

Full Paper

Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system

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Fifteen mesophilic bacteria with high C_x cellulase activities were isolated and purified from a mixed-culture enriched from a flower stalks-vegetable waste co-composting system. A CMCase test showed that the enzyme activity of these isolates ranged from 7.9 to 28.0 U ml⁻¹. Although filter paper degrading capability was low in single culture, significant synergetic cellulose degradation were detected in four groups of mixed cultures, their degradation rates were 23.5%, 26.3%, 19.4% and 24.5%, respectively. Study of morphological and physiological characters of five predominant isolates which possess high CMCase and had positive effect on synergetic cellulose degradation in mixed culture system showed that two of them were closely related to *Bacillus pasteurii* and *Bacillus cereus*, whereas the rest belong to the genus *Halobacillus*, *Aeromicrobium* and *Brevibacterium*, respectively.

Key Words—cellulose-degrading bacteria; characterization; composting; identification

Introduction

The substrates of composting with primary components of plant material such as cellulose, hemicellulose and lignin are rather difficult to biodegrade and reduce the availability of the other polymers by means of a physical restriction (Ladisch et al., 1983). Studies have showed that it is of great importance that increase of the microbial populations especially the lignocellulose-degrading microorganisms in the compost will help in enhancing lignocellulose-degrading waste decomposition and thus hasten the process of composting with different substrates (Beguín and Aubert, 1994; EL-Din et al., 2000; Hart et al., 2002; Iiyami et al., 1996; Kakezawa et al., 1992; Lu et al., 2004;

Straatsma et al., 1994; Tengerdy and Szakacs, 2003). Moreover, agronomical study showed that inoculation of compost with lignocellulose-degrading microorganisms is a potentially successful strategy for improving the product for agronomic purposes (El-Din et al., 2000; Lynch, 1986).

Yunnan province is the biggest flower production base and an important vegetable production area in China. Pollution of plantation wastes, mainly as flower stalk and vegetable wastes have become an increasing problem to the environment, especially to the surface water bodies of Dianchi Lake, the most famous plateau lake in Southwest China. As cellulose is a major component of these agricultural wastes, a technology using extrinsic inocula to remove or reduce ligno-cellulose during waste composting was developed in a previous study (Lu et al., 2004). The scope of this study is to isolate and characterize the predominant mesophilic cellulose-degrading bacteria (CDB) from a previous lignocellulose-degrading enrichment, thus to provide biological information for their potential

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application.

Materials and Methods

Isolation of mesophilic cellulose-degrading bacteria.

A mixed culture of lignocellulose-degrading bacteria was enriched with compost materials from a pilot scale composting ($1 \times 1 \times 2 \text{ m}^3$), which was prepared from chopped material (5–8 cm in length) of flower stalks and vegetable waste (4:6, wt/wt) at the demonstrate pilot of the ongoing project in Yunnan Province. They were continually cultured at 30°C in liquid medium containing: yeast extract 1 g; peptone 5 g; CaCO_3 2 g; NaCl 1.2 g; dried rice straw 10 g, distilled water 1 L. The effectiveness of the primary mixed-culture in enhancing lignocellulose biodegrading and improving the composting process of flower stalk and vegetable waste had been validated in previous studies (Huang et al., 2004; Lu et al., 2004). In this study, 10 ml of the above mixed culture with 3 replicates were taken and subjected to serial dilution using sterilized saline water (0.85%), an aliquot of 100 μl of each dilution was spread on cellulose-Congo red agar plates and incubated at $30 \pm 2^\circ\text{C}$ for 3–5 days to obtain single colonies, and those that generated a clearing zone around the colonies were picked out and purified on LB agar.

C_x cellulase-producing activities of the isolates were estimated by the carboxymethylcellulose hydrolysis capacity (HC value) on the cellulose Congo-red agar, i.e. ratio of diameter of clearing zone and colony (Hankin and Anagnostakis, 1977; Hendricks et al., 1995; Reese et al., 1950), and those with high HC values were selected and stored on slants at 4°C for further studies.

Cellulase activity test of the isolates. The chosen isolates were grown in flasks for 3–7 days in medium containing CMC-Na 5.0 g, NaNO_3 2.5 g, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6 g, NaCl 0.1 g, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 g, FeCl_3 0.01 g, gelatin 2.0 g, yeast extract 0.1 g, distilled water 1,000 ml, pH 6.8–7.2. Samples were taken and supernatant cellulase, here specified as CMCase, were determined using the method of Somogyi (1952). One unit of enzyme activity was determined as the amount of enzyme producing reducing sugars corresponding to 1 μmol glucose per minute.

