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Full Length Research Paper

Studies on cellulose degrading bacteria in tea garden soils

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Cellulose degrading bacteria of tea garden soil were isolated, screened *in vitro* and its characterization, in relation to cellulase activity, was studied. Among the 25 isolates, the five strains showed higher enzyme activity when compared to other strains. Cellulase activity was expressed at a higher level by strain CDB 12 when blotting paper was used as a cellulose source in comparison with the other two substrate sources incorporated with minimal salt medium and followed by CDB 13 and CDB 21 in blotting paper. Maximum growth of cellulose degradation bacteria (CDB) was recorded at 30°C and pH 7.0. Among the carbon sources tested, maximum growth was observed in glucose amended mineral salts medium followed by fructose and maltose. Ammonium sulphate, ammonium nitrate and potassium nitrate were good nitrogen sources for better survival of CDB isolates. The biomass were continuously removed and placed as such into the tea field, then native and proven CDB strains were applied and they played an important role on the degradation of harvested biomass, which required replenishment to maintain the sustainable productivity of tea.

Key words: Cellulase, cellulose degrading bacteria, tea.

INTRODUCTION

Tea is one of the most popular and inexpensive beverages. It is manufactured from young shoots comprising two to three leaves and a bud of commercially grown tea plant (*Camellia*.sp.) (Baby, 2004). The majority of fungi and bacteria present in soils are considered to be beneficial to higher plants by breakdown and release of minerals from organic matter present in the soil resulting in essential element availability increases to higher plants. Degradation of plant and animal matter (the releasing and binding of nutrients and trace elements) is one of the most important functions of soil microorganisms. When organic matter, such as compost or wood residuals, is added to topsoil, the natural organic matter content is raised considerably and it increases food source (carbon) availability for beneficial soil microorganisms (Munten, 2005). Cellulose from major land plants as forest trees and cotton is assembled from glucose, which is produced in the living plant cell from photosynthesis (Bernfeld, 1955).

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Microorganisms bring about most of the cellulose degradation occurring in nature. They meet this challenge with the aid of a multi-enzyme system (Aubert et al., 1987). Aerobic bacteria produced numerous individuals and extra-cellular enzymes with binding modules for different cellulose conformations, while anaerobic bacteria possess a unique extracellular multi enzyme complex, called cellulosome. However, the main cellulose utilizing species are the aerobic and anaerobic hemophilic bacteria, filamentous fungi, basidiomycetes, thermophilic bacteria and actinomycetes (Wright, 2003). At the first step, the microorganisms responsible for cellulose decomposition bring about an enzymatic hydrolysis of the complex polymer, that is, the enzymes system which involves a group of different enzymes, is collectively known as cellulase. Microorganisms are very important for the enzymatic degradation of complex organic substances to nutrients. As such, this present study revealed the isolation, screening and biology of cellulose degrading bacteria isolated from tea garden soil. It could be used to finally degrade the huge amount of cellulosic biomass deposited during pruning time in tea plantation.

MATERIALS AND METHODS

Isolation of cellulose degrading bacteria (CDB) by enrichment method

Top soil samples (0 to 10 cm) were collected from the tea farm of UPASI Tea Research Institute using auger. The soil samples were air dried under shade and sieved through a 2.0 mm width mesh to remove stones and plant debris. Cellulose degrading bacteria (CDB) were isolated by enrichment method. Soil samples (10.0 g) obtained from a tea field were added in minerals salts medium broth containing 0.1% of carboxyl methyl cellulose and incubated at 30°C and 100 rpm for two days of incubation under the shaking condition. From this stock, 10 ml of the sample was taken and transferred to fresh MSM broth containing 0.1% CMC substrate as carbon source. After overnight incubation at 30°C, a 10 ml portion of supernatant fluid were serially diluted up to 10^7, and then the 10^3, 10^4 and 10^5 dilutions were plated into the mineral salt medium (100 ml) with CMC (0.1%) as carbon source. Plates were incubated at 30°C and examined periodically for colonies which showed areas of clearing. Representative colonies were transferred to nutrient broth, allowed to grow for 24 h, diluted and plated again on cellulose agar. This procedure was repeated until a pure culture of the cellulolytic bacterium was obtained. The cellulolytic isolate was maintained at 4°C on cellulose agar. Spread plate technique was followed and colonies which appeared on CMC were amended on MSM media. Again, they were streaked on mineral salts medium with 0.1% cellulose media in order to bring pure cultures of CDB.

