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EXTRACELLULAR CELLULASE ACTIVITY OF TERMITE GUT BACTERIA ISOLATED FROM DIFFERENT GEOGRAPHICAL LOCATIONS OF CHHATTISGARH (C.G.), INDIA

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Abstract

The present investigation was carried out to isolate and screen the Cellulase producing bacteria from the gut of termites, which were collected from different geographical locations of Chhattisgarh (C.G.), India. Termite gut bacteria were screened for Cellulase activity in cellulose basal medium supplemented with Esculin, Filter paper and Carboxymethyl cellulose. Out of 33 bacterial isolates, 20 isolates showed Esculin hydrolysis by forming black zone around colony, whereas, 17 isolates showed maceration of filter paper. Among all the isolates, L isolate showed maximum β-glucosidase activity (Esculin hydrolysis) forming black zone of 8 mm. On the basis of halo zone formation on CMC agar medium, 16 isolates were found positive for Cellulase activity after staining with two different stains. Gram's iodine stain was found best as it formed larger halo zones as compared to Congo red stain. Bacterial isolate B1 showed maximum halo zone of size 12 mm.

Key words : Termite, bacteria, cellulase, CMC.

Introduction

Lignocellulose or fiber is the main compound of plant cell wall that consists of cellulose, hemicelluloses and lignin (Haltrich and Steiner, 1994). The degradation of lignocellulosic biomass into fermentable or otherwise utilizable sugars is a complex process that requires a diversity of enzymes (Taggar, 2015). Cellulases are the class of enzymes that perform several enzymatic activities, namely endo-β-1, 4-glucanase (also known as endocellulase) or carboxymethyl cellulases (CMCase), exo-β-1,4-glucanase (exocellulase) and β-glucosidase activity (Fry, 1995).These enzymes convert complex cellulose into simple sugars by hydrolyzing the bonds present between the cellulose chains. In this way, degradation of lignocellulosic biomass can be achieved by applying source of Cellulase enzyme.

Termites are one of the most Lignocellulose digesting insects and can act as one of the best source for cellulolytic systems. Their ability to digest lignocellulosic material depends on the different enzymes produced by the microorganisms including bacteria, yeasts and protists which live in symbiotic relationship in the intestinal tract. Termites play an important role in the turnover and mineralization of complex biopolymers, such as wood and other cellulose and hemicelluloses containing materials (Wenzel, 2002). Termite guts have been considered as the "world's smallest bioreactors" (Brune, 1998). Termites are said to dissimilate a significant proportion of cellulose (74-99%) and hemicellulose (65-87%) components of lignocelluloses they ingest (Ohkuma, 2003).

Studies on the lignocellulolytic systems may elucidate mechanisms of efficient Lignocellulose degradation in termites as well as offer novel enzyme sources. Microbial composting is one of the upcoming technologies for agricultural waste disposal in which biodegradation of agricultural biomass is carried out using efficient microbial communities of lignocellulolytic microorganisms. Therefore, the main objective of this study was to isolate cellulose degrading bacteria from termites.

Materials and Methods

Collection of termites

Termites used for the isolation of cellulolytic bacteria were collected from different geographical regions of Chhattisgarh, India (table 1). Approximately 100-150 termites were picked from termitarium and brought to the laboratory in plastic petriplates.

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Table 1 : List of bacterial isolates used in the present study.

