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Identification of a Novel Cellulolytic Bacteria as Cotton Stalk Degrader

Preeti R. Parmar[1*], Himani K. Patel[2], B. K. Rajkumar[1] and D. H. Patel[1]

[1]*Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Athawa Farm, Surat, Gujarat State, India*
[2]*Bhagahawan Mahavir College of Science and Technology, Survey No. 149, New City Light Road, Bharthana-Vesu, Surat, Gujarat State, India*

**Corresponding author*

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A B S T R A C T

Isolation of total 18 bacterial strains were carried out from the decayed wood samples collected from farm of MCRS, NAU, Surat and Surat Municipal Waste site at Khajod, Surat on nutrient agar plates. Among 18 isolates, four cellulose degrading bacterial strains (CDB) *viz.*, CDB9 (4.0), CDB5 (3.0), CDB15 (3.0) and CDB18 (2.0) showed significant cellulolytic index on Carboxymethyl Cellulose (CMC) agar plate within 48 hrs after flooding with congo red stain. Further, CDB9, CDB5, CDB15 and CDB18 were studied to determine the quantitative cellulose degradation activity in CMC broth and examined for thier FPase and CMCase activity. Maximum FPase and CMCase activity observed with CDB9 (FPase: 0.72 FPU ml^{-1}, CMCase: 1.28 IU ml^{-1}) followed by CDB15 (FPase: 0.50 FPU ml^{-1}, CMCase : 0.78 IU ml^{-1}), CDB5 (FPase : 0.15 FPU ml^{-1}, CMCase : 0.27 IU ml^{-1}) and CDB18 (FPase : 0.002 FPU ml^{-1}, CMCase : 0.008 IU ml^{-1}) at 48 hrs in static condition. Based on quantitative assay, CDB9 and CDB 15 were selected to study biodegradation of cotton stalk. CDB9 isolate showed significant FPase (2.79 FPU ml^{-1}) and CMCase (3.20 IU ml^{-1}) activities with 17 % weight loss of cotton stalk within 30 days than CDB15 (FPase: 0.30 FPU ml^{-1}), CMCase:: 2.30 IU ml^{-1} and 11 % weight loss); while decreased cellulose amount was observed with both the strains (CDB9: 35.67 mg/ml, CDB15 : 40.27mg/ml). Thus, CDB9 was found to be a remarkable cotton stalk degrader. On the basis of qualitative plate assay for extracellular enzymes production and antibiotic disc assay, it was found CDB9 was able to produce lipase and inhibited by the antibiotics *viz.*, Ampicillin, Amoxyclav, Cefotaxime, Co-Trimoxazole, Gentamicin and Tobramycin within 24 hrs, respectively. On the basis of 16S-rDNA sequencing, CDB9 bacterial strain was identified as *Kosakonia oryziphila.*

Introduction

Photosynthesis is the process of fixation of carbon dioxide to cellulose in the plants and thus cellulose is one of the more abundant resource produced on the earth. Cotton and fibers are the major source of cellulose. Enormous amount of agricultural, industrial and municipal cellulose possessing wastes are accumulating due to lack of knowledge of their utilization into the economical and eco-friendly

products (Saha, 2003; Nishida *et al.,* 2007; Waghmare *et al.,* 2018). Agriculture is the backbone of Indian economy as majority of peoples depends on farming for their livehoods. Among different crops, cotton is one of the commercial crop grown in India. China, United state, Pakistan, Brazil and Australia are major cotton growing nations across globe countries (Hepbasli *et al.,* 2007). Cotton residue contains 58.5 % cellulose, 14.4% hemicelluloses and 21.4% lignin which are suitable feedstock for thermochemical conversion process (Daud *et al.,* 2013). There were few studies in which cotton stalk were used to produce bioethanol, biofertilizers, paper and feed for cattles (Poore and Rogers 1995; Ververis *et al.,* 2004; Holt *et al.,* 2004; Osama Abdel-Twab Seoudi 2013; Afif *et al.,* 2019). Diverse group of cellulose degrading microbes have been isolated and identified. Due to high production of cellulase and easy extraction of cellulase enzymes fungi *viz*., *Aspergillus*, *Trichoderma*, *Phanerochaete*, *Fomitopsis* and *Penicillium* have been prominently studied than bacteria (Gusakov and Sinitsyn, 2012). Now a days, researchers accent and draw attention to cellulose degrading bacteria because of their rapid growth, multienzyme complex, resistance to extreme environment and ability to grow in consortium. Different genera of bacteria such as *Cellulomonas*, *Cellulosimicrobium*, *Acetovibrio*, *Clostridium*, *Ruminococcus*, *Bacillus*, *Paenibacillus* and *Thermomonospora* etc. able to degrade cellulose in aerobic and anaerobic condition (Maki *et al.,* 2009; Lo *et al.,* 2009; Wilson, 2011). Further, a simple cost effective eco-friendly method to convert cotton stalk into biofertiliers or other products will help to earn supplementary income from the cotton farming (Gurjar *et al.,* 2007). Cellulose made up of polymer of glucose molecules connected with beta glycosidic bond, which indicates that abundance of carbohydrates within the cell wall of the plant and thus decaying wood is the richest source to isolate the potential cellulose degrading bacteria (Gbenro *et al.,* 2019; Ma *et al.,* 2020). Thus, the present study was made to isolate the cellulose degrading bacteria from the rotten or decayed wood samples and exploit it into cotton stalk degradation.

Materials and Methods

Collection of the Samples

Decaying wood samples from Surat Municipal Corporation (SMC) waste disposal site, Khajod, Surat and Farm of Main Cotton Research Station, Athwafarm, Navsari Agricultural University, Surat, Gujarat, India were collected for the isolation of cellulose degrading bacteria. The samples were carefully taken into the sterile polythene bags and brought to the laboratory and kept at 4ºC.

