Exploration of the key functional proteins from an efficient cellulolytic microbial consortium using dilution-to-extinction approach

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A B S T R A C T

In the present study, the cellulose binding proteins (CBPs) secreted by a putative cellulolytic microbial consortium were isolated and purified by affinity digestion. The purified CBPs were subsequently separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Using mass spectrometric analyses, eight CBPs were identified and annotated to be similar to known proteins secreted by Clostridium clariflavum DSM 19732 and Paenibacillus sp. W-61. In addition, in combination with dilution-to-extinction approach and zymogram analysis technique, CBPs 6 (97 kDa) and 12 (52 kDa) were confirmed to be the key functional proteins that influence cellulolytic activities. Moreover, structural domain analyses and enzymatic activity detection indicated that CBPs 6 and 12 contained glycoside hydrolase families (GH) 9 and 48 catalytic modules, which both revealed endoglucanase and xylanase activities. It was suggested that the coexistence of GH9 and GH48 catalytic domains present in these two proteins could synergistically promote the efficient degradation of cellulose.

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Introduction

Lignocellulose, the most abundant renewable resource biomass in nature, is widely used in industrial applications and agricultural productions. A large number of different types of microorganisms such as bacteria, fungi and actinomycetes are capable of degrading cellulose materials (Lynd et al., 2002). Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin, which form a recalcitrant lingo–cellulose complex that hampers their efficient biological conversion (Himmel et al., 2007). Biological conversion of lignocellulosic to value-added products by microbial co-cultures has caused a worldwide concern, because it can avoid the feedback regulation and metabolite repression problems posed by isolated single strains (Haruta et al., 2002; Yang et al., 2004). The use of microbial co-cultures or complex microbial consortium to bio-transform lignocellulose to value-added products has become a hot research topic in recent years (Tuesorn et al., 2013; Zhang et al., 2011). Studies have shown that compared with the sole cultivation of the model cellulolytic strains, microbial co-cultures can utilize cellulose materials more efficiently and harvest the fermentation products with...
higher concentrations, revealing apparent advantages over the conventional methods (Kato et al., 2004). In recent years, a large number of researches have focused on the utilization of microbial consortium in the biotransformation of cellulosic materials into value-added products and achieved significant results (Kalyani et al., 2013; Lü et al., 2011; Wen et al., 2015).

Similarly, in a previous study, a microbial consortium with highly efficient cellulolytic activity was successfully constructed and applied in enhancing the anaerobic digestion of cassava fibers (Zhang et al., 2011). The said microbial consortium increased the final methane yield and producing velocity, suggesting great application potentials. However, the underlying mechanisms on the high efficiency of cellulolytic activity were still unclear. It has been suggested that efficiency of lignocellulose degradation depends on the cooperation and interactions between microorganisms that produce fibrolytic enzymes (Haruta et al., 2002). Thus, understanding the key functional microbes and the significant enzymes they secrete will provide insights on how to possibly exploit the use of such consortia.

Investigations on some functional microbes from a naturally occurring consortium was conventionally done by isolation and culturing of single strains. However, the use of conventional plating technique presents a complicated and time-consuming screening process, and could only culture in vitro a small fraction of the naturally existing microbes in a microflora (Tajima et al., 1999). Due to the un-culturability of certain key functional strains in natural consortium, it would be challenging to clarify the mechanisms corresponding to a specific biological process through traditional screening scheme (Wang et al., 2010). Therefore, the utilization of a novel method that is easy to implement in practice and without losing some key functional strains, seems particularly necessary.

Recently, a novel dilution-to-extinction approach, which does not necessitate isolation of pure strains, has been proposed to isolate the simplest biological consortium that can exhibit the target function of a mixed culture, revealing extensive application prospects in the investigation of some natural microbial consortia (Wang et al., 2010). This proposed approach has also been successfully utilized in revealing the key functional microbes in a previously constructed cellulolytic consortium where bacteria similar to Clostridium clariflavum DSM 19732 and Paenibacillus significantly contributed to the cellulolytic activity (Zhang et al., 2013). However, the key functional enzymatic proteins secreted by these microbes are still unknown. Isolation and investigation of these key proteins are vital to the understanding of the degradation mechanisms of the cellulolytic consortium.