Screening of CDB

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microbes produce significant quantities of cell- free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro*. A loopful of overnight grown culture of native isolated colonies streaked on MSM agar plates were amended with 0.1% cellulose. The plates were incubated at 35 ± 2°C for 24 to 48 h and observed for growth. Following incubation, the plates were flooded with grams iodine solution and the zone of clearance was observed around the line of growth while the zone of diameter was determined. Consequently, they were graded based on diameter of zone production.

Quantification of cellulase by plate assay

The colony which was isolated from the soil sample that degraded cellulose was compared with the commercial cellulase enzyme. Zone of inhibition was analyzed at different concentration. Water agar medium (0.2%) was prepared with 0.1% carboxyl methyl cellulose and was sterilized at 121°C for 15 min. The sterilized whatman paper discs were placed on water agar after saturating with different concentration of standard enzymes and culture filtrates, and then they were incubated at 37°C for 24 h. After incubation period, grams iodine solution was added for visualizing the zone of inhibition. The production of cellulase by culture filtrates was quantified by a comparison of zone production and standard enzyme.

Colorimetric assay of cellulase activity

Cellulase activity in cell-free culture filtrates were determined by the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) through the determination of the amount of sugars reduction. The cellulase activity was determined by a standard graph with glucose in the concentration range of 50 µg to 1000 µg/mL.

Determination of the ability to utilize cellulose (as c-source) and cellulase activity of isolates

CDB bacterial isolates were streaked onto MSM agar media, amended with the mentioned different cellulose sources and were incubated at 37°C for 5 days. Their growth on cellulose supplemented media was taken as an indication of cellulose degradation ability. Those isolates showing the ability to utilize cellulose were inoculated into MSM broth with different cellulose sources and their cellulase activity was determined.

Two hundred milliliters of mineral salts broth was prepared and 10 ml was dispensed into test tubes. Cellulose sources (filter paper, blotting paper and carboxy methyl cellulose) was added to the test tubes and sterilized at 121°C for 15 lbs/20 min. After sterilization, cellulose degrading bacteria were inoculated into the test tubes with different cellulose sources, incubated at 30°C and maintained for 5 to 7 days of incubation under the shaking condition. Cellulose and cellulase enzyme activity was determined by the Updegroff (1969) and Dension and koehn (1977) method. Three milliliters of acetic/ nitric acid reagent was added to a known amount (0.5 or 1.0 ml) of the sample in a test tube and mixed with the help of a vortex mixer. Subsequently, tubes were kept in a boiling water bath at 100°C for 20 min, then cooled and centrifuged at 8000 rpm for 20 min under laboratory conditions. After centrifugation, the supernatant was discarded and the residue washed with distilled water, then 10 ml of 67% sulphuric acid was added and allowed to stand for 1 h. After 1 h, 1 ml of this solution was diluted to 100 ml.

In the diluted solution (1 ml), 10 ml of anthrone reagent was added and mixed well. Afterwards, the tubes were kept in a boiling water bath for 10 min and were allowed to cool down, then the colour change was observed at 630 nm. Cellulose estimation was done with a standard solution and the amount of cellulose in the sample was calculated with the standard graph.

A similar procedure was followed for both filter and blotting paper degradation study, for which strips of filter (Whatman No. 1) and blotting paper were substituted for CMC-Na, respectively. After 7 days of incubation, residual cellulose of the papers was spectrophotometrically determined (Tailliez et al., 1989).