Isolation, Enrichment and Primary screening of cellulose degrading bacteria

For the isolation of bacteria, each of the decayed wood samples were inoculated into the 100 ml of sterile distilled water in 250 ml flask and kept on shaker for one hour at 120 rpm at room temperature. After proper mixing of the samples with water, serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were carried out and diluents of each samples spread on sterile nutrient agar plates for 24 hrs at room temperature (Harrigan and McCance, 1966). Bacterial colonies were purified using four flame technique on sterile nutrient agar plates and each bacterial strains were stored at 4ºC and further used for the screening of cellulose degrading activities.

For enrichment, 10 ml of sterile minimal broth medium (K_2HPO_4;7.0gms,KH_2PO_4; 2.0 gms, Sodium citrate; 0.5gm, Magnesium Sulphate; 0.1 gm, Ammonium Sulphate; 1.00gms, pH;7.0, D/W;1000ml) embedded with 1.0% Carboxymethyl cellulose (CMC) powder inoculated by each bacterial strains and were kept at room temperature for 48 hrs in static condition.

Enriched cultures were spot inoculated on CMC agar plates and kept at room temperature for 48 to 72 hrs. Congo red (0.1 % aqueous solution) was added on plates and kept for 15 min and washed with 1M NaCl solution to observe the zone of hydrolysis (Apun *et al.,* 2000). Colonies that showed discoloration of congo red were suspected as

cellulose degrading bacteria and cellulolytic index was measured by the following formula (Ferbiyanto *et al.*, 2015);

Cellulolytic index = (Diameter of zone - Diameter of bacterial colony) / Diameter of bacterial colony

Further, bacterial strains that showed significant cellulolytic index were used for secondary screening.

Secondary screening for cellulolytic activity

Secondary screening of the bacteria was performed using quantitative assay as described by Sherief *et al.,* (2010). For that, bacterial strains with significant cellulolytic index were inoculated into 100 ml of sterile minimal broth embedded with 1.0 % CMC as a sole source of carbon into 250 ml capacity flask. All flasks were kept at room temperature for seven days in static condition. At time interval of 48 hrs, 10 ml of samples were withdrawn and centrifuged at 1500 rpm for 15 minutes. The supernatant was analyzed for the FPase and CMCase activities.

Measurement of enzyme activity

Total cellulase activity was determined using FPase activity by Filter paper assay (Ghose, 1987). For that, one ml of supernatant as a crude enzyme source was added to filter paper strip (1 x 6 cm; 50 mg) immersed into one ml of 0.05 M sodium citrate buffer (pH – 4.8). After incubation at 55 ± 2ºC for 30 minutes, the reducing sugar released was estimated using dinitrosalicyclic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing one µmol of reducing sugar from filter paper per ml per min.

Endoglucanase activity (CMCase) was measured by addition of one ml crude enzyme into the one ml of 1 % CMC in 0.05M sodium citrate buffer (pH – 4.8). The reaction mixture was incubated at 55 ± 2ºC for 30 minutes and the reducing sugar released was estimated using dinitrosalicyclic acid (DNS) method (Miller 1959). One unit (IU) was defined as the amount of enzyme releasing one µmol of reducing sugar per min.

Bacterial strains that showed significant enzyme activities were used for degradation of cotton stalk.

Biodegradation Study of cotton stalk by cellulose degrading bacterial isolates

Cotton stalks preparation

Cotton stalk was collected from the field of Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Athwa Farm Surat, Gujarat, India and was sundried for several days.

The dried cotton stalks were crushed and sieved into 3 mm particle size using mill and stored in air-tight containers for its further use (Meehnian *et al.,* 2016).

Seed culture preparation and bacterial degradation of cotton stalk in submerged condition

Degradation of cotton stalk by the significant strains was carried out according to method of Zheng *et al.*, (2003). For that, enrichment of the culture was carried out by inoculating each bacterial colony into the 10 ml of sterile minimal broth embedded with 1 % of cotton stalk. Tubes were kept at room temperature for ten days in static condition to enriched the culture and act as seed culture. Five ml of seed culture was inoculated into 95 ml of sterile minimal medium embedded with one gram of cotton stalk powder.

Control flasks were kept without inoculation of the bacteria. All flasks were kept in static condition at 30±1ºC. At time intervals of five days, 10 ml of the samples withdrawn from both test and control flasks and filtered using qualitative filter paper (size- 9.0 cm dia; Hi-media). Filtrate was used to measure the FPase and CMCase activity, while samples remaining on the filter paper were used to determine weight loss and moisture content.

Weight loss and moisture content analysis

Weight loss (%) of the sample was calculated according to following formula (Gautam *et al.*, 2012);

Weight loss (%) = (B-C)/(B-A) x 100

Where, Weight of filter paper = A, Weight of filter paper + moisture sample = B and Weight of filter paper + oven dried sample = C

Moisture content was calculated according to Thimmaiah (2009) as follow;

Moisture content (%) = Weight of sample-Weight of dry sample/Weight of sample x 100

Microbiological characterization and molecular identification of cotton stalk degrader

Significant cotton stalk degrading bacterial strain was characterized using standard microbiological method according to Bergey's Manual of determinative bacteria (Krieg and Hold 1986).

Phenotyping characterization

Bacterial morphological characters *viz.*, size, shape and its arrangement was recorded by performing gram staining. Cultural characters such as size, shape, surface, elevation, opacity, margin and pigment production was recorded on the basis of colonies appeared on nutrient agar plate, while MacConkey's agar plate was used to observed lactose fermenting characteristics of the bacteria.

