Variation in susceptibility to microbial lignin oxidation in a set of wheat straw cultivars: influence of genetic, seasonal and environmental factors

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KEYWORDS: Microbial lignin degradation; Wheat straw; *Sphingobacterium*; *Rhodococcus jostii* RHA1; *Pseudomonas putida*

ABSTRACT: An alkali lignin preparation from wheat straw was found to be compatible with a previously developed nitrated lignin assay for microbial lignin oxidation, allowing comparison of the susceptibility of different wheat lignins to microbial oxidation. Alkali lignin was prepared from a panel of 18 wheat varieties, and was analysed by FT-IR spectroscopy, revealing minor variations in the lignin structure. Samples of nitrated alkali lignin were assayed against five lignin-degrading bacteria: *Streptomyces viridosporus*, *Rhodococcus jostii* RHA1, *Pseudomonas putida* mt-2, *Microbacterium phyllosphaerae*, and *Sphingobacterium* sp. T2. Up to 5-fold variation in rates of lignin oxidation were observed between different wheat varieties, and between different bacterial strain, with *Sphingobacterium* sp. T2 showing consistently highest absorbance changes. Testing of wheat varieties from two consecutive harvests, and from different locations, revealed that the observed variation was due to a combination of genetic, seasonal and environmental factors, but that some wheat varieties showed consistently higher rates of oxidation. Samples of wheat straw treated with *Sphingobacterium* sp. T2 were analysed by FT-IR spectroscopy after 7 and 14 days' treatment, showing changes in lignin structure versus time, consistent with lignin breakdown.

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Introduction

Wheat straw is readily available agricultural residue that is produced in large quantities in the UK, and many other countries in *Europe* and around the world, that could potentially be used as a feedstock for a lignocellulose-based biorefinery (Talebnia et al 2010). Depending on the type of pretreatment used, sugar yields of 74-99% can be obtained from wheat straw, hence the sugar content of wheat straw is amenable to cellulosic ethanol production (Talebnia et al 2010). Organosolv fractionation of wheat straw has been shown to generate lignin, hemicellulose and digestible cellulose streams for biorefinery applications (Wildschut et al 2013; Reisinger et al 2014). Higher value products such as polyhydroxyalkanoates could also be derived from wheat straw as a feedstock (Xu et al 2010).

Wheat straw is also an attractive source of lignin for conversion into aromatic chemicals. Recent studies have shown that bioconversion by bacteria genetically modified in lignin breakdown can generate high value chemicals from wheat straw lignocellulose: vanillin can be generated at 96 mg/L yield (2% overall yield from lignin) via using a gene deletion strain of *Rhodococcus jostii* RHA1 (Sainsbury et al 2013); and pyridine-dicarboxylic acids can be generated at 80-125 mg/L yields using strains of *R. jostii* RHA1 in which aromatic degradation pathways had been re-routed by genetic modification (Mycroft et al 2015).

If wheat straw is to be used as a feedstock for a lignocellulosic biorefinery, one question that is important for process optimisation is whether there is variation in bioprocessing of different wheat varieties. Previous literature studies have examined the genetic variation in wheat cultivars relevant to degradability and cellulosic ethanol production, showing up to 2-fold variation in degradability (Jensen et al 2011), and up to 26% difference in sugar release between cultivars (Lindedam et al 2012). Compositional studies have also shown up to 3.5-fold variation in phenolic acid content in wheat flour from a range of wheat cultivars (Li et al 2008), and variability in anti-oxidant properties of the wheat bran (Zhou et al 2004). A recent study has examined a set of 90 wheat cultivars, finding that sugar content can vary by up to 1.8-fold, and lignin content by up to 2-fold, but that this is primarily due to differences in the ratio of component tissues (Collins et al 2014).

To date there has been no study of the variation in susceptibility of lignin from different wheat varieties to valorization. In order to examine this question, we aimed to use a UV-visible spectroscopic assay method for evaluation of microbial lignin oxidation, using chemically nitrated lignin samples, which can be carried out in a 96-well microplate reader (Ahmad et al 2010). Nitration is likely to occur *ortho*- to free phenolic units in the lignin structure, and bacterial degradation has been shown to release nitrophenol-containing products with

increased absorbance at 430 nm (Ahmad et al 2010). This assay was verified using culture supernants from a known bacterial lignin degrader *Streptomyces viridosporus*, and with *Pseudomonas putida* mt-2 and *R. jostii* RHA1 (Ahmad et al 2010), and was subsequently extended to bacterial lignin degraders identified from environmental samples, including *Sphingobacterium* sp. T2, and *Microbacterium phyllosphaerae* (Taylor et al 2012). Our aim was to study a set of 18 wheat cultivars, examining the variation in lignin structure by Fourier transform infra-red (FT-IR) spectroscopy, and the susceptibility to bacterial lignin oxidation using the nitrated lignin assay.

Materials and Methods

Plant materials

Wheat straw samples were obtained from plants grown as described previously (Miller et al 2016).

Lignin preparation methods

Wheat straw samples were milled in a Glen Creston (Stanmore) bench top ball milling apparatus. Initial testing of lignin preparation methods was carried out using Hereward wheat straw.

Alkali lignin from wheat straw

The method was modified from Scalbert et al 1985.

Wheat straw powder (12.5 g) was stirred for 2 hours in 1.0 M NaOH (375 ml) under nitrogen gas in a round flask at 35 °C. The solution was filtered twice through a Buchner funnel and Whatman 1 filter paper to remove the remaining solid. The filtered solution was acidified to pH 1 with 6M HCl and left overnight to precipitate. The precipitate was recovered by centrifugation at 4000 rpm for 20 min, and the supernatant was again centrifuged and the second precipitate was combined with the first, and freeze-dried overnight. Yield was 19.1% per g wheat straw powder.

Alkali lignin with autoclave from wheat straw

The method was modified from Scalbert et al 1985.

Wheat straw powder (3.0 g) was autoclaved in 20 ml of distilled H_2O at 121 °C for 15 min under 1.1 bar pressure, then 1.0 M NaOH (75 ml) was added, and the mixture was transferred to a 250 ml round bottom flask. The above procedure was then followed. Yield 32.8% per g of wheat straw powder.

Ethanosolv lignin from wheat straw

The method was modified from Pan et al 2006.

Wheat straw powder (4.0 g) was added to 65% ethanol (28 mL) and 0.060 g H_2SO_4 (1.5 % w/w of wheat powder weight), and the mixture was stirred under reflux for 80 min. After cooling to room temperature, the solution was filtered through a Buchner funnel and Whatman 1 filter paper, and washed three times warm 65% ethanol. The filtrate and the washings were combined and precipitated by addition of 3-fold cold distilled water. The solution was left to precipitate overnight at 4 °C. The solution was evaporated at reduced pressure, then freeze-dried. Yield 2.0% per g of wheat straw powder.

Ionic liquid lignin from wheat straw

The method was modified from Lee et al 2009.

Wheat straw powder (0.10 g) was added to 2.0 g [emim]Ac (1-ethyl-3-methylimidazolium acetate) [ratio 50 mg wheat per g of [emim]Ac]. The solution was incubated at 130 °C under stirring for 90 min. After cooling to room temperature, the solution was diluted with 10.0 mL 1.0 M NaOH and centrifuged at 11000 rpm for 20 min. The supernatant was removed and the pH was adjusted to 2.0 with 20 % H_2SO_4. The solution was left overnight to precipitate at 4 °C, and readjusted to pH 2.0 and centrifuged at 11000 rpm for 20 min. The supernatant was removed and the precipitate was dried for 3 h at 150 °C. Yield 23.5% per g of wheat straw powder.

Lignin Characterisation by FTIR ATR Spectroscopy

FT-IR spectra of a sample (5-10 mg) of each lignin type were recorded using a Perkin Elmer FT-IR Spectrum One spectrometer with a Universal attenuated total reflectance (ATR) sampling accessory. Each sample's transmittance signal was corrected for background.

Molecular Weight Characterisation by Gel Permeation Chromatography (GPC)

GPC was performed on a Polymer Laboratories 390-LC machine, using a PLgel Mixed-D column 650 mm, a Refractive Index and a PDA detector. Eluent: 95% tetrahydrofuran (THF), 5% triethylamine. Lignin samples were dissolved in dry THF at a concentration of 0.2 % w/v. Flow rate: 1.00 ml/ min, injection volume 100 µl, run time 31 min, temperature 30 °C. Calibration of the method was carried out with polystyrene standards.

Nitrated lignin assays

Nitration of Lignin Samples: A sample of each lignin (10 mg) was dissolved in 2.0 ml glacial acetic acid, with the aid of an ultrasonic bath. When a precipitate was formed, the solution was filtered through filter paper. Concentrated nitric acid (6 drops) was added, and the solution was left to react in a water-ice bath for 1 h under stirring in a 50 ml Falkon tube. After the temperature of the solution returned to room temperature, 4.0 ml distilled water was added, and the pH was neutralised to pH 7 with 1.0 M NaOH. A stock solution of each nitrated lignin type was prepared (2 ml lignin stock/ 20 ml buffer) by 10-fold dilution in 750 mM Tris-Cl buffer pH 7.4 containing 50 mM NaCl.

UV-Vis Screening assays against bacterial supernatants: Cultures of *M. phyllosphaerae, Sphingobacterium sp. T2, P. putida mt-2* and *Streptomyces viridosporus* were inoculated in 2 mL of Luria-Bertani broth (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract) at 30 °C, shaking at 180 rpm (except *Sphingobacterium* culture was inoculated at 45 °C at 77 rpm). When the bacteria reached their log phase (A_{595} = 0.6), the cloudy bacterial solution was spun down (10000 rpm for 5 min), and the supernatant containing the extracellular solution was collected and kept at 4 °C for no longer than two days.

Assays (200 µl total volume) were carried out in 96 well plates, using a TECAN GENios microplate reader. To each well was added 30 µl of bacterial supernatant, 160 µl nitrated lignin in Tris buffer, then either 10 µl of 40 mM H_2O_2 (2 mM final concentration) solution, or 10

µl of Tris-Cl buffer. A control containing no bacteria (in triplicate) was carried out on each plate, where the bacterial supernatant was replaced with 30 µl Tris-Cl pH 7.4 buffer, and this absorbance was subtracted. Measurements at λ = 430 nm were taken over 20 min. Triplicate assays were carried out for each sample, with and without H_2O_2. The difference in absorbance from 1-20 min was measured, and the mean absorbance of the control assays was subtracted.