S. no.	Bacterial isolate	Collection area
1.	B1	Champaran road, Raipur
2.	B2	Champaran road, Raipur
3.	B3	Champaran road, Raipur
4.	C1	Champaran road, Raipur
5.	C2	Champaran road, Raipur
6.	C3	Champaran road, Raipur
7.	M1	Airport road, Raipur
8.	A1	Airport road, Raipur
9.	Z1	Chhattisgarh Tourism Board, Raipur
10.	Z	Chhattisgarh Tourism Board, Raipur
11.	L	Durg Road
12.	R	Ghatarani forest
13.	N	Dhamtari forest
14.	B1 plate	IGKV University, Raipur
15.	B	Ghatarani forest
16.	C	Gangrel dam forest
17.	Q	Airport road, Raipur
18.	G	Gangrel dam forest
19.	O	Airport, Raipur
20.	W	Chhattisgarh tourism board, Raipur
21.	D	VIP road, Raipur
22.	E	VIP road, Raipur
23.	F	Gangrel dam, Dhamtari
24.	GH1	Ghatarani forest
25.	K	Aarang road, Raipur
26.	M2	Chhattisgarh tourism board, Raipur
27.	S	Mandir hasuad
28.	Y	Energy park, VIP road, Raipur
29.	T	Mandir Hasaud
30.	U	Dharampura road, Raipur
31.	A	Ghatarani forest
32.	H	Farmers hostel, IGKV, Raipur
33.	P	Mana road, Raipur

Preparation of termite homogenate

5-6 termites were picked up from plastic petriplates and surface sterilized by 70% ethanol. Then, they were washed with distill water several times and allowed to air dry for 1-2 minute. Under sterilized conditions, each termite was separated into two parts, head and body. After beheading the termites, their bodies were crushed with help of sterilized plastic pestles. The slurry obtained after crushing the termites was spread on Nutrient agar plates and incubated in the incubator at 37°C for 48 hrs.

Isolation of pure colonies

Pure bacterial colonies were obtained by repeated streaking on Nutrient agar plates. Bacterial isolates were then stored in nutrient agar slants at 30°C for further use.

Identification of Cellulolytic bacteria

Cellulose degrading bacteria were identified by different methods described by Pointing (1999). First method consists of preparation of cellulose basal medium ($C_4H_{l2}N20_6$ 5g; KH_2PO_4 1g; $MgSO_4.7H_2O$ 0.5 g; Yeast extract 0.1 g; $CaCl_2.2H_2O$ 0.001 g/liter) supplemented with 0.5% w/v Esculin and 1.6% w/v agar and was autoclaved. Later 1 ml of sterile 2% w/v aqueous ferric sulphate solution was added and mixed well. Media was aseptically transferred to sterile petriplates and allowed to cool. Then plates were streaked with pure bacterial isolates and incubated at 25°C in the darkness. Second method consists of preparation of Basal salt media ($C_4H_{l2}N20_6$ 5g; KH_2PO_4 1g; $MgSO_4.7H_2O$ 0.5 g; Yeast extract 0.1 g; $CaCl_2.2H_2O$ 0.001 g/liter). Media with the above ingredients was prepared and poured into 50 ml conical flasks containing filter paper of size 1x3cm (Whatman filter paper no.1). Filter paper strips were completely submerged in the media and then it was autoclaved. After autoclaving, pure bacterial cultures were inoculated in it. These cultures were incubated in the darkness and examined daily for 10 days. Third method consisting preparation of Cellulose basal medium ($C_4H_{l2}N_20_6$ 5 g; KH_2PO_4 1g; $MgSO_4.7H_2O$ 0.5 g; Yeast extract 0.1 g; $CaCl_2.2H_2O$ 0.1 g; 1% CMC and 1.6% w/v agar) was prepared and autoclaved. Plates were inoculated with pure cultures and incubated at 37°C in darkness for 48-72 hrs.

Preparation of staining solutions

After incubation plates containing CMC were treated with two different stains. These stains were prepared by the method described by Hardik (2014). Congo red stain was prepared by dissolving 0.1% Congo red dye in 100 ml of distilled water. Petriplates were flooded with 0.1% dye and left for 20 minutes. Then the plates were washed with 1M NaCl. Gram's Iodine Stain was prepared by dissolving 0.665 g KI and 0.335 g Iodine in 100 ml distilled water. Petriplates were flooded with Gram's iodine stain and washed with distilled water. The Cellulolytic activity was indicated by the appearance of halo zone around bacterial isolate. The halo zones or clear zones can be seen against the red colour of Congo red.

