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Isolation of high quality lignin as a by-product from ammonia percolation pretreatment of poplar wood

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Abstract

A two-step process combining percolation-mode ammonia pretreatment of poplar sawdust with mild organosolv purification of the extracted lignin produced high quality, high purity lignin in up to 31% yield and 50% recovery. The uncondensed fraction of the isolated lignin was up to 34%, close to that the native lignin (40%). Less lignin was recovered after pretreatment in batch mode, apparently due to condensation during the longer residence time of the solubilised lignin at elevated temperature. The lignin recovery was directly correlated with its molecular weight and its nitrogen content. Low nitrogen incorporation, observed at high ammonia concentration, may be explained by limited homolytic cleavage of β-O-4 bonds. Ammonia concentrations from 15% to 25% (w/w) gave similar results in terms of lignin structure, yield and recovery.

1. Introduction

The pretreatment step, in which lignin is dissociated from cellulose, is the key to economic conversion of lignocellulosic biomass to liquid biofuel (Agbor et al., 2011). The most important factor in the choice of pretreatment technology is therefore how efficiently the lignin is solubilised or otherwise rendered innocuous towards the enzymatic depolymerisation of cellulose. However it is widely agreed that the economic viability of biofuel production will depend on adding value to the by-products, including lignin. Efficient solubilisation will then improve the yield of both the primary fuel product and the lignin as a secondary product, but it is necessary to ensure that the quality of the lignin is not degraded.

The best prospects for adding value to lignin are through its conversion to aromatic fine chemicals by controlled depolymerisation (Bozell et al., 2007; Zakzeski et al., 2011). The quality of the isolated lignin can then be defined in terms of its structural suitability for depolymerisation. Recent research on lignin model compounds has suggested that lignin should be efficiently broken down into low molecular weight compounds if the starting material contains a high proportion of alkyl-aryl ether (β-*O*-4) bonds (Parsell et al., 2013). This bond accounts for up to 60 % of the *in-situ* linkages in hardwood lignins. Lignins also contain more stable carbon-carbon (β-5, β-β, β-1 and 5-5) and diaryl ether (4-*O*-5) bonds (Ralph, 2004), and are termed *condensed* if the carbon-carbon bonds are abundant and the polymer structure is heavily cross-linked.

For efficient delignification it is necessary, although not sufficient, to maximise the aryl-ether linkages that are broken (Pan et al., 2006). Unfortunately, severe thermal conditions, required in wood pretreatment, increase the degree of condensation, leading to lignin that is harder to break down (Choi and Faix, 2010; Li et al., 2007). For example the well-established organosolv process has proved to be efficient for hardwood delignification and the isolated

46   lignin, with low molecular weight and high phenolic hydroxyl content, has interesting anti-

47   oxidant properties, but high temperature and acidity led to significant structural alteration,

48   including loss of the γ-methylol group and increased condensation (El Hage et al., 2010; Pan

49   et al., 2006), reducing its suitability for conversion to fine chemicals.

50   The experiments on poplar wood that we describe here were focused on the optimisation

51   of the pretreatment step to combine minimal chemical alteration of the solubilised lignin with

52   high cellulose digestibility in the residue. Ammonia pretreatments are known to increase

53   cellulose digestibility while avoiding condensation of the lignin (Yoon et al., 1995). The

54   efficiency of ammonia-based pretreatments (Ammonia Fibre Expansion, Soaking Aqueous

55   Ammonia, Ammonia Recycling Percolation) for biofuel production has been extensively

56   studied (Bals et al., 2011; Gupta and Lee, 2009; Kim et al., 2003; Kumar et al., 2009), but

57   few studies have considered the lignin structure after ammonia treatment (Chundawat et al.,

58   2011; Liu et al., 2013). In a recent patent (Balan et al., 2013), lignin fractions were

59   characterised after batch-mode ammonia extraction of corn Stover, and 2D NMR analysis of

60   a fraction enriched in lignin showed that a moderate percentage of β-aryl ether linkages were

61   preserved. AFEX pretreatment of corn stover at 130°C, which retained the lignin in the

62   residue, altered its structure only to a moderate extent (Chundawat et al., 2011). The mild

63   temperatures (120-130°C) used in the ammonia pretreatment of corn stover partially

64   explained the limited structural alteration of the lignin. However in the case of hardwoods,

65   higher temperatures (160-180°C) are generally needed to produce residues suitable for

66   enzymatic saccharification. Under these conditions the lignin will suffer more condensation.