Bioconversion of lignin and lignocellulose

Wheat straw (processed through a 5 mm mesh using a rotary mill, CRN laboratory), alkali Kraft lignin or Organosolv lignin (6.25 g) and 5.0 g of either fish meal (Sigma-Aldrich F 6381, containing protein: 62 %, ash: 18 % and fat 9 %) or corn steep solids (Sigma-Aldrich C 8160) were added to M9 salt solution (250 ml). Organosolv lignin, also known as Biolignin™, was supplied by CIMV (Champagne-Ardenne, France). Starter cultures (50 ml) of *Sphingobacterium sp.*, *R. erythropolis* and *M. phyllosphaerae* were grown in LB broth over 48 h to OD_{600} 0.9 – 1.3. Each culture was centrifuged (5000 rpm, 30 min) and the cells washed twice with 20 mM potassium phosphate buffer (pH 7.0). The cells (0.9 g) were then re-suspended in fresh 20 mM phosphate buffer (10 ml). Re-suspended cells of each strain were added to each different medium and the cultures incubated at 30 °C, or 45 °C for *Sphingobacterium sp.*, with regular shaking at 180 rev min^{-1}. Aliquots of each culture (10 ml) were removed after 48, 96, 168, 240 and 336 h.

FT-IR analysis of treated samples

FT-IR measurements of the liquid fractions were performed on 96–well silicon plates. Acetone (2:1 ratio) was added to the samples, which were then vortexed for 5 s and centrifuged (10000 rpm, 3 min) in a microcentrifuge. Each sample (5 µl) was spotted onto marked locations in a 96-well format and the plate dried on an inverted hot block ingot at 30 °C for 10 min. Analysis of the solid fractions was performed via attenuated total reflectance (ATR) from 4,000 – 600 cm^{-1}, using an Equinox 55 FT-IR Spectrometer (Bruker Optik GmbH, Germany) fitted with a Golden Gate ATR accessory (Specac, UK). Each sample was dried at 50 °C for 1 h using a CHRIST 2 – 18 RVC vacuum concentrator at 5000 rpm, and then pressed onto a diamond crystal mounted as a window in a tungsten base plate. All spectra were averaged over 32 scans at a resolution of 4 cm^{-1} and corrected for background absorbance by subtracting the spectrum of the empty ATR crystal. Absorbance spectra were initially processed using Opus version 4.2 (Bruker Optik GmbH, Germany) and saved as text files.

Results

Comparison of four wheat lignin preparations in nitrated lignin assay

In order to screen lignins from a set of wheat varieties, it was necessary to identify a suitable lignin preparation that could be prepared in good yield from biomass, and which performed reliably in the nitrated lignin assay. We had previously used ball-milled lignin for the nitrated lignin assay, however, the ball-milling isolation method is time-consuming and proceeds in low overall yield. Therefore, we investigated four methods for lignin isolation reported in the literature: 1) an alkali lignin preparation involving treatment of biomass with sodium hydroxide, then precipitation after acid treatment (Scalbert et al 1985); 2) the same alkali lignin method after initial autoclaving of the biomass (Scalbert et al. 1985); 3) an ethanosolv lignin preparation obtained via extraction into ethanol and 1.5% (w/w) H_2SO_4 (Pan et al 2006); 4) an ionic liquid lignin preparation obtained after treatment with 1-ethyl-3-methylimidazolium acetate ([emim]Ac) (Lee et al 2009).

Yields of 19% and 33% were obtained from the two alkali lignin methods respectively, while the ethanosolv lignin was prepared in only 2% yield, and the ionic liquid lignin was prepared in 23% yield. Since wheat straw contains 15-20% lignin content, it is likely that the lignin preparations still contain some residual carbohydrate content.

The isolated lignins were characterized by gel permeation chromatography (GPC), giving M_w and M_n values in the range 830-950 g mol^{-1} for all four samples (see *Table 1*). Analysis by MALDI mass spectrometry also gave peaks in the range *m/z* 900-1250 (data not shown). These M_W values are somewhat lower than values of 1000-2000 g mol^{-1} observed in our previous study of the nitrated lignin assay (Ahmad et al 2010), and a further recent study of a range of lignin preparations found M_W values of 1700-5200 g mol^{-1} (Lancefield et al 2016), hence these lignin preparations give relatively small lignin fragments.

Each sample of lignin was chemically nitrated, and the nitrated lignin tested for changes in absorbance at 430 nm using supernatants obtained from cultures of *Pseudomonas putida* mt-2 and *Sphingobacterium* sp. T2. Absorbance changes were observed for each lignin preparation (see *Table 1*), but alkali lignin and ionic liquid lignin preparations showed somewhat higher absorbance changes than those observed previously using ball-milled lignin (Ahmad *et al*, 2010). As the alkali lignin method was a more convenient method to carry out on many samples of biomass, this preparation was selected to prepare lignins from a set of 18 wheat straw varieties.

Characterisation of set of wheat alkali lignins by FT-IR spectroscopy

Each sample of alkali lignin was analysed by FT-infrared spectroscopy (see Supporting Information S1-S19). All samples showed an intense absorption band at 1035-1043 cm^{-1}, assigned to a C-H deformation of guaiacyl (G) units present in the lignin (Faix, 1991). Nearly all samples also showed a small, sharp absorption at 1647-1653 cm^{-1}, due to a carboxylic acid carbonyl stretch, and some samples showed an additional weak absorption at 1504-1509 cm^{-1} due to an aromatic C=C stretch. All samples showed a broad absorption of variable intensity at 3100-3300 cm^{-1} due to O-H stretch of free hydroxyl groups in the lignin structure (Faix, 1991).

Table 1 - Analysis and testing of wheat straw lignin preparations. Weight average (M_w) and number average (M_n) molecular weights determined by gel permeation chromatography (in g mol^{-1}). Nitrated lignin assay data with supernatant from *Sphingobacterium* sp T2 and *Pseudomonas putida* mt-2, in the presence and absence of 1 mM hydrogen peroxide (change in absorbance units at 430 nm over 20 min assay).

Lignin preparation	M_w (g mol^{-1})	M_n (g mol^{-1})	*Sphingobacterium* sp.		*P. putida*	
			+ H_2O_2	- H_2O_2	+ H_2O_2	- H_2O_2
Alkali lignin	855	836	0.031	0.040	0.002	0.005
Alkali lignin with autoclave	930	916	0.017	0.025	0.003	0.005
Ethanosolv lignin	945	925	0.017	0.023	0.002	0.001
Ionic liquid lignin	934	921	0.009	0.040	0.004	0.005

Testing of nitrated wheat alkali lignins against bacterial lignin degraders

Each sample of wheat alkali lignin was chemical nitrated, and the nitrated alkali lignin samples were assayed for changes in absorbance at 430 nm over a 20 min assay, using supernatants of the following lignin-degrading bacteria: *Streptomyces viridosporus*, *Pseudomonas putida* mt-2, *Rhodococcus jostii* RHA1, *Microbacterium phyllosphaerae*, and *Sphingobacterium* sp. T2. Culture supernatants were used in this study and previous assay work (Ahmad et al 2010), because the enzymes catalysing oxidative attack on lignin are known to be extracellular (Bugg, Rahmanpour 2015). Assays were carried out in the presence and absence of 1 mM hydrogen peroxide, since lignin-oxidising peroxidase DypB has been identified in *R. jostii* RHA1 (Ahmad et al, 2011), though strains *Sphingobacterium* sp. T2 and *M. phyllosphaerae* were found previously to show higher activity in the absence of hydrogen peroxide (Taylor et al 2012). The assay data is shown in *Fig 1*.

The data in *Fig 1* show a significant amount of variation between different wheat straw cultivars. In the presence of hydrogen peroxide, four cultivars (Joss Cambier, Oakley, Palur, Capelle Desprez) showed up to 5-fold higher absorbance than the mean value of 0.004 absorbance units, with particular bacterial supernatants (in the first three cases, *Pseudomonas putida*, in the latter case, *Rhodococcus jostii*). 5-10 fold higher rates of reaction were observed using *Sphingobacterium* sp. T2 (see *Fig 1* C,D), as observed previously. Variation in rate was also observed for this bacterial supernatant across different cultivars, of 2-3 fold above the mean value. Slightly higher rate of reaction was observed for *Sphingobacterium* in the absence of hydrogen peroxide, as observed previously (Taylor et al 2012).

In order to investigate whether the variation in rate observed was due to genetic factors or environmental factors, wheat was collected from 12 varieties in the following year's harvest (2012 harvest), and samples of nitrated lignin were compared with the samples obtained from the 2011 harvest. The assay data are shown in *Fig 2*.

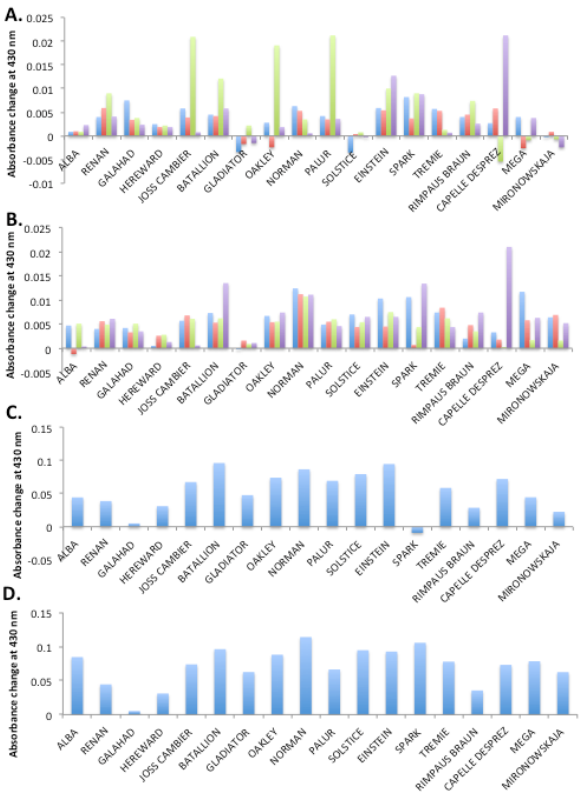

Fig 1 - Nitrated lignin UV-visible assay data for 18 wheat cultivars from 2011 harvest. A. With H_2O_2 (*S. viridosporus* (blue), *M. phyllosphaerae* (red), *P. putida* (green), *R. jostii* (purple)); B. without H_2O_2; C. *Sphingobacterium* with H_2O_2; D, *Sphingobacterium* without H_2O_2. Data were calculated from triplicate assays, standard errors are in the range 5-10% for the data shown.