Biochemical characterization

Hicarbohydrate™ kit was used to test carbon utilization profile as described by manufacturer (Himedia

Laboratories, Mumbai, India). Bacterial cells were grown in Nutrient broth overnight. An aliquot of 50 ìl of this suspension was inoculated to each well of Hicarbohydrate™ kit, inoculated at 30°C and kept for 24 hrs. The results were recorded according to the instructions given by the manufacturer. Various biochemical tests were carried out which included Methyl red test, Voges-Proskauer test, starch hydrolysis, gelatin liquefaction, nitrate reduction, Catalase test, oxidase test, urease test, Litmus milk test, Hugh-leifson's test, phenylalanine test, Triple Iron Sugar test, Lipase test and Casein hydrolysis test.

Results and Discussion

Isolation and screening of Cellulolytic bacteria

Different methods were performed for screening Cellulase producing bacteria. Activity of β-glucosidase was detected by the growth of bacterial isolate on agar plate containing Esculin as sole carbon source. Out of 33 bacterial isolates, 20 isolates showed β-glucosidase activity (table 2). Formation of black colour around the bacterial colony is an indication of β-glucosidase activity. Bacterial isolate L showed highest β-glucosidase activity forming black zone of size 8 mm. similar findings were also reported by Veena (2011) who isolated β-glucosidase producing bacteria which showed blackening of the medium around the bacterial colony. Blackening of the medium around the colony was an indication of β-glucosidase activity. Degradation of Filter paper, which contains crystalline and amorphous cellulose is indicative of Cellulolysis (Pointing, 1999). Filter paper used in this experiment was made up of 100% cellulose. In this test, 17 bacterial isolates showed maceration of the filter paper (Filter paperase activity) and considered as Cellulase positive (table 2). Maceration of the filter paper was occurred after 15 days of inoculation and basal media showed increased cloudiness as compared to uninoculated controls. Similar results were observed by Ramin (2008), who conducted an experiment which showed filter paper degradation activity within 10-15 days of inoculation and medium became colloidal after one month.

Table 2 : Screening of bacterial isolates for determining their cellulolytic activity.

Bacterial isolate	β-Glucosidase activity (mm)	Maceration of filter paper (Filter paperase activity)	Cellulase activity (mm) Congo red	Cellulase activity (mm) Gram's Iodine
Potential isolates with high β-Glucosidase, filterpaperase and cellulase activity				
Bl	2	+	7	12
B3	2	+	7	10
M1	5	+	7	8
Z	6	+	4	10
C3	7	+	6	7
L	8	+	5	11
Cl	4	+	5	8
A1	6	+	5	8
Z1	2	+	5	8
N	+	+	7	8
B1 Plate	+	+	7	8
C	+	+	5	8
Bacterial isolates with medium β-Glucosidase, filterpaperase and cellulase activity				
B2	3	+	5	7
C2	3	+	5	7
R	3	+	5	6
B	3	+	5	6
Bacterial isolates showing β-Glucosidase and filterpaperase activity				
Q	6	+	-	-
Bacterial isolates showing only β-Glucosidase activity				
G	2	-	-	-
O	3	-	-	-
W	6	-	-	-

