



# Cellulase production on easy-to-handle solid media containing agricultural waste and its application for enzymatic hydrolysis of cellulosic biomass

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## Abstract

The process of bioethanol production has several economic drawbacks such as the cost of enzymes, i.e., cellulase, which hydrolyze cellulose to produce fermentable sugars. In this study, to reduce the production cost of cellulase, agricultural wastes such as wheat straw (WS) and rice straw (RS) were used as carbon sources for cellulase production through solid-state fermentation. First, the standard cellulase production conditions were optimized, the initial moisture content of the solid medium was 60%, the biomass particle size was 100 µm to 4 mm size mixed, and the optimal carbon/nitrogen (C/N) ratio in the medium of WS and RS was 59.0 and 41.2, respectively. Subsequently, to simplify process handling, eliminating trace elements such as MgSO<sub>4</sub>, CaCl<sub>2</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub>, ZnSO<sub>4</sub>, CoCl<sub>2</sub>, and phosphorous compounds such as KH<sub>2</sub>PO<sub>4</sub> in the general Mandel's medium was used for cellulase production medium and compared with the standard medium. Maximum 5.4 filter paper unit (FPU)/g-substrate (WS) and 5.3 FPU/g-substrate (RS) were attained under trace element eliminating medium; these were similar to that from the standard medium of WS (4.8 FPU/g-substrate) and RS (5.7 FPU/g-substrate). Moreover, the produced crude cellulases from RS were evaluated for their potential in hydrolysis of alkaline-treated RS. The performances of the crude cellulases were similar to those of commercial cellulases.

**Keywords** Solid-state fermentation · Wheat straw · Rice straw · Cellulase · Enzymatic hydrolysis

## 1 Introduction

Lignocellulosic biomass as the carbon-neutral resources is a promising resource for the production of bioethanol and other beneficial chemicals [1, 2]. The conversion of lignocellulosic biomass to bioethanol entails the following three main steps: pretreatment to remove lignin and reduce the recalcitrance of cellulose, enzymatic hydrolysis to release fermentable sugars such as glucose, and fermentation to

produce bioethanol [3]. Enzymatic hydrolysis is conducted using cellulase, which breaks down cellulose into glucose [4]. Cellulase is an industrially important enzyme for bioethanol production, food processing (more optimized extraction and clarification of fruit juice), the detergent industry (accelerating detergency), the pulp and paper industry (biomechanical pulping), and the textile industry (biopolishing of textile fibers) [5–7]. However, the cellulase production process is quite costly; it accounts for 50% of the overall cost of hydrolysis [6, 7]. Therefore, the production cost of bioethanol is greatly affected by the cost of cellulase [8].

Solid-state fermentation (SSF) has numerous advantages, such as high volumetric productivity and low moisture content in the medium, and occurs in a natural state for enzyme production [9, 10]. SSF has numerous economic and practical advantages over submerged fermentation. Particularly, SSF can utilize heterogeneous byproducts such as agricultural wastes and industrial wastes based on cellulosic materials [11]. As the expression of the produced cellulase is greatly influenced

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by physical state and chemical properties of the carbon source [12], identical microorganisms growing on different types of cellulosic materials can yield varying amounts of cellulase [13].

Wheat straw (WS) and rice straw (RS) are lignocellulosic agricultural wastes that are abundantly available worldwide and are considered to be potential feedstocks for the production of ethanol. Approximately 850 million tons of WS and 669 million tons of RS are produced in Asia per annum. [14]. Therefore, these straw wastes are promising resources for the production of ethanol and cultivation of cellulase-producing fungi. Recently, herbaceous biomass solid among various special biomass solids was used as cellulase production medium nutrients by SSF [15–21]. In these studies, a general standard nutrient additive called Mandel's medium containing nitrogen and various mineral salts was used with solid biomass. Scaling up and manufacturing on a large scale with low cost and easy handling necessitate the use of the popular and abundant cellulosic biomass worldwide as well as limiting the additives into the biomass medium.

In this study, we used the poplar herbaceous cellulose biomass, wheat straw (WS), and rice straw (RS) as natural cellulosic substrates for cellulase production through SSF using *Trichoderma reesei* as cellulase producer. First, the fermentation parameters such as the initial medium moisture content, particle sizes, and carbon/nitrogen ratio in the medium were optimized, and the total cellulase titer and detailed activities, i.e., endoglucanase, carboxymethyl cellulase (CMCase),  $\beta$ -glucosidase, and xylanase activities, of the cellulolytic enzymes produced from WS and RS were evaluated. Next, under optimized condition, to reduce handling nutrient, elements in the WS and RS production medium were minimized. Finally, the detailed activities of cellulase produced from the minimized biomass medium were evaluated, and the hydrolysis ability was tested.

## 2 Materials and methods

### 2.1 Cellulosic substrate materials for the production of cellulase

Cellulosic materials for the production of cellulase were WS and RS. The WS and RS were provided by a local farm in Tokushima City; they were cut to pieces measuring 20 mm  $\times$  10 mm  $\times$  1 mm and then ground using a model D3V-10 cutter mill (Osaka Chemical Co. Ltd., Osaka, Japan) for 5 s to obtain particles ranging from 100  $\mu$ m to 4 mm in size. Furthermore, 100 g of ground RS was washed with 2 L of ion-exchange water after being dried in an oven at 100 °C; the residue was ground for 15 s.

### 2.2 Microorganism

SSF was carried out using *Trichoderma reesei* (*T. reesei*), which is commonly used for commercial cellulase production and has been researched extensively because of its high cellulase production capability [22–24]. *T. reesei* ATCC 56765 was purchased from the American Type Culture Collection. The strain was cultivated on potato dextrose agar (PDA) medium in Petri dishes at 28 °C for 1 week.

