Bioresource Technology 173 (2014) 352–360

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Kenaf biomass biodecomposition by basidiomycetes and actinobacteria in submerged fermentation for production of carbohydrates and phenolic compounds

Ivana Brzonova [a], Evguenii Kozliak [b], Alena Kubátová [b], Michelle Chebeir [c], Wensheng Qin [d], Lew Christopher [e], Yun Ji [a,*]

[a] Department of Chemical Engineering, University of North Dakota, Grand Forks, ND, USA
[b] Department of Chemistry, University of North Dakota, Grand Forks, ND, USA
[c] Department of Chemical and Material Engineering, California State Polytechnic University, Pomona, CA, USA
[d] Department of Biology, Lakehead University, Thunder Bay, ON, Canada
[e] Center for Bioprocessing Research and Development, South Dakota School of Mines & Technology, Rapid City, SD, USA

HIGHLIGHTS

- Release and removal of monosaccharides occur prior to lignin biodecomposition.
- Monosaccharide products may be collected separately from phenolics.
- TD–Pyr–GC–MS confirmed that lignin decomposition as a result of microbial action.
- Biomass and lignin degradation were similar for fungi and bacteria.

ARTICLE INFO

Article history:
Received 1 July 2014
Received in revised form 12 September 2014
Accepted 13 September 2014
Available online 20 September 2014

Keywords:
Biomass
Lignin
Kenaf
Basidiomycetes
Actinobacteria

ABSTRACT

The efficiency and dynamics of simultaneous kenaf biomass decomposition by basidiomycetous fungi and actinobacteria were investigated. After 8 weeks of incubation, up to 34 wt.% of the kenaf biomass was degraded, with the combination of fungi and bacteria being the most efficient. Lignin decomposition accounted for ~20% of the observed biomass reduction, regardless of the culture used. The remaining 80% of biomass degradation was due to carbohydrate based polymers. Major monosaccharides were produced in tangible yields (26–38%) at different times. Glucose, fructose and xylose were then fully consumed by day 25 while some galactose persisted until day 45. Once monosaccharides were depleted, the production of laccase, manganese-dependent peroxidase and lignin peroxidase enzymes, essential for lignin decomposition, was induced. The products of lignin biodecomposition were shown to be water-soluble and characterized by thermal desorption–pyrolysis–gas chromatography.

1. Introduction

Lignocellulosic biomass (LCB) is one of the most abundant and economical sources of valuable chemicals and biofuels. LCB, whose production in the US is near 1.4 billion dry tons per year (Limayem and Ricke, 2012), is currently used for manufacturing of methanol and ethanol (Limayem and Ricke, 2012; Sarkar et al., 2012) from its cellulose portion. By contrast, lignin is currently utilized mostly as a source of heat and electricity by its combustion, although several pioneering applications were recently envisioned for its use to produce replacement petrochemicals (De la Torre et al., 2013). This study focuses on the use of kenaf (*Hibiscus cannabinus* L.) as a biomass source. Kenaf is a readily grown plant both in warm and colder climate zones, thus being a reasonable candidate for production of renewable chemicals.

The use of lignin-degrading fungi and/or cellulase-producing bacteria is essential for efficient biomass conversion. Based on the previous studies conducted with biomass sources other than kenaf, white rot fungi are considered to be the most efficient wood-degrading organisms (Evans and Palmer, 1983; Sun et al., 2004). Kenaf was also successfully degraded by white rot fungi (Halis et al., 2012). Table 1 lists the essential information on the previous work on microbial degradation of lignocellulosic biomass. The presented information is not complete due to the large number

* Corresponding author.

http://dx.doi.org/10.1016/j.biortech.2014.09.057
0960-8524/

Table 1
Overview of studies targeting microbial lignin degradation in lignocellulosic biomass.

Biomass source	Microorganism used		Incubation time (day)	Lignin loss (%)	Biomass loss (%)	Analysis of degradation products	References
	Fungi	Bacteria					
Wood	*T. trogii MT*	–	84	22	–	DEM, IR, LC–MS	Ji et al. (2012)
	C. subvermispora	–	84	20	15–20	Gravimetric	Tanaka et al. (2009)
	P. ostreatus	–	180	9	–	Gravimetry	Piškur et al. (2011)
	C. versicolor	–	84	75	–	UV VIS, HPLC, NMR, gravimetry	Evans and Palmer (1983)
Kenaf	*P. sanguineus* *O. latemarginatus*	–	91	15 10		FTIR, SEM, gravimetry	Halis et al. (2012)
	O. latemarginatus *R. vinctus* *P. chrysosporium* *C. versicolor*	–	28	–	28 14 8 12	Gravimetry	Mohamed et al. (2013)
Agricultural waste (straw, stalk)	–	*Bacterial community XDC-2*	12	45 9 16	25 38 20	GC–MS	Hui et al. (2013)
	P. chrysosporium	–	21	30	–	Gravimetric, NMR, FTIR, Py–GC MS	Singh et al. (2011)
			7	25	27	SEM, gravimetry, FTIR, Pyr–GC, X ray diffraction	Zeng et al. (2011)
	–	*S. viridosporuis*	56	APPL (acid precipitable polymer lignin) 20	36	Gravimetric, GPC, NMR, titration	Crawford et al. (1983)
	P. chrisosporium	*S. viridosporus* *S. badius* *S. setonii*	21	APPL 19–27	–	HPLC, GPC	Pometto and Crawford (1986b)
	P. chrysosporium	–	10	14–36	–	Gravimetric	Yao and Nokes (2014)
	T. hirsuta	–	42	71	73	Gravimetric	Sun et al. (2011)
	P. chrysosporium	–	15	34	10	Gravimetric	Zhao et al. (2012)
	Irpex lacteus	–	28	60	25	Gravimetric	Song et al. (2013)
	–	*Bacillus* sp. *Bacillus* sp. and *Lactic acid bact.*	10 10	20 62	–	Gravimetric	Chang et al. (2014)

of pertinent publications. Those articles that are not included in Table 1 addressed only the degradation of carbohydrates rather than that of both carbohydrates and lignin. Similarly, publications are not cited that focused on bioethanol production, with only cursory, e.g., gravimetric, information on lignin removal.

