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59	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 (degradation into fermentable monomeric sugar molecules). 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. This confirmed the suitability of Biolog microplate-based assays as an alternative screening method for lignocellulolytic bacteria. 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.

60	Keywords separated by ' - '	Biolog (MT2) microplate - Lignocellulose degradation - Correlation analysis - Ball-milled straw - Bacterial screening
61	Foot note information	<ul style="list-style-type: none">• Traditional enzyme assay (lignocellulosic waste) is time-, cost- and labor-consuming• BIOLOG microplates are a cheaper, faster and more effective tool for screening bacteria• Significant correlation observed between traditional and Biolog (MT2) plate assays• Biolog plate successfully used to quantitatively screen lignocellulolytic bacteria• Biolog plate successfully used to assess microbial degradation of different straws

The online version of this article (doi:10.1007/s13213-015-1044-y) contains supplementary material, which is available to authorized users.

Electronic supplementary material

Supplementary Table 1

Enzyme activities (cellulase, xylanase and strawase U mL⁻¹) of the 30 different bacterial isolates using the traditional crude enzyme production assay method (DOCX 949 kb)

Supplementary Figure 1

Phylogenetic tree of selected bacterial isolates. Note: tree was constructed from 1,000–1,200 nucleotide positions. Distances were calculated with the maximum likelihood model using PhyML. Bootstrap values > 0.50 are shown. *Asterisks* ~~Lowest~~ enzyme activity (based on strawase assay) by the traditional assays and to the “negative isolates” by Biolog (MT2) microplates.(DOCX 18 kb)

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 (degradation into fermentable monomeric sugar molecules). 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. This confirmed the suitability of Biolog microplate-based assays as an alternative screening method for lignocellulolytic bacteria. 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 bioconverted to monosaccharides 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.

Highlights

- Traditional enzyme assay (lignocellulosic waste) is time-, cost- and labor-consuming
- BIOLOG microplates are a cheaper, faster and more effective tool for screening bacteria
- Significant correlation observed between traditional and Biolog (MT2) plate assays
- Biolog plate successfully used to quantitatively screen lignocellulolytic bacteria
- Biolog plate successfully used to assess microbial degradation of different straws

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

Introduction

The annual production 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

58 produced worldwide (Chandrakant and Bisaria 1998;
 59 Tuomela et al. 2000). In fact, it has been estimated that about
 60 200 billion tons of lignocellulosic materials are produced an-
 61 nually (Michelin et al. 2013). Lignocellulosic biomass is a
 62 major component of plant materials, and is composed primar-
 63 ily of structural carbohydrates such as cellulose and hemicel-
 64 lulose surrounded by lignin. This complex combination of
 65 biopolymers renders lignocellulosic materials less biodegrad-
 66 able (Ball and McCarthy 1988; Jørgensen et al. 2007).
 67 Lignocellulosic straws (LCS) or materials can cause serious
 68 air pollution when subjected to open-air burning. This process
 69 reduces local air quality, creating a variety of health concerns
 70 from the discharge of carcinogenic oxides (NO_x , SO_2 and
 71 CO_x) leading to asthma or pulmonary morbidity in humans
 72 (Ball and McCarthy 1988; Domínguez-Escribá and Porcar
 73 2010). ~~Accumulation of these wastes can also result in aes-~~
 74 ~~thetic insult.~~ Proper treatment and disposal of lignocellulosic
 75 wastes is therefore beneficial to both the environment and
 76 human health.

77 Lignocellulosic waste treatment can be carried out using
 78 physico-chemical and biological approaches. However, bio-
 79 logical approaches are preferred, primarily because they are
 80 more environmentally friendly and ~~can result in significant~~
 81 ~~biodegradation of lignocellulosic residues~~ (Galbe and Zacchi
 82 2007; Taherzadeh and Karimi 2008; Dashtban et al. 2009;
 83 Kumar et al. 2009). In addition to ~~eliminating or~~ reducing
 84 the environmental and health concerns associated with ligno-
 85 cellulosic waste treatment, the reuse of ~~these~~ agro-wastes can
 86 result in the generation of useful products such as proteins,
 87 enzymes, biogas and bioethanol (Das and Singh 2004). The
 88 global nature of lignocellulosic biomass makes it a cheap and
 89 sustainable source for bioethanol production (Banerjee et al.
 90 2010). Therefore, cellulosic bioethanol can be a suitable alter-
 91 native to fossil fuel, free from conflict with food production
 92 (Galbe and Zacchi 2007). However, the generation of
 93 bioethanol and other useful products from LCS at a commer-
 94 cial scale ~~faces a great challenge; the bioconversion step (en-~~
 95 ~~zymatic hydrolysis or saccharification) of lignocellulosic bio-~~
 96 ~~polymers into reducing sugars~~ is problematic (Das and Singh
 97 2004; Galbe and Zacchi 2007) as its cellulose, hemicellulose
 98 and lignin components are difficult to degrade (Ball and
 99 McCarthy 1988; Pérez et al. 2002). ~~The varying chemical~~
 100 ~~composition of LCS from one straw to another can also have~~
 101 ~~a detrimental impact on straw biodegradation~~ (Alvira et al.
 102 2013).