There is some correlation between the 2011 and 2012 data (linear correlation coefficient 0.86 for *R. jostii* without H_2O_2, 0.61 for *R. jostii* with H_2O_2). However, the highest levels of activity observed in specific cultivars from the 2011 harvest were not observed in samples from the 2012 harvest, hence there is evidence for seasonal variation in lignin oxidation rate. Some cultivars did show consistently higher rates of lignin oxidation in both harvests (Mega and Alba in *Fig 2A*, using *R. jostii*), which was found to be statistically significant using the ANOVA significance test (Eriksson et al 2008), while other varieties showed consistently lower than average rates of oxidation (Norman, Palur in *Fig 2A*). Pairwise significance tests were carried out using Tukey's honestly significant difference test (Sokal, Rohlf, 1969) between data collected for Alba vs Norman, Alba vs Palur, Mega vs Norman, and Mega vs Palur, indicating that there is a pairwise significant difference between these sets of data.

Samples of these 12 wheat varieties were also collected from two different locations in the same harvest (2012), in order to examine the effect of environmental factors (soil, pH, nutrient availability). The two samples were assayed separately, using *Rhodococcus jostii* supernatant, and the data are shown in *Fig 3*.

The linear correlation coefficient between the two sets of data in *Fig 3* is 0.51, indicating a significant variation in rate in some cases, consistent with environmental

factors influencing the rates of lignin oxidation. However, cultivars Alba and Mega did again show consistently high rates, while Norman and Palur showed consistently lower rates.

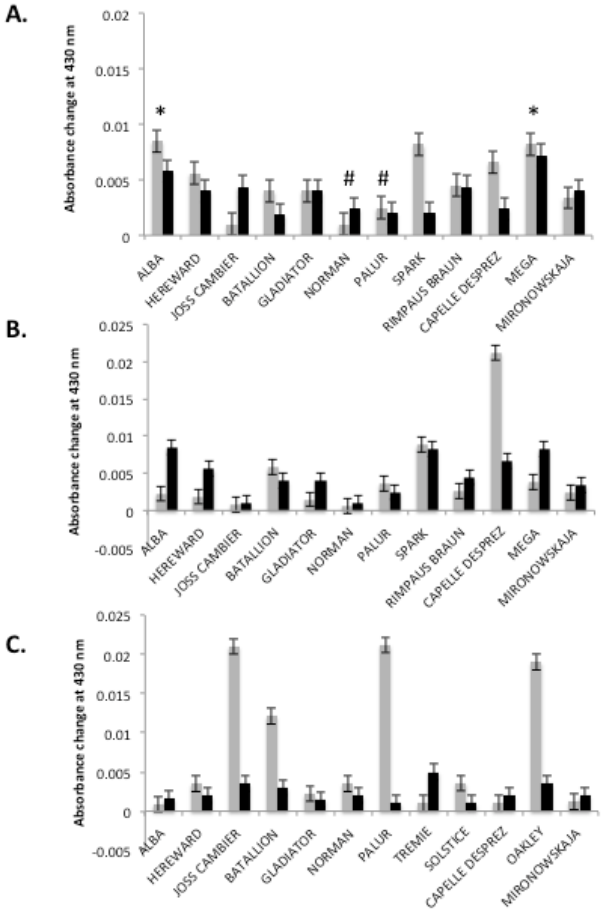

Fig 2 - Nitrated lignin UV-visible assay data for alkali lignin samples prepared from 2012 harvest (black), compared with 2011 harvest (grey). A. *R. jostii* with H_2O_2; B., *R. jostii* without H_2O_2; C. *P. putida* with H_2O_2. Error bars indicate the standard error. * and # indicate cultivars with statistically higher or lower readings across both harvests and locations.

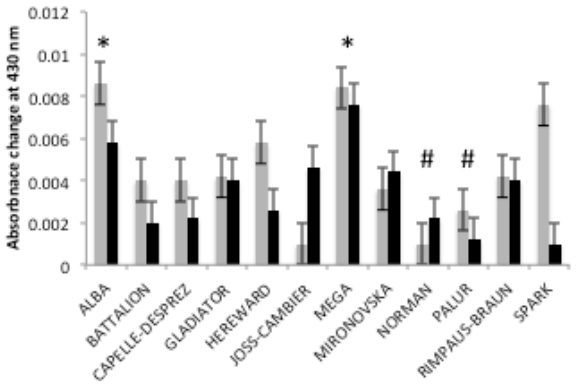

Fig 3 - Nitrated lignin UV-visible assay data for alkali lignin samples from wheat cultivars collected from different locations in the 2012 harvest. Assays carried out using *Rhodococcus jostii* supernatant, in the presence of hydrogen peroxide. Error bars indicate the standard error. * and # indicate cultivars with statistically higher or lower readings across both harvests and locations.

Use of FT-IR to study lignin degradation by Sphingobacterium sp. T2

Sphingobacterium sp. T2 was found to grow on M9 minimal media containing 2.5% (w/v) milled wheat straw, and that growth was enhanced by addition of 2.0% (w/v) corn steep liquor as nitrogen source (see Supporting Information for growth curves), reaching 4 x 10^8 colony forming units/ml after 7 days. Under these conditions, growth was also observed using 2.5% (w/v) organosolv lignin as carbon source, forming 8 x 10^7 colony forming units/ml after 7 days.

We have previously reported that *Sphingobacterium* generates low molecular weight products oxalic acid and protocatechuic acid when grown on minimal media containing wheat straw lignocellulose (Taylor et al 2012). In order to seek further evidence for lignin metabolism in both liquid and solid fractions, we used Fourier transform infra-red (FT-IR) spectroscopy, which gives information regarding functional group changes in lignin (Faix, 1991), and has been used to study lignin breakdown in white-rot fungi *Stropharia rugosoannulata* (Buta et al 1989) and *Coriolus versicolor* (Gilardi et al. 1995).

Shown in *Fig 4* are FT-IR data obtained from liquid fractions removed from fermentation of *Sphingobacterium* T2 with either wheat straw lignocellulose (A), Organosolv lignin (B), or alkali Kraft lignin (C) over 14 days at 45 °C. In each case, significant increases in absorbance were observed versus time. Peaks at 3300-3500 cm^{-1} (O-H stretch) increased 2-3 fold, peaks at 2500-3000 cm^{-1} (COOH stretch) increased 2-3 fold, peaks at 1600-1680 cm^{-1} (carbonyl C=O stretch, aromatic C=C stretch) increased 2-5 fold, and peaks at 1000-1200 cm^{-1} (C-O stretch) increased 2-4 fold, compared with untreated samples, indicating the release of soluble lignin fragments containing O-H, C=O and/or C=C bonds.

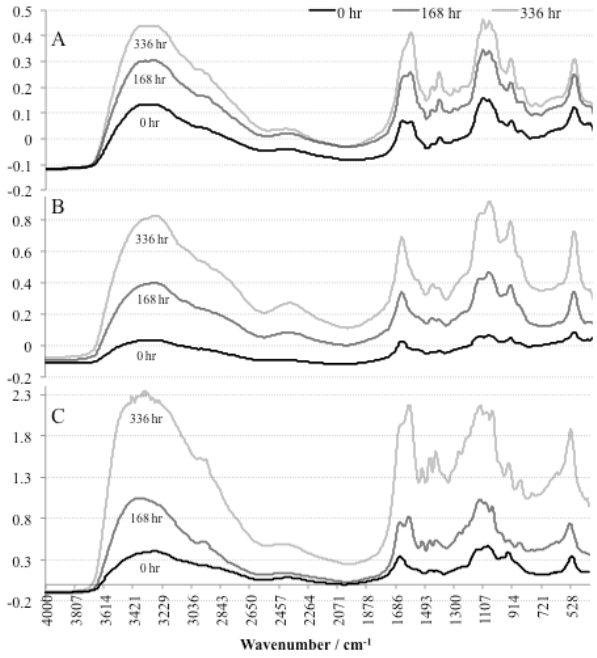

Fig 4 - .FT-IR spectra of liquid fractions from *Sphingobacterium* sp. T2 treatment of (A) wheat straw (B) organosolv lignin (C) alkali Kraft lignin in M9 media containing 2% CSL after 7 and 14 days.

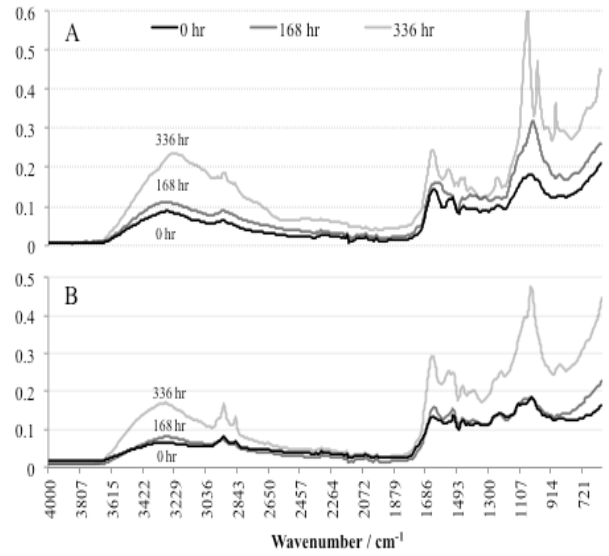

Fig 5 - FT-IR spectra of solid fractions from *Sphingobacterium* sp. T2 treatment of (A) wheat straw (B) organosolv lignin in M9 media containing 2% CSL after 7 and 14 days.

Analysis of the solid fractions from treatment of wheat straw or organosolv lignin with *Sphingobacterium* sp. T2 after 7 and 14 days are shown in *Fig 5*. Only small changes were observed after 7 days, but after 14 days, 2-3 fold increases were observed in peaks at 3300-3500 cm^{-1} (O-H stretch), 1600-1680 cm^{-1} (carbonyl C=O stretch, aromatic C=C stretch), and 1000-1200 cm^{-1} (C-O stretch), compared with untreated samples, indicating oxidative modification of the polymeric fraction.

Discussion

We have prepared alkali lignin samples from a set of 18 wheat cultivars, and examined the rates of microbial lignin oxidation using a UV-visible continuous assay. The alkali lignins were characterized by FT-IR spectroscopy, showing similarities in structure (see Supporting Information S1-S19), but also minor differences in IR absorption wavelength and peak intensity, indicative of minor variations in lignin structure. Previous literature studies have also shown up to 2-fold in lignin content in a set of wheat cultivars (Collins et al 2014), and up to 3.5-fold variation in phenolic acid content (Li et al 2008), due to the presence of ferulic acid and p-coumaric acid in grass lignins.