As can be seen from Table 1, most of the prior work focused on the degradation of agricultural wastes with lower lignin content. Most of the studies used fungi as lignin-degrading cultures; furthermore, most of the work was conducted using *Phanerochaete chrysosporium* (Zeng et al., 2011; Zhao et al., 2012; Yao and Nokes, 2014). A potential contribution of indigenous microorganisms was merely mentioned but not documented. Gravimetry was used in most of the publications as the main technique monitoring the lignin decomposition. Only a few studies involved the use of protocols assessing the conversion of both carbohydrates and lignin using more sophisticated analyses of lignin decomposition products (Crawford et al., 1983; Zeng et al., 2011).

As a step toward efficient and comprehensive LCB biochemical conversion, this study explores the application of both white rot fungi and cellulase producing bacteria. The white rot fungi used in this study, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), are known to degrade all three major biomass components (i.e., lignin, cellulose and hemicellulose) at similar rates (Sun et al., 2004). *Microbacterium* sp. (MB) and *Streptomyces* sp. (SM) are cellulase producing bacteria (Hong et al., 2011; Maki et al., 2011) but may also degrade lignin (Seo et al., 2009) and phenolics (Zhang et al., 2013).

A possibility of simultaneous efficient decomposition of both lignin and cellulose into smaller organic molecules was explored here for the first time, focusing on the dynamics of product evolution. To address this objective, the biomass treatment was conducted using several combinations of lignin converting basidiomycetous fungi and cellulase producing bacteria. The specific goal of this study was to apply *mixtures* of lignin-degrading fungi (CV and TG) and lignocellulose-degrading microorganisms (MB and SM). We postulated that such a treatment would facilitate lignin utilization for generation of valuable chemicals due to potential synergy.

A comprehensive suite of analytical techniques was applied in this study, not only to assess the biomass conversion efficiency but also to analyze the main lignin degradation products. The traditional gravimetrical analysis was supplemented with field emission scanning electron microscopy (SEM), high process liquid chromatography (HPLC) and thermodesorption–pyrolysis–gas chromatography–mass spectrometry (TD–Pyr–GC–MS), along with pH and enzyme activity monitoring. The TD–Pyr–GC–MS method in this study utilized the innovative use of different temperature steps, which showed that the observed phenolics were the low-MW products of lignin degradation rather than non-decomposed lignin itself.

2. Methods

2.1. Feedstock and microorganism

Air dried kenaf biomass was provided by the Department of Plant Sciences, North Dakota State University (Fargo, North Dakota,

Table 2
Experimental setup for kenaf biodegradation experiments. The microorganisms used are labeled with X while the applied operational conditions are labeled with Y.

Fungi		Bacteria		Operational conditions		
Coriolus versicolor	*Trametes gallica*	*Mycobacterium* sp.	*Streptomyces* sp.	Stationary/dark/room temp	Stationary/light/[a] Room temp	Stirring/with less light/[b] at 29 °C
X	–	–	–	Y	Y	Y
–	X	–	–	Y	Y	Y
X	X	–	–	Y	Y	Y
X	–	X	–	Y	Y	Y
X	–	–	X	Y	Y	Y
–	X	X	–	Y	Y	Y
–	X	–	X	Y	Y	Y
X	X	X	–	Y	Y	Y
X	X	–	X	Y	Y	Y
–	–	X	X	Y	Y	Y
X	X	X	X	Y	Y	Y
–	–	–	–	Y	Y	Y

[a] Sunlight passed through a window glass.
[b] In an incubator which was placed in a room with sunblinds.

USA). Kenaf biomass was pulverized in a Thomas Wiley mill to yield the average particle size distribution between 2.0 and 1.0 mm. Cellulase producing bacteria (MB and SM) and basidiomycetous fungi (CV and TG), were obtained from the Department of Biology of Lakehead University, Thunder Bay, Ontario, Canada. All other chemicals were purchased from Sigma Aldrich, St. Louis, MO, USA.

2.2. Biodecomposition setup

Erlenmeyer flasks (200 mL) used for biodecomposition experiments contained 2.0 g of kenaf and 75 mL of liquid medium. A medium for cultivation of both fungi and bacteria was prepared by mixing 2.7 g dextrose, 5.0 g sucrose, 1 g $MgSO_4$, 2.5 g peptone, 1.5 g yeast extract, 2.5 g NaCl and 1.5 g KH_2PO_4 in 1 L of deionized water (i.e., phosphate buffer, pH 5.5). Kenaf was sterilized along with the liquid medium for 20 min at 121 °C. Once cooled down, the microbial mixtures were inoculated. For fungal inoculation, eight 4×4 mm fragments of agar were applied, which were removed from the plates with fully grown cultures were applied. For bacterial inoculation, 1 mL of bacterial medium with an OD_{600} of 1.0 was added.

Experiments were conducted while varying three environmental parameters, stirring, light and temperature. Three different setups were applied: (1) with both stirred and non-stirred media, (2) either under light or in the dark and (3) at room temperature vs. the incubator temperature, 29 °C. For experiments in the dark, the Erlenmeyer flasks were completely covered with aluminum foil. The experiments with stirred media were performed in an incubator at 29 °C for 57 days, with a rotating speed of 60 rpm. The samples for analysis were taken on days 6, 11, 18, 25, 36, 46 and 57.

Both CV and TG were used in all binary and ternary combinations among themselves and/or with MB and SM bacteria, plus the combination of all four strains and a mixture of two bacteria as specified in Table 2. Control samples included (1) sterile kenaf biomass with the corresponding dead (sterilized) microorganism(s) and (2) non-sterile kenaf biomass without any specific bacteria or fungi. Experiments were conducted in triplicate unless the variation in operational conditions resulted in no difference. In those cases, the values obtained with and without illumination and/or shaking were combined.

2.3. Chemical characterization

2.3.1. Gravimetric analysis

The overall analysis setup is shown in Fig. 1A, with the chromatographic analysis detailed in Fig. 1B. The biomass amount along with its lignin and ash contents were measured gravimetrically using a standard method, NREL TP510-42618 (Sluiter et al., 2011).