103 LCS treatment requires pre-treatment designed to disrupt
 104 the straws' hemicellulose-lignin complexes (Mosier et al.
 105 2005; Sanchez 2009; Maki et al. 2012) after which enzyme
 106 application (saccharification) is carried out. Enzyme applica-
 107 tion can be an expensive process as large (and costly) enzy-
 108 matic doses are required to breakdown the polysaccharides
 109 (cellulose and hemicellulose) into fermentable sugars
 110 (Howard et al. 2004; Jørgensen et al. 2007). The main

111 enzymes used, such as cellulases, hemicellulases, ligninases
 112 and other related enzymes, are well-reviewed (Pérez et al.
 113 2002; Saha 2003; Wu et al. 2007; Sanchez 2009). The main
 114 sources of these enzymes are microorganisms such as bacteria,
 115 actinobacteria and fungi (Gusakov 2011). Bacterial cellulases
 116 and hemicellulases have been reported to be more effective
 117 than those from other microorganisms in terms of ease of
 118 culturing, potential for accelerated production and expression
 119 systems (Tuomela et al. 2000; Maki et al. 2012; Pandey et al.
 120 2013). Bacterial isolates also show more adaptation and flex-
 121 ibility to different environmental stresses and harsh conditions
 122 such as oxygen limitation, temperature variations, salinity and
 123 change in pH than fungi (Maki et al. 2012; Pandey et al.
 124 2013). However, to date, there is no report of bacterial isolates
 125 that can secrete a complete array of multi-enzyme systems to
 126 bioconvert the lignocellulosic biomass components into fer-
 127 mentable sugars effectively (Wang et al. 2012). Currently
 128 there are two steps for the screening and isolation of ~~micro-~~
 129 ~~organisms capable of breaking down LCS into monomeric~~
 130 ~~sugars~~. The first is through qualitative assay involving the
 131 use of techniques such as agar-plate assay methods while the
 132 second is a quantitative step designed to measure the hydro-
 133 lytic capacities of the crude enzymes produced (Ball and
 134 McCarthy 1988; Ibrahim and El-diwany 2007; Brito-Cunha
 135 et al. 2013).

136 This conventional approach however faces many chal-
 137 lenges. Agar-plate screening methods are neither sensitive
 138 nor quantitative enough because many factors (temperature,
 139 moisture, agar concentration and media type) influence the
 140 size of the clear zone. This makes deducing a clear correlation
 141 between enzyme activity and size of the clear zone difficult
 142 (Kasana et al. 2008), necessitating the need for additional
 143 flask-based assays for accurate quantitative assessments of
 144 the crude enzymes produced by a new bacterial isolate.
 145 Unfortunately this process is time consuming, taking around
 146 12–15 days for the crude enzymes to be induced on different
 147 lignocellulosic substrates (Brito-Cunha et al. 2013).
 148 Consequently, the main disadvantages of the conventional ap-
 149 proach include high costs relating to labor and assay consum-
 150 ables and the long time frame required for these assays to be
 151 completed. In addition, the traditional approaches also involve
 152 the use of potentially toxic chemicals such as dinitrosalicylic
 153 acid or arsenate, making them less environmentally friendly
 154 resulting in the creation of additional waste management is-
 155 sues (Nelson 1944; Miller 1959; Wu et al. 2007, 2011;
 156 Dashtban et al. 2010).

157 The development of an accurate, rapid and efficient screen-
 158 ing assay for highly efficient lignocellulose-degrading bacte-
 159 rial strains is urgently required. Enzyme costs are estimated to
 160 be 50 % of the total LCS hydrolysis cost (Howard et al. 2004)
 161 and the long time frame required for quantitative assessments
 162 is undesirable and presents additional expense for large scale
 163 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) has been used successfully 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 quantitatively 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 1.2×10^{-6} Torr, emission current 96 μ A, filament current 2.49 A and the filament voltage was 1.88 V.