### 2.3 Chemical analysis of wheat straw and rice straw

The cellulosic materials were analyzed for their chemical compositions as follows. The cellulose content in the sample was determined based on the monomer content (glucose) measured after hydrolysis (72 wt% H<sub>2</sub>SO<sub>4</sub> followed by dilution). The sample was evaluated using the Klason lignin measurement method [25], where 1 g of the material was added to 15 mL of 72 wt% H<sub>2</sub>SO<sub>4</sub> and stored at room temperature for 4 h. The residue was then placed in a 1 L conical flask containing 560 mL of distilled water and autoclaved for 1 h at 121 °C. After cooling, the solid and liquid portions were separated by centrifugation, and the glucose in the liquid was analyzed by high-performance liquid chromatography with a refractive index detector and a model HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 °C. The mobile phase was 5.0 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL/min. The solid portion was washed with distilled water, dried at approximately 105 °C with a heater to a constant weight, and weighed as an acid-insoluble material (lignin). The amount of hemicellulose was calculated by subtracting the cellulose content from the total sugar content. The total sugar content was determined using the phenol–sulfuric acid method [26]. To determine the ash content, 5 g of the sample was placed in a sintered crucible, which was placed inside a muffle furnace at 600 °C for 2 h followed by cooling in a desiccator. Ash content was calculated using the following formula:

$$\text{Ash (\%)} = [(\text{Weight of ash with crucible (g)} - \text{Weight of dry crucible (g)}) / \text{Weight of the sample}] \times 100$$

All analytical determinations were performed in triplicate, and the calculations are shown.

The mineral K was measured by atomic absorption spectrometry. The minerals Ca, Mg, Fe, Zn, Mn, and P were measured by inductively coupled plasma optical emission spectroscopy. The minerals analyses were performed by Japan Functional Food Analysis and Research Center (Fukuoka, Japan).

### 2.4 Carbon-to-nitrogen ratio measurement

The total carbon and nitrogen of WS and RS samples were measured using the combustion method by MICRO

CORDER (JM10, J-SCIENCE LAB, Osaka, Japan). The total carbon and nitrogen content on the weight basis of samples was used to calculate the carbon-to-nitrogen (C/N) ratio. The C/N ratio of the medium was calculated using the following formula:

$$\text{C/N ratio} = \frac{\text{[Weight of carbon in WS or RS (g) + weight of carbon in pepton, urea, and ammonium sulfate (g)]}}{\text{[Weight of nitrogen in WS or RS (g) + weight of nitrogen in pepton, urea, and ammonium sulfate (g)]}}$$

## 2.5 Inoculum preparation

Inoculum preparation was performed in 300 Erlenmeyer flasks containing 100 mL of Mandels and Weber growth medium [27] with slight modifications consisting of the following compositions (g/L):  $\text{KH}_2\text{PO}_4$ , 2.0;  $(\text{NH}_4)_2\text{SO}_4$ , 1.4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0016;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0015;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.004;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3; urea, 0.3; peptone, 0.1; glucose, 5.0; and Avicel, 10. Five loopfuls of mycelial conidia cultures from the PDA medium of the Petri dish were added and shaken at 140 rpm at 30 °C in an incubator shaker for 48 h. After incubation, the inoculum was placed in a test tube and separated by centrifugation (3500 rpm for 15 min). The collected solid cells were used for SSF.

## 2.6 Solid-state fermentation

Dry substrate (5.0 g) was added to a 100 mL Erlenmeyer flask. The flask was autoclaved at 121 °C for 20 min for sterilization. After cooling, 0.06 g of the cells (dry cell weight) was suspended in the concentrated production medium (5 mL) and aseptically added to the substrates. The concentrated production medium had the following composition (in g/L):  $\text{KH}_2\text{PO}_4$ , 20;  $(\text{NH}_4)_2\text{SO}_4$ , 14;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.016;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.015;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.04;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3; urea, 3.0; and peptone, 1.0. The moisture content was adjusted with 2.5 mL and 14 mL of sterilized distilled water to levels of 60% or 80%. The cultures were incubated at 30, 32, or 36 °C for 9 days. The incubated samples were collected at 24-h intervals and checked for cellulase and xylanase activities.

## 2.7 Enzyme extraction

At the end of incubation, the fungal enzyme was extracted. For each 5 g of fermented substrate, 50 mL of sodium citrate buffer (50 mM and pH 4.8) was added and agitated in a shaker for 1 h. The mixture was centrifuged at room temperature (25 °C) at 3500 rpm for 15 min to collect the clear supernatant as a crude enzyme solution.

## 2.8 Enzyme assays

The total cellulase activity was determined by filter paper unit (FPU) activity according to the standardized National Renewable Energy Laboratory analytical procedure [28]. The assay was carried out by adding 0.5 mL of enzyme solution to a test tube containing 1 mL of sodium citrate buffer (50 mM and pH 4.8) and a Whatman No. 1 filter paper strip (1 cm × 6 cm and 50 mg). The mixture was incubated at 50 °C for 60 min, and the released reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method [29]. One unit of the activity was defined as the amount of enzyme that releases 1 μmol of glucose per min under assay conditions. Endoglucanase activity, that is, carboxymethyl cellulase activity (CMCase), was measured with 1% (w/v) carboxymethyl cellulose in 50 mM sodium citrate buffer (50 mM and pH 4.8) [30]. The definition of activity in this case is similar to that of FPU activity. For the xylanase assay, suspension of beech wood xylan of 1% (w/v) in the buffer mentioned above was used as a substrate. One unit of xylanase activity was defined as the amount of enzyme that releases 1 μmol of xylose per min under assay conditions [31]. The β-glucosidase assay was carried out with 1 mL of *p*-nitrophenyl -β-D-glucopyranoside (pNPG, 2 mM, Sigma-Aldrich) as substrate, which was digested using 0.1 mL of enzyme solution at 50 °C for 5 min. The reaction was stopped by adding 2 mL of sodium carbonate solution (1 M), and the amount of *p*-nitrophenol was determined by reading the absorbance at 405 nm [32]. One unit of β-glucosidase activity was defined as the amount of enzyme that liberated 1 μmol of *p*NPG per min under assay conditions.