Effect of pH and temperature on the growth of cellulose degrading bacteria

Mineral salts broth with 0.1% cellulose was prepared and dispensed in test tubes and was adjusted to pH 4.0, 5.0, 6.0, 7.0 and 8.0. These were inoculated with test cultures and incubated at 37°C for 48 h. MSM broth with 0.1% cellulose was prepared and dispensed in test tubes and was incubated at different temperatures (25, 30, 35, 40 and 45°C). Following the incubation, growth of the cultures was measured by observation of the optical density at 560 nm.

Utilization of different carbon and nitrogen sources

CDB strains were used to study the utilization of different carbon and nitrogen sources. Mineral salts broth with 0.1% of different carbon sources, such as: glucose, fructose, maltose, arabinose, starch, inosital, xylose, trehalose, malate and citrate, were prepared and dispensed in test tubes and their pH were adjusted to 7.0, then they were incubated at 37°C. These were inoculated with test cultures and incubated at 37°C for 48 h. Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm using specord S 100.

MSM broth with 0.1% of different nitrogen sources such as: ammonium sulphate, sodium nitrate, ammonium nitrate, potassium nitrate, ammonium tartarate, ammonium chloride and urea, were prepared and dispensed in test tubes and their pH were adjusted to 7.0, then incubated at 37°C. Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm by using specord S 100.

RESULTS AND DISCUSSION

Isolation and screening of CDB by enrichment method

Among the 25 CDB isolates that were screened, the zone

Table 1. Estimation of cellulose using filter and blotting paper by CDB strains after incubation period (Mean of 4 replications).

Strains	Cellulose estimation	
	Blotting paper amount of cellulose (mg)	Filter paper amount of cellulose (mg)
Initial quantity	695.30	403.0
CDB1	242.47	169.16
CDB7	179.62	159.16
CDB12	302.11	201.08
CDB13	294.70	200.67
CDB21	272.30	169.75
CD @ 5%	10.48	11.85

production in cm formed the cellulose degrading bacteria in MSM medium. However, enzyme production was estimated by the plate assay method, in order to study the cellulose degrading bacteria, which were isolated by using mineral salt medium containing 0.1% carboxy methyl cellulose. The enzyme activity was determined by zone production in cm which was compared with the standard cellulase. The result indicated that the selected five strains (CDB1, CDB7, CDB12, CDB13 and CDB21) produced higher level of enzyme activity when compared to other strains. This reflected that these CDB microbes utilized the available cellulose sources in soil environment and proceeded with its degradation process.

Estimation of cellulase from the culture filtrates of CDB

Cellulase estimation was determined by using the culture filtrates of all 25 isolates and the results were noted by optical density (OD) values. Among the 25 isolates, the five strains showed higher enzyme activity when compared to other strains which were tabulated in Table 2. Many fungi were able to break down polysaccharides such as celluloses and were able to convert these polymeric compounds into sugars due to their capability to produce extracellular enzyme and cellulose (Abdelnasser and Ahmed, 2007).

Utilization of cellulose substrates (CMC, filter and blotting papers) by CDB strains

The efficient five strains were chosen for the study of cellulose utilization by using both filter and blotting paper. Among the five strains, CDB12, CDB13 and CDB21 strains showed higher production of cellulase while using blotting paper compared to filter paper. Strain CDB 12 utilized CM cellulose in high level compared to the other four strains (302.11 mg), followed by CDB 13 (294.70 mg), CDB 21 (272.30 mg), CDB 1 (242.47 mg) and CDB 7 (179.62 mg). This result indicated that cellulose was high in blotting paper (Table 1). This is because, for it to survival, it may degrade blotting paper efficiently due to its simplicity nature of chemical structure than filter paper and carboxy methyl cellulose. As such, CDB isolates showed the ability to utilize different cellulose sources inoculated into MSM broth by cellulase production. Cellulase activity was expressed at a higher level in blotting paper when compared to other substrate sources incorporated with MSM. In the biological system, every activity is gene specific and time specific in nature. Hence, according to the previous biological rule, cellulase enzymes expressed its activity whenever it is specific and whenever preferable substrates are available. The cellulose degrading bacterial organisms will select and fix its carbon and nutritional sources based on its availability and physical factors (Abdelnasser and Ahmed, 2007).

