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Cellulolytic activity of bacteria from the gut of termites (*Macrotermes michaelseni)* from Eldoret and Kakamega

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Cellulose degrading bacteria in termites are important plant degraders as they play an essential role in digesting cellulose for termites. This study aimed to identify cellulose degrading bacteria in termites (*Macrotermites michaelseni)* collected from Kakamega and Kapsabet region. Six termites were aseptically crushed in an Eppendorf tube containing distilled water. To distinguish cellulose degrading bacteria from non-cellulose degrading isolates, the homogenates were inoculated on nutrient agar and carboxymethyl cellulose media. As a result, 14 isolates from Kakamega termites and 3 isolates from Kapsabet showed cellulolytic activity on carboxymethyl cellulose media based on the existence of a clear zone around their colony out of 24 obtained from both termites. The highest cellulolytic index obtained was 5.8, while the lowest cellulolytic index obtained was 1.5. These findings suggest that termites harbor cellulose-degrading bacteria that can be used in cellulose degradation.

Key words: Cellulose, cellulose degrading bacteria, termites, *Macrotermes*.

INTRODUCTION

Cellulose is the most prevalent component of plant cell walls and the world's most abundant renewable bio-resource (Behera et al., 2017). Cellulose is a glucose-based polysaccharide containing glycosidic connections (Kameshwar and Qin, 2016) It accounts for 35-50% of the plant's dry weight, while hemicelluloses and lignin account for 20-35% and 5-30% of the plant's dry weight, respectively, (Behera et al., 2017). Cellulase is a high-capacity enzyme that works in tandem with three other cellulases to hydrolyze cellulose into glucose. Endo-1,4-glucanase, exoglucanase, and -D-glucosidase are the three forms of cellulose (Sakolvaree and Deevong, 2016). To digest cellulose, bacteria produce the enzyme cellulase during their development on cellulose (Sreena et al., 2015). Endo-glucanases, cellobiohydrolases (or exo-glucanases), and -glucosidases all work together to break down cellulose completely into glucose (Sharma et al., 2015; Sreena et al., 2015). The endoglucanses attack the various interior sites of the cellulose fiber amphorous area at random. This frees up potential attack sites for

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the exoglucanase in the future. By removing mono and dimers from the reducing and non-reducing ends of the glucose chain, cellobiose and oligosaccharides are produced. Finally, -glucosidase breaks down the cellobiose into glucose monomers. Glucose is transported across the membrane to engage in energy-generating metabolic processes. Carboxymethyl cellulose (CMC) has been utilized extensively in the investigation of gut microbes that produce endo-1,4-glucanase. Due to the rise of cellulase applications in various sectors, cellulase is currently preferred as the third enzyme for industrial demand in the world, and it is predicted to become the largest amount of industrial enzyme in the future (Sakolvaree and Deevong, 2016).

Bacteria in soils, mammals, and termites have all been demonstrated to be capable of degrading complex cellulose in extensive investigations (Kameshwar and Qin, 2016; Sreeremya et al., 2016). Termite bacteria, on the other hand, are the most efficient cellulose degraders (Kudo, 2009; Mikaelyan et al., 2015), as evidenced by their ability to devour wood that is difficult to breakdown in nature (Auer et al., 2017). Macrotermitinae is a subfamily of the termites knows as fungus-growers (Vesala et al., 2017). They are among the most numerous and dominant insects in Asia's tropical and subtropical habitats, as well as the African rainforest (Ali et al., 2019; Femi-Ola and Oyebamiji, 2019). In several areas of Africa's savanna, they are the main decomposers of plant biomass (Vesala et al., 2017). Macrotermitinae are distinguished by their massive mound structures and underground gallery networks (Dangerfield et al., 1998; Sileshi et al., 2009). They build mounds to keep moisture, humidity, gas exchange, and temperature conditions stable, while the underground gallery system is used to gain access to foraging areas (Dangerfield et al., 1998; Vesala et al., 2017). They build mounds to maintain moisture, humidity, gas exchange, and temperature condition while the underground gallery system is used for accessing foraging areas (Dangerfield et al., 1998; Vesala et al., 2017). Remarkably is the termite's unique symbiotic relationship with the fungus termitomyces (Femi-Ola and Oyebamiji, 2019; Nobre, 2010). The symbiotic fungus grows on a fungus comb made by the termite.

The accumulation and underutilization of plant biomass in the ecosystem has been a global issue, prompting many scientists to perform research that will contribute to the reuse of this plentiful and renewable resource into valuable goods that will lead to the world's sustainable development. Researchers observed that the symbiotic micro-organisms in the intestines of termites assist in digesting termites.

