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ORIGINAL ARTICLE

An effective microplate method (Biolog MT2) for screening native lignocellulosic-straw-degrading bacteria

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Abstract Lignocellulosic wastes such as straw are attractive resources for biofuel production when subjected to biological treatment (hydrolysis). However, their complex lignocellulosic structure can hinder saccharification. The urgent need for microbial groups with high levels of straw saccharifying activities is therefore a key step in the bioconversion of lignocellulosic straw into fermentable monosaccharides. Existing traditional methods of qualitative and quantitative screening of lignocellulolytic microbial isolates are costly, time consuming and largely not environmentally friendly. In this study, a Biolog (MT2) microplate-based assay was evaluated for potential use as an alternative screening method. This was carried out using three commercially available substrates (cellulose, xylan and lignin) and four native lignocellulosic straws (wheat, rice, sugarcane, and pea ball-milled straws). Selected bacterial isolates from soil, compost and straws were screened quantitatively using both traditional crude enzyme and Biolog (MT2) microplate methods. Positive correlations (R^2 values up to 0.86) between Biolog and the traditional enzyme methodologies were observed with respect to these isolates and their lignocellulosic activities. Quantitative assays were less labor intensive and faster (3–7 days) in Biolog microplates than in traditional assays which lasted for 12–15 days. Ball-milled rice and sugarcane straws were bio-converted to monosaccharaides more readily than wheat and pea straws and the commercially available substrates (cellulose, xylan and lignin). Environmental scanning electron microscopy (ESEM) analysis of ball-milled rice and sugarcane straws suggested that this was due to their higher silica content. Overall, the Biolog (MT2) microplate system was shown to be an effective, time saving and inexpensive alternative method for the screening of both lignocellulose-degrading bacteria and different substrates for saccharification.

Keywords Biolog (MT2) microplate · Lignocellulose degradation · Correlation analysis · Ball-milled straw · Bacterial screening

Introduction

The annual production rates of crop residues such as wheat, rice and sugarcane straws globally from 1997 to 2001 were 354, 731 and 181 million tons, respectively (Dashtban et al. 2009). When combined with forestry residues, these agricultural residues account for more than 60 % of the total plant materials produced worldwide (Chandrakant and Bisaria 1998; Tuomela et al. 2000). In fact, it has been estimated that about 200 billion tons of lignocellulosic materials are produced annually (Michelin et al. 2013). Lignocellulosic biomass is a major component of plant materials, and is composed primarily of structural carbohydrates such as cellulose and hemicellulose surrounded by lignin. This complex combination of biopolymers renders lignocellulosic materials less biodegradable (Ball and McCarthy 1988; Jørgensen et al. 2007). Lignocellulosic straws (LCS) or materials can cause

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serious air pollution when subjected to open-air burning. This process reduces local air quality, creating a variety of health concerns from the discharge of carcinogenic oxides (NO_x, SO_2 and CO_x) leading to asthma and pulmonary diseases in humans (Ball and McCarthy 1988; Domínguez-Escribá and Porcar 2010). Proper treatment and disposal of lignocellulosic wastes is therefore beneficial to both the environment and human health.

Lignocellulosic waste treatment can be carried out using physico-chemical and biological approaches. However, biological approaches are preferred, primarily because they are more environmentally friendly and highly effective (Galbe and Zacchi 2007; Taherzadeh and Karimi 2008; Dashtban et al. 2009; Kumar et al. 2009). In addition to reducing the environmental and health concerns associated with lignocellulosic waste treatment, the reuse of agro-wastes can result in the generation of useful products such as proteins, enzymes, biogas and bioethanol (Das and Singh 2004). The global nature of lignocellulosic biomass makes it a cheap and sustainable source for bioethanol production (Banerjee et al. 2010). Therefore, cellulosic bioethanol can be a suitable alternative to fossil fuel, free from conflict with food production (Galbe and Zacchi 2007). However, the generation of bioethanol and other useful products from LCS at a commercial scale is problematic (Das and Singh 2004; Galbe and Zacchi 2007) as its cellulose, hemicellulose and lignin components are difficult to degrade (Ball and McCarthy 1988; Pérez et al. 2002) with the LCS composition varying from one straw to another (Alvira et al. 2013).

LCS treatment requires pre-treatment designed to disrupt the straws' hemicellulose-lignin complexes (Mosier et al. 2005; Sanchez 2009; Maki et al. 2012) after which enzyme application (saccharification) is carried out. Enzyme application can be an expensive process as large (and costly) enzymatic doses are required to breakdown the polysaccharides (cellulose and hemicellulose) into fermentable sugars (Howard et al. 2004; Jørgensen et al. 2007). The main enzymes used, such as cellulases, hemicellulases, ligninases and other related enzymes are well-reviewed (Pérez et al. 2002; Saha 2003; Wu et al. 2007; Sanchez 2009). The main sources of these enzymes are microorganisms such as bacteria, actinobacteria and fungi (Gusakov 2011). Bacterial cellulases and hemicellulases have been reported to be more effective than those from other microorganisms in terms of ease of culturing, potential for accelerated production and expression systems (Tuomela et al. 2000; Maki et al. 2012; Pandey et al. 2013). Bacterial isolates also show more adaptation and flexibility to different environmental stresses and harsh conditions such as oxygen limitation, temperature variations, salinity and change in pH than fungi (Maki et al. 2012; Pandey et al. 2013). However, to date, there is no report of bacterial isolates that can secrete a complete array of multi-enzyme systems to bioconvert the lignocellulosic biomass components into fermentable sugars effectively (Wang et al. 2012). Currently there are two steps for the screening and isolation of LCS degrading microorganisms. The first is through qualitative assay involving the use of techniques such as agar-plate assay methods while the second is a quantitative step designed to measure the hydrolytic capacities of the crude enzymes produced (Ball and McCarthy 1988; Ibrahim and El-diwany 2007; Brito-Cunha et al. 2013).

