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Newly Isolated and Characterized Bacteria with Great Application Potential for Decomposition of Lignocellulosic Biomass

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Key Words
Biodegradation · Lignocellulose-producing bacteria · Xylanase · Lignase · *Bacillus* · *Pseudomonas*

Abstract
This study focuses on the isolation and characterization of bacteria from municipal waste and peat to determine those bacteria with good potential for modification and decomposition of lignocellulosic biomass for industrial application. Twenty cellulase-producing bacteria belonging to four major phyla – Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes – were found when screened on carboxymethyl cellulose-containing agar. Six isolates also exhibited activities towards filter paper as the sole carbon source in salt media, while 12 exhibited activities towards xylan when screened on xylan-containing plates. Moreover, 5 isolates survived in and increased the absorbance of 1% black liquor in salt media by an average of 2.07-fold after 21 days of incubation. Similarly, these 5 isolates increased the absorbance of 0.1% pure lignin at 280 nm in salt media, indicating modification of lignin. Additionally, the Fourier transform infrared spectroscopy analysis of 1% barley straw treated for 21 days with these 5 strains showed a preference for consumption of hemicelluloses over lignin; however, a change in lignin was observed. A *Bacillus* strain (55S5) and a *Pseudomonas* strain (AS1) displayed the greatest potential for lignocellulose decomposition due to a variety of cellulase activities, as well as xylanase activity and modification of lignin. Several of these isolates have good potential for industrial use in the degradation of lignocellulosic biomass.

Introduction

In recent years, global climate change and rising fuel costs have caused an increased awareness and potential for renewable fuel sources such as biofuels from lignocellulosic biomass [Schneider, 1989]. There are many reports of microorganisms such as bacteria and fungi which possess cellulose hydrolytic activities, the major component of lignocellulosic biomass. However, there are several limitations to this approach; for example, lignocellulosic biomass is also comprised of some hemicelluloses (heterologous polymers of 5- and 6-carbon sugars), the majority of which have a xylan backbone [Scheller and Ulvskov, 2010]. Furthermore, celluloses and hemicelluloses are entrapped by lignin, a more complex aromatic polymer. The combination of mainly these components make up the structure of plant cell walls and provide the plants with structural integrity and protect

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plants from such things as disease, pests, wind and mechanical wounds.

Lignocellulosic biomasses such as agricultural residues and energy crops currently undergo extensive pretreatment using acid hydrolysis and steam or high-temperature treatments to remove lignin and hemicellulose to expose the cellulose for enzymatic hydrolysis [Galbe and Zacchi, 2007]. Thus, microorganisms with abilities to decompose or modify lignin and hemicellulose in addition to cellulose have greater potential in the industrial production of biofuels, as they can help reduce the associative costs with pretreatment.

Researchers have typically focused on one group of enzymes during isolation, such as cellulases, hemicellulases or lignases. For example, white rot fungi are among the greatest microorganisms which can degrade lignin and the most well studied [Otjen and Blanchette, 1982]. However, anaerobic bacterium *Clostridium thermocellum* and aerobic fungi *Trichoderma reesei* are among some of the greatest cellulase-producing microorganisms [Ng and Zeikus, 1981]. Nonetheless, none of these microorganisms are efficient at cellulolytic, hemicellulolytic and ligninolytic activities simultaneously, rendering the opportunity for discovery of better lignocellulase-producing isolates. Here, we define 'lignocellulase producing' as microorganisms which can produce different enzymes such as cellulases, hemicellulases and lignases collectively, for the decomposition of lignocellulosic biomass.

We have focused on the isolation of lignocellulolytic bacteria, i.e. bacteria which produce a greater variety of enzymes including, in our case, cellulases, xylanases and lignases, in the hopes of finding bacteria which can have an overall greater decomposing impact on complex biomass which could thus be potentially applied in industrial practices. Use of such strains in industry could thereby reduce the need for extensive pretreatments or reduce limitation on the types of biomass used for cost-competitive markets.

Fungi and yeasts have frequently been applied in the development of industrial enzymes. However, bacteria have several advantages over the use of such microorganisms, for example, many strains have short generation times and can be easily cultured, making the use of bacteria in the biofuel industry more amiable. Additionally, bacteria also have increased resilience to environmental stresses due to their biochemical versatility (i.e. temperature variations, salinity, oxygen limitation and change in pH) [Daniel and Nilsson, 1998].

Several studies have focused time and again on isolation strategies for targeting bacteria with more specific activities such as efficient cellulase-producing bacteria, yet some studies isolated bacteria with cellulase and xylanase activity, and very few with lignase activities in addition to the previous [Maki et al., 2009, 2011; Sizoza et al., 2011]. In this study, we developed a strategy for finding bacteria which can produce cellulases, xylanases and potential lignases, and therefore, can be more efficient in decomposing lignocellulosic biomass, making them more practical in industrial use.

In this study, several efficient aerobic cellulase-producing microorganisms were isolated from various sites within a municipal landfill and peat core samples from a poor fen. The purpose was to characterize lignocellulosic abilities of all cellulase-positive isolates and identify those isolates displaying the greatest variety of activity towards lignocellulosic biomass for the possibly more practical use in large-scale biorefining.