Traditional isolation and purification of enzymatic proteins is mainly performed through ammonium sulfate precipitation and column chromatography. These methods however are complicated and time-consuming with low yield. Some studies reported that the degradation of cellulose by anaerobic cellulolytic microbes is mainly realized through the formation of cellulose, a very specific and defined multi-enzyme complex with distinct structural features such as a scaffoldin protein with cohesin domains that bind to enzymes containing dockerin domains (Morrell-Falvey et al., 2015; van Dyk et al., 2010). Different types of cellulases in cellulose are then tightly combined and bound together by the scaffoldin proteins. This makes the separation and purification of the different enzymatic components from a cellulosome challenging especially by using the traditional isolation methods. Additionally, specific carbohydrate-binding modules (CBMs) are also found existing in the scaffoldin proteins of cellulosome, and these modules can specifically anchor on the cellulosic substrates (Boraston et al., 2004). Affinity digestion, an approach which could almost totally recover enzymes without much loss, can be utilized to effectively purify the cellulase complex. It works by adsorbing the cellulosome to an acid-treated cellulose polymer which was subsequently hydrolysed in toto by the enzymatic components, and the highly active complex was finally left in a purified state (Morag et al., 1992). This approach was found to be very efficient in the purification of cellulose binding proteins (CBPs) of some cellulosomal consortia (Toyoda et al., 2009; Wongwilaivanita et al., 2010).

In the present study, a metaproteomic analysis was used to focus on the identification of CBPs in a constructed cellulolytic microbial consortium in order to extend our knowledge of the fibrolytic systems in the cellulolytic microbial consortium. Further, combined with a dilution-to-extinction approach and zymogram analysis technique, the key functional CBPs were also clarified. Specifically, this study aims to (1) to isolate and purify the CBPs of the cellulolytic microbial consortium by affinity digestion; (2) to identify the CBPs through mass spectrometry; (3) to explore the key functional CBPs of this microbial consortium through the combination of a metaproteomic analysis and a dilution-to-extinction approach. To date, the combined utilization of the metaproteomic technique and dilution-to-extinction approach to identify the key functional proteins from a natural cellulolytic consortium has been limited, making results of this study contribute to the investigation of other natural functional consortia.

1. Materials and methods

1.1. Medium

The subcultivation and fermentation media in the present study both used peptone-cellulose solution (PCS) medium, which was composed of the following components: 5 g peptone, 1 g yeast extract, 5 g NaCl, 3 g CaCO₃, 6 g cassava residues, 4 g filter paper and 1 L H₂O (pH 7.5). The dilution of the microbial consortium was performed using PCS basal medium, which was composed of the following components: 5 g peptone, 1 g yeast extract, 5 g NaCl and 1 L H₂O (pH 7.5).

1.2. Preparation of amorphous cellulose

Five grams of Avicel was dissolved in 250 mL of concentrated phosphoric acid at 25°C to prepare amorphous cellulose. After the Avicel completely dissolved for 2 hr, six volumes of distilled water were added into the mixture, and the precipitated amorphous cellulose was centrifuged at 6000 r/min for 10 min, and then resuspended in distilled water and re-centrifuged. This procedure was repeated for several times until the pH reached 7.0.

1.3. Investigation of the cellulolytic microbial consortium using dilution-to-extinction approach

A total of 10 mL of the activated cellulolytic microbial consortium liquor was inoculated into the flasks with 100 mL of PCS medium.
These flasks with loose caps were then incubated at 55°C under static conditions for 48 hr until the filter paper totally degraded. The degradation broth was used as inoculums of the original consortium. Subsequently, 10 mL of the broth was transformed into the flask containing 90 mL of sterilized PCS basal medium. After complete mixing, $10^{-1}$ of the diluted microbial consortium was obtained. Accordingly, the microbial consortia with the dilution ratios of $10^{-2}$, $10^{-3}$ until $10^{-11}$ were prepared using the same procedures. Further, 10% ($V/V$) of the above different diluted microbial consortia were separately inoculated into the PCS fermentation medium. These inoculated media were stationarily incubated at 55°C for different times until the filter paper totally degraded or still kept intact after 8 days of incubation. The PCS fermentation medium inoculated or un-inoculated with the activated original microbial consortium was used as the control and blank treatments, respectively.

1.4. Purification of CBPs using affinity digestion

When filter papers of the flasks (inoculated with the original or diluted microbial consortia) were totally degraded or still kept intact after 8 days of incubation, the crude enzyme was purified using affinity digestion for separation of the CBPs following the methods suggested by Morag et al. (1992). In brief, the broths were first filtered through a 6-layered gauze, and the supernatant was centrifuged at $10,000 \times g$ for 10 min and then further filtered through a 0.45 μm of nitrocellulose membrane. The crude enzyme was concentrated by ultrafiltration using a diether sulphone ultrafiltration membrane (Millipore) with 10 kDa cutoff. The concentrated enzyme was then bound on acid swollen carboxymethyl cellulose (CMC) or xylan substrate were stained with 0.3% ($W/V$) congo red for 30 min and then destained with 1 mol/L NaCl until bands appeared. These gels were further counterstained with 0.5% ($V/V$) of acetic acid solution.