This conventional approach however faces many challenges. Agar-plate screening methods are neither sensitive nor quantitative enough because many factors (temperature, moisture, agar concentration and media type) influence the size of the clear zone. This makes deducing a clear correlation between enzyme activity and size of the clear zone difficult (Kasana et al. 2008), necessitating the need for additional flask-based assays for accurate quantitative assessments of the crude enzymes produced by a new bacterial isolate. Unfortunately this process is time consuming, taking around 12–15 days for the crude enzymes to be induced on different lignocellulosic substrates (Brito-Cunha et al. 2013). Consequently, the main disadvantages of the conventional approach include high costs relating to labor and assay consumables and the long time frame required for these assays to be completed. In addition, the traditional approaches also involve the use of potentially toxic chemicals such as dinitrosalicylic acid or arsenate, making them less environmentally friendly resulting in the creation of additional waste management issues (Nelson 1944; Miller 1959; Wu et al. 2007, 2011; Dashtban et al. 2010).

The development of an accurate, rapid and efficient screening assay for highly efficient lignocellulose-degrading bacterial strains is urgently required. Enzyme costs are estimated to be 50 % of the total LCS hydrolysis cost (Howard et al. 2004) and the long time frame required for quantitative assessments is undesirable and presents additional expense for large scale LCS treatment. One method, the use of Biolog (MT2) microplate offers the potential for the direct screening and quantitative assessment of different bacterial strains against a range of carbon sources in a single microplate within a comparatively shorter time-frame compared to traditional assays (Kadali et al. 2012). Biolog (MT2) plates have been successfully used for the screening and evaluation of bacterial strains growing on selected carbon sources (dos Santos et al. 2002; Lu et al. 2008; Kadali et al. 2012). Biolog (MT2) has also been used successfully as a rapid means of identifying bacteria that are able to metabolize microcystin-LR (Manage et al. 2009), biodegrade hydrocarbon fractions (Lu et al. 2008; Kadali et al.

2012) and for acute toxicity screening (dos Santos et al. 2002). However, to the best of our knowledge, there is no report of this tool being used to screen LCS-degrading microorganisms.

Therefore, the main aims of this study were to (1) develop a rapid screening tool that is quantitively accurate, environmentally friendly and cost effective for the screening of bacterial strains capable of straw saccharification; and (2) statistically validate the approach through analysis of the correlation relationship (R^2) between both the traditional assays and Biolog (MT2) microplate-based methods.

Materials and methods

Lignocellulosic straws

Four different lignocellulosic straws (wheat, rice, sugarcane and pea straws) were collected from an Australian market (Adelaide, South Australia), cut into small pieces (10 cm), washed with sterile water and air-dried. All straws were subjected to grinding using a coffee grinder (Breville, Melbourne, Australia) and milled to a fine powder using a ball-mill (Retsch Mixer Mill MM 200, Haan, Germany). Dried ball-milled straw was sterilized at 121 °C for 15 min and kept at 4 °C for further investigations.

Environmental scanning electron microscopy for different straws

Straw specimens were glued on specimen stubs and coated with a thin layer of gold in a sputter coater for 60 s to ensure conductivity (Taherdanak and Zilouei 2014). The straw specimen used for environmental scanning electron microscopy (ESEM) study was placed on 1-cm stubs with the size of the straws ranging from 4 to 10 mm. Ball-milled straw images (from different straw types) were captured digitally using a Quanta 200 Environmental Scanning Electron Microscopy ESEM (FEI, Melbourne, Australia). The ESEM was operated at 3,000 x magnification, high voltage (15 kV), pressure $1.2e^{-6}$ Torr, emission current 96 μA, filament current 2.49 A and the filament voltage was 1.88 V.

Isolation and purification of bacterial strains

Bacterial strains were isolated from soil, compost and straws by selective enrichment medium using a mineral salts medium (BH medium) supplemented with 0.5 % (w/v) of an equal concentration of ball-milled mixed straws (wheat, rice, sugarcane and pea straw) as the sole carbon and energy source (Bushnell and Haas 1941). Bushnell Haas (BH) mineral salts medium containing 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $CaCl_2 \cdot 2H_2O$, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} $(NH_4)_2HPO_4$, 1.0 g L^{-1} $(NH_4)_2SO_4$, 0.05 g L^{-1} $FeCl_3 \cdot 6H_2O$, pH 6.8 was supplemented with the desired carbon source and sterilized at 121 °C for 15 min. The samples were serially diluted (10^{-1} to 10^{-6}) using phosphate buffered saline (PBS, 0.1 M). An aliquot (150 μL) of each dilution was spread onto BH straw-agar plates [agar amended with 0.5 % (w/v) milled-straws]. These plates were incubated for 6 days at 28 °C and 55 °C. Bacterial colonies were purified by repeated sub-culturing before being re-streaked onto BH straw-agar plates to revalidate their abilities to utilize straw as a sole carbon/energy source.

Screening of bacterial isolates for cellulase and xylanase activities

All pure isolates from BH medium (60) were further screened for cellulase and xylanase activities on BH medium agar plates (pH 6.8) supplemented with 0.5 % (w/v) carboxymethycellulose (CMC) or xylan, (Sigma-Aldrich, Melbourne, Australia). The plates were incubated for 4–6 days at the desired temperatures of 28 °C and 55 °C for mesophilic and thermophilic bacterial growth, respectively. To visualize the hydrolysis zone, the plates were stained with Congo red (0.1 %, w/v) for 30 min and then de-stained with NaCl (1 M). The diameter of the clear halo around the bacterial colony was measured (Pandey et al. 2013). The bacterial isolates that had the largest clear-zone (as an indicator of cellulase and xylanase production) were selected (15 isolates each) for quantitative screening and correlation studies. The formation of clear-zone diameter (for cellulases and xylanases) was assessed as follows: ≤ 0 cm^2 no activities detected, 0–1.5 cm^2 "low activities", 1.5–3 cm^2 "medium activities" and ≥ 3 cm^2 "high activities". All pure isolates were stored in 20 % (v/v) glycerol/nutrient broth at −80 °C pending further investigation (Ball and McCarthy 1988). Strawase activities could not be carried out successfully using BH-agar plates with straw mixture as a sole carbon source because of the dark brown color of the straws-mixture.