Results

Carboxymethyl Cellulose Activity

A total of 57 isolates were described based on size, colour and morphology, labelled and photographed for a database (data not shown here). From the database, 25 of 57 isolates were removed due to similar size, colour and morphological characteristics. The remaining 32 isolates were then tested on carboxymethyl cellulose (CMC) agar for cellulase activity; 20 of the 32 isolates exhibited cellulase activity and are shown in the photographs of figure 1 along with positive *(Cellulomonas xylanilytica)* and negative (*Escherichia coli* JM109) controls. The following 7 cellulase-producing isolates had the greatest halos after 48 h of incubation on CMC agar: CDS1B, CDS2B, AS2B, CTS1A, CTS2, GH2OS1 and 6S4 (fig. 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 rRNA Gene Amplification

Genomic DNA was successfully isolated from all 20 cellulase-producing isolates using Gram-positive DNA isolation methods. The universal 16S rRNA gene primers were used in conjunction with PCR to successfully amplify 16S rRNA gene fragments from all 20 isolates. Confirmation of the 16S rRNA gene fragments was validated by a band on a 1% agarose gel with an approximate expected size of 800 bp.

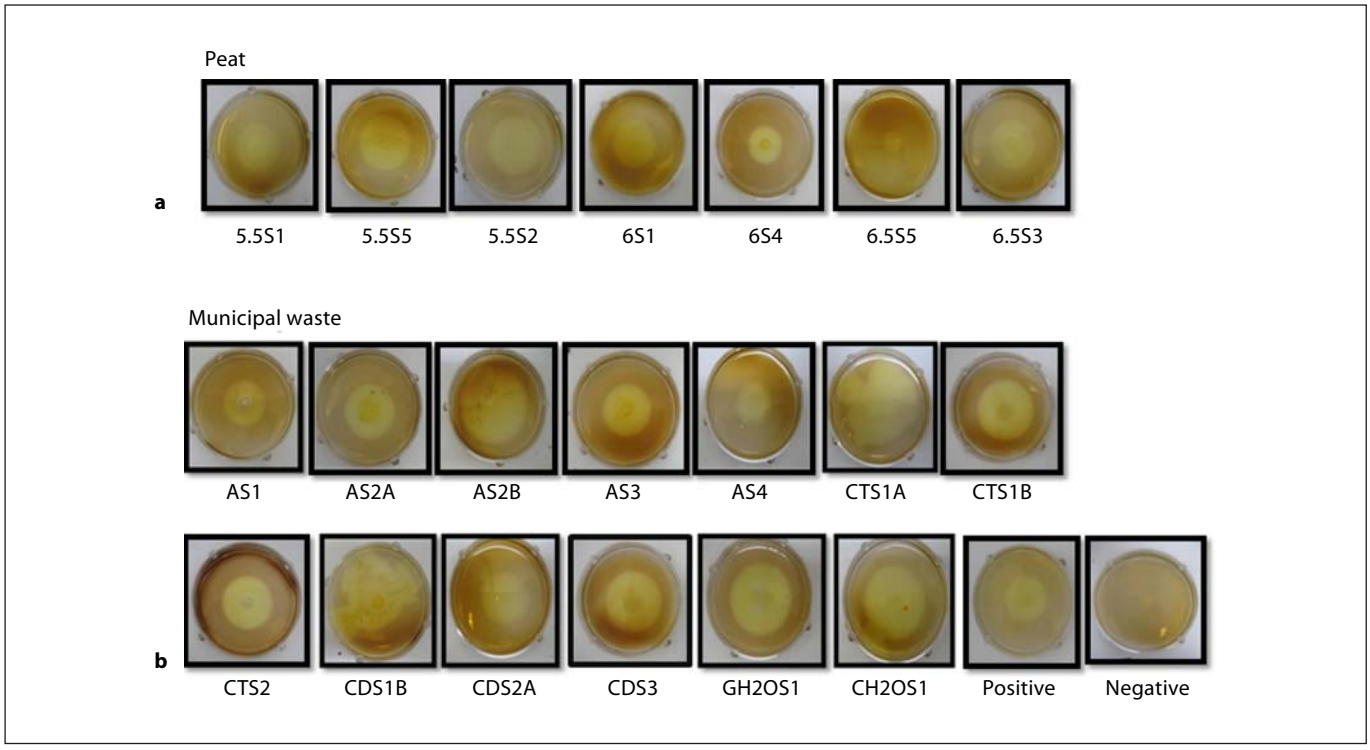

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Fig. 1. Twenty cellulase-producing isolates were grouped based on bacteria isolated from peat (**a**) and derived from municipal waste (**b**). Additionally, a positive and a negative control, *C. xylanilytica* and *E. coli* JM109, are included.