## 2.9 Enzymatic hydrolysis of alkaline-treated rice straw

Alkaline-treated RS was used as the substrate for the enzymatic hydrolysis test by producing cellulase from RS.

After grinding and washing with water treatment of RS, alkaline treatment was conducted at a ratio of 1:10 using 3% (w/v) of NaOH, that is, 10 g of RS in 100 mL of 3% (w/v) of NaOH. The RS in the NaOH solution was then autoclaved at 121 °C for 30 min. Afterwards, the treated residue was neutralized to an approximate pH of 7.0 using a diluted HCl solution, washed with distilled water, and oven dried at 100 °C for 24 h and stored at room temperature (25 °C) prior to use.

The crude enzyme mixture produced by *T. reesei* ATCC 56765 from RS medium was produced under optimal conditions of an incubation temperature of 30 °C and substrate moisture content of 60% and extracted after 6 days of growth. Commercial cellulases were used as controls. *Acremonium* cellulase (derived from *Acremonium cellulolyticus*,

269 FPU activity/g-enzyme) was purchased from Meiji Seika Pharma Co., Ltd. (Osaka, Japan).

Enzymatic hydrolysis was performed using 10 mL of 0.1 M sodium acetate buffer (pH 5.0) at 50 °C in a rotary shaker operating at 160 rpm. The substrate concentrations and enzyme loading were 50 g/L and 26 FPU/g of dry substrate, respectively. The hydrolyzed samples were obtained at 24-h intervals and centrifuged to remove the solid residue. The supernatant was analyzed for glucose using the mutarotase glucose oxidase method (Glucose C-II test, Wako Pure Chemicals Co., Ltd., Japan). All enzymatic hydrolysis experiments were performed in triplicate, and the means were calculated.

The hydrolysis yield (%) by enzymatic hydrolysis was calculated using the following equation:

$$\left(\frac{\text{Amount of glucose produced (g)}}{\text{Amount of cellulose in the substrate (g)}} \times 1.1\right) \times 100.$$

### 3 Results and discussion

#### 3.1 Chemical composition of WS and RS

To confirm the chemical component in the experimental material, chemical analyses of WS and RS (washed with water) were performed. The amounts of cellulose, hemicellulose, lignin, crude protein, and ash were determined. Moreover, elemental composition, i.e., carbon content and nitrogen content in the WS and RS washed with water were also determined. The results are summarized in Table 1. The cellulose contents of WS and RS were 35.9% and 35.1%, respectively. The carbon content (%) and nitrogen content (%) in the WS and RS washed with water were as follows: 40.0 and 0.23, 41.1, and 0.55.

#### 3.2 Optimization of solid-state fermentation conditions for cellulase production by *T. reesei* using wheat straw and rice straw

##### 3.2.1 Initial moisture content

For the assessment of the effective utilization of agricultural byproducts, WS and RS were used as solid substrate carbon sources for the production of cellulase.

Initial moisture content is an important parameter for cellulase production by SSF. In this study, initial moisture content of 60% and 80% using WS as incubation medium was investigated. Figure 1 shows the time course of cellulase activity as FPU activity during incubation. Many researchers observed that the optimum temperature for cellulase production using *T. reesei* species was approximately 30 °C [33, 34]. Verma et al. reported that temperatures above or below 30 °C were somewhat unfavorable for *T. reesei*, cellulase producer, which might be due to their physiological nature and protein synthesis capability. At a higher temperature (35 °C), enzyme biosynthesis decreases owing to thermal deactivation. At a lower temperature (25 °C), the transport of nutrients in the cells is hindered, and the microbial enzyme production capacity decreased [6]. Therefore, subsequent experiments were performed at 30 °C. The maximum activity of 4.8 FPU/g-dry WS was obtained on day 9 under 60% of moisture content. Conversely, for the moisture content of 80%, although they have similar cellulase activity to that of moisture content of 60%, the activities were variable (large error bars). Many studies have reported on the initial moisture content of cellulase production by SSF using

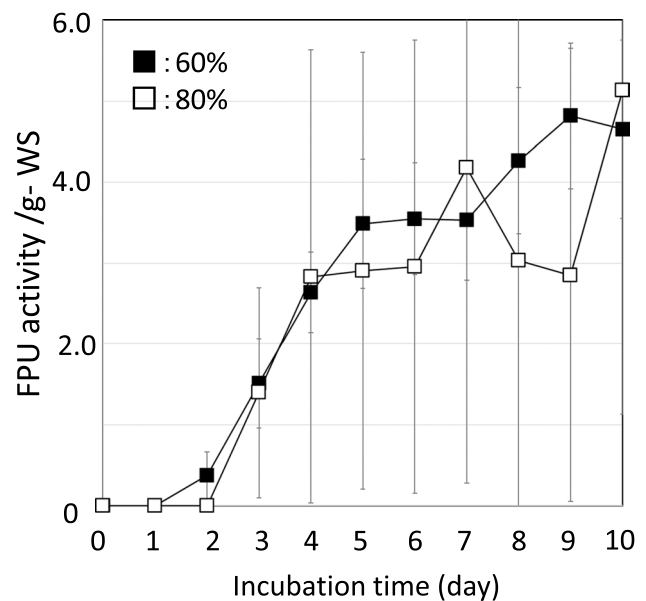


Fig. 1 Effect of the initial moisture content on cellulase filter paper unit (FPU) activity using WS medium under SSF