67   An interesting question is whether lignin is more vulnerable to condensation in its native,

68   solid state or after solubilisation. If condensation occurs more readily when the lignin is

69   soluble, then removing the solubilised lignin immediately by adopting a percolation mode

70   should lead to less condensed lignin than with batch mode, where the lignin remains in

71 solution at high temperature for longer. In this study poplar sawdust was subjected to both

72 batch and percolation ammonia pretreatments to extract lignin. The extracted lignin was then

73 purified by a very mild organosolv post-treatment to produce high purity, high quality lignin

74 in excellent yield.

75 **2. Materials and Methods**

76

77 **2.1.Materials**
78
79   Hybrid poplar sawdust was provided by a UK sawmill. The sawdust was sieved and

80 the particle size range from 125µm to 1080 µm was used. The dry matter content of the

81 sawdust was 92.6 %. All reagents and solvents were purchased from Sigma-Aldrich and used

82 without further purification.

83   The poplar sawdust was subjected to batch and percolation (continuous) ammonia

84 pretreatments to extract lignin. Each extracted lignin was then purified by mild organosolv

85 post-treatment (see section 2.3) to produce an ethanol-soluble, purified lignin after

86 precipitation in water. The multistep process is illustrated in Fig. 1.
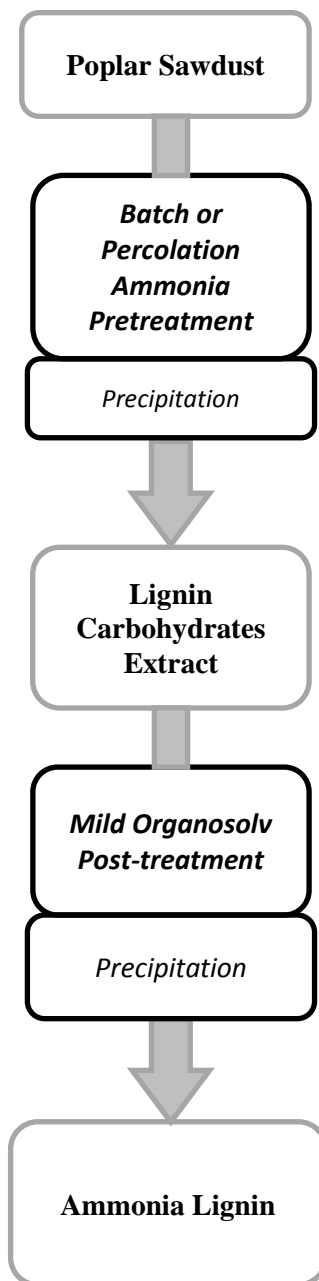
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                    ┌─────────────────────┐
                    │   Poplar Sawdust    │
                    └─────────────────────┘
                              │
                    ┌─────────────────────┐
                    │     Batch or        │
                    │    Percolation      │
                    │     Ammonia         │
                    │   Pretreatment      │
                    ├─────────────────────┤
                    │    Precipitation    │
                    └─────────────────────┘
                              │
                              ▼
                    ┌─────────────────────┐
                    │      Lignin         │
                    │   Carbohydrates     │
                    │      Extract        │
                    └─────────────────────┘
                              │
                    ┌─────────────────────┐
                    │  Mild Organosolv    │
                    │   Post-treatment    │
                    ├─────────────────────┤
                    │    Precipitation    │
                    └─────────────────────┘
                              │
                              ▼
                    ┌─────────────────────┐
                    │   Ammonia Lignin    │
                    └─────────────────────┘
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Fig 1. Global flow-chart for ammonia lignin isolation from poplar sawdust

2.2. Ammonia extraction of poplar lignin

2.2.1. Batch mode

   A stainless steel reactor (75 ml) was packed with 5-6g of poplar sawdust. The reactor

was filled with 15% (w/w) aqueous NH_3 (60 ml) and soaked for 2h at 40 °C. The pressure

124   was then increased to 20 Bar and the temperature was raised to the cooking temperature at 25

125   °C/min. The cooking temperature was held for 120 min, then dropped to 80 °C in order to

126   flush the reactor with nitrogen. The residue was washed twice with water at 80 °C. The

127   residue was transferred to a sealed bag and weighed. Aliquots were taken for measurement of

128   moisture and lignin content (2 x 2g). The extracted lignin was recovered by precipitation in

129   water.

