

***Trichoderma reesei* Cellulase Complex in Hydrolysis of Agricultural Waste of Grapefruit Peel and Orange Peel**

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Previous attempts have already been performed for the production of sugars and, later, bioproducts from orange peel using different *Trichoderma reesei* commercial cocktails in combination with other kinds of enzymes. In this study, the feasibility of simple pretreatments combined with enzymatic treatments was compared between grapefruit inner peel (GFIP), orange inner peel (OIP), grapefruit whole peel (GFWP), and orange whole peel (OWP). The four biomaterials were characterized with respect to the contents of cellulose and hemicellulose, elemental analysis, and Fourier transform infrared (FTIR) spectrometry. The 3,5-dinitrosalicylic acid assay demonstrated that GFIP and OIP produced 31.7% and 34.9% more reducing sugar than GFWP and OWP, respectively. Further investigation of the bioprocess showed the optimal conditions include the following: (i) a solid to liquid ratio of 4%, (ii) enzymatic activity of 0.075 U/mL, and (iii) reaction at 55 °C and a pH of 5.0. Moreover, the major products after cellulolytic hydrolysis were fructose, glucose, and cellobiose. This study provides an alternative and effective approach to extend the utilization of agricultural waste in the fields of food and energy.

Keywords: Grapefruit peel; Orange peel; *Trichoderma reesei* cellulase; Agricultural waste; Sugar

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INTRODUCTION

Recently, attention has been directed toward biomass, which is an alternative energy source, as there is a large amount of agricultural waste for industrial operations and trials (Sud *et al.* 2008). Agricultural biomass is a relatively broad category of biomass that includes food-based crops (corns and canes), non-food based crops (leaves, stalks, and peels of fruit), perennial grasses, and animal waste (Chandra *et al.* 2012). Furthermore, agricultural biomass can only be considered sustainable if it is economically efficient and profitable, socially viable, and provides a net benefit in improving the environmental performance and rural development. It is compatible with policy goals for agriculture, environment, energy, industry, and in the wider context of trade liberalization and sustainability (Hamelinck *et al.* 2005). Thus, agricultural waste materials are economical and eco-friendly due to their unique chemical composition, availability in abundance, renewability, and low cost. Cellulose, hemicellulose, and simple sugars from lignocellulosic biomass (LCB) show potential capacity for sugar production. Moreover, the residue of LCB can be further used for heavy metal bio-sorption, as well as nutritional and functional components for some kinds of microorganism (Kim and Pan 2010; Ng *et al.* 2013).

One of the most abundant agricultural wastes is the fruit peel. It is typically generated in large quantities by the fruit juice industry. These materials have received more and more scientific attention, especially grapefruit peel and orange peel. Orange crops are one of the most popular fruits and are cultivated all around the world. The orange whole peel (OWP) represents roughly 20 to 30% of its total biomass (Hou *et al.* 2013). In contrast, grapefruit juice processing is a much smaller industry than orange juice processing as the agricultural areas were 1 million metric tons of grapefruit *versus* 10 million metric tons of oranges in 2003 and 2004 (Saeed *et al.* 2010). However, grapefruit is cultivated in all tropical and subtropical regions of the world with approximately 4 million metric tons annual production of grapefruit whole peel (GFWP). It represents approximately 40% of the total biomass and can be used to produce ethanol or other products. In addition, grapefruit and oranges are often processed simultaneously in processing plants, resulting in peel waste from both fruits being blended together (Saeed *et al.* 2010). The OWP and GFWP contain water-soluble and -insoluble monomers and polymers. The water-soluble fraction contains glucose, fructose, sucrose, and some xylose, while pectin, cellulose, hemicellulose, and lignin constitute between 50 and 70% of the insoluble fraction (Namasivayam *et al.* 1996; Mostaedi *et al.* 2013). Therefore, they are an abundant, cheap, and readily available lignocellulosic biomass and may be hydrolyzed to produce sugars through cellulases and other enzymes (Jourdiier *et al.* 2013).

Cellulosic enzymatic hydrolysis of biomass, coupled with fermentation to convert the lignocellulosic agricultural wastes into bioproducts, is widely applied (Hamelinck *et al.* 2005; Chandra *et al.* 2012). Cellulase production from agricultural wastes by bacteria, fungi, yeast, and other microorganisms has also been reported (Maki *et al.* 2009; Pagán *et al.* 2010; Yoon *et al.* 2013). Among them, the filamentous fungus *Trichoderma reesei* (TR) is currently used for the industrial production of cellulolytic enzyme cocktails because of its high capacity for secretion (Singhania *et al.* 2010; Scott-Craig *et al.* 2011). However, few studies have focused on the use of cellulolytic enzyme cocktails in hydrolysis of fruit peels to directly produce sugar, which can be fermented by *Saccharomyces cerevisiae* to produce ethanol (Grohmann *et al.* 1994; Wilkins *et al.* 2007; Zhao *et al.* 2012).