Biochemical characteristics was performed using KB009 Hi25 *Enterobacteriaceae* Identification and HiCarbo (35 carbohydrates) kit which includes tests *viz*., ONPG test, lysine, ornithine, urease, phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, Vogus Proskauer's, methyl red, Indole, Malonate and Esculin hydrolysis. Thirty five different carbohydrates like Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Rabinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium Gluconate, Glycerol, Salicin, Glucosamine, Dulcitol, Inocitol, Sorbitol, Mannitol, Adonitol, α-Methyl-D-glucoside, Ribose, Rhamnose, Cellobiose, Melezitose, α-Methyl-D-mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate, Sorbose were tested.

Biochemical tests were performed according to the instructions given in the kit literature. For that, 50 µl of bacterial culture (24 hrs old) was inoculated in each well of the kit and incubated at 37ºC temperature for 24 hrs. On the basis of pH change due to substrate utilization results were interpreted as positive and negative.

Plate assay for evaluation of other extracellular enzymes

Lipase, protease and amylase production was performed according to the method of Gupta *et al.*, (2003); Zhou *et al.*, (2009) and Abd-Elhalem *et al.*, (2015). For that, bacterial culture was spot inoculated on spirit blue agar plate for lipase, Casein agar plate for protease and Starch agar plate for amylase activity. Plates were observed for the zone of solubilization after incubation at room temperature for 24 hrs. The conversion of blue color of spirit blue agar into yellow indicated lipase production, while clear zone after flooding of iodine on starch agar plate indicated amylase production. Clear zone around colony on casein agar plate indicated protease production.

Determination of antibiotic susceptibility test

The antibiotic susceptibility was determined using Hexa G-minus 1 and Hexa G- plus 8 (Hi-media antibiotic discs). The antibiotic disc concentration was given in Table 1. For that, 0.1 ml of 24 hrs old culture of bacterial culture was spreaded on sterile nutrient agar plate and kept for 15 minutes. After that, an antibiotic disc was kept aseptically on the surface of the medium. After 24 hrs, plates were examined for the zones of inhibition (Padaria *et al.*, 2013).

Molecular identification of cotton stalk degrader

Bacterial genomic DNA was extracted using the single colony of the isolate. The DNA quality was confirmed on 1.0% agarose Gel. Amplification of 16S rDNA gene was done by PCR. The PCR amplicon was purified by column purification to remove contaminants. Further, DNA sequencing for PCR amplicon was done with 357F and 1391R primers using BDT v3.1 Cycle sequencing kit on ABI 3500xl Genetic Analyzer.

BLAST with the database of NCBI Genbank database of 16S rDNA sequence was carried out. First fourteen sequences were selected on the basis of its maximum identity score, selected and aligned using multiple alignment software programs. Neighbor-Joining method (Saitou and Nei, 1987) was used for the evolutionary history while Maximum Composite Likelihood method (Nei and Kumar, 2000) were used to computed evolutionary distances. The evolutionary analyses were conducted in programe MEGA7 (Kumar *et al.,* 2016).

Results and Discussion

Isolation and Screening of bacteria for its cellulolytic activity

Initially, decayed wood samples collected from the Surat Municipal Corporation waste site, Khajod, Surat and Farm of Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Surat were serially diluted and each diluents were spreaded on Nutrient agar plates to achieve intense number of bacterial strains from the samples. Total 18 different bacterial strains were isolated, among them 8 (CDB1 to 8) were from Surat Municipal Corporation waste site while 10 (CDB9 to CDB18) from the MCRS, NAU, Surat decayed wood sample (Table 2).

Each isolates were enriched in CMC broth and then spot inoculated on CMC agar plate. Bacterial strains showed discoloration of congo red or halo zones on CMC agar plates after 48 hrs were suspected as cellulose degrading bacteria. Cellulolytic Index (CI) shown that CDB9 has the largest CI (4.0) followed by CDB5 (3.0), CDB15 (3.0) and CDB18 (2.0) while other isolates had CI less than 1.0 (Table 3). Bacterial strains that showed greater than one CI *viz*., CDB9, CDB5, CDB15 and CDB18 were selected as significant cellulose degraders. Cellulolytic bacteria from decayed wood samples were also isolated by Gbenro *et al.,* (2019) and Ma *et al.,* (2020) on CMC agar plates. Gbenro *et al.,* (2019) found 22 bacterial strains from three different wood industries in Chennai; while Ma *et al.,* (2020) isolated about 55 bacterial isolates from the five rotten wood samples of Qinling Mountain, China.

Evaluation of FPase and CMCase activity for secondary screening

Cellulose degrading bacteria have inherent potentiality to produce cellulase enzymes, which converts cellulose to glucose. Cellulase is a combination of multiple enzymes *viz.,* total cellulase (FPase), endoglucanase (CMCase) and exoglucanase. Thus, each of the bacterial strains selected during primary screening were inoculated into the CMC broth for 48 hrs at 30ºC temperature. The crude enzyme extract was subjected to determine their total cellulase activity (FPase) and endoglucanase (CMCase) activity (Gautam *et al.,* 2011). In our study, total cellulase activity through FPase assay was ranged from 0.002 IU mL^{-1} to 0.72 FPU mL^{-1} while endoglucanase activity through CMCase assay ranged from 0.008 IU mL^{-1} to1.28 IU mL^{-1}. FPase and CMCase assay was performed by numerous researchers to screen the significant cellulolytic strains. For example, Ekperigin (2007) reported CMCase activity (0.48 IU mL^{-1}) by *Acinetobacter anitratus*; while Rastogi *et al.,* (2009) showed CMCase activity of 0.02 and 0.058 IU mL^{-1} by *Brevibacillus* sp. DUSELG12 and *Geobacillus* species DUSELR7 on 10^{th} and 7^{th} days respectively. Da Vinha *et al.,* (2011) and Sheng *et al.,* (2012) reported CMCase activity of 2.0 and 1.432 IU mL^{-1} by *Streptomyces viridobrunneus* SCPE-09 and *Pseudomonas* sp. HP207, respectively. Saini *et al.,*

