Int J Biochem Mol Biol 2011;2(2):146-154
www.ijbmb.org /ISSN:2152-4114/IJBMB1104001

Original Article

Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers

Miranda L Maki[1,2], Michael Broere[2], Kam Tin Leung[2], Wensheng Qin[1,2]

[1]Biorefining Research Initiative, Lakehead University, 955 Oliver Rd, Thunder Bay, Ontario P7B5E1, Canada; [2]Department of Biology, Lakehead University, 955 Oliver Rd, Thunder Bay, Ontario P7B5E1, Canada.

Received April 1, 2011; accepted April 15, 2011; Epub April 19, 2011; Published April 30, 2011

Abstract: The wide variety of bacteria in the environment permits screening for more efficient cellulases to help overcome current challenges in biofuel production. This study focuses on the isolation of efficient cellulase producing bacteria found in organic fertilizers and paper mill sludges which can be considered for use in large scale biorefining. Pure isolate cultures were screened for cellulase activity. Six isolates: S1, S2, S3, S4, E2, and E4, produced halos greater in diameter than the positive control (*Cellulomonas xylanilytica*), suggesting high cellulase activities. A portion of the 16S rDNA genes of cellulase positive isolates were amplified and sequenced, then BLASTed to determine likely genera. Phylogenetic analysis revealed genera belonging to two major Phyla of Gram positive bacteria: Firmicutes and Actinobacteria. All isolates were tested for the visible degradation of filter paper; only isolates E2 and E4 (*Paenibacillus* species) were observed to completely break down filter paper within 72 and 96 h incubation, respectively, under limited oxygen condition. Thus E2 and E4 were selected for the FP assay for quantification of total cellulase activities. It was shown that 1% (w/v) CMC could induce total cellulase activities of 1652.2±61.5 and 1456.5±30.7 μM of glucose equivalents for E2 and E4, respectively. CMC could induce cellulase activities 8 and 5.6X greater than FP, therefore CMC represented a good inducing substrate for cellulase production. The genus *Paenibacillus* are known to contain some excellent cellulase producing strains, E2 and E4 displayed superior cellulase activities and represent excellent candidates for further cellulase analysis and characterization.

Keywords: Biodegradation, cellulase-producing bacteria, firmicutes, actinobacteria

Introduction

Increasing demand and the rising cost of fossil fuels, as well as a concern for global climate change have shifted global efforts to utilize renewable resources for the production of a 'greener' energy replacement [1]. Lignocellulosic biomass, ('plant biomass'), is a renewable, abundant and inexpensive resource for the bioconversion to biofuels and bioproducts. It is comprised of mainly cellulose, a homologous polymer of glucose molecules connected by β-1,4 linkages (the most abundant organic polymer in the world). It also contains some hemicellulose (a heterologous polymer of 5- and 6-carbon sugars) and even less so lignin (a complex aromatic polymer). Known as abundant, there are a great many sources to derive lignocellulosic biomass from such as municipal waste, agricultural residues, forestry or pulp and paper excesses, and, energy crops (i.e. Switchgrass) [2].

Several microorganisms including both bacteria and fungi have been found to produce a variety of cellulases for the degradation of cellulose. Primarily, cellulases are classified into three main groups: the exoglucanases, endoglucanases (cleaving β-1,4-glycosidic bonds from chain ends and internally within chains, respectively) and β-glucosidases (cleave the final β-1,4 linkage of cellobiose or small polysaccharides) [3]. Bacteria and fungi have been found to produce and secrete these enzymes freely in solution; however, some microorganisms have also been found to produce cell-bound enzymes and multi-protein complexes expressing cellulases and hemicellulases called cellulosomes. The

cellulosome was first discovered in 1983 from the anaerobic, thermophilic spore-forming *Clostridium thermocellum* [4].

One major obstacle facing the development of lignocellulosic biofuels is the cellulose hydrolysis stage. Generally speaking, there is a lack of microorganisms which can produce sufficient amounts of all three types of cellulases to efficiently breakdown crystalline cellulose to glucose. Moreover, the biorefining process remains economically unfeasible due to a lack of biocatalysts that can overcome costly hurdles such as cooling from high temperature, pumping of oxygen/stirring, and, neutralization from acidic or basic pH. The extreme environmental resistance of bacteria permits screening and isolation of novel cellulases to help overcome these challenges.

Although molecular engineering is leading researchers in the field of biorefining towards developing microorganisms which can produce a greater number of and more efficient cellulases, the traditional microbiological technique of isolation still plays an important role. New isolates lay the foundation for molecular engineering strategies, perhaps a new cellulase-degrading strain may represent a good host or framework to further improve or add additional enzyme genes for further improvement. Similarly, a cellulase produced by an isolate may be more efficient and may be worth cloning and introducing to an already good industrial cellulase producer to further improve its cellulose-degrading repertoire. Each small step will make biorefining and ethanol production more economically feasible and will help take reliance off of petroleum based fuels and allow progression towards a more renewable fuel source.