Isolation and purification of bacterial strains

Bacterial species 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 MgSO₄·7H₂O, 0.02 g L⁻¹ CaCl₂·2H₂O, 1.0 g L⁻¹ KH₂PO₄, 1.0 g L⁻¹ (NH₄)₂HPO₄, 1.0 g L⁻¹ (NH₄)₂SO₄, 0.05 g L⁻¹ FeCl₃·6H₂O, pH 6.8 was prepared as a selective medium 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 μ L) 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 for mesophilic and thermophilic bacterial growth, respectively. 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 strains isolated from BH medium (60 isolates) were further screened for cellulase and xylanase activities on BH medium agar plates (pH 6.8) supplemented with 0.5 % (w/v) carboxymethylcellulose (CMC) or xylan, (Sigma-Aldrich, Melbourne, Australia). 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. 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 strains that had the highest 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 cm² no activities detected, 0–1.5 cm² “low activities”, 1.5–3 cm² “medium activities” and > 3 cm² “high activities”. All pure isolates were stored in 20 % (v/v) glycerol/nutrient broth at –80 °C pending further investigation (Ball and McCarthy 1988).

Preparation of bacterial inoculum

All pure isolates (30 strains) were cultured in nutrient broth (liquid broth for culturing bacteria; 100 mL) for 36 h at 28 °C