2.3.2. Monosaccharide analysis

Samples were filtered through a 0.2 μm filter and run through an Agilent 1200 HPLC (Waldbronn, Germany) with a refractive index detector, RID (Shanghai, China) and a 300×7.8 mm Transgenomic CHO-Pb column (Omaha, NE). The mobile phase was deionized water with a flow rate of 0.6 mL min^{-1}. Prior to the sample analysis, a set of external calibration standards were analyzed to calibrate the HPLC RID detector. The concentrations of standards ranged from 0.5 to 18 g L^{-1}. In addition, an internal sugar recovery standard with a concentration of 4.0 g L^{-1} was run every 8 injections to test for column parameter drift and validate the RID readings. The external standard and recovery standard solutions consisted of D-(+)glucose, D-(+)xylose, D-(+)galactose, L-(+)arabinose, and D-(+)mannose.

2.3.3. Scanning electron microscopy (SEM)

At the end of experiments (day 57), the liquid medium was filtered through a 0.2 μm filter; then, one droplet of this sample was placed on a glass cover plate and air-dried at room temperature (24 ± 3 °C). The resulting dry residue was examined under a field emission scanning electron microscope, SEM (Hitachi, SU8010, Japan). The solid fraction of the remaining kenaf biomass was washed three times with distilled water, centrifuged (13,000 rpm) and dried at 65 °C prior to the SEM examination.

2.3.4. Analysis of biomass (lignin) decomposition products

Lignin decomposition products were analyzed by a thermal desorption–pyrolysis system (CDS Analytical) connected to an Agilent 7890 gas chromatograph equipped with a mass spectrometric detector, 5975C (Santa Clara, CA) MS with electron ionization (TD–Pyr–GC–MS). The filtered solid phase (Fig. 1B) was analyzed at three allotted times: (1) at the start of biodecomposition experiments, then (2) on day 25, after the depletion of the bulk of carbohydrates and (3) at the end of experiments, on day 57. Liquid fractions of selected samples were centrifuged (10,000 rpm, 5 min) and filtered through a 0.2 μm filter. After the pH in the liquid fraction was adjusted to 6.0 with 0.1 mM H_2SO_4, a brown precipitate appeared. This precipitate and liquid fractions were dried and analyzed separately on TD–Pyr–GC–MS.

The pyroprobe program consisted of three temperature steps: TD at 350 °C for 3 min, 450 °C for 1 min (thermodesorption of adsorbed species and pyrolysis of oligomers) and 700 °C for 1 min (pyrolysis of high-molecular weight (MW) chemicals).

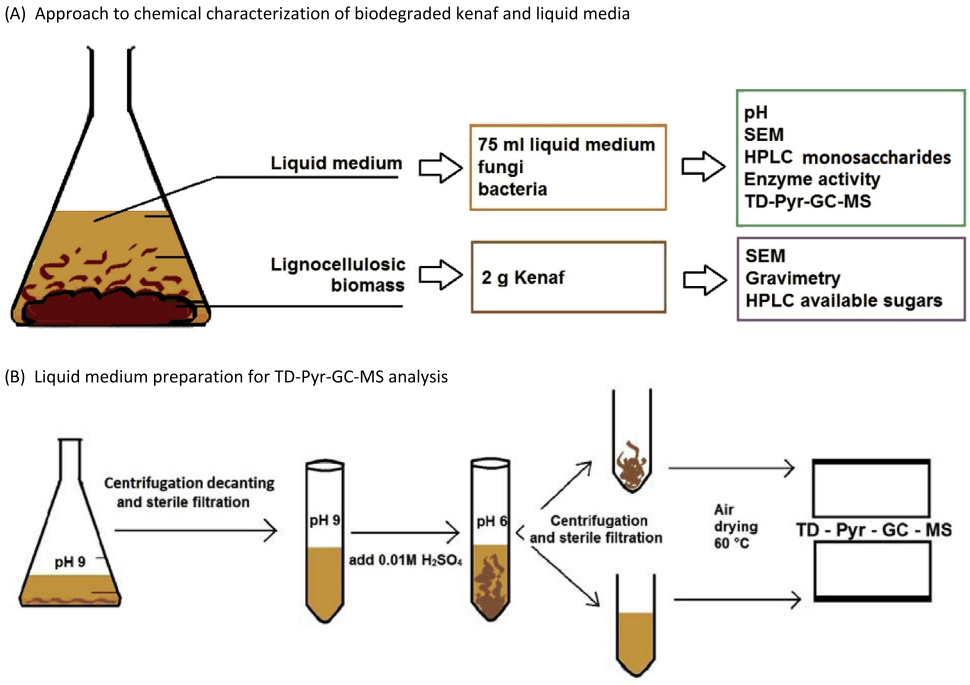

Fig. 1. The experimental setup. (A) Decomposition experiment and analytical methods applied to biomass and medium. (B) Detailed protocol for sample preparation for the TD–Pyr–GC–MS analysis of lignin decomposition products.

Samples were collected after each temperature step on a TENAX trap and evolved at 300 °C through a heated transfer line to GC–MS. The analyses were performed using a 30 m long DB-5MS column with 0.25 mm I.D. and 0.25 mm film thickness. Ultra-pure helium (99.999%) was used as the carrier gas with a constant flow rate of 1.1 mL min^{-1} and split ratio 10:1. The GC oven temperature program started at 40 °C min^{-1}, followed by a gradient of 35 °C min^{-1} to 80 °C then 15 °C min^{-1} gradient to 320 °C and hold for 7 min, with a total analysis time of 25 min. The lignin decomposition was assessed as a percent response of the sum of all three TD–Pyr (350, 450, 700 °C) steps.

2.3.5. Enzyme activity measurements

The enzyme activity measurements were conducted using a UV–vis Evolution™ 600 Spectrophotometer (Thermo Fisher Scientific, Madison, WI) according to the procedures described by Hong et al., 2011. The laccase activity was determined based on the product absorption at 436 nm with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) as a substrate. Manganese peroxidase (MnP) activity was measured at 469 nm using 2,6-dimethoxyphenol (DMP) as a substrate. Lignin peroxidase (LiP) was assayed at 651 nm with azure B as a substrate after a 1 h reaction at room temperature. For all of these assays, 250 μL of a filtered sample, 250 μL of a substrate (100 mM) and 500 μL of 0.01 M phosphate buffer were mixed. The reaction was initiated by the enzyme addition. The corresponding blanks contained no enzyme and consisted of 250 μL of the original sterile growth medium with 750 μL of 0.01 M phosphate buffer. One unit (U L^{-1}) of enzyme activity was defined as the amount of enzyme that transformed 1.0 μmol of a substrate per minute.

