cellulolytic bacteria from termites, termites hind gut had 9.0cfu/ml of colonies on nutrient agar media compared with the mid gut of termites with 5.0 cfu/ ml.

3.2 Morphological characteristic of the isolates

Table 2.0 and 2.1 shows the result of morphological and other features of the isolates, twenty six isolates were identified, gram staining result show that all the fourteen isolate from the hind gut were gram negative bacteria while four isolate from mid gut are gram positive and eight gram negative. Gram staining results from the Table also present twelve of the isolates as cocci and fourteen were bacilli. This result is in conformity with that of (Screena *et al*., 2015 and Bholay *et al.,* 2014). That most bacteria associated with gut of Termites are gram negative cocci and bacilli.

3.3 DETERMINATION OF CELLULOLYTIC ACTIVITY OF THE ISOLATE

Cellulolytic activity of the isolates were determined on SCB agar media where sugarcane bagasse stand as source of carbon, flushed with 0.1percent of methyl orange as indicator, Table 3 reveals that fourteen (14) of the isolates were cellulose positive, nine (9) from the hind gut while (4) four from the mid gut with highest halo zone measure three point one (3.1cm) from the hind gut while the least with the zero point two (0.2cm) from the mid gut, this implies that isolate from hind gut are capable to produce enzyme that can utilize cellulose more than the isolates in the mid gut of Termites. This results is in conformity with that of Brain, (2013) where the highest halo zone was three point five (3.5cm) observed in media with carboxyl methyl cellulose as source of carbon and congo red used as indicator.

Table 3: Show the cellulose utilizing bacteria isolated at hid and mid gut of Termite

Anatomical portion of Termite	ISOLATES	CELLULOSE UTILIZATION	HALO ZONE (CM)
HID GUT	Dipolcocci species	4	6.30
	Diplobacilli species	4	8.00
	Streptobacilli species	1	29.00
	Staphylococci species	0	43.30
	TOTAL	9.00	86.60
MID GUT	Dipolcocci species	1	0.00
	Diplobacilli species	0	0.00
	Streptobacilli species	4	0.00
	Staphylococci species	0	0.00
		5.00	0.00

3.4 METHANOGENIC DETERMINATION OF ISOLATES

Table 4.0: Shows the methanogenic activity of the isolate on blended water melon peel medium, six (6) of the isolates from the hind gut produced gas while three of the isolates at the mid gut produced gas. The highest volume of gas produced was sixty (60) ml from the hind gut of the isolate with the highest halo zone while ten (10) ml was the least from the mid gut of termite observed with the least halo zone 0.2cm

Table 4: Show the volume of gas produced in the hid and mid gut of Termite

Sample	Volume of gas produce (cm^3)	Isolate
H1	58	*Diplococci spp*
H5C	45	*Diplobacilli spp*
H6A	43	*Diplobacilli spp*
H6B	60	*Diplobacilli spp*
H9	55	*Diplococci spp*
H10	40	*Diplococci spp*
TOTAL	**301**	
MEAN	**50.16**	
M2A	50.00	*Streptococci*
M4	58.00	*Diplococci*
M10	10	*Diplococci*
TOTAL	**118.00**	
MEAN	**39.33**	

3.5 PERCENTAGE OCCURANCE OF ISOLATES AT HID AND MID GUT OF TERMITES

Table 5 shows the percentage occurrence of isolates which reveals that 8 (57%) of the isolates are Diplococci, 5 (35.70%) of the isolates are Diplobacilli and 1 (7.00%) are streptobacilli. This implies that Diplococi with 57% are the dominate isolates at the hind gut of termites which is in line with the work of Screena *et al*. (2015) which isolate enterococci as the dominated isolate.