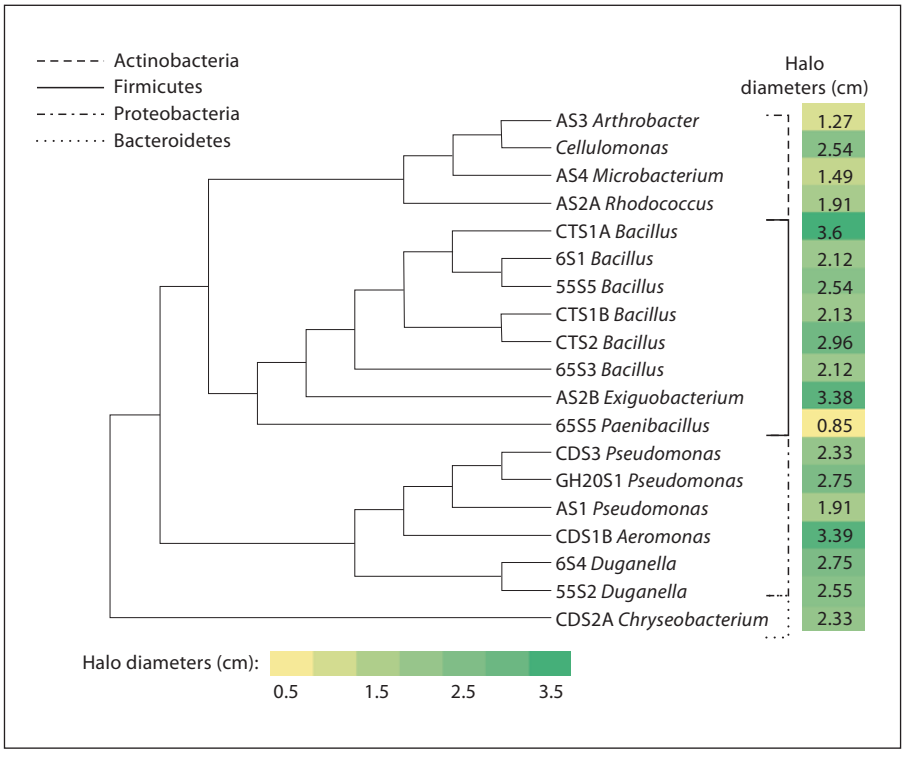

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Fig. 2. Phylogenetic tree produced from the alignments of 16S rRNA gene fragments from the isolates, presented in TreeView. Closer related isolates and their respective phyla are outlined as indicated in the legend. The diameter of the halos which the isolates produced on CMC agar is shown with a colour scale, indicating small to large halos.

Table 1. Likely genera of all cellulase-positive isolates determined by the BLAST search

	Isolate	Identity, %	Likely genus
Municipal waste	AS1	100	*Pseudomonas*
	AS2A	97	*Rhodococcus*
	AS2B	100	*Exiguobacterium*
	AS3	100	*Arthrobacter*
	AS4	100	*Microbacterium*
	CDS1B	99	*Aeromonas*
	CDS2A	97	*Chryseobacterium*
	CDS2B	100	*Bacillus*
	CDS3	100	*Pseudomonas*
	CTS1A	100	*Bacillus*
	CTS1B	100	*Bacillus*
	CTS2	100	*Bacillus*
	GH201	100	*Pseudomonas*
Peat	55S1	99	*Bacillus*
	55S2	98	*Duganella*
	55S5	100	*Bacillus*
	65S3	97	*Bacillus*
	65S5	96	*Paenibacillus*
	6S1	100	*Bacillus*
	6S4	100	*Duganella*

Sequencing and Sequence Analysis of 16S rRNA Gene PCR Products

Sequencing results were successfully obtained for all 20 different 16S rRNA gene PCR products. The resulting sequences were put into the nucleotide BLAST feature of the National Center for Biotechnology Information database to obtain possible identities based on sequence similarity. The genera of all 20 isolates were determined by the BLAST search, based on a high sequence similarity ranging from 96 to 100%. The nucleotide BLAST results are shown in table 1. The majority of sequences yielded a 100% similarity in the nucleotide database, with very few with a sequence similarity as low as 96 and 97%. Many of the 20 isolates belong to genera of Gram-positive bacteria, such as *Bacillus, Paenibacillus*, *Rhodococcus*, *Arthrobacter*, *Exiguobacterium* and *Microbacterium*. Some strains of Gram-negative bacteria were also found, belonging to the genera *Pseudomonas, Aeromonas, Duganella* and *Chryseobacterium* (table 1).

Phylogenetic Analysis of 16S rRNA Gene Sequences

The phylogenetic tree displays two main groups of more closely related Gram-positive bacteria – the Firmicutes and the Actinobacteria – while the more distantly related Gram-negative bacteria were grouped into two main phyla, the Proteobacteria and Bacteroidetes. *Chryseobacterium,* the only genus belonging to the phylum Bacteroidetes, was least related to all of the isolates. The yellow to green colour legend represents the halo diameter (cm) from the smallest to the greatest halo on CMC agar, respectively. In this study, isolates belonging to the phyla Firmicutes and Proteobacteria contain genera of bacteria with relatively larger halos than the genera of bacteria found belonging to the phyla Actinobacteria and Bacteroidetes (fig. 2).

Filter Paper Activity

All of the positive cellulase-producing isolates were grown with filter paper as a sole carbon with one drop of 10 mmol/l 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. The following 6 strains – 6S1, 55S5, AS1, CDS1B, CH2OS1 and CTS1B – were found to completely degrade filter paper cellulose within 96 h of incubation, similar to the positive control, as can be seen in figure 3. Degradation was confirmed by the amount of sugars released into solution after complete degradation for 6S1 *Bacillus*, 55S5 *Bacillus, C. xylanilytica* (+), AS1 *Pseudomonas,* CDS1B *Aeromonas,* CH2OS1 *Bacillus* and CTS1B *Bacillus* and were found to be similar at 782.6 ± 123, 978.0 ± 92, 1,043.5 ± 10, 847.8 ± 31, 913.0 ± 215, 608.7 ± 245, and 1,065 ± 92 nmol of glucose equivalents, respectively. No sugars were detected in the negative control *(E. coli).*

Xylanase Activity

The 20 cellulase-positive isolates were further screened for evidence of hemicellulase activity on xylan-containing media. It was observed that 12 of 20 isolates were able to use xylan as a sole source of carbon by producing halos after staining. Isolates 55S5 *Bacillus,* 6S1 *Bacillus,* CDS2B *Bacillus,* CDS3 *Pseudomonas* and GH2OS1 *Pseudomonas* produced the greatest halo diameters, suggesting a high production of xylanase, as can be seen in figure 4.

