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Biogas Producing Bacteria from Termites

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Abstract – The human demands for energy and conservation of healthy environment have driven the development of cellulosic wastes into new generation of bioenergy. Ethanol was the first generation of biofuel developed to lessen dependence on fossil fuels. Efficiency of conversion of cellulosic material into simple sugars was the driving force behind search for strong cellulose-degrading microbes; Termites was chosen to be an excellent candidate to find such microbes. Six bacteria were isolated from the Termite's guts where their cellulose degrading activities were screened using carboxymethyl cellulose as a substrate and Congo red as an indicator. Only one isolate showed a relatively higher cellulolytic activity and biogas production. This isolate was identified by a variety of methods including the MALDI-TOF MS and turned out to be *Enterobacter cloacae*. The ability of this bacterium to degrade cellulose and produce biogas qualifies it for further development at genetic levels and biotechnological levels to produce biogas from cellulosic wastes.

Keywords – Cellulose degrading bacteria, Termite, MALDI-TOF MS and Biogas.

I. INTRODUCTION

Cellulose is a crystalline polymer composed of β-1, 4 glycosidic bonds and it is the most abundant biopolymer on earth **[1]**. The accumulation of agricultural residues and the municipal wastes compounded environment problems associated with their disposal. These solid waste material composed of 40–50% cellulose, 9–12% hemicelluloses, and 10–15% lignin on a dry weight basis **[2]**.

Cellulose hydrolysis to glucose and soluble sugars requires the synergistic action of three enzymes: 1,4-β-endoglucanases, 1,4-β-exoglucanases, and β-glucosidases **[3]**. One of the best sources for a cellulolytic enzymes was found to be harbored by symbiotic microorganisms in the intestinal tract of organism (e.g. Termits and cattles) which their lives depend on metabolizing cellulosic materials **[4]**. Termites as an insect have evolved effective strategies to use lignocellulosic substrates as energy sources, making them an ideal target for new cellulolytic enzymes **[5, 6]**. The symbiotic relationship between the microbial community within the termite's guts enable them to degrade cellulose from complex natural woods consisting of lignin, hemicellulose, and cellulose with great efficiency **[7]**. Recent studies have indicated that their gut microbiota may provide a suitable model system to understand the electron flow involved during cellulose degradation by anaerobic microbial communities **[2]**. The cellulolytic enzymes from termites were capable of withstanding high temperatures, low specific activity, and inhibitory agent generation during hydrolysis and susceptibility to changes in pH.

In this study, we have investigated cellulolytic bacteria isolated from the gut of a wood-fed termite that could be a possible source of cellulases for hydrolysis of cellulosic biomass and bioconversion biofuels and/or biogases.

II. MATERIAL AND METHOD

Collection of Termites

The termites were collected in a sterilized plastic container from apparently decomposed wood and/or tree samples in three different localities of Faculty of Agricultural Alexandria University, Mansoura University campus, and from Aswan Governorate, Egypt. All collected termites were used for bacterial isolation with the help Prof. Dr. Ahlam Alfazairy, College of Agriculture, Alexandria University, Egypt.

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Isolation of bacteria from termite gut

All termites were surface sterilized by 70% alcohol in a petri dish. The head of each termite was separated and the guts were removed from the body with sterile forceps. The pulled guts were out crushed with glass rods and used for bacterial isolation. A series of sequential dilutions up to 10^{-8} were performed to reduce the density cells before being inoculated onto nutrient agar plates. The inoculated plates were incubated in an anaerobic chamber for 3-5 days at 37°C **[2]**.

Screening for cellulose-degrading bacteria

For the isolation of cellulolytic bacteria, the grown colonies from previous procedure were re-inoculated onto Carboxymethyl Cellulose (CMC) agar plates, containing per liter: $MgSO_4.7H_2O$ 0.5 g, NaCl 2.3 g, $Na_2HPO_4.2H_2O$ 5 g, yeast extract 2 g, CMC 10 g and agar 15 g. The cultures were kept for two days at 37°C. The culture agar plates were flooded with 0.1 % Congo red reagent and left at room temperature for 20 minutes. Then the plates were washed with 1M NaCl. Colonies displaying Congo-Red discoloration around them are considered cellulose-degrading ones and chosen for further studies **[2]**.

Morphological and cultural characteristics

Morphological and cultural characteristics of the isolates were examined; such as the diameters, colors, texture, size, cell arrangement, elevation and pigmentation. Moreover, all the isolates were also subject to microscopic examination after Gram staining.