The same medium was used for the test of filter paper degradation, except the carbon source, for which $1 \times 6 \text{ cm}$ strips of filter paper (Whatman No. 1)

were substituted for CMC-Na. After 7 days cultivation, residual cellulose of filter paper was gravimetrically determined (Tailliez et al., 1989; Updegraff, 1969).

Morphological, physiological and biochemical characteristics of predominant isolates. The morphological characterization of each isolate was performed, including color, size, and colony characteristics (form, margin, and elevation). Cell wall hydrolysate analysis was demonstrated using thin layer chromatography to investigate wall chemotype of each isolate by testing the composition of cell wall diaminopimelic acid isomers and whole-cell sugars (Hasegawa et al., 1983). Physiological and biochemical tests were processed based on Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The Biolog test followed those outlined in the Biolog Manual (MicroLog System, Biolog, Inc., Hayward, CA) was also performed to compare with the result of systematic bacteriology identification.

Results and Discussion

Isolation of mesophilic cellulose-utilizing bacteria

Fifteen bacteria that grew vigorously and showed the ability to develop clearing zones around their colonies on cellulose Congo-red agar during aerobic incubation were isolated from the previous lignocellulose-degrading enrichment, and designated as CDB in this paper (Fig. 1). The clearing zone size and colony diameter of these isolates were measured daily when incubated aerobically at 30°C ; the result showed that maximum clearing zone ranged between 2.55 and 6.40 cm, and the average HC value, i.e. ratio of zone size to colony diameter ranged between 4.24 and 10.36 cm, demonstrating that all the isolates have the ability of CMC degrading and indicating high ability of C_x cellulase production (Table 1). Distinct C_x cellulase production was detected in strain CDB19 as evidenced by its max HC value of 13.11, other strains with higher HC values were CDB1, CDB10, CDB13, CDB15, and CDB18. The time course for C_x cellulase activity of these isolates showed that the maximum HC occurred between 4 and 8 days (Fig. 2).

A temperature stability test of these isolates showed that most of them grow between 30 and 40°C ; only five of them can survive up to 50°C , indicating that all of the isolates are mesophilic bacteria.

Cellulase activity test of the isolates

The result of CMCase determination is shown in Table 1. All tested isolates produce CMCase and their activities ranged from 7.9 to 28.0 U ml⁻¹. However, except CDB19, no distinct filter paper degradation occurred in a single culture in which filter paper was used as sole carbon source over the incubation period; neither were reducing sugars were detected, indicating that their filter paper cellulase (FPase) activity with single cultures is relatively low.

It is known that cellulase system contain endoglucanase (1,4- β -glucan glucanohydrolase, EC 3.2.1.4), exoglucanase (1,4- β -glucan cellobiohydrolase, EC 3.2.1.91) and β -glucosidase (β -D-glucoside glucohy-

drolase or cellobiase, EC 3.2.1.21). Exoglucanase is necessary for splitting off the elementary fibrils from the crystalline cellulose (Fan and Lee, 1983; Schewale, 1982; Woodward and Wiseman, 1983), but only the synergy of the above enzymes makes possible the cellulose hydrolysis to glucose (Ryu and Mandels, 1980; Sandhu and Bawa, 1992; Wood, 1989; Wood and McRae, 1978, 1979) or a thorough mineralization to H₂O and CO₂. An orthogonal test (L₁₆C₂¹⁵) thus was designed in this study to test the synergetic degrading of cellulose by mixed-culture of these isolates. The result revealed an enhanced synergetic

Table 1. C_x cellulase production of the isolates as estimated by ratio of clearing zone size to colony diameter (HC value) and their CMCase activities.

Strains	Max clearing zone size (cm)	Average HC	Max HC	CMCase (U ml ⁻¹)
CDB1	4.60	7.05	9.00	17.0
CDB2	6.15	5.08	5.85	14.6
CDB3	2.55	4.93	6.20	10.0
CDB6	3.60	4.97	6.40	12.9
CDB10	4.09	8.08	9.70	22.3
CDB11	3.80	5.56	6.25	11.0
CDB13	6.30	6.58	8.29	16.5
CDB15	5.20	5.82	6.50	10.2
CDB18	5.20	8.42	11.23	15.8
CDB19	6.40	10.36	13.11	28.0
CDB23	6.20	4.24	4.85	7.9
CDB26	6.15	4.76	5.88	9.2
CDB29	5.70	4.49	5.14	11.0
CDB30	2.75	4.96	5.80	12.1
CDB32	3.60	4.33	5.50	9.6

