Microwave subcritical water pre-treatment and enzymatic hydrolysis of Geographical Identification (GI) tag Indian black rice (*Chakhao Poireiton*) straw for fermentable sugar production

Kamaljit Moirangthem *[a, b], Abdelrahman S. Zaky [c], Gregory A. Tucker [a]

[a] School of Biosciences, University of Nottingham, Loughborough LE12 5RD, UK

[b] Department of Engineering, Aarhus University, Aarhus N 8200, Denmark

[c] School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FF, UK

* Corresponding author: kamaljit.moirangthem@hotmail.com

Abstract

Rice straw is an important feedstock for second-generation biorefineries, the majority being conventional white rice. This research investigated the potential of the straw of black rice *Chakhao Poireiton*, a rice variety of increasing importance, for biorefining. Pre-treatment of black rice straw was carried out in a pressurized microwave reactor and free sugar release was measured following digestion with Cellic® CTec3. This was compared to white rice straw. Pre-treatment (100 - 200° C for 5 min) did not drastically impact the sugar composition but brought about an enhanced release of glucose after enzymatic hydrolysis from 25 to 50% (black rice) and from 26 to 55% (white rice). For xylose digestibility, the increase was around seven-fold for black rice (8 to 57%) and five-fold for white rice (13 to 64%). This improvement in digestibility of the straw samples could have come from modification in the lignocellulose structural features making the polysaccharides more susceptible to hydrolysis. Black rice could be a suitable feedstock for a combined biorefinery – food and nutraceuticals from grain (much higher than white rice) and the straw for fermentable sugars comparable to white rice straw for biofuels.

Keywords

Black rice; *Chakhao Poireiton*; Straw; Microwave Hydrothermal Pre-treatment; Enzymatic Saccharification; Biorefining.

Introduction

As we strive towards self-reliance in terms of energy requirements, bioenergy is becoming more attractive, especially, for its positive impact on the environment [1-4]. The residual straw from rice, which is the main staple crop for many Asian countries and the third most grown cereal in the world, with a global production of over 748 million tons [5], is currently a major biomass for the production of bioenergy. However, research for other unconventional feedstock is necessary to meet the demand for biofuel production. Rice can be differentiated based on its colours such as brown, white (polished brown rice), red, purple or black, and more importantly based on its geographical origin. White rice is the most widely consumed rice, however, pigmented rice such as black rice has higher overall health-beneficial components [6,7] (Table 1). Geographical origin or place of production influences the characteristics or quality of food, and ultimately the purchasing decision of consumers [8]. This is why certain food products are given Geographical Identification (GI). Black rice *Chakhao Poireiton* from Manipur (India) which was awarded a GI tag in 2020 [9]. Figure 1 shows the production process for rice. The unpolished grain and the husk are obtained from paddy rice (d) following industrial milling. For conventional rice, the unpolished grain is what is called brown rice, which gets polished to give white rice. However, black rice is usually not polished to maintain its nutritious bran and colour characteristics [10].

Figure 1 Production of black rice (*Chakhao Poireiton*) in Manipur, India. a) Three-month-old Black Rice field in Manipur; b) Mature crop harvested and sundried in the field; c) Sundried crop (pile on left) threshed to get grain (collected behind the machine) and straw (pile on right); d) Black rice paddy; e) Black rice grain de-husked (or brown rice); f) Black rice husk; g) Black rice paddy bag and straw pile in the field; h) Black rice straw; i) Burning of straw in the field. (e) and (f) are obtained after processing the paddy (d) in a rice mill where it undergoes dehusking.

Table 1 Nutritional and phytochemical profiles of black, red and white rice

Phytochemicals[a]	**Black rice**	**Red rice**	**White rice[+]**
Total Phytochemical content (µg/g)	4,351	297	32
Anthocyanins (%)[1]	80	1	0
Flavone and flavonols (%)[1]	11	7	75
ɣ oryzanol (%)[1]	1	27	25
Flavan-3-ols (%)[1]	8	65	0
Nutritional component[b]	**Black rice**	**Red rice**	**White rice[+]**
Carbohydrate (%)[2]	73	75	75
Ash (%)[2]	1.56	1.36	1.26
Protein (%)[2]	10.15	7.86	10.12
Fat (%)[2]	2.85	2.48	2.92
Water (%)[2]	13.01	13.32	11.08

[a] represents data obtained from Pereira-Caro et al [11]; [b] represents data obtained from Fatchiyah et al [12]. [1] is expressed as a percentage of the total phytochemical content. [2] is expressed as a percentage of the grain biomass.

Black rice is generally cultivated in Southeast Asian countries [13], with China accounting for 62% of the global black rice production followed by Sri Lanka, Indonesia, India, and the Philippines [14]. Owing to their health beneficial properties, interest in black rice consumption has been rapidly increasing [15]. In India, along with the awarding of GI, recent developments such as 100% *Chakhao Poireiton* beer that showed enhanced oxidative stability [10] and the search for food with bioactive components have renewed the interest in this aromatic black rice. Straw from black rice (*Chakhao Poireiton*) has also been reported as a source of bioactive compounds such as anthocyanin with anticancer and antioxidant properties similar to anthocyanin from the black rice grain [16]. The residual straw may also represent a source of biomass for bioenergy.

In India a surplus amount (44.5 Mt) of rice straw is burned annually, contributing to around 0.05% of greenhouse gas emissions in India [17]. Hence, economically viable, socially acceptable and eco-friendly solutions for the alternative uses of rice straw are needed [18]. Additionally, with Asia, led by China and India, expected to drive nearly 50% (416 quadrillions Btu) of the global energy demand in 2050 [19], advancements in low carbon energy such as bioenergy will need to have a stronger region-centric approach. This makes rice straw an attractive lignocellulosic feedstock for biorefinery such as bio-ethanol production in India and China. Currently, the production cost for bioethanol from rice straw is (23–26 $/GJ) which is comparable to that of fossil fuels (20–30 $/GJ) [20]. However, by improving the pre-treatment, enzyme hydrolysis and productive utilization of residual biomass, the bioethanol production process cost (1.19 $/L) could be lowered to 0.45 $/L as shown in Vietnam (plant size, 200 ML per year) [21].

