

Enhanced Biological Straw Saccharification Through Coculturing of Lignocellulose-Degrading Microorganisms

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Abstract Lignocellulosic waste (LCW) is an abundant, low-cost, and inedible substrate for the induction of lignocellulolytic enzymes for cellulosic bioethanol production using an efficient, environmentally friendly, and economical biological approach. In this study, 30 different lignocellulose-degrading bacterial and 18 fungal isolates were quantitatively screened individually for the saccharification of four different ball-milled straw substrates: wheat, rice, sugarcane, and pea straw. Rice and sugarcane straws which had similar Fourier transform-infrared spectroscopy profiles were more degradable, and resulted in more hydrolytic enzyme production than wheat and pea straws. Crude enzyme produced on native straws performed better than those on artificial substrates (such as cellulose and xylan). Four fungal and five bacterial isolates were selected (based on their high strawase activities) for constructing dual and triple microbial combinations to investigate microbial synergistic effects on saccharification. Combinations such as FUNG16-FUNG17 (*Neosartorya fischeri*–*Myceliophthora thermophila*) and RMIT10-RMIT11 (*Aeromonas hydrophila*–*Pseudomonas poae*) enhanced saccharification (3- and 6.6-folds, respectively) compared with their monocultures indicating the beneficial effects of synergism between those isolates. Dual isolate combinations were more efficient at straw saccharification than triple combinations in both bacterial and fungal assays. Overall, co-culturing can result in significant increases in saccharification which may offer significant commercial potential for the use of microbial consortia.

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Introduction

Currently, the main source (80~88 %) of energy supply worldwide is fossil fuels (crude oil, coal, and natural gas) which are non-renewable, finite, and costly resources [1]. The environmental effects of using fossil fuels such as gasoline as a primary source of energy include air, water, and soil pollution together with negative impacts on public health [2]. Sustainable bioenergy generated from biomaterials (e.g., bioethanol from starch commonly referred to as biofuels) not only results in reduced reliance on fossil fuels, but the final product may have a more economic and superior net performance. This is especially important in the transportation sector where the combustion properties of fuels such as octane number, heat of vaporization, power outputs, and oxygen content are important [3, 4].

The production of biofuels as a sustainable source of fuel has been reported since the 1880s and was established as an alternative and sustainable bioenergy (bioethanol) approach by 1970s (following a fuel crisis) in many countries such as USA and Brazil [5]. Bioethanol production is well established globally, but unfortunately, this is currently based on the use of expensive and edible crops (known as first generation of biofuels from corn, wheat, rice, and sugarcane) as substrates. This has led not only to a limitation in the amount of material available but also to food versus fuel conflicts [2, 5]. Therefore, although the capacity for biofuel production is growing every year (\$4 billion in 2007), the contribution of sustainable biofuels to the world's transportation fuel was only 1.8 % in 2008 [6]. Consequently, replacement with cheap, abundant, and nonedible feedstocks such as lignocellulosic waste (LCW) is an area of current research with the aim of (1) making bioethanol production more economically competitive and (2) reducing the dependency on fossil fuels.

LCW, an abundant (10^9 tons annually), inedible, and renewable biomass accounting for around 50 % of the total sustainable biomass in the biosphere worldwide, is a cheap and promising alternative substrate for bioethanol production. The majority of LCW is considered as an agro-waste and subject to open-field burning, leading to significant environmental, health, and economic impacts [2, 5]. The chemical composition of LCW varies from one substrate to another and significantly affects the potential of the biomass to undergo enzymatic saccharification. Lignocellulosic biomass is primarily composed of structural carbohydrates such as cellulose and hemicellulose surrounded by lignin which adds extra strength, protection, and resistance against microbial or enzymatic degradation. In general, LCW consists of three main components: cellulose (~45 % of dry weight), hemicellulose (~30 % of dry weight), and lignin (~25 % of dry weight) [5]. This complex combination of the three biopolymers renders lignocellulosic materials less biodegradable [7, 8]. Currently, bioethanol production from lignocellulosic biomass is a great challenge and still not produced commercially due to technical barriers mainly associated with saccharification.

Unlike the linear structure of cellulose which consists of β -1-4-linked glucose, hemicellulose is more branched and heterogeneous requiring a more diverse set of hydrolytic enzymes including β -xylanases, β -xylosidases, α -arabinofuranosidases, acetylxylanesterases, and α -glucuronidases acting synergistically [9]. Therefore, enzymatic hydrolysis of LCW requires the use of expensive enzyme complexes which act synergistically to saccharify the polysaccharide biopolymers (cellulose and hemicellulose) into monomers [6, 10, 11].

There are four steps for the production of bioethanol from LCW: pretreatment, hydrolysis, fermentation, and distillation [10]. The main burden is the hydrolysis step which requires the degradation of cellulose and hemicellulose biomolecules into their monomers. The hydrolysis of biomass can be carried out by physical, chemical, or biological means. However, biological approaches using microorganisms (bacteria or fungi) or their enzymes are preferred primarily because they are more efficient, environmentally friendly, and selective in their hydrolysis. Biological approaches can result in significant bioconversion of LCW into reducing sugars which can be used for bioethanol production from cheap substrates under ambient conditions [12–16].

The main technical challenge of the commercialization of cellulosic bioethanol is enhancing efficient and economic bioconversion or complete saccharification of lignocellulosic materials into reducing sugars using either robust hydrolytic enzymes or microbial isolates. The cost of lignocellulosic enzymes required for the complete hydrolysis is very expensive and considered to be the key challenge commercially. It has been estimated that the cost of these hydrolytic enzymes is around 50 % of the total hydrolysis cost; about 19 kg of cellulases (11 million FPU; filter paper unit) are required to saccharify only one ton of lignocellulosic material to produce 84 gallon (318 l) of cellulosic bioethanol. The enzyme loading rate is 25 FPU g⁻¹ of cellulosic substrate with an estimated cost of US\$ 3 per gallon (US\$ 0.8 per liter) [17]. Therefore, to enhance the economic feasibility, enzyme costs must be brought down to less than US\$ 0.07 per gallon (US\$ 0.027 per liter) [16, 17]. Production cost of hydrolytic enzymes can be reduced either by using cheap lignocellulosic materials as an inducer or the use of novel microbial isolates or developed microbial consortia with the desired saccharification properties. The main sources of lignocellulose-degrading enzymes are bacteria, actinomycetes, and fungi [9, 18]. The complete biodegradation of LCW enzymatically is a poorly understood process. To date, there is no report of an individual microbial isolate that can secrete a complete array of multienzyme systems to convert LCW components into fermentable sugars effectively [11].

The bioconversion step bottleneck in the commercial production of cellulosic biofuels may be overcome through new initiatives. Van Dyk and Pletschke (2012) stated that a large number of enzymes (up to 80) are required to convert lignocellulose carbohydrates into monomeric sugars effectively [19]. In addition, the hydrolytic activity of multiple enzyme combinations (working together) results in increased saccharification compared with the addition of individual enzymes. However, the hydrolysis yield depends on the specific characteristics of the enzymes involved, their ratios, and the surface characteristics of the substrate [19]. The main enzymes such as cellulases and xylanases involved in the saccharification of cellulose and hemicellulose are well reviewed [20–25].