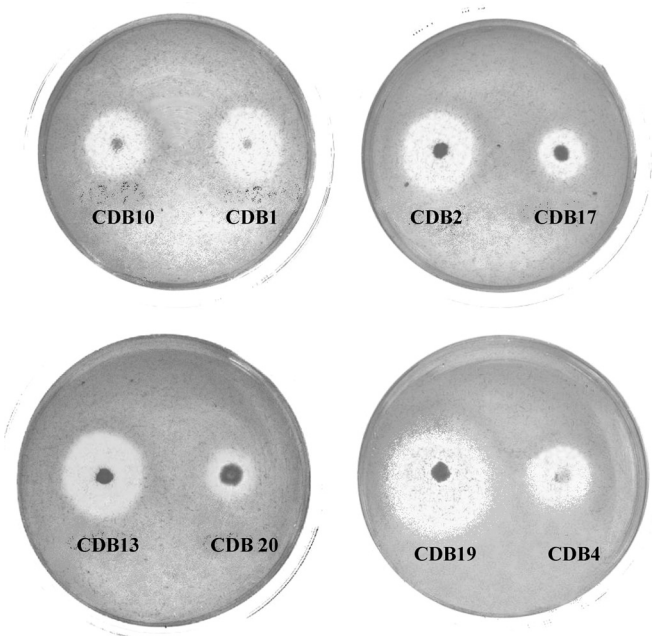


Fig. 1. Photo image of clearing zone generated by isolates of cellulose-degrading bacteria on cellulose Congo-red agar.

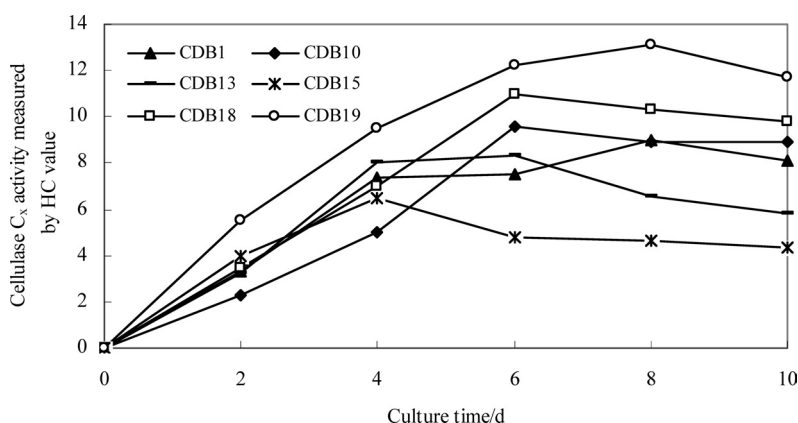


Fig. 2. Cellulase C_x activities of cellulose-degrading bacteria isolated from the composting.

Table 2. Synergetic cellulose degradation by mixed-cultures of bacterial isolates from the composting.

Run	Strains														Filter paper activity
	CDB1	CDB30	CDB3	CDB10	CDB6	CDB11	CDB15	CDB13	CDB23	CDB32	CDB18	CDB26	CDB29	CDB2	CDB19
I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
II	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
III	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
IV	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
V	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
VI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
VII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
VIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
IX	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
X	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XIV	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XV	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
XVI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

1 for presence of the strain; 0 for absence of the strain; + for degrading of filter paper; - for no degradation.

degradation of filter paper by complementary combination of these isolates (Table 2). Four groups of mixed-culture demonstrated the ability of initiating cellulose degradation in 3 days when cultured in broth medium with filter paper as the sole carbon source, and 23.5%, 26.3%, 19.4% and 24.5% of filter paper were degraded respectively after 7 days' cultivation (Figs. 3 and 4).

Many fungi are able to break down polysaccharides such as celluloses and convert these polymeric compounds into sugars due to their capability to produce extracellular enzymes, and cellulase research was mainly focused on fungi in the past (Akin, 1987; Mandels, 1981; Petre et al., 1999; Rosevear, 1984; Wood, 1992). Few bacteria possess a complete multi-enzyme system for lignocellulose degradation; however, there has been increasing interest in cellulase production by bacteria because of the fast growth rate and many cellulolytic bacteria were isolated from various environments (Coughlan and Mayer, 1992; Li and Gao, 1997; Mandels and Reese, 1999; Petre et al., 1999). As previous studies had showed enhanced lignocellulose biodegradation during the composting of flower stalk and vegetable waste when inoculated with the primary mixed culture (Huang et al., 2004; Lu et al., 2004), the result in this study indicates that enhanced cellulose degrading ability was due to the complementarity of cellulases from different strains.

