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Comparison of different stains in determination of extracellular cellulose activity of *Odontotermes obesus* gut bacteria

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Abstract
Termites (Isoptera) are known to hydrolyse the polysaccharide: cellulose, hemicellulose and lignocellulose into glucose monomers with the help of symbiotic bacteria of their gut. Fifty termite (*Odontotermes obesus*) guts were extracted then homogenized to culture the bacteria on nutrient agar. Then the isolated bacterial colonies were cultured on CMC media at 37^0 C for 24 hrs. A total of 12 isolates grew on CMC but only 9 have shown cellulolytic activity. Out of 9, 4 were obtained from soldiers and 5 from workers. Then these colonies were isolated for cellulolytic activity. The potential of bacteria to degrade cellulose was measured with the help of different stains like Congo Red, Comassie Brilliant Blue R 250 stain, Safranin and Gram's Iodine stain. The soldiers showed more cellulolytic activity than workers. On the basis of these staining techniques, it was found that the Gram's Iodine is the best stain to measure the cellulolytic activity. So it forms significantly higher zone of clearance. Congo Red also showed poor stain retention with degraded polymer. Clear zone formation depends on binding of degraded polymer with stain and on bacterial isolate.

Keywords: Termites, *Odontotermes obesus*, Congo Red, Comassie Brilliant Blue R 250 stain, Safranin and Gram's Iodine

1. Introduction
Cellulose is the most abundant biopolymer on Earth and important source of carbon on this planet (Sreeja *et al.* 2013) [13]. It is a linear polysaccharide (Gupta et al., 2012) assembled from glucose monomer units, and it is the main constituent of plant cell walls. Termites play an important role in terrestrial ecosystem by recycling lignocellulosic biomass which refers to a mixture of cellulose, hemicellulose and lignin (Upadhyaya *et al.* 2012) [15]. Lignocellulose is the predominant component of woody plants and dead plant material, as well as being the most abundant biomass on earth, especially in terrestrial ecosystem. Termites are said to dissimilate or depolymerize a significant proportion of cellulose (74-99%) and hemicellulose (65-87%) components of lignocellulose they ingest (Ohkuma, 2003) [10]. The gut of these termites contains endosymbionts i.e. Protozoa and Bacteria. They are helpful in cellulose digestion, acetate formation and the formation of methane (Brauman *et al.* 1992) [3]. Termites maintain an anaerobic microbial flora in their gut and are dependent on these microbes for effective digestion of their diet, lignocellulose (Kudo, 2009) [8].
Cellulose is the main plant constituent. It is a crystalline polymer consisting of D- glucose residues connected by -1, 4 glucosidic linkages. Cellulose is produced by microorganisms (moulds, fungi and bacteria) during their growth on cellulolytic material. Cellulolytic enzymes account for 20% of world's enzyme markets (Reka and Ananthi 2013) [11].
It has been found that this cellulose is present in the hind gut of termites, which could be derived from symbiotic bacteria (Tokuda and Watanabe, 2007) [14]. It is very important to find out a rapid and easy screening method to differentiate between cellulolytic and non- cellulolytic bacteria. In this study, an attempt was made to determine the staining efficiency of various stains for cellulose activity on solid CMC agar media and the potential of bacterial isolates to bind with stains.

2. Material and Methods
2.1 Bacterial Isolation
Approximately 50 individuals of a single termite species i.e. *Odontotermes obesus* were collected from Kurukshetra University Campus, Kurukshetra, Haryana. They were brought to the laboratory and surface sterilized with distilled water and 70% alcohol. Further all the steps were performed under Leminar Air Flow. The guts of approximately 50 termites were extracted and and kept in 1ml PBS. They were disrupted by sterilized homogenizer and passing through pippett and then vortexed for uniform distribution. This homogenate was used for the isolation of termite gut bacteria.Gut homogenate was directly used for spreading on nutrient agar media. After spreading NA plates were incubated at 37ºC for 48 hours. After 48 hours the isolated bacterial colonies were appeared on agar.