In this study, several efficient aerobic cellulase-producing microorganisms were isolated from different pulp and paper mill sludges and one commercial microbially enhanced soil amendment sample. The purpose was to identify and characterize those isolates displaying the greatest cellulase activity for the possible use in large scale biorefining.

Methods

Lignocellulosic samples and media used

The lignocellulosic samples for isolation of cellulase-producing bacteria were obtained from a few sources. Two samples were obtained from the area of Red Rock, Ontario, Canada and were labeled B (black-coloured solid sludge) and W (wood-like solid sludge). Both B and W were dry, aged waste products leftover from pulp and paper mill processing. The exact stage and treatment of the sludge is not known. Additionally, a sludge material (S) produced from the kraft processing of fine paper was obtained from a paper mill in Thunder Bay, Ontario, Canada. The center of the sludge sample displayed microbial activity which was observed by a change in colour of the sludge from white to grey. Finally, a commercial fertilizer was analyzed which is called Efficient Microorganism Dust (E).

The growth media used in the experiments include R2A agar (0.5g l^{-1} yeast extract, 0.5g l^{-1} protease peptone, 0.5g l^{-1} casamino acids, 0.5g l^{-1} glucose, 0.5g l^{-1} soluble starch, 0.3g l^{-1} dipotassium phosphate, 0.5g l^{-1} magnesium sulfate $7H_2O$, 0.3g l^{-1} sodium pyruvate, 15.0g l-1 agar), LB liquid media (10.0g l^{-1} peptone, 5.0g l^{-1} yeast extract, 5.0g l^{-1} NaCl), and carboxymethyl cellulose agar (0.5g CMC, 0.1g $NaNO_3$, 0.1g K_2HPO_4, 0.1g KCl, 0.05g $MgSO_4$, 0.05g yeast extract, 1.5g agar, per 100 ml ddH_2O) [5].

Isolation of bacteria from lignocellulosic samples using R2A

To isolate bacteria, 1 g of each sample was suspended in 20 ml of sterile potassium phosphate buffer solution (PBS) by vortexing for 2 min on maximum speed. Following, a 10X serial dilution of the suspension was made in PBS. Thereafter, 200µl of each dilution in the series was spread onto the surface of R2A agar using the standard spread plate technique. All plates were incubated at 28°C for 24 h before sampling and then they were incubated for an additional 48 and 72 h to allow growth of slower growing microorganisms for further sampling. From the growth observed over 24, 48 and 72 h, various colonies were selected based on their morphology, size and colour. The colonies selected were then streaked out on separate R2A plates to ensure purity. Colonies were further subcultured on R2A if more purification was required. After purification, the cultures were compared visually to eliminate those of similar size, morphology and colour. The plates were then photographed and described for a database

(database not shown here).

Screening for carboxymethyl cellulose activity

Isolates were grown in 10 ml of LB broth for 24 h, shaking at 28°C, slower growing isolates were left to incubate for an additional 48 h. The positive control used was *Cellumonas xylanilytica.* This strain was also grown in the LB medium; however it required incubation for a 5 day period using the same growth conditions. The negative control used was *Escherichia coli* JM109, also grown in LB broth overnight; however, it grows at 37°C for 18 h. All resulting broth cultures (isolates, positive control and negative control) were tested for cellulase activity via the Gram's iodine method [5]. In this method, 5µl of each broth culture were singly dropped onto a plastic Petri dish containing carboxymethyl cellulose (CMC) agar and then incubated for 48 h at 28°C. The positive control was incubated for an additional 60 h longer than the isolates and negative control due to its slow growth rate. After the allotted growth time, the CMC agar plates with the isolates and controls were stained with Grams iodine solution (2.0g KI and 1.0g I, per 300ml ddH_2O) to visualize the cellulase activity. This solution stains the agar containing CMC brown and leaves areas without CMC clear, described here as halos. The appearance of clear halos around the drops confirms cellulase activity by the bacteria. Each plate was flooded completely with approximately 5 ml of the Grams iodine solution using a Pasteur pipette. The plates were allowed to sit for 5 minutes until the dye settled into the media and then they were photographed for a database not shown here. The cellulase positive isolates were then re-grown in LB broth and drop plated onto smaller CMC plates (50mm × 9mm) using the same techniques and conditions previously described, shown in **Figure 1**. From the new, smaller, CMC plates, the halo diameters were measured using a ruler for a semi-qualitative comparison of cellulase activity among the isolates. The halo measurement is used to relate cellulase activity to bacteria position on the phylogenetic tree as shown in **Figure 2**.