Black Liquor Decolourization and Cell Survival

From 20 isolates screened for growth and survival in 1% (w/v) black liquor, a total of 11 isolates were capable of surviving 21 days of incubation. Absorbance at 425 nm allowed observation of change in colour of the black liquor. No isolates were capable of decreasing the absorbance after 21 days. However, 5 strains – AS1 *Pseudomonas,* AS4 *Microbacterium,* 65S3 *Bacillus,* 65S5 *Paenibacil-*

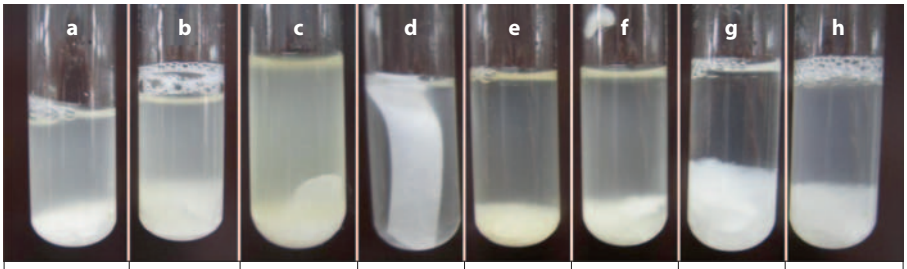

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Fig. 3. Qualitative results for the 6 isolates capable of completely degrading filter paper within 10 days of incubation: 6S1 *Bacillus* (**a**), 55S5 *Bacillus* (**b**), positive control *C. xylanilytica* (**c**), negative control *E. coli* (**d**), AS1 *Pseudomonas* (**e**), CDS1B *Bacillus* (**f**), CH20S1 *Bacillus* (**g**), and CTS1B *Bacillus* (**h**). Data are nmol glucose equivalents after degradation.

lus and CH2OS1 *Bacillus* – were capable of increasing the absorbance of black liquor after 21 days of incubation of an average 2.07-fold greater than the absorbance at day 0. The pH value was recorded after 21 days for the 5 previously mentioned strains, and an increase in colour was related to an increase in pH from 6.5 to 9.5 (fig. 5a). Similarly, these 5 strains could also grow and proliferate, increasing their initial cell densities on average by 0.47-fold and maintaining these cell densities up to 21 days of incubation, as determined by cell survival in black liquor (fig. 5b).

Absorbance of Lignin at 280 nm

Modification of 0.1% (w/v) pure lignin extracted from black liquor was observed by measuring the absorbance at 280 nm after 21 days of incubation for each strain, displaying modification of black liquor: AS1, AS4, 65S3, 65S3 and CH2OS1 (fig. 6). All 5 strains increased the absorbance of lignin at 280 nm compared to the untreated control sample. Isolates AS1 and AS4 were capable of increasing absorbance by 23.8 and 21.8%, respectively, compared to the control, whereas isolate CH2OS1 only increased absorbance by 6.3% compared to the control. The remaining 2 isolates, 65S5 and 65S3, increased absorbance by 13.9 and 11.1%, respectively, compared to the control.

Fourier Transform Infrared Spectroscopy Analysis of Isolates on Barley Straw

The Fourier transform infrared spectroscopy (FTIR) spectrum data were used to analyse the preference of all 5 previously mentioned potential lignin-modifying isolates (AS1, AS4, 65S3, 65S5 and CH2O) for hemicellulose and lignin compared to cellulose, as shown in figure 7. For comparison, the cellulase-positive control *(C. xylanilytica)* which could not survive in black liquor and which displayed minimal hemicellulose activity was used. The results showed that *C. xylanilytica* had no preference for lignin and a 43.2% preference for hemicellulose compared to cellulose, as was expected. Also displaying relatively low preferences for lignin were isolates CH2OS1 and 65S5, with 6.5 and 27.2%, respectively, whereas they preferentially consumed hemicellulose with preferences of 93.2 and 92.7%. In contrast, strains AS1, 65S3 and AS4 had greater preferences for lignin of 56.5, 49.0 and 42.0%, respectively. Additionally, they consumed hemicellulose with greater preferences of 97.6, 68.7 and 61.8%.

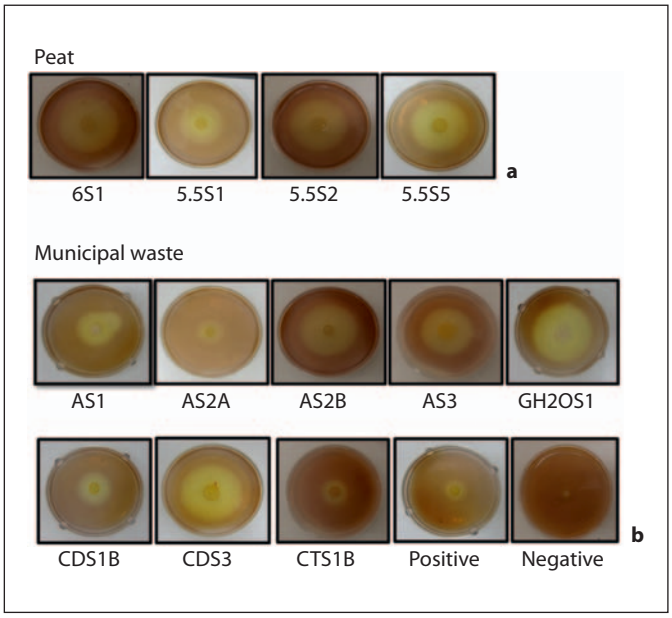

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Fig. 4. Xylanase-positive isolates on agar shown by the appearance of halos after staining with Gram's iodine solution. Grouped into bacteria isolated from peat (**a**) and municipal waste (**b**), including positive and negative controls (*C. xylanilytica* and *E. coli* JM1O9, respectively).