130   **2.2.2. Percolation mode**

131

132   The percolation device, inspired by previous works, is illustrated in Fig. 2 (Kim et al.,

133   2009). The stainless steel reactor (75 ml) was packed with 18-19g of poplar sawdust, filled

134   with 15% (w/w) aqueous NH_3 (40 ml) and soaked for 1h at 40°C. The pressure was increased

135   to 20 Bar and the temperature was raised to the cooking temperature at 25°C/min. Once the

136   temperature of the oven reached 180°C, more liquid extractant was percolated through the

137   reaction vessel at 3ml/min for 90 min. Deionised water was then percolated at 5 ml/min for

138   40 min, after which the temperature was reduced to 60 °C before flushing the reactor with

139   nitrogen. The residue was transferred to a sealed bag and weighed. Aliquots were taken for

140   measurement of moisture and lignin content (2 x 2g). The extracted lignin was recovered by

141   precipitation in water.
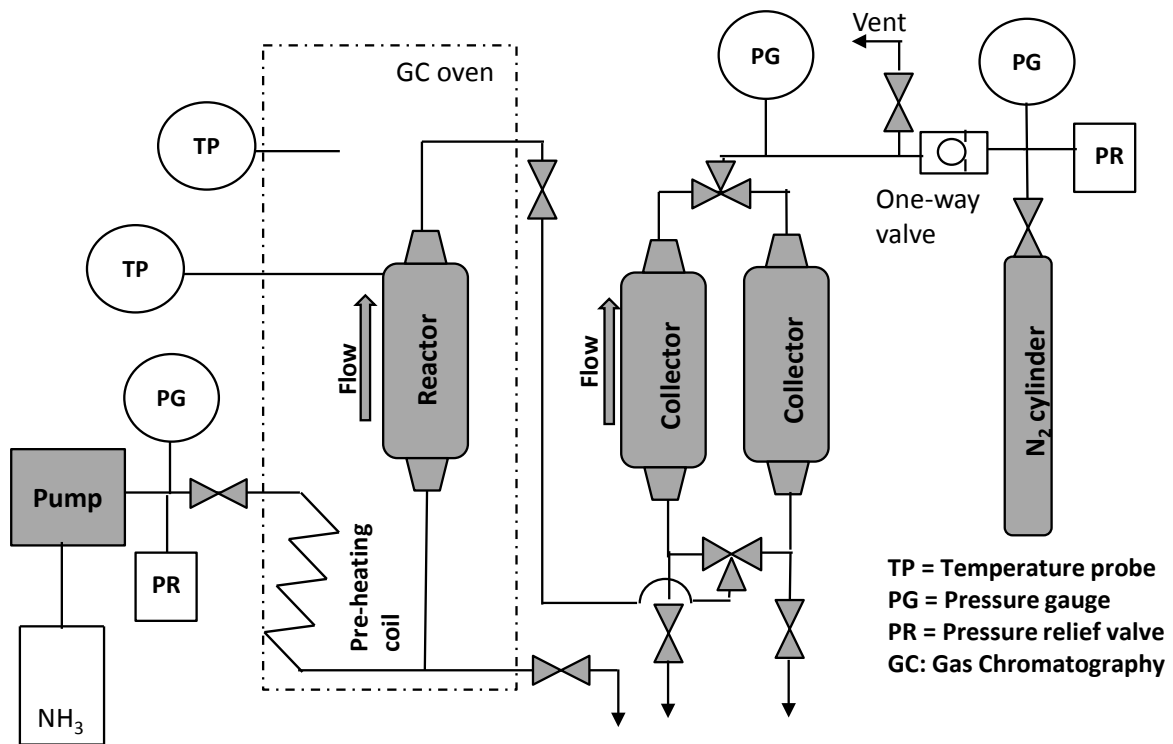
142

143 Fig 2. Schematic of percolation system for poplar ammonia pretreatment

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145

146 **2.2.3. Extracted lignin recovery**

147 The liquor was reduced in pH to 7-8 by evaporation of the ammonia and then acidified to pH

148 2 (HCl 2M). After leaving overnight at 4 °C, the precipitate was recovered by centrifugation,

149 washed three times with HCl at pH2 and freeze dried.

150

151 **2.3.Organosolv hydrolysis of Lignin-Carbohydrates precipitate**

152

153 The extracted lignin containing polysaccharide impurities was dissolved in ethanol/water

154 solution (6/4 : v/v) with varying H_2SO_4 concentrations (0.1 to 1N) and heated for 2 h at

155 temperatures ranging from 80 to 100°C. After this mild hydrolysis, the insoluble fraction was

156 recovered by centrifugation and washed with ethanol/water. Ethanol-soluble lignin was then

157 precipitated from the supernatant in three volumes of acidified water (HCl, pH 2). After

158 leaving overnight at 4°C the purified lignin was recovered by centrifugation, washed three

159 times with acidified water (HCl pH 2) and freeze dried.