Table 1 Component and elemental analysis of wheat straw (WS) and rice straw (RS) washed with water

	Component analysis (%)					Elemental analysis (%)		
	Cellulose	Hemicellulose	Lignin	Ash	Crude protein	Carbon	Hydrogen	Nitrogen
Wheat straw	35.9±0.7	23.2±1.6	20.1±0.3	7.5±0.01	1.4±0.01	39.95	5.86	0.23
Rice straw (washed with water)	35.1±0.3	31.7±1.4	22.4±0.4	19.2±0.01	4.7±0.01	41.09	5.94	0.55

*Trichoderma* spp. Salomão et al. reported that relatively high enzymatic activities were obtained at an incubation temperature of 28 °C to 50% of initial moisture content of sugarcane bagasse solid medium using *Trichoderma koningii* [35]. Verma et al. also reported that optimal initial moisture content was approximately 60% using wheat bran as solid substrate for cellulase production by *T. reesei* and *Neurospora crassa* co-culture [36].

### 3.2.2 Particle sizes

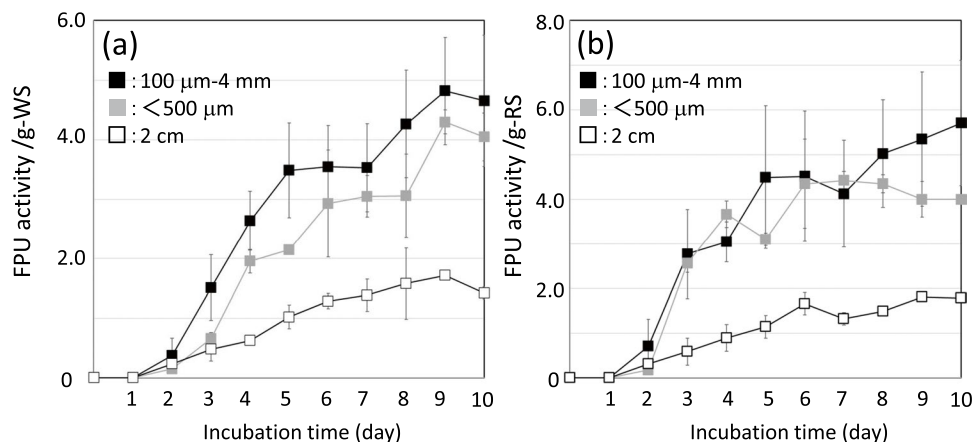
The effect of the particle sizes of WS and RS on cellulase production by *T. reesei* were evaluated using powder particles (< 500 µm, sieved after grinding), powder and fiber particles mixed (100 µm to 4 mm, only grinding), and pieces (measuring 20 mm × 10 mm × 1 mm) as substrates. With RS as a solid substrate, the residue after washing with water was used as substrate, because no production of cellulase was observed in cases where RS was not washed with water (data not shown). Figure 2 shows the time course of cellulase activity as FPU activity during incubation using WS and RS as substrates. For both WS and RS, FPU activity profiles with powder, and fiber particles and only powder particles were similar; however, with pieces, there was no bigger increase than those obtained with powder and fiber or powder particles.

A relatively small substrate size provides a relatively large surface area for growth and action of microorganisms and is beneficial for heat transfer and uptake of oxygen [37]. Verma et al. reported that a particle size of 850 µm (in the case of wheat bran) was effective for cellulase activity [36]. Vanajakshi et al. also suggested that both interparticle porosity and surface area exhibit a high medium particle size range, which encourages the growth of microorganisms and cellulase production owing to more optimized mass and heat transfer [38]. A mixture of powder and fiber particles (100 µm to 4 mm) was used throughout this study to simplify the preparation of the substrate because it is necessary to sieve the sample to produce powder particles (< 500 µm).

### 3.2.3 Carbon-to-nitrogen ratio

According to the modified Mandel's medium used in this study (standard medium), the C/N ratios of WS and RS were 59.0 and 41.2, respectively. The compositions of different cultivation media and their C/N ratios of WS and RS mediums are summarized in Table 2. In the nitrogen-reduced medium (C/N ratio; 14.2 and 13.1 for WS and RS), urea and ammonium sulfate were half of the standard medium. In the nitrogen-increased medium (C/N ratio; 86.7 and 52.7), urea and ammonium sulfate were six times higher than those of the standard medium. Figure 3 shows the time course of cellulase activity as FPU activity during incubation using WS and RS as substrates under various C/N ratios. Unstable cellulase production was observed at C/N ratios of 14.2 (WS) and 13.1 (RS). Conversely, no drastic increments were observed at C/N ratios of 86.7 (WS) and 52.7 (RS). These results showed that C/N ratios of 59.0 (WS) and 41.2 (RS), i.e., standard medium composition, were favorable for the production of cellulase. It is important to calculate the C/N ratio using biomass solid substrate and other medium additives (Mandel's medium), because carbon content and nitrogen content differ depending on the biomass solid substrate. For example, Moran-Aguilar et al. used sugarcane bagasse and brewery spent grain as cellulase fermentation substrate by *Aspergillus niger* (*A. niger*), carbon content, and nitrogen content in the biomass substrates which are reported as 42.55% and 0.25%, 44.38%, and 3.61%, respectively. Using these carbon and nitrogen contents, C/N = 149 (sugarcane bagasse) and C/N = 12.1 (brewery spent grain) could be calculated [39]. Although the nitrogen content varies greatly depending on the substrate, only a few studies have analyzed carbon and nitrogen contents of the substrates and calculated and discussed the C/N ratio values. Amaro-Reyes et al. carried out co-culture cellulase fermentation of *A. niger* and *T. reesei* using Bermuda grass and corn cob mixture as solid-state substrate. Maximum FPU activity (23.2 FPU/g-substrate) was observed at C/N = 30, and no