There is a current need for microbial isolates or unique consortia with new or superior saccharifying activities. To date, most of the isolations and subsequent culturing have involved single strains [6, 9, 26–30]. In natural ecosystems, the biodegradation of lignocellulosic biomass is achieved by the synergistic cooperation of a variety of microorganisms (bacteria and fungi); however, the reconstruction of defined microbial consortia for certain lignocellulose substrate degradation is challenging since ~99 % of known microorganisms are currently classified as nonculturable. Coculturing has, however, been successfully used in several industrial applications, for example, in the production of synergistic enzymes, dairy fermentation, antibiotics, and composting throughout mixing of at least two microbial isolates as an inoculum [27]. Microbial consortia represent a promising approach that could be useful for understanding the complex interaction and the synergistic relationship between mixed enzymes generated in nature for the biodegradation of lignocellulosic biomass. To date, there have been very few studies on the impact of coculturing lignocellulolytic microorganisms in order to increase lignocellulose-degrading capabilities.

Therefore, the main aims of this study were to (i) isolate lignocellulose-degrading microorganisms (bacteria and fungi) grown on four different lignocellulosic straws as a carbon source and (ii) to develop microbial combinations of selected microbial isolates (bacteria–bacteria and fungi–fungi) in order to construct an efficient hydrolytic enzyme-producing consortium to improve straw saccharification.

Materials and Methods

Chemicals and Media

All chemicals, commercial enzymes (cellulase and xylanase), and media used in this study were of analytical grade and were purchased from Sigma–Aldrich Chemical Company (NSW, Australia) and Oxoid Ltd (Basingstoke, Hampshire, UK) unless otherwise mentioned in the text.

Lignocellulosic Straws (LCS)

Four different LCSs (wheat, rice, sugarcane, and pea straw) were collected from an Australian local market (Adelaide, South Australia), cut into small pieces (10 cm), washed with sterilized water, and air-dried. All straws were subjected to grinding using a coffee grinder (Breville, Australia) and milled to a fine powder using a ball mill (Retsch Mixer Mill MM 200, Germany). These were kept at 4 °C for further use as a carbon source, crude enzyme induction, and for Fourier transformer infrared analyses.

Microbial Source Collection

Compost (EZI-WET, Melbourne, Australia) and soil samples (sampled from a farm straw piles) were collected from Australia (Adelaide and Melbourne) as a rich source of microorganisms (bacteria and fungi). These samples were kept in zip-lock plastic bags and stored at either 4 or –20 °C for further investigation.

Screening, Isolation, and Purification of Lignocellulosic Degrading Microorganisms

Lignocellulose-degrading microorganisms (bacteria and fungi) were isolated from compost, soils, and straws at mesophilic (30 °C) and thermophilic (50 °C) temperatures using selective enrichment medium. The medium used was a mineral salts medium (BH-medium) supplemented with an equal mix of ball-milled straws (0.5 % *w/v* wheat, rice, sugarcane, and pea straw) as the sole carbon and energy sources [31]. The collected samples (compost, soils, and straws) were serially diluted (10^{-1} to 10^{-6}). An aliquot (150 μ l) of each dilution was spread onto BH straw agar plates (amended with 0.5 % ball-milled straws). These plates were incubated for 6 days at 30 and 55 °C for mesophilic and thermophilic microbial growth, respectively. For fungal isolation, an antibiotic solution containing 0.015 g l^{-1} of tetracycline was added to the media (containing 12–15 g l^{-1} of Oxoid bacteriological agar no. 1) to inhibit bacterial growth [32].

Bacterial and fungal isolates were purified by repeated subculturing in the appropriate media (nutrient agar (NA) or potato dextrose agar (PDA)) before being re-cultured onto BH straw agar plates to revalidate their ability to utilize straw as the sole carbon and energy source. All purified bacterial and fungal species after growth in their respective liquid media (nutrient or

potato dextrose broth) were stored in 20 % (v/v) glycerol/nutrient broth at $-80\text{ }^{\circ}\text{C}$ pending further investigation [8, 28].

Sixty bacterial isolates and 50 fungal strains were further screened qualitatively for cellulase and xylanase activities on BH-medium plates supplemented with 0.5 % cellulose or xylan as a sole carbon source. The plates were incubated for 4–6 days at the desired temperatures. To visualize the hydrolysis zone, the plates were stained with Congo red (0.1 %) for 30 min and then destained with NaCl (1 M). The diameter of the “clear halo” around the bacterial colony and the fungal plug was measured [33]. Microbial isolates that exhibited the highest halo zone diameter around standard microbial inoculation (50 μl of OD_{600} 0.9 for bacteria and 1 cm^2 plug for fungi) were selected (as the halo zone was indicative of cellulase and xylanase activities). The clear zone diameter assessment was made based on a defined standard: $\leq 0\text{ cm}^2$ represented “no activity detected for both bacteria and fungi,” 0–1.5 cm^2 (bacteria) and 0–2.0 cm^2 (fungi) represented “low activity,” 1.5–3.0 cm^2 (bacteria) and 2.0–4.0 cm^2 (fungi) represented “medium activity,” and $\geq 3.0\text{ cm}^2$ (bacteria) or $\geq 4.0\text{ cm}^2$ (fungi) represented “high activity.” The bacterial and fungal strains that had the highest clear zone (as an indicator of cellulase and xylanase production) were selected (30 bacteria and 18 fungi) for quantitative screening for crude enzyme production (cellulase, xylanase, and strawase).

Standardization and Inoculation of Microbial Inocula for Crude Enzyme Production

All pure bacterial isolates (30 strains) were cultured in nutrient broth (100 ml), agitated on a rotary shaker at 150 rpm for 48 h at 30 and 55 $^{\circ}\text{C}$ for mesophiles and thermophiles, respectively. Optical density (OD_{600}) of bacterial cultures were normalized at 600 nm (Pharmacia LKB Ultrospec II Spectrophotometer, CT, USA) to attain an absorbance of 0.9 after being harvested by centrifugation at 4700 rpm for 10 min at 4 $^{\circ}\text{C}$. Bacterial pellets were aseptically washed three times to remove all the nutrients and resuspended in sterilized water to get the optimum concentration (3 ml of resuspended pellets from each bacterial isolate; 0.9 at OD_{600}). This optimum concentration was used for the traditional crude enzyme assays carried out using individual isolates in BH liquid media supplemented with an individual straw (wheat, rice, sugarcane, or pea straw) as the carbon source. Adjustment was made when necessary by the addition of bacterial colonies or sterilized water aseptically. Fungal inoculums (18 isolates) were inoculated using normalized fungal biomass based on dry weight. The fungal application rate used in this study was 0.7 g l^{-1} (dry weight) [34].

For the combination studies, five bacterial and four fungal species were used with these isolates being selected on the basis of their high strawase activities (two bacterial species) and ability to grow on lignin supplemented plates (three bacterial isolates). Fungal species selected for use were the highest producers of cellulase, xylanase, strawase, and laccase enzymes. User-determined bacterium–bacterium or fungus–fungus combination methods were used. For bacteria (five isolates), ten combinations were used for the two isolates' combination approach and nine different combinations for three isolates' combinations for a cumulative total of 19 combinations (supplementary Table 1). For fungi (four isolates), six different combinations were used for two isolates' combinations and four different combinations for three isolates' combinations resulting in a ten cumulative combinations (supplementary Table 1). Inoculations were carried out with the different combinations' inoculum load being the same as used for individual isolate's assays. For example, for two fungal isolates' combinations, each individual isolate was inoculated at 0.35 g l^{-1} (dry weight) for a total inoculum load of 0.7 g l^{-1} (dry weight) per sample so that the inoculum load was the

same as used for inoculations with single isolates. All the experiments were carried out in triplicate.