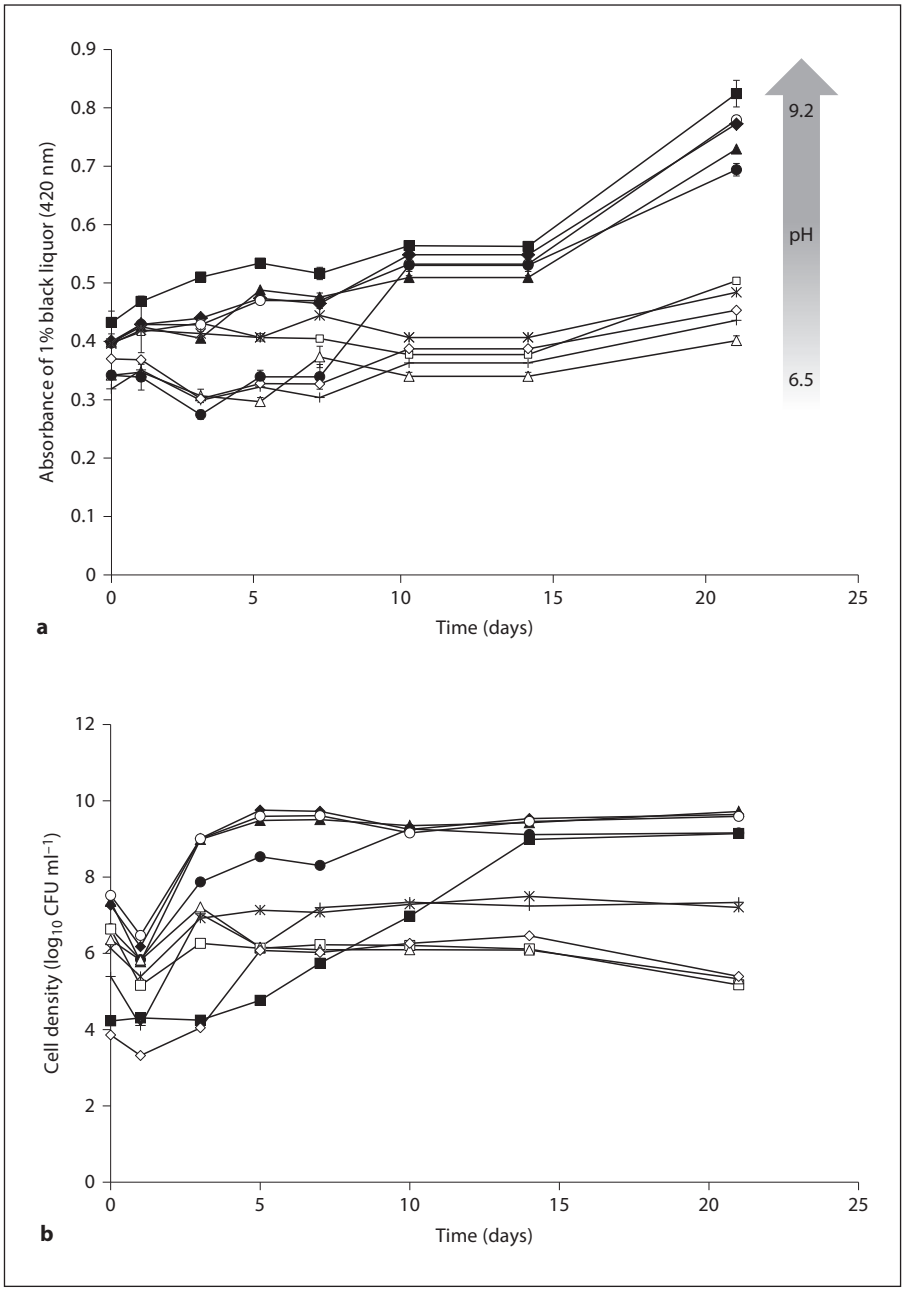

Fig. 5. Change in colour (Abs_{420nm}) of 1% black liquor with recorded final pH (**a**) and survival of all isolates reported as cell density (log CFU ml^{-1}), for all isolates which can tolerate 1% black liquor for 21 days of incubation (**b**). Isolates: AS1 (■), AS4 (◆), CH2OS1 (●), 65S3 (▲), CTS1B (□), CTS1A (◇), 65S5 (○), 55S1 (△), 55S5 (x), 6S1 (+).

Discussion

A variety of bacterial isolates were recovered from different samples of municipal waste and peat. Phylogenetic analysis of 16S rRNA gene sequences of all bacteria displaying cellulase activity on CMC plates revealed four main phyla of bacteria: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. Within these four phyla, various genera of bacteria were isolated including *Bacillus, Paenibacillus, Exiguobacterium, Rhodococcus, Arthrobacter, Microbacterium, Pseudomonas, Aeromonas, Duganella* and *Chryseobacterium.* Little research has been done on cellulase, let alone lignocellulase production from genera such as *Duganella* and *Chryseobacterium;* however, it is not surprising that several of these bacteria produce cellulases, as these four phyla contain important genera of bacteria capable of biodegradation of organic compounds, and these species can be found ubiquitously in the environ-

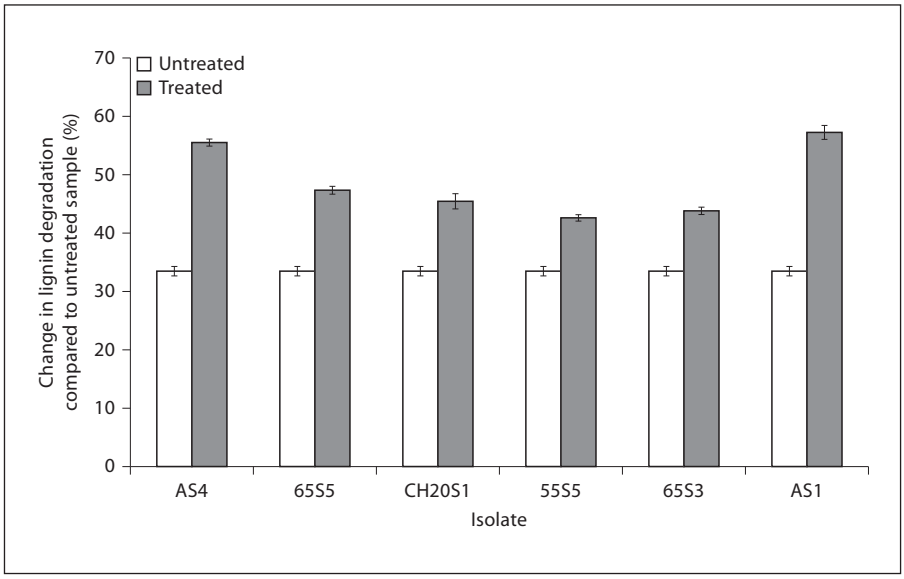

Fig. 6. Percent change in absorbance at 280 nm of 0.1% pure kraft lignin after 21 days of incubation with black liquor-tolerant isolates and compared to untreated sample.