DNA isolation and 16S rDNA amplification

The cellulase producing isolates as well as the positive control were grown up in LB broth for 24 h at 28°C. DNA was isolated from each isolate broth culture using the Fungi/Yeast Genomic DNA Isolation Kit from Norgen Biotek Corporation, Canada. The resulting isolated DNA was used as a template in a PCR reaction to amplify a region of the 16S rDNA. Universal primers designed within conserved regions of the 16S rDNA for Eubacteria were used: HAD-1 (5´-GACTCCTACGGGAGGCAGCAGT) and E1115R (5´-AGGGTTGCGCTCGTTGCGGG), they amplify approximately a 796 bp fragment [6]. The PCR reaction mixtures contained 10 ng of genomic DNA individually from each positive isolate, 10 pmol of both forward and reverse primers, 10x Taq buffer with KCl, 25 mmol l^{-1} $MgCl_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 U DNA polymerase per 50 µl reaction. The PCR program was as follows: primary denaturation 3 minutes at 95°C, followed by 33 amplification cycles consisting of denaturing at 95°C for 1 minute, annealing for 1 minute at 63°C, and extension at 72°C for 1 minute, upon completion of 33 amplification cycles a final extension step was done at 72°C for 10 minutes. The PCR products were then viewed on a 1% agarose gel to confirm size, quantity and purity. Then, PCR products were sequenced using standard run modules on the ABI 3730xl automatic sequencer (Eurofins MWG Operon, Canada).

Isolate Identification and Relatedness

Sequencing results were individually inputted online into the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov/) to identify the possible genera of the isolates. Sequencing results of the isolates and positive control were also inputted into a sequence alignment program called ClustalX to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA algorithm, which considers the rate of evolution to be constant between species, to develop a phylogenetic tree based on sequence homology. The resulting alignment was opened into a program called TreeView which allowed the phylogenetic tree to be viewed.

Qualitative filter paper activity

Isolates displaying cellulase activity on the CMC plates were further screened for quality of cellulase activity by transferring 100 µl of an overnight culture to 5 mL of Dubois salts media (K2PO4 1g l^{-1}, KCl 0.5 g l^{-1}, MgSO4 0.5g l^{-1}, NaNO3 0.5g l^{-1}, FeSO4 0.01 g l^{-1}, pH 7.4) with a 7 mm wide strip of filter paper (FP) and two drops of 10 mM glucose in glass culture tubes.