Quantification of Crude Enzyme Production from the Individual and the Combination's Supernatant

The microbial growth of individual (30 bacterial and 18 fungal isolates) or microbial combinations (four fungal and five bacterial isolates used to form 10 and 16 different microbial combinations, respectively) were determined as earlier described for synergy studies. Enzyme assays for individual and mixed culture of selected microbial strains were carried out in Erlenmeyer flasks (150 ml) containing BH-medium (100 ml) supplemented with individual ball-milled straw (0.5 % of wheat, rice, sugarcane, and pea straw) for the induction of crude lignocellulolytic enzymes. The flasks were inoculated with the desired inoculum (3 ml) of each bacterial strain (optimum concentration of 0.9 at 600 nm) or 0.7 g l⁻¹ dry weight of fungal isolate or an equal combination of different microbial isolates with appropriate controls (without inoculum). All flasks were incubated in triplicate (at either 30 or 55 °C) on a rotary shaker at 150 rpm for 12 days. Samples (5 ml) were collected periodically (0, 3, 6, 9, and 12 days), centrifuged at 4700 rpm for 20 min at 4 °C to remove microbial biomass and residual lignocellulosic materials and the supernatants (crude enzyme) were filtered through sterile 0.22- μ m filters. The cell-free supernatants (crude enzyme) were analyzed for (hydrolytic) lignocellulase enzyme activity (cellulase, xylanase, and strawase) and used for further investigations of the synergistic studies of the combination of individual isolate's cell-free supernatant [22].

Lignocellulase Enzyme Assays

Hydrolytic activities of cellulase, xylanase, and strawase were measured in the individual bacterial, fungal, and the cell-free supernatants of combinations based on the release of reducing sugars from different substrates. The substrates used for total cellulase were modified and developed as filter paper strips (6 × 1 cm ~ 60 mg of Whatman no. 1) while xylan (1 % in Na-citrate buffer 0.05 M, pH 4.8) was used for xylanase assays. An equal mix of the four different straws in slurry suspension (1 %, wheat, rice, sugarcane, and pea straw) in Na-citrate buffer (0.05 M, pH 4.8) was used for strawase assays. These were incubated with the crude enzyme (0.5 ml) at 50 °C for 60 min alongside respective controls (without crude enzymes and with 0.5 ml of Na-citrate buffer instead) [35, 36]. Reducing sugars released by crude enzymes were estimated using the 3,5-dinitrosalicylic acid (DNS) method [37]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of the reducing sugar per minute under assay conditions [35, 36]. For comparison, the reducing sugar released (mg ml⁻¹) by commercially sourced xylanase and cellulase preparations, at three concentrations (cellulase 2, 5 and 5 U ml⁻¹ and xylanase, 0.25, 0.5 and 1 U ml⁻¹) from the saccharification (1 %) of four different ball-milled straws in Na-citrate buffer 0.05 M, pH 4.8 at 50 °C was also carried out. Reducing sugar concentrations (mg ml⁻¹) were estimated through the use of standard curves using xylose and glucose [38]. Enzyme activities for oxidative enzymes, laccase, and lignin peroxidase (LiP) were carried out at 30 °C as described by Tien and Kirk (1984) and others [39–41].

Identification of Microbial Isolates (Bacteria and Fungi)

Based on the screening results, representative fungal and bacterial candidates were selected for further investigations. For the selected microorganisms (five bacterial and four fungal species),

genomic DNA was extracted from the broth cultures using either a phenol-chloroform-isoamyl-alcohol-bead beating method or a MoBio DNA extraction kit.

Nucleic Acid (Bacterial and Fungal DNA) Extraction and PCR Amplification

Genomic DNA was extracted from selected overnight bacterial cultures grown on nutrient broth using a MoBio DNA extraction kit according to the manufacturer's guidelines. Fungal DNA was extracted by the transfer of ~0.5 g of the centrifuged fresh fungal biomass into sterile tubes (2-ml tubes) containing sterilized glass beads and 0.5 ml of sterilized DNA extraction buffer [42]. The extracted (bacterial and fungal) DNA (2 μ l) was used as a template for PCR amplification using 63f and 1389r and ITS1 and ITS4 primers for bacteria and fungi respectively [43, 44]. All PCR reactions were performed in a thermocycler (BioRad, Australia). PCR amplicons were purified using a PCR clean up kit (Promega, USA), before being sent for sequencing according to Australian Genome Research Facility (AGRF) requirements (<http://www.agrf.org.au/>). The sequenced data were edited with Sequencher (Version 5.0). The edited data were subject to BLAST analysis on the National Centre for Biotechnology Information website (NCBI) (<http://www.ncbi.nlm.gov/>) for the determination of bacterial and fungal putative identities. Selected bacterial and fungal 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). Bacterial and fungal phylogenetic trees were constructed using maximum likelihood algorithm using Molecular Evolutionary Genetics Analysis (<http://www.megasoftware.net/>) (version 6) [45].

Characteristics of Different Straws

Molecular analyses and surface characterization of the four different ball-milled straws (wheat, rice, sugarcane, and pea straw) were carried out qualitatively by Fourier transformer-infrared (FT-IR) spectroscopy [46].

Statistical Analyses

Analyses of variance (ANOVA) between the microbial supernatant (individuals and the combination) were performed using an SPSS statistical program. Mean value separation was performed using the least significant difference (LSD) test ($p=0.05$), where the F value was significant. The standard deviation and standard error (SE) were used where required.

Results and Discussion

Isolation and Purification of Lignocellulosic Degrading Microorganisms

Sixty bacterial and 30 fungal candidates were isolated and purified from compost, straws, and soil as lignocellulose-degrading microorganisms using selective BH-medium supplemented with an equal mix of four different ball-milled straws (wheat, rice, sugarcane, and pea straw). Approximately 60 % of bacterial and fungal strains were isolated from compost. Compost tends to be the main source for lignocellulose-degrading microorganisms (Table 1) [47]. All microbial isolates were grown either at 30 and 55 °C for the growth of mesophilic and thermophilic microorganisms, respectively; the clear zone diameter method was applied for

Table 1 Microbial isolates from different environmental sources and their enzyme activities ($U\ ml^{-1}$) after 12-day incubation