3. Results and discussion

3.1. Kenaf biodecomposition

The major effects caused by bacteria and/or fungi on kenaf biomass were investigated prior to chemical analyses and dynamic studies. The gravimetric information obtained in kenaf biodecomposition experiments is provided in Table 3. The biodecomposition was statistically different from the blank in all of the runs. It was also statistically similar with the control containing only indigenous microorganisms (no specific microorganisms were added). The combination of all four microbial species turned out to be most efficient; 34 ± 2% of the biomass was degraded in those runs after 57 days. Similar values were previously reported for bacterial (*Streptomyces*) biomass treatment, 36% of corn stover

Table 3
Gravimetric characterization of kenaf biomass samples after 57 days of biodecomposition.

Biodecomposition organism	Solid fraction [a]		Soluble fraction [b]
	Biomass decomposition (wt.%)	Lignin decomposition (wt.%)	(wt.%)
CV	24 ± 1	19 ± 1	25 ± 1
TG	21 ± 1	20 ± 1	24 ± 1
CV, TG	29 ± 2	19 ± 1	26 ± 1
CV, TG, MB, SM	34 ± 2	20	31 ± 1
Biomass with only indigenous microorganisms (control)	22 ± 1	12 ± 2	17 ± 1
Biomass sterilized with no specific microbial pretreatment (blank)	3 ± 1	3 ± 1	3 ± 1

[a] Calculated using a sample preparation protocol described in Section 2.3.1 as compared to the original sample.
[b] Calculated as the mass of dissolved matter (after liquid evaporation) as compared to the mass of the original sample (kenaf biomass plus solid medium components).

decomposition after 56 days (Pometto and Crawford, 1986a; Crawford et al., 1983).

The gravimetrically measured lignin biodecomposition percentages listed in Table 3 show similar results for both fungal species and their combination. The simultaneous application of lignin degrading fungi thus does not appear to result in a significant enhancement of biomass decomposition. However, the biomass decomposition was enhanced when both fungi and bacteria were applied, similar to the trends previously published in literature. Crawford et al. (1983) reported 19%, 13%, 5% and 3% lignin decomposition using *Streptomyces viridosporus* for corn stover, quackgrass, maple and spruce, respectively showing that the biomass source plays a major role. Alternatively, lignin degradation may also depend on operational conditions. Evans and Palmer (1983) did not observe any lignin decomposition by *C. versicolor* in nitrogen-rich media (milled wood lignin), as well as in the absence of cellulose as a co-substrate. By contrast, 45% lignin decomposition in 14 days was reported under optimized conditions (nitrogen limitation) using solid-phase fermentation, with a lignin disc as a substrate.

SEM of the residual biomass showed a significant difference between the biomass incubated with specific fungal and bacterial strains and kenaf incubated only with indigenous microorganisms (control). The SEM image of the control was similar to the sterile blank. By contrast, the biomass particles were broken down into smaller pieces in the samples treated with specific microorganisms, perceivably as a result of biodecomposition. Particle size before the microbial decomposition, 1.0 ± 0.2 mm, was similar to the particle size of kenaf incubated with indigenous microorganisms only (data not shown). By contrast, the biomass particle size after the microbial decomposition became smaller, around 0.2 mm (data not shown).

Structural changes were also observed. The original kenaf particles maintained their structure upon a shearing stress whereas the biomass particles after microbial decomposition readily fell apart into dust when merely touched. In the samples that underwent microbial decomposition, crystals were observed that may represent phenolic compounds, salts, or lipids. By contrast, these crystals were absent in the samples without a specific microbial treatment. These changes indicate significant kenaf biomass degradation.

3.2. Cellulose and hemicellulose decomposition

Results from low-MW carbohydrate analysis (Fig. 2) showed the time-dependent release of glucose, xylose, galactose and fructose into the medium, which occurred at different times. At the beginning of incubation, only glucose and galactose (apparently produced during the sterilization) were present in concentrations of 8.0 and 0.4 g L^{-1}, respectively. During the microbial growth the glucose concentration increased by 1.8–6.3 g L^{-1} for different cultures until day 6, with the highest yield (38%) obtained using a combination of all four strains. The glucose concentration then gradually declined, approaching zero between days 11 and 18. The only exception occurred during the incubation with the mixed culture of *Microbacterium* and *Streptomyces*. In this experiment, the starting glucose concentration did not change until day 18 and then gradually declined to reach statistical zero on day 25. The galactose concentration increased until day 18 to 1.6 and 2.1 g $L^{-1}$and then gradually declined until day 46. The highest observed yield of galactose was 38% with *T. gallica* and *Microbacterium* sp.

Xylose was first detected on day 18 reaching the value between 0.2 and 1.7 g L^{-1} on day 18. The highest xylose yield, 26%, was observed when the biomass was incubated with both *Microbacterium* sp. *and T. gallica*. After day 18 the concentration of xylose gradually declined and the complete removal of xylose was recorded on day 36.

Fructose appeared on day 6, where the highest concentration was almost 6.3 g L^{-1} for all of the microbial combinations except for the fungus-free bacterial mixture. In that case, the fructose concentration increased up to 2.2 g L^{-1}on day 11. The % yield of fructose could not be calculated as it was produced as a result of isomerization of other carbohydrates. The concentration then decreased until day 25, when no fructose was observed in the medium. At this time, near day 25, the medium lost its fluidity in most of the samples becoming gel-like. This effect is apparently due to a massive mucus formation resulting from sugar conversion. By day 50, the mucus disintegrated and the medium regained its fluidity.