Preparation of bacterial inoculum

All pure isolates (30) were cultured in nutrient broth (100 mL) for 36 h at 28 °C and 55 °C at 150 rpm or 36 h. Optical density of bacterial cultures was determined at 595 nm (OD_{595}, Pharmacia LKB Ultrospec II Spectrophotometer, Norwich, CT) to obtain an absorbance of 0.9 and used as inoculum for crude enzyme production. To prepare the inoculum for Biolog (MT2) microplates screening, the cells were harvested by centrifugation of the cultures at 4,700 rpm for 10 min at 4 °C. The pellets were washed three times aseptically to remove all nutrients and resuspended in sterilized water to get the optimum concentration ($OD_{595} = 0.9$) (Kadali et al. 2012).

Growth of selected isolates in liquid medium containing lignocellulose for crude enzyme production

Growth of selected isolates for the production of lignocellulolytic enzymes was carried out in Erlenmeyer flasks (150 mL) containing BH medium (100 mL) supplemented with the desired individual ball-milled straw [either 0.5 % (w/v) of wheat or rice or sugarcane or pea straw] as the sole carbon and energy source. The flasks were inoculated with 3 mL of each culture (optimum concentration $OD_{595} = 0.9$). Appropriate controls were without inoculum). All flasks were incubated in triplicate (at either 28 °C or 55 °C) on a rotary shaker at 150 rpm for 12 days. Previous studies have confirmed that 12–15 days of incubation of were required for the crude enzymes to be induced on different lignocellulosic substrates (Brito-Cunha et al. 2013). Samples (5 mL) were collected periodically (0, 3, 6, 9 and 12 days), centrifuged at 4,700 rpm for 20 min at 4 °C and the supernatant sterilized through a 0.22 μm sterile filter. The crude enzyme supernatants were kept at 4 °C for downstream enzymatic assays (cellulase, xylanase and strawase) (Soni et al. 2010).

Enzyme assays

Total cellulase, xylanase and strawase activities were measured based on the release of reducing sugars from different substrates. The substrates used for total cellulase, xylanase and strawase activities were filter paper strips (6 × 1 cm, ~ 60 mg Whatman No. 1), xylan (1 % w/v) and an equal mix of four straw slurry suspensions (1 % w/v of wheat, rice, sugarcane and pea straw) in Na-citrate buffer solution (0.05 M, pH 4.8), respectively. The mixtures were incubated with the desired crude enzyme (0.5 mL withdrawn under sterilized conditions) at 50 °C for 60 min (Ghose 1987; Bailey et al. 1992; Dashtban et al. 2010; Soni et al. 2010). Reducing sugars released by crude enzymes were estimated using the 3,5 dinitrosalicylic acid (DNS) method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar per minute under assay conditions (Ghose 1987; Bailey et al. 1992). Hexose and pentose standards were made using pure glucose and xylose (Sigma-Aldrich) in the range 0–5 mg mL^{-1}.

Biolog (MT2) microplates screening

Traditionally Biolog (MT2) microplates (Biolog, Hayward, CA) are 96-well microplates used for the evaluation and identification of different bacterial species through assessment of the individual bacterial isolate's ability to utilise a range of different carbon substrates (Kadali et al. 2012). Each well contains a buffered nutrient medium (except carbon source) and an equal concentration of tetrazolium violet dye, which is sensitive to the oxidation of a carbon source and bacterial respiration (dos Santos et al. 2002). In this study, the Biolog carbon source substrates supplied were: sterilized slurry suspensions of (1 % w/v in H_2O) cellulose, xylan (from Beechwood; X4252), lignin (alkali; 471003), wheat straw, rice straw, sugarcane straw and pea straw. Aliquots of the individual substrate suspension (40 μL) were loaded into the wells of the Biolog (MT2) microplate. To ensure homogeneous substrate suspension, the slurry was stirred during substrate inoculation into the 96-well microplates in a laminar flow cabinet. The wells (in replicates) were then inoculated with the resuspended bacterial pellet (150 μL). Appropriate controls were set up for each isolate by loading the strain (150 μL) into wells without any carbon source and loading (40 μL) sterilized water instead. Biolog microplates were incubated at 30 °C in the dark for 7 days. The color change was monitored every 2 h using a microtiter plate reader (iMark, Bio-Rad, Richmond, CA) over the 1st day, then every 6 h for the 2nd day and every 24 h from the 3rd day until the end of the experiment. The final OD used was determined by subtraction of the recorded OD_{595} from the test of the initial readings and any increased absorbance found in the control wells (Kadali et al. 2012).

Correlation between Biolog (MT2) microplate and crude enzyme assays

The relationship between the enzyme activities determined (cellulase, xylanase and strawase) using the traditional crude enzyme (after 12 days) and the relevant substrates biodegradation/utilization using the Biolog (MT2) microplate assay (after 7 days) was assessed statistically according to Pearson's correlation coefficient (R^2) using IBM-SPSS (version 21) and Microsoft Excel 2010. All other statistical analyses were carried out with SPSS version 21 using *t*-test or one-way ANOVA for test of significance. Statistical significance was taken at $P \leq 0.05$.