Bacterial isolates	Temperatures			Isolation sources			Enzyme activities					
	Mesophilic (30 °C)		Thermophilic (55 °C)	Compost	Straws	Soil	Hydrolytic enzymes			Oxidative enzymes		
	+	-	-	+	-	-	Cellulases ($U\ ml^{-1}$)	Xylanases ($U\ ml^{-1}$)	Strawase ($U\ ml^{-1}$)	Laccase ($U\ ml^{-1}$)	LiP ($U\ ml^{-1}$)	
RMIT1	+	-	-	+	-	-	1.65(±0.01)	0.56(±0.00)	0.79(±0.04)	-	-	
RMIT2	+	-	-	+	-	-	2.46(±0.01)	0.43(±0.00)	0.80(±0.04)	-	-	
RMIT3	+	-	-	+	-	-	2.02(±0.01)	0.30(±0.02)	0.85(±0.05)	-	-	
RMIT4	+	-	-	-	+	-	2.32(±0.01)	0.48(±0.00)	1.29(±0.10)	-	-	
RMIT5	+	-	-	+	-	-	3.57(±0.01)	0.48(±0.00)	1.10(±0.04)	-	-	
RMIT6	-	+	-	+	-	-	4.10(±0.01)	0.48(±0.00)	0.76(±0.04)	-	-	
RMIT7	-	+	-	+	-	-	2.61(±0.01)	0.43(±0.00)	1.28(±0.04)	-	-	
RMIT8	-	+	-	+	-	-	3.35(±0.01)	0.38(±0.00)	0.94(±0.03)	-	-	
RMIT9	-	+	-	+	-	-	4.24(±0.01)	0.46(±0.00)	1.00(±0.04)	-	-	
RMIT10**	+	-	-	+	-	-	3.42(±0.01)	0.31(±0.00)	0.68(±0.41)	-	-	
RMIT11**	+	-	-	+	-	-	2.69(±0.01)	0.05(±0.00)	0.44(±0.04)	-	-	
RMIT12	+	-	-	-	+	-	1.26(±0.01)	0.44(±0.01)	0.34(±0.04)	-	-	
RMIT13	+	-	-	-	-	+	1.83(±0.01)	0.04(±0.00)	0.54(±0.04)	-	-	
RMIT14	+	-	-	-	+	-	2.64(±0.01)	0.30(±0.00)	0.58(±0.03)	-	-	
RMIT15	+	-	-	+	-	-	2.14(±0.01)	0.32(±0.00)	0.39(±0.04)	-	-	
RMIT16	+	-	-	-	-	+	1.37(±0.01)	0.29(±0.00)	0.63(±0.03)	-	-	
RMIT17	+	-	-	-	-	+	1.56(±0.01)	0.03(±0.00)	0.56(±0.04)	-	-	
RMIT18	+	-	-	-	-	+	2.25(±0.01)	0.03(±0.00)	0.19(±0.04)	-	-	
RMIT19	+	-	-	+	-	-	1.61(±0.01)	0.02(±0.02)	0.26(±0.05)	-	-	
RMIT20	+	-	-	-	-	+	1.89(±0.01)	0.03(±0.00)	0.35(±0.04)	-	-	

Table 1 (continued)

	Temperatures				Isolation sources			Enzyme activities				
	Mesophilic (30 °C)		Thermophilic (55 °C)		Compost	Straws	Soil	Hydrolytic enzymes			Oxidative enzymes	
	+	-	+	-				Cellulases (U ml ⁻¹)	Xylanases (U ml ⁻¹)	Strawase (U ml ⁻¹)	Laccase (U ml ⁻¹)	LiP (U ml ⁻¹)
RMIT21	+	-	-	-	-	-	+	2.17(±0.01)	0.02(±0.00)	0.52(±0.05)	-	-
RMIT22	+	-	-	+	-	-	-	2.78(±0.01)	0.46(±0.00)	0.56(±0.04)	-	-
RMIT23	+	-	-	-	-	-	+	1.76(±0.01)	0.04(±0.00)	0.39(±0.05)	-	-
RMIT24	+	-	-	+	-	-	-	2.65(±0.01)	0.26(±0.00)	0.78(±0.11)	-	-
RMIT25	+	-	-	-	-	-	+	2.10(±0.01)	0.03(±0.00)	0.49(±0.06)	-	-
RMIT26	+	-	-	-	-	-	+	1.80(±0.01)	0.28(±0.00)	0.58(±0.04)	-	-
RMIT27	-	+	-	+	-	-	-	2.08(±0.01)	0.20(±0.00)	0.65(±0.04)	-	-
RMIT28	-	+	-	+	-	-	-	1.93(±0.01)	0.14(±0.01)	0.52(±0.04)	-	-
RMIT29	-	+	-	+	-	-	-	1.60(±0.01)	0.23(±0.00)	0.54(±0.04)	-	-
RMIT30**	-	+	-	+	-	-	-	1.51(±0.01)	0.25(±0.00)	0.66(±0.03)	-	-
Fungal isolates												
FUNG1	+	-	-	+	-	-	-	5.41(±0.11)	0.58(±0.00)	1.73(±0.00)	-	0.01(±0.00)
FUNG2	+	-	-	-	+	-	-	4.43(±0.09)	0.58(±0.00)	1.44(±0.00)	-	-
FUNG3	+	-	-	+	-	-	-	9.36(±0.20)	0.60(±0.00)	2.33(±0.00)	-	0.09±0.00)
FUNG4	+	-	-	+	-	-	-	6.64(±0.13)	0.53(±0.00)	1.62(±0.00)	-	0.05(±0.00)
FUNG5	+	-	-	+	+	-	-	3.24(±0.07)	0.31(±0.03)	1.22(±0.00)	0.88(±0.03)	0.07(±0.00)
FUNG6	+	-	-	-	-	-	+	4.41(±0.09)	0.39(±0.00)	1.11(±0.00)	-	-
FUNG7	+	-	-	+	-	-	-	4.77(±0.10)	0.52(±0.00)	1.69(±0.00)	-	0.01(±0.00)
FUNG8	+	-	-	+	-	-	-	4.47(±0.09)	0.52(±0.00)	1.33(±0.00)	-	-
FUNG9	+	-	-	-	-	+	+	5.09(±0.11)	0.50(±0.00)	1.82(±0.00)	-	-
FUNG10	+	-	-	-	-	-	FU*	6.69(±0.11)	0.38(±0.00)	2.22(±0.00)	-	-

Table 1 (continued)

		Isolation sources				Enzyme activities			
Temperatures		Thermophilic (55 °C)		Soil		Hydrolytic enzymes		Oxidative enzymes	
Mesophilic (30 °C)	Thermophilic (55 °C)	Compost	Straws	Soil	Cellulases (U ml ⁻¹)	Xylanases (U ml ⁻¹)	Strawase (U ml ⁻¹)	Laccase (U ml ⁻¹)	Lip (U ml ⁻¹)
-	+	+	-	-	3.16(±0.00)	0.58(±0.00)	1.59(±0.00)	-	-
-	+	+	-	-	3.38(±0.00)	0.83(±0.00)	1.12(±0.00)	-	-
-	+	-	-	+	2.83(±0.00)	0.90(±0.01)	1.04(±0.00)	-	-
-	+	+	-	-	4.45(±0.00)	0.97(±0.00)	1.40(±0.00)	-	-
-	+	-	+	-	6.34(±0.00)	0.93(±0.00)	1.20(±0.00)	-	-
-	+	-	-	+	7.78(±0.00)	0.98(±0.00)	1.46(±0.00)	-	-
+	+	-	-	-	3.62(±0.00)	0.97(±0.00)	1.14(±0.00)	2.19(±0.02)	-
+	-	-	+	-	4.80(±0.00)	0.95(±0.00)	1.16(±0.00)	-	-

FU* *Phanerochate chrysosporium* (ATCC 24725) was a kind gift from Professor David Catcheside, Flinders University

"-" Isolated not obtained or growth not observed, "+" isolated obtained and growth observed

** Bacterial isolates grew on lignin supplemented BH-plate but no ligninolytic enzyme production detected within 12 days

selection of microorganisms for further cellulolytic and xylanolytic screening qualitatively (for cellulase and xylanase activities) using BH-medium plates supplemented with cellulose or xylan (0.5 %) as a sole carbon source after being stained with Congo red. Approximately 35 to 50 % of bacterial and fungal isolates (30 bacteria; RMIT1 to RMIT30 and 18 fungi; FUNG1 to FUNG18) (Table 1) were selected as microorganisms classified as producing high cellulolytic and xylanolytic (according to the halo zone diameter; data not shown). The main purpose of the preliminary screening techniques (using BH-medium and Congo red) was to reduce the number of microbial isolates identified which were going to be used for further investigations. Other studies have carried out similar procedures. For example, 19 fungal isolates were selected out of 137 fungal strains screened for cellulase activity based on Congo red staining and clearance zone observation in a study by Kalyani et al. [28].