One pre-treatment method is the use of microwave heating which can be conducted at atmospheric pressure or under high pressure. High-pressure microwave pre-treatment takes place in pressurised reactors at temperatures ranging from 150 to 250° C [22]. At elevated temperature (>160° C) microwave pre-treatment is in essence, an acid-catalysed reaction as acidity increases with increasing temperature during the treatment [23]. In rice straw, microwave heating disrupts the silica waxy surface, breaks down the lignin–hemicellulose complex, partially removing silicon and lignin and thus improving enzymatic hydrolysis [24]. When used with water it also acts as a hydrothermal treatment dissolving part of the lignin and hemicellulose of the rice straw [25]. Microwave pre-treatment is reported to have a greater impact on structural features of the lignocellulose, such as reducing the cellulose crystallinity and/or decreasing the size of cellulose crystals thus improving its enzymatic hydrolysis, than on the composition of the biomass [26,27]. Microwave pre-treatment has been applied to conventional rice straw with varying efficiencies. This includes glucose yields of 37.8% (680 W, 24 min, water) [24]; glucose yields of 11.2% (100° C, 5 min, solid acid catalyst) [28]; total sugar yields of 71.41% (230 W, 5 min, acetic acid) and 80.08% (230 W, 5 min, propionic acid) [29]; glucose yields of 65% (700 W, 30 min, 1 % NaOH) [30]; total sugar yields of 10 - 19% (160° C, 15 min, 1% H_2SO_4), 18-40% (160° C, 15 min, 1% NaOH) and 9-14% (160° C, 15 min, water) depending on rice variety [31]. Microwave hydrothermal extraction at low severity (100° C, 5 min, water) has also been used to extract bioactive compounds from black rice *Chakhao Poireiton* straw [16].

The majority of rice grown worldwide is white rice and as such has been the focus of previous studies into exploitation for biorefining. To the best of our knowledge, this is the first and foremost study on microwave subcritical water pre-treatment of black rice straw for sugar production. The objective was to evaluate the sugar composition and its saccharification following microwave pre-treatment and enzymatic hydrolysis by Cellic® CTec3. The released renewable sugars could be used for biofuels and high-value chemical production.

Materials and Methods

Plant Material

The black rice (*Chakhao poireiton*) straw was obtained from a rice field in Imphal (India) located at 24° 53′ 27.762′′ N and 94° 6′ 26.800′′ E with an elevation of 799.51 m. The straw was air-dried and milled to 2 mm mesh size using a Pulverisette 19 knife mill (FRITSCH GmbH, Germany) and stored at 4° C in bags with airtight seals. White *Brojen* rice straw, from a neighbouring field in Imphal (India) located at 24° 53′ 27.768′′ N and 94° 6′ 16.610′′ E with an elevation of 798.34 m, was used for comparison and was treated as above.

Microwave pre-treatment

Microwave pre-treatment was conducted in a pressurised Monowave 300 microwave generator (Anton Paar, Germany) with 850 W maximum magnetron output power [32]. Accurate temperature control was maintained using both external infrared and internal ruby based fibre optic thermometers. Output power and pressure were not controllable, but controlled heating to a given set temperature in a given time was achieved by choosing the inbuilt "as-fast-as-possible" mode followed by rapid cooling with a flow of nitrogen. 0.5 g of plant material was added to 10 mL of water, steeped at room temperature for 2 min and then heated under pressure for 5 min at six different temperatures 100° C, 120° C, 140° C, 160° C, 180° C and 200° C. The samples were then centrifuged at 4472 x G for 10 min. The supernatants were filtered through 0.22 μm syringe filters, the pH recorded and were stored at -20° C for further analysis. The residues were air-dried for 72 h, weighed and were stored at 4° C for further analysis.

Sugar composition using High-Performance Anion-Exchange Chromatography (HPAEC)

The sugar composition of raw biomass and the pre-treated residues was determined by Saeman hydrolysis [33]. 1 mL of 12 M H_2SO_4 was added to 30 mg of the sample at 37° C for 1 h. This was followed by the addition of 11 mL of MilliQ water (reducing the molarity to 1 M) and incubation at 100° C for 2 h. The hydrolysate was analysed for monomer sugars by high-performance anion-exchange chromatography (HPAEC) on a Dionex ICS-3000 comprised of a high-pressure GD 50 gradient pump, a guard column (Carbopac PA1, 4 mm × 50 mm), an analytical column (Carbopac PA20, 4 mm × 250 mm) and a pulsed amperometric detector (PAD). 10 mM NaOH was used as the mobile phase and the column was flushed with 200 mM NaOH between runs. The injection volume was 10 µL and all chromatographic analyses were carried out at 30° C with a flow rate of 0.5 mL min^{-1}. Samples and standards were diluted 1:100 in 10 mM NaOH and centrifuged at 4472 x G for 10 min before loading to Dionex vials. Sugar standards ranged from 0.250 to 2 g L^{-1} of arabinose, galactose, glucose and xylose.

Digestibility Assay

Air-dried pre-treated biomass residue was hydrolysed using Cellic® CTec3 (kindly provided by Novozyme A/S, Denmark). This was carried out using a slight modification to the method described by the National Research Energy Laboratory (NREL) [34]. The enzyme solution was made up using 5 mL of Cellic® CTec3 in 1 L of 50 mM sodium citrate buffer, pH 3.76. 40 mL of the enzyme solution was added to 0.2 g of pre-treated residue and incubated at 50° C, 150 rpm for 72 h in a shaking incubator (MaxQ 4358 shaking incubator, Thermo Scientific, UK). Aliquots taken at 6 different time points; 0 h, 0.5 h, 3 h, 24 h, 48 h and 72 h were assessed for monomer sugar composition using HPAEC. The sugar concentration present in the enzyme solution was also analysed and subtracted to allow accurate calculation of the percentage of glucose released.