2.2 Cellulolytic activity assay
These isolated bacterial colonies were picked up and inoculated on CMC agar at 37ºC for 24-48 hours in four replicates as to stain with four dyes. After incubation media plated were flooded with different dyes: Congo Red, Comassie Brilliant Blue R 250 stain, Safraninand Gram's Iodine stain (Gohel *et al.* 2014) [5]. The colonies plated on CMC agar were flooded with the respective dyes for 10-12 minutes then washed with distilled water(Gram's Stain and Coomassie Brilliant Blue)and 1M NaCl (Congo Red and Saferenin).The zone of cellulose hydrolysis as a clear zone was appeared with all the dyes.Then the colonies were compared for the best results with different dyes. Clear zone appeared around growing bacterial colonies indicating cellulose hydrolysis (Irfan *et al.* 2012). Cellulolytic potential of the cellulose-

hydrolysisng bacteria isolates was determined by calculation their Hydrolysis Capacity (HC) (Lloyd and Tarun, 2016). Cellulolytic activity was calculated by using formula as follows (Andri et al, 2015).

$$\text{Cellulolytic Index} = \frac{\text{Diameter of zone} - \text{Diameter of bacterial colony}}{\text{Diameter of bacterial colony}}$$

3. Results & Discussion

A termite species was collected from Kurukshetra University Campus and identified as *Odontotermes obesus* belonging to order Isoptera. A total of 12 bacterial isolates were obtained from termite worker and soldier guts when grown on Nutrient agar. Bacterial colonies isolated on nutrient agar were grown on CMC to check the cellulolytic activity of bacteria. These cellulose-degrading bacteria form a clear zone around the colony that shows their cellulose degrading ability (Ariffin *et al*, 2006, Butera *et al.* 2016) [2, 4]. (Table 1, Fig.1). The efficiency of cellulose degradation was checked by the use of different stains. Different types of stains were used to visualize the zone of clearance that indicates the ability of the bacteria to hydrolyse cellulose. Different stains were used to compare their efficiency by forming clear zone. Cellulases are known to convert cellulose into monomeric or dimeric structure hence; CMC was used as a carbon source that is a soluble form of cellulose (Vimal *et al* 2016) [16].

Out of 12 bacterial isolates, 9 isolates showed cellulolytic activity. Out of 9 isolates, 4 were obtained from the soldiers. In Gram's Iodine staining T_6S_2 isolate from soldier showed maximum cellulolytic activity of 3.43 and maximum Zd/Cd ratio of 4.43. Out of 9, 5 isolates were obtained from workers. Isolate T_6W_3 showed the maximum activity of 1.0 and maximum Zd/Cd ratio of 2.0 (Table 1). In Congo Red, T_6S_4 isolate from soldiers showed the maximum activity of 1.17 and maximum Zd/Cd ratio of 2.17. In workers not very significant activity was observed i.e. maximum of 0.75 with Zd/Cd ratio of 1.75 in T_6W_6 (Table 1).

Table 1: Showing the Cellulolytic activity and Zd/Cd ratio of bacterial isolates with different stains

S. No.	Bacterial strain ➔ Stain ⬇	Cellulolytic Activity ⬇								
		T_6S_1	T_6S_2	T_6S_3	T_6S_4	T_6W_2	T_6W_3	T_6W_4	T_6W_6	T_6W_9
1.	Gram's Iodine	1.5	3.43	0.73	1.0	0.22	1.0	0.7	0.88	0.5
	Zd/Cd	2.5	4.43	1.73	2.0	1.22	2.0	1.7	1.88	1.5
2.	Congo Red	0.9	0.63	0.67	1.17	0.5	0.17	0.5	0.75	0.11
	Zd/Cd	1.9	1.63	1.67	2.17	1.5	1.17	1.5	1.75	1.11
3.	Coomassie Brilliant Blue	0.5	0.25	0.11	0.5	0.11	0.14	0.1	-	0.11
	Zd/Cd	1.5	1.25	1.11	1.5	1.11	1.14	1.1	-	1.11
4.	Safrenin	1.25	0.88	0.11	0.17	0.11	0.17	0.09	-	0.14
	Zd/Cd	2.25	1.88	1.11	1.17	1.11	1.17	1.09	-	1.14