The molecular weights of protein bands with activities were determined by excising the section of the gel containing molecular marker and separately stained with Coomassie Brilliant Blue R250. After destaining, the marker portion of the gel was placed next to the zymogram to determine the molecular weights of the active bands.

1.6. Identification of CBPs by mass spectrometry

The purified CBPs were further separated by SDS-PAGE, followed by in-gel digestion and protein identification by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS; Biosystems 4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA). The spectra were acquired in linear mode, and the matrix used was alpha-cyano-4-hydroxycinnamic acid (CHCA). A 10 mg/mL solution of the matrix was made in 50:50 ($V/V$) water:acetoniitre containing 0.1% formic acid by volume. Equal volumes of the matrix and CBP solutions were mixed, which was spotted onto the MALDI plate. A total of 2000 laser shots were combined to produce the final spectrum. Searches of the peptide mass fingerprint data against NCBI-nr databases were performed with the MASCOT search engine at http://www.matrixscience.com.

1.7. Prediction of the structural domain of CBPs

The pfam software was utilized to predict the structural domains of CBPs: http://pfam.sanger.ac.uk/.

1.8. Detection of enzyme activity

The filter paper cellulase (FPase), carboxymethyl cellulase (CMCase) and xylanase activities were measured by dinitrosaliclyclic acid (DNS) method at pH 6.0, 60°C (Zhang et al., 2011). One unit of xylanase activity was defined as the amount of enzyme that releases 1 μmol of xylose per minute, while one unit of FPase or CMCase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar under assay conditions per minute.

2. Results and discussion

2.1. SDS-PAGE and zymogram analysis

The purified CBPs of the degradation liquor pretreated by the original cellulolytic consortium were isolated through SDS-PAGE, and their CMCase and xylanase zymogram were also analyzed. As shown in Fig. 1a, thirteen protein bands (named as CBP 1 to CBP 13) appeared on SDS-PAGE gel with molecular weights ranging from 50 to 200 kDa. In addition, the CMCase and xylanase activities of the purified CBPs were
observed by spotting CBPs into agarose gels either containing CMC or xylan, respectively. As shown in Fig. 1b, most of the above separated protein bands either contained CMCase (CBPs 1–9 and 12) or xylanase activities (CBPs 1–4, 6 and 12), while others both had CMCase and xylanase activities (CBPs 1–4, 6 and 12) as revealed by zymogram analysis.

2.2. Identification of CBPs using MALDI-TOF mass spectrometry

To identify and annotate the CBPs secreted by the microbial consortium, these CBPs were firstly isolated by SDS-PAGE. After protein separation, the SDS-PAGE gel was cut down and digested in gel, and the resultant peptide mixture from each gel piece was subjected to MALDI-TOF mass spectrometry. As shown in Table 1, a total of 8 types of proteins were identified. These CBPs were similar to seven kinds of proteins secreted by C. clariflavum DSM 19732, i.e., three kinds of beta-1,4-xylanase protein, a putative carbohydrate binding protein, a dockerin-like protein and two kinds of unnamed protein products. In addition, a xylanase 5 protein secreted by Paenibacillus sp. W-61 was also identified.

The theoretical isoelectric points and molecular weights of these proteins are located from the periods of 4.46–5.60 and 50,654–221,017 Da. The theoretical and actual molecular weights of several proteins revealed some differences (CBPs 3, 8, 9, 11 and 12), while other some gel bands with different molecular weights were identified to be the same proteins (CBPs 5 and 6, CBPs 7–9 and 11–12). Such characteristics may be attributed to the phosphorylation and glycosylation modification of some proteins, which further results in the change of isoelectric point and molecular weight (Lü, 2010).

Table 1 – Identification of the CBPs by mass spectrum.