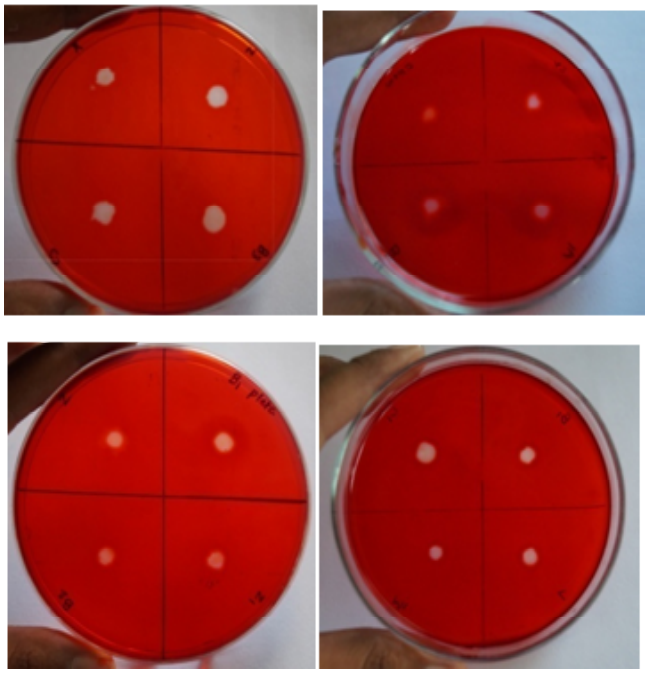

Fig. 1 : Results of Congo red staining.

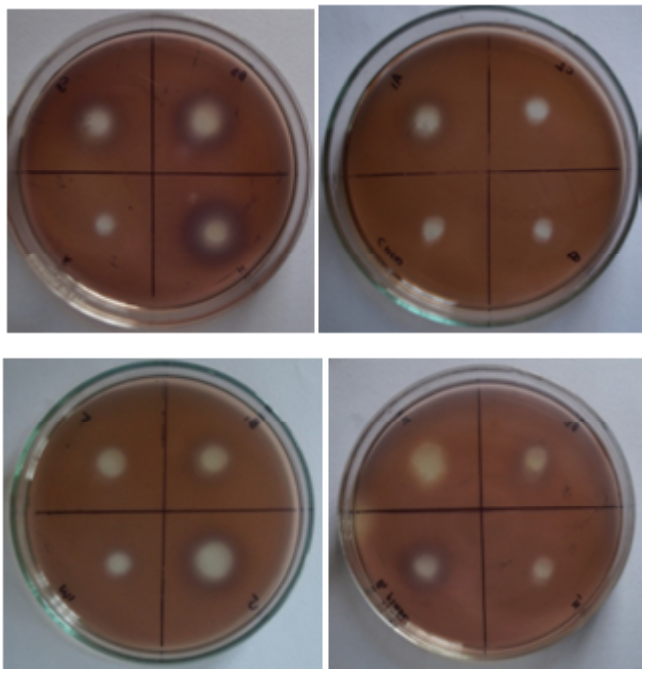

Fig. 2 : Results of Grams iodine staining.