Statistical analysis

Statistical analysis of variance (ANOVA) and standard deviations were calculated using Prism 7.0b for Mac (GraphPad Software, Inc) and Microsoft Excel 2016. All experiments were carried out in triplicates and the results are mean ± standard deviations. Differences were considered significant at $p < 0.05$, with * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

Results and Discussion

Compositional analysis of straw

The results of the sugar composition of the two straw samples are presented in Figure 2. Overall black rice straw had a higher percentage of sugars than white straw. The glucose content of the straw was 40.2 ± 1.4 and 30.7 ± 1% for black and white rice, respectively. This being statistically significant (p = 0.0003). Whilst the values for xylose were 20.4 ± 1 and 16.7 ± 1.6% for black and white rice, respectively, again this was statistically significant (p = 0.0230). These values were in the range of the reported sugar composition of rice straw which were 36-42% for glucose and 21-26% for xylose [35]. The other two sugars measured were, as expected, only minor components of the straw. The differences between galactose of 1.4 ± 0.2 and 1.1 ± 0.1% (p = 0.0377) were statistically significant while that for arabinose of 4.0 ± 0.4 and 3.3 ± 0.3% (p = 0.0728) was not significant, for the black and white rice, respectively. The ratios of the sugars in each case are very similar and this may suggest that black rice straw possesses a similar lignocellulosic make-up to that of white rice straw.

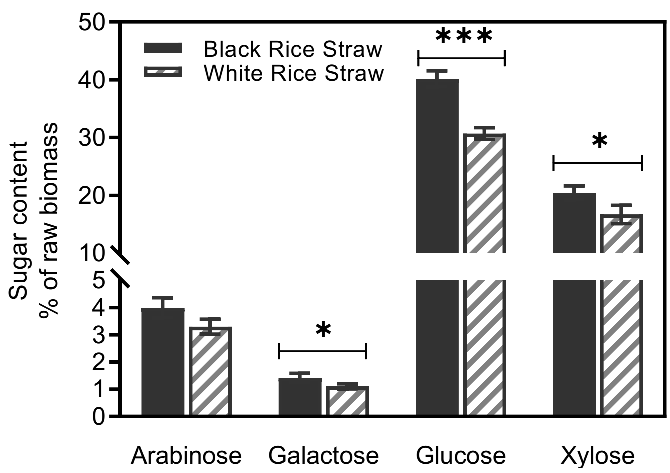

Figure 2 Monomeric sugars composition of raw black rice straw compared against 'normal' white rice straw. Results are expressed as percentage of biomass (mean ± SD, n = 3). * = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001.

Microwave pre-treatment

Straw was subjected to pre-treatments at 100 - 200° C for 5 min. A short pre-treatment time was used since it has been shown that the effect of temperature is more important than residence time for reducing recalcitrance [36] and also avoids drastic degradation of the hydrolysate. Additionally, a previous study using autohydrolysis of rice straw by compressed liquid hot water at various temperatures (160, 180 and 200° C) and time (5, 10 and 15 min), found that the maximum glucose yield of 42.5% (percentage of pretreated biomass) was achieved at 200° C for 5 min [37].

The monomeric compositional changes in the pre-treated liquor are shown in Table 2. Increasing severity of the pre-treatment was accompanied by an increase in hemicellulosic derived arabinose in the pretreated liquor in both straw samples. At 200° C, galactose and xylose were detected, and the arabinose levels were double those found at 180° C. Glucose was detectable at 100° C in both straw samples and levels seemed to decline with increasing severity. This may represent free glucose or glucose derived from residual starch but in both straw samples, the glucose levels in the liquor at 200° C were very low. These values were in the range of the reported sugar composition of microwave pretreated rice straw (at 140° C - 210° C for 2.5 min to 160 min) which were up to 0.2 % for glucose, 0.5 % for xylose, 0.35 % for galactose and 0.4 % for arabinose [38].

Table 2 Changes in the composition of pre-treated rice straw liquor with different pre-treatment regime

Temp (° C) 5 min	Black Rice				White Rice			
	Sugars in pre-treated liquor (% of original biomass)				Sugars in pre-treated liquor (% of original biomass)			
	Ara	**Gal**	**Glu**	**Xyl**	**Ara**	**Gal**	**Glu**	**Xyl**
100	0	0	1.82 ± 0.06	0	0	0	0.14 ± 0.01	0
120	0	0	1.74 ± 0.10	0	0	0	0.11 ± 0.01	0
140	0.03 ± 0	0	1.77 ± 0.08	0	0	0	0.12 ± 0.01	0

160	0.08 ± 0.01	0	1.75 ± 0.07	0	0.04 ± 0	0	0.11 ± 0.01	0
180	0.40 ± 0.06	0	1.57 ± 0.05	0.01 ± 0.02	0.30 ± 0.01	0	0.07 ± 0.01	0
200	0.96 ± 0.03	0.08 ±0.01	1.00 ± 0.08	0.21 ± 0.09	0.6 ± 0.01	0.06 ±0	0.04 ± 0	0.13 ± 0

Results are expressed as percentage of biomass (mean ± SD, n =3). Ara = arabinose; Gal = galactose; Glu = glucose; Xyl = xylose

The pH of the hydrolysate liquor was recorded and the solid residues were assessed for recovery and sugar composition as shown in Table 3. Overall, with an increase in severity of treatment, we observed a decrease in both the pH of the hydrolysate liquor and recovered weight of the solid residue along with subtle changes to the sugar composition of the recovered residue for both straws (black and white). In both straws pre-treatment up to 180° C does not seem to have a drastic impact on the composition of the pre-treated residue. However, between 180° C and 200° C there was a sudden decrease in the weight of the pre-treated biomass 80% at 180° C - 67% at 200° C for black rice and 84 at 180° C - 73% at 200° C for the white rice. This compares with the changes seen in the sugar content of the pretreated liquor at 200° C (Table 2). This could be due to a combination of solubilisation of cell wall components (predominantly hemicelluloses and salts), hydrolysis and volatilisation of some wall components [39] as the severity was raised. The primary effect of the hydrothermal pre-treatment on biomass composition of both straw samples seems to have been partial but, incremental removal of hemicelluloses.