<table>
<thead>
<tr>
<th>Gel section no. a</th>
<th>MW b</th>
<th>pI b</th>
<th>Protein name or description</th>
<th>Organism</th>
<th>Sequence coverage (%)</th>
<th>Scores</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>199,489</td>
<td>4.46</td>
<td>Beta-1,4-xylanase</td>
<td>C. clariflavum DSM 19732</td>
<td>14</td>
<td>1212</td>
<td>gi359825408</td>
</tr>
<tr>
<td>2</td>
<td>161,418</td>
<td>4.63</td>
<td>Beta-1,4-xylanase</td>
<td>C. clariflavum DSM 19732</td>
<td>4</td>
<td>287</td>
<td>gi359824250</td>
</tr>
<tr>
<td>3</td>
<td>221,017</td>
<td>4.92</td>
<td>Beta-1,4-xylanase</td>
<td>C. clariflavum DSM 19732</td>
<td>4</td>
<td>165</td>
<td>gi359826237</td>
</tr>
<tr>
<td>4</td>
<td>142,675</td>
<td>4.76</td>
<td>Xylanase 5</td>
<td>Paenibacillus sp. W-61</td>
<td>2</td>
<td>81</td>
<td>gi27227837</td>
</tr>
<tr>
<td>5, 6</td>
<td>98,290</td>
<td>5.30</td>
<td>Putative carbohydrate binding protein</td>
<td>C. clariflavum DSM 19732</td>
<td>17</td>
<td>584</td>
<td>gi359827584</td>
</tr>
<tr>
<td>7, 8, 9, 11, 12</td>
<td>81,659</td>
<td>5.14</td>
<td>Dockerin-like protein</td>
<td>C. clariflavum DSM 19732</td>
<td>21</td>
<td>599</td>
<td>gi359827670</td>
</tr>
<tr>
<td>10</td>
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<td>5.60</td>
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<td>C. clariflavum DSM 19732</td>
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<td>87</td>
<td>gi374295465</td>
</tr>
<tr>
<td>13</td>
<td>50,654</td>
<td>4.88</td>
<td>Unnamed protein product</td>
<td>C. clariflavum DSM 19732</td>
<td>7</td>
<td>183</td>
<td>gi374296818</td>
</tr>
</tbody>
</table>

a Sections 1, 2, 3, 4, 6, 7, 10 and 13 approximately correspond to 200, 162, 153, 97, 76, 55 and 50 kDa, respectively.

b Values are theoretical; MW, molecular weight; pI, isoelectric point; CBPs: cellulose binding proteins.
sections (CBPs 5 and 6, CBPs 7–9 and 11–12), this could be related to the products of degradation by proteases from the same protein, present in the cellulolytic microbial consortium (Miwa et al., 2000).

2.3. Degradative characteristics of the cellulolytic microbial consortia

The degradation characteristics of cassava residues and filter paper pretreated with the original or diluted consortia were investigated. The activated original and diluted microbial consortia ($\leq 10^{-5}$) could totally degrade filter paper in 1 to 4 days of incubation (data not shown). Meanwhile, the activities of FPase, xylanase and CMCase in the broths with low dilution ratios ($10^{-1}$ to $10^{-5}$) could reach a comparative level to the ones produced by the original consortium (Fig. 2). At the same time, the change of FPase, xylanase and CMCase in these broths where filter papers were totally degraded (the original and diluted consortia $\leq 10^{-5}$) showed the same tendency, i.e., the FPase, xylanase and CMCase activities can reach their highest levels with 1 to 5 days of incubation, and then slowly decreased to a very low level (FPase and CMCase) or kept almost constant (xylanase) with prolongation of the incubation time. However, it was also observed that even with further increase of the incubation time to 8 days, the filter paper in the broths pretreated by the diluted consortia ($10^{-7}$ and $10^{-9}$) did not degrade. Meanwhile, the activities of the three enzymes in these broths without cellulolytic abilities (dilution ratios of $10^{-7}$ and $10^{-9}$) were always kept at very low levels (near to 0) for the duration of the entire experiment. These results indicate that $10^{-5}$ is the critical dilution point for effective degradation of filter paper of the original consortium. The above phenomenon confirmed that some key functional microbes were washed out from the system and accompanied with the loss of the degradation enzymes (FPase, xylanase and CMCase) during the dilution process, which further resulted to decreased cellulolytic activity of the microbial consortium.