Table 5.0 Shows the percentage of Bacteria isolated at Hind gut of Termite

Bacterial isolates	Frequency of bacterial	Percentage
Diplococci species	8	57.10
Diplobacilli species	5	35.70
Streptobacili species	1	7.00
Total	14	100.00

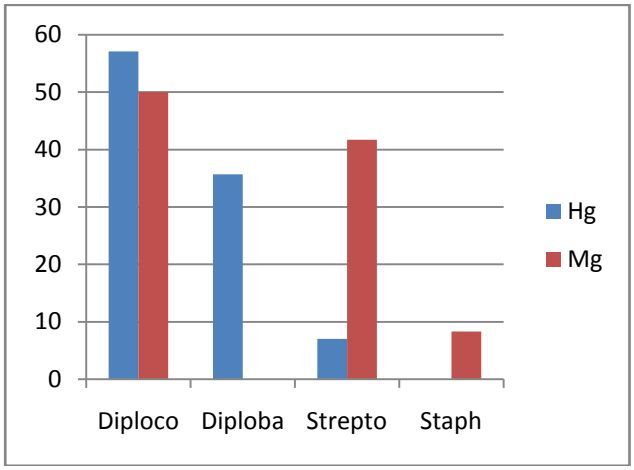
Table 6 shows that in the mid gut of termites also 6 (50%) of the isolate are Diplococci, 5 (41.70%) are streptobacilli and 1 (8.30%) are staphylococcus. Result reveals that Diplococci dominated the mid gut of termite which is contrary with the work of Bholay *et al.,(*2014) which isolate *Bacillus species* as the dominated bacteria.

Table 6.0 shows the percentage of Bacteria isolated at mid gut of Termite

Bacterial isolates	Frequency of bacterial	Percentage
Diplococci species	6	50.00
Streptobacillus species	5	41.70
Methanococci species	1	8.30
Total	12	100.00

Table 7 shows the percentage of total bacteria at hind and mid gut of termite which reveals that a total of 26 bacteria were isolated. Out of the 26 bacteria isolates, Diplococci and bacilli (Diplobacilli and strptobacilli species) had frequency of 25 (96.10%) which account for the most dominated bacterial in the gut of termite with high potential to utilize cellulolytic material by producing various enzymes for the activity. This result is in agreement with the work of Bholay *et al., (*2014) and Screena *et al.,* (2015) which isolated long rods, short rods and cocci bacteria in gut of termites.

Figure1: shows the percentage occurrence of isolates on both mid and Hid Gut of Termites



KEY: Diploco = Diplococci species , Diploba = Diplobacilli species, Strepto = Streptobacilli species and Staph = Staphylococci species

Table 7: Shows the Percentage occurrence of Total Bacteria Isolated from both mid and Hind gut of Termite

Bacterial isolates	Frequency of bacterial	Percentage
Diplococci species	14	53.80
Diplobacilli species	5	19.20
Streptobacili species	6	23.10
Methanocci species	1	3.80
Total	26	100.00

Conclusion and Recommendations

Cellulolytic bacteria from Termites gut were successfully isolated. Isolates from hind gut show a high potential in utilization of cellulose were identified as H1A,H4A,5B,5C,6A, and7B, and mid gut isolates identified as M3A,M4A,M5A,M6A,M7AM8A,M9A and M10B. The highest cellulose utilization activity was shown by isolate 4A from hind gut. Research on biotechnological aspect of these isolates should be carried out. Biochemical and molecular test of the isolates should be considered in other to identify the isolates to species level and molecular level.

Table 1.0 shows the plate count in colony forming unit per ml from mid and hid gut of Termites.

