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TECHNICAL NOTE

Biological pretreatment and bioconversion of agricultural wastes, using ligninolytic and cellulolytic fungal consortia

Rathinam Ramarajan and Cathrine Sumathi Manohar

Biological Oceanography Division, CSIR–National Institute of Oceanography, Dona Paula, Goa, India

ABSTRACT

Agricultural wastes have attractive potential as alternate energy sources. However, a major bottleneck is to identify eco-friendly treatment methodologies to utilize them. The large diversity of unexplored, novel, and potential microorganisms hold great promise and require periodic isolation and characterization of microorganisms for bioprospection. In this study, approximately 100 fungal isolates were tested for their lignocellulolytic enzyme activities, based on plate assay, followed by quantification of enzyme activity. From this, M2E (*Inonotus tropicalis*) and 2a (*Cerrena unicolor*) showed good growth and proficient ligninolytic activity; isolates GK1 (*Chaetomium globosum*) and GK2 (*Chaetomium brasiliense*) exhibited exceptional cellulolytic activity on lignocellulosic substrates such as rice straw and sugarcane bagasse. Consortia of the potential ligninolytic and cellulolytic isolates were set up to determine their ability to biodegrade the lignocellulosic substrates such as rice straw and sugarcane bagasse. The efficiency of the consortia was determined on the basis of the increase in enzyme activity; it was also evident through scanning electron microscopy, x-ray diffraction analysis of the degraded substrates, and the sugar yield. Experiments were also carried out to compare the biological with the physical pretreatment methods. The consortium of ligninolytic and cellulolytic marine-derived fungi developed in this study prove to have the potential for application in the effective utilization of agricultural wastes.

KEYWORDS

Agricultural wastes; biological pretreatment; enzymes; fungal consortia, marine

Introduction

Annually, 200 billion tons of lignocellulosic biomass is generated across the globe from agriculture, forestry, paper industries, and agro-based processing units (Chandel and Singh 2011). Disposal of these wastes is an environmental concern, as they are accumulated rapidly and the natural rate of degradation is very slow. Lignocellulosic agricultural by-products such as rice straw, sugarcane bagasse, and corn cobs are complex polymeric structures composed of cellulose, hemicellulose, and lignin (Chen 2014). They have a wide range of applications as raw materials for the synthesis of various products, such as organic acids, food additives, single-cell proteins, and enzymes and as feedstock for the production of biofuels (Ragauskas et al. 2006; Viikari et al. 2007). The most crucial step for the utilization of lingocellulosic waste is its delignification, through pretreatment processes.

Industries follow various pretreatment methods through physical processes such as comminution, irradiation, steam explosion, etc. Chemical treatment uses alkali, acid, organic solvent hydrolysis, ano/or ozonolysis, and various physicochemical methods are adapted by combining steam explosion with sulfur dioxide treatment, ammonia fiber explosion, carbon dioxide explosion, microwave-assisted chemical treatment, etc. Some of the disadvantages of the physical and chemical methods are the requirement of harsh operating conditions, energy-intensive processes (Sun and Cheng 2002), and the formation of inhibitors such as furans and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000). Although biological methods could overcome some of these problems, their poor efficiency and treatment rates reduce their application on an industrial scale (Taherzadeh and Karimi 2008). All these drawbacks make lignocellulosic biomass a constricted energy source.

CONTACT Cathrine Sumathi Manohar cathrine@nio.org Biological Oceanography Division, CSIR—National Institute of Oceanography, Dona Paula, Goa, 403 004, India.
The current address of Rathinam Ramarajan is Richcore Lifesciences Pvt. Ltd., Bengaluru, Karnataka, India.
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Various strategies are being developed to improve the efficiency for bioutilization of the lignocellulosic substrates. In nature, microorganisms with their unique ecological significance survive synergistically on the same substrate. Similarly, consortiums of potential microbes are shown to be efficient in the degradation of the natural substrates in comparison with a single organism (Lin et al. 2011; Kausar et al. 2013). The lignocellulosic substrates are recalcitrant in nature, and their effective biodegradation requires synergistic action of microbes, which can provide optimal combination of enzymes. Studies have also shown that the biodegradation of lignocellulosic substrates requires a complex set of enzymes that includes peroxidases, laccases, endoglucanases, exoglucanases, β-glucosidases, fucosidases, and xylanases in different concentrations and proportions (Jiménez, Dini-Andreote, and van Elsas 2014; Brossi et al. 2016). Interaction between the cultures forming the consortium, and their synergistic enzyme activity, has been shown to greatly improve the biodegradation process. Use of microbial consortia can also help in overcoming the disadvantages of biological treatment, such as improving the capacity to breakdown toxic end products and augmenting the otherwise slow reaction processes. Studies have also shown that microbes from different ecological habitats can form consortia with high potential to efficiently bioutilize agricultural wastes (Cortes-Tolalpa et al. 2016; Jiménez et al. 2016). In this study, we have examined the efficiency of marine-derived ligninolytic and cellulolytic fungi in consortia to carry out simultaneous biological pretreatment and saccharification to release fermentable sugars.