**Fig. 2** Effect of substrate size of wheat straw (WS) (a) and rice straw (RS) (b) medium on cellulase FPU activity under SSF



significant activity difference was observed at C/N ratios of 60 and 120. However, the lowest FPU activity was observed at a C/N ratio of 3 (half of the highest) [40]. Determining a suitable C/N ratio is essential to producing cellulase for each substrate. The growth of *T. reesei* on WS and RS medium during 9 days of incubation at optimal culture condition (initial moisture 60%, mixture of powder and fiber particles (100  $\mu\text{m}$  to 4 mm), C/N = 59.0 for WS and 41.2 for RS) is shown

in Fig. 4. Both WS and RS medium growths of hyphae were observed within 4 days, forming white fluffy aerial mycelia. Finally, the mycelia turned greenish.

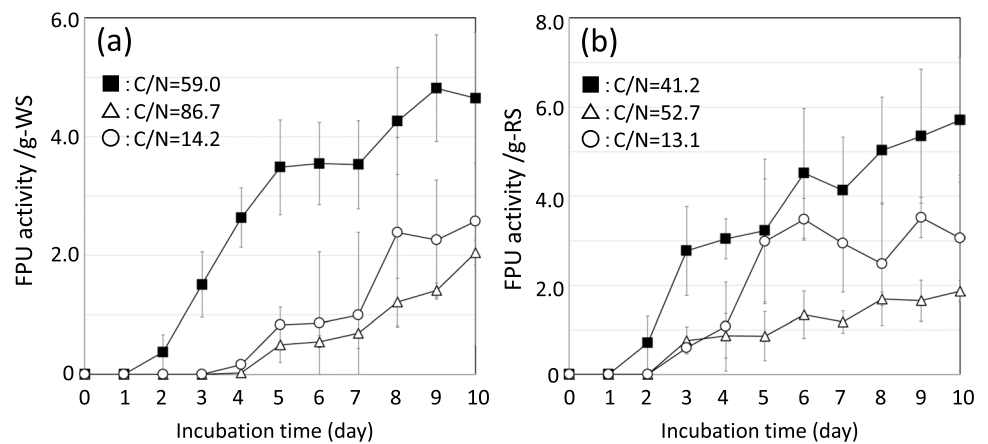
Furthermore, a cellulolytic enzyme system is complex and comprises endoglucanase (CMCase), exoglucanase,  $\beta$ -glucosidase, and xylanase that acts synergistically to degrade cellulosic substrate [41, 42]. An efficient enzyme system should contain balanced activities of these

**Table 2** Compositions of different cultivation media and carbon/nitrogen ratio of wheat straw medium and rice straw medium

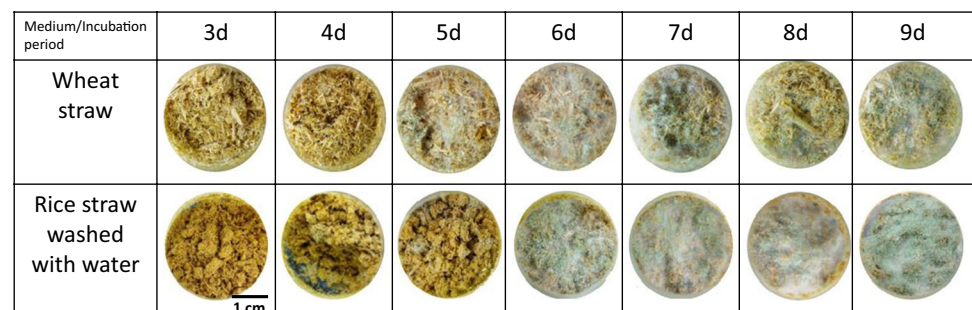
Substance (mg/g-solid medium substrate)	Standard medium (modified Mandel's medium)	Nitrogen-reduced medium	Nitrogen-increased medium
Pepton <sup>a</sup>	5.0	5.0	5.0
Urea	15.0	7.5	90.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	70.0	35.0	420.0
KH <sub>2</sub> PO <sub>4</sub>	100.0	100.0	100.0
MgSO <sub>4</sub> ·6H <sub>2</sub> O	0.25	0.25	0.25
CaCl <sub>2</sub> ·2H <sub>2</sub> O	20.0	20.0	20.0
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.25	0.25	0.25
MnSO <sub>4</sub> ·H <sub>2</sub> O	0.08	0.08	0.08
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.07	0.07	0.07
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.19	0.19	0.19
C/N ratio			
Wheat straw	59.0	14.2	86.7
Rice straw (washed with water)	41.2	13.1	52.7

<sup>a</sup>C content (%) and N content (%) by elemental analysis were 42.61 and 14.77, respectively

**Fig. 3** Effect of carbon/nitrogen (C/N) ratio of WS (a) and RS (b) medium on cellulase FPU activity under SSF



**Fig. 4** Growth of *Trichoderma reesei* on WS or RS medium under optimal culture condition (initial moisture 60%, mixture of powder and fiber particles (100  $\mu\text{m}$  to 4 mm), C/N = 59.0 for WS and 41.2 for RS) at 30 °C during 9-day culture period



