Electronic Supplementary Information (ESI) for

“Carbon dioxide as a pH-switch anti-solvent for biomass fractionation and pre-treatment with aqueous hydroxide solutions”

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Materials and methods

All commercial reagents were purchased from Sigma-Aldrich (Castle Hill, Australia). Rice husks (from Oryza sativa) were kindly donated from SunRice Australia. All hazardous materials were handled and kept from the environment following UNSW guidelines (document HS321), and following the plans and policies outlined in UNSW’s Green Lab Environmental Compliance Program.

Pre-treatment of rice husks in 4.95 M NaOH (11.5 mol H₂O to 1 mol of OH⁻) solution

In a 50 mL polypropylene centrifuge tube, air-equilibrated rice husks (average moisture 7.56 ± 0.24 wt%) were soaked in 4.95M NaOH solutions at 2.5 wt% loading (1 g in 40 mL of NaOH solution). The tube was then placed in a shaker at room temperature for 3 days. The treated rice husks were then separated from the NaOH liquor using a 10 µm pore size nylon Millipore filter, rinsed with water (3 x 10mL) and transferred immediately for enzymatic hydrolysis. This yielded 280 ± 6.8 mg glucose per g of the original rice husk (relative to 0 mg per g for untreated rice husks), highlighting the effectiveness of the alkali pre-treatment.

Pre-treatment of rice husks in 4.95 M NaOH (11.5 mol H₂O to 1 mol of OH⁻) solution with MeOH as the antisolvent

The pre-treatment set up was the same as above. When the pre-treatment finished, methanol was added (40 mL) and the mixture was stirred and left standing for 20 minutes. The rice husks, together with any extra precipitate, were separated using 10 µm pore size nylon Millipore filter, rinsed with water (3
x 10 mL) and transferred immediately for enzymatic hydrolysis. This yielded only 19 ± 5 mg glucose per g of the original rice husk, highlighting the need to physically isolate the pre-treated rice husks prior to anti-solvent addition.

**Pre-treatment of rice husks in 4.95 M NaOH (11.5 mol H₂O to 1 mol of OH⁻) solution with CO₂ as the antisolvent**

The pre-treatment set up was the same as above. Small pieces of dry ice were added to a two-necked flask, which was fitted with a cannula. Upon sublimation of the dry ice at room temperature, CO₂ gas came out of the cannula and was bubbled through the pre-treatment mixture. The reaction was stopped when the pH of the solution stopped decreasing (pH typically 8.3).

The pre-treated rice husks, together with any extra precipitate, were isolated using a 10 µm pore size nylon Millipore filter, rinsed with water (3 x 10 mL) and transferred immediately for enzymatic hydrolysis. This yielded no detectable glucose (0 mg glucose per g of the original rice husk), highlighting the need to physically isolate the pre-treated rice husks prior to anti-solvent addition.

**Enzymatic hydrolysis of pre-treated rice husks**

Enzymatic hydrolysis of the various samples was performed by slightly modifying and scaling down the standard method of enzymatic hydrolysis for lignocellulosic biomass,¹ at a fixed loading of 20 mg wet pre-treated biomass per mL enzymatic broth. The slightly wet pre-treated rice husks were weighed and a portion equal to 200 mg of the original rice husks was transferred to a centrifuge tube (i.e. since the starting weight was 1 g rice husks, 1/5th of the mass of wet
pre-treated rice husks were transferred for enzymatic hydrolysis; e.g. after isolating 1.5 g of wet pre-treated rice husks, then 300 mg were taken for enzymatic hydrolysis). To this centrifuge tube, 9.728 ml of 50 mM sodium acetate buffer of pH 4.8, 400 µg of tetracycline hydrochloride (40 µl from 10 µg µL⁻¹ in 70 % ethanol solution), 300 µg of cycloheximide (30 µl from 10µg µL⁻¹ in water solution) and 25 FPU g⁻¹ cellulase (71.4 µl of as received Celluclast 1.5L, Novozymes Biologicals, Australia, from Trichoderma reesei) were added. The tubes were placed in an incubated shaker at a constant temperature of 50 °C (Maxi rotator, USA). The converted glucose concentration after 48 h was measured using a commercial glucose meter, Onetouch Ultra (Life First), for selective quantification of glucose. Each experiment was performed in duplicate or triplicate.

**Complete pre-treatment cycle of rice husks and recycle of KOH**

In a 50 mL polypropylene centrifuge tube, air-equilibrated rice husks (average moisture 7.56 ± 0.24 wt%) were soaked in 2.42 M KOH solutions at 2.5 wt% loading (1 g in 40 mL of KOH). The tube was then placed in a shaker at room temperature for 2 hours. When finished, the treated rice husks were separated from the KOH liquor using 10 µm pore size nylon Millipore filter, rinsed with water (3 x 10 mL) and transferred immediately for enzymatic hydrolysis. Subsequent detailed investigation of the washing step post-KOH treatment revealed that washing was essential (or the pH of the enzymatic broth was altered), but only one washing step was strictly required, rather than three.

The dark brown KOH liquor was bubbled with CO₂ until its pH stopped decreasing (end pH ~8.3). At this point, white flakes of silica precipitated out of
the solution and were removed using centrifugation (10 min at 6000 rpm / 4423 g). The silica was rinsed with water twice (and recovered from the suspension by centrifugation) to remove excess KHCO$_3$ residue before being dried in the oven at 110 °C.

