



Microbial degradation of lignocellulosic biomass: discovery of novel natural lignocellulolytic bacteria

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Abstract

Lignocellulosic biomass is the most abundant biomaterial on Earth and holds enormous potential as a source of fermentable sugars for cost-effective biofuel production. The main obstacle is the intractable and convoluted structure and composition of lignocellulosics which are mainly made of lignin, cellulose and hemicellulose. However, nature has equipped microbes with enzymes that can deconstruct lignocellulose and release fermentable sugars. In this study, we selected bacteria (belonging to 6 different genera) from nature that had been grown in minimal culture media with cellulose or lignin as the sole carbon source, and assessed their lignocellulolytic activities in qualitative and quantitative assays. Through sequence analysis of the 16S rRNA gene, we discovered a novel lignocellulolytic bacterial species, *Chryseobacterium gleum*, capable of degrading both cellulose and lignin. In addition, a few other bacterial species previously known to degrade either cellulose or lignin were found to have lignocellulolytic activities. The majority of these bacterial species were found to produce extracellular lignocellulolytic enzymes. Lignocellulolytic bacteria identified in this study may serve as sources of genes/enzymes which might find applications in biofuel production and other industrial applications.

Key words: biofuel, lignocellulosic biomass, cellulose, lignin, lignocellulolytic bacteria

Introduction

The global consumption of fossil fuels (coal, gas and oil) is over 20 billion tons per year (BP Energy Outlook-2017). In the International Energy Outlook 2016 report, the U.S. Energy Information Administration (EIA) projected a 48% increase in the world energy consumption rate by 2040. This ever-increasing demand for energy, rapid depletion of fossil fuel reserves, global warming and associated climatic changes have brought the issue of fuel production from renewable sources to the forefront of discussions worldwide (Yoshikawa et al., 2013; Zhang et al., 2010; Tiwari et al., 2013; Talebnia et al., 2010). One of the most promising alternatives to fossil fuels is biofuel, which is derived through the conversion of biomass (Huang et al., 2013; Talebnia et al., 2010; Ooi et al., 2004). Many countries have already set specific goals in their national strategies to adopt biofuel for

sustainable development (Zhang et al., 2010; Abbas and Ansumali, 2010; Viikari et al., 2012).

Lignocellulosic biomass is the most abundant and renewable natural resource on Earth (Zhang et al., 2010; Zhou et al., 2011). The net photosynthetic production of dry lignocellulosic biomass by plants on Earth has been estimated to be 155 billion tons per year (Talebnia et al., 2010). Lignocellulosics from sources other than food, therefore, can provide significant economic and strategic benefits for the production of biofuel (Viikari et al., 2012). This not only offers a cost-effective way to tackle the energy crisis, but it also means that it may be possible to manage environmental issues accrued from lignocellulosic waste accumulation and carbon dioxide emissions (Zhang et al., 2010; Zhou et al., 2011).

Lignocellulose is primarily composed of cellulose, hemicellulose and lignin (Yoshikawa et al., 2013; Zhou

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et al., 2011). In general, lignocellulosic biomass contains approximately 35–50% of cellulose, 20–35% of hemicellulose, and 10–25% of lignin (Zhou et al., 2011). Cellulose is an organic polymer of D-glucose units connected through $\beta(1-4)$ glycosidic bonds, while hemicelluloses contain many different sugar monomers (Murphy and McCarthy, 2005; Zhou et al., 2011). These can be used as a cheap and easily available source of fermentable sugars to produce biofuel. Cellulose and hemicellulose present in the plant cell wall remain tightly bound to lignin, which is a complex of chemically heterogeneous high molecular weight polymers composed of alkylphenol units (Huang et al., 2013; Yoshikawa et al., 2013; Talebnia et al., 2010). Lignin is highly recalcitrant to degradation and forms a physical barrier to biological and chemical hydrolysis of cellulose and hemicellulose (Huang et al., 2013; Yoshikawa et al., 2013; Talebnia et al., 2010; Nobre and Aanen, 2012; Woo et al., 2014; Bandounas et al., 2011). Therefore, lignin must be broken efficiently before fermentable sugars can be liberated to make biofuel production from lignocellulose economically viable.

Although several physicochemical pretreatment technologies have been developed to disrupt the non-cellulosic matrix to render cellulose and hemicellulose more accessible for enzymatic hydrolysis, these pretreatment technologies are generally expensive, require significant energy inputs and long reaction times, and/or generate compounds that are inhibitory to fermentation in the subsequent step (Huang et al., 2013; Bandounas et al., 2011; Talebnia et al., 2010). Efficient but cost-effective methods for selective lignin degradation are, therefore, in high demand (de Gonzalo et al., 2016; Huang et al., 2013). Enzymatic hydrolysis offers a more specific and effective alternative for lignin depolymerization as well as cellulose degradation (Bandounas et al., 2011). Moreover, enzymatic breakdown generally takes place under milder conditions with lower energy inputs and fewer negative environmental impacts (Bandounas et al., 2011; Huang et al., 2013).