The nitrated lignin assay data shows considerable variation in the rates of microbial lignin oxidation (see *Fig 1*), with rates of up to 5-fold higher than mean values in certain cases. There appears to be selectivity for certain bacterial supernatants for certain types of wheat lignin, implying some form of molecular recognition of structural features of the lignin. The different bacteria tested show different behaviour in the assays: *Sphingobacterium sp*. T2 showed approximately 10-fold higher activity than other strains, as observed previously (Taylor et al 2012), The high activity of this strain has been found to be due to the presence of two extracellular manganese superoxide dismutase enzymes with activity for lignin oxidation (Rashid et al 2015). Supernatants from *Sphingobacterium* sp. T2, *S. viridosporus* and *M. phyllosphaerae* generally showed higher activity in the absence of hydrogen peroxide, whereas *P. putida* mt-2 and *R. jostii* RHA1 in several cases showed higher activity in the presence of hydrogen peroxide, consistent with the presence of a DyP-type peroxidase DypB in *R. jostii* RHA1 with activity for lignin oxidation (Ahmad et al 2011). The enzymes responsible for bacterial lignin degradation have only been recently elucidated, but involve extracellular peroxidase and laccase enzymes (Bugg and Rahmanpour, 2015). Although lignin-degrading bacteria show somewhat lower activity than white-rot basiodiomycetes (Ahmad et al 2010), the ability to readily overexpress recombinant bacterial enzymes and to genetically modify lignin-degrading bacteria offer new applications for biotechnology (Sainsbury et al 2013; Mycroft et al 2015).

The especially high activity of *P. putida* mt-2 and *R. jostii* RHA1 was not observed in alkali lignins prepared from the same wheat cultivars from the following year's harvest (see *Fig 2*), implying that there is significant seasonal variation in the lignin oxidation behaviour, which could be due to changes in the lignin structure. However, two cultivars (Mega and Alba in *Fig 2A*) did show consistently higher rates of lignin oxidation in samples from both years, while two other cultivars (Norman, Palur in *Fig 2A*) showed consistently lower than average rates of oxidation. We note that Li et al (2008) report Alba to have a lower than average phenolic content, which potentially might result in a less complex lignin structure that is easier to oxidise. In a separate study, we have also found that different types of lignin preparations show variable yields and products of lignin oxidation from biocatalytic and chemocatalytic valorization methods, hence differences in the lignin structure can have a major impact on rates of lignin oxidation (Lancefield et al 2016). Lignin samples prepared from the same cultivars grown in different sites also showed some variation in rates of oxidation (see *Fig 3*), indicating that there is also an influence of environmental factors that would affect the growth of the plant, but the same cultivars noted above showed either higher or lower rates of oxidation. Hence there do appear to be some underlying genetic factors influencing lignin structure that could affect rates of lignin oxidation, but seasonal and environmental factors are equally significant.

Analysis of lignin fractions from *Sphingobacterium* sp. T2 treatment of wheat straw lignocellulose or organosolv lignin by ATR FT-IR spectroscopy show changes in IR absorption versus time, consistent with lignin oxidation. There is an increase in soluble lignin fragments (see *Fig 4*), consistent with lignin depolymerisation into oligomeric and monomeric fragments (Rashid et al 2015). There are also structural changes to the solid fraction (see *Fig 5*), consistent with an increase in free hydroxyl groups, which might be caused by demethylation of lignin, an activity observed for *Sphingobacterium* manganese superoxide dismutase (Rashid et al 2015); and an increase in carbonyl group absorption, perhaps due to generation of additional carboxylic acid groups via lignin oxidation.

Conclusions

The study shows that there are significant differences in rates of lignin oxidation between different wheat cultivars, which should be considered in the development of a wheat straw-based biorefinery. The data indicate that a combination of genetic, seasonal and environmental factors can influence rates of lignin oxidation, presumably due to minor differences in lignin structure

Acknowledgements

The authors thank the Warwick Systems Biology Doctoral Training Centre (P.J.S.) and the Tuck Foundation Enerbio (C.R.T.) for financial support. The authors would like to thank Mr Matt Mitchell (Warwick HRI) for assistance with wheat milling, and Dr Rajan Randev (University of Warwick) for assistance with GPC analysis. This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC grants BB/H004351/1 and BB/H004270/1 (IBTI Club)).

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Manuscript received May 26, 2017
Accepted September 7, 2017

Supporting Information

Fig S1-S18. FT-IR spectra of alkali lignins from 18 wheat cultivars (2011 harvest)
Fig S19 Summary table of FT-IR peaks observed for different wheat cultivars
Fig S20-S22. Growth of *Sphingobacterium sp.*, *R. erythropolis* and *M. phyllosphaerae* on M9 media containing 2.5 % (w/v) ground wheat straw lignocellulose (*Fig S20*), 2.5 % (w/v) Organosolv lignin (*Fig S21*) or 2.5 % (w/v) alkali Kraft lignin (*Fig S22*).

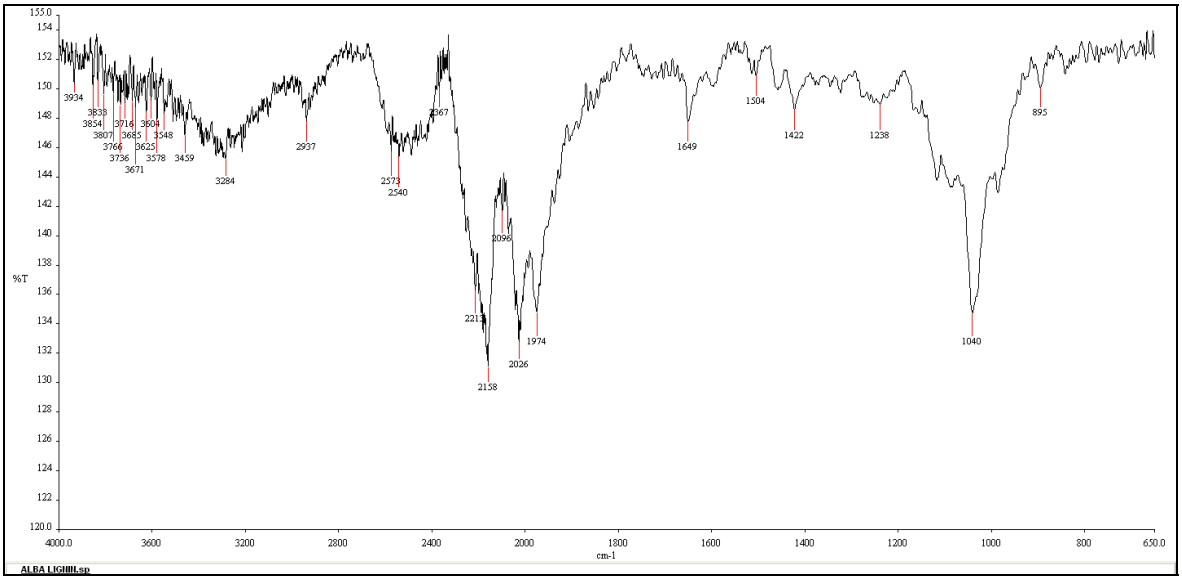

Fig S1 - FTIR spectrum of Alba wheat lignin.

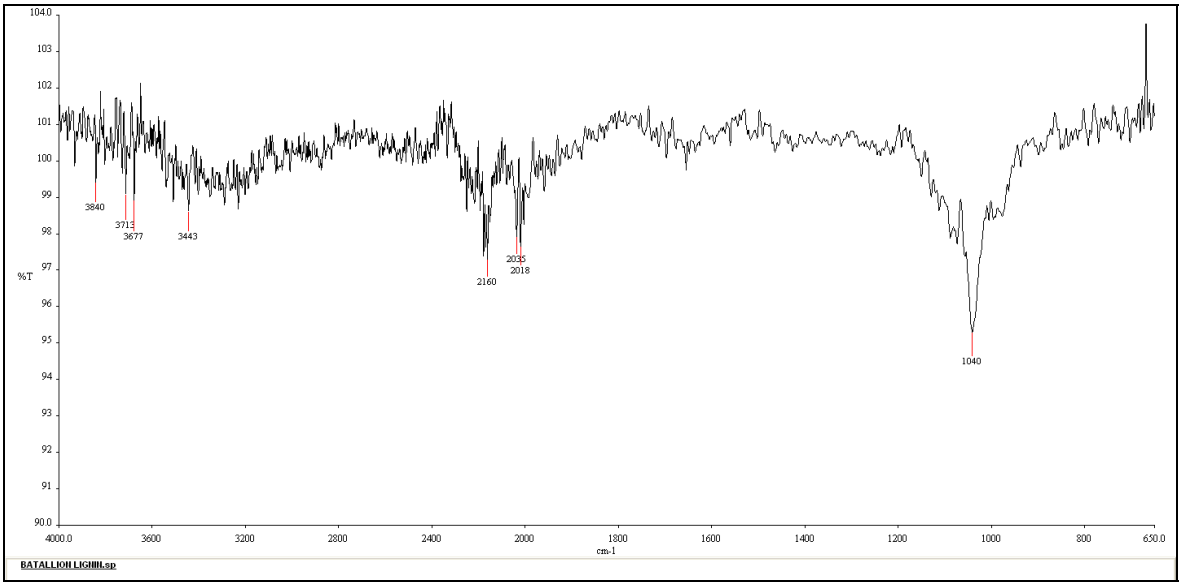

Fig S2 - FTIR spectrum of Batallion wheat lignin.

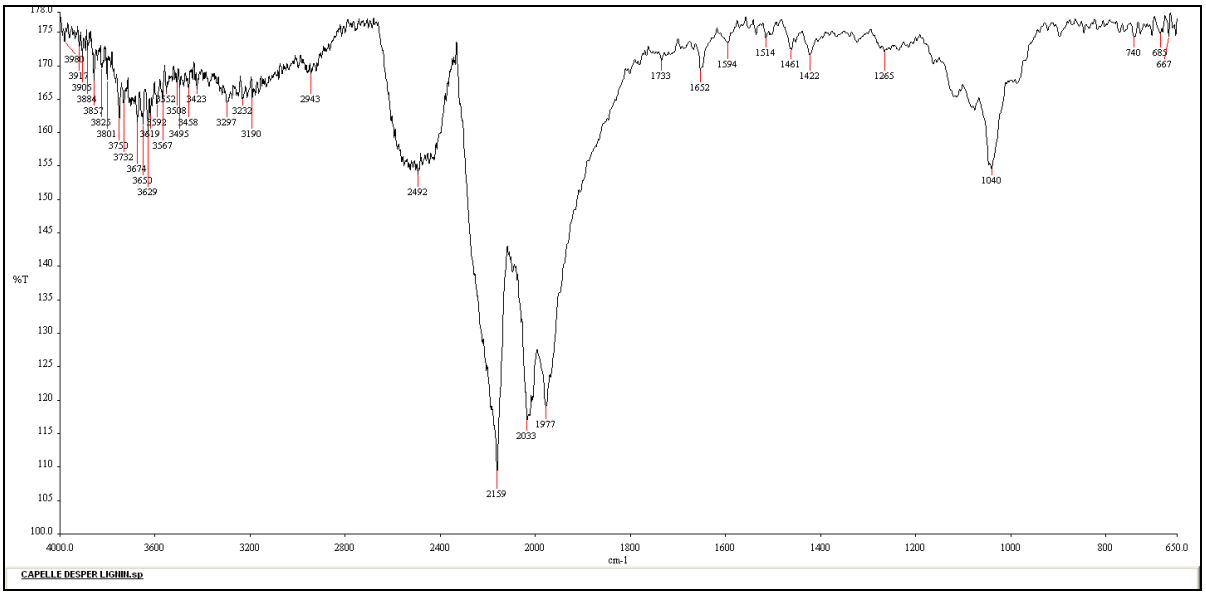

Fig S3 - FTIR spectrum of Capelle Desprez wheat lignin.