Materials and methods

Sampling, isolation, and molecular identification of fungal isolates

Fungi were isolated from mangrove sediments and coral sand using particle-plating technique (Bills and Polishook 1994). They were grouped into morphotypes, purified, and maintained on microbiological media, as described in Jebaraj and Raghukumar (2009). A qualitative analysis of the fungal isolates was carried out for the lignocellulolytic enzymes by plate assay on Boyd and Kohlmeyer (BK) agar medium (Kohlmeyer and Kohlmeyer 1979). Model compounds such as guaiacol (D'Souza-Ticlo et al. 2006), carboxymethyl cellulose (CMC), and birch wood xylan (Teather and Wood 1982) were used for the assay. Potential isolates with prominent lignocellulolytic activity are deposited with the National Collection of Industrial Microorganisms, Pune, India. The molecular identification of the isolates was carried out based on the partial 18S rRNA gene sequence, and they are deposited in the National Center for Bioinformation (NCBI) GenBank under accession numbers KU720997–KU721003. Isolate 2a, a known ligninolytic isolate *Cerrena unicolor* MTCC 5159 (Raghukumar et al. 1999), was also considered for the study.

Enzyme activity of isolates grown on simple liquid media

Isolates were grown in BK medium for the determination of laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) enzyme activities. They were grown in basal salt solution containing 1% birch wood xylan and CMC for estimating xylanase and CMCase activities, respectively. Homogenized mycelial mats, approximately 10% (*v*/*v*), from 7-day-old cultures grown on BK medium, were used as inoculum for all the experiments. Pure cultures were incubated in the respective media for 14 days at 28 $\pm$ 0.5°C under static conditions. At the end of the incubation period, cultures were harvested on moisture free, preweighed, Whatmann Grade 1 Filter Paper (GE Healthcare, Little Chalfont, UK). The culture filtrates were collected in clean, sterile tubes for quantification of enzymes.

Enzyme activity of isolates and consortia grown on lignocellulosic substrates

Rice straw (RS) and sugarcane bagasse (SCB) were chosen as solid substrate for SSF. They were cut into 2–3 cm long pieces, sun dried, and left in the hot air oven at 60°C for 24 h to remove the moisture content completely. They were ground into powder, and particles that passed through a sieve of 1 mm pore size were used for the experiments. Individual isolates were grown on these substrates amended with chicken manure (CM) (80% substrate and 20% chicken manure) to attain the recommended C: N ratio (Agbogbo and Holtzapple 2007). Each of the fungal cultures was allowed to grow on fine-powdered, sterilized substrates (about 3 g amended with CM) in 250-ml Erlenmeyer flasks, and an initial moisture level in

the ratio 1:4 (*w*/*v*) of the substrate was maintained. After 14 days of growth at 28 ± 0.5°C, enzymatic hydrolysate containing the lignocellulolytic enzymes and fermentable sugars was collected following the protocol of Lin et al. (2011). In short, 20 ml sodium phosphate buffer (pH 7.0) per gram of the substrate was added to all the flasks and incubated in a rotary shaker for 1 h at 200 rpm. Then the cell-free filtrate was collected by centrifugation at 7000 rpm for 10 min at 4°C, and a final filtration was performed through Whatmann filter paper. The treated substrates were incubated again with 40 ml of the respective filtrates, and the slurry was incubated on a rotary shaker (120 rpm) at 50°C for 24 h. The lingocellulolytic enzyme activity and sugar concentration were estimated in the hydrolysate before and after incubation. Uninoculated substrates were taken as negative controls and processed following the same protocol of the experimental setups.

The fungal isolates selected were evaluated for their interactions with each other through compatibility tests as described by Molla et al. (2001). To study the biodegradation potential of the consortia, the sterilized substrates were co-inoculated with both the fungal isolates of each consortium and incubated. All other experimental procedures followed were similar to the protocol followed to determine the enzymatic activity and sugar yield by single isolates.

Comparison between physical and biological pretreatment methods

Experiments were carried out to compare the biological pretreatment with physical pretreatment through mild steam explosion or autoclave treatment (AC) and microwave irradiation treatment (MW). The substrates were subjected to AC treatment at 121°C, 15 psi for 1 h and irradiated at 270 W for 10 min for MW pretreatment. Only the best consortium that gave the highest sugar yield on RS and SCB were considered for this study. The combined effect of biological and physical pretreatments on the sugar yield was also studied. All the procedures followed for inoculation, incubation, and harvesting were the same as described earlier.

Quantitative estimation of lignocellulolytic activity

Ligninolytic enzymes laccase (Niku-Paavola et al. 1988), LiP (Tien and Kirk 1988), MnP (Paszczyński, Crawford, and Huynh 1988), and cellulolytic enzymes CMCase, Avicelase, FPase, and xylanase were determined in the cell-free culture filtrates (Ghose 1987) following standard procedures. The enzyme units were expressed as micromole of substrate transformed per minute per liter of culture filtrate, i.e., as enzyme units per liter of culture filtrate (U L^{-1}). The sugar yield was determined by dinitrosalicylic acid (DNS) method (Miller 1959). All estimates were carried out in triplicate.

Scanning electron microscopy (SEM) analysis

SEM analysis was carried out to observe the physical changes in the native untreated and treated substrates. Images of the substrates were taken using a JEOL (Tokyo, Japan) JSM-5800 SEM at magnification 600×. The dried samples to be analyzed were mounted on a conductive carbon tape and coated with gold palladium using sputter coater module (Structure Probe, Inc., West Chester, PA, USA) for 140 s at DC (direct current) 47 mA and 20 kV.