A number of fungi are known to produce lignocellulose degrading enzymes (Ponnambalam et al., 2011; Woo et al., 2014). However, the use of fungi requires more time and means a higher cost of enzyme production, purification and engineering compared to bacterial use (Huang et al., 2013; Ponnambalam et al., 2011; Woo et al., 2014; Taylor et al., 2012). The discovery of novel lignocellulolytic bacteria may constitute the source of

novel enzymes to be used in biofuel production and other industrial applications. In this study, we isolated and identified bacteria from nature which can degrade both lignin and cellulose.

Materials and methods

Sources of bacteria

Soil from dumping areas for domestic kitchen wastes (Ramna area, Dhaka, Bangladesh), decaying plant material in garden soil (Curzon Hall area, University of Dhaka), cow dung and woodworm gut were used as the starting materials to isolate and characterize lignocellulolytic bacteria. 1g of each of the soil samples and cow dung were mixed together and the volume was made up to 30 ml with the addition of sterile 0.9% (w/v) NaCl solution. The suspension was mixed by mild vortexing and incubated at 35 °C for 30 minutes. Woodworm gut was macerated separately in 0.9% (w/v) NaCl solution under sterile conditions, mixed by mild vortexing and incubated at 35 °C for 30 minutes. After incubation, clearer solutions from the top were collected, mixed and serially diluted (10^{-1} to 10^{-5} times) with 0.9% (w/v) NaCl solution.

Selection and screening of cellulose degrading bacteria

100 μ l of each dilution was inoculated in a minimal liquid culture medium (NaCl 6.0 g/l, $(\text{NH}_4)_2\text{SO}_4$ 1 g/l, KH_2PO_4 0.5 g/l, K_2HPO_4 0.5 g/l, MgSO_4 0.1 g/l and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l) with carboxymethyl cellulose (CMC) (0.1% (w/v)) (039-01335, Wako Chemicals) as the sole carbon source and incubated at 35 °C for 72 h at 140 rpm. Following bacterial growth, 100 μ l of culture was spread on a solid selection medium (NaCl 6 g/l, $(\text{NH}_4)_2\text{SO}_4$ 1 g/l, KH_2PO_4 0.5 g/l, K_2HPO_4 0.5 g/l, MgSO_4 0.1 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l, CMC 0.1% (w/v) and 1.5% agar (w/v)) and incubated at 35 °C for 72 h. Well separated colonies with different morphological characteristics were sub-cultured on LB-agar medium (LB 2.0% (w/v) and 1.5% agar (w/v)) and incubated at 35 °C for 48 h.

Selected pure cultures of bacterial samples were grown separately in liquid media (NaCl 6 g/l, $(\text{NH}_4)_2\text{SO}_4$ 1 g/l, KH_2PO_4 0.5 g/l, K_2HPO_4 0.5 g/l, MgSO_4 0.1 g/l and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l) with Whatman[®] grade1 filter paper (1001 110, GE Healthcare-Whatman), which is made of cellulose, as the only source of carbon. In this assay, 12 pieces of $\sim 0.04 \text{ mm}^2$ sized sterilized Whatman[®] filter

papers were added to 5 ml of medium in each tube. The cultures were incubated at 35 °C for 48 h in an incubator at 140 rpm. The growth of bacteria was compared to the control culture medium without bacterial inoculation.

Qualitative analyses of cellulolytic activity

Qualitative analyses of cellulolytic activity of selected bacterial samples were performed using the Congo Red zone clearing assay (Kaur and Arora, 2012; Behera et al., 2014; Ponnambalam et al., 2011). In the first approach, selected bacteria were cultured on CMC-LB-agar media (CMC 0.1% (w/v), LB 2.0% (w/v) and agar 1.5% (w/v)) at 35 °C for 48 h and then flooded with 0.1% (w/v) Congo Red (0379, Amresco LLC) solution in water. After incubation for 15 min, the culture media were de-stained with 1M NaCl solution. In the second approach, the bacteria were inoculated on CMC-LB-agar solid media along with Congo Red (CMC 0.1% (w/v), LB 2.0% (w/v), agar 1.5% (w/v) and Congo Red 0.1% (w/v)) and incubated at 35 °C for 48 h. The plates were then de-stained with 1 M NaCl solution.