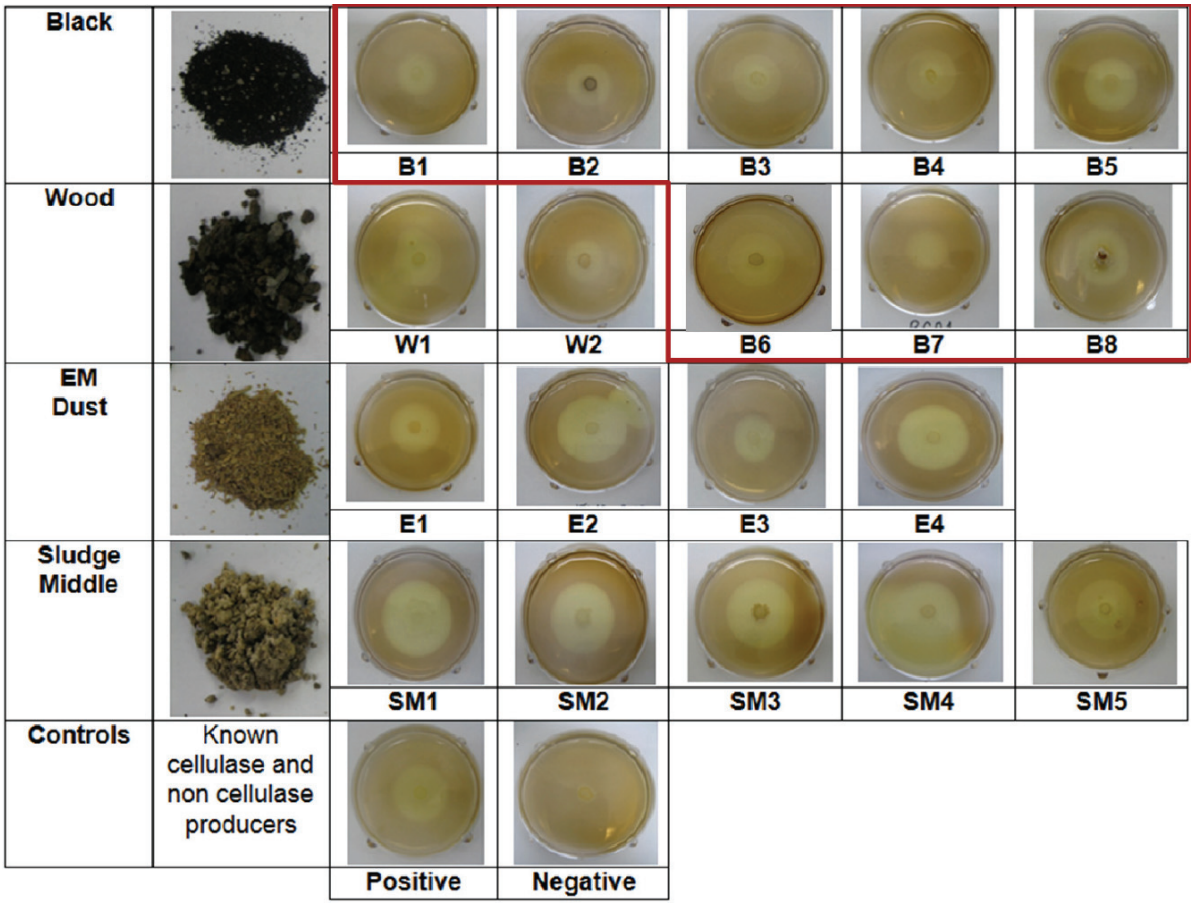

Figure 1. Nineteen cellulase-producing isolates grouped based on the samples they were derived from, and a positive and negative control, *C. xylanilytica* and *E. coli* JM109, respectively.

The cultures were incubated for a maximum of 10 days and viewed daily for visual evidence of filter paper degradation. Those strains capable of completely degrading the filter paper within 96 h were selected for further quantitative analysis. The test was done using aerobic culture techniques and repeated under limited oxygen conditions by sealing the tubes with parafilm.

Total cellulase activity assay

Two isolates (E2 and E4), displaying the greatest cellulase activity qualitatively were selected for further study and quantification of total cellulase activity. Isolate E1 and E2 were grown as similarly described in 5 mL of Dubois salts media with FP (1%, w/v) or CMC (1%, w/v) in glass culture tubes, under limited oxygen conditions. The cultures were incubated for 48 h and cellulase activities were measured. A microplate-based filter paper assay using the DNS method to measure reducing sugars, modified from Xiao *et al.* 2004 [7], was used to measure the total cellulase activity for the four isolates displaying the highest cellulolytic activity. Modifications included the use of 50mM TrisHCl buffer, pH 7 in place of 50 mM NaAc buffer, pH 4.8. Bacterial enzymes do not work efficiently at such low pH. Additionally, the enzymes with the buffer and filter paper substrate were allowed to incubate at 50°C for 2 h instead of 1 h, due to the known smaller quantities of enzymes produced by bacteria.