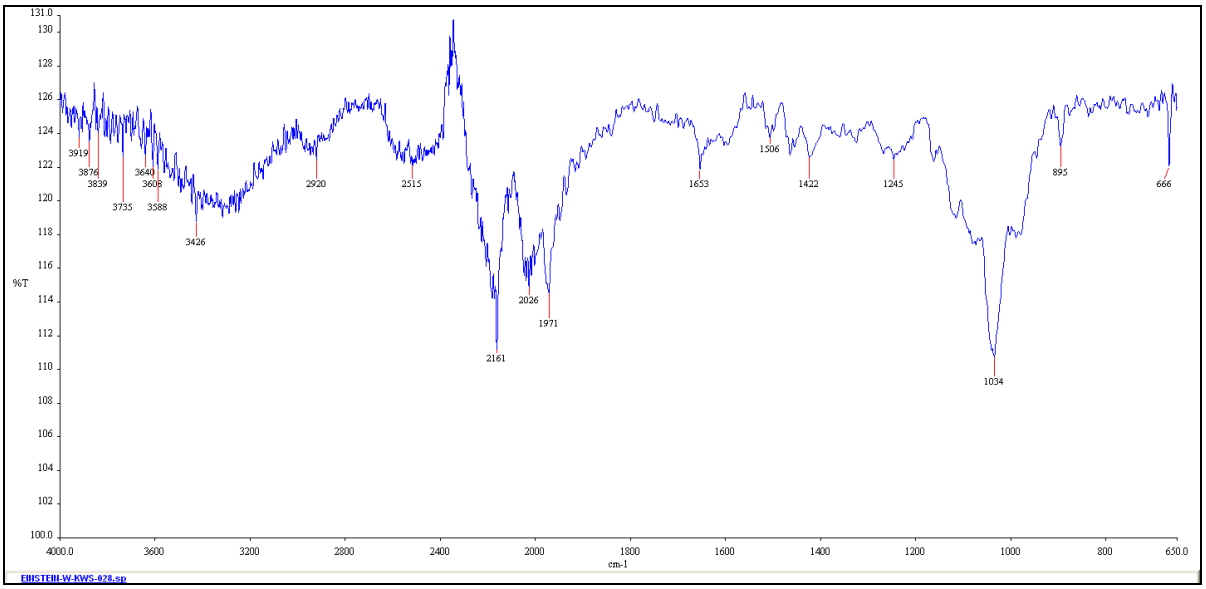

Fig S4 - FTIR spectrum of Einstein wheat lignin.

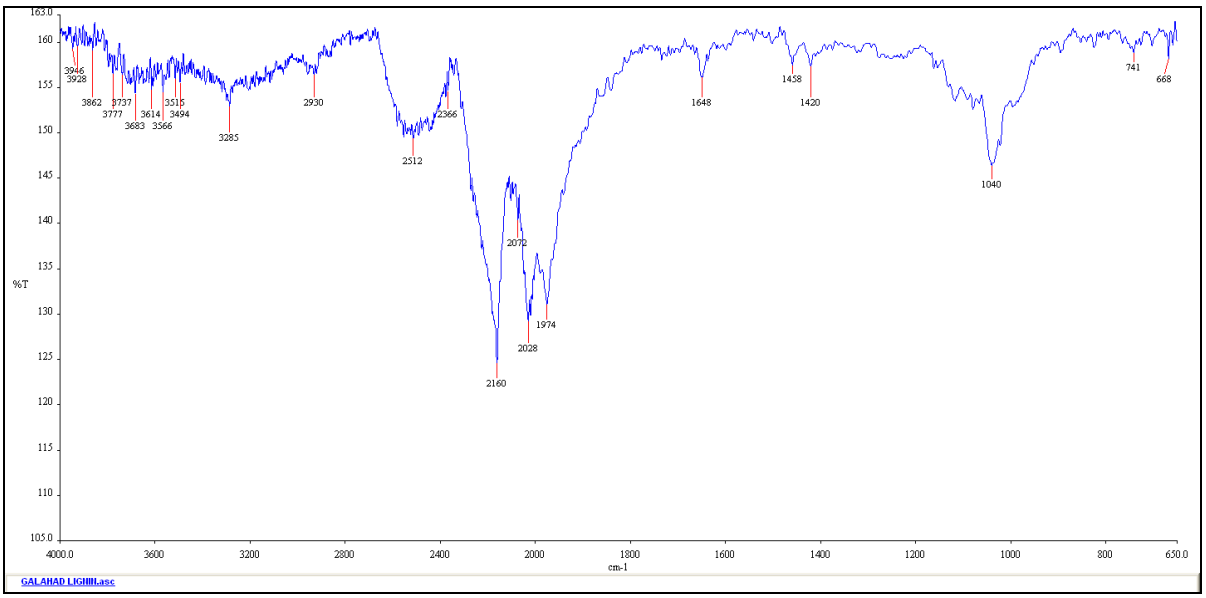

Fig S5 - FTIR spectrum of Galahad wheat lignin.

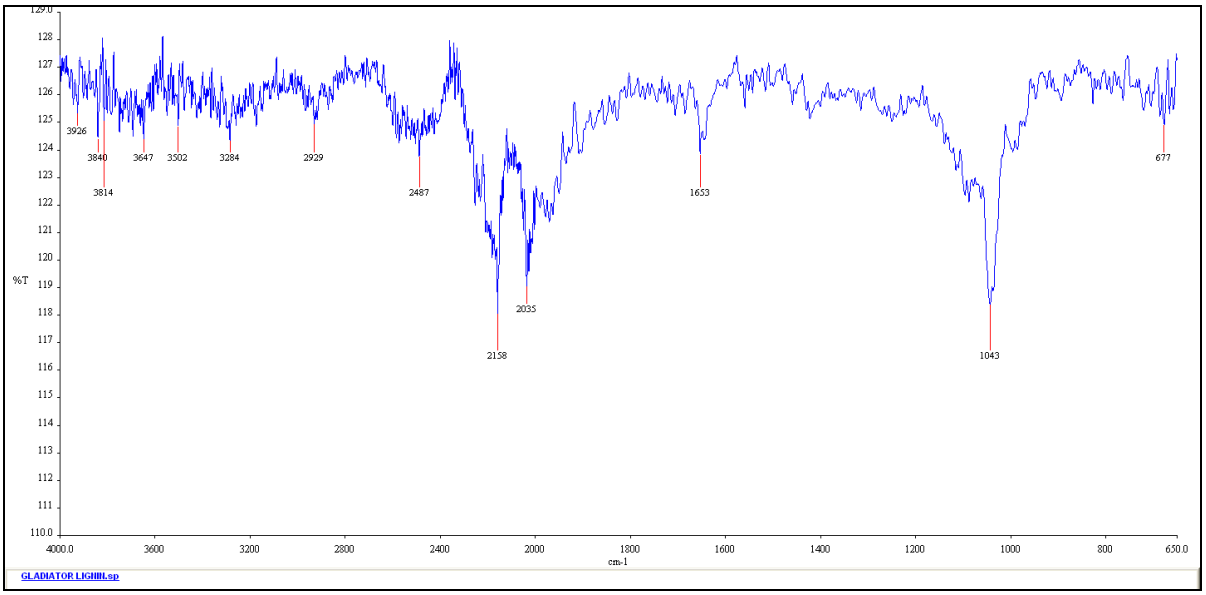

Fig S6 - FTIR spectrum of Gladiator wheat lignin.

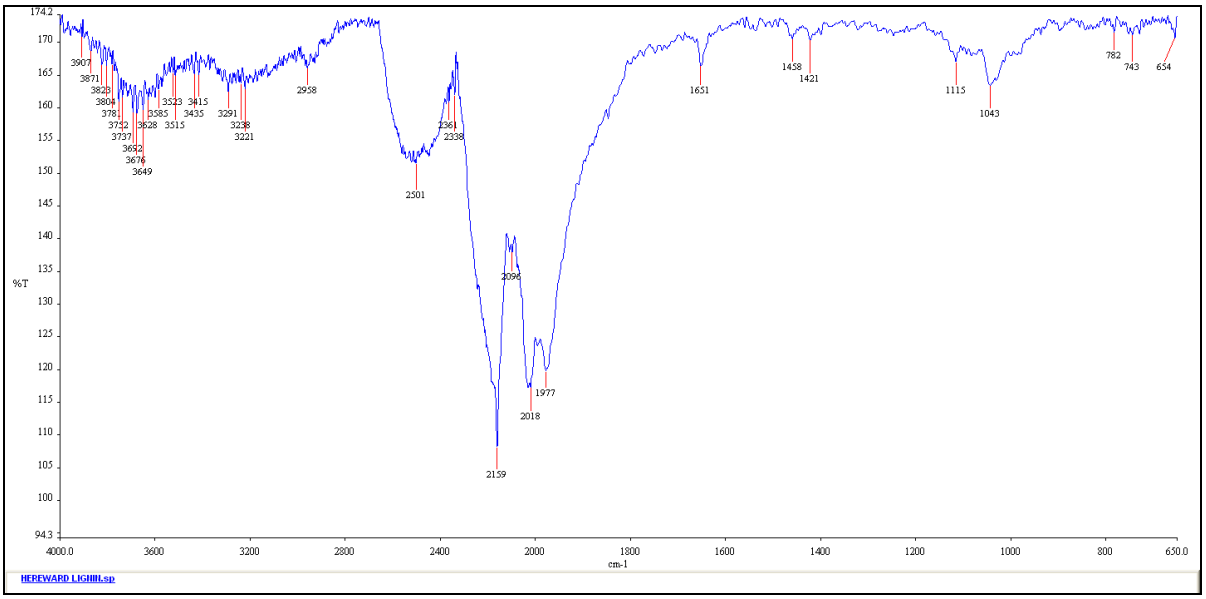

Fig S7 - FTIR spectrum of Hereward wheat lignin.