Table 3 Changes in rice straw residues composition associated with different pre-treatment regimes

Temp. (° C, 5 min)	Black Rice						White Rice					
	Solid recovery (wt. %)	pH of hydrolysate	**Sugar in pre-treated residue** (% of residue biomass)				Solid recovery (wt. %)	pH of hydrolysate	**Sugar in pre-treated residue** (% of residue biomass)			
			Ara	Gal	Glu	Xyl			Ara	Gal	Glu	Xyl
100	86.4 ±1	6.1 ±0.01	3.8 ±0.2	1.2 ±0.1	43.7 ±1.4	33.9 ±1.0	90.3 ±0	6.7 ±0.01	4.2 ±0.1	1.4 ±0.1	40.4 ±1.9	22.6 ±0.8
120	84.7 ±0	6.0 ±0.01	3.8 ±0.2	1.2 ±0.1	42.1 ±1.9	32.8 ±1.3	89.3 ±1	6.5 ±0.10	4.1 ±0.3	1.4 ±0.1	40.3 ±2.3	22.3 ±1.5
140	83.5 ±1	5.7 ±0.03	4.0 ±0.2	1.2 ±0.0	43.6 ±1.4	34.2 ±0.8	88.3 ±1	6.2 ±0.06	3.8 ±0.4	1.3 ±0.1	38.8 ±1.8	21.0 ±1.0

160	82.5 ±1	5.2 ±0.03	3.8 ±0.2	1.1 ±0.1	43.1 ±2.1	23.4 ±1.2	88.9 ±0	5.7 ±0.03	3.8 ±0.3	1.3 ±0.1	39.3 ±2.7	21.3 ±1.2
180	80.4 ±1	4.7 ±0.02	3.3 ±0.2	1.0 ±0.1	42.8 ±1.9	23.1 ±1.0	84.1 ±1	5.1 ±0.03	3.4 ±0.1	1.1 ±0.0	40.0 ±1.3	21.3 ±1.0
200	67.5 ±2	4.1 ±0.02	2.0 ±0.4	0.3 ±0.4	46.0 ±2.6	18.4 ±2.5	73.1 ±1	4.6 ±0.04	2.0 ±0.1	0.7 ±0.0	43.0 ±2.7	17.2 ±0.8

Results are expressed as the percentage of biomass (mean ± SD, n =3). Ara: arabinose; Gal: galactose; Glu: glucose; Xyl: xylose

Enzymatic saccharification

Glucose and xylose released after 72 h of hydrolysis of the pre-treated biomass are shown in Figure 3. Digestibility was expressed as the percentage of available sugar in the pre-treated biomass released after 72 h. Associated with the drastic loss of weight between treatments at 180° C and 200° C, the residues showed reduced recalcitrance from 180° C as demonstrated by an increase in digestibility. For both straw samples, pre-treatment from 100° C to 200° C for 5 min resulted in a two-fold increase in glucose digestibility; 24.9 ± 0.35 to 49.6 ±1.4 and 26.81 ±1.08 to 55.1 ± 2.4% for black and white rice straw, respectively. For xylose digestibility, the increase was around seven-fold for black rice (7.6 ± 1.3 to 57.0 ± 4.3%), while the increase was around five-fold for white rice (13.2 ± 0.1 to 64.1 ± 3.1%). Galactose was detectable only from black rice straw pre-treatment at 180° C and 200° C. This could be due to the low amounts of galactose in the pre-treated residue. These differences in digestibility between the two straw samples under the same pre-treatment and enzymatic hydrolysis further highlights the importance of rice variety on digestibility [31,40]. Depending on rice variety, the digestibility of rice straw pre-treated with water at 160° C for 15 min has been shown to range from 9 - 14% [31].

The highest enzymatic digestibility was obtained after pre-treatment at 200° C, with glucose digestibility of 49.6 ± 1.4% and 55.1 ± 2.4% for black and white rice straws, respectively ($p = 0.03$). These values equate to a glucose yield of around 147 and 173 mg g^{-1} of original straw for black and white straw, respectively. While the xylose yield equates to around 67 and 80.2 mg g^{-1} of original straw for black and white straw, respectively. The theoretical yield of bioethanol from black rice straw would thus be about 110 g kg^{-1} straw, based on the theoretical ethanol yield per gram of biomass [41]. Hence, black rice straw is a lignocellulosic biorefinery feedstock comparable to white rice straw. These values are also similar to a previously reported glucose saccharification efficiency of 45% using steam explosion (220° C for 10 min) for rice straw [39].

The increase in digestibility could be due to a multitude of changes in the lignocellulose matrix brought about by the pre-treatment. A) Reduction in cellulose crystallinity and/or decrease in the size of cellulose crystals [26,27]. B) Alterations or changes in the surface area of pre-treated rice biomass [42]. C) Removal of barriers to enzymatic hydrolysis such as hemicellulose and lignin [43]. In rice straw, microwave heating has been shown to disrupt the silica waxy surface, break down the lignin–hemicellulose complex, partially remove silicon and lignin thus improving enzymatic hydrolysis [24]. D) Creation of an explosion effect in the biomass matrix due to microwave's selective heating of the more polar parts of the lignocellulosic feedstock which facilitates the degradation of recalcitrant structures more efficiently [44]. This has been reported to provide 12 times higher sugar yield within half the time compared to conventional heating [45].

The observed glucose yields for both the black and white rice straws are comparable to previous reports of white straw under similar treatment conditions. For example, a yield of 42.5% was obtained with compressed liquid hot water pre-treatment at 200° C for 5 min [37]; 46.6% after a two-stage 0.5% H_2SO_4 at 15 bar for 10 min and 30 bar for 3 min pre-treatment [46]; 57.7% after a compressed liquid hot water pre-treatment at 180° C for 10 min with 0.25% Oxalic acid [42]; 31.7% after a microwave pre-treatment at 680 W for 24 min with water [24] and 45% after steam explosion pre-treatment at 220° C for 10 min [39].