The data showed that strains designated as CDB1, CDB2, CDB10, CDB13 and CDB19 seemed to have positive effects on cellulose degradation in the mixed-culture systems, combine with their higher CMCase activity, we therefore chose these five bacteria for detailed morphological and physiological characterization.

Morphological, physiological and biochemical characterization of five predominant isolates

Main morphological, physiological and biochemical characteristics of these five CDBs and their cultivation characteristics on different media are summarized in Table 3. The result showed morphological diversity of colonies on culture media. Microscopic tests revealed that all of them are rod-shaped Gram-positive bacteria, with 3 of them motile and spore-forming. In the assimilation test of mono-, oligo- and polysaccharides, it was shown that CDB1 and CDB13 assimilated all carbon sources tested, whereas CDB2 and CDB10 assimilated only some of the tested carbon sources. CDB19



Fig. 3. Photo image of enhanced synergetic degradation of filter paper by four groups of mixed-culture of cellulose-degrading bacteria after 4 days' cultivation.

(1) Control, (2) group VI, (3) group VII, (4) group IX, (5) group XIII.

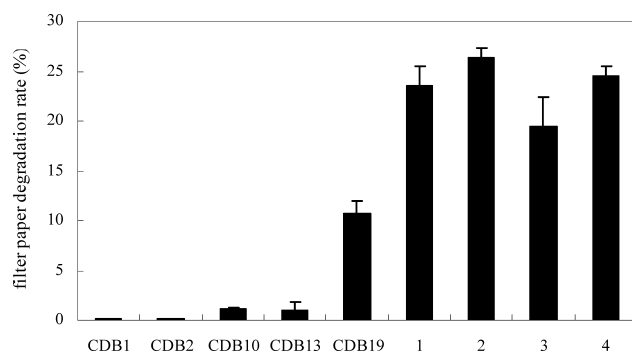


Fig. 4. Filter paper degradation rates of pure- or mixed-cultures.

$n=3$; error bars indicate SD. (1) Group VI, (2) group VII, (3) group IX, (4) group XIII.

could ferment some of the tested saccharide to produce a small amount of acid, including D-glucose, L-arabinose, D-mannose, and D-fructose. Assimilation of cellulose and xylan/starch coincided with the good growth of these isolates on cellulose Congo-red agar. Optimum temperatures for growth of these CDBs range between 25 and 40°C, indicating that they are mesophilic bacteria, while maximum growth was observed at initial pH ranging from 5.7–7.5.

Thin layer chromatography analysis of cell wall hydrolysates of these isolates revealed the presence of *meso*-diaminopimelic acid in all isolates except CDB13, and no diagnostic sugars were identified in the whole-cell sugar analysis of all isolates (Table 4). Thus CDB1, CDB2, CDB10 and CDB19 were classified under cell wall type II, whereas CDB13 was classified under cell wall type I due to occurrence of LL-diaminopimelic acid in its cell hydrolysates. The above morphological characteristics together with the results of physiological tests revealed that CDB1 and CDB2 are closely related to *Bacillus pasteurii* and *Bacillus*

cereus respectively, where CDB10 and CDB13 belong to the genus *Halobacillus* and *Aeromicrobium*, respectively. Physiological and biochemical characteristics and additional 16S rDNA sequencing (data not shown) identified CDB19 as *Brevibacterium linens*. However, the result of the Biolog test showed that except for CDB1, the similarity indices of the test strains were <0.50, which is not high enough to identify at either genus or species level, and the primary identities of all the isolates are quite different from the result gained by systematic bacterial identification (data not shown). Other researchers also have found that the majority of microorganisms tested did not match the Biolog database (Atkinson et al., 1997; Boulter et al., 2002; Lu et al., 2002). It may be concluded that the Biolog system is not suitable for bacterial identification regarding complex microbial communities with wide range from the compost due to the lack of a sufficient database.