* Zd = Zone Diameter and Cd= Colony Diameter

In Comassie Brilliant Blue R 250 stain (CBR) and Saferenin not much cellulolytic activity was observed i.e. in CBR, in soldiers maximum of 0.5 and Zd/Cd ratio maximum of 1.5 was shown by T_6S_1 and T_6S_4. In workers cellulolytic activity was very poor i.e. 0.14 of T_6W_3 and maximum Zd/Cd ratio od 1.14. In Saferenin maximum activity was shown by T_6S_1 i.e. 1.25 with Zd/Cd ratio of 2.25 in soldiers. In workers the maximum activity is of 0.17 and Zd/Cd ratio was 1.17 of T_6W_3 (Table 1). Staining efficiency also depends on the degree of cellulose degradation. While in case of Gram's iodine and Congo red the stain retention is poor with degraded polymer so the isolates show significantly higher zone of clearance (Fig.1). The diameter of clear zone varies with stains as well as with bacterial isolates. Zone of clearance showed that Gram's iodine has greatest efficiency as compared to Congo red and other like Saferenin and Coomassie Brilliant Blue (Table 1 and Fig. 1). These cellulolytic bacterial stains show variable cellulolytic potential, distinct colony and morphological characteristics and genetic makeup (Lloyd and Tarun, 2016). The clarity of zone depends on the binding of the different stains with the degraded polymer.

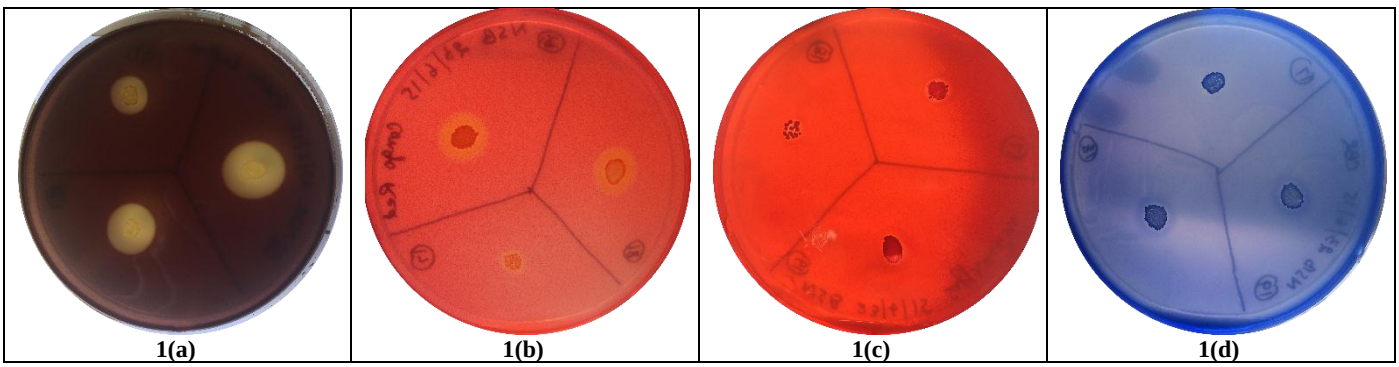

Fig 1: Comparative Staining techniques: showing clear zone with 1(a) Gram's Iodine, 1(b) Congo Red, 1(c) Safrenin and 1(d) Coomassie Brilliant Blue.

4. Conclusion

Conclusion drawn from the above research that the termite workers and soldiers harbor some symbiotic bacterial community inside their gut. These bacteria secrete cellulose, which help them to digest cellulose. These cellulose degrading bacteria can be isolated and checked for their potential to digest cellulose. It was found that all the bacterial isolates from soldiers showed cellulolytic activity. Various stains were used for this so that the efficiency of the stains could also be analysed. It was found that Gram's Iodine is the best stain to measure the activity. The clear zone formation depends on the binding of stain with the degraded polymer as well as on the bacterial isolate.

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