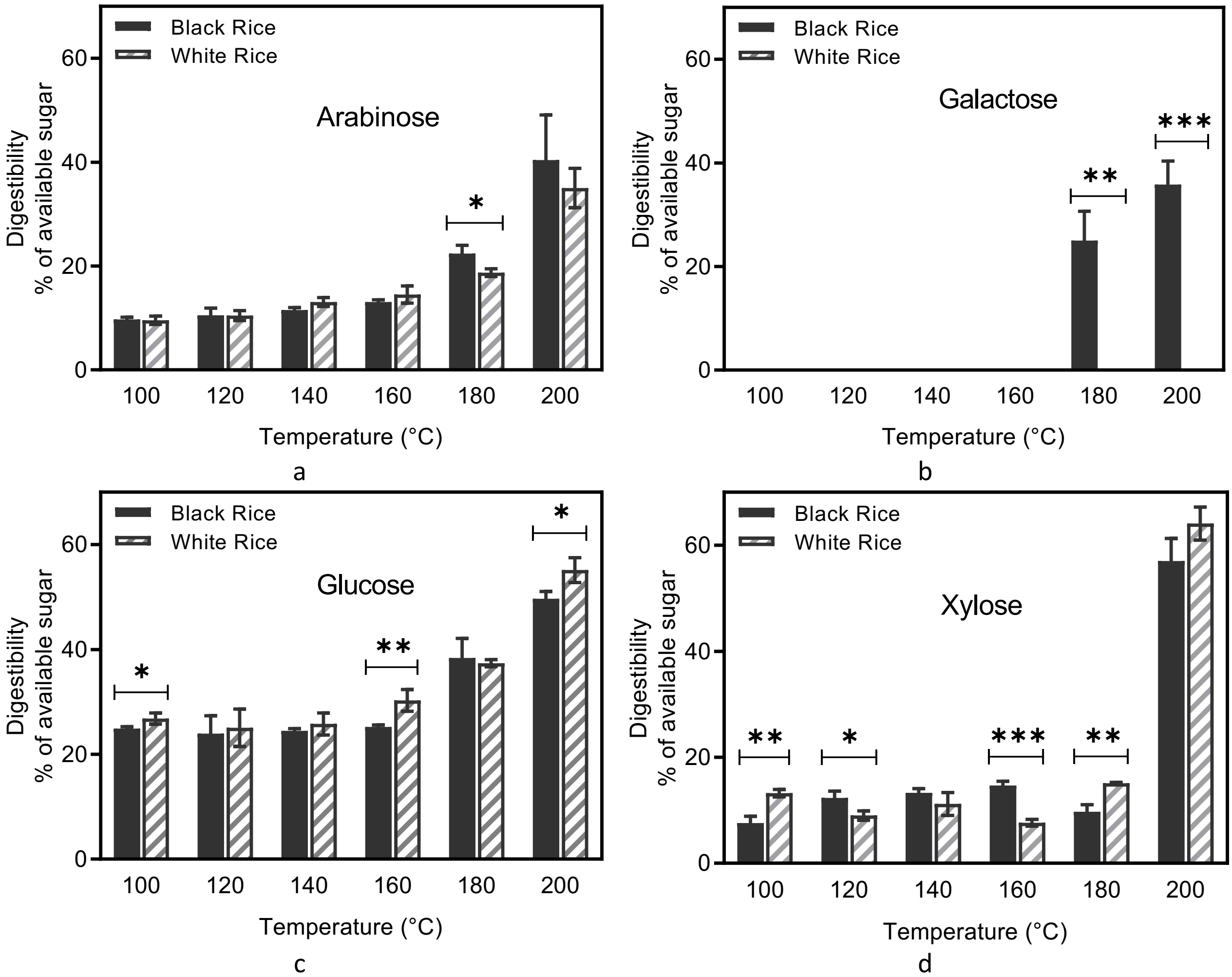

Figure 3 Monomeric sugar release from pre-treated black rice and white rice straw residues after 72 h of enzymatic saccharification (mean ± SD, n = 3). a) Arabinose; b) Galactose; c) Glucose and d) Xylose digestibility expressed as a percentage of available sugar in the pre-treated biomass. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

However, these observed glucose yields for both black and white rice straws were lower than reported using other pre-treatments. The glucose yields for white rice straw include 85% after pre-treatment with 0.8% H_2SO_4 for 4 min at 190° C [47]; 76.3% after pre-treatment with 0.015% HCl at 200° C for 60 min and 85% after a hot-compressed water pre-treatment of 180° C for 30 min [48]; 95.4% after Glycerol-$AlCl_3$ pre-treatment at 146.8° C for 20 min [49]; 94% after 0.055 mol/L AlCl3 pre-treatment at 150° C for 0.5 h [50] and 90.2% after a 3 h pre-treatment at 100° C and two-stage deep eutectic solvent pre-treatment [51]. Even though these reported yields were higher, these treatments were often conducted at higher severity or used chemicals that would involve neutralisation or other additional steps downstream before fermentation.

The impact of microwave pre-treatment on black rice husk was also explored (Table 4). Black rice husk showed good sugar composition in both raw (glucose 33.7% ± 1.1, expressed as a percentage of original biomass) and pre-treated states (glucose 41.3% ±3.4, expressed as a percentage of pre-treated residual biomass). However, the digestibility of the husk following pre-treatment at 200° C for 5 min was only 15.5% ± 1.6, expressed as a percentage of available sugars. This observed value for black rice husk is similar to that reported for white rice husk (16.3% glucose digestibility after microwave pre-treatment at 210° C for 2 h) [52]. This high recalcitrance in the husk may be related to the fact that white rice husk has almost twice the lignin content of rice straw [52].

Table 4 Sugar composition of raw black rice husk, its sugar composition and digestibility following pre-treatment at 200° C for 5 min

	Arabinose	Galactose	Glucose	Xylose
Sugar composition of raw husk (% of original biomass)	2.31 ± 0.04	0.94 ± 0.08	33.69 ± 1.06	16.15 ± 0.5
Sugar composition of pre-treated residue (% of pre-treated biomass) Wt. of recovered residue 76 % ± 1	1.05 ± 0.10	0.00 ± 0.00	41.25 ± 3.37	14.30 ± 1.34
Sugar composition of pre-treated liquor (% of pre-treated biomass) pH of pre-treated liquor 4.4 ±0.05	0.4 ± 0.01	0.05 ± 0	0.13 ± 0.01	0.08 ± 0.06
Digestibility (% of available sugar)	0.00 ± 0.00	0.00 ± 0.00	15.48 ± 1.59	11.93 ± 0.46

Results are expressed as the percentage of biomass (mean ± SD, n =3). Raw sugar composition is expressed as a percentage of the original biomass. Sugar in the pre-treated biomass is expressed as a percentage of the pre-treated biomass. Digestibility of the pre-treated biomass is expressed as a percentage of available sugar in the pre-treated biomass measured via HPAEC.

