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 (Beguin 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...
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 x 1 x 2 m³), 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; CaCO₃ 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±2°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.

Cx 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 (Han-kin 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, NaNO₃ 2.5 g, KH₂PO₄ 1.0 g, MgSO₄·7H₂O 0.6 g, NaCl 0.1 g, CaCl₂·6H₂O 0.1 g, FeCl₃ 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 x 6 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 dianaminopimelic 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 Cx cellulase production (Table 1). Distinct Cx 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 Cx 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 glucohydrolase 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 H\(_2\)O and CO\(_2\). An orthogonal test (L\(_{16}(C^2)\)) 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>
<thead>
<tr>
<th>Strains</th>
<th>Max clearing zone size (cm)</th>
<th>Average HC</th>
<th>Max HC</th>
<th>CMCase (U ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDB1</td>
<td>4.60</td>
<td>7.05</td>
<td>9.00</td>
<td>17.0</td>
</tr>
<tr>
<td>CDB2</td>
<td>6.15</td>
<td>5.08</td>
<td>5.85</td>
<td>14.6</td>
</tr>
<tr>
<td>CDB3</td>
<td>2.55</td>
<td>4.93</td>
<td>6.20</td>
<td>10.0</td>
</tr>
<tr>
<td>CDB6</td>
<td>3.60</td>
<td>4.97</td>
<td>6.40</td>
<td>12.9</td>
</tr>
<tr>
<td>CDB10</td>
<td>4.09</td>
<td>8.08</td>
<td>9.70</td>
<td>22.3</td>
</tr>
<tr>
<td>CDB11</td>
<td>3.80</td>
<td>5.56</td>
<td>6.25</td>
<td>11.0</td>
</tr>
<tr>
<td>CDB13</td>
<td>6.30</td>
<td>6.58</td>
<td>8.29</td>
<td>16.5</td>
</tr>
<tr>
<td>CDB15</td>
<td>5.20</td>
<td>5.82</td>
<td>6.50</td>
<td>10.2</td>
</tr>
<tr>
<td>CDB18</td>
<td>5.20</td>
<td>8.42</td>
<td>11.23</td>
<td>15.8</td>
</tr>
<tr>
<td>CDB19</td>
<td>6.40</td>
<td>10.36</td>
<td>13.11</td>
<td>28.0</td>
</tr>
<tr>
<td>CDB23</td>
<td>6.20</td>
<td>4.24</td>
<td>4.85</td>
<td>7.9</td>
</tr>
<tr>
<td>CDB26</td>
<td>6.15</td>
<td>4.76</td>
<td>5.88</td>
<td>9.2</td>
</tr>
<tr>
<td>CDB29</td>
<td>5.70</td>
<td>4.49</td>
<td>5.14</td>
<td>11.0</td>
</tr>
<tr>
<td>CDB30</td>
<td>2.75</td>
<td>4.96</td>
<td>5.80</td>
<td>12.1</td>
</tr>
<tr>
<td>CDB32</td>
<td>3.60</td>
<td>4.33</td>
<td>5.50</td>
<td>9.6</td>
</tr>
</tbody>
</table>
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.

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Filter paper activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDB1</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB2</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB3</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB4</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB5</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB6</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB7</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB8</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB9</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB10</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB11</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB12</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB13</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB14</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB15</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB16</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB17</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB18</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>CDB19</td>
<td>+ + + + + + + + + + +</td>
</tr>
</tbody>
</table>

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

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
could ferment some of the tested saccharides 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 L,L-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
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 petals. Table 3. Main morphological, physiological and biochemical characteristics of the CDB isolates from the composting.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDB1</td>
</tr>
<tr>
<td>Cell diameter (μm)</td>
<td>1.2–1.8</td>
</tr>
<tr>
<td>Spores</td>
<td>oval</td>
</tr>
<tr>
<td>Sporangium</td>
<td>swollen</td>
</tr>
<tr>
<td>Flagellum</td>
<td>+</td>
</tr>
<tr>
<td>Gram stain</td>
<td>G⁺</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharide utilization</td>
<td></td>
</tr>
<tr>
<td>d-Glucose</td>
<td>+</td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>ND</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>+</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
</tr>
<tr>
<td>Inositol</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolyzing ability</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of carbon sources</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
</tr>
<tr>
<td>Propionate</td>
<td>ND</td>
</tr>
<tr>
<td>Cellulose</td>
<td>(+)³</td>
</tr>
<tr>
<td>Degradation of tyrosine</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
</tr>
<tr>
<td>Formation of indole</td>
<td>–</td>
</tr>
<tr>
<td>Growth on 7% NaCl</td>
<td>+</td>
</tr>
<tr>
<td>Milk coagulation</td>
<td>+</td>
</tr>
<tr>
<td>Milk proteolysis</td>
<td>–</td>
</tr>
<tr>
<td>H₂S production</td>
<td>–</td>
</tr>
<tr>
<td>Optimum pH condition for growth</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND: not determined.

² Ability of saccharide fermentation.
³ Weak growth with single culture.
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.

Acknowledgments

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References