258	and 55 °C for mesophiles and thermophiles, respectively, at	assay conditions (Ghose 1987; Bailey et al. 1992). Hexose	307
259	150 rpm. Optical density (OD ₅₉₅) of bacterial cultures was	and pentose standards were made using pure glucose and xy-	308
260	determined (after normalization) at 595 nm (Pharmacia LKB	lose (Sigma-Aldrich) in the range 0–5 mg mL ⁻¹ .	309
261	Ultrospec II Spectrophotometer, Norwich, CT) to <u>attain</u> absor-		
262	bance of 0.9, and harvested by centrifugation at 4,700 rpm for		
263	10 min at 4 °C. Adjustment was made when necessary by the	Biolog (MT2) microplates screening	310
264	addition of bacterial colonies or sterilized water. The pellets		
265	were washed three times aseptically to remove all nutrients	Traditionally Biolog (MT2) microplates (Biolog, Hayward,	311
266	and resuspended in sterilized water to get the optimum con-	CA) are 96-well microplates used for the evaluation and iden-	312
267	centration (0.9 at 595 nm) for use in the traditional crude	tification of different bacterial species through assessment of	313
268	enzyme assays and Biolog (MT2) microplate methods	the individual bacterial isolate's ability to utilise a range of	314
269	(Kadali et al. 2012).	different carbon substrates (Kadali et al. 2012). Each well	315
		contains a buffered nutrient medium (except carbon source)	316
270	Growth of selected strains in liquid medium containing	and an equal concentration of tetrazolium violet dye, which is	317
271	lignocellulose for crude enzyme production	sensitive to the oxidation of a carbon source and bacterial	318
		respiration (dos Santos et al. 2002). In this study, the Biolog	319
272	Growth of selected strains for the production of	carbon source substrates supplied were; sterilized slurry sus-	320
273	lignocellulolytic enzymes was carried out in Erlenmeyer	pensions of (1 % w/v in H ₂ O) cellulose, xylan (from	321
274	flasks (150 mL) containing BH medium (100 mL) supple-	Beechwood; X4252), lignin (alkali; 471003), wheat straw,	322
275	mented with the desired individual ball-milled straw [either	rice straw, sugarcane straw and pea straw. Aliquots of the	323
276	0.5 % (w/v) of wheat or rice or sugarcane or pea straw] as	individual substrate suspension (40 µL) were loaded into the	324
277	the sole carbon and energy source. The flasks were inoculated	wells of the Biolog (MT2) microplate. To ensure homoge-	325
278	with the inoculum (3 mL) of each strain (optimum concentra-	neous substrate suspension, the slurry was stirred during sub-	326
279	tion 0.9 at 595 nm) together with appropriate controls (without	strate inoculation into the 96-well microplates in a laminar	327
280	inoculum). All flasks were incubated in triplicate (at either	flow cabinet. The wells (replicated three times) were then	328
281	28 °C or 55 °C) on a rotary shaker at 150 rpm for 12 days.	inoculated with the resuspended bacterial pellet (150 µL).	329
282	Previous studies have confirmed that 12–15 days of incuba-	Appropriate controls were set up for each isolate by loading	330
283	tion of were required for the crude enzymes to be induced on	the strain (150 µL) into wells without any carbon source and	331
284	different lignocellulosic substrates (Brito-Cunha et al. 2013).	loading (40 µL) sterilized water instead. Biolog microplates	332
285	Samples (5 mL) were collected periodically (0, 3, 6, 9 and	were incubated at 30 °C in the dark for 7 days. The color	333
286	12 days), centrifuged at 4,700 rpm for 20 min at 4 °C and	change was monitored every 2 h using a microtiter plate	334
287	the supernatant sterilized through a 0.22 µm sterile filter.	reader (iMark, Bio-Rad, Richmond, CA) over the 1st	335
288	The crude enzyme supernatants were kept at 4 °C for down-	day, then every 6 h for the 2nd day (or as required)	336
289	stream enzymatic assays (cellulase, xylanase and strawase)	and every 24 h from the 3rd day until the end of the	337
290	(Soni et al. 2010).	experiment. The final OD used was determined by sub-	338
		traction of the recorded OD ₅₉₅ from the test of the ini-	339
291	Enzyme assays	tial readings and any increased absorbance found in the	340
		control wells (Kadali et al. 2012).	341
292	Total cellulase, xylanase and strawase activities were mea-		
293	sured based on the release of reducing sugars from different	Correlation between Biolog (MT2) microplate and crude	342
294	substrates. The substrates used for total cellulase, xylanase	enzyme assays	343
295	and strawase activities were filter paper strips (6 × 1 cm~		
296	60 mg Whatman No.1) , xylan (1 % w/v) and an equal mix	The relationship between the enzyme activities determined	344
297	of four straw slurry suspensions (1 % w/v of wheat, rice,	(cellulase, xylanase and strawase) using the traditional crude	345
298	sugarcane and pea straw) in Na-citrate <u>buffer</u> (0.05 M,	enzyme (after 12 days) and the relevant substrates	346
299	pH 4.8), respectively. The mixtures were incubated with the	biodegradation/utilization using the Biolog (MT2) microplate	347
300	desired crude enzyme (0.5 mL withdrawn under sterilized	assay (after 7 days) was assessed statistically according to	348
301	conditions) at 50 °C for 60 min (Ghose 1987; Bailey et al.	Pearson's correlation coefficient (<i>R</i> ²) using IBM-SPSS (ver-	349
302	1992; Dashtban et al. 2010; Soni et al. 2010). Reducing sugars	sion 21) and Microsoft Excel 2010. All other statistical anal-	350
303	released by crude enzymes were estimated using the 3,5	yses were carried out with SPSS version 21 using <i>t</i> -test or one-	351
304	dinitrosalicylic acid (DNS) method (Miller 1959). One unit	way ANOVA for test of significance. Statistical significance	352
305	of enzyme activity was defined as the amount of enzyme	was taken at <i>P</i> ≤ 0.05.	353
306	required to liberate 1 µmol reducing sugar per minute under		