The current study shows the potential of black rice as a source of lignocellulosic biomass suitable for second-generation biofuels by generating fermentable sugars comparable to white rice straw. The search for economical biofuels is crucial as they will not only contribute to reducing emission in road and aviation but also in marine and inland transport [53]. Fermentation and recovery of bioethanol were not attempted in this study. However, the potential theoretical yield of bioethanol from black rice straw from this study would be about 110 g kg^{-1} straw. However, further studies for pre-treatment and hydrolysis optimisation are required to further enhance digestibility and sugar release. This could include optimising the microwave pre-treatment with acid and alkali [31] or application of green pre-treatment method such as ionic liquids to further improve its yield. However, these higher severity processes would also encompass higher cost and challenges downstream through the potential production of fermentation inhibitors. The production cost for second-generation biofuels is two to three times more expensive than petroleum fuels on an energy equivalent basis [54]. With the black rice, the economics of its straw biorefinery could potentially be balanced by its high-value grain. Valorising the learnings from white rice straw, it is reasonable to envisage that the different components present in the black rice straw such as cellulose, hemicelluloses, lignin and extractives would also need to be capitalised on to make the process economically viable.

Plants like black rice could help us move from a food vs fuel debate to a combined food, health and fuel solution, within the limits of our planetary boundary. A combined biorefinery where black rice grain provides food with health-promoting compounds, while the straw providing fermentable sugars for the production of bioproducts such as biofuels could be further explored on a small scale. Similar concepts are available for wheat - improving wheat to give better grain yield while also possessing straw suitable for biorefinery [55,56]. White rice straw has been reported to contain a variety of bioactive compounds such as water-soluble phenolic acids (ferulic, p-coumaric and protocatechuic) and flavonoids [57,58]. Through response surface methodology (RSM) the microwave subcritical water pre-treatment process could be optimised to develop a two-step process where these bioactive compounds such as anthocyanin [17,70] could be extracted at a lower severity from the black rice straw followed by pre-treatment at higher severity for fermentable sugars. Black rice grain is popular for its nutraceutical properties and its production could further be fuelled by the renewed interest in health-promoting foods and the award of GI tag. With the growing interest in the link between health and food, exploring the biorefinery potential of by-products of plants such as black rice would be a timely study.

In this research, we have explored the potential for producing fermentable sugars from black rice straw. These sugars could be converted to various products such as liquid fuels, furfurals and hydroxymethylfurfurals (HMFs). However, within this biochemical route, the straw's potential for producing biogas and aromatic products could also be explored. Then, there is the thermochemical route, which could be more suitable for black rice husk. Under this route, both the straw and the husk could be explored as a feedstock for producing synthetic gas, bio-oil and heat and electricity [59]. Exploring the biorefinery potential of black rice straw would be an important and timely step to develop an economically feasible biorefinery concept. Reduction in greenhouse gas (GHG) from this concept could be delivered in three ways – i) reducing our reliance on fossil fuels by providing biofuels, ii) reducing the burning of rice straws by diverting them to the biorefinery, and iii) reutilising the by-products of Agri-Food Industry which is also a major contributor of GHGs.

Conclusion

In this study, a lab-scale microwave subcritical water pretreatment experiment followed by enzymatic hydrolysis was conducted on the GI tag Indian black rice (*Chakhao Poireiton*) straw obtained from Imphal, Manipur and compared white rice straw also obtained from Imphal. Following enzyme hydrolysis, the black rice straw yielded fermentable sugars comparable to, or slightly higher than that obtained from white rice straw. The sugar content of black rice straw was higher than white rice straw for all measured sugars (arabinose, galactose, glucose and xylose). This indicates the potential of black rice straw as an additional biomass substrate for the production of biofuels and biobased chemicals. However, this biomass required a pre-treatment above 160° C for 5 min to obtain a significant impact on the subsequent enzyme hydrolysis, with a glucose digestibility of 49.6 ±1.4% at 200° C for 5 min compared to 55.1 ± 2.4% observed with white rice straw. This indicates the need for further study into the optimisation of pre-treatment technologies to improve the digestibility of the black rice straw. Optimisation studies on the present process or alternative routes such as thermochemical processes are needed for efficient utilisation of this feedstock which would otherwise be burnt on the field creating environmental and health issues, and loss of biological resource. With the growing interest in the link between health and food, exploring the biorefinery potential of plants residues, such as black rice, would be a timely study.

Acknowledgments

We would like to thank the University of Nottingham's Bioenergy and Brewing Sciences (BABS) technical team for the use of analytical facilities such as HPLC and the Microwave reactor. Dr Kamaljit Moirangthem is grateful to Dr Ranjana Rajkumari, Dhanamanjuri University (India) for providing the black rice samples; Dr Emily Fong, University of Nottingham (UK) for coordinating the import of the samples to the UK; and Novozyme A/S, Denmark for kindly providing the Cellic CTec3 enzyme.

Disclosure statement

The authors declare that they have no conflict of interest.

References

1. David Samuel O, Adekojo Waheed M, Taheri-Garavand A, *et al.* Prandtl number of optimum biodiesel from food industrial waste oil and diesel fuel blend for diesel engine. *Fuel*. 285, 119049 (2021).

2. Verma P, Dwivedi G, Behura AK, Patel DK, Verma TN, Pugazhendhi A. Experimental investigation of diesel engine fuelled with different alkyl esters of Karanja oil. *Fuel*. 275, 117920 (2020).

3. Rajak U, Nashine P, Chaurasiya PK, Verma TN, Patel DK, Dwivedi G. Experimental & predicative analysis of engine characteristics of various biodiesels. *Fuel*. 285, 119097 (2021).