Quantitative analysis of the production of reducing sugars

A quantitative analysis of the production of reducing sugars from the hydrolysis of cellulose polysaccharides by bacterial enzymes was performed using 3,5-Dinitrosalicylic acid (DNS) (Ponnambalam et al., 2011; Kaur and Arora, 2012). Briefly, selected bacteria were grown on culture medium (NaCl 6 g/l, $(\text{NH}_4)_2\text{SO}_4$ 1 g/l, KH_2PO_4 0.5 g/l, K_2HPO_4 0.5g/l, MgSO_4 0.1 g/l and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l) containing Whatman® grade 1 filter paper (1001 110, GE Healthcare-Whatman) strips of 6 cm² (~50 mg). Then, the cultures were incubated for 48 hours at 35 °C in an incubator at 140 rpm. Optical densities (OD) of all cultures at 600 nm were equalized to the culture with the lowest OD using phosphate buffered saline (PBS) (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l and KH_2PO_4 0.24 g/l, pH 7.4). The cultures were centrifuged at 2000 × g for 5 min and supernatants (culture media) were collected from each of the cultures. 1 ml of the supernatants was mixed with 2 ml of water and 3 ml of DNS in test tubes and boiled for 5 min. Once cooled, 1 ml of sodium potassium tartrate (also known as Rochelle salt) (40% w/v) solution was added to each tube and ODs were measured at 540 nm using a spectrophotometer. The concentration of reducing sugars in the

samples was measured and compared to those of standard glucose solutions.

Screening for ligninolytic activity

Bacterial samples primarily selected on CMC-minimal medium were inoculated in another medium (Kraft lignin 0.25 g/l, KH_2PO_4 0.25 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01g/l, MgSO_4 0.25g/l, NaCl 0.1g/l, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g/l and agar 1.5% (w/v)), in which Kraft-lignin (Sc-489763, Santa Cruz Biotechnology, Inc.) was used as the sole carbon source, and these were then incubated at 35 °C for 7 days.

The ligninolytic activity of these bacterial samples was analyzed using 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Mabrouk et al., 2010; Floch et al., 2007; Huang et al., 2013). This assay was performed following 2 different approaches. In the qualitative approach, the bacteria were cultured on a medium containing ABTS (LB 2.0% (w/v), agar 1.5% (w/v), ABTS 1.0% (w/v), and CuSO_4 0.1 g/l) at 35 °C for 48 hours. Following incubation, the appearance of a greenish color surrounding the cultures was observed. In the relative-quantitative approach, the bacterial samples were cultured in tryptone-yeast extract broth (Tryptone 0.5% (w/v) and yeast extract 0.3% (w/v)) and incubated for 48 h at 35 °C in a shaking incubator at 140 rpm. Optical densities (OD) of all cultures at 600 nm were equalized using PBS to the culture with the lowest OD. The cultures were centrifuged at 2000 × g for 5 min and supernatants (culture media) were collected from each of the cultures. Cell pellets were suspended in 5 ml of cold PBS and lysed by sonication. Cell extracts were collected following centrifugation at 8400 × g for 10 min. Optical densities of the cell extracts at 280 nm were equalized using PBS to the extract with the lowest OD. 150 µl of ABTS solution along with 50 µl of the cell extracts or supernatants (culture media) were added to the wells in a 96-well plate. ABTS solutions were prepared fresh at pH 4, 5 and 6 using PBS. After vigorous mixing, absorption at 420 nm was measured in a micro-plate spectrophotometer at 1 min intervals for 3 min and compared to the controls.

Identification of bacteria by 16S rRNA gene sequencing

The bacterial samples were cultured in LB broth at 35 °C in a shaking incubator for 24 h. The cultures were centrifuged at 5000 rpm for 5 min and the pellets were

washed twice with phosphate buffered solution (PBS). The pellets were mixed with 150 μ l of 10% (w/v) of Chelex[®]-100 (C7901, Sigma) in sterile water and vortexed vigorously for 10 s. The suspensions were incubated at 95 °C for 20 min, vortexed again and then centrifuged at 14 000 \times g for 10 min. The supernatants were collected and quantified for DNA in a spectrophotometer. The amount of 0.5 μ l of the DNA samples (~100 ng) were mixed with 2.5 μ l of 10 \times PCR buffer, 1.5 μ l of dNTP mix (10 mM), 0.75 μ l of each forward (16S_357F: CTCCTA CGGGAGGCAGCAG) and reverse (16S_1100R: AGGGT TGCGCTCGTTG) primers (10 μ M), 18.8 μ l of nuclease-free water and 0.2 μ l of Taq DNA polymerase (EP0702, Thermo Fisher Scientific). The sequences were amplified in a thermal cycler under the following conditions: an initial denaturation step at 94 °C for 3 min., then 30 cycles – each with denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min., and elongation at 72 °C for 1 min., followed by the final extension at 72 °C for 5 min. The amplified products were resolved in 1.2% (w/v) agarose gels using 1 \times Tris Acetate EDTA (TAE) buffer along with a DNA size marker (300003, GeneON). Amplified DNA sequences were visualized in a gel documentation system following incubation with ethidium bromide in 1 \times TAE buffer. PCR products were purified using FavorPrep[™] GEL/PCR Purification Kit (FAGCK 001, Favorgen Biotech Corp.) following the manufacturer's protocol. Purified PCR products were sequenced following the Sanger sequencing technique at 1st Base DNA Sequencing Services, Malaysia. The bacteria were identified by comparing the sequences in the databases using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website.