354 Bacterial DNA extraction, amplification and identification

355 Genomic DNA was extracted from overnight bacterial cul- 402
 356 tures grown on nutrient broth using a MoBio DNA extraction 403
 357 kit (MoBio Power Soil, Carlsbad, CA). The extracted DNA 404
 358 (2 µL) was used as a template for PCR amplification using 63f 405
 359 (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1389r (5'- 406
 360 ACG GGC GGT GTG TAC AAG-3') bacterial primers in a 407
 361 50 µL PCR Promega mastermix (Promega, Madison, WI) to 408
 362 obtain a product of approximately 1,500 bp (Kadali et al. 409
 363 2012). ~~The PCR reaction mixture (50 µL) was composed of~~ 410
 364 ~~27.75–29.75 µL sterile water, 10 µL GoTaq flexi buffer (10×),~~ 411
 365 ~~3–5 µL MgCl₂ (25 mM), 1 µL dNTPs mixture (2 mM), 2 µL~~ 412
 366 ~~of each primer (10 µM) and 0.25 µL Taq DNA polymerase~~ 413
 367 ~~enzyme (5 U/µL).~~ The following thermocycling profile was 414
 368 used for PCR; 1 cycle 5 min at 95 °C; 30 cycles of 1 min at 415
 369 95 °C, 1 min at 65 °C, 1.5 min at 72 °C; and a final extension 416
 370 at 72 °C for 10 min. PCR amplicons were purified using a 417
 371 PCR clean up kit (Promega), sequenced according to AGRF 418
 372 requirements (<http://www.agrf.org.au/>) and the sequenced 419
 373 data was edited with Sequencher (version 5.0). The edited 420
 374 data was subject to BLAST analysis on the National Center 421
 375 for Biotechnology Information website (NCBI) ([http://www.](http://www.ncbi.nlm.gov/) 422
 376 [ncbi.nlm.gov/](http://www.ncbi.nlm.gov/)) for the determination of bacterial putative 423
 377 identities (Kadali et al. 2012). Selected bacterial sequences 424
 378 were aligned to desired sequences from GenBank with 425
 379 CLUSTALW2 (version 2.1) ([http://www.ebi.ac.uk/Tools/](http://www.ebi.ac.uk/Tools/msa/clustalw2/) 426
 380 [msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) and edited with Bioedit ([http://www.mbio.](http://www.mbio.ncsu.edu/bioedit/bioedit.html) 427
 381 [ncsu.edu/bioedit/bioedit.html](http://www.mbio.ncsu.edu/bioedit/bioedit.html)) (version 7.2.5). A 428
 382 phylogenetic tree was constructed using the maximum 429
 383 likelihood algorithm and viewed with Tree Dyne in PhyML 430
 384 (<http://www.phylogeny.fr/>) (version 2) (Dereeper et al. 2008; 431
 385 Adetutu et al. 2012). 432

386 Results and discussion

387 Isolation and purification of bacterial strains (agar-plate 433 388 screening) 434

389 Sixty bacterial strains were obtained from soil (19), compost 435
 390 (35) and straw (6) samples using a mineral salts medium (BH) 436
 391 containing 0.5 % (w/v) of an equal mix of all four straw types 437
 392 (wheat, rice, sugarcane and pea straw) as the sole carbon and 438
 393 energy source. Previous studies had identified that compost 439
 394 and soil tend to be the main source of lignocellulosic bacteria 440
 395 (Plecha et al. 2013). This explains why the majority of the 441
 396 bacterial isolates (58 %) obtained in this study were from 442
 397 compost. Following purification on BH-straw agar plates, 443
 398 the selected bacterial isolates were further screened on BH 444
 399 medium supplemented with carboxymethylcellulose (CMC) 445
 400 and xylan as individual carbon sources. This was carried out 446
 401 to re-validate their cellulolytic (endoglucanase activity) and 447
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xylanolytic abilities. The clear-zone diameter method was ap-
 plied 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 strains (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 quantitatively high cellulolytic specific activity; Filter
 Paperase 0.032, CMCase 0.5, Cellobiase 0.6, unit mg⁻¹ protein (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
 the desired 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).

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)

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 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 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 also requires no hazardous chemicals such as DNS or arsenate, which are toxic to the humans and the environment (Wu et al. 2011). Additionally, the Biolog (MT2) microplate offers the potential

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

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, 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 purified

artificial 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 artificial 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 purified artificial 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 artificial 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

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.

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 ($P\leq0.05$), 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 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 ($P\leq0.05$). 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 ($P\leq0.05$; 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 (Holtzaple et al. 1990).

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			References
						Cellulose	Xylan	Straw	
t1.1	Q1	RMIT1	Proteobacteria	KC853308.1	100	0.121±0.0025	0.593±0.1745 ^a	0.062±0.0205	(Waghmare et al. 2013; Weinstein 2013)
t1.2	Q5/Q4/Q3/Q2	RMIT2	Firmicutes	AY308758.1	100	0.173±0.0127	0.022±0.0116	0.139±0.0173	(Rastogi et al. 2009; Lee et al. 2010; Eida et al. 2012; Lupan et al. 2014)
t1.3	Q7/Q6	RMIT3	Proteobacteria	JQ336959.1	100	0.083±0.0032	0.211±0.0058	0.170±0.0169	(Kumar and Farrell 1987; Vicuña 1988; Zimmermann 1990; Bandonas et al. 2011)
t1.4	Q9/Q8	RMIT4	Proteobacteria	KC139460.1	100	0.128±0.0152	0.326±0.0133	0.356±0.0095 ^a	(Lee 1997; Xu et al. 2010; Saratale et al. 2013; Tran and Lin 2013)
t1.5		RMIT5	Firmicutes	KC778385.1	100	0.258±0.0088	0.125±0.0064	0.279±0.0041	(Bandonas et al. 2011)
t1.6		RMIT6	Firmicutes	KF387713.1	100	0.311±0.0072 ^a	0.164±0.0153	0.090±0.0439	(Haruta et al. 2002; Rastogi et al. 2009)
t1.7	Q11/Q10/Q1	RMIT7	Firmicutes	KF475870.1	100	0.241±0.0040	0.161±0.0253	0.351±0.0318	(Mabrouk and El Ahwany 2012; Maki et al. 2012)
t1.8	Q12/Q11	RMIT8	Firmicutes	KF158801.1	100	0.202±0.0000	0.265±0.0767	0.264±0.0089	(Archana and Satyanarayana 2007; Vargas-Garcia et al. 2010)
t1.9		RMIT9	Firmicutes	KC422649.1	100	0.284±0.0039	0.095±0.0132	0.281±0.0070	(Haruta et al. 2002; Bandonas et al. 2011)