Results

Carboxymethyl cellulase activity

A total of 53 isolates were described based on

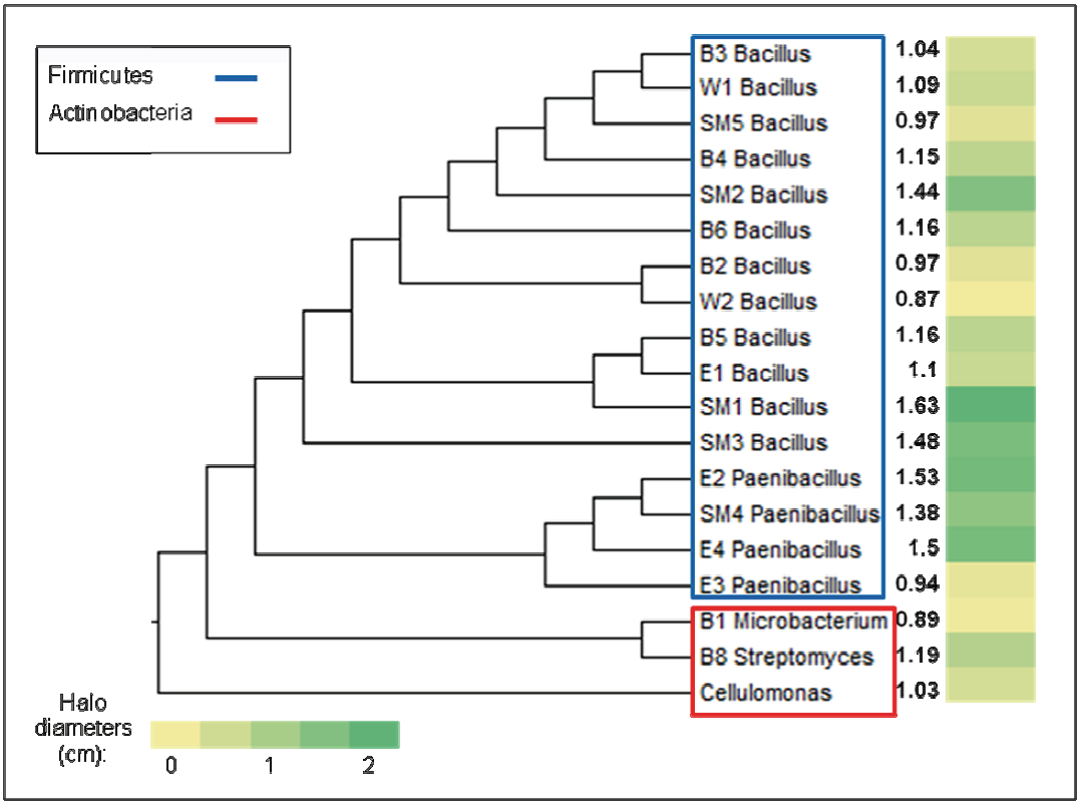

Figure 2. Phylogenetic tree produced from the alignments of 16S rDNA fragments from the isolates, presented in TreeView. Closer related isolates outlined in blue belong to the phylum Firmicutes and the most distantly related isolates outlined in red belong to the phylum Actinobacteria. The diameter of halos the isolates produced on CMC agar is respectively shown with a colour scale indicating small to large halos, qualitative cellulase activity.

size, colour, and morphology, labeled and photographed for a database (not shown here). From the database 30 of 53 isolates were removed due to similar size, colour and morphological characteristics. The resulting 23 isolates were then tested on CMC agar for cellulase activity; 19 of the 23 isolates exhibited cellulase activity and are shown in the photographs of **Figure 1** along with positive (*Cellulomonas xylanilytica*) and negative (*E. coli* JM109) controls. The following 6 cellulase-producing isolates had the greatest halos after 48h incubation on CMC agar: S1, S2, S3, S4, E2, E4 (**Figure 1**). The halos were measured in centimeters using a standard ruler. The diameters of the halos can be seen in **Figure 2** plotted beside each genus in the phylogenetic tree.

16S rDNA amplification

Genomic DNA was successfully isolated from all 19 cellulase-producing isolates using Gram positive DNA isolation methods. The universal 16S rDNA primers were used in conjunction with PCR to successfully amplify 16S rDNA gene fragments from all 19 isolates. Confirmation of the 16S rDNA gene fragments was validated by a band on a 1% agarose gel with an approximate expected size of 800 bp.

Sequencing and sequence analysis of 16S rDNA PCR products

Sequencing results were successfully obtained for all 19 different 16S rDNA PCR products. The resulting sequences were inputted to the nucleotide blast feature of the NCBI database to obtain possible identities based on homology. From BLAST search results, genera of all 19 isolates were determined based on 97-99% homology. The nucleotide BLAST results are shown in **Table 1**. The majority of sequences

Table 1. The BLAST search results for the sequenced cellulase producing isolates

Isolate	Homology (%)	Genus
B1	99	*Microbacterium*
B2	99	*Bacillus*
B3	99	*Bacillus*
B4	99	*Bacillus*
B5	98	*Bacillus*
B6	99	*Bacillus*
B7	99	*Streptomyces*
E1	99	*Bacillus*
E2	98	*Paenibacillus*
E3	99	*Paenibacillus*
E4	98	*Paenibacillus*
SM1	97	*Bacillus*
SM2	98	*Bacillus*
SM3	98	*Bacillus*
SM4	99	*Paenibacillus*
SM5	97	*Bacillus*
WC1	99	*Bacillus*
WC2	98	*Bacillus*

yielded 99% homology in nucleotide database with very few as low as 97% homology. All 19 isolates belong to genera of Gram positive bacteria, several of which were shown to belong to the genus *Bacillus* and *Paenibacillus*, while one strain from the genus *Microbacterium* and *Streptomyces* were also found (**Table 1**).

Phylogenetic analysis of 16S rDNA sequences

The sequences were then inputted into a sequence alignment program called ClustalX. An alignment was then done using UPGMA algorithm which finds the relatedness between the isolates assuming that the rate of evolution is constant. The aligned sequences were then uploaded into a program called TreeView which allows us to view the phylogenetic tree produced from the alignment information using the UPGMA algorithm. The phylogenetic tree displays two main groups of Gram positive bacteria; the Firmicutes (blue) more closely related than the Actinobacteria (red). The Firmicutes are made up of *Bacillus* and *Paenibacillus* sp., while the Actinobacteria include the genera *Streptomyces, Microbacterium* and positive control *Celluomonas xylanilytica.* The yellow to green colour legend represents a visual of the halo diameter (cm) from smallest to greatest halo, respectively. Data was collected from the CMC plates. The *Paenibacillus* sp. all have relatively larger halos and the *Bacillus* sp. has halos of varying sizes. The Actinobateria exhibits a variety of halo sizes and *Streptomyces* seems to be the greatest producer of cellulases in this Phylum (**Figure 2**).