Data analysis

The data were analyzed using Microsoft Excel and GraphPad Prism (version 6.1) software. All data are presented as a mean \pm standard error of mean (SEM).

Results and discussion

The deconstruction of lignin to enhance the release of fermentable sugars from plant cell walls presents a major challenge for biofuel production from lignocellulosic biomass. Nature has devised ways to degrade lignocellulosic biomass via microbes (de Gonzalo et al.,

2016). These microbes can be used as sources of enzymes for industrial applications to effectively degrade lignocellulosic biomasses for biofuel production. In this study, lignocellulolytic microorganisms with potential abilities to degrade cellulose and/or lignin were selected from various natural sources, as the microbes present in decaying leaves, in garden soil and in household wastes, or living in the guts of organisms that thrive on lignocellulosic biomasses are supposed to have lignocellulolytic enzymes (Dubey et al., 2014; Ahmad et al., 2013).

Our study was based on the premise that if bacteria were allowed to grow in a minimal medium containing cellulose as the sole carbon source, then only those bacteria capable of utilizing cellulose would survive. As cellulose is insoluble in a culture medium, in this study carboxymethyl cellulose (CMC) was used as an alternative substrate (Ponnambalam et al., 2011; Anand et al., 2009; Behera et al., 2014). CMC, an amorphous and esterified form of cellulose, is considered a perfect analog for assessing cellulolytic activities (Ponnambalam et al., 2011).

Bacteria belonging to 6 different genera were selected in minimal media, with CMC as the sole carbon source (Fig. 1). Pursuant to the homology of 16S rRNA gene sequences to those in the database repositories, these bacteria belonged to *Acinetobacter spp.*, *Klebsiella variicola*, *Chryseobacterium gleum*, *Bacillus spp.*, *Proteus mirabilis* and *Stenotrophomonas maltophilia* (Table 1). 16S ribosomal RNA gene (16S rRNA) sequence analysis is a standard approach to identify bacteria (Bandounas et al., 2011; Klindworth et al., 2013). At the species level and on the basis of 16S rRNA gene sequences, 4 bacterial species (*K. variicola*, *C. gleum*, *A. baumannii*, *P. mirabilis*, and *S. maltophilia*) were identified (Table 1). Using this approach, the identity of the 2 remaining lignocellulolytic bacteria (*A. calcoaceticus/pittii* and *B. pumilus/altitudinis/stratosphericus/xiamenensis*) could not be resolved at the species level, although the query sequences found 100% identical matches to several sequences in database repositories. These sequences have been repositied and are available at GenBank, NCBI (accession numbers: MG738223, MG738224, MG738225, MG738226, MG738227, MG738228 and MG738229).

All selected bacteria, except for *P. mirabilis*, grew in minimal media, with Kraft-lignin as the only carbon source. Similar to an earlier report (Prasad et al., 2014), we, too, observed a slow growth of bacteria. In fact, the