^a Highest utilization of each substrate

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 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 (SiO₂) 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 63 F 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 63 F-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, xylan and lignin, demonstrating

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

645 their versatility (Fig. 5). However, only four isolates, RMIT1,
 646 RMIT5, RMIT7 and RMIT 8, were able to utilize lignin
 647 (Fig. 5). Lignin is especially difficult to utilize or break down
 648 due to its highly stable chemical structure, which is composed
 649 of aromatic compounds (Pérez et al. 2002).

650 Although all the bacterial isolates were able to use cellulose
 651 as a carbon source, two bacterial strains, RMIT6
 652 (*Brevibacterium frigoritolerans*) and RMIT9 (*Bacillus* sp.)
 653 were found to be the highest degraders (utilizers) of cellulose
 654 as a sole carbon and energy source over the 7-day experiment.
 655 One of the isolates, *Brevibacterium* sp. is a well-known cellu-
 656 lose-degrading species (Haruta et al. 2002). The same isolates
 657 (RMIT6 and RMIT9) were amongst the best cellulase-
 658 producing bacteria when screened by the traditional assay
 659 method (Supplementary Table 1). In contrast, isolates
 660 RMIT4 (*Klebsiella oxytoca*) and RMIT7 (*Bacillus*
 661 *amyloliquefaciens*) were able to utilize cellulose moderately,
 662 yet were still capable of growth on lignocellulose (Table 1).
 663 Strain RMIT1 (*Klebsiella variicola*) was able to utilize xylan
 664 as the sole carbon source more effectively than other isolates
 665 (Table 1) and was also classified as the best xylanase producer
 666 in the conventional crude enzyme production (Supplementary
 667 Table 1) (Saratale et al. 2013). A recent study developed a co-
 668 culture system by applying *Bacillus* sp. and *Klebsiella oxytoca*
 669 for enhancing ethanol production from lignocellulosic materi-
 670 als (Tran and Lin 2013). It was therefore not unexpected that
 671 *Bacillus* sp. (RMIT5, 7, 8 and 9) were putatively identified as
 672 lignin or cellulosic waste degrading isolates in this study
 673 (Table 1, Fig. 5).

674 Having used an efficient and cost effective Biolog MT2
 675 microplate based screening method to select an array of
 676 lignocellulosic-waste-degrading microorganisms, future stud-
 677 ies should involve the use of these isolates for the saccharifi-
 678 cation of these wastes in laboratory-based and large-scale
 679 studies. In addition, there is a need to assess whether the in-
 680 duction of the desired enzymes from these isolates can be
 681 improved by co-culturing or using a consortium of these mi-
 682 croorganisms (which mimics what happens in the natural bio-
 683 degradation of agro-wastes). Some studies have shown that
 684 the use of microbial co-cultures can lead to improved degra-
 685 dation of different kinds of wastes (Pohlschroeder et al. 1994;
 686 Chou et al. 2011), although only limited information regarding
 687 the use of a consortium *Bacillus* sp. for lignin and cellulose
 688 containing wastes appears in the literature.

689 Conclusions

690 This study has demonstrated that the Biolog (MT2) microplate
 691 technology developed here represents a cheap, environmental
 692 friendly and effective tool for the rapid screening and quanti-
 693 tative assessment of bacterial species capable of degrading
 694 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 de-
 gradability 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.
 The Biolog (MT2) microplate-based assay therefore repre-
 sents an effective method for the quantitative screening of
 the abilities of bacterial strains to degrade a variety of straw
 substrates.

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