X-ray diffraction (XRD) analysis

The change in crystallinity of the samples before and after treatment was estimated by the XRD analysis of the substrates. Microcrystalline cellulose (Avicel PH-101; Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India) was used as a positive control. Scanning was performed on a Rigaku x-ray diffractometer (Ultima IV; Rigaku Corp., Tokyo, Japan) instrument set at 40 kV, 20 mA; radiation was Cu Kα ($\lambda = 1.54$ Å) and a range between 5° and 40° with a step size of 0.02°. Crystallinity index (CrI) was calculated according to the empirical method described by Segal et al. (1959):

$$\mathrm{CrI} = (I_{002} - I_{\mathrm{AM}}) / I_{002} \times 100 \quad (1)$$

where CrI expresses the relative degree of crystallinity, I_{002} is the maximum intensity of the 002 lattice diffraction, and I_{AM} is the height of the minimum between the 002 and the 101 peaks. The crystallite size was calculated from the Scherrer equation, where the width of the crystalline peak (002) at half height has been directly related to the crystallite size:

$$D(hkl) = K\lambda / \beta_0 \cos\theta \quad (2)$$

where $D(hkl)$ is the size of crystallite, innm is the Scherrer constant (0.94),λ is the x-ray wavelength

Table 1. Lignocellulolytic enzyme activities (U L^{-1}) of the fungal isolates.

	Enzyme activities (U L^{-1})				
	BK			BSS-C	BSS-X
Isolate	Laccase	LiP	MnP	CMCase	Xylanase
GK1	ND	ND	ND	400.7 (41.6)	277.3 (54.1)
GK2	ND	ND	ND	270.9 (20.3)	403.4 (16.9)
G4	ND	2.2 (0.1)	ND	15.4 (4.3)	59.3 (29.6)
G10	ND	2.0 (0.3)	ND	11.6 (3.1)	57.7 (4.6)
G13	ND	0.4 (0.1)	2.1 (0.6)	35.4 (12.3)	77.5 (27.6)
M155	0.1 (0.0)	0.2 (0.0)	2.4 (0.7)	51.0 (13.8)	122.7 (39.5)
M158	ND	0.2 (0.1)	3.0 (3.1)	17.4 (6.4)	48.6 (22.2)
M2E	12.2 (1.0)	3.8 (0.3)	37.5 (1.9)	161.5 (21.9)	238.5 (53.2)
2a	0.4 (0.0)	1.4 (0.8)	25.6 (2.3)	17.9 (9.5)	102.0 (16.4)

Note. BK: Boyd and Kohlmeyer medium; BSS-C and BSS-X = basal salt solutions with carboxymethyl cellulose and birch wood xylan, respectively; ND = not detectable under employed assay conditions. Standard deviation values of the triplicates are given in parentheses.

(0.15418 nm for Cu), β_0 is the full width at half maximum of the reflection *hkl*, and 2θ is the corresponding Bragg angle (Oh et al. 2005).

Results and discussion

Isolation, screening, and enzyme activity of isolates

Of the 100 fungal isolates screened on plate assay, 7 fungal isolates—GK1 (*Chaetomium globosum*) and GK2 (*Chaetomium brasiliense*) from the Gulf of Kutch region; G4 (*Engyodontium album*), G10 (*Metarhizium anisopliae*), and G13 (*Engyodontium album*) from the Grande Is. off Goa; M155 (*Acremonium persicinum*) and M158 (*Acremonium minutisporum*) from Gulf of Mannar; and M2E (*Inonotus tropicalis*) from the Goa mangrove habitats—exhibited good lignocellulolytic activity. The activity of these isolates and isolate 2a (Raghukumar et al. 1999) was evaluated in the liquid culture medium based on the laccase, LiP, MnP, CMCase, and xylanase activities. These enzymes play a crucial role in degrading the lignocellulosic biomass to release fermentable sugars, and the isolates exhibited different levels of enzyme activity (Table 1).

All the three major ligninolytic enzyme, laccase, LiP, and MnP, activities were recorded only in the isolates M155, M2E, and 2a. Among these isolates, M2E showed very good activity levels of 12.2, 3.8, and 37.5 U L^{-1} for laccase, LiP, and MnP, respectively. LiP and MnP are the important peroxidases and were produced by nearly five isolates, but the activity of all the isolates was less than M2E. Based on the quantification of the lignin-degrading enzymes of the different isolates grown on BK broth, M2E could be designated as the highest potential lignin degrader (Table 1). CMCase and xylanase activity levels reflect the ability of the isolates to be actively involved in the saccharification process of the delignified or cellulose substrates. Isolates GK1 and GK2 showed significant CMCase and xylanase activities, followed by M2E. Isolate M2E, which showed good activity for all the five enzymes, seems to have a good potential for industrial applications (Table 1).