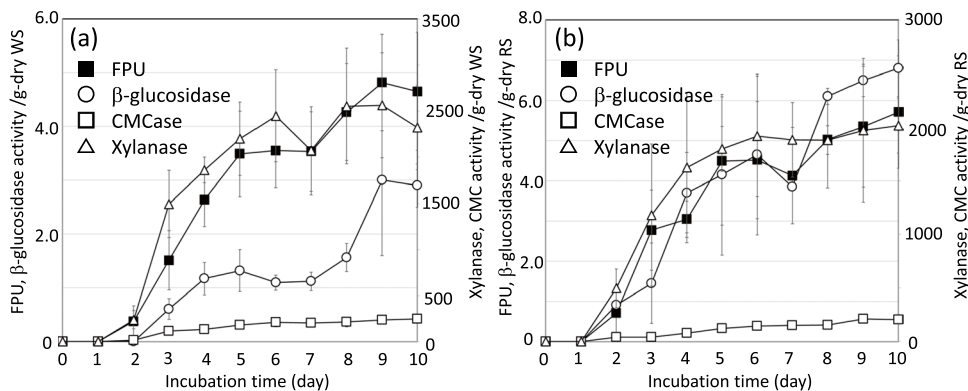
cellulases; therefore, FPU (total cellulase titer), CMCCase,  $\beta$ -glucosidase, and xylanase activities were evaluated in this study. Figure 5 shows the time course of the produced FPU, CMCCase,  $\beta$ -glucosidase, and xylanase activities from WS and RS as the carbon source under optimized fermentation conditions, initial moisture content of 60%, particle size ranging from 100  $\mu$ m to 4 mm, and C/N ratio of 59.0 (WS) and 41.2 (RS). With CMCCase, the maximum activities of 246 U/g-WS and 205 U/g-RS were obtained on day 10. The  $\beta$ -glucosidase activity increased to 3.0 U/g-WS and 3.5 U/g-RS on day 9; the xylanase activity increased to 2563 U/g-WS and 2111 U/g-RS on days 9 and 10. These production amounts were similar to that reported by Dhillon et al. using RS as the solid fermentation substrate by *T. reesei* Rut C-30 [43]; 16.1 FPU/g-RS of FPU activity, 58.2 U/g-RS of CMCCase, 12.6 U/g-RS of  $\beta$ -glucosidase activity, and 1299 U/g-RS of xylanase activity were attained at 96 h of incubation time at 30 °C. Taherzadeh-Ghahfarokhi et al. also reported that 2.5 FPU/g-WS and 1.9 FPU/g-RS, 7.5 U/g-WS and 1.8 U/g-RS of CMCCase activity, and 400 U-g-WS and 200 U/g-RS of xylanase activity were obtained from WS and RS solid medium by *T. reesei* ATCC 13631 at 28 °C (data of  $\beta$ -glucosidase activity not shown) [15]. However, these activity values varied depending on the microorganism and fermentation parameters used. In this study, high CMCCase

activity and xylanase activity were observed compared to other studies.

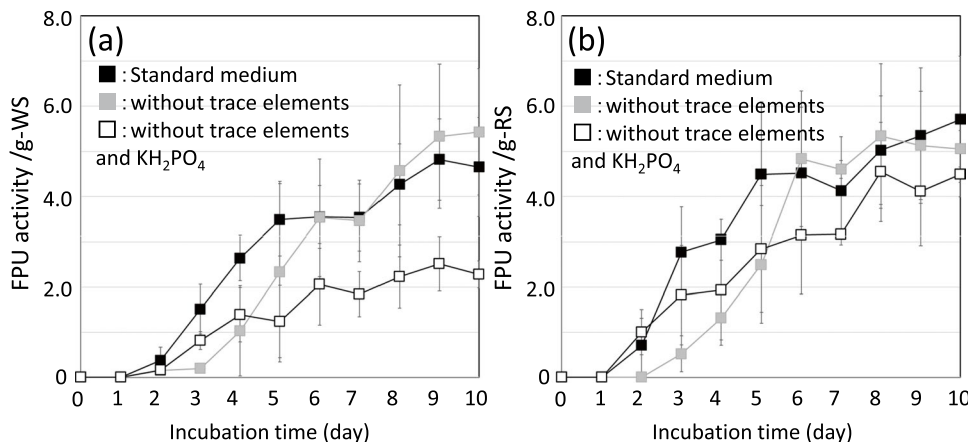
### 3.3 Easy-to-handle medium for cellulase production by *T. reesei* using wheat straw and rice straw

To reduce handling steps, the simplification of medium components was carried out using WS and RS as solid fermentation substrates under optimized fermentation conditions: initial moisture content of 60%, 100  $\mu$ m to 4 mm of particle size mixed, and C/N ratio of 59.0 (WS) and 41.2 (RS). Figure 6 shows the time courses of cellulase activity as FPU activities during incubation using WS (a) and RS (b) standard medium (with Mandel’s medium), WS and RS standard medium without trace elements compounds ( $MgSO_4$ ,  $CaCl_2$ ,  $FeSO_4$ ,  $MnSO_4$ ,  $ZnSO_4$ ,  $CoCl_2$ ) in the Mandel’s medium, and WS and RS standard medium without trace elements and  $KH_2PO_4$ . Both WS and RS mediums without trace element compounds, with similar activity production behavior, and with maximum FPU activities were observed to those of standard medium of WS and RS. Conversely, both WS and RS medium without trace element compounds and  $KH_2PO_4$  and with relatively low maximum FPU activities than that of the standard medium were observed. These results showed