160

161 **2.4.   Analytical Methods**

162 Moisture content was determined after oven drying at 105˚C for 16h. Polysaccharide

163 composition was determined after two-step hydrolysis (Remond et al., 2010) . Acid insoluble

164 lignin was determined by a two-step acid hydrolysis (Monties, 1984).

165 Monosaccharides were derivatised for GC as described (Blakeney et al., 1983; Bouxin et

166 al., 2014) and quantified on a HP 5890 gas chromatograph fitted with a Supelco SLB-5ms

167 capillary column (30m x 0.32mm, 1mm thickness). A volume of 1 µl was injected in splitless

168 mode. The oven temperature was increased from 180˚C to 280˚C at 2.5˚C/min and held at

169 280˚C for 30 min. Flow pressure was set to 12 psi. The injector temperature was set at 250˚C

170 and the flame ionisation detector temperature was set at 280˚C. Quantification was performed

171 using pure monosaccharide standards with myo-inositol as internal standard.

172 Thioacidolysis of the extracted or isolated lignins was performed as described

173 (Lapierre et al., 1995). The trimethylsilyl (TMS) derivatives were separated on a Supelco

174 SLB-5ms capillary column (30m x 0.32mm, 1mm thickness) and quantified by FID. The

175 GPC analysis was performed on a Gilson2 system, equipped with a UV detector. A set of

176 PS/DVB columns (5 µm, 300x7.5mm, 50 Å and 500 Å, Polymer Lab) was used. The

177 injection volume was 100µL. The temperature of the column was 30°C. The ChromPerfect

178 software package was used to manage the data.

179   CHN analysis was performed on an Exeter CE-440 Elemental Analyser.

180

181   **3. Results and Discussion**

182

183   **3.1.Ammonia pretreatment of Poplar : Batch vs Percolation**

184

185   **3.1.1. Quantitative analysis of lignin extracts**
186

187   To determine whether lignin was more sensitive to high-temperature condensation in

188   solution state, poplar sawdust was subjected to two modes of ammonia pretreatment

189   (percolation or batch) at two different temperatures. The lignin was recovered by

190   precipitation after adjusting the pH to 2 (cf. section 2.2.3.).

191   Table 1: Extraction yield, xylan content and lignin recovery in percolation and batch mode

	Yield of extract (g/100g of dry poplar)	Xylan content in the extract (g/100g of extract)	Lignin content in the residue (g/100g of dry poplar)	Lignin recovery (g/100g of solubilised lignin)
Percolation at 170 °C	10.3	19.4 (0.1)	12.6 (0.2)	79.8
Percolation at 180 °C	13.7	18.1 (1.5)	10.1 (0.1)	87.0
Batch at 170 °C	5.3	32.6 (1.1)	14.2 (0.4)	40.6
Batch at 180 °C	6.6	31.3 (0.2)	13.3 (0.1)	46.7

192

193   As illustrated in Table 1, more lignin was extracted by the percolation approach but the

194   extracted lignin was associated with substantial amounts of carbohydrate, largely xylan. The

195   lignin content in the extract may be estimated as (100% - % carbohydrate). Increasing the

196   temperature from 170°C to 180°C increased the total mass solubilised, did not significantly

197   change its xylan content, but slightly increased the lignin recovery. Twice as much

198   solubilised lignin was recovered in percolation mode as in batch mode. The lower yield of

199   lignin in batch mode may be explained at least partly by *in-situ* redeposition after cooling,

200   and is consistent with the higher lignin content in the residue from the pretreatment step.

201   Potentially an additional explanation for the lower lignin recovery in the batch reaction is

202   degradation of the lignin fragments of lower molecular mass and greater solubility, leading to

203   reduced precipitation in acidic water. From a practical viewpoint, the percolation mode led to

204   higher lignin yield and reduced co-precipitation of xylan.

205

206   **3.1.2.   Qualitative analysis of lignin extracts**

207

208   Thioacidolysis, which cleaves β-*O*-4 bonds, can be used to estimate the degree of

209   condensation of lignin. If thioacidolysis leads to a high proportion of monomers the lignin

210   can be said to be relatively uncondensed, with good potential for controlled depolymerisation

211   to useful products on an industrial scale. After pretreatment at 180°C, the soluble and

212   insoluble lignin fractions were subjected to thioacidolysis and the thioacidolysis products

213   were separated by GPC. Fig. S1 shows that the yield of low-molecular (dp1, dp2)

214   thioacidolysis products was substantial and quite similar to the poplar native lignin (black

215   line). In both pretreatment modes the low-molecular fraction was abundant in the insoluble

216   lignins (blue and purple lines) but there was a relative increase in thioacidolysis products of

217   higher molecular mass in the lignin solubilised by batch mode pretreatment (red line)

218   suggesting that condensation reactions had occurred once the lignin was in solution.