Filter paper activity

All of the positive cellulase-producing isolates were grown with FP as a sole carbon with one drop of 10 mM glucose to possibly induce cellulase production, for qualitative observation of filter paper activity. This was done in both aerobic and oxygen limited environments. The ability to degrade filter paper, more than likely represents the production of more than one type of enzyme and the ability to degrade crystalline cellulose, thereby being a more efficient cellulase-producing isolate. The following two strains: E2 and E4 were found to completely degrade the filter paper cellulose in 72 and 96 h incubation, respectively, as can be seen in **Figure 3**.

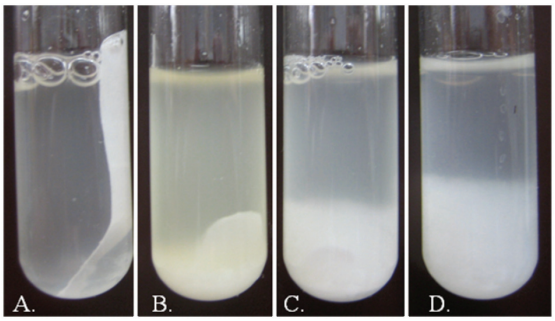

Figure 3. Qualitative results for the 2 isolates capable of completely degrading filter paper within 96 h incubation: **A**) Negative control (*E. coli* JM109), **B**) Positive control (*C. xylanilytica*), **C**) *Paenibacillus* E2 and D) *Paenibacillus* E4.

Total cellulase activity

The total cellulase activity is determined by the amount of glucose and cellobiose released from filter paper after 2 h incubation and is referred to in glucose equivalents. Similarly, the CMCase activity is also a measure of the glucose and cellobiose released however after 20 min incubation; it is also referred to as glucose equivalents. The activity for total cellulases was evaluated for whole cells of *Paenibacillus* sp. E2 and E4. It was found that using CMC as the culture carbon substrate, E2 and E4 total cellulase activity was 1587.0±215.2 and 1652.2±61.5 μM

Table 2. Total cellulase activity of *Paenibacillus* sp. E2 and E4 shown in glucose equivalents (mM) after 48 h growth with CMC (1%,w/v)

	Total Cellulase Activity (glucose equivalents (μM))	
Substrate (1%, w/v)	*Paenibacillus* E2	*Paenibacillus* E4
Carboxymethyl cellulose	1652.2 ±61.5	1456.5±30.7
Filter paper	202.9±66.4	260.9±157
Quotient (CMC/FP)	8.143±0.36	5.581±0.13

of glucose equivalents, respectively, not significantly different. However, when FP was used as the cellulase inducing substrate, total cellulase activity of E2 and E4 was 202.9±66.4 and 260.9±157 μM of glucose equivalents. No significance difference in total cellulase activity was observed between E2 and E4; however, CMC induced greater activity. The quotient of CMC to filter paper was 8.143±0.36 and 5.58±0.13 for E2 and E4, respectively (**Table 2**).

Discussion

Several isolates could be recovered by aerobic spread plates from the different industrial and commercial samples. For those isolates displaying cellulase activity on the CMC containing plates four different genera of isolates including *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Streptomyces* species were found. According to our phylogentic analysis, these bacteria can be grouped into two main Phyla based on sequence homology: Actinobacteria and Firmicutes. Both Phyla consist of Gram positive bacteria distinguished by high and low GC content, respectively; and both groups of bacteria contain species capable of degrading organic materials. Thus, it is not surprising that many of the genera can produce cellulases. Several strains of *Paenibacillus*, *Bacillus, Microbacterium* and *Streptomyces* have been found to produce cellulases and their cellulases have been well studied; these strains represent important cellulase degrading genera.

For example, researchers have characterized a novel endoglucanase (Cel9P) from a newly isolated *Paenibacillus* sp. BME-14. Endoglucanase Cel9P displayed 65% of its maximal activity at 5°C, which could be beneficial for some industries which have processes at lower temperatures [8]. Similarly, in other newly isolated *Paenibacillus* sp., multienzyme complexes called cellulosomes have been characterized in the degradation of lignocellulosic substrates [9-11]. Cellulosomes, such as the cellulosome of *Clostridium thermocellum* can have high efficiency for the degradation of crystalline celluloses, higher than that of *Trichoderma reesei* [12]. In addition, cellulases have also been well characterized in *Bacillus* species; most recently a unique *Bacillus* sp. was observed to maintain up to 70% stable CMC activity at a range of pH 6-8 [13]. Similarly, many of the modular enzymes present in *Paenibacillus* are also present in *Bacillus* species [10].