Although effective isolation and identification with cellulolytic activity of these microbes were attempted, some species remained uncultured and therefore uninvestigated. As a result, there is a need to research efficient cellulolytic bacteria for the conversion of cellulose into glucose.

The purpose of this work was to isolate and detect active cellulolytic bacteria in the complete body of *Macrotermes michaelseni* that can be exploited in plant cellulose breakdown.

MATERIALS AND METHODS

Sample collection and processing

The termite samples were collected in January, 2018 in Vihiga that lies between 0.0768N latitude and 34.7078E longitude in the eastern part of Kakamega forest that is 1500-1600 m above sea level. The other termite samples were collected from the termite mound surrounding Kimondi River in Kapsabet. Kapsabet is a town in Nandi County located 40 km southwest of Eldoret on the way to Chavakali. It lies between latitude 0034N and longitude 34045E. The termite hills in Vihiga and Kapsabet were dug with a hoe and a piece of termite mound was selected. Six mound fragments were placed in separate boxes and transported to the laboratory for further examination. The boxes were kept in a cabinet at 25°C in the laboratory. Soldier castes were stored in 70% ethanol and utilized for morphology-based termite identification, while worker castes were employed for bacterial isolation within 24 h after sampling (Sakolvaree and Deevong, 2016).

Six worker termite were randomly chosen from the six mound fragments and sterilized with 70% ethanol for ten minutes and rinsed in sterile distilled water for another minute (Femi-Ola and Oyebamiji, 2019). Each termite were crushed with a glass rod in an Eppendorf tube with 1.5 µl microliters of distilled water to create a paste for bacteria isolation. The tubes were shaken for 24 h at 37°C at a speed of 150 rpm on a shaker (Kavitha et al., 2014).

Isolation and purification of bacteria

Each homogenous solution were plated on nutrient agar composed of (peptone, beef extract, sodium chloride, agar and distilled water) for the cultivation of bacteria and incubated at 37°C for seventy-two hours. Single distinct colonies that appeared on the plate were picked and re- grown on a new nutrient agar plate for twenty-four hours until pure isolates were obtained (Kavitha et al., 2014).

Morphological characteristics of bacterial isolates

Morphological characteristics of the pure isolates' colonies, such as form, surface, margin, and color, of the colonies on the plate were observed (Sharma et al., 2015). Gram staining was also done to see whether the twenty-four isolates were Gram-negative or Gram-positive (Ayitso and Onyango, 2016). This process was performed by the collection of a portion of the colony using an inoculating loop under aseptic conditions, which was transferred to a water-containing slide to form a thin coating. The slide was then passed through the Bunsen burner to fix the bacteria. The slide cooled in the air and then poured crystal purple stain for a minute, followed by a wash through flowing tap water. Following that, the slides were drenched with Gram iodine for a minute before being rinsed with water. To prevent the cells from bleaching, the decolorizer was poured for a few seconds and promptly washed with water. Finally, the saffron stain was put on the slide to stain the Gram-negative bacteria for two minutes, rinsed with water, and dried. The slides were viewed under a microscope by applying oil immersion and analyzed under 100x objectives lens to determine whether or not the bacteria were Gram-positive (purple or blue) or Gram-negative

Table 1. Morphological characteristics of the bacterial isolates

S/N	Isolate	Termite	Shape	Surface	Colour	Shape of bacteria	Gram stain
1	KG11	Macrotermes	Irregular	Raised	Cream	Bacillus	Negative
2	KG12	Macrotermes	Spherical	Raised	Cream	Coccus	Positive
3	KG13	Macrotermes	Spherical	Raised	Orange	Coccus	Negative
4	KG14	Macrotermes	Irregular	Raised	Brownish	Coccus	Negative
5	KG15	Macrotermes	Irregular	Raised	Cream	Bacillus	Positive
6	KG16	Macrotermes	Spherical	Raised	Red	Bacillus	Negative
7	KG21	Macrotermes	Spherical	Raised	Yellow	Coccus	Positive
8	KG22	Macrotermes	Spherical	Raised	Cream	Coccus	Negative
9	KG23	Macrotermes	Spherical	Raised	Cream	Coccus	Negative
10	KG24	Macrotermes	Spherical	Raised	Greyish	Coccus	Negative
11	KG25	Macrotermes	Spherical	Raised	Greyish	Bacillus	Positive
12	KG26	Macrotermes	Spherical	Raised	Red	Bacillus	Negative
13	KG31	Macrotermes	Spherical	Raised	Yellow	Bacillus	Positive
14	KG32	Macrotermes	Spherical	Raised	Orange	Bacillus	Positive
15	KG33	Macrotermes	Spherical	Raised	White	Coccus	Positive
16	KG34	Macrotermes	Spherical	Raised	Cream	Coccus	Positive
17	KG35	Macrotermes	Irregular	Raised	Yellow	Coccus	Negative
18	KG36	Macrotermes	Irregular	Raised	Cream	Coccus	Negative
19	EG11	Macrotermes	Spherical	Raised	Cream	Bacillus	Positive
20	EG12	Macrotermes	Spherical	Raised	Cream	Bacillus	Positive
21	EG13	Macrotermes	Spherical	Raised	Orange	Coccus	Positive
22	EG21	Macrotermes	Oval	Flat	Greyish	Bacillus	Positive
23	EG31	Macrotermes	Filamentous	Flat	Orange	Coccus	Positive
24	EG32	Macrotermes	Spherical	Raised	Orange	Coccus	Positive