Validation of the Potential Activity of Microbial Isolates

Thirty bacterial isolates and 18 fungal strains were selected for quantitative screening individually using traditional crude enzyme production (submerged mode) on individual ball-milled lignocellulosic straws used as substrate (inducers). The term “crude enzyme” refers to the natural enzymes produced either on solid or submerged state fermentation of the microorganisms (individual or combinations) without genetic or downstream modifications [48]. The majority of lignocellulases are extracellular and inducible enzymes which are produced by microorganisms during the growth on carbon-limited medium using lignocellulosic residues as a carbon source [49].

Individual enzyme activities from the growth of each fungal and bacterial isolate were determined throughout the 12-day incubation; the enzyme activities of all individual isolates in culture supernatant harvested at the end (12 days) of the incubation are shown in Table 1. In terms of the effect of microbial growth on enzyme activity in culture supernatants, all enzyme activities (cellulase, xylanase, and strawase) peaked after 6 days of cultivation and remained at similar activities from 6 to 12 days (Fig. 1) for all isolates. For both individual bacterial and fungal isolates, cellulase showed the highest activity compared to xylanase and strawase activities (Table 1). Bacterial cellulase activity ranged between 1.26 U ml^{-1} (RMIT12) to 4.24 U ml^{-1} (RMIT9) while the activities of fungal cellulases were more than double (e.g., 9.36 U ml^{-1} ; FUNG3) compared to bacterial cellulases. RMIT1 was found to secrete the highest bacterial xylanase (0.56 U ml^{-1}) while FUNG16 was the best fungal xylanase producer (0.98 U ml^{-1}). Previous studies showed similar xylanase activity from bacteria such as *Streptomyces thermoviolaceus* grown on sugarcane bagasse (0.42 U ml^{-1}) for the same period of incubation (12 days) [50]. In general, enzyme production in individual fungal representatives was almost double (cellulase, xylanase, and strawase activities) that of bacterial enzymes. Figure 1 highlights this point by comparing the mean enzyme activities of all bacterial (a) and fungal (b) isolates. The mean of cellulase, xylanase, and strawase activities (U ml^{-1}) of all fungal isolates (b) are almost double of those observed in all bacterial isolates (a); fungal cellulase represents the highest (5 U ml^{-1}) in comparison to bacterial cellulase (2.5 U ml^{-1}). The same trend was observed in strawase and xylanase activities although the enzyme activities were comparatively lower (Fig. 1). Kuhad et al. [17] reported that fungi are the main sources of lignocellulosic enzymes; Fig. 1 corroborates this conclusion [9, 17, 27]. Control incubations based on the use of purified commercial preparations of cellulose and xylanase, at the same concentration as those detected in bacterial (2.5 and 0.25 U ml^{-1} cellulase and xylanase activity, respectively) and fungal (5.0 and 0.5 U ml^{-1} cellulase and xylanase activity, respectively) culture supernatants were also run to compare their activity against the four straw samples. The results confirmed that the culture supernatants, with similar enzyme activities to

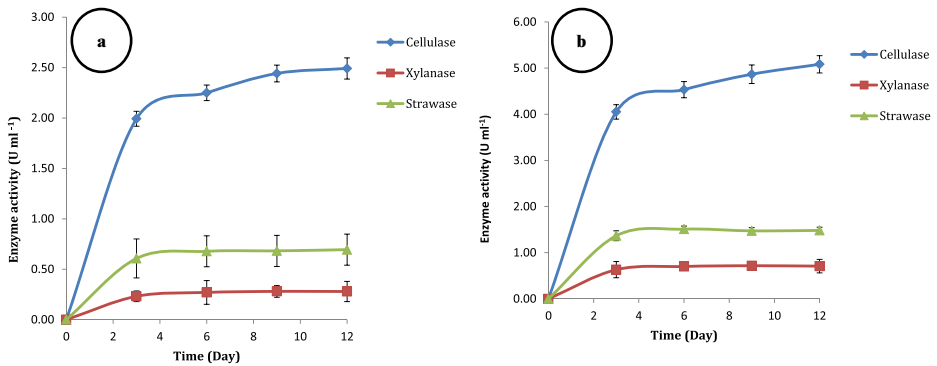


Fig. 1 The mean of cellulase, xylanase, and strawase activities (U ml^{-1}) of all isolates (30 bacteria and 18 fungi) over 0, 3, 6, 9, and 12 days at two different temperatures (30 °C) mesophilic and (55 °C) thermophilic. Bacteria (**a** on the left) and fungi (**b** on the right). All assays were conducted at 50 °C

the commercial preparations, showed increased reducing sugar levels following incubation (30 and 12 mg ml^{-1} for fungi and bacteria compared with 25 and 9 mg ml^{-1} for fungal- and bacterial-equivalent xylanase/cellulase combinations) (data not shown). These results suggest the importance of additional hydrolytic and perhaps oxidative enzymes present in the culture supernatant but not in purified enzyme preparations in the saccharification of straw. Although oxidative enzymes (laccase; Lac and lignin peroxidase; LiP) were not detected in any bacterial isolates, both laccase and lignin peroxidase activities were detected in a number of fungal isolates; some of the levels detected for laccase were high compared to those reported in the literature, for example *Agaricus bisporus*'s laccase activity was reported to be 0.108 U ml^{-1} after 6 days of incubation and *Pleurotus ostreatus* activity was reported to be 0.57 U ml^{-1} after 9 days [51]. In this study, only two isolates produced laccase; FUNG17 was the highest laccase producer (2.19 U ml^{-1}) (Table 1) followed by FUNG5. Laccase productivity has been reported to be affected by the carbon source which acts as laccase inducer. Galhaup et al. [52] concluded that glucose and cellobiose were the best laccase inducers when inoculated with *Trametes pubescens* for 12 days of cultivation which resulted in enhanced higher laccase activity (60 and 65 U ml^{-1}) compared to when lactose and α -cellulose were used as laccase inducers (11 and 4 U ml^{-1}) [52]. Out of 18 fungal isolates, 5 produced LiP but in extremely low concentrations (Table 1).

Effect of Different Substrates (Straws) on Enzyme Induction and Biodegradability

Saccharification activity (strawase) reflects the cooperative interactions and synergism between lignocellulolytic enzymes (crude enzyme) of each microbial isolate or their combination together. Individual fungal strawase activity was found to be almost double those found in bacteria: 1.28 and 1.29 U ml^{-1} in RMIT7 and RMIT4 compared to 2.22 and 2.33 U ml^{-1} in FUNG10 and FUNG3, respectively (Table 1). In addition, despite a similar complexity and heterogeneity present in LCS's structure (LCS) from different sources, significant differences in enzyme activities and biodegradability were observed in different substrates (Fig. 2). Rice straw and sugarcane straw consistently resulted in greatest enzyme activity for both bacteria and fungi, suggesting that rice and sugarcane straws represent the best source of lignocellulose for the production of saccharifying enzymes. There are three main factors that affect the enzymatic saccharification of LCSs and the productivity of crude enzyme on different substrates. These factors are (1) substrate characteristics such as the lignin and hemicellulose

content, the extent of crystallinity of the cellulose component, and the type of hemicellulose present [24, 53]; (2) enzyme-related factors including enzyme activity, synergism among enzymes, end-product inhibitors, and the adsorption of enzymes to lignin [24, 53]; (3). The presence of noncatalytic proteins (isolated from microorganisms) such as swollenins, expansins, and GH61 proteins can make the substrate more accessible to microbial or enzymatic attack by disrupting the crystalline structure (by breaking the hydrogen bonds) and therefore weakening the lignocellulosic structure leading to increased hydrolysis [24, 53]. The lignin and hemicellulose content of rice straw (11.9 and 25 %) is almost half of the reported content in wheat straw (23.4 and 50 %) which may result in higher enzyme production (Fig. 2), leading to increased biodegradability of rice straw in comparison to wheat straw ($P \leq 0.05$) [7].