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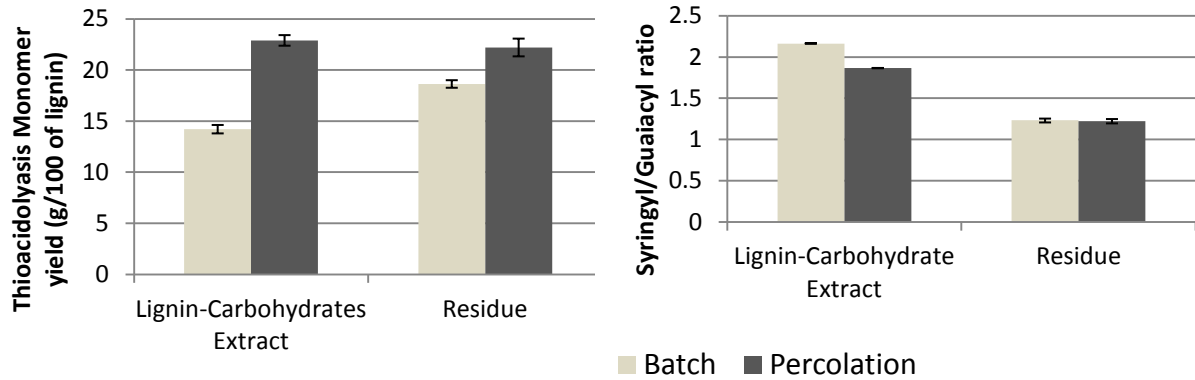
221   Fig 3. Thioacidolysis monomers and G/S ratio of the lignin in the extract and the residue

222
223
224   Condensation of the soluble lignin during batch mode pretreatment is also evident

225   from Fig. 3 where the thioacidolysis monomer yield was only 14 % compared to 38% for

226   native lignin. Minimising the cooking residence time by using percolation mode (Fig. S1,

227   green line) significantly limited the condensation of the soluble lignin fraction, which

228   generated 23 % of thioacidolysis monomers. Nevertheless it is interesting that the lignin

229   remaining in the insoluble residue retained a substantial proportion of alkyl-aryl bonds, with

230   19 % and 22 % monomer yields for batch and percolation mode respectively. This

231   observation is consistent with data on ammonia pretreatment of Miscanthus (Liu et al., 2013).

232   The Syringyl/Guaiacyl ratio of the extracted lignin was 2.2 and 1.9 respectively for

233   batch and percolation mode, compared to the native lignin with S/G ratio of 1.2. Syringyl

234   enrichment has previously been observed in the syringyl-guaiacyl lignin of AFEX-pretreated

235   corn stover residue (Chundawat et al., 2011). The increase could in principle be explained

236   either by preferential extraction of S-rich lignin, or by more condensation of the G units. The

237   lignin remaining in the residue had the same S/G ratio as native lignin, 1.2 for both modes,

238   which suggests that the increased relative proportion of S units released by thioacidolysis

239   from the extracted lignins can be explained mainly by preferential condensation of the G

240   units after solubilisation. This explanation is consistent with the greater increase in S/G value

241   for the more condensed soluble lignin extracted in batch mode.

242

243   **3.2.Purification of the lignin**

244

245   To reduce their undesirably high carbohydrate content (20 % of xylan after

246   percolation mode pretreatment), the extracted lignin fractions were subjected to mild acid

247   hydrolysis in aqueous ethanol. The conditions used somewhat resembled a very mild

248   organosolv treatment, but with the objective of hydrolysing a small number of the relatively

249   acid-labile xylopyranosyl linkages to release the carbohydrate component, rather than

250   hydrolysing the β-O-4 linkages in lignin. A range of conditions were tested and the resulting

251   condensation of the lignin was monitored by the coupled thioacidolysis/GPC technique.

252   Under all the conditions tested, the purified lignin contained less than 1 % of residual

253   carbohydrate and the ethanol-insoluble fraction contained most of the xylan, still in

254   polymeric form, with no residual lignin. When the purified lignin was separated by GPC (Fig.