Similar maximum monosaccharide yields were reported in literature. Tanaka et al., 2009 obtained 50% and 33% glucose yields from rice straw using *P. chrysosporium* and *Ceriporiopsis subvermispora*, respectively. Wan and Li, 2012 reported up to a 66% glucose yield with *C. subvermispora* from corn stover. Even though the microorganisms used in the current work are not known to be the most efficient polysaccharide degraders, the maximum glucose yield obtained by us, 38 ± 6% for the combination of all four microorganisms, was of a similar order of magnitude.

Thus, different monosaccharides were produced sequentially rather than simultaneously within the first 1–3 weeks of incubation, apparently reflecting the order of biodecomposition of several carbohydrate-based polymers. This observation suggests that they may be harvested sequentially if sugars can be readily separated from the rest of the mixture. By day 28 most of the monosaccharides were removed from the medium, although the observation of abundant mucus formation indicated that some of them could be stored as readily digestible polymers.

3.3. Occurrence and analysis of lignin decomposition products

The chromatograms of the dried filtered media on day 57 were obtained using TD–Pyr–GC–MS at temperatures of 350, 450 and 700 °C. Different temperatures were used based on the fact that for most organic chemicals, thermodesorption of monomers occurs below or near 400 °C whereas chemicals evolving at much higher temperatures are products of pyrolysis of oligomers and polymers (Beránek et al., 2013). Adsorption of monomers on both organic and inorganic residues could slightly shift the evolution temperatures higher. Thus, most of the monomers including phenolics were expected to appear at 350 °C; the rest of the monomers and either dimers or products of their decomposition should evolve at 450 °C whereas the decomposition products of polymers would be seen at 700 °C.

When comparing the TD–Pyr–GC–MS profile of the original kenaf biomass with those of the samples that underwent microbial decomposition, the occurrence of some of the observed phenolic compounds was similar. However as expected, some of them shifted from the high-temperature fractions in kenaf to lower-temperature fractions in the microbial treatment products suggesting decomposition of high-MW species to lower-MW species. Furthermore, several other phenolics (marked in Table 4) were observed, with specific profiles, only in the samples obtained after the microbial treatment, indicating lignin biodegradation.

As expected, no lignin biodecomposition products were observed on day 1. No phenolics were observed in TD–Pyr–GC–MS fractions on day 25 either (data not shown). Only early-eluting nitrogen-containing compounds, perceived as protein decomposition products, were observed along with furans and furfurals (common products of sugar thermolysis). By contrast, phenolic compounds were observed on day 57 as abundant late-eluting peaks at 350 and 450 °C but not at 700 °C. Similar phenolic products of pyrolytic lignin decomposition were reported in litera-

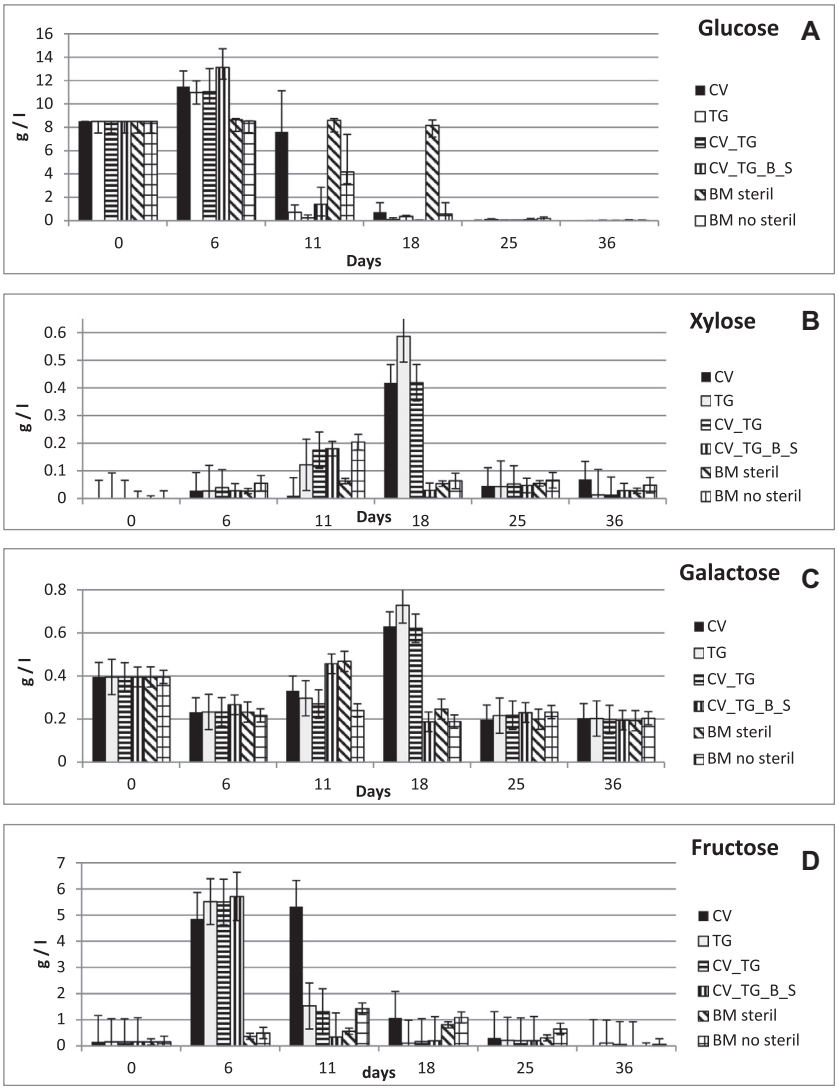

Fig. 2. Specific monosaccharide yields during the decomposition study: glucose (A), xylose (B), galactose (C), fructose (D). On day 46, no sugars were detected in the medium in detectable amounts.

Table 4
Distribution of lignin decomposition products among the TD–Pyr–GC fractions in both the precipitate and supernatant obtained upon acid precipitation at day 57.

Temperature steps	Supernatant			Precipitate		
	CV, TG	CV, TG, B, S	Nonspecific MO	CV, TG	CV, TG, B, S	Nonspecific MO
350 °C	9 ± 2	9 ± 3	10 ± 3	2 ± 1	8 ± 2	8 ± 2
450 °C	52 ± 3	33 ± 5	12 ± 5	20 ± 4	15 ± 4	17 ± 5
700 °C	0 ± 2	1 ± 1	2 ± 2	1 ± 2	1 ± 3	4 ± 3
Sum of lignin derivatives	61 ± 4	43 ± 4	24 ± 2	23 ± 4	24 ± 3	29 ± 3

On days 1 and 25, no quantifiable lignin products were observed.

ture (Jiang et al., 2010; Gosselink, 2011; Koncsag et al., 2012; El Mansouri and Salvadó, 2006), thus confirming our data obtained by TD–Pyr–GC–MS.