Bacterial DNA extraction, amplification and identification

Genomic DNA was extracted from overnight bacterial cultures grown on nutrient broth using a MoBio DNA extraction kit (MoBio Power Soil, Carlsbad, CA). The extracted DNA (2 μL) was used as a template for PCR amplification using 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1389r (5′-ACG GGC GGT GTG TAC AAG-3′) bacterial primers in a 50 μL PCR Promega mastermix (Promega, Madison, WI) to obtain a product of approximately 1,500 bp (Kadali et al. 2012). The following thermocycling profile was used for

PCR; 1 cycle 5 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 65 °C, 1.5 min at 72 °C; and a final extension at 72 °C for 10 min. PCR amplicons were purified using a PCR clean up kit (Promega), sequenced according to AGRF requirements (http://www.agrf.org.au/) and the sequenced data was edited with Sequencher (version 5.0). The edited data was subject to BLAST analysis on the National Center for Biotechnology Information website (NCBI) (http://www.ncbi.nlm.gov/) for the determination of bacterial putative identities (Kadali et al. 2012). Selected bacterial sequences were aligned to desired sequences from GenBank with CLUSTALW2 (version 2.1) (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and edited with Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) (version 7.2.5). A phylogenetic tree was constructed using the maximum likelihood algorithm and viewed with Tree Dyne in PhyML (http://www.phylogeny.fr/) (version 2) (Dereeper et al. 2008; Adetutu et al. 2012).

Results and discussion

Isolation and purification of bacterial strains (agar-plate screening)

Sixty bacterial isolates were obtained from soil (19), compost (35) and straw (6) samples using a mineral salts medium (BH) containing 0.5 % (w/v) of an equal mix of all four straw types (wheat, rice, sugarcane and pea straw) as the sole carbon and energy source. Previous studies had identified that compost and soil tend to be the main source of lignocellulosic bacteria (Plecha et al. 2013). This explains why the majority of the bacterial isolates (58 %) obtained in this study were from compost. Following purification on BH-straw agar plates, the selected bacterial isolates were further screened on BH medium supplemented with carboxymethycellulose (CMC) and xylan as individual carbon sources. This was carried out to re-validate their cellulolytic (endoglucanase activity) and xylanolytic abilities. The clear-zone diameter method was applied to all the 60 isolates that were able to grow on CMC and xylan plates. Based on the results obtained from the use of the clear-zone formation method, only 30 bacterial isolates (50 %, 15 isolates from each substrate) were selected according to their high cellulase (from 2.2 to 4.4 cm) and xylanase (from 2 to 4.16 cm) activities on CMC and xylan agar-plates, respectively (data not shown).

Earlier studies have shown that the presence of clear zones around the bacterial strains after staining with Congo red or Gram staining is indicative of an isolate's potential for cellulose and xylan degradation (Ibrahim and El-diwany 2007; Maki et al. 2012; Brito-Cunha et al. 2013; Pandey et al. 2013). One advantage of the preliminary screening carried out in this study was that it allowed for a reduction in the number of isolates through the selection of only those strains exhibiting significant zone clearing. Other similar studies using this method have shown that only 20 different bacterial isolates out of 300 strains showed a clear zone on CMC agar-plates as an indicator for cellulase activity (Pandey et al. 2013). Another study reported that, out of 59 isolates (30 bacterial and 29 fungal isolates) qualitatively screened, only 3 showed quantitively high cellulolytic specific activity (U mg^{-1} protein): filter paperase 0.032, CMCase 0.5, cellobiase 0.6 (Boonmee 2009). Therefore, it is not unusual as observed in this study that only 50 % of the total isolates were found to produce substantial levels of cellulase and xylanase enzymes. However, this agar-based screening technique is not aimed at assessing the quantitative degradation of the carbon sources used (CMC and xylan). Consequently, a further quantitative screening is required.

Quantitative assays with traditional crude enzyme production and Biolog (MT2) microplate methods

One of the main challenges in studies of lignocellulosic waste degradation by microorganisms is the length of time taken to validate the lignocellulolytic abilities of a new candidate quantitatively using conventional traditional flask-based assay methods. Currently, it takes between 12 and 15 days (12 days to induce enzyme production and up to 3 days for enzyme assays) to validate this ability in microbial candidates, with associated costs for reagents, standards and labor (Brito-Cunha et al. 2013). Therefore, any screening tool that substantially reduces the time-frame needed for this validation will be advantageous, especially in screening experiments designed for large-scale microbial degradation of lignocellulosic wastes. Consequently, in this study, the 30 bacterial isolates selected for further studies were screened quantitatively using both traditional crude enzyme production and Biolog (MT2) microplate methods. The results obtained from the use of both methods were compared and analyzed statistically using correlation curves (Fig. 1).

Results from the conventional assay showed that the bacterial isolates tested produced a disparate amount of cellulase, xylanase and strawase activities induced on different straws (during the period from 0 to 12 days). The concentrations of enzymes produced by the bacterial isolates are shown in Supplementary Table 1. However, using the Biolog (MT2) microplate only 17 positive isolates were able to utilize at least one of the carbon substrates (cellulose, xylan, lignin, wheat, rice, sugarcane and pea straw) as the sole carbon source (data not shown). In the Biolog (MT2) microplate technique, the "negative isolates" (13 isolates) represented the isolates showing the lowest enzymes activity using the traditional assay