(red) and cocci or bacillus.

Screening for carboxymethyl cellulose hydrolyzing bacteria

The isolates obtained were grown on the agar medium, added by 1% level CMC at 37°C for 48 h. Thereafter, a gram's iodine solution was poured onto the plates for 15 min. Afterward, the solution was poured out and observed in the presence of a clear zone around the colonies. A clear zone around colonies suggested the bacterial synthesis of extracellular cellulase. The cellulolytic potential of the positive isolates was assessed using the cellulolytic index (CI), which is defined as the ratio of the diameter of the zone of hydrolysis to the diameter of the colony mentioned by Saini et al. (2017).

RESULTS

Sample collection and processing

Termites used in this study were collected from two different locations in Kakamega and Kapsabet. In both sites, termites were found in mounds that were stronger than normal soil. In Kakamega, the termites mound contained fungus combs in them with and the mound was constructed in with plant stems as opposed to the Kapsabet termite's mound, which was made up of networks linked to each other. The termites collected were identified as *Macrotermes michaelseni* by an entomologist at the University of Nairobi.

Isolation and purification of bacteria

A total of twenty four isolates were obtained from Kakamega and Kapsabet termite after successful purification of the isolates. The isolates were denoted as KG from Kakamega and EG from Kapsabet (Table 1).

Morphological characteristics of bacterial isolates

Colony characteristics of each isolates showing different characteristics in shape, elevation margin and color are presented in Table 1. 17 of the twenty-four isolates had circular shapes and elevated surfaces, 5 were irregular, and the other two had oval and filamentous shapes, respectively. The gram staining procedure revealed that the majority of the isolates stained gram positive, while 9 isolates stained gram negative (Table 1). On the other

Table 2. Cellulolytic activity of the seventeen isolates.

Isolate	Location	Diameter of clear zone (mm)	Diameter of colony (mm)	Cellulolytic index
KG12	Kakamega	7	5	0.15
KG13	Kakamega	8	6.9	0.40
KG14	Kakamega	29	16	0.81
KG15	Kakamega	29	23	0.26
KG16	Kakamega	79	45	0.76
KG21	Kakamega	18	5	2.60
KG23	Kakamega	15	3	4.00
KG24	Kakamega	24	18	0.33
KG25	Kakamega	32	15	1.13
KG26	Kakamega	67	41	0.63
KG31	Kakamega	20	10	1.00
KG32	Kakamega	10	5	1.00
KG33	Kakamega	17	7	1.43
KG35	Kakamega	16	5	2.20
EG11	Kapsabet	31	6	4.17
EG12	Kapsabet	41	6	5.83
EG21	Kapsabet	22	17	0.29

hand, morphological presence on the microscope showed that 10 isolates were rods and 14 isolates were spherical.

Screening for carboxymethyl cellulose hydrolyzing bacteria

To assess cellulolytic activity, all twenty-four isolates were subjected to a carboxymethyl cellulase activity. As a result, 17 isolates had a clear zone surrounding their colony, suggesting carboxymethy cellulose activity. The cellulolytic activity of the isolates after staining with gram's iodine is shown in Table 2.

All the twenty four isolates were screened for carboxymethyl cellulase activity to determine their cellulolytic activity. As a result, 17 isolates showed presence of clear zone around their colony thus indicating carboxymethy cellulose activity. Among 17 isolates that showed clear zone, 14 isolates came from termites in Kakamega while 3 isolates were from Kapsabet. Based on cellulolytic index among Kakamega termites, isolate KG23 showed the highest cellulolytic index of 4.00 while the lowest was KG12 with cellulolytic index of 0.16 respectively. In contrast, isolate EG 12 from Kapsabet termite had the highest cellulolytic index (5.83), while isolate EG21 had the lowest (0.29).