Enzyme activity of isolates on lignocellulosic substrates

The lignin-degrading enzyme activities of M2E and 2a increased considerably when the isolates were grown on

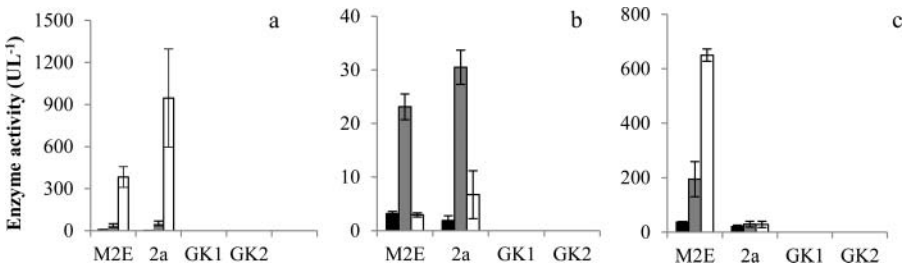

Figure 1. Ligninolytic enzyme laccase (a), LiP (b), and MnP (c) activities of four selected isolates grown on plain BK medium (black), rice straw (gray), and sugarcane bagasse (white).

lignocellulosic substrates RS and SCB. Laccase activity in isolates M2E and 2a increased considerably from approximately 10 and 1 U L^{-1} to 37.1 and 52.5 U L^{-1} respectively, when they were grown on RS. A more significant increase to 383.3 and 946.4 U L^{-1} was observed when the isolates were grown on SCB (Figure 1a). A 10-fold increase in LiP activity was observed in isolates M2E and 2a on RS, and there was not much increase on SCB substrate (Figure 1b). M2E showed increased MnP activity when grown on RS and SCB, from 37.5 to 194.6 and 649.8 U L^{-1}, respectively. However, 2a did not show any increase in MnP activity on both the substrates (Figure 1c). The isolates GK1 and GK2 were able to grow on RS and SCB (visual observation), but they did not show any quantifiable ligninolytic activity (Figure 1).

CMCase (endoglucanase), Avicelase (exoglucanase), and FPase (total cellulase activity) assays were performed to understand the cellulolytic activity of the isolates. Xylanase activity in isolates is important, as hemicellulose is composed of about 70% xylan. Only when the hemicellulose is broken down do the cellulose regions become accessible to the cellulase enzymes, which depolymerase it to pentose sugars, which will then be available for the fermentation process. The four isolates studied for cellulolytic activity on untreated lignocellulosic substrates did not show much change in their endoglucanase and exoglucanase activities (Figure 2a and b). Isolates GK1 and GK2, with the highest cellulolytic activity on BSS medium (Table 1), showed an increase in activity of FPase from approximately 70 to 170 U L^{-1} and <500 to approximately 1500 U L^{-1} when they were grown on RS (Figure 2c and d). However, no change in activity was observed when the isolates were grown on SCB. This lack of change could be because the substrates were not pretreated; delignification is crucial for efficient utilization of the substrates.

Lignocellulolytic activities of fungal isolates have an enormous potential for bioutilization of lignocellulosic substrates, as they are the major group of microorganisms capable of synthesizing enzymes to degrade these substrates (Cohen and Hadar 2001). The application of crude enzyme cocktails from a consortium is also explored for its direct application in biodegradation, as sugar loss due to consumption when whole cultures are used can be avoided (Jiménez, Dini-Andreote, and van Elsas 2014). Dias et al. (2010) recorded a maximum laccase production of 0.10, MnP about 0.82, xylanase and CMCase of 0.08 and 0.15 U ml^{-1}, respectively, in a basidiomycetous fungi grown on wheat straw. Studies carried out using nine strains of *Phanerochaete chrysosporium* for their lignocellulolytic enzyme production on paddy straw reported CMCase activity of 0.36–0.42 and filter paper units (FPU) of 0.55–0.88 U ml^{-1} (Mishra and Pandey Lata 2007). However, isolates 2a and M2E from this study showed a much better increase in ligninolytic activity on the substrates (Figure 1a). Based on the enzyme activity and growth characteristics, M2E and 2a were identified as

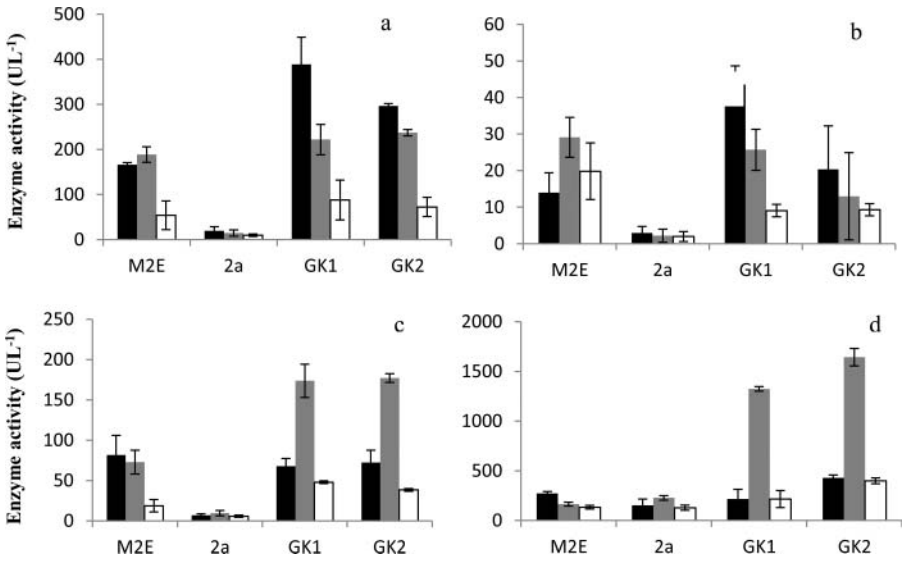

Figure 2. Cellulolytic enzyme CMCase (a), Avicelase (b), FPase (c), and xylanase (d) activities of four selected isolates grown on basal salt solution containing the respective substrates (black), on rice straw (gray), and sugarcane bagasse (white).