Determination of biogas production abilities

CMC Broth medium were prepared and inoculated with the cellulosic bacteria, then layered with mixture of Vaseline and melted wax (1:1 w/w) and incubated for 3 days at 37°C.The amount and production of biogas were estimated as the space appearing above the surface of the liquid culture **[8]**.

Identification of biogas-producing isolate

The cellulose-degrading bacterium was identified using MALDI-TOF MS technology. A single colony was spotted on a stainless-steel target plate as a thin layer, allowed to dry, then covered with a 1ul of matrix solution, allowed to dry again and then inserted into the MALDI-TOF MS instrument (Biotyper® 4.0.19 Bruker; **Model:** Microflex LT) for analysis using the default parameter settings. Results were classified following the standard identification scores provided by the Bruker Biotyper® software: ID score < 1.7 indicates "not reliable ID," "Genus level ID" is a reliable identification and probable genus level ID with an ID score > 1.7, and ID score > 2.0 indicates a reliable identification secure Genus level ID (probable Species level) **[9]**.

III. Results

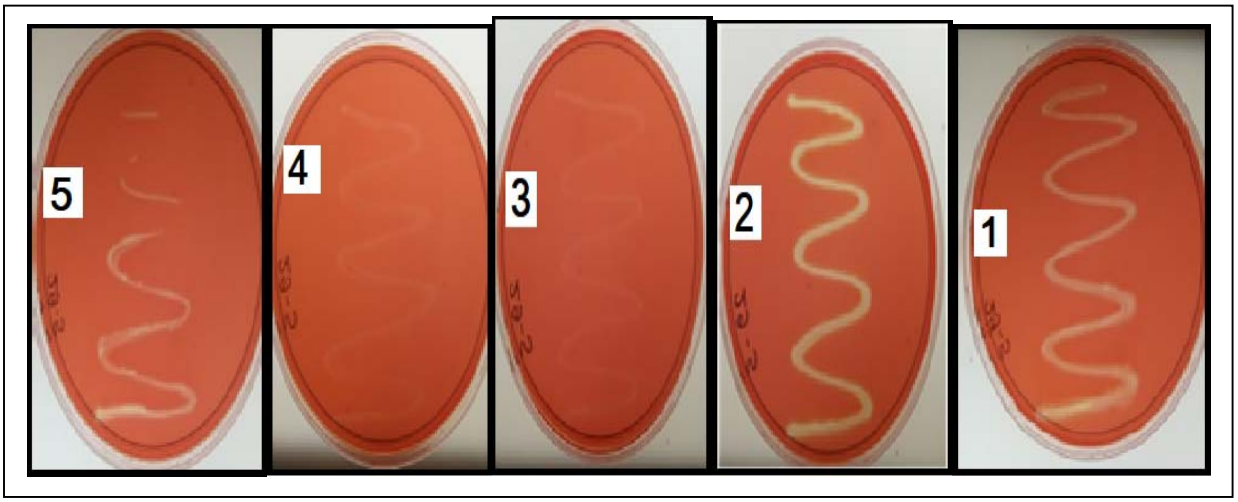
A total of 10 bacteria were isolated from the midguts of the termites. The isolates were further screened for their cellulolytic abilities, and biogas production as detailed below. The 10 isolates were grouped based on the colony appearance to five only. These five colonies showed different shape, elevation, margin and color as summarized in Table (1).

Table 1: Colony characteristics of five isolates from termite gut.

Number of isolates	Margin	Color	Elevation	Form	Gram Stain	Shape
1	Entire	Buff	Raised	Circular	Rod	Positive
2	Entire	Buff	Raised	Circular	Rod	Negative
3	Entire	Buff	Raised	Round	Rod	Positive
4	Entire	Buff	Raised	Round	Rod	Positive
5	Entire	Buff	Raised	Round	Rod	Positive