**Fig. 5** Time profiles of the FPU, CMCCase,  $\beta$ -glucosidase, and xylanase activity production derived from WS (a) and RS (b) medium under SSF optimized condition



**Fig. 6** Time profiles of the FPU activity derived from standard biomass medium (with Mandel’s medium), trace element eliminating medium, and trace element and  $KH_2PO_4$  eliminating medium



that trace element compounds in the Mandel's medium could be removed from the WS and RS medium; however,  $\text{KH}_2\text{PO}_4$  must be added into the WS and RS medium. Wen et al. also reported that when dairy manure (with sufficient content trace elements) was used as cellulase production nutrients, the elimination of  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ , and other trace elements such as Fe, Zn, Co, and Mn from the original salt solution (Mandel's medium) had no negative impact on the cellulase production, and  $\text{KH}_2\text{PO}_4$  elimination did reduce cellulase production [44]. The contents of potassium and phosphorus from the manure were low. Therefore, metal elemental features (except for Co) in WS and RS washed with water were determined and are presented in Table 3. Regarding trace elements such as Ca, Mg, Fe, Zn, and Mn, all the metals except for Ca present enough in the WS and RS as solid fermentation substrate compared with standard medium (modified Mandel's medium). Belén et al. reported that Fe, Mn, and Zn are regarded as cofactors in the growth and development metabolism of the microorganism [45]. Shu et al. found Mn is crucial in cellular functions such as cell wall synthesis, sporulation, and the production of secondary metabolites [46]. As *T. reesei* could utilize these trace elements from WS or RS, the elimination of trace elements via the standard Mandel medium had no negative influence on cellulase production. Based on the result, medium composition using WS and RS for the cellulase production can be simplified and cost-effective. Conversely, K and P were significantly less than those of the standard medium; their contents were 13.3 mg/g-WS and 3.83 mg/g-RS and only 0.71 mg/g-WS and 0.47 mg/g-RS, respectively. This necessitates the compensation of K and P using  $\text{KH}_2\text{PO}_4$  into the natural WS and RS as cellulase fermentation medium. Generally, because  $\text{KH}_2\text{PO}_4$  (with  $\text{K}_2\text{HPO}_4$ ) is added to buffer the pH of the medium (in liquid medium case) [47], elimination of  $\text{KH}_2\text{PO}_4$  may not directly affect the quality of secondary metabolites, i.e., cellulase ability. Therefore, in the subsequent hydrolysis ability test, cellulase hydrolysis ability and the detailed activities such as CMCase,  $\beta$ -glucosidase, and xylanase activities derived from standard Mandel's medium,

the medium without trace element, and the medium without trace elements and  $\text{KH}_2\text{PO}_4$  were compared. The cellulase activity (FPU) of RS was slightly higher than that of WS; therefore, hydrolysis tests were carried out using cellulase obtained from RS.

### 3.4 Hydrolysis ability test with cellulase produced from RS medium by *T. reesei*

Hydrolysis tests for cellulosic materials were performed using a cellulase solution prepared from RS via the standard Mandel's medium (with trace elements), RS via a standard medium without trace elements, and RS via a standard medium without trace elements and  $\text{KH}_2\text{PO}_4$ , respectively, to confirm the cellulose in the RS biomass hydrolysis ability of the cellulase produced by *T. reesei* ATCC 56765 under SSF. Cellulase was extracted from each medium on day 6. As a reference for hydrolysis ability, commercial cellulases such as Acremonium cellulase (AC) was used. Before the hydrolysis test of the cellulose in RS, to reduce the high lignin content, alkaline treatment with 3 wt% of NaOH solution was performed on RS at 121 °C for 30 min. The chemical compositions of the raw and alkali-treated RS are summarized in Table 4. After the treatments, the content of lignin in the RS decreased to 1.5%; however, the cellulose content increased to 64.3%. Low lignin content is advantageous for enzymatic hydrolysis by cellulase [48, 49]. Figure 7 shows the hydrolysis yield time profiles from the alkali-treated RS at incubation times during 120 h by produced cellulase and commercial cellulase (AC). The substrate concentration and cellulase loading were 50 g/L and 26 FPU/g-substrate, respectively. A similar high hydrolysis yield was observed, and almost 100% of hydrolysis yield were attained at 96 h for cellulose produced from RS via a standard medium without trace elements and RS via standard medium without trace elements and  $\text{KH}_2\text{PO}_4$ . With the cellulase dosage of 26 FPU/g-substrate, corresponding individual cellulase activities from cellulase produced from 3 kinds of medium and AC were summarized in Table 5. The produced cellulase contained similar CMCase and higher xylanase activities than those of commercial cellulase AC. In contrast, AC contained

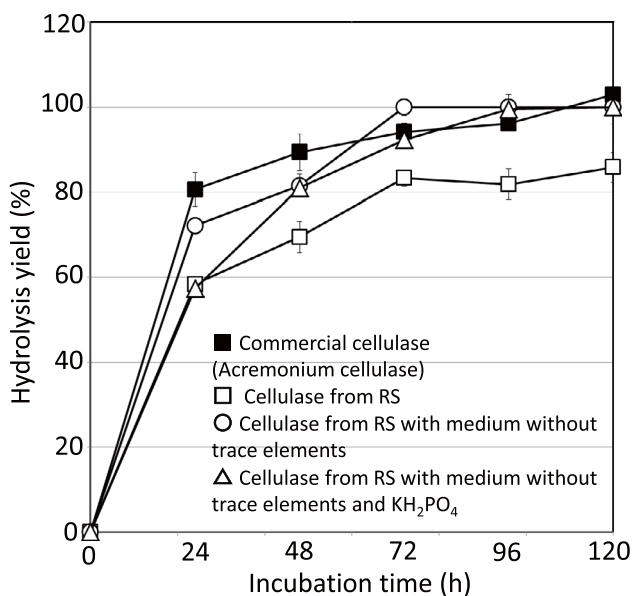
**Table 3** Metal elemental characterization of the raw wheat straw and rice straw washed with water