ligninolytic isolates. Both belong to phylum Basidiomycota and are studied for their lignolytic activity. Although reports say that *Inonotus* sp. are difficult to be grown under laboratory conditions, M2E isolate grew luxuriantly and exhibited good enzyme activity in the experimental setup. Similarly, isolates GK1 and GK2 *Chaetomium* spp., selected for their cellulolytic activity, are also widely studied for their biotechnological potentials (Cohen and Hadar 2001). Fungal consortia between these potential isolates were set up to study their bioconversion efficiency.

Enzyme activity of fungal consortia grown on lignocellulosic substrates

Consortiums were set up between the ligninolytic isolates (M2E or 2a) and the cellulolytic isolates (GK1 or GK2). Molla et al. (2001) have defined that interactions between isolates can be categorized as mutual intermingling, partial intermingling, inhibition at contact point, inhibition at a distance, and replacement. Compatibility evaluation of the isolates showed that none of the combinations showed inhibition or replacement, so all the possible combinations of consortia were selected for further studies. The interaction between M2E and GK1 clearly showed mutual intermingling, and all the other combinations M2E with 2a and GK2, 2a with GK1 and GK2, and GK1 with GK2, were found to be partially intermingling (Figure S1).

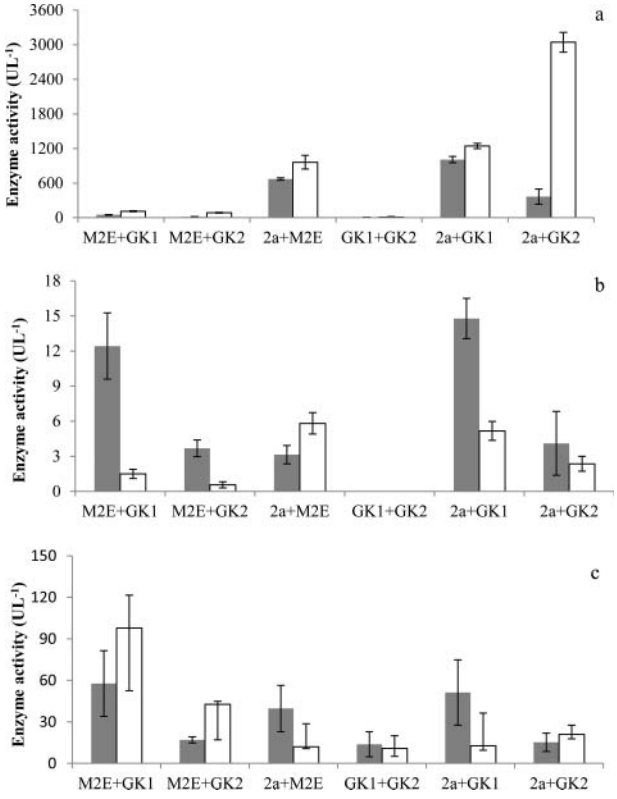

Figure 3. Ligninolytic enzyme laccase (a), LiP (b), and MnP (c) activities of consortia on rice straw (gray) and sugarcane bagasse (white).

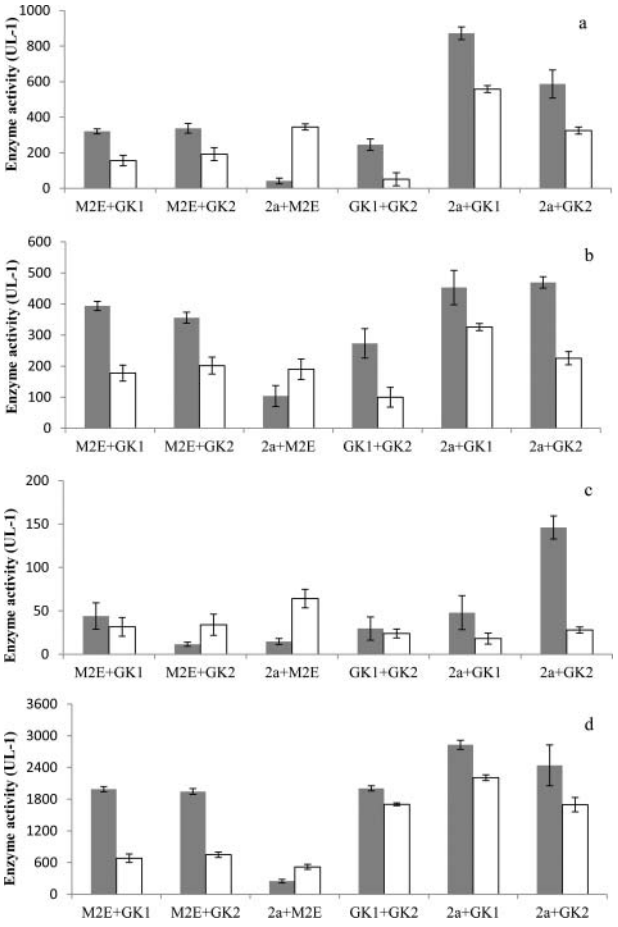

Figure 4. Cellulolytic enzyme CMCase (a), Avicelase (b), FPase (c), and xylanase (d) activities of consortia on rice straw (gray) and sugarcane bagasse (white).