(2017) isolated 124 cellulose degrading bacteria, among them NAB37 shown 0.948±0.011 IU mL^{-1} of CMCase and 0.125±0.005 FPU mL^{-1} of FPase activity. *Streptomyces* S-G20 isolated by Majidi *et al.,* (2011) shown highest FPase (0.091 IU mL^{-1}) and CMCase (0.079 IU mL^{-1}) after 72 hrs. Further, Liang *et al.,* (2014) isolated strain ME27-1 produced CMCase of 2.08 IU mL^{-1}. Recently, Ma *et al.,* (2020) found the FPase activity of 0.0133 IU $mL^{-1}$and CMCase activity of 0.0368 IU mL^{-1} by *Bacillus subtilits* 1BJ4 strain isolated from decayed wood samples. Data variation observed with FPase and CMCase activities in different studies by researchers. In our study, highest FPase activity was found with two bacterial isolates CDB9 (0.72 IU mL^{-1}) and CDB15 (0.50 IU mL^{-1}) than the CDB5 (0.15 IU mL^{-1}) and CDB18 (0.002 IU mL^{-1}). CDB9 and CDB15 had remarkable CMCase activity of 1.2 IU mL^{-1} and 0.78 IU mL^{-1}, respectively (Table 4). The present data was similar with the report of Saini *et al.,* (2017) and Elperigin (2007).

On the basis of CI and enzyme activities, two strains CDB9 and CDB15 were used for the cotton stalk degradation study.

Biodegradation of cotton stalk by the isolates

Cotton is one of the most important commercial crop in India and cotton stalk is the byproduct produced after cotton harvesting. Due to similar dimension of fibre of cotton stalk with most common species of hardwood, it is prefer to manufacture of hard board, paper and pulp, boxes as well as for the production of microcrystalline cellulose (Gurjar *et al.,* 2007). Beside this, using pretreatment with acid or other techniques, high cellulose content of cotton stalks can be converted into fermentable sugars (Meehnian *et al.,* 2016). Today, techniques like bioethanol, biofertilzer and green manure production from the agricultural waste is of the great interest for researchers. Day by day, studies to isolate novel type of microbe and its use for the utilization of waste biomass is increasing. Thus, an attempt was carried to exploit the selected cellulose degrading bacterial strains (CDB9 and CDB15) into cotton stalk degradation. Each strain was separately inoculated into 95ml of minimal medium with 1% cotton stalk powder with five ml of seed culture at 30±1 ºC temperature in static condition for 30 days and data was taken at five days interval (Fatima *et al.,* 2018).

Data showed that FPase activity was ranged from 0.007 to 2.79 FPU mL^{-1} by CDB9 strain, while 0.002 to 0.30 FPU mL^{-1} by the strain CDB15 within 30 days. Similarly, CMCase was in the range of 0.009 to 3.20 IU mL^{-1} by CDB9 bacteria and 0.003 to 2.30 IU mL^{-1} by CDB15 isolate (Fig. 1). Detectable FPase and CMCase activity was started from 15^{th} day upto 30^{th} day incubation by the strain CDB9 while in case of CDB15 bacterial strain it was from 25^{th} day to 30^{th} day. Significant FPase activity was not observed with CDB15 isolate. Further, results reveled with cotton stalk weight loss shown in Fig. 2 indicated that 17% weight loss by CDB9 strain while 11% by the isolate CDB15 within 30 days. Beside this, cellulose estimation (mg mL^{-1}) was also performed initially and after 30 days. It was found that initial cellulose amount was 47.25 mg mL^{-1}while it was decreased upto 35.65 mg $mL^{-1}$and 40.27 mg mL^{-1} with CDB9 and CDB15 bacterial strains after 30 days, respectively. Significant difference in moisture content (%) was not observed (Table 5). Additionally, remarkable change observed with particles size of cotton stalk during the study (Fig. 3). Multifaceted work carried out on cotton stalk degradation. For example, Zheng *et al.,* (2003) studied with exploded bast, branch and stem of cotton stalk degradation by *Bacillus* NT-19, with weigh loss of 24%, 20% and 14% respectively. Iscia and Demirerb (2007) used cotton stalks, cotton seed hull and cotton oil cake for biogas production. In order to increase the efficiency of degradation, diluted acid pretreatment cotton stalk was subjected to degrade by cellulose degrading microbial community screened by Linqiao *et al.,* (2012). Osama Abdel-Twab Seoudi (2013) used *Phanerochaete chrysosporium* and *Azotobacter chroococcum* inocula to achieve good quality biofertilizer from the cotton stalk. Effect of particle size, moisture content and media supplements of

cotton stalk during delignification by *Daedalea flavida* MTCC 145(DF-2) in solid state fermentation was studied by Meehnian *et al.,* (2016) and found highest cellulolytic enzyme activities with decrease particle size and increased moisture content. Fatima *et al.,* (2018) reported CMCase activity of 0.850 IU mL^{-1} and FPase activity of 0.878 FPU mL^{-1} by *Bacillus subtilis* on cotton stalk pretreated with 1% sulfuric acid. Biochar as a carrier material for the growth of *Bacillus subtilis* SL-13 was prepared from cotton stalk by Tao *et al.,* (2018). Bano and Irfan (2019) studied bioethanol production from cotton stalk. Majority of work on cotton stalk was with optimized physiochemical parameters in laboratory condition shows significant results but in natural environment it may not give the same potential results. Utilization of cotton stalk into biofertilizer or other products, development of simple and cost effective method that give supplementary income from cotton farming and to help entrepreneurship creation in rural area is necessary. Thus, here an attempt was made to study the degradation of cotton stalk with isolates in static condition without any optimized conditions.