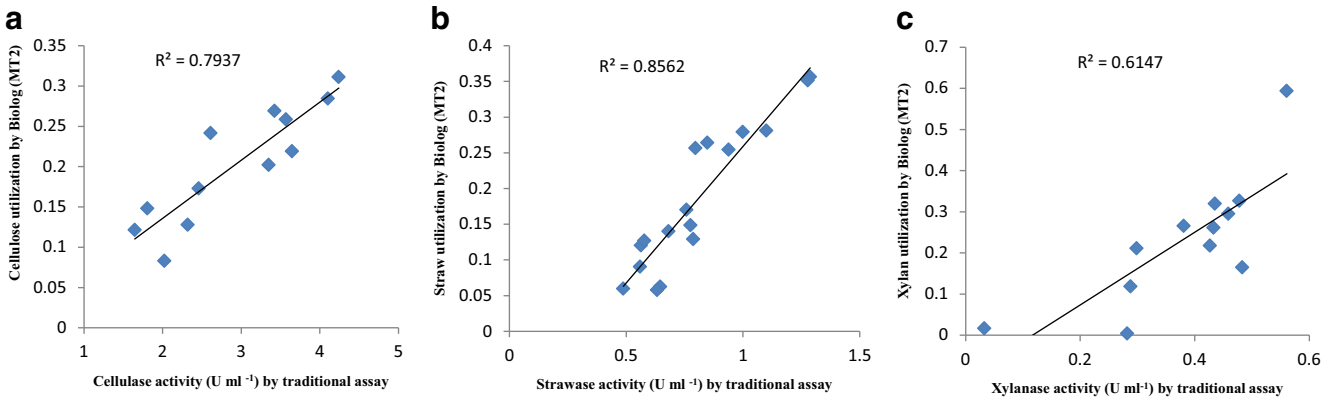

Fig. 1 Correlation coefficient between traditional enzyme assays (**a** cellulase, **b** strawase, **c** xylanase activities after 12 days) and Biolog (MT2) microplate screening assays (utilization of **a** cellulose, **b** straws, **c** xylan as a carbon source after 7 days)

technique (Supplementary Table 1). One advantage of the Biolog (MT2) microplate screening technique appears to be the fact that it is less sensitive than the traditional assay, resulting in the exclusion of inefficient degrader strains. Therefore the technique quickly identifies ineffective strains, thereby reducing quantitative screening time and costs. Nevertheless, a positive and strong correlation exists between both methods in the cellulase, strawase and xylanase activities when the activities of the remaining 17 strains are compared (Fig. 1). The R^2 value describes the strength of the linear relationship between both screening method activities. For all three enzyme assays (cellulase, strawase and xylanase), a positive correlation can be observed with R^2 values of 0.79, 0.86 and 0.61, respectively.

The strong correlation results (Fig. 1) validate the Biolog (MT2) microplate approach; the Biolog (MT2) microplate assay is a cost-effective tool and is about five times cheaper than the traditional assay. Based on the authors' estimates, it costs about AUD$ 0.35 for individual screening using the Biolog microplate compared to AUD$1.8 for the traditional assay. The Biolog assay also requires significantly less labor (it takes about 1 h to set up and only 5–10 min per day to measure the microplates) compared to traditional assays. It requires no hazardous chemicals such as DNS or arsenate, which are toxic to humans and the environment (Wu et al. 2011). Additionally, the Biolog (MT2) microplate offers the potential for the immediate screening of different bacterial strains from different habitats against a range of LCS to be saccharified in a single microplate instead of waiting (12–15 days) for the crude enzyme to be produced.

Quantitative screening of positive bacterial strains by Biolog (MT2) microplate

Having established a significant correlation between the traditional and the Biolog methodologies, further analysis of the Biolog data was carried out. The average of well color development with the different carbon sources utilized (cellulose,

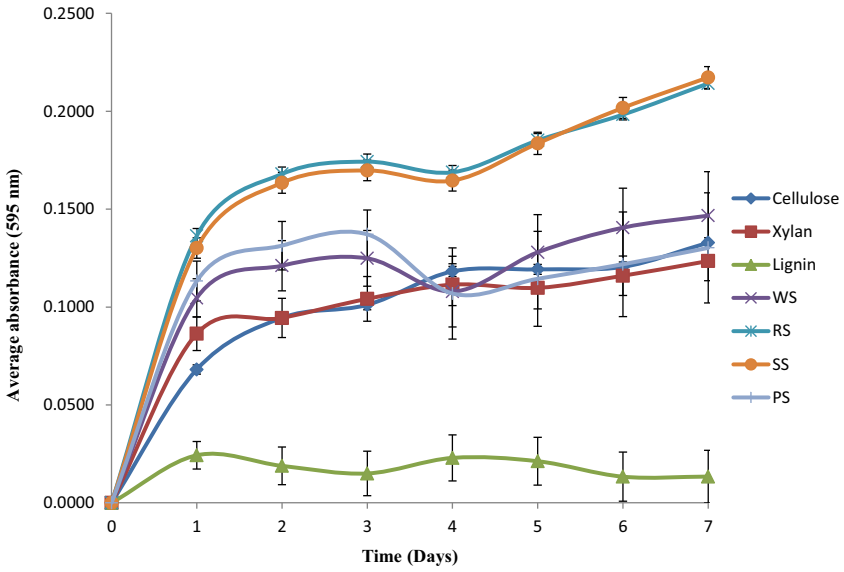

Fig. 2 Average utilization of different carbon sources by bacterial strains (absorbance at 595 nm) using the Biolog (MT2) microplate method. Readings from a total of 17 strains were used as average utilization of ball-milled wheat straw (WS), rice straw (RS), sugarcane straw (SS), pea straw (PS); 12 strains for cellulose and xylan and 5 for lignin