255   S2a), a shift to lower molecular mass was observed for higher H_2SO_4 concentrations. This

256   shift cannot be attributed to the cleavage of lignin-carbohydrate bonds because even the

257   lowest acid concentration reduced the carbohydrates content to less than 1%. The shift to

258   lower molecular weight may be attributed to inadvertent cleavage of β-O-4 linkages in lignin.

259   These results are consistent with previous observations on organosolv pretreatment of

260   Miscanthus (El Hage et al., 2010).

261

262   As illustrated in Fig. S2b, increasing both temperature and acid concentration during

263   the purification step (green and red lines) led to more condensed lignin fractions. The milder

264   hydrolysis conditions (black line) avoided condensation of the lignin, which showed

265   molecular mass distribution profiles similar to the extracted lignin before hydrolysis (purple

266   line). Even at low temperature and acid concentration, analysis of the minor fraction that

267   remained insoluble showed that all the lignin had been solubilised.

268   This organosolv-type post-hydrolysis step under mildly acidic conditions led to lignin

269   of high purity. If incorporated into a biorefinery process it would allow the production of high

270   purity lignin (low sugar content) with high depolymerisation potential (high β-*O*-4 content).

271   In order to reduce the number of steps, the mild organosolv post-treatment can be directly

272   applied to the concentrated ammonia liquor without a precipitation step. This simplification

273   will reduce the use of water and the number of soluble fractions.

274

275   **3.3.Effect of the temperature and ammonia concentration**

276

277   **3.3.1.   Extractability, recovery and structure**

278   A series of ammonia percolation experiments were performed at varying ammonia

279   concentrations and temperatures to assess the effects on lignin extractability, recovery and

280   structure. Aqueous ammonia solutions ranging from 2.5 to 25% (w/w) were pumped through

281   poplar sawdust at 1ml/min at 180°C. The extracted lignin was collected by precipitation and

282   purified by mild acid hydrolysis (0.1N/80°C). The amount of solubilised lignin

283   (delignification) was determined by the difference in Klason lignin content before and after

284   the ammonia pretreatment.

285   Table 2: Yield, recovery and structural analysis of ammonia lignin for different pretreatment

286   conditions

| Exp.	NH3/Temp (w%/C)	Yield of isolated lignin (g/100g of total lignin)	Recovery of lignin (g/100g of solubilised lignin)	Extracted lignin Mw (g/mol)	Isolated lignin Mw (g/mol)	Monomers yield (g/100g of lignin)
1	2.5/180	5.0	13.7	3875	4367	23.0
2	5/180	11.7	28.9	4458	4967	27.0
3	10/180	22.1	42.8	5210	5941	30.2
4	15/180	30.5	53.5	5913	6588	32.9
5	20/180	30.1	47.9	6181	6854	33.0
6	25/180	31.4	54.0	5798	6515	32.8
7	15/140	20.6	69.2	6802	6971	32.8
8	15/160	21.4	41.9	6695	7224	33.3

287

288   As illustrated in table 2, the recovery i.e. the fraction of the solubilised lignin that was

289 subsequently precipitated, increased from 14% to 53% as the ammonia concentration was

290 increased from 2.5% to 15% w/w, and then was stable up to 25% w/w ammonia. The low

291 yield of lignin at low ammonia concentrations was due to both reduced solubilisation, and

292 reduced precipitation of the solubilised lignin in water. The reduced precipitation of the

293 lignin extracted under these conditions was associated with lower molecular mass (Fig. 4A),

294 and reduced yield of monomers released by thioacidolysis (Table 2) indicating that β-O-4

295 bonds had been cleaved. The β-O-4 bond cleavage is unlikely to have been hydrolytic as it

296 increased with decreasing ammonia concentration. Low initial concentrations of free

297 ammonia are likely to have been further reduced by the hydrolytic release of acetic acid. It

298 seems likely, therefore, that homolytic β-O-4 bond cleavage was facilitated at low ammonia

299 concentrations (Li and Gellerstedt, 2008). The reasons are not clear but the practical

300 consequence is that increasing the ammonia concentration not only brought more lignin into

301 solution but also reduced its depolymerisation and condensation. Reducing the pretreatment