DISCUSSION

Sample area

This study successfully isolated cellulose degrading bacteria in *Macrotermites michaelseni* collected from Kakamega and Kapsabet. This study supports the idea that termites harbour celluloytic bacteria inside their gut (Gupta et al., 2012; Kakkar et al., 2015). After culturing on nutrient agar, a total of twenty-four isolates were collected. Kakamega termites had a higher bacterial population than Kapsabet termites. This research backs up previous findings showing the presence of bacteria in termites (Ferbiyanto et al., 2015; Pourramezan et al., 2012). While it is known that one termite has a large number of microbial species in its gut, only a few were culturable during this research. One possible explanation for this may be the difficulty in simulating natural environments in the gut atmospheres.

Isolation and purification of bacteria

The morphological characteristics of the bacterial isolates obtained in this study varied and differed between the two sites. When opposed to Kapsabet termites, Kakamega termites had a higher diversity of culturable bacteria. These findings are consistent with those of Kavitha et al. (2014) and Ntabo et al. (2010), who isolated diverse bacteria in soil feeding termites and surrounding soil from Juja and Kakamega forest termites in Chennai, respectively.

Some of the isolates grew faster and were visible after twenty four hours while other became clearly visible after forty eight hours of incubating. The majority of the cells were able to maintain violet stain, suggesting gram positive organisms, whereas fewer cells were able to keep primary stain, indicating gram negative organisms.

However, these findings contradict those of Ayitso and Onyango (2016), who found that gram negative microbes outnumber gram positive ones. Microorganisms may be endemic to certain geographical regions due to differences in soil composition, food type, rainfall received, and agricultural activity carried out in the area, all of which may influence the sort of bacteria found in termites (Ayitso and Onyango, 2016).

As previously observed in other termites, the majority of the isolates collected in this study were found to be rods, indicating that they belonged to the genus *Bacilli*. Other isolates were cocci in form. These findings are consistent with previous research that has consistently found *bacillus* to be the dominant isolate. For example, the frequency of *Bacillus* in the *M. michaelseni* worker and soldier was observed by Ayitso and Onyango (2016). *Bacillus* in subterranean *Psammotermes hypostoma Desneux* has also been discovered in another study by Ali et al. (2019).

Screening for carboxymethyl cellulose hydrolyzing bacteria

Cellulolytic bacteria produce cellulases enzymes that break down the glycosidic bonds between cellulose microfibrils, releasing oligosaccharides and improving cellulose digestion (Hidayat, 2021). This enzyme aids in the breakdown of complex cellulose compounds into smaller molecules, allowing bacteria to digest them (Peristiwati and Herlini, 2018). All plates were screened using 1% carboxymethyl cellulose media to test the isolates' cellulolytic capability, as described above. The presence of endoglucanase was demonstrated by the creation of a clear zone after pouring gram's iodine (Hidayat, 2021).

Only seventeen isolates had clean zones surrounding their colonies, despite the fact that 24 isolates developed effectively on carboxymethyl cellulose media. The cellulose in the media was hydrolyzed as a result of the bacteria's cellulolytic enzymes. As a result of the binding of gram's iodine with polysaccharide during the hydrolysis process, clear zones formed around the colony, forming a clear zone (Gohel et al., 2014).

Cellulolytic index was used to test the cellulolytic potential of the positive isolates. This was achieved by dividing the diameter of the clear zone by the diameter of the colony. According to Hidayat (2021), isolates with cellulolytic index greater than 1.50 are regarded as potential cellulose producers. The cellulolytic index of the 17 isolates that formed clear zone around their colony varied from one organism to another. The highest cellulolytic index obtained was 5.8, while the lowest cellulolytic index is 1.5 as mentioned above. These findings are in agreement with those obtained by Kakkar et al. (2015), who determined cellulolytic activity in *Odontotermes parvidens* guts. However, the highest cellulolytic index (3.50) value reported in their study was lower than the one obtained from this study. Another study by Hidayat (2021) obtained similarly cellulolytic index results ranging from 1.16 to 4.89 as obtained from this study.

Conclusion

This study indicated the existence of cellulose degrading bacteria in termites to break down carboxymethyl cellulose media, indicating their potential in cellulose degradation. The cellulolytic bacteria obtained in this study can be employed in plant waste, production biofuel thus leading to sustainable development.

Recommendation

To better understand the mechanism of plant cellulose degradation, more research should be done to evaluate the efficacy of these isolates in degrading plant biomass in order to improve its long-term use in biotechnology, biofuel, and bio products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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