The genus *Bacillus* consists of a group of aerobic or facultatively anaerobic bacteria with a wide diversity of physiological ability with respect to heat, pH and salinity. Many species are normally present in soil and in decaying animal and vegetable matter (Holt, 1994). Other studies demonstrated that species from *Bacillus* species played important roles in biodegradation and bioconversion of big molecular compounds (Akin, 1987; Holt, 1994; Rosevear, 1984), and *Bacillus subtilis* as well as *Bacillus licheniformis* are frequently reported cellulolytic species (Liu et al., 2004; Petre et al., 1999). The isolation of *Halobacillus* and *Aeromicrobium* spp. from the macrocosm of this research is probably another evidence for adaptation and predominance of resistant species in the adverse environment of composting. Besides, a cellulolytic bacterium with a cellulose (filter paper) degradation rate of 10.7% was isolated; it was closely related to *Brevibacterium*

Table 3. Main morphological, physiological and biochemical characteristics of the CDB isolates from the composting.

Characteristics		Strains				
		CDB1	CDB2	CDB10	CDB13	CDB19
Cell diameter		1.2–1.8 μm	1.0 μm	1.2–3.0 μm	0.9–1.7 μm	0.9–3.5 μm
Spores		oval	round	oval	–	–
Sporangium		swollen	swollen	swollen	–	–
Flagellum		+	+	+	–	–
Gram stain		G ⁺	G ⁺	G ⁺	G ⁺	G ⁺
Catalase		+	+	+	–	+
Aerobic growth		+	+	+	+	+
Anaerobic growth		–	–	–	–	ND ^a
Oxidase		ND	+	+	+	–
Saccharide utilization	D-Glucose	+	+	+	+	+ ^b
	L-Arabinose	+	–	–	+	+ ^b
	D-Xylose	+	–	–	+	– ^b
	D-Mannitol	+	–	–	+	– ^b
	Sucrose	+	+	+	+	ND
	Raffinose	+	–	+	+	– ^b
	D-Mannose	ND	ND	+	ND	+ ^b
	D-Galactose	+	ND	+	+	– ^b
	D-Fructose	+	ND	+	+	+ ^b
	Sorbitol	–	ND	ND	ND	– ^b
	Inositol	–	ND	–	+	– ^b
	Starch	–	+	+	+	–
Hydrolyzing ability	Casein	+	+	–	ND	ND
	Gelatin	+	+	+	+	+
	Cellulose	(+) ^c	(+)	(+)	(+)	(+)
Utilization of carbon sources						
	Citrate	–	+	–	–	+
	Propionate	ND	ND	ND	ND	–
	Cellulose	(+) ^c	(+)	(+)	(+)	(+)
Degradation of tyrosine		–	+	–	ND	ND
Nitrate reduction		+	–	–	–	–
Formation of indole		–	–	–	–	–
Growth on 7% NaCl		+	+	+	+	–
Milk coagulation		+	ND	ND	+	+
Milk proteolysis		–	ND	ND	–	+
H ₂ S production		–	–	ND	–	–
Optimum temperature for growth		30–40°C	25–35°C	25–33°C	25–35°C	25–35°C
Optimum pH condition for growth		ND	5.7–7.5	6.8–7.5	6.5–7.5	ND

^aND: not determined.^bAbility of saccharide fermentation.^cWeak growth with single culture.

linens. The result demonstrated that significant synergistic cellulose degradation can be achieved in mixed culture systems of cellulolytic bacteria and non-cellulolytic bacteria, in which non-cellulolytic bacteria enhanced cellulolytic activity probably through consuming metabolites derived from cellulose as well as providing essential growth factors for cellulolytic bacteria;

in fact, enhanced bacterial growth with no lag time and faster growth speed was observed more often in mixed cultures than in any single culture under the same incubation conditions (data not shown).

Isolation and characterization of predominant mesophilic cellulose-utilizing bacteria were performed from a previous bacterial enrichment from flower

Table 4. Cell wall chemotypes of the CDB isolates from composting.

Strains	CDB1	CDB2	CDB10	CDB13	CDB19
Diagnostic diamino acid	<i>meso</i> -DAP, ^a Glycin	<i>meso</i> -DAP, Glycin	D-Ornithine, D-Aspartic acid	LL-DAP, Glycin	<i>meso</i> -DAP, Glycin
Diagnostic sugars	—	—	—	—	—
Cell wall type	Type II	Type II	Type II	Type I	Type II

^a Diaminopimelic acid.

stalks-vegetable wastes co-composting, and their cellulase activities were investigated. The result showed that a high cellulose degradation rate can be achieved by mixed-cultures via suitable grouping due to their enzyme complementarity of cellulase. The development and application of large-scale composting inoculants as biocatalysts is now underway.

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