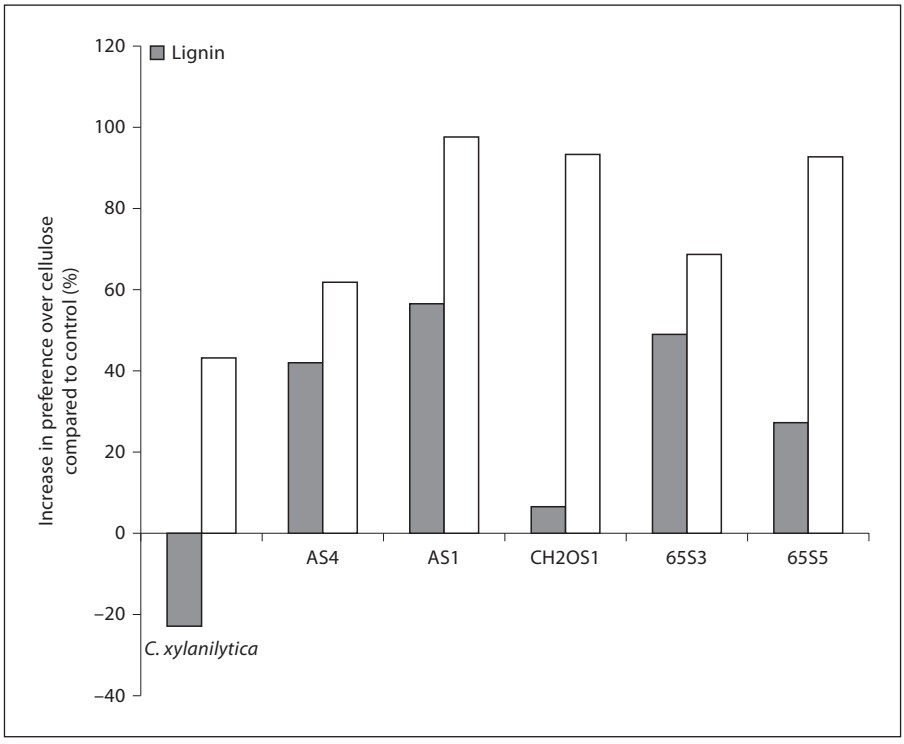

Fig. 7. FTIR analysis of all potential lignin-modifying isolates comparing percentage preference for hemicellulose and lignin to cellulose consumption.

ment. Therefore, these strains represent good potential candidates for greater lignocellulolytic activities including degradation of crystalline cellulose, xylanase activities and abilities to modify or even degrade lignin.

Generally speaking, all of the isolates displayed good industrial potential for degradation of lignocellulosic biomass; however, some displayed greater potential. For example, from 20 isolated bacteria, a total of 6 could degrade filter paper in addition to soluble cellulose. Moreover, 12 isolates could degrade xylan, 5 of which were among those capable of degrading filter paper. Additionally, 5 isolates displayed modification of lignin, while 2 of

these isolates, 55S5 and AS1, a *Bacillus* sp. and a *Pseudomonas* sp., respectively, displayed all 3 activities including degradation of crystalline cellulose, xylanase activity and modification of lignin. In the near future, our lab will focus particular attention on isolates 55S5 and AS1 for the optimization of lignocellulolytic abilities of these strains on complex biomass such as barley straw and paper mill sludge. These two isolates displayed the greatest variety of activities and have great potential for industrial applications in the degradation of more complex biomass such as agricultural residues and energy crops.

The characterization of cellulase- and xylanase-producing bacteria has gained an immense amount of attention due to the readily available abundance of cellulosic and hemicellulosic carbon sources in the world, which can be degraded into reducing sugars and ultimately fermented to value-added by-products such as bioethanol [Ragauskas et al., 2006]. Therefore, evaluating these activities in our isolates is pertinent to finding an efficient lignocellulosic bacterium. As a result, it was important for us to distinguish those strains which can degrade amorphous and crystalline cellulose in addition to xylanase activity. Hence, we could obtain 5 isolates displaying activities towards soluble and crystalline cellulose in addition to activities on beechwood xylan, ultimately leading us to distinguish the greatest lignocellulase-producing isolates: 55S5 and AS1.

Furthermore, it is no surprise that 5 of the isolates (including 55S5 and AS1) displayed evidence for modification of lignin in black liquor and of pure lignin extracted from black liquor. Several researchers reported strains of *Bacillus* and *Paenibacillus* which displayed abilities to decolourize kraft black liquor [Chandra et al., 2007, 2008; Hassan and Amr, 2009]. Moreover, Raj et al. [2010] reported the decolourization of black liquor by a newly isolated bacterium, *Aneurinibacillus aneurinilyticus*. Incredibly, after 6 days of incubation, *A. aneurinilyticus* was able to reduce the colour of black liquor by 58% and the lignin content by 43% [Raj et al., 2010]. Similarly, Chandra and Abhisshek [2011] recently reported that mixed cultures of *Citrobacter* sp. could increase the reduction in colour in black liquor to 79% and the reduction in lignin to 60% after just 6 days of incubation. Perhaps mixed cultures could serve to improve the activities and collective effects of our isolates on lignin modification and carbohydrate degradation lending greater lignocellulosic abilities. Most of our isolates were derived from similar sources, suggesting they co-exist in the environment and may therefore have great potential in the production of a lignocellulose-degrading bacterial consortium, which may be examined in the future.