2.4. SDS-PAGE of the purified CBPs under different dilution ratios

The CBPs purified from the broths with different dilution ratios were separated through SDS-PAGE, using equal amount of CBP samples in each lane. The distribution of gel bands from the broths with cellulolytic abilities (dilution ratio $\leq 10^{-5}$) was almost constant (Fig. 3). However, the sum of gel bands drastically decreased with the increase of dilution ratios (i.e. higher than $10^{-5}$). Specifically, bands A and B corresponding to CBP6 and CBP12, disappeared from the gels when the dilution ratio increased to $10^{-7}$ or even higher, accompanying the loss of the cellulolytic ability of the microbial consortium (Fig. 2). Combined with the results of domain analysis (Appendix A Fig. S1), CBPs 6 and 12 were observed to belong to GH9 and GH48, which could indicate endo- and exo-cellulase activities, respectively (Luciano Silveira et al., 2012). Endo- and

![Fig. 2 – Change of enzymatic activities (FPase, CMCase and xylanase) in the broths pretreated by the original or diluted consortia ($10^{-1}$ to $10^{-5}$) under different incubation times. FPase: filter paper cellulase; CMCase.](image-url)
exo-acting cellulases could efficiently and synergistically hydrolyze crystalline cellulose (Wood and McCrae, 1978). It was generally assumed that endoglucanases nick the cellulose surface, and the resulting chain ends serve as an entry point for exoglucanases (Lynd et al., 2002). These further indicate that CBPs 6 and 12 secreted from this microbial consortium play the key roles in the efficient degradation of cellulose, and the coexistence of the GH9 and GH48 catalytic domains of these two proteins could significantly strengthen their cellulolytic abilities. This finding was in accordance with the previous report demonstrated by Izquierdo et al. (2010).

2.5. Zymogram analysis of the purified CBPs under different dilution ratios

Assays on the purified CBPs from the broths with different dilution ratios were performed with CMCase and xylanase zymogram analyses. As shown in Fig. 4, a lot of similar active bands existed on CMCase (CBPs 1–6 and 12) and xylanase (CBPs 1–4 and 6) zymograms when the CBPs were purified from the broths with cellulolytic abilities (dilution ratios \( \leq 10^{-5} \)). Additionally, the sum of active bands drastically decreased and the majority of active bands even disappeared on the CMCase and xylanase zymograms, when the CBPs were purified from the broths without cellulolytic activities at higher dilution ratios (\( 10^{-7} \) and \( 10^{-9} \)).

Furthermore, two active bands (97 and 52 kDa) with higher intensities (corresponding to CBPs 6 and 12) were found to be located on the CMCase zymogram when the CBPs were purified from the broths that have cellulolytic abilities (dilution ratios \( \leq 10^{-5} \); Fig. 4a). The band CBP 6 (97 kDa) was determined to be a very important protein, since it can always reveal obvious xylanase activity in the broths with lower dilutions (\( \leq 10^{-5} \); Fig. 4b). Although the proteins with molecular weights ranging from 116 to 200 kDa (i.e., CBPs 1–4) were invisible on SDS-PAGE analysis (Fig. 3), these proteins could still be existing on CMCase and xylanase zymograms of the corresponding cultures. This phenomenon indicates that the sensitivity of zymogram analysis is higher than SDS-PAGE. In addition, the intensity of the bands (CBPs 1–4) on zymogram indicated the concentration and potential level of expression of these proteins which were relatively low in the corresponding cultures. Based on these observations, it could be concluded that the presence of proteins with lower enzymatic activity (CBPs 1–4 etc.) can promote the degradation of cellulose, however these proteins are believed not the key factors for degradation.

In this study, structural domain analyses revealed that CBPs 1 to 4 all own the structural domains of glycoside hydrolase family 10 (GH10; Appendix A Fig. S1), all of which can reveal obvious xylanase activities (Do et al., 2013). The GH10 family xylanases also show catalytic activities towards certain cellulosic substrates, such as aryl cellobioside and are characterized by higher yields of reducing sugars after the exhaustive hydrolysis of the polymeric substrates (Biely et al., 1997). Meanwhile, CBPs 1, 2 and 4 also possessed typical CBMs. These CBMs play a critical role in enzymatic hydrolysis of plant structural and storage polysaccharides (Cosgrove, 2000; Selvaraj et al., 2010), and the connection of catalytic domain with CBM could obviously enhance the Avicel degradation ability of the enzymes (Maglione et al., 1992). The contribution of CBMs to the activity of cellulases against insoluble cellulose was attributed to the characteristics of increasing enzyme-substrate proximity and enhancement of accessibility of the substrate by modifying its surface structure (Telke et al., 2012). The removal of CBM(s) from modular glycoside hydrolases such as cellulases and xylanases (Sakka et al., 2011) or the artificial connection of a CBM and a catalytic domain (Kittur et al., 2003) sometimes could affect enzyme activity towards insoluble and even soluble substrates. It has been also observed that more drastic effects