Production of cellulose by CDB strains

Those isolates showing the ability to utilize cellulose were inoculated into MSM broth with different cellulose sources to determine cellulase activity. The result was observed at 560 nm and OD values were calculated spectrophotometrically. The result indicated that CDB 12 showed the highest level of cellulase activity followed by CDB 13 and CDB 21 in blotting paper. Overall, the result showed that cellulase enzyme activity was high in blotting paper when compared to the other two substrate sources tested (Table 2).

Effect of pH and temperature on cellulose degradation bacterial growth

The various range of pH 4.0 to 8.0 tested, shows that the maximum growth of cellulose degradation bacteria (CDB) was recorded at pH 7.0 and that even all the strains grew at pH 4.0 to 8.0. In this study, those five strains survived at both acidic and alkaline environments (Table 3). Although CDB organisms preferred a neutral pH range,

Table 2. Estimation of cellulase using filter paper, blotting paper and CMC by CDB strains (Mean of 4 replications).

Cellulase estimation (mg glucose released/ min)			
Strains	**Filter paper**	**Blotting paper**	**CMC**
CDB1	479	489	492
CDB7	473	477	470
CDB12	539	637	564
CDB13	511	533	550
CDB21	485	508	494
Control	-	-	-
CD @ 5%	25.05	43.33	46.49

Table 3. Growth of CDB strains at different pH (Values indicate OD at 560 nm).

	pH				
Strains	**4.0**	**5.0**	**6.0**	**7.0**	**8.0**
CDB1	0.211	0.350	0.562	0.687	0.113
CDS7	0.123	0.289	0.402	0.555	0.200
CDB12	0.205	0.328	0.419	0.443	0.272
CDB13	0.187	0.414	0.503	0.575	0.217
CDB21	0.222	0.300	0.532	0.612	0.240

Poor growth (0.0 to 0.5); Medium growth (0.6 to 1.0); Good growth (1.1 to 1.5).

Table 4. Growth of CDB strains at various temperature (Values indicate OD at 560 nm).

Strains	**Temperature (°C)**				
	20º	**25º**	**30º**	**35º**	**40º**
CDB1	0.374	0.417	0.749	1.103	0.805
CDB7	0.326	0.408	0.853	0.959	0.792
CDB12	0.702	0.862	1.025	1.325	0.753
CDB13	0.635	0.720	0.937	1.112	0.888
CDB21	0.570	0.716	0.898	0.963	0.669

Poor growth (0.0 to 0.5); Medium growth (0.6 to 1.0); Good growth (1.1 to 1.5).

they adopted themselves to survive at slightly basic and acidic pH conditions in the environment. Reese et al. (1950) said that the ability to produce enzymes capable of hydrolyzing the 1, 4- β- modified celluloses was widespread among micro-organisms. The CDB organisms can grow well at a pH ranging from 5.7 to 7.5 which was observed by Lu et al. (2005). The temperature ranges between 20 and 40°C were tested for growth of cellulose degradation bacteria, while the maximum growth of CDB was observed at 35°C. The tested CDB strains were withstood and were able to tolerate the temperature from 20 to 40°C. In this present study, we can conclude that all five CDB strains can grow at low and high temperature tested (Table 4). A temperature specificity of these isolates showed that most of them grow between 30 and 40°C indicating that all of the isolates are mesophilic in nature. Based on these properties, the enzyme may be suited to particular conditions. The effect of pH and temperature on the activity of cellulose was similar to that observed in previous studies (Chung et al., 2009; Singh et al., 2004).

Utilization of carbon and nitrogen sources

Results indicated that all the organisms could grow in different nutritional sources. Among the carbon source, maximum growth was observed in glucose amended MSM, followed by fructose and maltose (Table 5). The tested CDB strains utilized glucose as a basic carbon source, and then used other sugar sources moderately. Also, they preferred other carbon sources when simple

Table 5. Utilization of carbon sources by cellulose degrading bacteria (Values indicate OD at 560 nm).