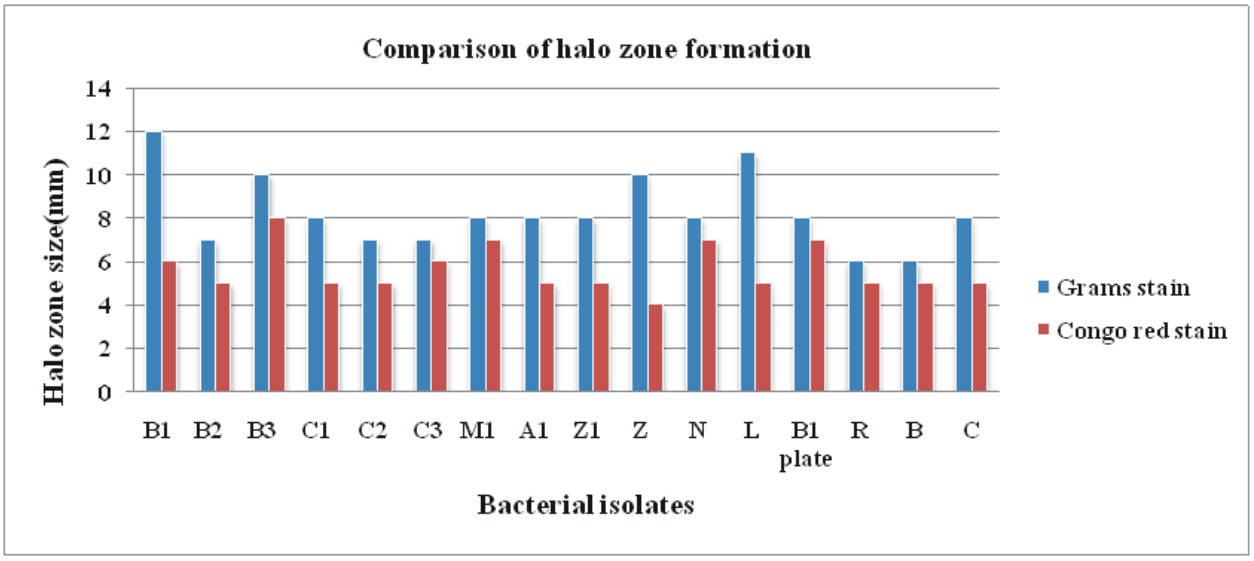

Fig. 3 : Comparison of halo zone formation after staining with two different dyes.

Plate assay for screening cellulolytic bacteria

Results of Congo red staining

Congo red staining was done for determination of Cellulase activity of the bacteria. Out of 33 isolates, 16 isolates showed Cellulase activity by forming the halo zone on agar plates supplemented with 1% CMC as sole carbon source (fig. 1). 16 bacterial isolates formed halo zones ranging from 4-7 mm after staining with Congo red dye (table 2). Similar findings were reported by some researchers. Kavitha (2014) isolated cellulose degrading bacteria from the termite guts on the basis of zone of clearance after staining the plates with Congo red dye. Gupta (2012) reported isolation of cellulose degrading bacteria from termite guts after staining the plates with Congo red dye. Upadhyaya (2012) isolated Cellulolytic bacteria from the gut of termite on the basis of halo zone formed in the CMC containing plates after stained with Congo red stain.

Results of Gram's iodine staining

Grams iodine staining was also done for screening Cellulase activity of bacteria. 16 isolates which showed halo zones after staining with Congo red dye also showed

Table 3 : Morphological characterization of Cellulolytic bacterial isolates

Bacterial Isolate	Shape	Surface	Margin	Colour
B1 isolate	Spherical	Raised	Entire	Creamish white
B2 isolate	Spherical	Raised	Entire	Creamish white
B3 isolate	Spherical	Raised	Entire	Creamish yellow
C1 isolate	Spherical	Raised	Entire	Creamish
C2 isolate	Spherical	Raised	Entire	Creamish white
C3 isolate	Spherical	Raised	Entire	Creamish
M1 isolate	Spherical	Raised	Entire	Creamish white
A1 isolate	Spherical	Raised	Entire	Creamish
Z1 isolate	Spherical	Raised	Entire	Creamish white
Z isolate	Spherical	Raised	Entire	Creamish white
N isolate	Spherical	Raised	Entire	Creamish white
L isolate	Spherical	Raised	Entire	Creamish yellow
B1 plate isolate	Spherical	Raised	Entire	Creamish white
R isolate	Spherical	Raised	Entire	Creamish yellow
B isolate	Spherical	Raised	Entire	Creamish yellow
C isolate	Spherical	Raised	Entire	Creamish white

Table 4 : Different biochemical characteristics revealed by sixteen cellulose degrading bacterial isolates.

Tests	B1	B2	B3	C1	C2	C3	M1	A1	Z1	Z	L	R	N	B	B1 Plate	C
1	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	+
14	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-
18	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
23	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-
24	+	-	-	+	-	+	+	+	-	+	+	+	-	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+

Table 1 continued....

Table 1 continued....