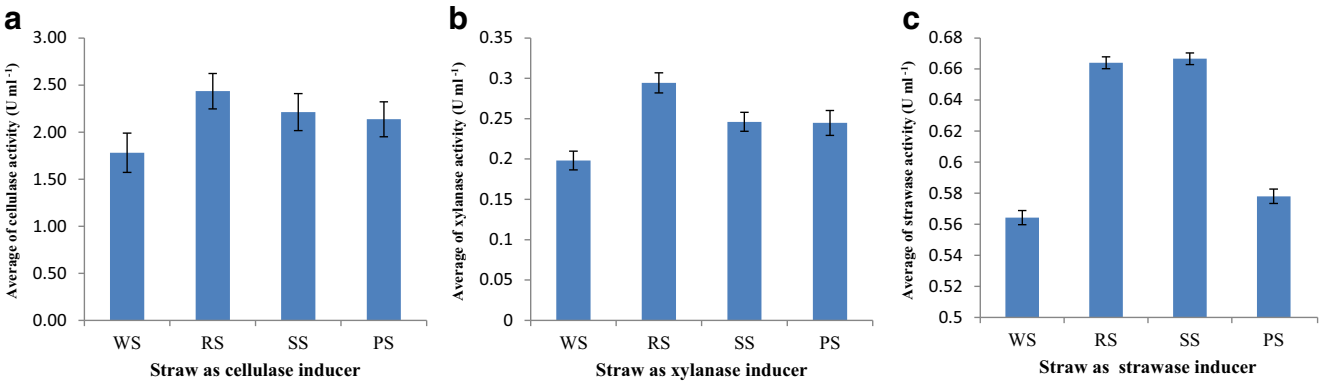

Fig. 3a–c Effect on the enzymes induced of different types of straws (WS, RS, SS and PS) as the sole carbon source, expressed as an average of 30 bacterial strains. **a** Cellulase, **b** xylanase, **c** strawase

xylan, lignin and four different straws) by the 17 isolates differed from one substrate to another (Fig. 2). Overall, the substrate utilization patterns of these micro-organisms differed significantly, suggesting differing approaches to lignocellulosic biodegradation. The utilization patterns of the synthetic substrates (xylan, cellulose and lignin) were observed to be lower than those detected in the natural substrates tested (Fig. 2). The structure of native straws is clearly different from that of the synthetic substrates; it has been suggested that the concentration of soluble inducers, which enhance the production of lignocellulose-degrading enzymes, is greater in lignocellulosic material than in the synthetic substrates (Obruca et al. 2012). Previous research suggested that the mineralization activities of microbial isolates on different native straws were often greater than those observed in the same isolates grown on synthetic substrates such as cellulose, xylan and lignin (Obruca et al. 2012).

As expected, lignin was the least utilized substrate as a sole carbon source followed by xylan and cellulose (Fig. 2). The extent of lignin utilization by the isolates in this study was significantly lower than that observed in the remaining synthetic compounds and the different straw types ($P \leq 0.05$). A previous study had reported that the biodegradation of filter paper was reduced by 60 % when lignin (15 % w/v) was added to the substrate (Sewalt et al. 1997). Lignin is an aromatic, biopolymer and very resistant to physical and microbial attack (Pérez et al. 2002). Very few bacterial isolates have the capability to break lignin down into its aromatic monomers (Zimmermann 1990). The biodegradation patterns of both cellulose and xylan were similar and not significantly different from each other ($P \leq 0.05$), although the biodegradation of cellulose was slightly better than that of xylan (a non-crystalline substrate). The reason might be because xylan is a more complex and branched structure compared to

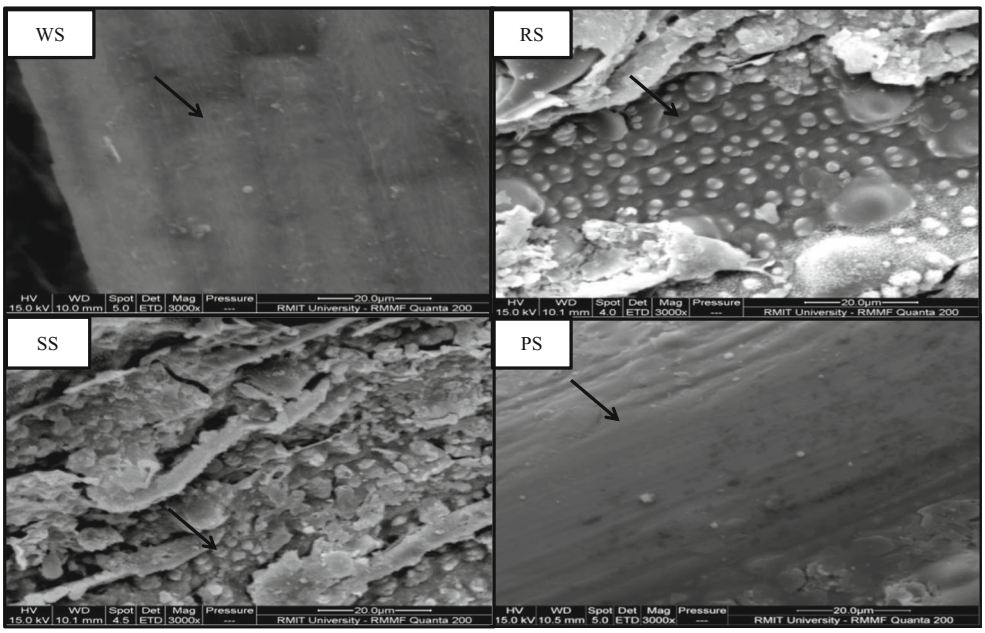

Fig. 4 Environmental scanning electron microscopy (ESEM) images of gold-coated ball-milled WS, RS, SS and PS. The *arrows* indicate the compact rigid structure in WS and PS and the distorted structure and visible cellulosic microfibrils in the structural network of the silica backbone in RS and SS. *WS* wheat straw, *RS* rice straw, *SS* sugarcane straw and *PS* Pea straw

cellulose; it requires a variety of different hydrolytic enzymes working synergistically for effective degradation (Pérez et al. 2002; Saha 2003; Alvira et al. 2013).

Sugarcane and rice straw samples had the greatest carbon source utilization levels, suggesting that ball-milled sugarcane and rice straws were degraded more readily than other straws (Fig. 2). The biodegradation of sugarcane and rice straw samples was significantly better than that observed in wheat and pea straw ($P \leq 0.05$). This may be due to the fact that the lignin (11.9 % w/w) and hemicellulose (25 % w/w) content of rice straw is reported to be almost half of the content in wheat straw (23.4 and 50 % w/w, respectively). This could have contributed to the significant increase observed in the biodegradability of rice straw ($P \leq 0.05$; Fig. 2) (Saha 2003; Jørgensen et al. 2007). The presence and the distribution of hemicellulose and lignin also inhibit the hydrolysis of certain lignocellulosic straws. Lignin can adsorb up to 60–70 % of the total enzymes produced, thereby impinging on their action and decreasing the biodegradation of certain LCS (Jørgensen et al. 2007).