Strains	Glucose	Fructose	Maltose	Arabinose	Starch	Inosital	Xylose	Trehalose	Malate	Citrate
CDB7	+++(1.058)	+(0.522)	++(0.658)	++(0.545)	+(0.401)	+(0.312)	+(0.416)	++(0.541)	++(0.589)	++(0.512)
CDB12	+++(1.366)	++(0.821)	++(0.806)	++(0.775)	++(0.638)	++(0.556)	++(0.618)	++(0.555)	++(0.725)	++(0.612)
CDB13	++(0.757)	++(0.647)	++(0.580)	+(0.449)	++(0.560)	+(0.423)	++(0.571)	+(0.456)	++(0.554)	++(0.525)
CDB21	++(0.925)	++(0.788)	++(0.862)	++(0.517)	++(0.679)	++(0.528)	+(0.381)	++(0.605)	+(0.458)	+(0.351)

+ Poor growth (0.0 to 0.5); ++ Medium growth (0.6 to 1.0); +++ Good growth (1.1 to 1.5).

Table 6. Utilization of nitrogen sources by CDB (Values indicate OD at 560 nm).

Strains	Ammonium sulphate	Sodium nitrate	Ammonium nitrate	Potassium nitrate	Ammonium tartrate	Ammonium chloride	Urea
CDB1	0.260	0.293	0.204	0.168	0.195	0.164	0.057
CDB7	0.182	0.197	0.162	0.106	0.332	0.179	0.092
CDB12	0.129	0.153	0.0161	0.160	0.363	0.305	0.065
CDB13	0.132	0.139	0.196	0.145	0.246	0.287	0.027
CDB21	0.158	0.188	0.188	0.305	0.186	0.284	0.093

Poor growth (0.0 to 0.5); Medium growth (0.6 to 1.0).

and basic nutrients are unavailable. So those organisms could be able to survive at different carbon sources available in the soil and substratum environment. All the nitrogen compounds supported the growth of CDB strains. Ammonium sulphate, ammonium nitrate and potassium nitrate were good nitrogen sources for utilization of CDB strains. Strains such as CDB12, CDB13 and CDB21 showed better growth in Ammonium sulphate amended media (Table 6). From this study, it was clearly understood that CDB utilized different sources of nitrogen obtained from fertilizers and those that were naturally available in soil. Most of the agricultural and plantation crops depend on different category of nitrogenous fertilizers for higher productivity. Based on the results achieved by this study, the tested five CDB strains were able to utilize different nitrogen sources (ammonium sulphate, ammonium nitrate and potassium nitrate) for better survival. As such, CDB gets nitrogen from external sources and improve the organic content in soil by degrading cellulose by production of cellulase enzyme.

Microorganisms are so small, that they have a very high ratio of surface area to volume. This enables them break complex substrates into simpler molecules. Some microbes absorb their nutrients through their cell membranes and as such, result in high metabolic rates (www.ineedcoffee.com). So, this present study comprised the tea biomass (dead wood, leaf litter, plant and other residues) which contains various types of carbon and nitrogen sources. During the pruning period, the selected five strains of cellulose decomposer, introduced into tea plantation pruning field, can easily utilize carbon and nitrogen sources, thereby converting raw materials into energy rich compounds. In nutrient recycling, they are important in maintaining sustainability and stability inside the tea ecosystem. More so, the vast amount of biomass on the floor of the tea plants can be recycled by microbes not only to conserve energy, but also to minimize biomass pollution, fungal disease and improve the organic matter in soil.

Although soil enzymes are available as an indicator of soil quality, McCarty et al. (1994) suggested that caution is required in such use because the relationship between soil enzyme activities and soil quality is complex. Soil fertility is not related to soil enzyme activities only, but also microbial properties (microbial biomass, microbial turnover, microbial population, etc.,), soil physiological properties (pH, organic carbon content, nutrient availability, etc.) and vegetation response (plant biomass or yield, nutrient uptake,

etc). In tea fields, the elements are continuously removed at each plucking round and thus in a year, considerable amount is removed from the soil which is required to be replenished in order to maintain the sustainable productivity of tea (Verma et al., 2001). Chemical fertilizers with instant ability to refurbish deleted nutrients in necessary quantities and forms have come to be recognized as a key component of soil fertility management and sustainable productivity.