Another reason why incubation on rice straw led to greatest enzyme production may be due to the differences in surface characteristics of the lignocellulosic materials. Obruca et al. (2012) observed that the highest amounts of hydrolytic enzymes (cellulases, xylanase) and oxidative enzyme (enzymes involved in lignin biodegradation; Lac, MnP, and LiP) were significantly enhanced (15 to 20 times higher) when lignocellulosic substrate (corn bran) was used as a substrate [25]. In addition, Murad and Azzaz [23] reported that the highest induction of cellulase enzymes was observed when *Aspergillus flavus* was grown on rice straw compared to wheat straw, corn stalk, and banana waste as different natural carbon sources for the intensification of hydrolytic enzymes [23]. Kalyani et al. [28] showed that in terms of cellulolytic enzymes, CBH, BGL, EG, and FPU activities increased significantly when rice straw was used as the carbon source providing 4.7-, 3.4-, 2.6-, and 1.5-fold increases compared to glucose, sucrose, carboxymethyl cellulose, and cellobiose as alternative cellulase inducers, respectively [28]. Pandey et al. [33] found that the bacterial strains *Bhargavaea cecembensis* (JQ71510.1) and *Bacillus sp.* (EF690432) produced appreciably more cellulase activity when grown on rice straw than produced on artificial purified substrates such as α -cellulose, avicelPH101, and sigmacell101 [33].

Moreover, the crude enzyme produced on native straw performed better than those induced on artificial substrates such as glucose, cellulose, xylan, and lignin in terms of saccharification activity [7]. Gupte and Madamwar [26] concluded that sugarcane straw was also a more favorable substrate than wheat straw (due to redistribution of lignin) which resulted in

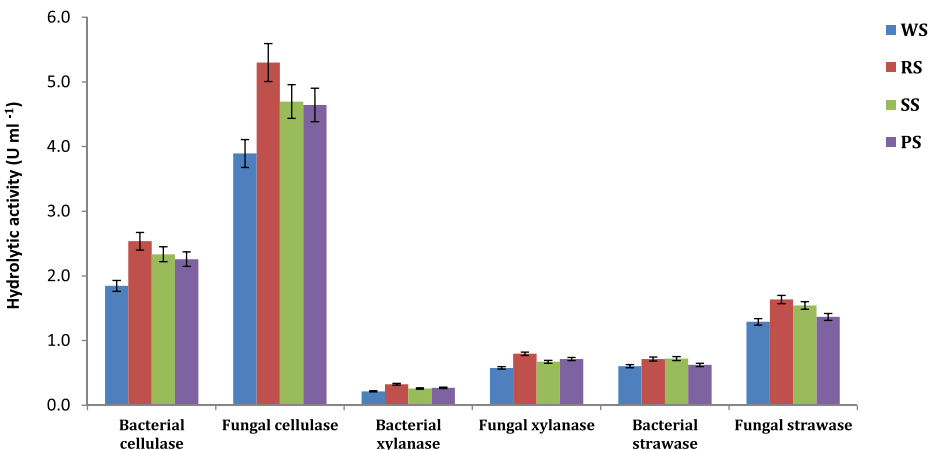


Fig. 2 Average of bacterial and fungal enzyme activities (U ml⁻¹) induced on four different ball-milled straws

enhanced cellulase activity (FPU; 4.38 U g^{-1}) [26]. While it is a fact that leading enzyme manufacturing companies (e.g., Novozymes and Genencor) have optimized efficient multi-enzyme mixtures for the efficient hydrolysis of one specific lignocellulosic substrate, these enzymes cannot necessarily be applied to another lignocellulosic substrate successfully. Kabel et al. [54] concluded that the efficiency of straw hydrolysis (strawase) was dependent on the surface characteristics of lignocellulosic substrate rather than on the standard of multi-enzyme activities measured [54]. Therefore, efficient enzyme mixtures (include specific auxiliary enzymes) should be designed for specific substrates individually [7]. The multi-enzyme mixture must contain an appropriate combination of different enzymes (cellulases, hemicellulases, ligninases, and other nonhydrolytic enzymes) working together in synergy (between each enzyme group and between each other) for the complete saccharification of the lignocellulosic substrate, and this multi-enzyme component should be compatible with specific substrate [24].

Differences of the Straw Surface by FT-IR

Differences in the surface structure of the four different ball-milled straws used in this study were characterized by FT-IR analyses to investigate why rice and sugarcane straws exhibited better degradability and enzyme induction than wheat and pea straws. Previous work on ESEM had shown that wheat straw (WS) and pea straw (PS) had similar relatively smooth surface and a compact rigid structure (due to high hemicellulose and lignin content) which limits saccharification. Rice straw and sugarcane straw had similar semi-degradable (backbone of silica network observed) surfaces than WS and PS which was probably beneficial to saccharification. In the FT-IR spectrum (Fig. 3), there are a number of absorption peaks from 4000 to 650 cm^{-1} . Each peak indicates a specific functional group (linkages) in the structure of straw. For example, peaks around 3350 cm^{-1} were assigned to bonded hydroxyl groups ($-\text{OH}$) on the straw surface or stretching of the hydrogen bonds of cellulosic substance. $\text{C}-\text{H}$ bonds in the methyl group were identified by observation of peaks located near 2970 cm^{-1} . Peaks observed at 1730 cm^{-1} were attributed to carbonyl ($\text{C}=\text{O}$) groups and could be linked to

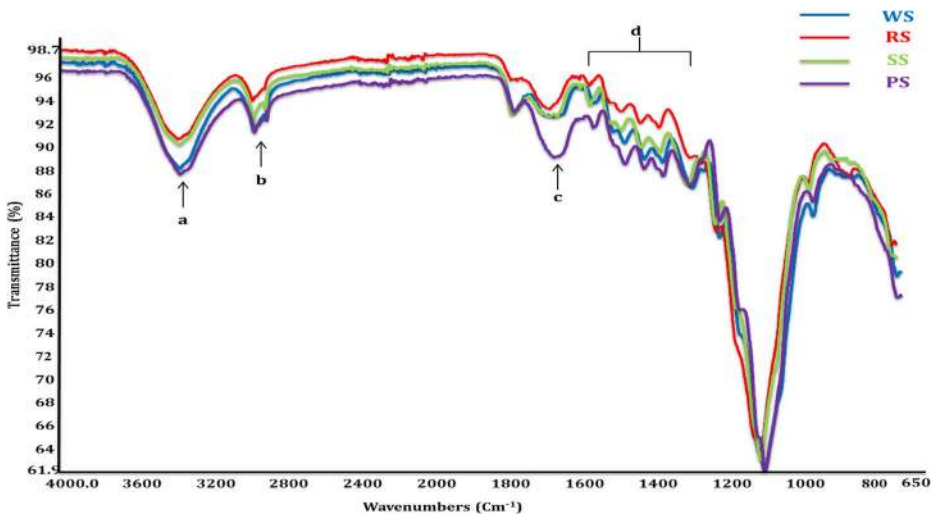


Fig. 3 Surface's characteristic differences between four different ball-milled straws using Fourier transformer-infrared (FT-IR): wheat straw (WS), rice straw (RS), sugarcane straw (SS), and pea straw (PS)

phenolic or aromatic rings or could be ester linkage of the hemicellulose–lignin complex. Finally, peaks located in the area from 1660 to 1300 cm^{-1} were characteristic of C–O bonds in phenols and indicative of the lignin polymeric structure [46]. Overall, the FT-IR spectrum of RS and SS were quite similar while the spectrum for WS and PS were also similar from 650 to 4000 cm^{-1} . These observations support our findings that the enzyme induction was varied and dependent on the type of LCSs used as substrate.