26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
29	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
30	-	+	-	-	+	-	+	+	+	+	-	-	-	+	-	-
31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+
33	-	-	+	-	+	-	+	-	+	+	+	-	-	+	-	-
34	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
35	+	+	+	+	-	+	+	+	-	-	-	+	+	-	+	-
36	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
46	-	-	-	-	+	-	+	+	+	+	+	+	+	+	-	+
47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
49	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Isolate N, B1 plate and C showed positive(+) results for Esculin hydrolysis on HicarbohydrateKit™. Different tests: 1: Lactose; 2: Xylose; 3: Maltose; 4: Fructose; 5: Dextrose; 6: Galactose; 7: Raffinose; 8: Trehalose; 9: Melbiose; 10: Sucrose; 11: L-Arabinose; 12: Mannose; 13: Inulin; 14: Sodium gluconate; 15: Glycerol; 16: Salicin; 17: Dulicitol; 18: Inositol; 19: Sorbitol; 20: Mannitol; 21: Adonitol; 22: Arabitol; 23: Erythritol; 24: Methyl D-glucoside; 25: Rhamnose; 26: Cellbiose; 27: Melezitose; 28: Methyl D-mannoside; 29: Xylitol; 30: ONPG; 31: Esculin; 32: D-Arabionse; 33: Citrate utilization; 34: malonate utilization; 35: Sorbose; 36: Lipolytic activity test in tween-80; 37: Gelatin Hydrolysis; 38: Phenyl-alanine test; 39: Casein Hydrolysis test; 40: Hugh-leifson's test (anaerobic condition); 41: Hugh-leifson's test (aerobic test); 42: Nitrate reduction (before Zn addition); 43: Nitrate reduction (after Zn addition); 44: starch hydrolysis; 45: Oxidase test; 46: TSI (Acid butt/alkaline slant); 47: TSI (Acid slant/acid butt); 48: TSI (bubbles/cracks); 49: TSI (black precipitate); 50: Urease test; 51: Methyl red test; 52: Voges-prsakauer test; 53: Catalase; 54: Litmus milk reaction (Lactose fermentation); 55: Litmus milk reaction (Acid coagulation); 56: Litmus milk reaction (acid reaction, gas and curd formation); 57: Litmus milk reaction (litmus reduction/redox reaction); 58: Litmus milk reaction (alkaline reaction/proteolysis).

halo zones after staining with gram's iodine solution (fig. 2). 16 bacterial isolates formed halo zones ranging from 6-12 mm (table 2). We found that less intensity of halo zones were formed after Congo red staining (4-7 mm) as compared to halo zones formed by staining with gram's iodine (6-12 mm). Bacterial isolate B1 showed maximum halo zone of size 12 mm. similar results were reported by some researchers. Kakkar (2016) determined extra cellular cellulase activity of termite gut bacteria after staining the CMC plates with different stains like Congo Red, Comassie Brilliant Blue R 250 stain, Safranin and Gram's Iodine stain. On the basis of these staining techniques, it was found that the Gram's Iodine is the best stain to measure the cellulolytic activity as it formed significantly higher zone of clearance. Hardik (2014) reported extra cellular cellulase activity of cellulose degrading bacteria on Carboxymethyl Cellulose (CMC) agar plates by staining with stains like Congo Red, Gram's Iodine, Safranin and Comassie Brilliant Blue R 250. It showed that gram's iodine stain formed larger halo zones as compared with other stains. In this study, the diameters of the halo zones produced by different bacterial colonies were used as a basis for comparison between various isolated strains (fig. 3). Those isolates that produced largest diameter of halo zones were considered to have the highest Cellulase activity. Bacterial isolates were characterized with the help of colony morphology (table 3).

Conclusion

In this study, termites were collected from different geographical locations of Chhattisgarh (India) and 16 cellulose degrading bacteria were isolated from termite gut. Cellulolytic activities of these bacteria were assessed by different methods, which include Esculin hydrolysis (β-glucosidase activity), Maceration of filter paper (Filter paperase activity) and CMC hydrolysis (CMCase activity). Bacterial isolate L showed maximum β-glucosidase activity whereas, B1 isolate was found potential Cellulase producer forming maximum halo zone on CMC plate. Gram's iodine stain was found more efficient than Congo red stain as it formed larger halo zones on CMC containing plates. Further studies are in progress to optimize Cellulase production at different parameters (pH, temperature and incubation period), to identify these isolates at molecular level and to decompose agricultural biomass (rice straw) using these potential isolates. Application of such compost can improve crop and soil quality. Keeping these facts in mind, this study was conducted for isolation of cellulolytic bacteria from termite gut.

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