Conclusion

The results from this research support the general conclusion that introduction of cellulose degrading bacterial (CDB) inoculants is a beneficial microbiological tool to aid recovery of energy from degraded ecosystems during pruning in tea plantation. In this study, the efficient five strains (CDB1, CDB7, CDB 12, CDB13 and CDB21) produced higher amount of cellulase enzyme actively among 25 strains isolated. All five CDB strains have relevance to shade grow eco-friendly tea in many ways. The vast amount of biomass on the floor of the tea plants can be recycled by microbes not only to conserve energy, but also to minimize biomass pollution, fungal disease and improve the organic matter in soil. In addition, these CDB strains are well adjusted in both acidic and alkaline pH and in the temperature range of 20 to 40°C. From this study, we clearly understood that these five strains will adopt themselves to various environmental conditions for maximum cellulose production. However, the effect of the cellulase activity was included in this study, that is, these five cellulose decomposers break down large organic molecules into smaller molecules that can be used by the biotic community. This in turn will lead to efficient use of renewable conventional resources inside the tea through microbiological processes rather than non renewable conventional sources. Consequently, this will open a new path of progressive thought, where tea planters will depend either less on synthetic fertilizers and chemicals and use both under integrated management approach of nutrient to increase tea productivity.

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REFERENCES

Abdelnasser SSI, Ahmed IE (2007). Isolation and identification of new celluloses producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. Austr. J. Basic App. Sci., 1(4): 473-478.

Aubert JP, Beguin P, Millet J (1987). Biochemistry and Genetics of cellulose Degradation, Fungal and Bacterial enzyme systems and their manipulation. FEMS Symposium. 43, Academic Press, New York, pp. 11-30.

Baby UI (2004). Dogmas and facts of bioinoculants. Planter's Chem., 100 (1): 12-15.

Bernfeld P (1955). Amylases, alpha and beta. Methods. Enzymol., 1: 149-158.

Chung-Yi W, Yi-Ru H, Chang-Chai NG, Helen C, Hsin-Tang L, Wen-Sheng T, Yuan-Tay S (2009). Purification and Characterization of a novel halostable cellulose from Salinivibrio sp. strain NTU-05. Enzyme and Microbial Technology-ELSEVIER, 44: 373-379.

Dension DA, Koehn RD (1977). Cellulase activity of *Poronia oedipus*. Mycologia, 69: 592-603.

McCarty GW, Siddaramappa R, Wright RJ, Codling EE, Gao G (1994). Evaluation of coal combustion by-products and soil liming materials-Their influence on soil pH and enzyme activities. Biol. Fert. Soils, 17: 167-172.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Munten W (2005). Beneficial Soil microorganisms. Soil and Plant Laboratory Inc., Bellevue, WA, pp. 1-2.

Reese E, Siu RGH, Levinson HS (1950). The Biological Degradation of Soluble Cellulose Derivatives and Its Relationship to the Mechanism of Cellulose Hydrolysis. J. Bact., 59: 485-497.

Singh J, Batra N, Sobti RC (2004). Purification and Characterization of alkaline cellulose produced by a novel isolates, *Bacillus sphaericus* js 1. Ind Microbiol. Biotechnol., 31: 51-56.

Tailliez P, Girard H, Millet J, Beguin P (1989). Enhanced cellulose fermentation by an asprogenous and ethanol-tolerant mutant of *Clostridium thermocellum.* Appl., 14: 172-176.

Updegroff DM (1969). Semi micro determination of cellulase in biological materials. Anal Biochem., 32: 420-424.

Verma DP, Palani P, Balasubramaniam K, Kumaraguru R, Venkatesan S, Ganapathy MNK (2001). Nutritional management of tea for sustainable productivity in South India. The planters chronicle, pp 215-227.

Wright SF (2003). The important of soil microorganisms in aggregate stability. Proc. North central extension. Industry soil fertility Conference, 19: 93-98.