The same trend was also observed in the traditional crude enzyme assay; both rice and sugarcane straws induced more hydrolytic enzymes (cellulase, xylanase and strawase) than wheat and pea straws (Fig. 3). The microbial treatment of sugarcane waste using four different bacterial strains also resulted in an increase (10-fold) in cellulase production using *Cellulomonas uda*, which enhanced the bioconversion of sugarcane waste into fermentable sugars (Singh et al. 2008). A similar trend has also been reported in fungi by Azzaz (2013), who showed that *Aspergillus flavus* grown on rice straw induced crude cellulases more successfully than when the fungus was grown on wheat straw, corn stalk or banana waste. This was because different native lignocellulosic structures influenced the induction of different hydrolytic enzymes and therefore the induction patterns varied (Azzaz 2013). Also, the generation of inhibitors can vary from one straw to another and may influence substantially the biodegradation of straws. For example, the concentration of cellulase inhibitors of *Trichoderma reesei* increased when wheat straw was used as an inducer when compared to rice straw (Holtzapple et al. 1990).

Analyses of straw surface by ESEM

From the data analyses of both the Biolog (MT2) microplate and the traditional crude enzyme assays, it was shown that ball-milled rice and sugarcane straws were more degradable and induced more enzymes than wheat and pea straws. Generally the physical pre-treatment (ball-mill) of the four straws increased the surface area available for enzymatic and microbial attack, as well as probably causing partial depolymerization of the cellulose and hemicellulose fractions. This would alter the physical structure of the straws by opening the lignin barrier (physical barriers) and reducing the cellulose crystallinity, leading to increased susceptibility to bacterial

Table 1 Bacterial identification of strains and the utilization pattern at day 7 of different carbon sources (1 % w/v cellulose, xylan and straws) using the Biolog (MT2) microplate method at 595 nm

Strain	Strain ID	Bacterial group	Accession No.	Identity (%)	Biolog (MT2) carbon source utilization		
					Cellulose	Xylan	Straw
RMIT1	*Klebsiella variicola* strain XF16	Proteobacteria	KC853308.1	100	0.121±0.0025	0.593±0.1745[a]	0.062±0.0205
RMIT2	*Paenibacillus favisporus* strain GMP03	Firmicutes	AY308758.1	100	0.173±0.0127	0.022±0.0116	0.139±0.0173
RMIT3	*Pseudomonas* sp. MML1910	Proteobacteria	JQ336959.1	100	0.083±0.0032	0.211±0.0058	0.170±0.0169
RMIT4	*Klebsiella oxytoca* strain GSC12206	Proteobacteria	KC139460.1	100	0.128±0.0152	0.326±0.0133	0.356±0.0095[a]
RMIT5	*Bacillus thuringiensis* strain BGB20	Firmicutes	KC778385.1	100	0.258±0.0088	0.125±0.0064	0.279±0.0041
RMIT6	*Brevibacterium frigoritolerans* strain BGa5	Firmicutes	KF387713.1	100	0.311±0.0072[a]	0.164±0.0153	0.090±0.0439
RMIT7	*Bacillus amyloliquefaciens*	Firmicutes	KF475870.1	100	0.241±0.0040	0.161±0.0253	0.351±0.0318
RMIT8	*Bacillus licheniformis* strain SCDB 1234	Firmicutes	KF158801.1	100	0.202±0.0000	0.265±0.0767	0.264±0.0089
RMIT9	*Bacillus* sp. PS23(2013)	Firmicutes	KC422649.1	100	0.284±0.0039	0.095±0.0132	0.281±0.0070

[a] Highest utilization of each substrate

or enzymatic attack (Ball and McCarthy 1988; Galbe and Zacchi 2007; Taherzadeh and Karimi 2008; Kumar et al. 2009). This pre-treatment does not require the addition of chemicals that generate inhibitors to enzymes or microorganisms. This process can also be achieved at a commercial scale for the production of large quantities of fine powder (Jørgensen et al. 2007). However, the effect of the ball-milled pre-treatment on the different straws surface is different and may affect the biodegradability of different straws. Therefore, the characteristic differences in the ball-milled straws' surface were investigated by ESEM. As seen in the ESEM micrograph presented in Fig. 4, wheat straw (WS) and pea straw (PS) have a compact rigid structure and the cellulosic fibrils were highly ordered (and not visible) in comparison to rice straw (RS) and sugarcane straw (SS). The interwoven cellulose microfibrils in WS and PS are embedded in the hemicellulose-lignin complex polymers (and were also not visible) and may cause biodegradability difficulties (Fig. 4) (Taherdanak and Zilouei 2014). However, rice straw (RS) and sugarcane straw (SS) exhibited visible cellulosic microfibrils and the structural network of the silica (SiO_2) backbone which therefore increases the external surface area and its porosity, with the hydrolysis efficiency being increased. The content of silica differs from one straw to another. RS is known to have the highest silica content (74 % w/w) followed by SS (70 % w/w) and WS has the lowest silica content (55 % w/w) (Frias et al. 2007; Binod et al. 2010). Silica can act as a crystalline quartz during the ball-milled process leading to distortion of the straw structure and therefore increasing the biodegradation of RS and SS more than WS (Fig. 2) (Prasanna and Patel 1987).