302 temperature led to a further increase in lignin recovery (up to nearly 70% at 140°C) and
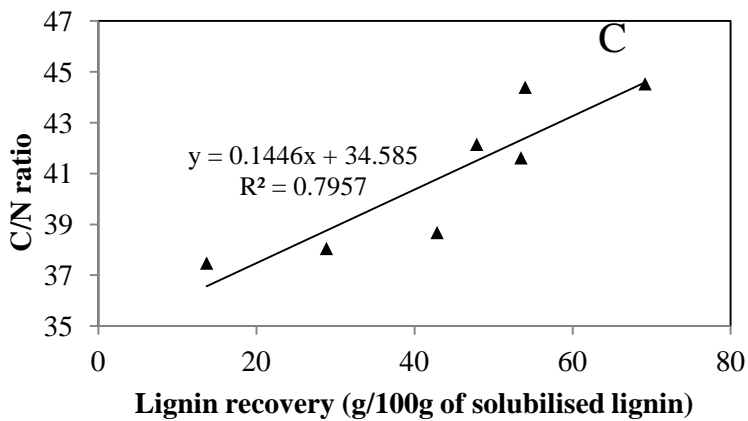
303 higher mean molecular mass.

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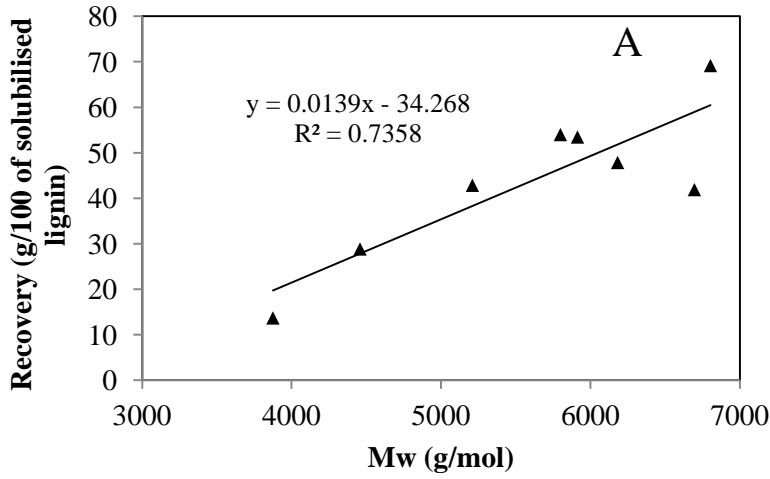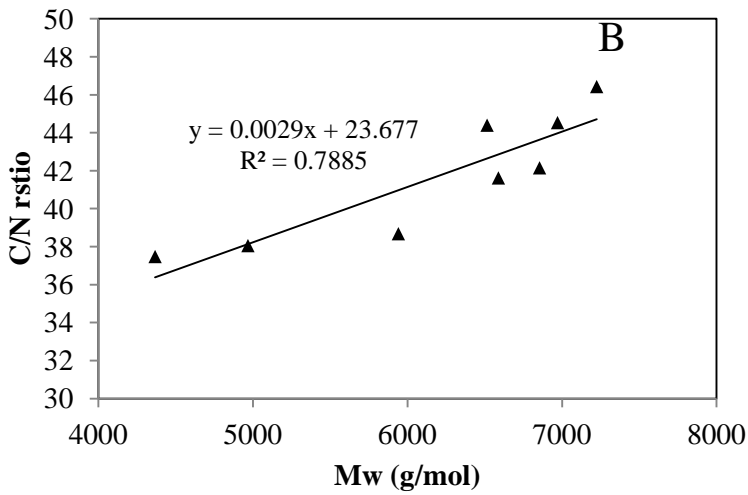
Fig 4. Lignin recovery as a function of the molecular mass of the extracted lignin (A), the

C/N ratio as function of the molecular mass of the isolated lignin (B) Lignin recovery (C)

310   For adequate recovery of poplar lignin after this type of pretreatment, the ammonia

311   concentration must be at least 15 % (w/w). Lowering the temperature below 180°C led to

312   reduced solubilisation of lignin that was not fully offset by improved recovery from solution.

313   At 180°C, 7.3 g of lignin per 100 g of dry poplar was isolated while only 4.9 g of lignin was

314   isolated at 140°C.