Although members of *Pseudomonas* sp. have not been as readily reported in the decolourization of black liquor, they are suggested to have ligninolytic activities. For example, some *Pseudomonas* sp. have recently been described for their abilities to degrade dyes such as Malachite Green and Direct Orange 39 (Orange TGLL) with such lignases as peroxidases [Du et al., 2011; Jadhav et al., 2010], thus explaining why our *Pseudomonas* sp. also has good potential for industrial degradation of lignocellulosic biomass.

Of 20 isolates, not all could survive and proliferate in 1% black liquor, due to the toxicity associated with lignins and modified kraft lignins, including the remaining components of black liquor. Ten strains most likely survived due to an activation of their stress survival response systems [Guiliodori et al., 2007], as can be seen by the initial decline and then rebound in cell density prior to 5 days of incubation. Although these 10 strains could adapt and survive 21 days of incubation in the presence of black liquor, only 5 strains caused a change in the colour of black liquor; however, the colour did not decrease in absorbance but increased, corresponding to an increase in pH. There is minimal discussion regarding such an increase in colour, because decreasing the colour in black liquor is important for paper mill industries which wish to detoxify the black liquor before release to the environment.

Additionally, for the same strains, there was an increase in the absorbance of lignin at 280 nm, while the FTIR analysis revealed that there was a greater preference for carbohydrates like hemicellulose as well as a notable change in lignin compared to our cellulase-positive control *C. xylanilytica* which could not use lignin, evident by the negative value. With the support of our FTIR analysis, we suggest that strains AS1, AS4, 65S3, 65S5 and CH2OS1 all have the ability to modify lignin. However, the exact mechanism is still unknown despite recent tests for lignase activities including manganese peroxidase, lipase and laccase (data not shown).

There are some speculations for the resultant increase in colour of black liquor after treatment. Increase in absorbance at 280 nm could be caused by increased concentration of proteins in the solution which also show absorbance in this range, as displayed in the Bradford assay [Bradford, 1976]. Moreover, some researchers have reported an increase in absorbance at 260 nm of white rot fungi-treated lignin from ultraviolet spectra, while others have reported an increase in peak absorbance to 360 nm during ultraviolet spectrum analysis of *Polyporus*

versicolor-treated fungi. They propose the increase in absorbance to be due to structural changes such as a possible increase in acidic moieties and an increase in benzyl carbonyl groups and phenolic units [Kirk and Lundquist, 1970; Thivend and Lebrevon, 1969]. It has been said that the structure, including intermonomer linkages, and various functional groups give microorganisms the opportunity to make limited changes without necessarily affecting significant decomposition [Kirk, 1971]. Structural changes or modifications and even possibly by-products in solution could be capable of increasing the pH value, thus suggesting that lignin is more soluble. This could be valuable information in building a unique bacterial system for the decomposition of lignocellulosic biomass.

According to this study, we have successfully identified two isolates: *Pseudomonas* sp. AS1 and *Bacillus* sp. 55S5, with exceptional potential for industrial use in the conversion of lignocellulosic biomass for the production of bioethanol and other valuable by-products such as organic acids. In addition, many of our isolates characterized here also have potential in industrial use, some of which are more efficient in cellulase and xylanase production. Others may possess undiscovered lignase genes. These isolates lay the foundation for current exploitation of these enzymes by further investigation. Also, these strains may have great potential for developing bacterial consortiums in the near future to enhance the decomposition of lignocellulosic biomass and help overcome costly hurdles being faced in the industrial production of biofuels.

Experimental Procedures

Lignocellulosic Samples and Media

The lignocellulose-producing bacteria were isolated from several samples of two main sources: municipal waste and peat. The first set of samples was obtained from the City of Thunder Bay Solid Waste and Recycling Facility, Ont., Canada. The second set of samples was obtained using a peat corer to 1.5 m deep of a poor fen near Raith, Ont., Canada.

The growth media used in the experiments include R2A agar (0.5 g/l yeast extract, 0.5 g/l protease peptone, 0.5 g/l casamino acids, 0.5 g/l glucose, 0.5 g/l soluble starch, 0.3 g/l dipotassium phosphate, 0.5 g/l magnesium sulfate $7H_2O$, 0.3 g/l sodium pyruvate, 15.0 g/l agar), Luria Bertani (LB) liquid media (10.0 g/l peptone, 5.0 g/l yeast extract, 5.0 g/l NaCl), and CMC agar (0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract and 1.5 g agar, per 100 ml ddH_2O) [Kasana et al., 2008].

Isolation of Bacteria from Lignocellulosic Samples

The method of Maki et al. [2011] was used to isolate bacteria. Briefly, 1 g of each sample was suspended in 20 ml of sterile potassium phosphate buffer solution (1× PBS) by vortexing for 2 min on maximum speed. Following, a 100× serial dilution of the suspension was made in PBS. Thereafter, 200 μl 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 isolating individual colonies, and then they were incubated for an additional 48 and 72 h to allow the growth of slower growing microorganisms. 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 until purity. After purification, the cultures were compared visually to eliminate those of similar size, morphology and colour [Maki et al., 2011].