4. Fazil Khan M, Garg A, Jain S, Dwivedi G, Nath Verma T. Optimization of low-temperature transesterification of low FFA blend of sunflower oil and algae oil. *Fuel*. 279, 118459 (2020).

5. FAO. Rice Market Monitor. XIX(4), 1–34 (2016).

6. Mbanjo EGN, Kretzschmar T, Jones H, *et al.* The Genetic Basis and Nutritional Benefits of Pigmented Rice Grain. *Front. Genet.* 11, 291 (2020).

7. Sapna I, Kamaljit M, Priya R, Jayadeep PA. Milling and thermal treatment induced changes on phenolic components and antioxidant activities of pigmented rice flours. *J Food Sci Technol*. 56(1), 273–280 (2019).

8. Teye E, Amuah CLY, McGrath T, Elliott C. Innovative and rapid analysis for rice authenticity using hand-held NIR spectrometry and chemometrics. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 217, 147–154 (2019).

9. Intellectual Property India. Manipur Black Rice (Chakhao). *Geographical Indications Registry* [Internet]. 126 (2020). Available from: http://ipindiaservices.gov.in/GirPublic/Application/Details/602.

10. Moirangthem K, Jenkins D, Ramakrishna P, Rajkumari R, Cook D. Indian black rice: A brewing raw material with novel functionality. *Journal of The Institute of Brewing*. 126(1), 35–45 (2020).

11. Pereira-Caro G, Cros G, Yokota T, Crozier A. Phytochemical Profiles of Black, Red, Brown, and White Rice from the Camargue Region of France. *J. Agric. Food Chem.* 61(33), 7976–7986 (2013).

12. Fatchiyah F, Ratih D, Sari D, Safitri A, Ketudat Cairns J. Phytochemical Compound and Nutritional Value in Black Rice from Java Island, Indonesia. *Systematic Reviews in Pharmacy*. 11, 414–421 (2020).

13. Kong L, Wang Y, Cao Y. Determination of Myo-inositol and d-chiro-inositol in black rice bran by capillary electrophoresis with electrochemical detection. *Journal of Food Composition and Analysis*. 21(6), 501–504 (2008).

14. Kushwaha UKS. Black Rice. In: *Black Rice: Research, History and Development*. Kushwaha UKS (Ed.). Springer International Publishing, Cham, 21–47 (2016).

15. Pandey S, MR A, A J. Changes in physical, cooking, textural properties and crystallinity upon iron fortification of red rice (Jyothi). *J Food Sci Technol*. 53(2), 1014–1024 (2016).

16. Moirangthem K, Ramakrishna P, Amer MH, Tucker GA. Bioactivity and anthocyanin content of microwave-assisted subcritical water extracts of Manipur black rice (Chakhao) bran and straw. *Future Foods*. 3, 100030 (2021).

17. Gadde B, Menke C, Wassmann R. Rice straw as a renewable energy source in India, Thailand, and the Philippines: Overall potential and limitations for energy contribution and greenhouse gas mitigation. *Biomass and Bioenergy*. 33(11), 1532–1546 (2009).

18. Bhattacharyya P, Bhaduri D, Adak T, *et al.* Characterization of rice straw from major cultivars for best alternative industrial uses to cutoff the menace of straw burning. *Industrial Crops & Products*. 143, 111919 (2020).

19. Energy Information Administration. International Energy Outlook 2019. 1–85 (2019).

20. van Eijck J, Batidzirai B, Faaij A. Current and future economic performance of first and second generation biofuels in developing countries. *Applied Energy*. 135, 115–141 (2014).

21. Diep NQ, Sakanishi K, Nakagoshi N, Fujimoto S, Minowa T. Potential for rice straw ethanol production in the Mekong Delta, Vietnam. *Renewable Energy*. 74, 456–463 (2015).

22. Li H, Qu Y, Yang Y, Chang S, Xu J. Microwave irradiation – A green and efficient way to pretreat biomass. *Bioresource Technology*. 199, 34–41 (2016).

23. Intanakul P, Krairiksh M, Kitchaiya P. Enhancement of Enzymatic Hydrolysis of Lignocellulosic Wastes by Microwave Pretreatment Under Atmospheric Pressure. *Energy Sources*. 23(2), 217–225 (2007).

24. Ma H, Liu W-W, Chen X, Wu Y-J, Yu Z-L. Enhanced enzymatic saccharification of rice straw by microwave pretreatment. *Bioresource Technology*. 100(3), 1279–1284 (2009).

25. Sasaki K, Okamoto M, Shirai T, *et al.* Precipitate obtained following membrane separation of hydrothermally pretreated rice straw liquid revealed by 2D NMR to have high lignin content. *Biotechnology for Biofuels*. 8(1), 88 (2015).

26. Urbaniec K, Grabarczyk R. Raw materials for fermentative hydrogen production. *Journal of Cleaner Production*. 17, 959–962 (2009).

27. Kucharska K, Rybarczyk P, Hołowacz I, Łukajtis R, Glinka M, Kamiński M. Pretreatment of Lignocellulosic Materials as Substrates for Fermentation Processes. *Molecules*. 23(11), 2937–32 (2018).

28. Victor A, Pulidindi IN, Kim TH, Gedanken A. Design of a selective solid acid catalyst for the optimization of glucose production from Oryza sativa straw. *RSC Adv.* 6, 31–38 (2016).

29. Gong G, Liu D, Huang Y. Microwave-assisted organic acid pretreatment for enzymatic hydrolysis of rice straw. *Biosystems Engineering*. 107(2), 67–73 (2010).

30. Zhu S, Wu Y, Yu Z, Liao J, Zhang Y. Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis. *Process Biochemistry*. 40(9), 3082–3086 (2005).

31. Sheetal KR, Prasad S, Renjith PS. Effect of cultivar variation and Pichia stipitis NCIM 3498 on cellulosic ethanol production from rice straw. *Biomass and Bioenergy*. 127, 105253 (2019).

32. Moirangthem K, Greetham D, Craigon J, Tucker G. Impact of mixed feedstock of wheat straw, willow and Miscanthus on enzymatic hydrolysis and inhibitor production after microwave hydrothermal pre-treatment in Europe. *Biofuels*. 1–8 (2021).