315   **3.3.2. Nitrogen incorporation**

316   Table 3 shows that the purified lignin contained approximately 1 nitrogen atom for every

317   4-5 lignin monomer units. This value is obtained by assuming an average of 10.65 carbons

318   per lignin unit deduced from the thioacidolysis G/S ratio. This assumption led to a calculated

319   nitrogen content ranging from 0.20 to 0.24 atoms per lignin unit. Covalent incorporation of

320   nitrogen into lignin during ammonia treatment of lignocellulosic materials has been proposed

321   previously (Chundawat et al., 2013), but the nitrogen-containing structures have not been

322   satisfactorily identified. One suggestion (Sewalt et al., 1997) is nucleophilic addition of

323   ammonia leading to an amino group at the benzylic position (C_α). This is not consistent with

324   our observations, for the following reason. As shown previously (**3.2**), the use of higher acid

325   concentrations for xylan hydrolysis led to condensation of the lignin. Lignin condensation

326   under acidic conditions is due principally to the formation of carbonium ions (Li and

327   Gellerstedt, 2008; Lunquist, 1976). The amine function is a better leaving group than the

328   hydroxyl function, so the formation of a benzylic carbonium ion in acid medium from

329   benzylamine-type structures should be favoured. Increased condensation would then be

330   associated with reduced nitrogen content. Table 3 shows that the nitrogen content remained

331   constant when increasing acid concentration led to increased condensation, inconsistent with

332   extensive prior ammoniation on the benzylic position.

333   Table 3: CHN elemental analysis of isolated lignins

Exp.	NH$_3$/Temp (w%/C)	H$_2$SO$_4$/Temp	%C	%H	%N	%O
1	15%/180	0.1N/100	60.89 (0.07)	6.02 (0.03)	1.49 (0.01)	31.6 (0.11)
2	15%/180	0.5N/100	61.72 (0.00)	6.05 (0.03)	1.48 (0.02)	30.76 (0.05)
3	15%/180	1N/100	62.14 (0.06)	6.12 (0.01)	1.46 (0.01)	30.29 (0.04)
4	2.5%/180	0.1N/80	60.71 (0.01)	6.19 (0.05)	1.62 (0.02)	31.50 (0.08)
5	5%/180	0.1N/80	60.5 (0.2)	6.04 (0.03)	1.59 (0.01)	31.88 (0.2)
6	10%/180	0.1N/80	58.79 (0.1)	5.81 (0.02)	1.52 (0.02)	33.89 (0.13)
7	15%/180	0.1N/80	59.51 (0.08)	5.91 (0.02)	1.43 (0.02)	33.15 (0.13)
8	20%/180	0.1N/80	59.85 (0.12)	6.02 (0.02)	1.42 (0.03)	32.72 (0.17)
9	25%/180	0.1N/80	59.93 (0.04)	6.01 (0.04)	1.35 (0.05)	32.72 (0.12)
10	15%/140	0.1N/80	59.45 (0.04)	5.81 (0.00)	1.34 (0.01)	33.41 (0.04)
11	15%/160	0.1N/80	59.89 (0.14)	5.90 (0.01)	1.29 (0.00)	32.92 (0.15)

334

335   Table 3 also shows that increasing the ammonia concentration reduced the nitrogen

336 content of the isolated lignin. This observation shows that the limiting factor was some

337 structural feature of the lignin itself, not the ammonia concentration, and suggests that the

338 structural feature concerned was less abundant after pretreatment at high ammonia

339 concentrations. High ammonia concentrations also led to isolated lignins of higher molecular

340 mass, so it is possible that with reduced lignin depolymerisation, fewer reactive loci were

341 formed at sites of cleavage. There is an interesting parallel with the deliberate oxidative

342 ammonolysis of lignin (Lapierre et al., 1994; Meier et al., 1994; Potthast et al., 1996).

343 Homolytic cleavage of lignin can lead to oxidised structures such as 3,4-

344 dimethoxyacetophenone (Li and Gellerstedt, 2008) which reacts with ammonia to generate

345 the corresponding benzonitrile (Potthast et al., 1996).

346   Consistent with this idea, Capanema et al (2001) suggested that oxidative degradation

347 of lignin introduced additional hydrophilic functions at the same time as reducing the

348 molecular mass. Both of these effects would make the lignin more water-soluble. Fig. 4B and

349 C show that both molecular mass and recovery of the solubilised lignin were inversely

350 correlated with its nitrogen content, and both were greatest after pretreatment at high

351 ammonia concentrations. Depolymerisation, nitrogen incorporation and increased water-

352 solubility all seemed to be linked in the experiments described here, whether or not the N-

353 containing functional groups included benzonitriles.

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355 **4. Conclusion**
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358 In a profitable biorefinery, cost-effective biofuel production needs to be combined with

359 high-value co-products. For producing biofuel, the ammonia percolation pretreatment is not

360 seen as competitive with dilute acid or AFEX pretreatment, but added value from the lignin

361 generated should be taken into account. AFEX pretreatment, more often used for

362 graminaceous feedstocks, is also likely to preserve the lignin structure. However extracting

363 hardwood lignin after AFEX pretreatment may need harsher conditions that risk altering the

364 lignin structure.

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