The enzyme activity in a consortium is highly influenced by the extent of compatibility between the organisms. The maximum recorded laccase activity by an individual isolate was less than 1000 U L^{-1} and increase in activity of all consortiums with 2a was recorded up to 3000 U L^{-1} in the 2a+GK2 setup (Figure 3a). None of the other consortia showed any increase in the laccase activity, and there was no increase in LiP and MnP activities in any of the consortia (Figure 3b and c). Increase in CMCase and Avicelase activities was observed in consortiums composed of ligninolytic (M2E and 2a) and cellulolytic (GK1 and GK2) isolates (Figure 4a and b) in

Table 2. Sugar yields (g L^{-1}) from rice straw and sugarcane bagasse hydrolysates.

	Sugar yields (g L^{-1})			
	Rice straw		Sugar cane bagasse	
Isolate/Consortium	0 h	24 h	0 h	24 h
GK1	0.41 (0.07)	0.46 (0.11)	0.55 (0.06)	0.68 (0.12)
GK2	0.28 (0.09)	0.42 (0.05)	0.60 (0.28)	1.35 (0.53)
M2E	0.46 (0.05)	0.57 (0.14)	0.18 (0.08)	0.53 (0.07)
2a	0.09 (0.05)	0.20 (0.07)	0.72 (0.06)	0.73 (0.09)
M2E+GK1	0.52 (0.17)	0.61 (0.08)	0.7 (0.09)	1.47 (0.42)
M2E+GK2	0.51 (0.13)	1.09 (0.27)	1.15 (0.22)	4.39 (0.18)
2a+GK1	0.26 (0.04)	0.83 (0.05)	0.12 (0.04)	0.65 (0.07)
2a+GK2	0.15 (0.04)	2.64 (0.17)	0.1 (0.04)	0.89 (0.07)
M2E+2a	0.1 (0.06)	0.12 (0.04)	0.39 (0.10)	0.41 (0.10)
GK1+GK2	0.61 (0.09)	0.88 (0.12)	0.93 (0.07)	2.37 (0.16)
Negative control*	0.18 (0.14)	0.23 (0.13)	0.29 (0.06)	0.31 (0.06)

* Without inoculum.

comparison with the activity of individual isolates. All the consortiums showed an increase in FPase and xylanase activities (Figure 4c and d).

Comparison of sugar yield by single isolates and consortia from lignocellulosic substrates

The sugar concentration yielded from RS and SCB when treated with individual isolates was less than 1 gL^{-1}, except GK2, which yielded the highest sugar concentration of 1.35 g L^{-1} from SCB (Table 2). There was a marginal increase in the sugar yield from all the consortia in comparison with the single isolates. Among the different consortia, M2E+GK2 gave the maximum sugar yield of 4.39 g L^{-1} on SCB, followed by 2a+GK2, which yielded 2.64 g L^{-1} on RS (Table 2).

SEM analysis

SEM images of the untreated and treated substrates with single isolates and the best consortia for each substrate gave evidence of the physical changes that occurred during the treatment (Figure 5). In the case of untreated RS, the structures appear smooth and the ordered arrangements can be observed on the surface (Figure 5a). But the rice straw treated by M2E (Figure 5b) and 2a (Figure 5c) showed disrupted surfaces and of flaking out. The rice straw treated by GK1 (Figure 5d) and GK2 (Figure 5e) did not show much change except for some uneven and worn out structures. Maximum disruption of the RS can be seen in the substrates treated by the consortium 2a+GK2 (Figure 5f) as compared with the single isolates. Similarly, untreated SCB has smooth and continuous surfaces, the external fibers are loosened during the treatment with M2E and 2a, minor disruptions by GK1 and GK2 are also evident, and intense flaking caused by the best consortia M2E+GK2 is reflected from the SEM images (Figure 5g–l).

XRD analysis

Cellulose in natural substrates is highly complex, as there are transition regions known as paracrystalline cellulose between crystalline and amorphous structures (Larson, Wickholm, and Iversen 1997). The amorphous forms are readily degraded compared with the crystalline components. It is also possible that the amorphous region is buried in the interior that is tightly packed by neighboring crystallites, thus making it sufficiently impenetrable to the enzymes. In such cases, the enzymes target the crystallites and break them down (Park et al. 2010).