In our study, significant FPase (2.79 FPU mL^{-1}) and CMCase (3.20 IU mL^{-1}) activities with 17 % weight loss of cotton stalk by CDB9 isolate followed by 0.30 FPU mL^{-1} of FPase, 2.30 IU mL^{-1} of CMCase and 11 % weight loss of cotton stalk by the stain CDB15 was observed. Decreased cellulose amount within 30 days by CDB9 and CDB15 during cotton stalk degradation showed that both strains were able to degrade cotton stalk but CDB9, a bacterial strain isolated from the decayed wood sample from MCRS, NAU, Surat, Gujarat was more efficient than CDB15 with respect to duration of time during degradation. Thus, microbiological characterization and molecular identification of CDB9 was carried out.

Characterization and Identification of the significant cotton stalk degrader bacteria

Among two bacterial isolates, CDB9 showed significant degradation of cotton stalk. Thus, CDB9 bacterial isolate was subjected to microbiological and molecular identification.

Microbiological characterization

Microbiological characters includes morphological, cultural and biochemical characteristics. An isolate, CDB9 was found gram negative with short rods. The appearance of colony on nutrient agar was medium, circular, entire, convex, glistening, sticky, transparent and non-pigmented while on MacConkey's agar, it was red in color that indicates its lactose fermenter colony. Further, CDB9 examined for its biochemical characterization through K009 Hi-carbohydrate kit and KB003 Hi25 *Enterobacteriaceae* identification kit (Hi-media). Tests for the utilization of carbohydrate or substrate were based on the principle of pH change. Metabolic changes gave spontaneous color change under incubation which interpreted either positive or negative based on color change, visually or by addition of reagents. Color change of the medium showed as positive reaction while no color changes as negative. Xylose, Maltose, Fructose, Dextrose, Galactose, Trehalose, L-arabinose, Mannose, Glycerol, Salicin, Sorbitol, Mannitol, Arabitol, Rhamnose, Cellobiose, ONPG, Esculin, D-arabinose and Citrate was utilized by the bacterium while other carbohydrates were not (Table 6 and Fig. 3). Here, Cellobiose (Zhang *et al.,* 2006), Esculin (Saquib and Whitney, 2006) and Salicin (Ramani *et al.,* 2012) used as key substrates to determine β-glucosidase activity, an important enzyme of cellulase complex. Hi25^{TM}*Enterobacteriaceae* Identification kit used to identify the bacteria belongs to *Enterobacteriaceae* family. Data of IMViC showed that Indole and Methyl red tests were negative, while Voges Proskauer's and Citrate utilization seen positive. Beside these, urease, oxidase and nitrate reduction tests were positive; Lysine utilization, Ornithine, Phenyl alanine and H_2S production was negative (Table 7 and Figure 5). As per identification index of Hi25^{TM}*Enterobacteriaceae* Identification kit, bacterial strain CDB9 might belongs to *Enterobacter* genus.

Table.1 Antibiotic disc and its concentration

Sr. No.	Name of Antibiotic	Concentration	Sr. No.	Name of Antibiotic	Concentration
Hexa G-minus 1 antibiotic disc			**Hexa G- plus 8 antibiotic disc**		
1	Ampicillin (AM)	10 mcg	1	Penicillin G (P)	10 unit
2	Amoxyclav (AMC)	30 mcg	2	Methicillin (MET)	5 mcg
3	Cefotaxime (CTX)	30 mcg	3	Vancomycin (VA)	30 mcg
4	Co-Trimoxazole (COT)	25 mcg	4	Oxacillin (OX)	1 mcg
5	Gentamycin (GEN)	10 mcg	5	Erythromycin (E)	15 mcg
6	Tobramycin (TOB)	10 mcg	6	Ampicillin (AMP)	10 mcg

Table.2 Details of bacteria isolated from the decayed wood samples, Surat

Sr. No.	Samples	Sites	Total bacteria isolated on nutrient agar	ID of isolates
1	Decayed Wood	Surat Municipal Corporation waste site, Khajod, Surat and.	8	Cellulose degrading bacteria 1 to 8 (CDB 1 to 8)
2	Decayed Wood	Farm of Main Cotton Research Station, Navsari Agricultural University, Surat	10	Cellulose degrading bacteria 9 to 18) (CDB 9 to 18)
Total bacterial isolates			**18**	

Table.3 Cellulolytic index (CI) of isolated cellulose degrading bacteria

Sr. No.	Isolates	Cellulolytic Index	Sr. No.	Isolates	Cellulolytic Index
1	CDB1	0.8	10	CDB10	--
2	CDB2	--	11	CDB11	--
3	CDB3	--	12	CDB12	0.2
4	CDB4	0.1	13	CDB13	0.2
5	**CDB5**	**3.0**	14	CDB14	0.8
6	CDB6	0.1	15	**CDB15**	**3.0**
7	CDB8	--	16	CDB16	0.5
8	**CDB9**	**4.0**	17	CDB17	--
9	CDB10	0.7	18	**CDB18**	**2.0**

Table4 Secondary screening of cellulose degrading bacteria

Sr. No.	Isolates	Enzyme activity (IU mL^{-1})	
		FPase	CMCase
1	**CDB9**	**0.72**	**1.28**
2	CDB5	0.15	0.27
3	**CDB15**	**0.50**	**0.78**
4	CDB18	0.002	0.008

Table.5 Data on moisture content and cellulose amount during degradation of cotton stalk

Days	Moisture content (%)		Cellulose (mg mL^{-1}) amount initial and after 30 days	
	CDB9	CDB15	CDB9	CDB15
Initial	--	--	47.25	47.25
5	72.00	70.19		
10	71.80	70.00		
15	69.00	69.00		
20	69.00	69.15		
25	68.15	68.20		
30	68.20	68.00	35.67	40.27

Table.6 Carbohydrate utilization by an isolate CDB9 based on K009 Hi-carbohydrate kit (Hi-media)