To the remaining liquor was added Ca(OH)$_2$ (10.72 g, 1.5 mol eq. of original [OH$^-$]). Since the solubility of Ca(OH)$_2$ and CaCO$_3$ are much lower than that of KOH (<2 g L$^{-1}$, <0.02 g L$^{-1}$ and >1,000 g L$^{-1}$, respectively), the calcium salts precipitated and were removed using a Whatman filter paper (pore size 11 µm). The regenerated KOH solution was used in further rice husk treatment.

Dissolution of the recovered calcium salt ‘cake’ in a suitable aqueous acid solution (such as HCl) allowed isolation of acid-insoluble lignin, and direct observation of acid-soluble lignin in solution.

**Acid washing, TEM and ICP-MS analysis of the CO$_2$-isolated silica**

The isolated silica was washed with HCl by adding 10 mL of 1 M aqueous HCl to 0.1 g silica in a 15 mL polypropylene centrifuge tube, and placing it in a shaker at room temperature overnight. The silica was isolated by centrifugation, and rinsed with water then centrifugation (3 x 10 mL).

As-isolated and acid-washed silica was analysed by dissolving 100 mg L$^{-1}$ of the sample in 10 mM aqueous tetramethylammonium hydroxide (570 mg L$^{-1}$, ca. 6.25 mM) in a 15 mL polypropylene centrifuge tube. Then 1 mL of this sample was made up to 10 mL with water in a separate tube. Both diluted and undiluted samples were diluted 100-fold just prior to analysis at the Mark Wainwright
Analytical Centre, UNSW, using a Nexion ICP-MS (Perkin Elmer), looking for the presence of 72 elements.

TEM characterisation of the acid-washed silica was performed on a Phillips CM200 with a Schottky field emission gun operated at 200 keV. TEM specimen was prepared by suspending the silica sample in water and dropping onto carbon-coated copper grids, which were then allowed to evaporate under ambient conditions.

**Thermogravimetric analysis (TGA)**

Approximately 10-15 mg sample was taken in an alumina crucible, and the heating rate was maintained at 10 °C min$^{-1}$ (from 35 °C to 1,000°C) in the presence of continuous flow of 30 mL min$^{-1}$ air. The steady weight at 1,000 °C was used to determine the silica content as a wt% value; the ash-content of untreated rice husks is known to be at least 94 wt% silica.$^{2,3}$

**Lignin extraction and determination**

Both the kinetics of lignin extraction and quantity of alkali-soluble lignin was monitored using a UV-Visible spectrophotomer (Varian Cary 50 Bio); 0.1 mL samples were taken at designated periods, diluted 30-fold with 2.4 M KOH (up to 3 mL), and measured in a 10 mm pathlength ES-quartz cuvette.

The lignin remaining in the pre-treated rice husks was determined following the NREL procedure.$^4$ Namely, 50 mg of the recovered rice husk was transferred to a pressure vessel. To this, 0.5 mL of 72% H$_2$SO$_4$ was added and the vessel placed in 30°C water bath for 1 hour, with frequent agitation. Then 14 mL
of water was added to dilute the solution to 4% H$_2$SO$_4$. The pressure vessel was then placed in oven at 121°C for 1 hour. The resulting mixture was filtered using 10 µm nylon Millipore filter, and the acid-insoluble lignin (AIL) was collected. The acid-soluble lignin (ASL) was quantified spectrophotometrically.$^4$

Lignin present in the CaCO$_3$ “cakes” was liberated by adding 1M HCl solution until all the CaCO$_3$ dissolved and no more CO$_2$ was released. The colour of the solution was pale yellow and observation under UV-Vis confirmed the presence of soluble lignin. The solid was collected and determined to be acid-insoluble lignin according to the NREL procedure.$^4$

References

Figure S1: Combined mass balance and flow chart, highlighting the individual steps taken. With good separation of the solids and liquids, the majority of the water and KOH was recovered and recycled. However, the rice husk-washing step prior to enzymatic hydrolysis (ca. 30 mL) represents a significant quantity of water that was not recycled (in this study).

The combined Ca(OH)$_x$(CO$_3$)$_y$ and lignin will also be challenging to effectively utilise. Acidification of the solid with acids such as HCl present significant corrosion issues, will only recover part of the lignin, and regenerate a corrosive, Ca-containing waste. Thermal treatment (or calcination) will recover
CO2 for re-use, and the exothermic combustion of lignin will also assist the actual calcination process. However, this would destroy the lignin in the process. Anaerobic and/or catalysed thermal treatment could first generate a range of reactive gases from the lignin (e.g. syngas), followed by aerobic higher temperature calcination to remove residual char and generate CaO, evolving only CO₂. All processes have their advantages and disadvantages; a novel process would likely be required to achieve effective utilisation of all of the materials.
Figure S2: Glucose yield after 48 h enzymatic hydrolysis, as a function of pre-treatment time (2.5 wt% rice husks in 2.4 M KOH). Error bars are twice the range from duplicate measurements. Top displays the full data, and bottom displays the same data with a logarithmic x-axis, to highlight the glucose values at shorter pre-treatment time.
Figure S3: Photographs of (a) the isolated ‘cakes’ of off-white Ca(OH)$_2$/CaCO$_3$ after step 3, and (b) a solution of some of this material in 1 M HCl, highlighting the distinctive presence of acid-soluble lignin and acid-insoluble lignin.
**Figure S4:** Absorbance (at the $\lambda_{\text{max}}$ between 326 and 340 nm) vs pre-treatment time for 2.5 wt% rice husks in 2.4 M KOH, demonstrating increasing lignin content in the pre-treatment media with extended time. Samples were diluted 30-fold with ultrapure water, before being analysed in a 10 mm quartz cuvette.