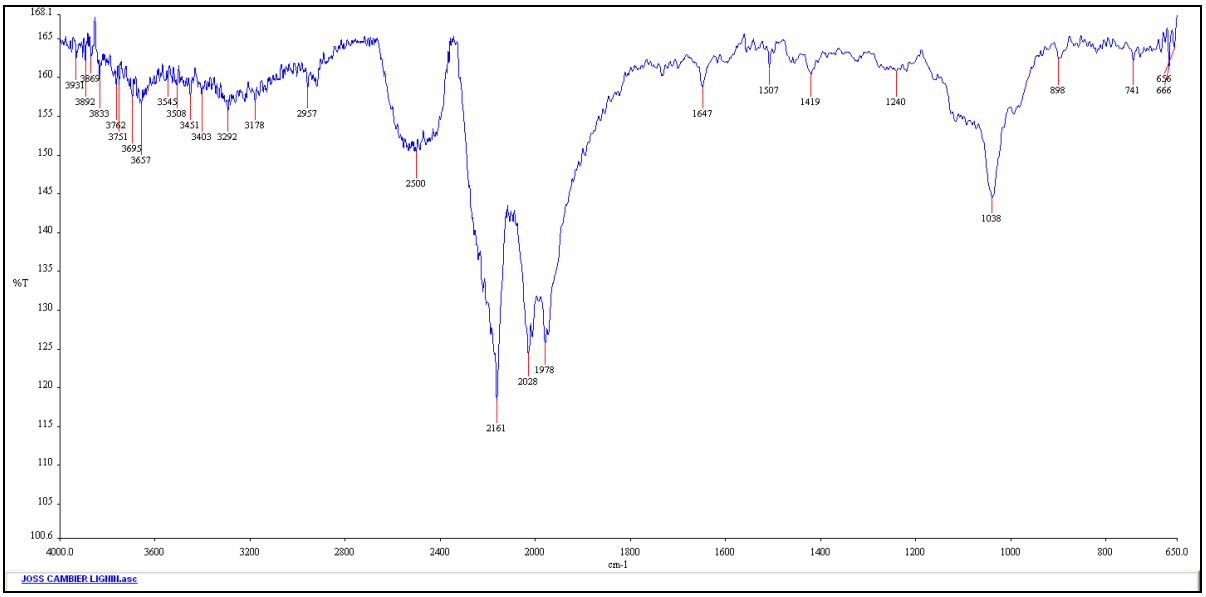

Fig S8 - FTIR spectrum of Joss Cambier wheat lignin.

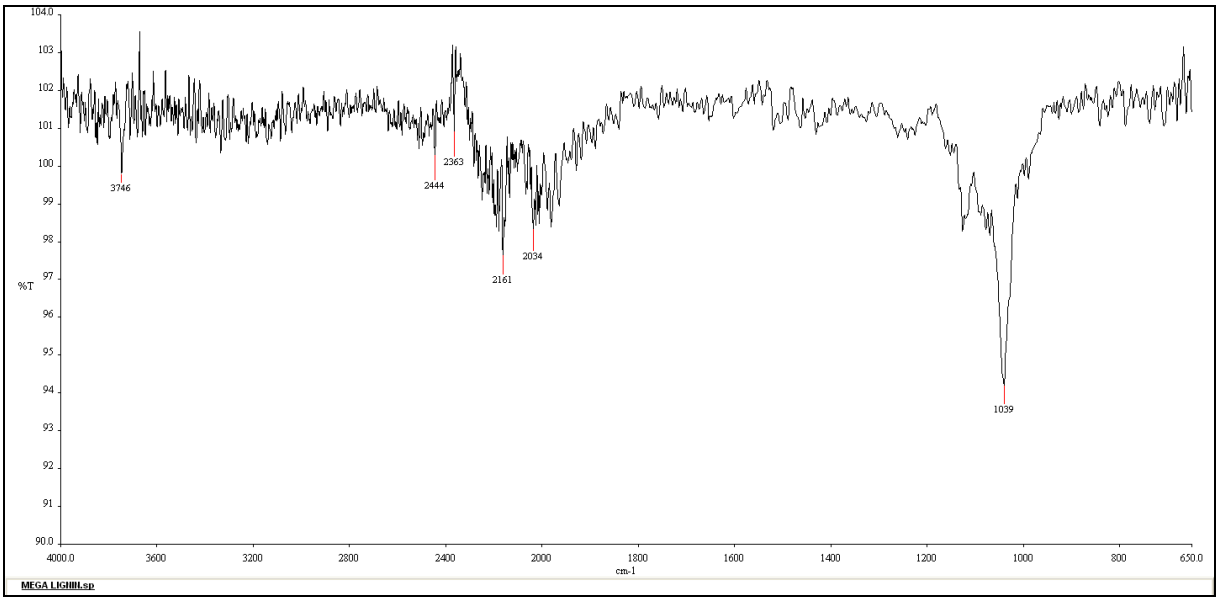

Fig S9 - FTIR spectrum of Mega wheat lignin.

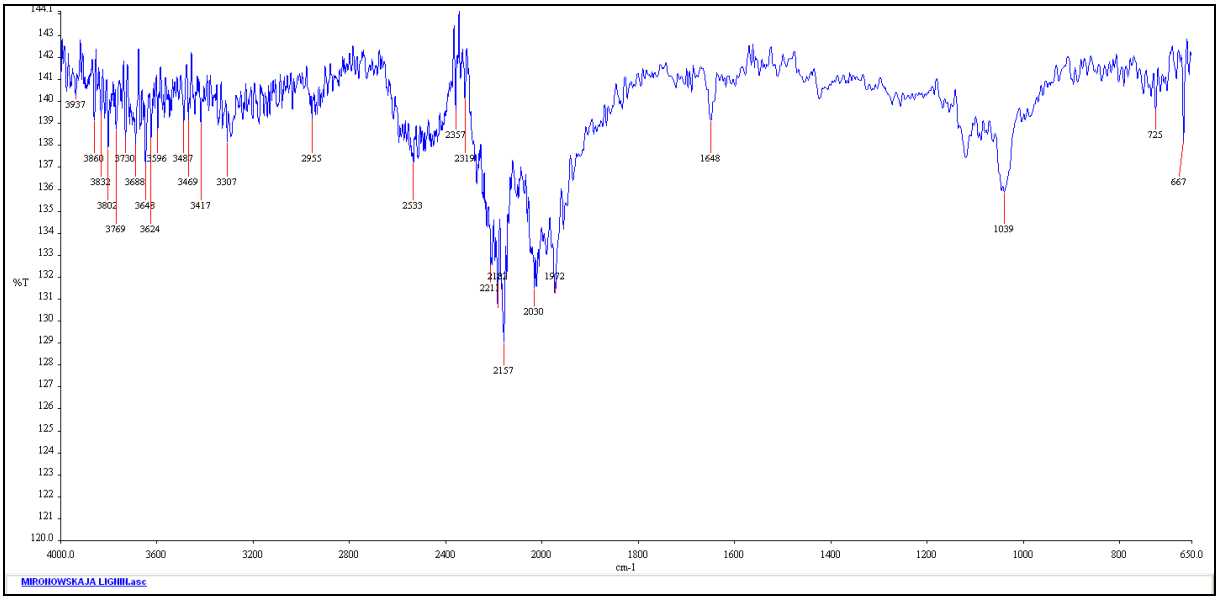

Fig S10 - FTIR spectrum of Mironowskaja wheat lignin.

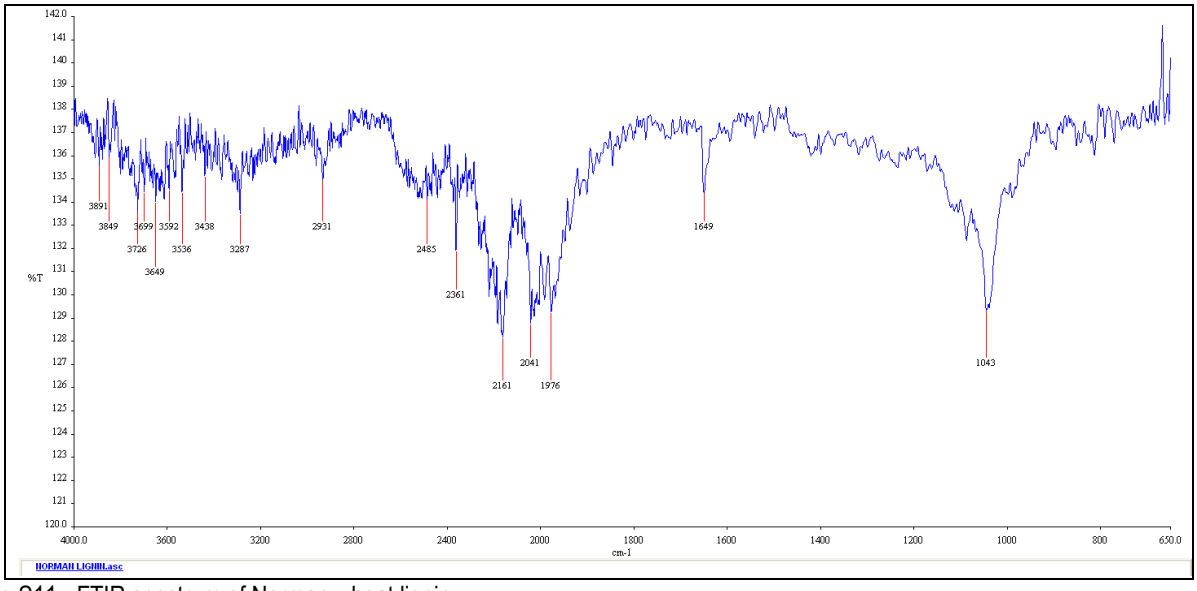

Fig S11 - FTIR spectrum of Norman wheat lignin.