The percentage of phenolic products among all eluted compounds (in all three temperature fractions) was estimated (Table 4, see the column labeled as "supernatant"). These semi-quantitative results corroborate the qualitative information obtained with TD–Pyr–GC–MS, in that the majority of phenolics were observed in the 450 °C fraction. This relatively high elution temperature indicates the thermal decomposition of either lignin (which could dissolve in the alkaline medium toward the end of experiments) or its lower-MW oligomeric decomposition products. To ascertain whether the non-degraded polymeric lignin remained in the solution as opposed to its partially degraded oligomeric fragments, an additional experiment was conducted.

In this additional experiment, sulfuric acid was added to the samples collected on day 57. The precipitates formed were subjected to TD–Pyr–GC–MS and the results were compared for the solution and precipitate. The pertinent classification of the peak sums is provided in Table 4. The precipitate turned out to

contain a much smaller fraction of phenolics than the corresponding solutions, eluting mostly the products of lipid and protein thermal decomposition. For example, for the combination of CV and TV, phenolics in the liquid medium accounted for 61 ± 4% of the total GC-eluted peaks whereas their fraction in the precipitate was only 24 ± 2%. Gutiérrez et al., 2006 observed significant amounts of lipids (wax) present in lignin obtained from kenaf. This observation corroborates the recovery of large amounts of lipids precipitated with lignin in the current study.

Thus, the phenolic compounds observed in TD–Pyr–GC–MS experiments should be attributed to water-soluble oligomers produced as a result of lignin biodecomposition as opposed to non-degraded lignin. The lack of their occurrence on day 25 corroborates the data on carbohydrate biodecomposition and lignin-degrading enzyme activities (covered in the next section). Lignin degradation commenced only after the bulk of readily digestible carbohydrates were depleted. It is of note that the non-specific control with indigenous microorganisms (i.e., with no specific fungi or bacteria added) showed less phenolics in the supernatant (Tables 4 and 5). By contrast, the fungal culture showed the greatest amount of phenolics (monomers and lower-MW oligomers) in the solution. This is to be expected for efficient lignin degrading cultures (Tables 4 and 5). A relatively smaller amount of low-MW phenolics found in the mixed culture containing both fungal and bacterial species compared to the strictly fungal culture may be explained by their further biodecomposition by bacterial species through their catabolism.

3.4. Enzyme activity profiles

Lignin degrading enzyme activities peaked at different times (Table 6). However, all of them peaked after the bulk of monosaccharides had been degraded (25 day). This was expected because the lignin decomposition would become significant only when most of the readily biodegradable carbohydrates were depleted. After the peak of each enzyme activity was passed, the activity did not decline completely until the end of experiments, apparently reflecting the continued lignin decomposition.

Oxidative enzymes require oxygen through aeration. As expected, the LiP activity was significantly greater for stirred samples in the incubator compared to the non-stirred samples run at room temperature. However, neither the other enzyme activities

Table 5
Specific products of lignin decomposition evolving in TD–Pyr–GC–MS experiments detected in the representative runs using CV and TG. The highlighted chemicals were not present in the TD–Pyr–GC–MS of original kenaf biomass.

				Precipitate			Supernatant		
#	Lignin decomposition products	m/z [a]	tr (min) [b]	350 [c]	450 [c]	700 [c]	350 [c]	450 [c]	700 [c]
1	Toluene	39, 65, 91	6.00			++			
2	Styrene	44, 72, 104	7.04		++				
3	Phenol	39, 66, 94	7.72		+++	++	++		
4	Methylphenol	39, 77, 107	8.64		+++	++			
5	Guaiacol	81, 109, 124	8.90		+++	+ +		+++	+
6	Benzenediol	64, 81, 110	9.82		++				
7	Methylguaiacol	95, 123, 138	9.89		+++	++			+
8	Coumaran	65, 91, 120	9.99				+++		
9	Methoxybenzenediol	97, 125, 140	10.58		+++			+++	
10	Vinylguaiacol	107, 135, 150	11.03	+++	+++		+++	+++	+
11	Syringol	111, 139, 154	11.32	+++	+++	+ +	++	+++	
12	Dihydroxybenzoquinone	54, 69, 138	11.59			++			
13	Methoxysalicylic acid	53, 125, 153, 168	12.12					+++	
14	Isoeugenol	55, 77, 103, 164	12.18				+++	+++	
15	Isopulegol acetate	43, 81, 136	12.49		+++				
16	Acetovanillilone	43, 84, 151, 166	12.54					+++	
17	Trimethoxy toluene	107, 139, 167, 182	12.76					+++	
18	Guaiacyl acetone	43, 122, 137, 180	12.88					+++	
19	Methyl syringaldehyde	77, 165, 180	13.13		++		+++	++++	
20	Propenylsyringol	77, 91, 119, 194	13.37				+++	++++	
21	Homovanilic acid	94, 122, 137	13.79		++				
22	Dimethoxyhydroxybenzaldehyde	65, 111, 182	13.87				++		
23	Dimethoxymethylphenol	125, 153, 168	14.13		+++				
24	Coniferyl aldehyde	51, 77, 135, 178	14.46				+++		
25	Coniferyl alcohol	77, 91, 137, 180	14.48					+++	
26	Homosyringic acid	43, 167, 210	14.67					+++	
27	Hydroxymethoxybenzenemethanol	70, 125, 154	15.29		++				

[a] Major ion (mass to charge ratio).
[b] Retention time.
[c] Abundance as measured by the units of the MS peak using ion current: ++++, >1.000.000; +++, >100.000; ++, >10.000; +, >1.000.

Table 6
Maximum enzyme activities and elapsed times at which they were observed.