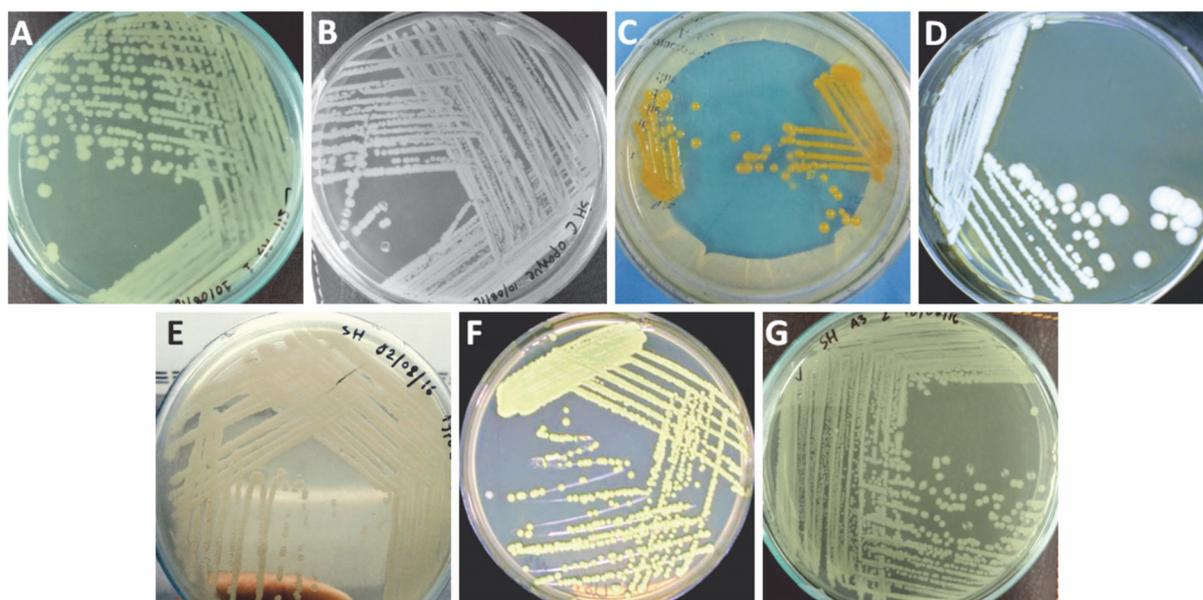


Fig. 1. Bacteria selected on minimal media with carboxymethyl cellulose (CMC) as the sole carbon source; six different bacterial genera with distinct morphological characteristics could grow in minimal media using CMC as the only carbon source; this figure shows culture of the selected bacteria on LB-Agar medium; A) *A. calcoaceticus/pittii*, B) *K. variicola*, C) *C. gleum*, D) *Bacillus spp.*, E) *A. baumannii*, F) *P. mirabilis*, and G) *S. maltophilia*

Table 1. Identification of the CMC-minimal medium selected bacteria based on 16S rRNA gene sequence homology

Bacterial spp.	Query size, bps	Query coverage	Identity
<i>Acinetobacter spp.</i> (possible species are <i>A. calcoaceticus</i> and <i>A. pittii</i>)	705	100%	100%
<i>Klebsiella variicola</i>	607	100%	100%
<i>Chryseobacterium gleum</i>	681	100%	100%
<i>Acinetobacter baumannii</i>	700	100%	100%
<i>Stenotrophomonas maltophilia</i>	707	100%	100%
<i>Bacillus spp.</i> (possible species are <i>B. pumilus</i> , <i>B. altitudinis</i> , <i>B. stratosphericus</i> , and <i>B. xiamenensis</i>)	689	100%	100%
<i>Proteus mirabilis</i>	694	100%	100%

growth of all tested bacterial species in the Kraft-lignin containing media was much slower when compared to that in the CMC-agar medium. All bacterial samples except *Bacillus spp.* were negative for Gram-staining (data not shown). Moreover, all of the CMC-selected bacteria grew in media with Whatman® grade 1 filter paper as the sole carbon source. According to the manufacturer, Whatman® grade 1 filter paper (1001 110, GE Healthcare-Whatman) is made of cellulose. The presence of cellulolytic enzymes in bacterial strains was further verified by the Congo Red zone clearing assay and estima-

tion of reducing sugars released from the cellulose. These tests are commonly used to assess cellulolytic activities (Ponnambalam et al., 2011; Behera et al., 2014; Anand et al., 2009; Kaur and Arora, 2012). The Congo Red zone clearing assay is a qualitative assay (Ponnambalam et al., 2011) based on the binding of Congo Red to cellulose and its release upon degradation, due to cellulolytic activity (Apun et al., 2000; Teather and Wood, 1982). In our study, clear zones were observed around the colonies of all bacterial species selected on CMC-minimal media (Fig. 2). Cellulolytic activities of

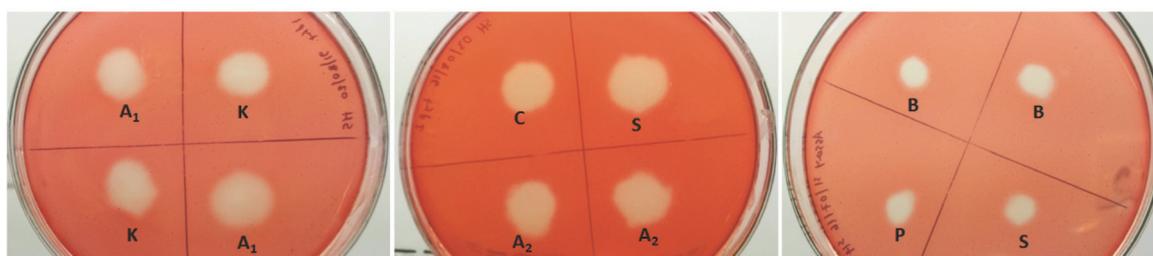


Fig. 2. Congo Red zone clearing assay. Clear zones were observed around the colonies of all CMC-selected bacterial species. A1) *A. calcoaceticus/pittii*, A2) *A. baumannii*, K) *K. variicola*, C) *C. gleum*, B) *Bacillus* spp., P) *P. mirabilis*, and S) *S. maltophilia*

these bacteria were quantitatively assessed by measuring the reducing sugars liberated from the cellulose (Whatman® grade 1 filter paper) using 3,5-dinitrosalicylic acid (DNS) based on the colorimetric method. *P. mirabilis* liberated the highest amount of reducing sugars from the cellulose (Fig. 3).