33. Saeman JF, Bubl JL, Harris EE. Quantitative saccharification of wood and cellulose. *Industrial Engineering Chemistry Analytical Edition*. 17(1), 35–37 (1945).

34. Selig M, Weiss N, (null) Y Ji. Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP). NREL- National Renewable Energy Laboratory.

35. EFSA. Scientific Opinion on the re-evaluation of anthocyanins (E 163) as a food additive. *EFSA Journal* [Internet]. 11(4), 3145 (2013). Available from: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2013.3145.

36. Alvira P, Negro MJ, Ballesteros I, González A, Ballesteros M. Steam Explosion for Wheat Straw Pretreatment for Sugars Production. *Bioethanol*. 2(1), 1–10 (2016).

37. Imman S, Arnthong J, Burapatana V, Laosiripojana N, Champreda V. Autohydrolysis of Tropical Agricultural Residues by Compressed Liquid Hot Water Pretreatment. *Applied Biochemistry and Biotechnology*. 170(8), 1982–1995 (2013).

38. Wu J, Elliston A, Le Gall G, *et al.* Optimising conditions for bioethanol production from rice husk and rice straw: effects of pre-treatment on liquor composition and fermentation inhibitors. *Biotechnology for Biofuels*. 1–13 (2018).

39. Wood IP, Cao H-G, Tran L, *et al.* Comparison of saccharification and fermentation of steam exploded rice straw and rice husk. *Biotechnology for Biofuels*. 1–9 (2016).

40. Galbe M, Wallberg O. Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. *Biotechnology for Biofuels*. 1–26 (2019).

41. Krishnan MS, Ho NWY, Tsao GT. Fermentation kinetics of ethanol production from glucose and xylose by recombinant Saccharomyces 1400(pLNH33). *Applied Biochemistry and Biotechnology*. 78(1), 373–388 (1999).

42. Imman S, Arnthong J, Burapatana V, Champreda V, Laosiripojana N. Effects of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw. *Bioresource Technology*. 171, 29–36 (2014).

43. Jin S, Zhang G, Zhang P, *et al.* Microwave assisted alkaline pretreatment to enhance enzymatic saccharification of catalpa sawdust. *Bioresource Technology*. 221, 26–30 (2016).

44. Chittibabu S, Saseetharan MK, Kalaivani MR, Rajesh MP. Optimization of Microwave-assisted Alkali Pretreatment and Enzymatic Hydrolysis of Banana Pseudostem. *Energy Sources*. 36(24), 2691–2698 (2014).

45. Zhu Z, Simister R, Bird S, McQueen-Mason S, Gomez L, Macquarrie D. Microwave assisted acid and alkali pretreatment of Miscanthus biomass for biorefineries. *AIMS Bioengineering*. 2, 449–468 (2015).

46. Karimi K, Kheradmandinia S, Taherzadeh M. Conversion of Rice Straw to Sugars by Dilute-Acid Hydrolysis. *Biomass and Bioenergy*. 30, 247–253 (2006).

47. Hsu T-C, Guo GL, Chen W-H, Hwang W-S. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. *Bioresource Technology*. 101(13), 4907–4913 (2010).

48. Yu G, Yano S, Inoue H, Inoue S, Endo T, Sawayama S. Pretreatment of Rice Straw by a Hot-Compressed Water Process for Enzymatic Hydrolysis. *Appl Biochem Biotechnol*. 160(2), 539–551 (2010).

49. Tang S, Dong Q, Fang Z, Cong W-J, Miao Z-D. High-concentrated substrate enzymatic hydrolysis of pretreated rice straw with glycerol and aluminum chloride at low cellulase loadings. *Bioresource Technology*. 294, 122164 (2019).

50. Tang S, Dong Q, Fang Z, Miao Z-D. Complete recovery of cellulose from rice straw pretreated with ethylene glycol and aluminum chloride for enzymatic hydrolysis. *Bioresource Technology*. 284, 98–104 (2019).

51. Hou X-D, Feng G-J, Ye M, Huang C-M, Zhang Y. Significantly enhanced enzymatic hydrolysis of rice straw via a high-performance two-stage deep eutectic solvents synergistic pretreatment. *Bioresource Technology*. 238, 139–146 (2017).

52. Wu J, Elliston A, Le Gall G, *et al.* Optimising conditions for bioethanol production from rice husk and rice straw: effects of pre-treatment on liquor composition and fermentation inhibitors. *Biotechnology for Biofuels*. 11(1), 62 (2018).

53. Moirangthem K. Alternative Fuels for Marine and Inland Waterways: An exploratory study. *JRC Technical Reports* [Internet]. 1–46 (2016). Available from: https://ec.europa.eu/jrc/en/publication/alternative-fuels-marine-and-inland-waterways-exploratory-study.

54. Carriquiry MA, Du X, Timilsina GR. Second generation biofuels: Economics and policies. *Special Section: Renewable energy policy and development*. 39(7), 4222–4234 (2011).

55. Moirangthem K, Waldron P, Tucker G, King I, King J. Dual purpose wheat: for a future that demands more food and energy. *Aspects of Applied Biology*. 131, 1–6 (2015).

56. Moirangthem K, Waldron P, Tucker G, King I, King J. Improving wheat straw composition and digestibility by alien introgression. ETA-Florence Renewable Energies, 330–334 (2016).

57. Karimi E, Mehrabanjoubani P, Keshavarzian M, Oskoueian E, Jaafar HZ, Abdolzadeh A. Identification and quantification of phenolic and flavonoid components in straw and seed husk of some rice varieties (Oryza sativaL.) and their antioxidant properties. *J. Sci. Food Agric.* 94(11), 2324–2330 (2014).

58. Menzel C, González-Martínez C, Vilaplana F, Diretto G, Chiralt A. Incorporation of natural antioxidants from rice straw into renewable starch films. *International Journal of Biological Macromolecules*. 146, 976–986 (2020).

59. Abraham A, Mathew AK, Sindhu R, Pandey A, Binod P. Potential of rice straw for bio-refining: An overview. *Bioresource Technology*. 215(C), 29–36 (2016).