Biodecomposition with	Laccase		Manganese Peroxidase		Lignin Peroxidase	
	Day	U L^{-1} (n = 3) [b]	Day	U L^{-1} (n = 3) [b]	Day	U L^{-1} (n = 2) [b]
CV	25	63 ± 11	25	7 ± 4	18	13–19
TG	18	25 ± 7	6–18	6 ± 1	18	7–11
CV, TG	6–18	25 ± 2	18	5 ± 2	18	6–16
CV, TG, MB, SM	18	33 ± 12	11–18	13 ± 4	18	5–21
Biomass with non-specific indigenous microorganisms (Control)	18	110 ± 25	18	22 ± 21	18	16–19
Biomass sterilized (Blank)	46	[a]	46	[a]	46	[a]

n – number of replicates.
[a] Statistical zero.
[b] U L^{-1} one unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of substrate per minute.

nor monosaccharide concentrations exhibited any dependence on this factor. Apparently, the oxygen demands were met even without stirring.

Akin et al. (1996) reported similar laccase, LiP and MnP activities for *T. gallica* degrading peat in low-nutrient mineral media. Hong et al. (2011) reported that *T. gallica* exhibited the highest laccase activity, 103 U L^{-1}, on day 25; the MnP activity by *C. versicolor*, 10 U L^{-1}, also peaked on day 25 whereas no significant LiP activity was observed. Our results obtained with the same fungi but with a different biomass source were qualitatively similar for laccase and MnP, although we also observed a significant LiP activity, between 13 and 19 U L^{-1}, on day 18. *T. gallica* consistently showed slightly lower values than *C. versicolor* for all enzyme activities. Pometto and Crawford (1986a) observed the peaks of lignin-degrading enzyme activities in *Streptomyces* much earlier than in the current study, on day 1 of cultivation. However, their entire measurement period was just 4 days.

Andreu and Vidal (2011) determined that products of lignin decomposition served as mediators for laccase activity. We also observed significant although irregular increases of all three monitored enzyme activities after day 40 when products of lignin decomposition started to accumulate in the medium (see the previous section). These data are not shown in Table 6 as they could not be interpreted unambiguously since significant microbial contamination became noticeable at those incubation times.

Surprisingly, the levels of lignin-degrading enzyme activities did not fully correlate with lignin decomposition. In particular, the highest enzyme activity observed (Table 6) was that expressed by non-specific indigenous microorganisms (originally present in the kenaf biomass), showing neither lignin nor biomass decomposition beyond the limits of statistics (Table 3). Similarly, *C. versicolor* exhibited the highest laccase and lignin peroxidase activities despite showing no increase in lignin decomposition (Table 3). However, the most efficient lignin degrading microbial combination including all four strains consistently showed relatively high levels of pertinent enzyme activities (Table 6).

3.5. pH profile

The pH decreased during the incubation for the first 11 days reaching the value of 3. The observed pH decrease can be explained by the accumulation of acidic products of sugar metabolism, e.g., pyruvic acid. However after day 11, pH gradually increased to 9. The observed pH increase is unlikely to be the result of cell lysis since cells continued to exhibit significant carbohydrate and lignin decomposition during and after the pH increase. Thus, it is more likely to reflect the formation of furfural and levoglucosan derivatives. The greatest pH change was from 2.9 on day 6 to 8.6 on day 59 observed with *C. versicolor* in combination with *Microbacterium* sp.

The literature recommends the use of acidic media when the main expected products are carbohydrates, such as pH = 4 (a lower pH might help with cellulose hydrolysis) for wheat straw biodecomposition by *Streptomyces* (Koncsag et al., 2012). However, whenever lignin biodecomposition was targeted, alkaline media were applied (e.g., pH = 8.4 for *S. viridosporus*). A higher pH might be essential to neutralize phenols and make them more accessible (Pometto and Crawford, 1986a). Thus, the pH profiles observed in the current work appear to be representative for both lignin and carbohydrate-based polymer biodecomposition.

4. Conclusion

The time profiles of carbohydrate and lignin biodecomposition by fungal and bacterial cultures show that the release and then removal of the bulk of monosaccharides occur prior to the onset of the lignin biodecomposition, even when using fungal cultures designed for fast lignin decomposition. This feature creates a potential of collecting the monosaccharide products separately from phenolics for their subsequent use in production of carbohydrate-based biofuels and lignin-based valuable chemicals. The TD–Pyr–GC–MS data confirmed that lignin decomposition occurs during the microbial action. Fungi and bacteria yielded similar results although the combination of all four strains featured the greatest biomass decomposition.

Acknowledgements

The authors are grateful to the students of Dr. Kubatova's group, R. Cochran, H. Jeong, J. Rousova and K. Voeller for technical assistance. The authors would like to acknowledge the National Science Foundation (NSF) for providing REU funding through Grant CHE 1156584. This material is based upon work supported by the NSF under Grant No. IIA-1330840.

Disclaimer: any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the NSF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.09.057.

References

Akin, D.E., Morrison, W.H., Rigsby, L.L., Gamble, G.R., Sethuraman, A., Eriksson, K.L., 1996. Biological delignification of plant components by the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus*. Anim. Feed Sci. Technol. 63, 305–321.

Andreu, G., Vidal, T., 2011. Effects of laccase-natural mediator systems on kenaf pulp. Bioresour. Technol. 102, 5932–5937.

Beránek, J., Kozliak, E., Kubátová, A., 2013. Evaluation of sequential solvent and thermal extraction followed by analytical pyrolysis for chemical characterization of carbonaceous particulate matter. J. Chromatogr. A 1279, 27–35.

Chang, Y.C., Choi, D.B., Takamizawa, K., Kikuchi, S., 2014. Isolation of *Bacillus sp.* strains capable of decomposting alkali lignin and their application in combination with lactic acid bacteria for enhancing cellulase performance. Bioresour. Technol. 152, 429–436.

Crawford, D.L., Pometto, A.L., Crawford, R.L., 1983. Lignin degradation by *Streptomyces viridosporus*: isolation and characterization of a new polymeric lignin degradation intermediate. Appl. Environ. Microbiol. 45, 898–904.

De la Torre, M.J., Moral, A., Hernández, M.D., Cabeza, E., Tijero, A., 2013. Organosolv lignin for biofuel. Ind. Crops Prod. 45, 58–63.