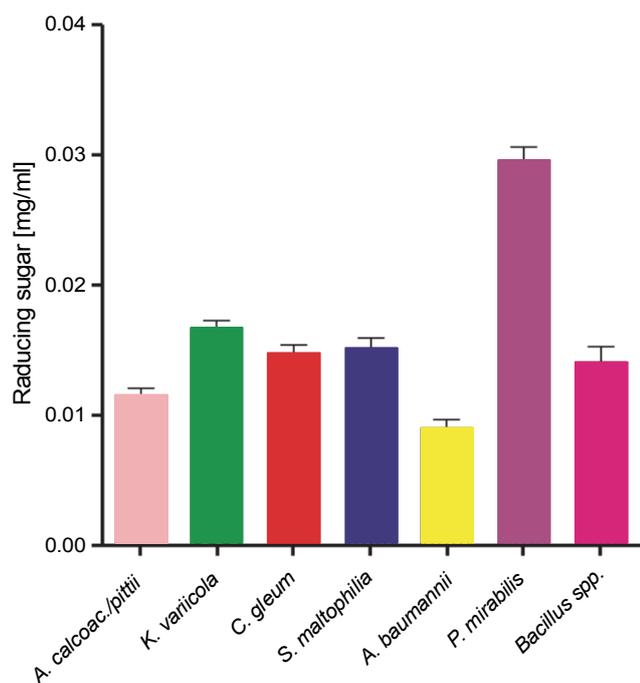


Fig. 3. Quantitation of reducing sugars released from cellulose by bacterial enzymes; *P. mirabilis* released the highest amount of reducing sugars from cellulose; all data are presented as mean \pm standard error of mean (SEM); data represents ≥ 3 replicates

The cellulolytic bacteria were cultured in minimal media with Kraft-lignin to select those with the ability to utilize Kraft-lignin as the carbon source. Unlike lignin, Kraft-lignin is water-soluble and is used as a carbon source for selection of ligninolytic bacteria (Bandounas et al., 2011; Huang et al., 2013). *Acinetobacter* spp.,

K. variicola, *C. gleum*, *Bacillus* spp. and *S. maltophilia* grew in minimal media with Kraft-lignin (Fig. 4A). Although *P. mirabilis* exhibited the highest cellulolytic activity, it neither grew in the Kraft-lignin selection medium nor showed ligninolytic activity in other assays.

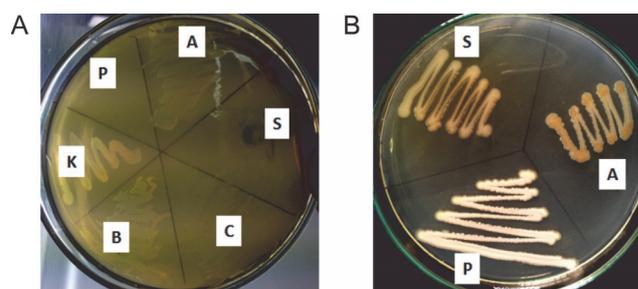


Fig. 4. Ligninolytic activity of bacteria: A) growth of bacteria in minimal media with Kraft-lignin as the only carbon source, B) in ABTS agar media assay greenish color was observed around the ligninolytic bacteria; in this representative image: A) *A. calcoaceticus/pittii*, B) *Bacillus* spp., C) *C. gleum*, K) *K. variicola*, P) *P. mirabilis*, and S) *S. maltophilia*

2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) is a substrate of laccase, an enzyme that can degrade lignin (Huang et al., 2013; Mabrouk et al., 2010). Unlike many oxidoreductases, laccases neither produce toxic hydrogen peroxide as a byproduct nor require the addition of cofactors – two features which make these enzymes industrially attractive (de Gonzalo et al., 2016). In an ABTS-agar media assay, the greenish color (indicative of laccase activity) was produced around the colonies of all species, except for *P. mirabilis* (Fig. 4B). The relative ligninolytic activities of the bacteria were assessed by measuring the ABTS oxidation level spectrophotometrically. In this assay, the cell extract and culture supernatant of all the CMC-selected bacteria, except *P. mirabilis*, became visibly green fol-