Saccharification Comparison Between Individual and Mixed Cultures

To assess the potential benefits of coculture in terms of saccharifying potential, four fungal isolates (FUNG3, FUNG10, FUNG16, and FUNG17; highest producer of cellulase, strawase, xylanase, and laccase, respectively) were selected. However, for bacteria, the focus was on strawase activities (important for saccharification), and two bacterial isolates RMIT4 and RMIT7 (highest strawase activities) were selected (Table 1). In addition, three isolates, RMIT10, RMIT11, and RMIT30, were selected because they were the only bacterial isolates that grew on lignin supplemented media (data not shown). However, these isolates were not able to produce detectable lignin peroxidase during our assay period (12 days) suggesting that a longer incubation period may be needed for accurate detection of ligninolytic enzymes (Lac and Lip) in these isolates. The identities of these microbial isolates are shown in Table 2 with the phylogenetic tree being shown in Fig. 4. Three of the bacterial species belong to the phylum Proteobacteria (RMIT4, 10, and 11) while the remaining two (RMIT7 and 30) belong to the phylum Firmicutes. Some of the putative genera these isolates were assigned to such as *Klebsiella* and *Bacillus* are known to contain isolates capable of carrying out extensive saccharification [55, 56]. FUNG3 was putatively identified as belonging to the genera *Trichoderma*, FUNG10, *Phanerochaete*, FUNG16, *Neosartorya*, and FUNG17,

Table 2 Sequence identities of microbial isolates (fungi and bacteria) used for individual and synergistic enzyme production assays

Microbial isolate	Most probable organism	Genbank	Microbial group	Identification (%)	Query coverage(%)
Fungal isolates					
FUNG3	<i>Trichoderma longibrachiatum</i>	EU401572.1	Ascomycota	100	97
FUNG10	* <i>Phanerochaete chrysosporium</i> -ATCC 24725	FJ805248.1	Basidiomycota	100	100
FUNG16	<i>Neosartorya fischeri</i>	EF669839.1	Ascomycota	99	99
FUNG17	<i>Myceliophthora thermophila</i>	JX868606.1	Ascomycota	99	95
Bacterial isolates					
RMIT4	<i>Klebsiella oxytoca</i> strain GSC12206	KC139460.1	Proteobacteria	99	100
RMIT7	<i>Bacillus amyloliquefaciens</i>	KF475870.1	Firmicutes	100	100
RMIT10	<i>Aeromonas hydrophila</i>	KF483981.1	Proteobacteria	100	100
RMIT11	<i>Pseudomonas poae</i>	JX515573.1	Proteobacteria	100	100
RMIT30	<i>Bacillus licheniformis</i>	KJ842096.1	Firmicutes	100	100

* *Phanerochaete chrysosporium* (ATCC 24725) was a kind gift from Professor David Catcheside, Flinders University

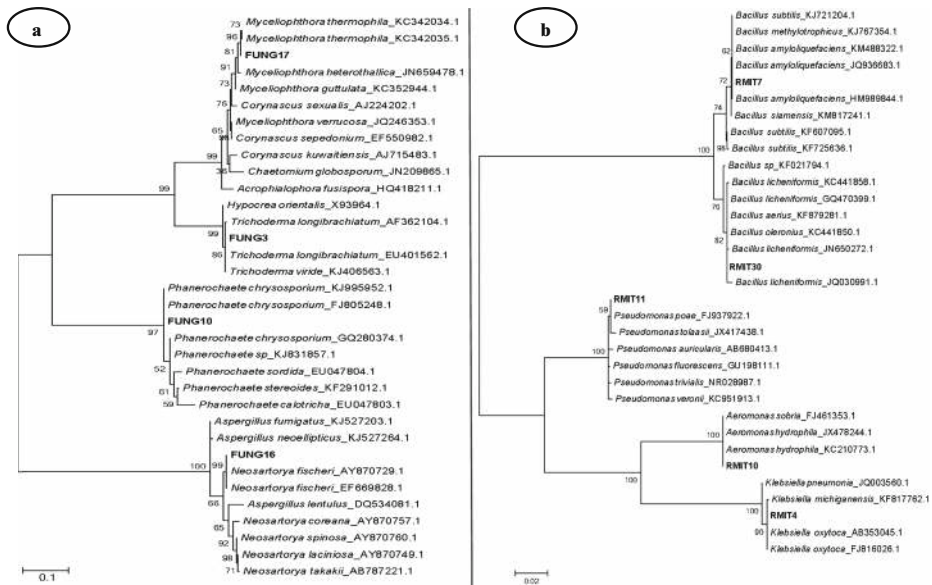


Fig. 4 The constructed evolution trees based on the PCR sequence identities of selected microbial isolates used for individual and synergistic enzyme production experiments. **a** represents selected fungal isolates and **b** refers to the selected bacterial isolates

Myceliophthora, all of which are known to contain fungal isolates with high enzymatic (saccharification) abilities [29, 57–59].

In this study, these strains cultured either individually or in different combinations (coculturing) in BH-media supplemented with individual straw as the carbon source and enzyme activities (cellulase, xylanase, and strawase) were investigated over the time (0, 3, 6, 9, 12 days). All cultures, both individual and mixed, showed maximum enzyme activities after 4 to 6 days (Fig. 5). Okeke et al. [9] also observed that highest cellulolytic and xylanolytic activities for a defined bacterial consortia (five different bacteria) reached the highest after 3 days of incubation when switch grass and Bermuda grass were used as substrates, respectively [9]. Some combinations were observed to have saccharifying rate (strawase) more than their individuals while other combinations in contrast showed poor hydrolysis efficiency than their monoculture. For example, in the fungal combination between FUNG16 (*Neosartorya fischeri*; highest in xylanase) and FUNG17 (*Myceliophthora thermophila*; highest in laccase) presented in Fig. 5, the highest rate of saccharification of the combined crude enzyme was approximately 3-fold greater than the saccharification activity of the strains when grown individually, representing excellent and exploitable properties of both isolates. FUNG3–FUNG17 combination was only slightly better than the individual isolates' saccharification activities while the remaining four (two isolates') combinations performed poorly when compared to individual isolates (data not shown). Combinations of three fungal isolates did not enhance the saccharification process as none of these combinations (supplementary Table 1) had higher enzymatic activities than any of the individual fungal isolates (data not shown).