Evans, C.S., Palmer, J.M., 1983. Ligninolytic activity of *Coriolus versicolor*. J. Gen. Microbiol. 129, 2103–2108.

Gosselink, R.J.A., 2011. Lignin as a renewable aromatic resource for the chemical industry(Ph.D. thesis). Wageningen University, Wageningen, NL. ISBN: 978-94-6172-100-5.

Gutiérrez, A., Rodríguez, I.M., del Río, J.C., 2006. Chemical characterization of lignin and lipid fractions in industrial hemp bast fibers used for manufacturing high-quality paper pulps. J. Agric. Food Chem. 54, 2138–2144.

Halis, R., Tan, H.R., Ashaari, Z., Mohamed, R., 2012. Biomodification of kenaf using white rot fungi. BioResources 7, 984–987.

Hong, Y., Dashtban, M., Chen, S., Song, R., Qin, W., 2011. Enzyme production and lignin degradation by four basidiomycetous fungi in submerged fermentation of peat containing medium. Int. J. Biol. 4, 172–180.

Hui, W., Jiajia, L., Yucai, L., Peng, G., Xiaofen, W., Kazuhiro, M., Zongjun, C., 2013. Bioconversion of un-pretreated lignocellulosic materials by a microbial consortium XDC-2. Bioresour. Technol. 136, 481–487.

Ji, X.L., Zhang, W.T., Gai, Y.P., Lu, B.Y., Yuan, C.Z., Liu, Q.X., Mu, Z.M., 2012. Patterns of lignocellulose degradation and secretome analysis of *Trametes trogii* MT. Int. Biodeterior. Biodegrad. 75, 55–62.

Jiang, G., Nowakowski, D.J., Bridgwater, A.V., 2010. A systematic study of the kinetics of lignin pyrolysis. Thermochim. Acta 498, 61–66.

Koncsag, C.I., Eastwood, D., Collis, A.E.C., Coles, S.R., Clark, A.J., Kirwan, K., Burton, K., 2012. Extracting valuable compounds from straw degraded by *Pleurotus ostreatus*. Resour. Conserv. Recycl. 59, 14–22.

Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog. Energy Combust. 38, 449–467.

Maki, M.L., Broere, M., Leung, K.T., Qin, W., 2011. Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. Int. J. Biochem. Mol. Biol. 2, 146–154.

El Mansouri, N.E., Salvadó, J., 2006. Structural characterization of technical lignins for the production of adhesives: application to lignosulfonate, kraft, soda-anthraquinone, organosolv and ethanol process lignins. Ind. Crops Prod. 24, 8–16.

Mohamed, R., Lim, M.T., Halis, R., 2013. Biodegrading ability and enzymatic activities of some white rot fungi on kenaf (*Hibiscus cannabinus*). Sains Malays. 42, 1365–1370.

Piškur, B., Bajc, M., Robek, R., Humar, M., Sinjur, I., Kadunc, A., Oven, P., Rep, G., Al Sayegh Petkovšek, S., Kraigher, H., Jurc, D., Pohleven, F., 2011. Influence of *Pleurotus ostreatus* inoculation on wood degradation and fungal colonization. Bioresour. Technol. 102, 10611–10617.

Pometto, A.L., Crawford, D.L., 1986a. Effects of pH on lignin and cellulose degradation by *Streptomyces viridosporus*. Appl. Environ. Microbiol. 52, 246–250.

Pometto, A.L., Crawford, D.L., 1986b. Catabolic fate of *Streptomyces viridosporus* T7A-produced, acid-precipitable polymeric lignin upon incubation with ligninolytic *Streptomyces* species and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 51, 171–179.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. Renew. Energy 37, 19–27.

Seo, J.S., Keum, Y.S., Li, Q.X., 2009. Bacterial degradation of aromatic compounds. Int. J. Environ. Health Res. 6, 278–309.

Singh, D., Zeng, J., Laskar, D.D., Deobald, L., Hiscox, W.C., Chen, S., 2011. Investigation of wheat straw biodegradation by *Phanerochaete chrysosporium*. Biomass Bioenergy 35, 1030–1040.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, Crocker, D., 2011. Determination of structural carbohydrates and lignin in biomass. Technical Report NREL/TP-510-42618.

Song, L., Ma, F., Zeng, Y., Zhang, X., Yu, H., 2013. The promoting effect of manganese on biological pretreatment with *Irpex lacteus* and enzymatic hydrolysis of corn stover. Bioresour. Technol. 135, 89–92.

Sun, F., Li, J., Yuan, Y., Yan, Z., Liu, X., 2011. Effect of biological pretreatment with *Trametes hirsuta* yj9 on enzymatic hydrolysis of corn stover. Int. Biodeterior. Biodegrad. 65, 931–938.

Sun, X., Zhang, R., Zhang, Y., 2004. Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. J. Basic Microbiol. 44, 220–231.

Tanaka, H., Koike, K., Itakura, S., Enoki, A., 2009. Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. 45, 384–390.

Wan, C., Li, Y., 2012. Fungal pretreatment of lignocellulosic biomass. Biotechnol. Adv., 1–11.

Yao, W., Nokes, S.E., 2014. P*hanerochaete chrysosporium* pretreatment of biomass to enhance solvent production in subsequent bacterial solid-substrate cultivation. Biomass Bioenergy 62, 100–107.

Zeng, J., Singh, D., Chen, S., 2011. Biological pretreatment of wheat straw by *Phanerochaete chrysosporium* supplemented with inorganic salts. Bioresour. Technol. 102, 3206–3214.

Zhang, L., Zhang, C., Cheng, Z., Yao, Y., Chen, J., 2013. Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by the bacterium *Mycobacterium cosmeticum* byf-4. Chemosphere 90, 1340–1347.

Zhao, L., Cao, G.L., Wang, A.J., Ren, H.Y., Dong, D., Liu, Z.N., Guan, X.Y., Xu, C.J., Ren, N.Q., 2012. Fungal pretreatment of cornstalk with *Phanerochaete chrysosporium* for enhancing enzymatic saccharification and hydrogen production. Bioresour. Technol. 114, 365–369.