Cellulose degrading bacteria

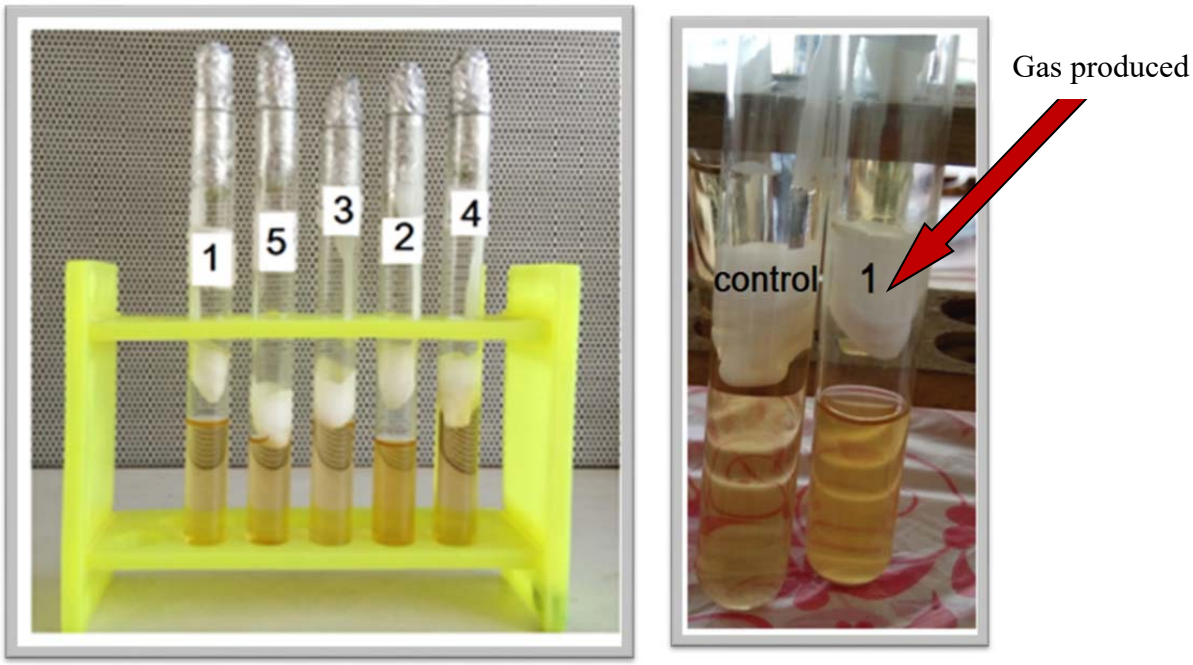
Three of the five bacterial isolates appeared to degrade CMC with varying degrees (Fig.1). The cellulolytic abilities can be arranged in a descending order as isolate two > isolate one > five. The arrangements depended on the degree of width and clarity around the bacterial streak.



(Fig.1): The clearing zone around the tested bacterial colonies.

Biogas production

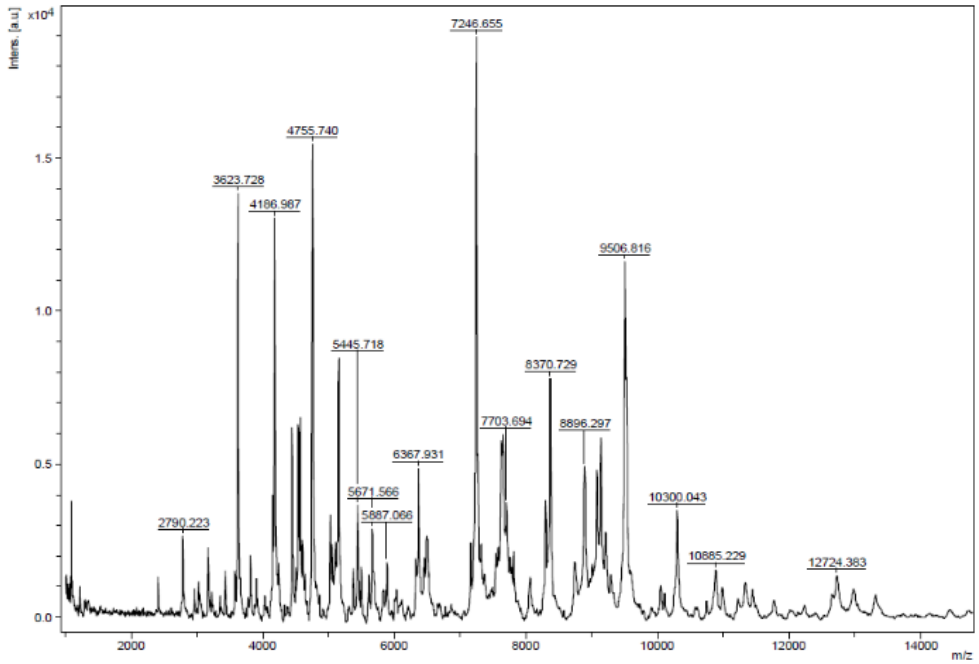
The three selected colonies, formed clear zone during their streaking on CMC agar plates, were further chosen to demonstrate their biogas production abilities using the creative Vaspar technique.