Dilution factor cfu/ml	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	TCC	ACC
10^{-4}	2	3	2	1	2	1	1	2	1	2	17	1.7
	HI	H2	H3	H4	H5	H6	H7	H8	H9	H10		
10^{-4}	2	1	2	1	2	2	2	2	2	2	18	1.8

KEY: M= Mid Gut, TCC= Total colony count, ACC= Average colony count, H = Hid Gut

Table 2.0 shows the identification of cellulolytic and methanogenic isolate from hid gut of Termite

Sample code	Phenotypic appearance of colony	GR	OX	CR	CU	HZ (cm)	GP	VG (cm^3)	Isolate
H1	Large, creamy, smooth, irregular shape with undulate margin	-	-	+	+	0.3	+	58	*Diplobacilli specie*
2a	Dot-like yellow,smooth,flat,circular in shapewith entire margin	+	-	+	-	-	-	-	*Streptobacilli specie*
B	Large, cream, rough, flat, irregular in shape with lobate margin	-	-	+	-	-	-	-	*Diplococci specie*
3a	Large, cream, smooth, irregular in shape, with lobate margin	-	+	+	-	-	-	-	*Diplococci specie*
4a	Small, whitish, smooth, flat, filamentous in shape with lobate margin	+	+	+	+	29	-	-	*Streptobacilli specie*
5a	Small, cream, rough, flat, irregular in shape with lobate margin	-	-	+	-	-	-	-	*Diplococci specie*
B	Large,cream,smooth, irregular in shape, with lobate margin	-	-	+	+	2.4	-	-	*Diplococci specie*
C	Large,whitish,rough,rhizoid in shape with filiform margin	-	-	+	+	3.0	+	45	*Diplobacilli*
6	Thin,whitish,smooth,flat,rhizoid in shape with filiform margin	-	-	+ve	+	1.6	+	43	*Diplobacilli*
7a	Small,yellow,smooth,raised,circular in shape with entire margin	-	-	+	-	-	-	-	*Diplobacilli*
B	Large,cream,smooth flat,irregular in shape, lobate margin shape, lobate margin	-	+	+	+	3.1	+	60	*Diplobacilli*
8	Small,whitish,smooth,concave,circular in shape with entire margin	-	-	-	-	-	-	-	*Diplococci*
9	Small,gray-whitish, rough, cratarformfilamantous in shape with cruzled shape	-	+	-	+	1.0	+	55	*Diplococci*
10	Small,pinkish,smooth,raised,circular in shape with entire margin	-	-	+	+	2.9	+	40	*Diplococci*

Table 2.1 shows the identification of cellulolytic and methanogenic isolate from mid gut of Termite

Sample code	Phenotypic appearance of colony	GR	OX	C	CU	HZ (cm)	GP	V (cm)	Isolate
M1	Large,cream,rough,lobate,filamentous ambonate	-	+	+	-	-	-	-	*Diplococci*
M2a	Small,yellow,smooth,undulate regular flat	+	+	+	-	-	+	50	*streptococci*
M3	Large,cream,lobate,smooth,regular,flat	+	+	_	+	.	-	-	*streptobacilli*
M4	Small,cream,smooth,filiform,rhizoid raised	-	+	+	-	-	+	58	*Diplococci*
M5	Small,rod,smooth,lobate,rhizoid,raised	+	_	+	-	-	-	-	*streptobacilli*
M6	Small,yellow,smooth entire,circular concave	-	_	+	+	_	-	-	*streptobacilli*
M7	Dot-like,light brown,smooth,entire,circular concave	-	_	+	+	_	-	-	*Diplococci*
M8	Dot-like yellow,smooth,entire circular lat	+	_	+	+	_	-	-	*stretobacilli*
M9a	Large,gray-whitish,rough,cuzled filamentous cretarform	+	_	+	+	_	-	-	*streptobacilli*
B	Small, pink,rough,entire,circular cretarform	+	-	_	-	-	-	-	*streptobacilli*
M10a	Large,cream,smooth,undulate,irregular,unbonate	+	-	_	-	-	-	-	*Staphylococci*
B	Small,yellow,smooth,raised,circular inshape with entire margin	-	-	+	+	_	+	10	*Diplococci*

KEY: GR= Gram reaction, OX= Oxidase, CU= Cellulose utilization, HZ= Halo Zone, GP= Gas production, V= Volume of Gas produce, - = Negative and + = positive

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