With bacterial combinations, only RMIT10–RMIT11 (*Aeromonas hydrophila* and *Pseudomonas poae*) (dual combinations) out of ten combinations and triple bacterial combinations (RMIT10–RMIT11–RMIT30) (*Aeromonas hydrophila*, *Pseudomonas poae*, and

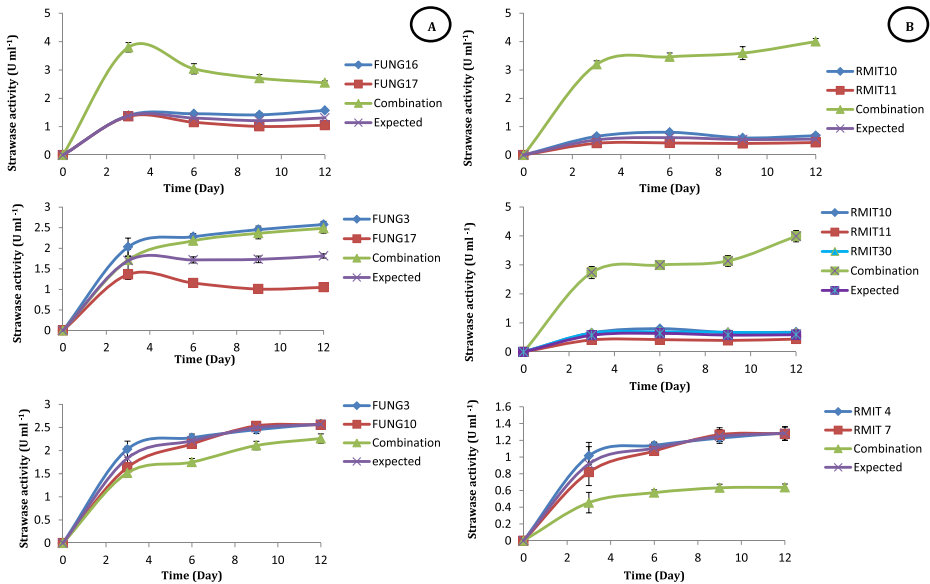


Fig. 5 Selected comparison between single pure isolates and some microbial combinations, different saccharification rate (in terms of strawase activity); **a** for fungal isolates and **b** for bacterial isolates. Note that combination refers to the enzyme activities obtained when two or more isolates were cultured together while expected values were obtained by finding the average of the isolate individual enzyme activities

Bacillus licheniformis (out of nine combinations) showed substantially enhanced activities with increased saccharification of 6.6- and 4.8-fold when compared to the activities displayed by their individual cultures (monocultures), respectively (Fig. 5), demonstrating the benefits of a coculture approach. These beneficial effects may be due to high compatibility among these fungal and bacterial isolates and/or to the synergistic nature of their enzyme activities, leading to enhanced LCS saccharification. Similarly, coculturing of *T. reesei* RUT-C30 and *A. niger* together on a cellulose-medium resulted in the greatest rates of saccharification in terms of concentration of glucose produced, 76.6 g l^{-1} compared to 37.1 g l^{-1} and the percentage of cellulose consumed (89.4 % compared to 79.3 %, respectively) [27]. Kalyani et al. [28] selected only two fungal isolates based on their cellulolytic efficiency to construct a simple consortium out of 137 fungal isolates, and they concluded that under the same conditions, cellulolytic enzyme activity (CBH, BGL, EG, and FPU activities) was increased 3-fold compared to cellulase activities obtained with individual strains due to the synergistic interaction between mixed cultures [28]. Therefore, the 4.8-fold to 6.6-fold increase in saccharification observed in this study was comparatively higher.

Advantages of Coculturing Lignocellulose-Degrading Microorganisms

There are many benefits of mixing a variety of selected isolates that might act cooperatively to enhance the complete biodegradation of a specific substrate. Other advantages have been reported by Kalyani et al. [28] which include increasing adaptability, improving productivity, better saccharification performance, control of pH during sugar utilization, and increasing substrate utilization [28]. Moreover, they also concluded that cellulolytic activity improved significantly when *T. reesei* and *A. niger* were mixed together; they suggested that the reason

might be due to the cooperative action among the two isolates leading to hypercellulase production (endo- β -glucanase and β -glucosidase increased substantially 4–5-folds); *T. reesei* was known for poor production of the cellobiose-degrading enzyme (β -glucosidase) and consequently the cellobiose that accumulated in the fermentation medium inhibited the process of saccharification; *A. niger* produced a large amount of β -glucosidase which therefore accelerated the bioconversion process [28, 30]. Ahamed and Vermette [27] reported that the cellobiose that accumulated in fermentation media is the main inhibitor for both endo- and exo-glucanases [27]. In contrast, some individual isolates showed greater hydrolytic activity than their combinations; for example, the mixed microbial culture between bacterial (RMIT4–RMIT7; *Klebsiella oxytoca* and *Bacillus amyloliquefaciens*) and fungal (FUNG3–FUNG10; *Trichoderma longibrachiatum* and *Phanerochaete chrysosporium*) isolates (Fig. 4) suggesting that the competition between these isolates or their metabolites was relatively high leading reduced saccharification.

Conclusions

In this study, the analysis of the enzyme activities from individual fungal isolates showed that they were almost 2-fold higher than those from bacteria with cellulase activity representing the dominant enzyme activity. The ability of the isolates to degrade the four different straws, wheat, rice, sugarcane, and pea varied. Based on the differences in LCS's surfaces observed with FT-IR analyses, RS and SS represented more preferable substrates than WS and PS in terms of biodegradability and crude enzyme induction. Some fungal (*Neosartorya fischeri*–*Myceliophthora thermophila*) and bacterial cocultures (*Aeromonas hydrophila*–*Pseudomonas poae*) led to increased saccharification (2.75- and 6.6-fold increase, respectively). Other microbial combinations (fungal; *Trichoderma longibrachiatum*–*Phanerochaete chrysosporium* and bacterial; *Klebsiella oxytoca*–*Bacillus amyloliquefaciens*) resulted in up to ~7-fold increases in the expected saccharification. Overall, the cooperation among mixing bacterial and fungal isolates substantially increased the ability to saccharify straw. Coculturing can result in significant increases in saccharification which may offer increased commercial potential.

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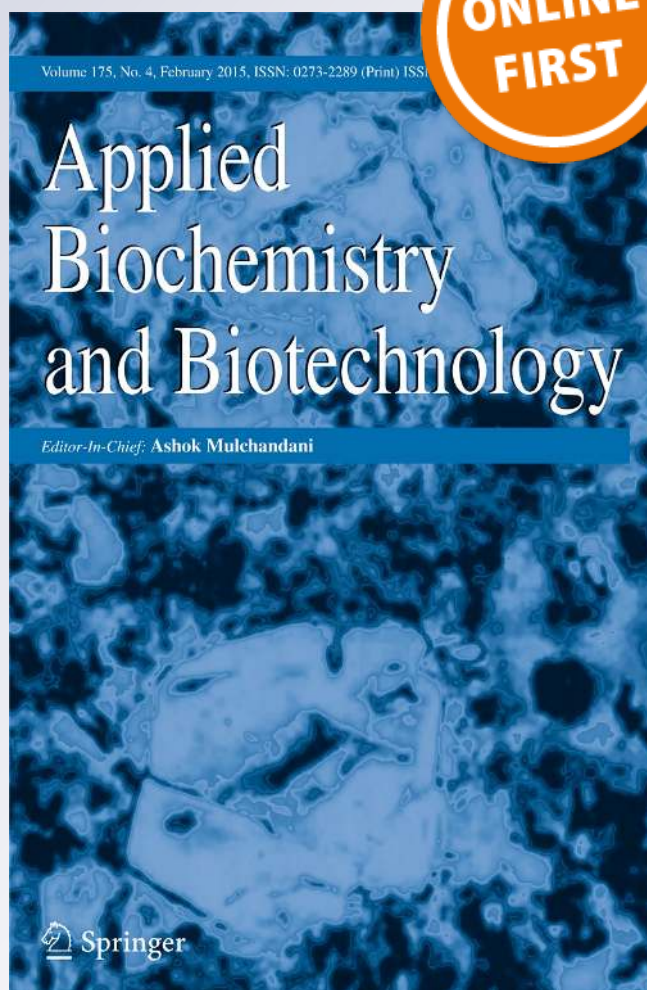
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