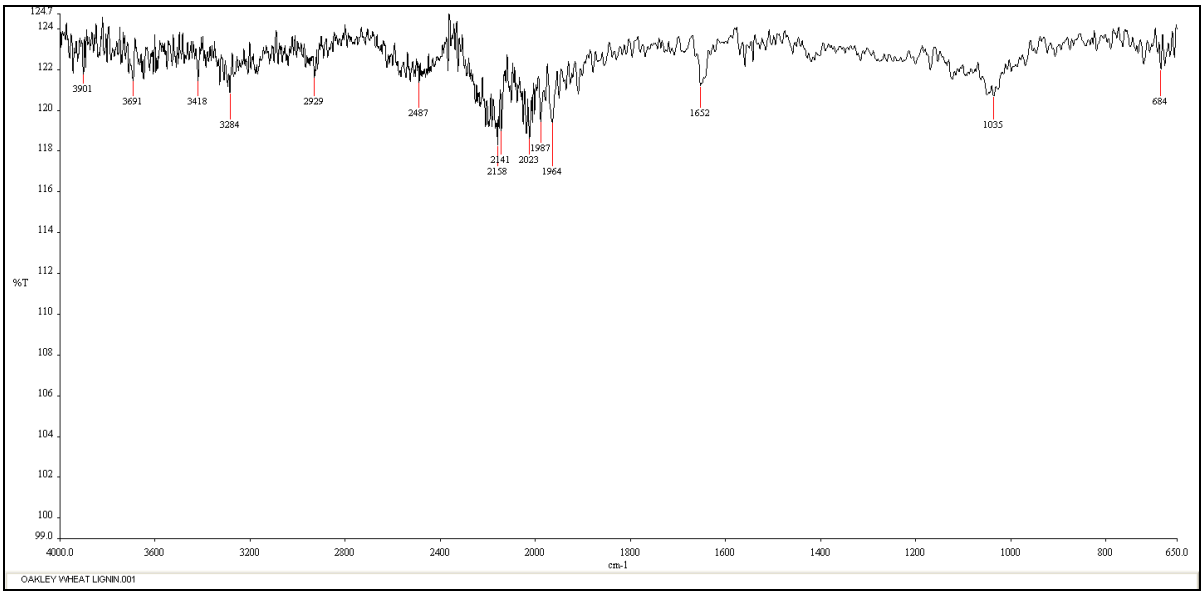

Fig S12 -FTIR spectrum of Oakley wheat lignin.

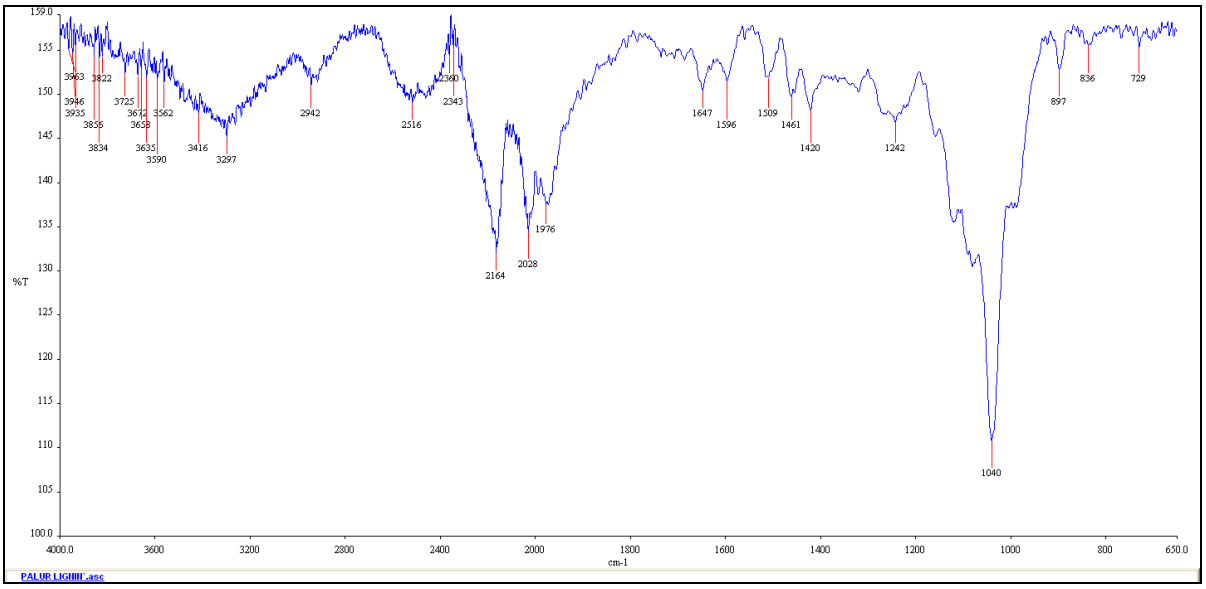

Fig S13 -FTIR spectrum of Palur wheat lignin.

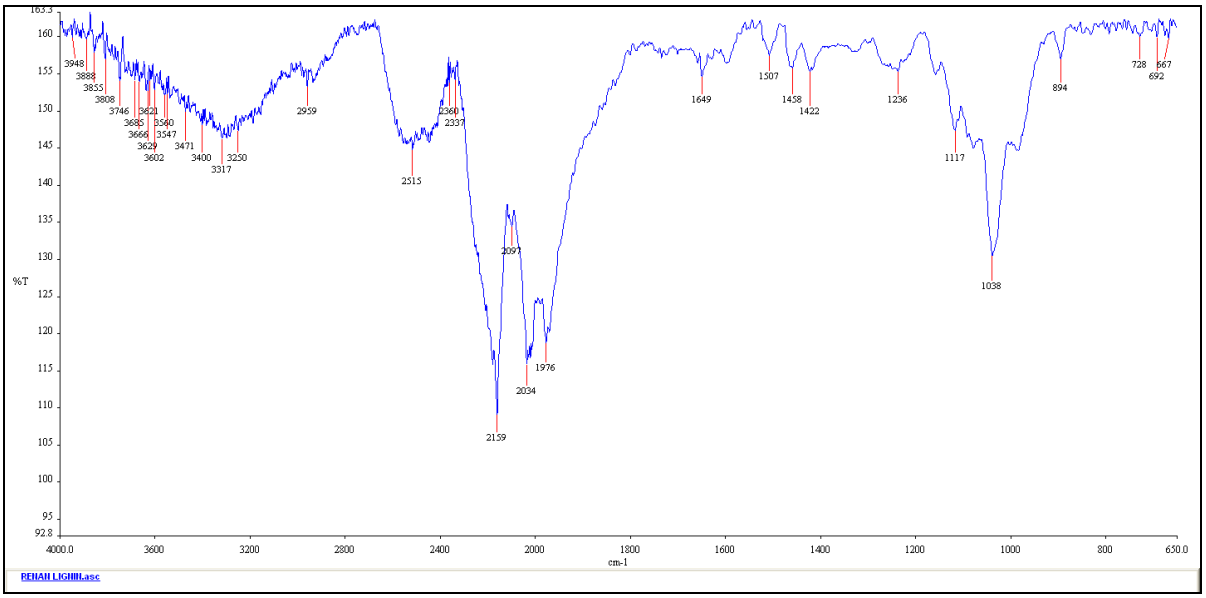

Fig S14 - FTIR spectrum of Renan wheat lignin.

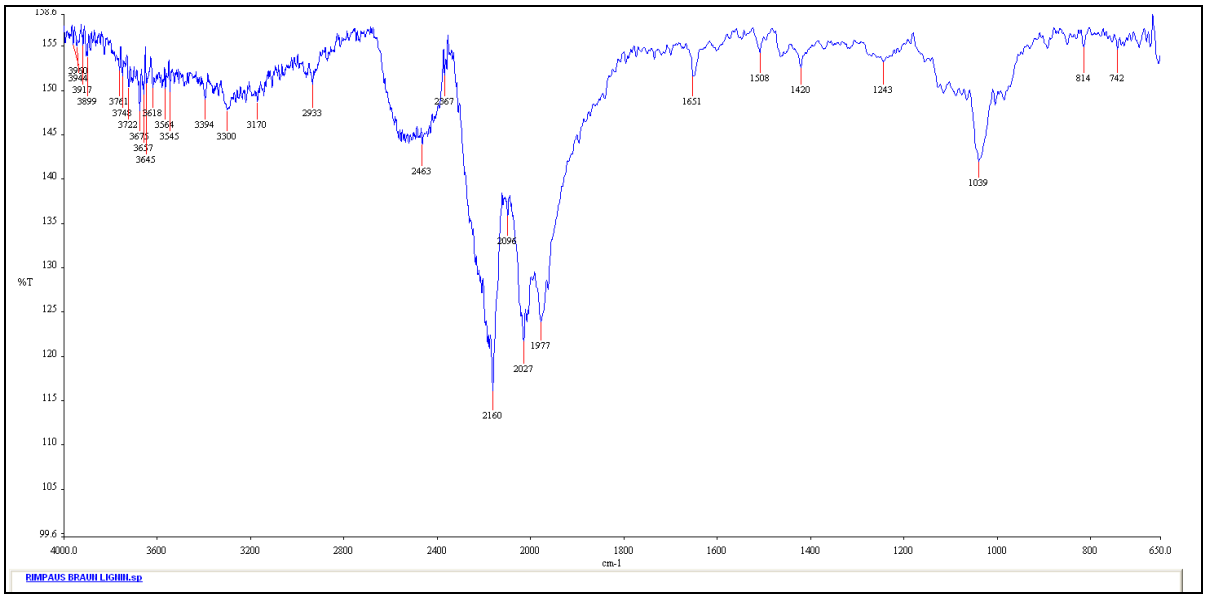

Fig S15 - FTIR spectrum of Rimpaus Braun wheat lignin.