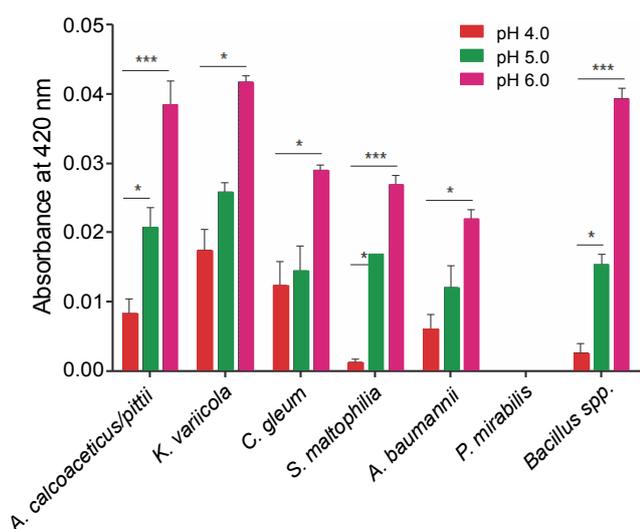


Fig. 5. Bacterial laccase activity at different pH; there was a gradual increase in laccase activity with higher pH; data are presented as mean \pm standard error of mean (SEM) of the activities of extracted intracellular laccase; data represents ≥ 3 replicates and absorbance was measured after 3 minutes; statistical significances were calculated using t-test (corrected for multiple comparisons using the Holm-Sidak method); * and *** indicate significance at $P < 0.05$ and $P < 0.0001$, respectively

lowing exposure to ABTS; an increase in absorbance at 420 nm was also observed. The ABTS-oxidizing activity of the samples was analyzed at pH 4, 5 and 6. All samples showed the highest activities at pH 6 (Fig. 5). The mean laccase activities of *A. calcoaceticus/pittii*, *K. variicola* and *Bacillus spp.* were greater than those of other bacterial species tested (Fig. 6).

Not all bacteria that can degrade cellulose can also degrade lignin (Bandounas et al., 2011). Many members of the genus *Bacillus* have been shown to have ligninolytic and/or cellulolytic activities. These include *B. pumilus*, *B. brevis*, *B. alcalophilus*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. circulans*, *B. atrophaeus*, *B. halodurans*, *B. tequilensis* and *B. polymyxa* (Anand et al., 2009; Dubey et al., 2014; Prasad et al., 2014; Woo et al., 2014; Bandounas et al., 2011; Behera et al., 2014; Ahmad et al., 2013; Huang et al., 2013). *K. variicola* has been reported to have not only lignocellulolytic activities but also the ability to utilize diverse types of carbohydrates (Jiménez et al., 2014; Dantur et al., 2015; Islam et al., 2017). *A. calcoaceticus* and other *Acinetobacter* members can utilize diesel and lignin as carbon sources (Ho et al., 2017; Ghodake et al., 2009; Ponnambalam et al., 2011). *Stenotrophomonas sp.* and *P. vulgaris* have been

shown to be capable of utilizing cellulose (Anand et al., 2009; Dubey et al., 2014). To date, there has been no published report on the ligninolytic and/or cellulolytic activities of *C. gleum*, nor on the ligninolytic activity of *S. maltophilia* and *P. mirabilis*. It has, however, been previously shown that *S. maltophilia* can degrade crystal violet (Kim et al., 2002), which may be indicative of its ligninolytic activity. In our study, *A. calcoaceticus/pittii*, *A. baumannii*, *K. variicola*, *C. gleum*, *Bacillus spp.*, and *S. maltophilia* were found to have the ability to degrade both lignin and cellulose.

The majority of the bacterial laccases already reported are located intracellularly (Huang et al., 2013; Bandounas et al., 2011; de Gonzalo et al., 2016). Only a few *Bacillus* species (*B. tequilensis*, *B. atrophaeus* and *B. pumilus*) have been shown to have both intracellular and extracellular laccase activities (de Gonzalo et al., 2016; Huang et al., 2013). In our study, both intra- and extracellular laccase activities of the bacteria were assessed by measuring the oxidation of ABTS. Among the laccase-positive bacteria, all but *C. gleum* showed intra- and extra-cellular activities (Fig. 6). In general, the extra-cellular laccase activity was relatively small for all tested bacterial species except for *Bacillus spp.*, which showed a higher activity.

In our study, a gradual increase in laccase activity was observed from pH 4 to pH 6 for all bacteria. The pH for the optimal activity of laccases varies depending on the source of the enzyme. A purified laccase from papaya leaves exhibited a pH optimum of 6 when measuring oxidation of ABTS (Jaiswal et al., 2015). Fungi grow, in general, at low pH and produce laccases which work best at acidic pH (2–4) (Kiiskinen et al., 2004; Santhanam et al., 2011). Bacterial laccases have characteristics different to those of their fungal analogs (Huang et al., 2013). The activity of most bacterial laccases (with ABTS) is at its optimum between pH 4 and 6, but some (*S. psammoticus*) display optimum activity at pH 8.5 (Santhanam et al., 2011). The data presented by Huang and coworkers (2013) showed an inconsistent relationship between pH and laccase activities from 2 *Bacillus* species. In the same study, the control laccase from a fungus, *Trametes versicolor*, showed a higher activity at a lower pH. Laccase activity from *Streptomyces sp.* has been shown to have a pH optimum at 6 (Demissie and Kumar, 2014). Zhang and coworkers (2012) reported that the efficiency of malachite green decolorization by a laccase