The crystallinity index (CrI) and crystallite size (nm) from the XRD spectra were analyzed for the untreated and treated samples (Segal et al. 1959). The peak height of the XRD spectrum was measured to calculate the CrI, which reduced from 56.6% to 42.52% in RS treated with 2a+GK2 consortium and SCB CrI reduced from 49.02% to 42.92% following treatment by M2E+GK2 consortium (Table 3). Avicel tested as a positive control and showed the highest CrI of 80.6% due to its microcrystalline structure. The crystallite size of untreated RS and that of SCB were in the range of 0.9266 and 0.8345 nm, respectively. The crystallite sizes of treated RS and SCB reduced and could be confirmed by the decrease in CrI, which is associated with the reduction of crystalline domains or an increase of the amorphous domains (Poletto,

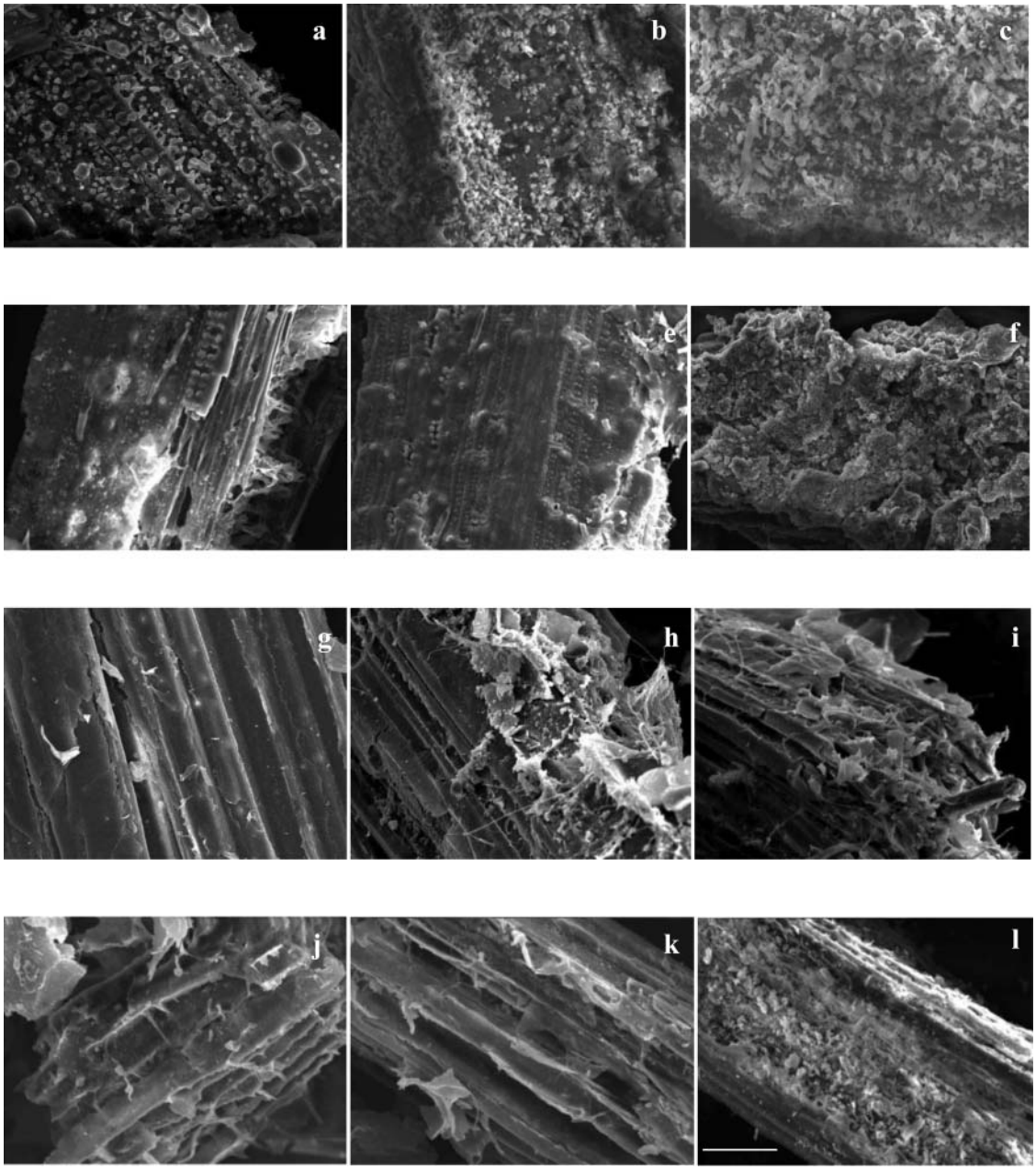

Figure 5. Scanning electron microscopy images of untreated rice straw (a); rice straw treated by M2E (b), 2a (c), GK1 (d), GK2 (e), and 2a+GK2 (f); untreated sugarcane bagasse (g); and sugarcane bagasse treated by M2E (h), 2a (i), GK1 (j), GK2 (k), and M2E+GK2 (l). Scale measures 50 μ.

Table 3. Crystallinity indices and crystallite sizes of samples that yielded the maximum sugar content.

Substrate	Crystallinity index (%)	Crystallite size (nm)
Untreated rice straw	56.56	0.9266
Rice straw treated with 2a+GK2	42.52	0.7178
Untreated sugar cane bagasse	49.02	0.8345
Sugar cane bagasse treated with M2E+GK2	42.92	0.7176
Avicel (positive control)	80.61	4.3653

Table 4. Comparison of sugar yields (g L^{-1}) from the rice straw and sugarcane bagasse hydrolysates using different pretreatment techniques.