Fig. 3 – SDS-PAGE of the purified CBPs under different dilution ratios. M: protein marker; 0: original; –1 to –9 corresponds to the dilution ratios from \( 10^{-1} \) to \( 10^{-9} \). SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
of the removal of CBMs from native enzymes sometimes resulted in an almost complete loss of the original activity even against soluble substrates (Burstein et al., 2009). Moreover, the structural domain of S-layer homology (SLH) was also found in CBPs 1, 2 and 4 (Appendix A Fig. S1). This domain could combine with N-acetylglucosamine, N-acetylmannosamine and pyroracemic acid of a secondary cell wall polymer, thus resulting in the enhancement of the adhesion of protein on cell surface (Mesnage et al., 1999). The presence of SLH-binding components with different specificities may probably influence the possibility for the bacterium to target exocellular proteins to different locations on the bacterial cell surface (Chauvaux et al., 1999).

The structural domains of GH9 were also found existing in CBPs 5 and 6. Previous reports indicated that enzymes containing this kind of catalytic domain (GH9) also have the abilities of secreting endoglucanase and cellobiase (Kanafusa-Shinkai et al., 2013). Such enzymes are thought to be endoglucanase with the highest activities in cellulosome secreted by Clostridium sp. (Garcia-Campayo and Béguin, 1997) which was consistent with our findings. For example, as shown in Fig. 1b, band 6 revealed apparent CMCase activity, and the corresponding protein was found to contain the catalytic domains of GH48. It has been suggested that proteins containing this domain have the abilities of producing exo-1, 4-β-glucanase and cellobiase (Sukharnikov et al., 2012). The enzymatic components of GH48 were found in almost all bacterial cellulase systems and widely regarded as the key components of bacteria for the effective degradation of cellulosic materials (Irwin et al., 2000). In this study, the combined dilution-to-extinction and proteomic approaches, CBPs 6 and 12 were found to be the key functional proteins secreted from this microbial consortium related to cellulosytic activity. These two proteins have GH9 and GH48 structural domain respectively and both exhibited endoglucanase and xylanase activities. Their coexistence can synergistically promote the degradation of lignocellulosic materials and has been observed previously (Izquierdo et al., 2010; Zhang et al., 2010).

Structural domain analysis on the other hand revealed that CBP10 containing a catalytic domain belongs to glycoside hydrolase family 8 (GH8), a family that displays an (α/α)6 fold (clan GH-M) and consists of some of the known enzymes like cellulases, lichenases, chitosanases and a number of other xylanases (Collins et al., 2005). Finally, the existence of dockerin protein in CBPs 5–12 indicated that enzymes secreted in this microbial consortium might be an extracellular macromolecular multi-enzyme complex, termed the cellulosome, which efficiently coordinated degradation of crystalline cellulose. Dockerin modules of cellulosomes are known to mediate the assembly of the various catalytic subunits onto one scaffoldin subunit thereby allowing synergistic breakdown of cellulose (Bayer et al., 1998). These proteins secreted from the cellulosolytic microbial consortium could accelerate the degradation of lignocellulosic materials.

In the present study, the CBPs were only purified from the supernatant of the degradation media. However, based on observations discussed, it could be suggested that the effective degradation of cellulose by this microbial consortium was mainly through formation of a multi-enzyme complex (i.e. cellulosome). These multi-enzyme complexes were found distributed on the surfaces of the corresponding microbes which then tightly adhered to the surfaces of the lignocellulosic substrates (such as cassava residues and filter paper). Thus, to fully explore their nature, it would be essential to collect these proteins from the surface of the substrates and further investigate their influences on cellulose degradation. Purification of CBPs from both the supernatants and substrates, combined with the dilution-to-extinction approach will generate more information to help understand the detailed underlying degradation mechanisms of the cellulosolytic microbial consortium.

3. Conclusions

In this study, affinity digestion was successfully utilized to separate and purify the CBPs from a previously constructed...
cellulolytic consortium. Eight proteins known to be secreted by Paenibacillus sp. and C. clariflavum DSM 19732 were also identified through mass spectrometry analysis. In addition, The combination of SDS-PAGE, zymogram analysis and dilution-to-extinction approach revealed the presence of CBPs 6 and 12 (belonging to GH9 and GH48 respectively) similar to those produced by C. clariflavum DSM 19732. These proteins may be playing key roles in the effective degradation of cellulose in the microbial consortium, and specifically the endoglucanase and xylanase produced by these two proteins can synergistically promote the degradation of cellulose.

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