Moreover, *Microbacterium* sp. displaying cellulase activities have been isolated from a variety of environmental samples and uniquely this strain has been isolated from the gut of termites [14]. One *Microbacterium* sp. exhibited particularly high filter paper activity and xylanase activity when a consortium of aerobic cellulase producing bacteria was studied [15].

Additionally, *Streptomyces* sp., have also been previously studied by researchers for cellulase production and found to produce a variety of unique cellulases including some of which were found to be thermoalkotolerant [16,17]. Also interestingly, *Streptomyces* sp. has been used in successful co-culturing trials. They have been found to work synergistically with *Thermomonospora fusca* and *Trichoderma reesei* to degrade cellulose [18].

All of the cellulase producing bacteria isolated and identified in this study have potential for further use and study, such as looking at individual enzyme activities to isolate efficient or novel cellulases with unique characteristics, or potential to use the strains to create microbial consortia with a high efficiency for degrading complex cellulose containing biomass such as lignocellulose.

Evaluating cellulase production between isolates can be a challenge because bacteria produce multiple types of cellulases (endoglu-

canase, exoglucanase, and b-glucosidase), which can be found to exist as free extracellular enzymes as well as found in enzyme complexes or cellulosomes expressed on the cell membrane [19]. Thus, we initially use qualitative tests such as the CMC test and filter paper degradation test. CMC agar allows us to identify isolates with cellulase activity on soluble cellulose such as CMC thus representing mainly endoglucanase and beta-glucosidase activities [5]. Secondly, we then screened isolates displaying cellulase activity on CMC for activity on crystalline insoluble cellulose such as filter paper. Due to the crystalline structure of filter paper, degradation of the filter paper would imply multiple cellulase activities including exoglucanase activities because these enzymes work in crystalline regions [20]. From these tests we could select isolates displaying the greatest activity based on ability to degrade soluble and crystalline cellulose for quantitative analysis of FP activity. All isolates may vary in growth properties which would not allow us to easily compare and quantify cellulase activities of all cellulase positive isolates in an equal manner. Difficulty also arises because some strains may secrete enzymes to solution while others may harbor enzymes on the cell surface or internally, and still some cellulase may end up in solution from cell lysis [21]. Additionally, some bacteria grow more rapidly than others, while still; cellulase production may be induced by different substrates for varying species. Thus, using qualitative screening methods is essential to narrow down isolates which may be more unique for further cellulase study in the future.

Narrowing down our isolates led us to the greatest cellulase producers, *Paenibacillus* sp. E2 and *Paenibacillus* sp. E4 for further analysis. These isolates displayed some of the greatest halos on CMC agar (**Figure 1**) and were the only strains capable of completely degrading filter paper after 72 and 96 h incubation, respectively, under oxygen limited conditions (**Figure 3**); qualitatively speaking cellulase activity in these strains was greater than the positive control. The cellulase activity could be further studied under facultative anaerobic conditions. Similarly, researchers have shown that under anaerobic conditions, *Paenibacillus* species will exhibit high levels of xylanases which can degrade xylan, a more branched portion of the cell wall [11].

Focusing on *Paenibacillus sp.* E2 and E4 for further study, it was shown that after 48 h shaking incubation in oxygen limited condition with CMC and FP as the cellulase inducing substrate isolate E2 displayed total cellulase activities of 1587.0±215.2 and 202.9±66.4 μM of glucose equivalents, respectively. Similarly, isolate E4 displayed total cellulase activity of 1652.2±61.5 and 260.9±157 μM of glucose equivalents for CMC and FP, respectively. Results show that 48h growth with CMC can induce more cellulases than 48 h growth with FP by approximately 8.1X and 5.5X for E2 and E4, respectively. There was no difference in total cellulase activity for either cellulosic substrate when whole cells were used versus when cells were lysed, thus data were not shown. This may suggest a high amount of cellulases are on the outside surface of the cells or secreted in the medium but not internalized.

Our results show that *Paenibacillus* species E2 and E4 may be good potential candidates for biorefining and the ultimate production of bioethanol and additional value-added bioproducts such as organic acids. These isolates are of particular interest because cellulase activities were higher and comparable to the well-known positive control, *Cellulomonas xylanilytica*. Future work will be done on E2 and E4 to optimize cellulase production and evaluate individual cellulase activities to look for novel cellulases.