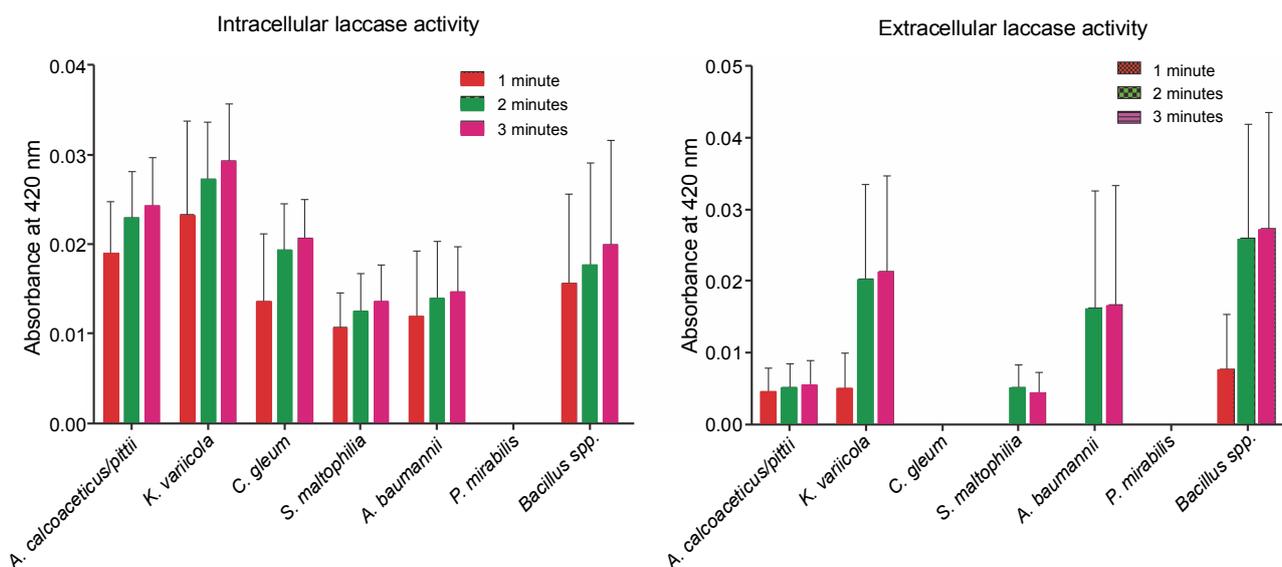


Fig. 6. Intra- and extra-cellular laccase activities of the bacteria; laccase activity was measured at pH 6.0; mean absorbance due to the oxidation of ABTS by laccases increased gradually with time, though the differences were not statistically significant; overall, *Bacillus spp.* and *K. variicola* showed higher mean laccase activities; all data are presented as mean \pm standard error of mean (SEM); data represents ≥ 3 replicates; statistical significances were calculated using t-test (corrected for multiple comparisons using the Holm-Sidak method)

from *B. vallismortis* spores improved as the pH increased and the maximum efficiency of oxidation of ABTS was obtained at pH 6.

Lignocellulolytic enzymes from bacteria may be used in industrial applications other than biofuel production. Cellulases have applications in starch processing, grain alcohol fermentation, extraction of fruit and vegetable juices, and the textile, pulp and paper industries (Kaur and Arora, 2012). On the other hand, ligninolytic organisms and enzymes can be used for the treatment of xenobiotic compounds (Sivakami et al., 2012). For example, laccases have broad substrate specificities and can oxidize a wide variety of organic and inorganic compounds including dyes in effluents from textile industries (Kiiskinen et al., 2004). Bacterial laccases have been shown to have better characteristics (high temperature, salt, and pH tolerance) than their fungal counterparts (de Gonzalo et al., 2016; Huang et al., 2013). Lignocellulolytic bacteria identified in this study may, therefore, serve as sources of enzymes which might find applications not only in biofuel production but also in other industrial applications.

Conclusions

Lignocellulosic biomass holds enormous potential as a source of fermentable sugars for cost-effective biofuel

production. In this study, we discovered a novel lignocellulolytic bacterial species, *C. gleum*, along with 5 other lignocellulolytic bacteria (previously known to degrade either cellulose or lignin) that belong to different genera (*Acinetobacter spp.*, *K. variicola*, *Bacillus spp.*, *P. mirabilis* and *S. maltophilia*). Earlier studies have reported better characteristics of bacterial enzymes (high temperature, salt, and pH tolerance) when compared to their fungal counterparts. Lignocellulolytic bacteria identified in this study may serve as sources of genes and/or enzymes which might find useful industrial applications. These genes may also be engineered to produce enzymes with better functionality and regulation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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