Sr. No.	Test	Reaction	Sr. No.	Test	Reaction	Sr. No.	Test	Reaction
1	Lactose	Negative	13	Inulin	Negative	25	Rhamnose	Positive
2	Xylose	Positive	14	Sodium gluconate	Negative	26	Cellobiose	Positive
3	Maltose	Positive	15	Glycerol	Positive	27	Melezitose	Negative
4	Fructose	Positive	16	Salicin	Positive	28	α – Methyl D-mannoside	Negative
5	Dextrose	Positive	17	Dulcitol	Negative	29	Xylitol	Negative
6	Galactose	Positive	18	Inositol	Negative	30	ONPG	Positive
7	Raffinose	Negative	19	Sorbitol	Positive	31	Esculin hydrolysis	Positive
8	Trehalose	Positive	20	Mannitol	Positive	32	D-Arabinose	Positive
9	Mellibiose	Negative	21	Adonitol	Negative	33	Citrate utilization	Positive
10	Sucrose	Negative	22	Arabitol	Positive	34	Malonate utilization	Negative
11	L-Arabinose	Positive	23	Erythritol	Negative	35	Sorbose	Negative
12	Mannose	Positive	24	α – Methyl D-glucoside	Negative			

Table.7 Results of biochemical test based on Hi25^{TM} *Enterobacteriaceae* Identification kit

Sr. No.	Test	Reaction	Sr. No.	Test	Reaction
1	Indole	Negative	7	Urease	Positive
2	Methyl red	Negative	8	Phenlalanine Deamination	Negative
3	Voges Proskauer's	Positive	9	Nitrate reduction	Positive
4	Citrate utilization	Positive	10	H_2S production	Negative
5	Lysine utilization	Negative	11	Oxidase	Positive
6	Ornithine utilization	Negative			

Table.8 Data on Antibiotic susceptibility test of CDB9 isolate based on Antibiotic Disc (Hi-media)

Isolate	**Hexa G-minus 1 antibiotic disc**					
CDB9	Ampicillin (AM)	Amoxyclav (AMC)	Cefotaxime (CTX)	Co-Trimoxazole (COT)	Gentamycin (GEN)	Tobramycin (TOB)
	+	+	+	+	+	+
	Hexa G- plus 8 antibiotic disc					
	Penicillin G (P)	Methicillin (MET)	Vancomycin (VA)	Oxacillin (OX)	Erythromycin (E)	Ampicillin (AMP)
	-	-	-	-	-	+

Fig.1 FPase and CMCase activity of CDB9 and CDB15 bacterial strain

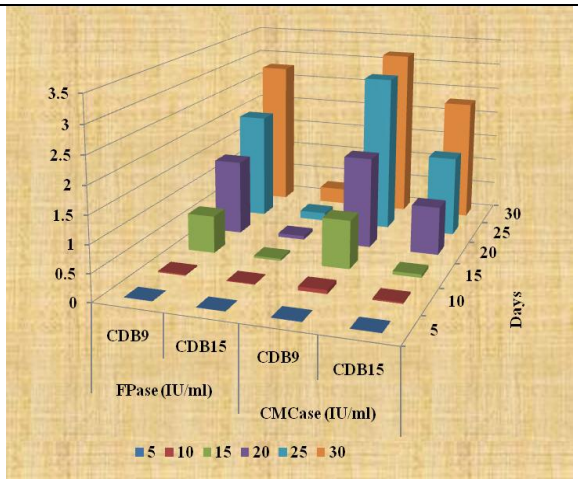


Fig.2 Weight loss (%) of cotton stalk by CDB9 and CDB15 bacterial strain

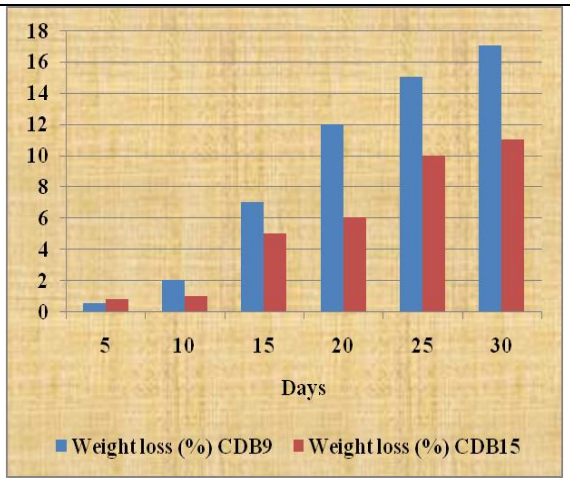


Table.9 DNA sequence of PCR amplicon with 357F, 1391R and RES1458_Consensus Sequence

Sample : >RES1458_357F (1057 bp)
GTCAATGGCGCAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAA AGCACTTTCAGCGGGGAGGAAGGTGCTGCGGTTAATAACCGCAGCAATTGACGTTACCCG CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAAT CCCCGGGCTCAACCCGGGAACTGCATCCGAAACTGGCAGGCTTGAGTCTCGTAGAGGGG GGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA AGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTA AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG CAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTTCAGAGATGGAAGGGTG CCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTT GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAA CTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCAT GGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTC GCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGGATTGGAGTCTGCAACTCG ACTCCATGAAGTCGGAAATCGCTAGTAATCGTGAAATCAGAATGTCACGGGTGAATACGT TCCCGGGTCCTTGCTACACAACCCGCCCCCCGGTCAGGAAGAAA
Sample : >RES1458_1391R (1050 bp)
TCGTCCTCCTTAACCGGGGGAGAGCAGCAGTGGGGAATATTGCACAAATGGGCGCAAGC TTGATGCAGCCATGCCCGCGTGTGTGAAGAAGGCCTTTCGGGTTTGTAAAGCACTTTCAG CGGGGAGGAAGGTGCTGTGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGC ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA TTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTC AACCCGGGAACTGCATCCGAAACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTGGAATTC CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCC TGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT GGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCG GAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA ACCTTACCTGGTCTTGACATCCACAGAACTTTTCAGAGATGGAAGGGTGCCTTCGGGAAC TGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGA CTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAC CAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAG CGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC GGAATCGCTAGTAATCGTGAATCAGAATCACCGACCC
Sample : >RES1458_Consensus Sequence (1110 bp)
TTTCTTCCTGACCGGGGGGCGGGTTGTGTAGCAAGGACCCGGGAACGTATTCACCGGGTC GGTGACATTCTGATTTCACGATTACTAGCGATTTCCGACTTCATGGAGTCGAGTTGCAGA CTCCAATCCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCT CTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGA CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACC

GCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACA CGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCCTTCCATCTC TGAAAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACC ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCG TACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAAC CTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC ACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCC TCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCCACCCCCCTCTACGAGACTCAA GCCTGCCAGTTTCGGATGCAGTTCCCGGGTTGAGCCCGGGGATTTCACATCCGACTTGAC AGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATT ACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGC GGTTATTAACCACAGCACCTTCCTCCCCGCTGAAAGTGCTTTACAAACCCGAAAGGCCTT CTTCACACACGCGGGCATGGCTGCATCAAGCTTGCGCCCATTTGAGCAATATTCCCCACT GCTGCTCTCCCCCGGTTAAGGAGGACGA

Table.10 Sequences showing significant alignment of CDB9 bacterial strain

Description	Max score	Total score	Query cover	E value	Ident	Accession
Salmonella enterica subsp. enterica strain Ty2 16S ribosomal RNA, partial sequence	1574	1574	99%	0	98.65 %	NR_074 799.1
Atlantibacter hermannii strain CIP 103176 16S ribosomal RNA, partial sequence	1568	1568	99%	0	98.54 %	NR_104 940.1
Enterobacter cloacae strain DSM 30054 16S ribosomal RNA, partial sequence	1567	1567	99%	0	98.54 %	NR_117 679.1
Salmonella enterica subsp. enterica strain LT2 16S ribosomal RNA, partial sequence	1563	1563	99%	0	98.43 %	NR_074 910.1
Kosakonia cowanii JCM 10956 = DSM 18146 strain 888-76 16S ribosomal RNA, partial sequence	1563	1563	100%	0	98.31 %	NR_025 566.1
Salmonella bongori strain NCTC 12419 16S ribosomal RNA, complete sequence	1557	1557	99%	0	98.31 %	NR_074 888.1
Enterobacter cloacae strain ATCC 13047	1552	1552	99%	0	98.20 %	NR_102 794.2

16S ribosomal RNA, complete sequence						
Salmonella enterica subsp. salamae strain DSM 9220 16S ribosomal RNA, partial sequence	1552	1552	99%	0	98.20 %	NR_044372.1
Enterobacter cloacae subsp. dissolvens strain LMG 2683 16S ribosomal RNA, partial sequence	1552	1552	99%	0	98.20 %	NR_044978.1
Kosakonia oryzendophytica strain REICA_082 16S ribosomal RNA, partial sequence	1546	1546	99%	0	98.09 %	NR_125586.1
Salmonella enterica subsp. diarizonae strain DSM 14847 16S ribosomal RNA, partial sequence	1546	1546	99%	0	98.09 %	NR_044373.1
Salmonella bongori strain DSM 13772 16S ribosomal RNA, partial sequence	1546	1546	98%	0	98.41 %	NR_116124.1
Kosakonia oryziphila strain REICA_142 16S ribosomal RNA, partial sequence	1537	1537	98%	0	98.18 %	NR_125587.1
Kosakonia oryzae strain Ola 51 16S ribosomal RNA, partial sequence	1537	1537	98%	0	98.18 %	NR_116033.1

Fig.3 Visual appearance of initial (control) cotton stalk and degraded cotton stalk after 30 days by isolates CDB9 and CDB15

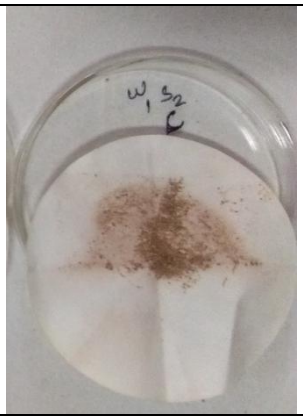
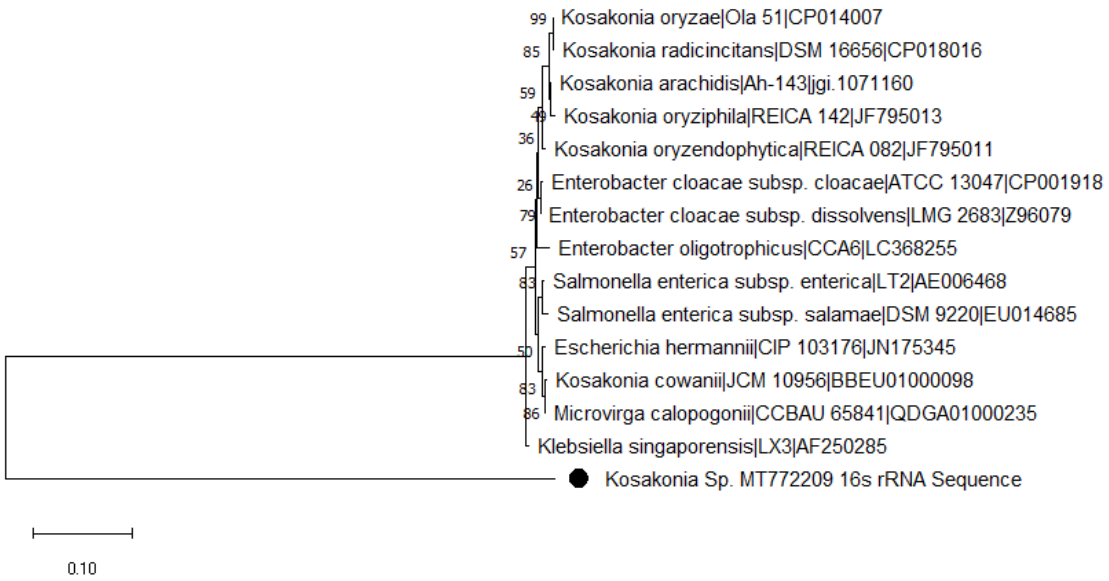
		