According to this study, our isolation, screening and identification methods were quick and efficient for allowing us to identify several good cellulase producing bacteria from a wide variety of samples. Moreover, we were able to distinguish the isolates displaying the greatest cellulase activity for future study. Finding naturally occurring cellulase degrading bacteria from the environment is important in the field of biorefining to help overcome costly hurdles in the biorefining process. All of our cellulase positive isolates may be an integral part of future work to develop good cellulases or produce efficient cellulase producing systems such as microbial consortia which can be used for industry. Isolation and characterization may provide a good starting point for the discovery of such beneficial enzymes.

Please address correspondence to: Wensheng Qin, PhD, Biorefining Research Initiative, Lakehead University, 955 Oliver Rd, Thunder Bay, Ontario P7B5E1, Canada.Tel: (807) 343-8840, Fax: (807) 346-7796, Email: wqin@lakeheadu.ca

References

[1] Schneider SH. The greenhouse effect: science and policy. Science 1989;243:771-781.

[2] Greene N. Growing energy. How biofuels can help end America's oil dependence. Nat Res Def Council Rep 2004:1-86.

[3] Davies G, Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 1995;3:853-859.

[4] Lamed R, Setter E, Kenig R, Bayer EA. The cellulosome: A discrete cell surface organelle of clostridium thermocellum which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol Bioeng 1983;13:163 -181.

[5] Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol 2008;57:503-507.

[6] Giannino ML, Aliprandi M, Feligini M, Vanoni L, Brasca M, Fracchetti F. A DNA array based assay for the characterization of microbial community in raw milk. J Microbiol Methods 2009;78:181-188.

[7] Xiao Z, Storms R, Tsang A. Microplate-based filter paper assay to measure total cellulase activity. Biotechnol Bioeng 2004;88:832-837.

[8] Fu X, Liu P, Lin L, Hong Y, Huang X, Meng X, Liu Z. A novel endoglucanase (Cel9P) from a marine bacterium Paenibacillus sp. BME-14. Appl Biochem Biotechnol 2010;160:1627-1636.

[9] Waeonukul R, Kyu KL, Sakka K, Ratanakhanokchai K. Isolation and characterization of a multienzyme complex (cellulosome) of the Paenibacillus curdlanolyticus B-6 grown on Avicel under aerobic conditions. J Biosci Bioeng 2009;107:610-614.

[10] Pastor FI, Pujol X, Blanco A, Vidal T, Torres AL, Diaz P. Molecular cloning and characterization of a multidomain endoglucanase from Paenibacillus sp BP-23: evaluation of its performance in pulp refining. Appl Microbiol Biotechnol 2001;55:61-68.

[11] Pason P, Kosugi A, Waeonukul R, Tachaapaikoon C, Ratanakhanokchai K, Arai T, Murata Y, Nakajima J, Mori Y. Purification and characterization of a multienzyme complex produced by Paenibacillus curdlanolyticus B-6. Appl Microbiol Biotechnol 2010;85:573-580.

[12] Ng TK, Zeikus JG. Comparison of Extracellular Cellulase Activities of Clostridium thermocellum LQRI and Trichoderma reesei QM9414. Appl Environ Microbiol 1981;42:231-240.

[13] Yang D, Weng H, Wang M, Xu W, Li Y, Yang H. Cloning and expression of a novel thermostable cellulase from newly isolated Bacillus subtilis strain I15. Mol Biol Rep 2010;37:1923-1929.

[14] Wenzel M, Schonig I, Berchtold M, Kampfer P, Konig H. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite Zootermopsis angusticollis. J Appl Microbiol 2002;92:32-40.

[15] Okeke BC, Lu J. Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. Appl Biochem Biotechnol 2010;163:869-881.

[16] George J, Arunachalam R, Paulkumar K, Wesely EG, Shiburaj S, Annadurai G. Characterization and phylogenetic analysis of cellulase producing Streptomyces noboritoensis SPKC1. Interdiscip Sci 2010;2:205-212.

[17] Alani F, Anderson WA, Moo-Young M. New isolate of Streptomyces sp. with novel thermoalkalotolerant cellulases. Biotechnol Lett 2008;30:123-126.

[18] Jung ED, Lao G, Irwin D, Barr BK, Benjamin A, Wilson DB. DNA sequences and expression in Streptomyces lividans of an exoglucanase gene and an endoglucanase gene from Thermomonospora fusca. Appl Environ Microbiol 1993;59:3032-3043.

[19] Maki M, Leung KT, Qin W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 2009;5:500-516.

[20] Dashtban M, Maki M, Leung KT, Mao C, Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 2010;30:302-309.

[21] Berg B. Cellulase location in Cellvibrio fulvus. Can J Microbiol 1975;21:51-57.