	Sugar yields (g L^{-1})			
	Rice straw		Sugarcane bagasse	
Treatment	0 h	24 h	0 h	24 h
Biological pretreatment				
GK2	0.28 (0.09)	0.42 (0.05)	0.60 (0.28)	1.35 (0.53)
M2E+GK2	0.51 (0.13)	1.09 (0.27)	1.15 (0.22)	4.39 (0.18)
2a+GK2	0.15 (0.04)	2.64 (0.17)	0.1 (0.04)	0.89 (0.07)
Autoclave pretreatment				
GK2	1.16 (0.16)	1.03 (0.10)	1.45 (0.16)	1.64 (0.07)
M2E+GK2	0.69 (0.09)	0.85 (0.14)	1.09 (0.08)	1.24 (0.22)
2a+GK2	0.36 (0.15)	0.58 (0.11)	0.48 (0.11)	0.66 (0.10)
Microwave pretreatment				
GK2	0.69 (0.26)	0.72 (0.59)	1.39 (0.11)	1.44 (0.17)
M2E+GK2	0.59 (0.19)	0.91 (0.14)	1.12 (0.08)	3.76 (1.46)
2a+GK2	0.40 (0.11)	0.50 (0.14)	0.50 (0.03)	0.62 (0.08)

Heitor, and Zattera 2014). The decrease in the CrI and crystallite size could be because the crystalline cellulose was converted to a less ordered amorphous form, and its accumulation was also reflected in the broadening of the peak in the XRD spectrum.

The reduction in the crystallinity of cellulose in the substrate is one of the crucial steps known to significantly affect hydrolysis. The x-ray diffraction profile of the treated substrates shows that the crystallinity indices and crystallite sizes were reduced and converted to amorphous regions (Table 3). The isolates employed in this study prove to be efficient in the biodegradation of lignocellulosic substrates. Optimization of this process would further increase its efficiency, thus making it an economically applicable method on industrial scales.

Comparative analysis of biological and physical pretreatments

It was observed that consortiums formed from efficient ligninolytic and cellulolytic fungi could enhance the final sugar yield obtained without the requirement of any other additional pretreatment methods. Experiments were also carried out to compare the biological with physical pretreatment methods; the combined effect of biological and physical pretreatments to increase the sugar yield was also tested. The sugar yield by GK2, when grown on RS, without any pretreatment was only 0.42 g L^{-1}, and in the two different physical pretreatments, the sugar yield increased to 1.03 and 0.72 g L^{-1}. For the same isolate, when grown on SCB, without pretreatment, the sugar yield was 1.35 g L^{-1}, and following physical pretreatment, it yielded 1.64 and 1.44 g L^{-1} (Table 4). The biological pretreatment by the two ligninolytic isolates M2E and 2a showed very good activity and maximum sugar concentrations of 2.64 and 4.39 g L^{-1} from RS and SCB, respectively, were obtained. High MnP activity by M2E+GK2 consortium on SCB and increased laccase activity of 2a+GK2 on RS followed by good cellulolytic of these consortia (Figures S2 and S3) could be the main reason for the increased sugar yield from the substrates. Although the physical pretreatment and the combination of physical and biological pretreatments were able to moderately increase the sugar yield from the substrates, it was less than those obtained through biological pretreatment using the developed

consortia. These results clearly demonstrate that potential fungal isolates can be used for biological pretreatment and effective bioutilization of lignocellulosic substrates.

Pensupa et al. (2013) compared the glucose concentration in the hydrolysate obtained by the hydrolysis of fermented wheat straw using the fungal culture filtrate and the commercial cellulose Ctec2. The former gave 4.34 and the latter gave 3.13 mg mL^{-1} of glucose, respectively. These results were comparable to the maximum sugar concentrations of 2.64 and 4.39 g L^{-1} obtained by the enzymatic hydrolysates of RS by 2a+GK2 and SCB by M2E+GK2, respectively (Table 3). Studies show that physical treatment such as microwave irradiation can enhance the saccharification process (Binod et al. 2012). In our results, the physical pretreatment and combination of physical and biological pretreatments were able to moderately increase the sugar yield from the substrates, but the maximum sugar yield was obtained through biological pretreatment in two of the best-developed consortia (Table 4). In the present work, we have shown that the use of consortia between ligninolytic and cellulolytic fungi could efficiently enhance the degradation of rice straw and sugarcane bagasse.

Conclusion

A consolidated bioprocessing technique, which combines enzyme production, saccharification, and fermentation, is gaining recognition. The simultaneous biological pretreatment and saccharification process tested in this study using a consortium of ligninolytic and cellulolytic marine derived fungi proves to have potential for effective utilization of agricultural wastes. The maximum sugar concentration of the enzymatic hydrolysate obtained was 2.64 mg/ml from RS by 2a+GK2 and 4.39 mg/ml from SCB by M2E+GK2. These levels were considerably higher than the results obtained for any individual isolate studied. It was also observed that autoclave and microwave treatment under specified employed conditions could not enhance the sugar concentration obtained by biological pretreatment and saccharification using the most efficient consortia. Also, the effect of these physical pretreatments alone could not produce similar results to biological pretreatments. This shows that microbial consortia of potential isolates can be utilized for effective biodegradation of lignocellulosic substrates. Identification of such potential isolates from natural habitats is an ongoing process and is required for developing a sustainable, efficient, and economically feasible technology.

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