Substance (mg/g-raw material)	Standard medium (modified Mandel's medium)	Wheat straw	Rice straw (washed with water)
Ca	5.48	1.97	3.65
K	28.7	13.3	3.83
Mg	0.053	0.63	1.56
Fe	0.05	0.17	0.16
Zn	0.016	0.012	0.035
Mn	0.026	0.04	0.18
P	22.7	0.71	0.47

**Table 4** Chemical composition of raw and alkaline-treated (3 wt% of the NaOH solution) RS

	Holocellulose		Lignin	Ash
	Cellulose	Hemicellulose		
Raw	35.1 ± 0.3	31.7 ± 1.4	22.0 ± 0.4	19.2 ± 0.01
Alkaline-pretreated	64.3 ± 0.3	22.2 ± 0.3	1.5 ± 0.2	2.3 ± 0.01





**Fig. 7** Hydrolysis ability test of alkaline-treated rice straw by the produced cellulase from RS and the commercial *Acremonium* cellulase (AC) at a substrate concentration of 50 g/L and cellulase loading of 26 FPU/g-substrate

higher  $\beta$ -glucosidase of 1169 U/g-enzyme than that of produced cellulase from RS, 20–55.6 U/g-enzyme. Based on this result, it was confirmed that the cellulase quality (hydrolysis ability) derived from Mandel’s medium without trace element and  $\text{KH}_2\text{PO}_4$  was similar to that derived from Mandel’s medium without trace element, and  $\text{KH}_2\text{PO}_4$  had no effect on the quality of cellulase. However, although the cellulase produced exhibited low  $\beta$ -glucosidase activities, the actual hydrolysis yields of alkali-treated RS among the cellulase produced from RS medium and AC were similar. Hu et al. reported that cellulase activity produced via SSF from textile waste as a fermentable substrate comprised a CMC<sub>50</sub> of 254 U/g-substrate and  $\beta$ -glucosidase of 31,500 U/g-substrate under 25 FPU/g-substrate, which were higher than those of a commercial cellulase, Celluclast 1.5 L from Novozymes (USA), with a CMC<sub>50</sub> of 114 U/g-substrate and

$\beta$ -glucosidase of 1633 U/g-substrate under 25 FPU/g-substrate. The actual hydrolysis yield of textile waste, cotton/polyester = 80/20, was lower (65%) than that of Celluclast 1.5 L (75%) at an incubation time of 72 h [21]. Kabel et al. reported that enzymatic hydrolysis is more dependent on the characteristics of the substrate than on measured standard enzyme activities [50].

### 4 Conclusion

This study demonstrated that agricultural wastes such as WS and RS are potential substrates for cellulase production through SSF using *T. reesei*. We developed a cellulase production medium that was easy to operate based on the amount of trace metal contents, i.e., ash, in WS and RS. Regarding the role of lignin in lignocellulosic materials, components besides cellulose, namely, lignin and hemicellulose, are also presumed to have certain functions in cellulase production in the cellulase producer. Wang et al. reported that cellulase production was performed by *A. niger* and *T. reesei* using cotton or a cotton and polyester mixture as carbon substrates. Where only cotton or the cotton and polyester mixture was used as substrates (no lignin and hemicellulose), cellulase activity was low at 1.5 FPU/g-cotton by *A. niger* and 1.0 FPU/g-mixture by *T. reesei*. Alternatively, cellulase activity from cotton medium with sawdust (0.1% w/v) or wheat bran (1% w/v) increased and was 8.5 FPU/g-cotton and 3.4 FPU/g-cotton, respectively. Moreover, the cellulase activity from cotton and polyester mixture medium with either sawdust (0.1% w/v) or wheat bran (1% w/v) increased and was found to be 7.1 FPU/g-mixture and 8.1 FPU/g-mixture, respectively [51].

Furthermore, the cellulase produced from easy-to-handle RS medium was found to be efficient in hydrolyzing agricultural wastes indicating their potential for sustainable utilization for the remediation of cellulose biomass into valuable products. Scale-up experiments and cost and energy estimations are necessary components for the commercialization of these processes. This will be the focus of the next study.

**Table 5** Enzyme dosages for the hydrolysis of alkaline-treated RS using commercial cellulase and produced cellulase from RS medium

Enzyme dosages	Commercial cellulase (AC)	Cellulase from RS with standard Mandel’s medium	Cellulase from RS with Mandel’s medium without trace elements	Cellulase from RS with Mandel’s medium without trace elements and $\text{KH}_2\text{PO}_4$
FPU (FPU/g-substrate)	26.0	26.0	26.0	26.0
CMC <sub>50</sub> (U/g-substrate)	2094	1038	2626	1669
$\beta$ -glucosidase (U/g-substrate)	1169	20.0	55.6	34.0
Xylanase (U/g-substrate)	9724	15,951	27,370	14,020

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**Author contribution** All authors contributed to the study conception and design. The roles are shown as follows: conceptualization, all of authors; methodology, C. Sasaki and K. Matsuura; formal analysis and investigation, C. Sasaki; writing — original draft preparation, C. Sasaki; writing — review and editing, all of authors; funding acquisition, K. Matsuura and T. Omasa; and supervision, T. Omasa. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data availability** Not applicable.

## Declarations

**Ethical approval** Not applicable.

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