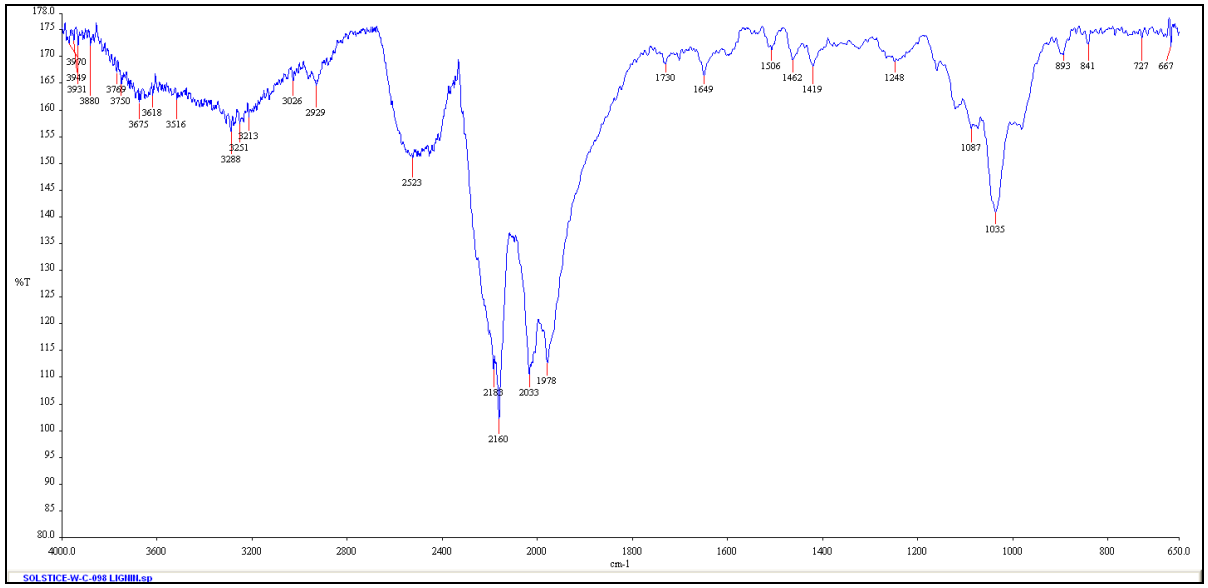

Fig S16 - FTIR spectrum of Solstice wheat lignin.

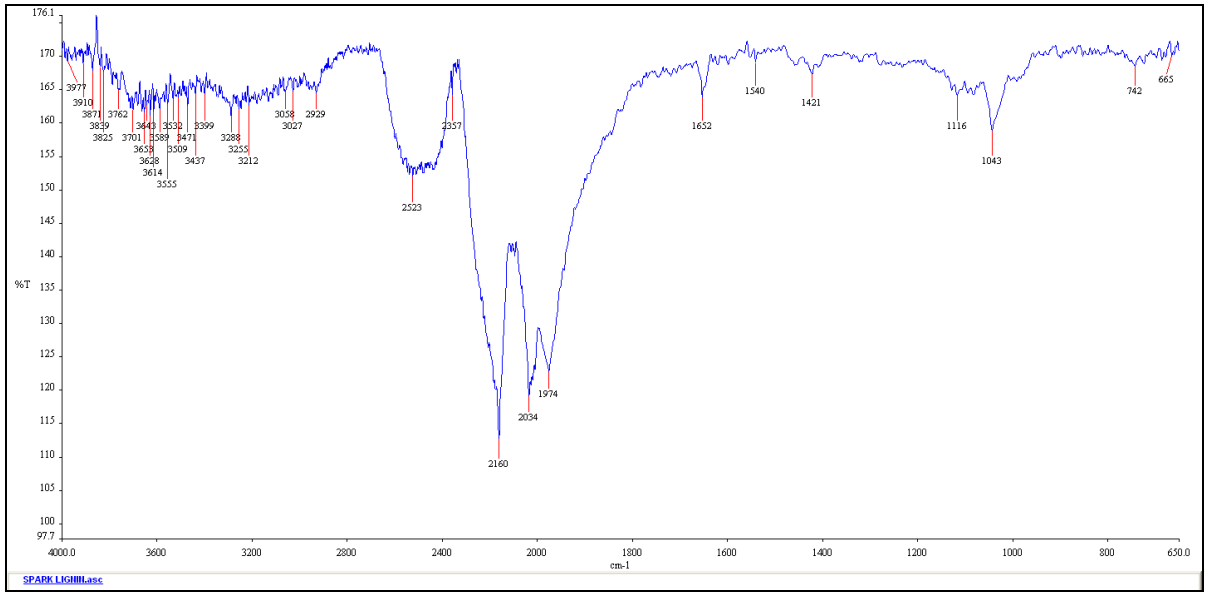

Fig S17 - FTIR spectrum of Spark wheat lignin.

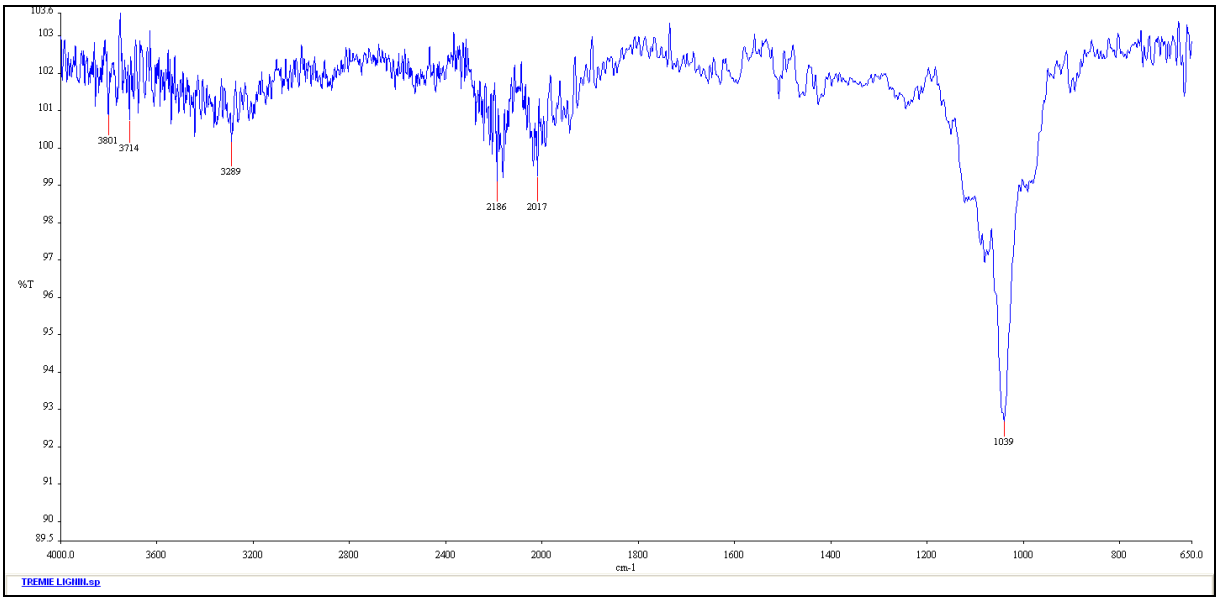

Fig S18- FTIR spectrum of Tremie wheat lignin.

Wheat variety (Country of origin)	**Absorption at 1000-1200 cm^{-1}**	**Absorption at 1500-1700 cm^{-1}**
Alba (Netherlands)	1040, 1110	1505, 1649
Battalion (UK)	1040, 1080	ND
Capelle Desprez (France)	1040	1594, 1652
Einstein (UK)	1034	1506, 1653
Galahad (UK)	1040	1648
Gladiator (UK)	1043	1653
Hereward (UK)	1043	1651
Joss Cambier (France)	1038	1507, 1647
Mega (UK)	1039	ND
Mironowskaja (Russia)	1039	1648
Norman (UK)	1043	1649
Oakley (UK)	1035	1652
Palur (Germany)	1040	1509, 1596, 1647
Renan (Austria)	1038	1507, 1649
Rimpaus Braun (Germany)	1039	1508, 1651
Solstice (UK)	1035, 1087	1506, 1649
Spark (UK)	1043	1540, 1652
Tremie (France)	1039	ND

Fig S19 - Summary of FT-IR peaks of alkali lignin samples from wheat straw varieties. ND, not detected

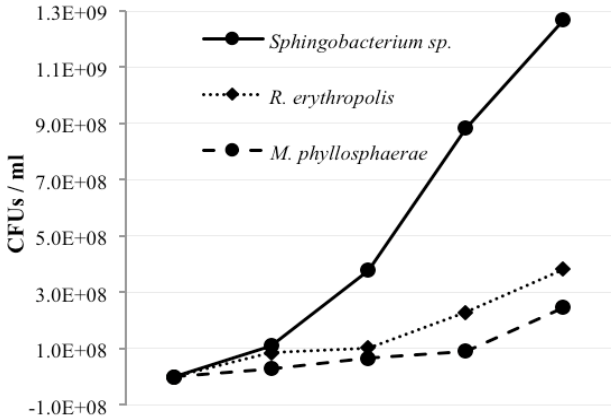

Fig S20 - Growth (measured by colony forming units per ml culture) of *Sphingobacterium sp.*, *R. erythropolis* and *M. phyllosphaerae* on M9 media containing 2.0 % (w/v) corn steep liquor and 2.5 % (w/v) ground wheat straw lignocellulose.

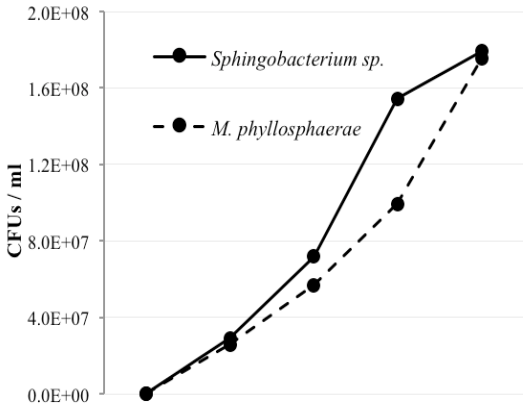

Fig S21 - Growth (measured by colony forming units per ml culture) of *Sphingobacterium sp.* and *M. phyllosphaerae* on M9 media containing 2.0 % (w/v) corn steep liquor and 2.5 % (w/v) Organosolv lignin

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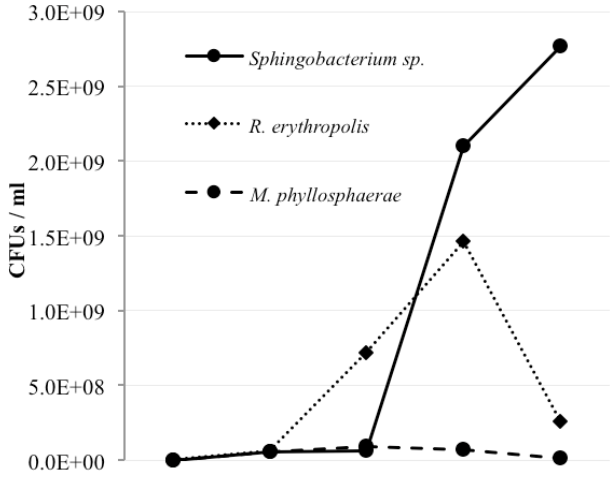

Fig S22 -. Growth (measured by colony forming units per ml culture) of *Sphingobacterium sp., R. erythropolis* and *M. phyllosphaerae* on M9 media containing 2.0 % (w/v) corn steep liquor and 2.5 % (w/v) Kraft lignin.