Screening for CMC Activity

The method described by Maki et al. [2011], was used to screen isolates for CMC activity. Isolates were grown in 10 ml of LB broth for 24 h, at 28°C, with shaking at 200 rpm, and slower growing isolates were left to incubate for an additional 48 h. The positive control used was *C. xylanilytica* XIL11 [Rivas et al., 2004]. 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 *E. coli* JM109, also grown in LB broth overnight, at 37°C for 18 h. All resulting broth cultures (isolates, positive control and negative control) were tested for cellulase activity via the Gram iodine method [Kasana et al., 2008]. Briefly, 5 μl of each broth culture was singly dropped onto a plastic Petri dish containing CMC agar, incubated for 48 h at 28°C and flooded with Gram iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH_2O) for 5 min to visualize and photograph the cellulase activity. The agar containing CMC stains brown, and areas without CMC are clear, described here as halos, as seen in figure 1. Halo diameters were measured using a ruler for a semi-qualitative comparison of cellulase activity among the isolates after 48 h of incubation. The halo measurement is used to relate cellulase activity to bacteria position on the phylogenetic tree as shown in figure 2, similarly done by Maki et al. [2011].

DNA Isolation and 16S rRNA Gene Amplification

The cellulase-producing isolates as well as the positive control were grown 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 rRNA gene. Universal primers designed within conserved regions of the 16S rRNA gene for Eubacteria were used: HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG), amplifying an approximately 796-bp fragment [Giannino et al., 2009]. The PCR reaction mixtures contained 10 ng of genomic DNA individually from each positive isolate, 10 pmol of both forward and reverse primers, 10× Taq buffer with KCl, 25 mmol/l $MgCl_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 units DNA polymerase per 50 μl reaction. The PCR program was as follows: primary denaturation for 3 min at 95°C, followed by 33 amplification cycles consisting of denaturing at 95°C for 1 min, annealing for 1 min at 63°C, and extension at 72°C for 1 min; upon completion of 33 amplification cycles, a final extension step was done at 72°C for 10 min. The PCR products were then viewed on 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 put online into the nucleotide blast tool through the National Center for Biotechnology Information 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 put into a sequence alignment program called ClustalX to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA (unweighted pair-group method with arithmetic mean) 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.

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 salt media (K_2PO_4 1 g/l, KCl 0.5 g/l, $MgSO_4$ 0.5 g/l, $NaNO_3$ 0.5 g/l, $FeSO_4$ 0.01 g/l, pH 7.4) with a 7-mm-wide strip of filter paper and two drops of 10 mmol/l glucose in glass culture tubes. The cultures were incubated for a maximum of 10 days and viewed daily for visual evidence of filter paper degradation. The release of reducing sugars by those strains capable of completely degrading the filter paper within 96 h was measured using the DNS method [Xiao et al., 2004] and was expressed as nmol glucose equivalents.

Screening for Xylanase Activity

Qualitative evidence for xylanase activities of all of the cellulase-positive isolates was evaluated using the same method described for the screening of cellulase activity. However, for the xylanase activity, 0.5 g of beechwood xylan (Sigma Aldrich) was substituted for CMC. Once again, the presence of halos after Gram staining indicated evidence of xylanase activities.

Black Liquor Decolourization and Cell Survival

From an overnight culture, 200 μl of cells for each isolate were inoculated in triplicate to 100 ml of Dubos salt media supplemented with 1% (w/v) black liquor (pH 6.5) and incubated at 30°C, with shaking at 150 rpm. Samples were collected at days 0, 1, 3, 5, 7, 10, 14 and 21. For decolourization experiments, 500 μl of cell suspensions and one untreated sample were diluted with 500 μl of PBS and then centrifuged at 17,000 *g* in a microcentrifuge tube for 4 min. In triplicate, 300 μl of the supernatant was loaded into a microtitre plate, and the absorbance (at 425 nm) of the samples was measured by an xMark Microplate Spectrophotometer (Bio-Rad, Canada). Simultaneously, to determine cell survival, 500 μl of cell samples were collected for the drop plate counting technique to determine cell density (CFU/ml). Additionally, a change in pH of black liquor after treatment with the isolates was measured using an Accument combination pH electrode with silver reference (Thermo Fisher Scientific, Canada).

Absorbance of Lignin at 280 nm

Isolates capable of modifying the colour of black liquor at 425 nm were selected for further analysis on 98% pure lignin extracted from black liquor. For each, 100 μl of an overnight culture was inoculated in triplicate to 6 ml of Dubos salt media supplemented with 0.1% (w/v) pure lignin, 0.3% (w/v) peptone and 0.5% (w/v) glucose, and incubated at 30°C, at 150 rpm for 21 days. After 21 days of incubation, 1-ml aliquots of each cell suspension and one untreated sample were centrifuged for 4 min at 17,000 *g*. Following, the supernatant was diluted 1,000× in 1× PBS before reading the absorbance at 280 nm.

FTIR Analysis of Isolates on Barley Straw

The isolates displaying modification of lignin were further analysed for lignase activities using FTIR analysis after 21 days of incubation with 1% (w/v) barley straw in Dubos salt media supplemented with 0.3% (w/v) peptone and 0.5% (w/v) glucose. In triplicate, 1 ml of isolate overnight cultures was inoculated to 100 ml of barley straw media and incubated at 30°C, with shaking at 150 rpm. After 21 days of incubation, isolate cultures and one untreated barley straw control were filtered through Whatman filter paper and washed once with 10 ml of distilled water. The barley straw was collected and oven dried at 60°C for 48 h prior to FTIR spectra analysis. Dried, treated and untreated barley straw samples were loaded in triplicate directly to a Bruker Tensor 37 Fourier Transform Infrared Spectrophotometer equipped with an InGaAs detector (Bruker Optics Ltd., Canada). Peak height and area were measured by constructing a baseline connecting the lowest data points on either side of the peak [Pandey and Pitman, 2003].

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