(Fig.2) Displacement of Vaspar layer showing production of gas.

MALDI-TOF identification

The most potent strain showing cellulose-degrading activity was the only one sent to the MALDI-TOF MS for identification (Fig.3). The biogas-producing isolate was identified to be *Enterobacter cloacae* bacterium with high degree of confidence; 2.17/3.0 score. It is highly similar to *E. cloacae* DSM 30054T_QC DSM type strain and NCBI identifier 336306. As stated with the instrument chart, this is the score to ensure the high confidence and secure genus and species identification.



(Fig.3): MALDI-TOF mass spectrum of *Enterobacter cloacae.* The measured range of 2,000 to 14,000 Da is displayed. The integrated MALDI software matches the pattern with entries of a database.

IV. Discussion

Termites belong to the group of terrestrial eusocial insects which thrive on cellulose rich sources like wood. Termite gut is known to harbor a wide variety of symbiotic microbes; where termites supply carbon, nitrogen and energy source to these microbes, and in turn microbes produce cellulases to help the termite feed on cellulose. All common cellulolytic bacteria species found in the digestive tract of termites differ proportionally to each other's. Each type of bacteria either individually or in association plays an important role in cellulose degradation and release of sugars for the welfare of termites.

The present study showed that amongst six bacteria, three isolates from termite's guts were cellulolytic and formed clearing zones, on CMC media plates (Fig.1). **Wood *et al.*, (1988)** established a simple and rapid method depended on the use of CMC as a selective media for preliminary screening of cellulolytic bacteria **[10]**.Qualitatively, the cellulolytic activities of the different isolates were visualized by the clear zone after the Congo red addition. Hence congo red strongly interacts with polysaccharides containing continuous β-1,4-linked D-glucopyranosyl units and β-1,3-D-glucans and some hemicellulosic galactoglucomannans possibly **[11]**. The data showed that not all *E. cloacae* isolates were equal in producing cellulases; isolate number two showed higher activity of cellulase production. On the other hand, isolates number three and four were the minimum cellulolytic index. This isolate that produced the largest diameter of halo zones were considered to have the highest cellulolytic activity. Our work is in accordance with previous data published by **Gohel *et al.*, (2014) and Angsana *et al.*, (2009) [12, 13]**

The cellulolytic activities were translated into the amount of biogas produced by each of the different *E. cloacae* isolates. The abilities to produce biogases can be

arranged in the following descending order; two > isolate one > five. This is the key issue we targeted for this study; since termites are voracious in the destruction of woods and now, we realized that was attributed to the microbial population within their guts. These microbes or the termite's microbiome are solely there for digestion of celluloses.

Morphological and biochemical characterizations were not enough to accurately identify the bacterial isolates. Therefore, we turned the most recent technology of the MALDI-TOF MS for identification of cellulose-degrading bacterium. This technology depended on ionization of the protein contents of the bacterial isolate after being hit by laser rays. It was identified as *Enterobacter cloacae* species; one the Enterobacteriaceae family. This was not a strange to find such microbe in the guts of termites since previous investigators such as **Sharma *et al.*, (2015)**, and **Chantarasiri (2015)** have isolated *Enterobacter cloacae* from termites' guts, as well **[14, 15]**. Moreover, **Wenzel *et al.*, (2002); Ramrn *et al.*, (2008); Gupta *et al.*, (2012)** had published several articles documenting *Enterobacter cloacae* as a high producer of cellulase enzymes **[16-18]**.

Moreover, the high cellulolytic potential of these selected bacterial isolates for decomposition of Carboxymethylcellulose and its fermentation for production of ethanol. The bacterial isolates showed a potential to convert cellulose into reducing sugars which could be readily used in many applications like feed stock for production of valuable organic compounds.

V. Conclusion

A termite gut harbor many potential cellulase producing bacteria which can be employed for treating cellulosic waste and producing biogas. Employing these cellulolytic bacteria to degrade cellulosic waste can be an ecofriendly and sustainable approach. The results were much encouraging for the application in biodegradation of cellulose based products. Development of potential consortium by screening more cellulase producing bacteria from insect gut is a commendable task which can help in reduction of cellulosic wastes.

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