Lignocellulosic bacterial species

From the Biolog data analyses, 9 isolates were selected (representing the most active isolates) from the 17 positive bacterial isolates. Given their commercial potential, the identity of the 9 strains was determined through the use of bacterial primers. The identities of these bacteria together with their activities as assessed using the Biolog system are presented in Table 1; a phylogenetic tree constructed based on their alignment with strains from GenBank is shown in supplementary Figure 1. The phylogenetic tree showed that these isolates belonging to two phyla: Firmicutes and Proteobacteria. From previous studies it has been reported that isolates in these phyla are involved in the biodegradation of lignocellulose substrates (Archana and Satyanarayana 1997; Bandounas et al. 2011; Maki et al. 2012; Obruca et al. 2012; Pandey et al. 2013).With regards to the identification of isolates belonging to the Firmicutes, different *Bacillus* species were identified using the 63f and 1389r primers used in a number of studies (Lucas et al. 2003; Ali et al. 2014). However, accurate discrimination of different *Bacillus* species would require the use of gyrase A and B genes (Chun and Bae 2000; Huang et al. 2012). Therefore, future studies should include a gyrase-genes-based PCR assay in addition to 63f-1389r PCR assays for *Bacillus* species. All nine selected isolates were able to degrade or utilize the individual non-synthetic straws, synthetic substrates such as cellulose and xylan, demonstrating their versatility (Fig. 5). However, only four isolates, RMIT1, RMIT5, RMIT7 and RMIT 8, were able to utilize lignin (Fig. 5). Lignin is especially difficult to utilize or break down due to its highly stable chemical structure, which is composed of aromatic compounds (Pérez et al. 2002).

Although all the bacterial isolates were able to use cellulose as a carbon source, two bacterial strains, RMIT6 (*Brevibacterium frigoritolerans*) and RMIT9 (*Bacillus* sp.) were found to be the highest degraders (utilizers) of cellulose as a sole carbon and energy source over the 7-day experiment. One of the isolates, *Brevibacterium* sp. is a well-known cellulose-degrading species (Haruta et al. 2002). The same isolates (RMIT6 and RMIT9) were amongst the best cellulase-

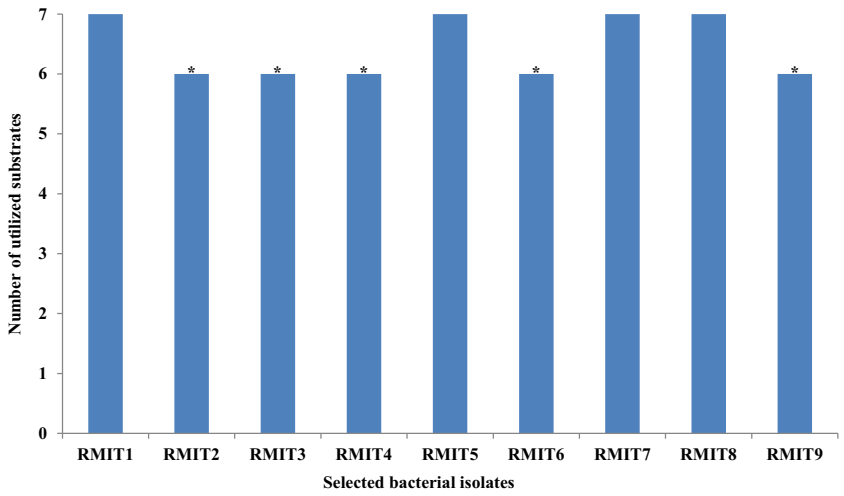

Fig. 5 Utilization of synthetic and non-synthetic substrates by selected bacterial isolates. Synthetic substrates were cellulose, xylan and lignin while non-synthetic substrates were ball-milled WS, RS, SS and PS. *Asterisks* - Isolates unable to degrade synthetic lignin

producing bacteria when screened by the traditional assay method (Supplementary Table 1). In contrast, isolates RMIT4 (*Klebsiella oxytoca*) and RMIT7 (*Bacillus amyloliquefaciens*) were able to utilize cellulose moderately, yet were still capable of growth on lignocellulose (Table 1). Strain RMIT1 (*Klebsiella variicola*) was able to utilize xylan as the sole carbon source more effectively than other isolates (Table 1) and was also classified as the best xylanase producer in the conventional crude enzyme production (Supplementary Table 1) (Saratale et al. 2013). A recent study developed a co-culture system by applying *Bacillus* sp. and *Klebsiella oxytoca* for enhancing ethanol production from lignocellulosic materials (Tran and Lin 2013). It was therefore not unexpected that *Bacillus* sp. (RMIT5, 7, 8 and 9) were putatively identified as lignin or cellulosic waste degrading isolates in this study (Table 1, Fig. 5).

Having used an efficient and cost effective Biolog MT2 microplate based screening method to select an array of lignocellulosic-waste-degrading microorganisms, future studies should involve the use of these isolates for the saccharification of these wastes in laboratory-based and large-scale studies. In addition, there is a need to assess whether the induction of the desired enzymes from these isolates can be improved by co-culturing or using a consortium of these microorganisms (which mimics what happens in the natural biodegradation of agro-wastes). Some studies have shown that the use of microbial co-cultures can lead to improved degradation of different kinds of wastes (Pohlschroeder et al. 1994; Chou et al. 2011), although only limited information regarding the use of a *Bacillus* sp. consortium for lignin and cellulose containing wastes appears in the literature.

Conclusions

This study has demonstrated that the Biolog (MT2) microplate technology developed here represents a cheap, environmental friendly and effective tool for the rapid screening and quantitative assessment of bacterial species capable of degrading different types of lignocellulosic straws. Results from traditional crude enzyme assays and Biolog (MT2) microplate methods were compared and a significant correlation between both methods was established. Differences between the degradability of different straws material were consistent with differences in the surface structure of these different straws after ball milling. The bacterial species with substantial LCS-degrading abilities detected in these studies belonged to two different phyla, namely Proteobacteria and Firmicutes.

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