Control	CDB9	CDB15

Fig.4 Carbohydrate utilization through K009 Hi- carbohydrate kit	**Fig.5** Biochemical test through KB003 Hi25 *Enterobacteriaceae* identification kit	**Fig.6** Plate assay for lipase, amylase and protease
		

Fig.7 Phylogenetic tree derived from sequences of 16S rRNA gene sequences using neighbor-joining method.



Evaluation for protease, amylase and lipase

Lipase, amylase and protease enzyme production by the CDB9 was performed qualitatively on spirit blue, starch and casein agar plates, respectively. Zone of hydrolysis was not observed with starch and casein agar plate indicates that CDB9 not produced amylase and protease; while yellow color zone on spirit blue agar indicates lipase production within 24 hrs at room temperature (Figure 6).

Antibiotic sensitivity test

Bacterial isolate CDB9 was tested for antibiotic sensitivity using antibiotic discs (Hexa G-minus 1 and Hexa G- plus 8, Hi-media antibiotic discs). Inhibition of growth of isolate was observed with Hexa G-minus 1 disc antibiotics (ampicillin, Amoxyclav, Cefotaxime, Co-Trimoxazole, Gentamicin and Tobramycin) indicated as "+" sign. Zone of inhibition was not observed (indicated as "-" sign) with Hexa G- plus 8 discs (Penicillin G, Methicillin, Vancomycin, Oxacillin, Erythromycin and Ampicillin) except ampicillin within 24 hrs (Table 8).

Molecular identification of the cotton degrader strain CDB9

Molecular techniques (16S-rDNA sequencing) is a very precise technique for species identification and distinguishing between closely related species of bacteria (Basavaraj *et al.*, 2014). Thus, 16S-rDNA sequencing of CDB9 was performed for its proper identification. DNA was isolated and its purity was observed on 1.0% Agarose Gel. A single band of high-molecular weight DNA was observed. DNA sequencing after PCR amplicon with 357F and 1391R primers and sequences producing significant alignments given Table 9 and 10.

A phylogenetic tree and distance matrix was prepared based on the comparison of 16S rRNA sequences of reference strains. The phylogenetic analysis for isolate CDB9 using the software MEGA 7 using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.15247930 is shown in Figure 7 (next to the branches).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei and Kumar, 2000) and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Data reveled with all positions that contain gaps and missing data were eliminated. Total of 1216 positions were in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

On the basis of 16S-rDNA sequencing, CDB9 was identified as *Kosakonia oryziphila* but microbiological data tentatively identified it as *Enterobacter* genus. The 16S rDNA gene sequences of *Kosakonia sp.* determined in this study were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession number MT772209. Literature shown that genetic techniques to study the microbial strains have revealed the complex nature of *Enterobacter* which leads to change in the taxonomy of *Enterobacter* genus or *Enterobacter* -like microbes (Brady *et al.,* 2013; Bhatti *et al.,* 2017).

Now, systematic classification of *Enterobacter* genus is re-classified into five distinct genera *viz; Enterobacter*, *Lelliottia*, *Pluralibacter*, *Cronobacter* and *Kosakonia* (Brady *et al.,* 2013).

On the basis of taxonomic position and concatenated partial rpoB, atpD, gyrB and infB gene sequence *Enterobacter oryziphilus* now re-classified as *Kosakonia oryziphila* (Li *et al.,* 2016). Related to cellulose degradation, few studies reported that some species of *Enterobacter* genus able to degrade cellulose.

For example, Waghmare *et al.,* 2018 reported that *Enterobacter* sp. SUK-Bio isolated from plant litter soil was able to utilized sugarcane trash powder, carboxymethyl cellulose, sorghum husk, wheat straw and water hyacinth (Waghmare *et al.,* 2018). Sawangjit (2017) isolated *Bacillus anthracis* and *Enterobacter cloacae* as cellulose degraders. To the best of our knowledge this would be the first report on cellulose degradation especially cotton stalk by *Kosakonia oryziphila;* as we have not come across any literature citing cellulose degradation especially cotton stalk by *Kosakonia.*

Among the isolated and screened bacteria for their cellulose degrading activity through CMC agar plate and enzyme activities (FPase and CMCase), the cellulose rich cotton stalk was efficiently degraded under static condition by the bacterial strain CDB9 that was identified as *Kosakonia oryziphila* based on the data FPase, CMCase, weight loss and cellulose estimation. *Kosakonia oryziphila* might be the first reported bacterial strain especially as cotton stalk degrader. Further, research to develop the simple technology with enhanced cotton stalk degradation with eco-friendly products that can utilize by the farmers is crucial.

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Enterobacter based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov.as *Lelliottia nimipressuralis* comb. nov.and *Lelliottia amnigena* comb. nov.,respectively, *E. gergoviae* and *E. pyrinus* into Pluralibacter gen. nov.as Pluralibactergergoviae comb. nov.and Pluralibacterpyrinus comb. nov., respectively, *E. cowanii*, *E.radicincitans*, *E.oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov.and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Syst Appl Microbiol* 36: 309–319

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