As the whole peels of fruit include phenolic and other compounds, inner peels are considered more attractive in renewable usage. To our best knowledge, most studies of the pretreatment of agricultural wastes have focused on the screening of novel enzymes and cocktails (Grohmann and Baldwin 1992; Wilkins *et al.* 2007), and only rare studies have compared such topics as whole peels and inner peels.

The aim of the present study is to evaluate and compare the efficiency of sugar production from grapefruit or orange peels and inner grapefruit or inner orange peels hydrolyzed by a *T. reesei* cellulases complex. The specific objectives are as follows:

- (1) to characterize the product using a variety of analytical methods, such as elemental analysis, component analysis, Fourier transform infrared (FTIR) spectroscopy, and high performance liquid chromatography (HPLC);
- (2) to investigate the effect of enzyme loadings, substrate concentrations, temperature, and pH on sugar yields; and
- (3) to provide an alternative approach for cost-effective usage of agricultural bioresources.

EXPERIMENTAL

Materials

Sample Preparation

The grapefruit and orange were bought from supermarkets, and the fruit was removed to obtain grapefruit whole peel (GFWP) and orange whole peel (OWP). The outside and yellow peels were pared to obtain grapefruit inner peel (GFIP) and orange inner peel (OIP). All the peels were dried in an oven at 70 °C for 24 h and then crushed into approximately 2-mm pieces and milled into small fractions by a sieve shaker. All the materials were screened by an 80-mesh sieve.

Composition Analysis

Elemental analysis

Elemental analyses of carbon (C), nitrogen (N), and hydrogen (H) were carried out on a Vario EL III Element Analyser (Germany). Approximately 3 to 5 mg of the biomaterials (GFIP, GFWP, OIP, and OWP) were wrapped in aluminum foil and placed in the analyzer at 900 °C (Ershova *et al.* 2012). Acetanilide was used as the calibration material.

Cellulose and hemi-cellulose

The extraction of crude alkali-soluble hemicellulose was performed according to Zhao *et al.* (2012) with some modifications. The holocellulose from GFWP, GFIP, OIP, and OWP was extracted for 2.5 h with stirring using a 10% NaOH solution at a solid to liquid ratio of 1:100 at 55 °C. The insoluble residues were filtered through a nylon cloth on a Büchner funnel and washed with distilled water until the pH of the filtrate was neutral. Each filtrate was concentrated to about 100 mL, and the pH was adjusted to 5.0 using 6 M HCl. The solution was left to stand for 12 h and centrifuged at 2000 x g for 20 min. The precipitate designated as crude hemicellulose was washed with 70% ethanol and freeze-dried.

FTIR spectrometry analysis

The biomaterials were analyzed by FTIR (Perkin Elmer, Spectrum one; USA) in the mid-IR region of 400 cm⁻¹ to 4,000 cm⁻¹ with a scan speed of 16 (Chittur 1998). The samples were mixed with spectroscopically pure KBr in the ratio of 5:9. Prior to the measurement with FTIR, all materials were placed into the oven at 70 °C for 24 h.

Characterization of *T. reesei* Cellulase Complex

Enzyme assays of FPase, CMCase, and beta-glucosidase

T. reesei cellulase, a commercial enzyme, was purchased from Sigma (C2730). The enzymatic activities of FPase, CMCase, and beta-glucosidase (BGL) or cellobiose hydrolase (CBU) were determined using 1% Whatman no.1 filter paper (FP), 1% carboxy-methyl-cellulose sodium salt (CMC), and 8 mM cellobiose in a sodium acetate buffer (50 mM, pH 5.0), respectively. A 3-μL amount of enzyme was added to 1 mL of solution and incubated at 55 °C for 15 min for CMC and 1 h for FP or cellobiose. The concentration of reducing sugar produced was determined with the dinitrosalicylic acid method (Miller 1959) at 540 nm (VersaMax™ microplate reader, Molecular Devices, CA). One unit of the activity corresponds to 1 μmol of glucose released *per* minute (Ng *et al.* 2010). Furthermore, SDS-PAGE, Native-PAGE with MUG-zymogram, and tandem

mass liquid chromatography were used to determine protein profiles of the *T. reesei* cellulase complex (Ng *et al.* 2011).

HPLC and DNS analysis for sugar content

Concentrations of cellobiose (CB), glucose, and fructose in reaction samples were determined as described previously (Ng *et al.* 2011). In brief, the analysis was performed using a 1200 series HPLC system (Agilent, USA), with a 3300 evaporative light scattering (ELS) detector (Alltech, USA) after nebulization of 80% at 60 °C and evaporation by nitrogen at 40 psi. Separation of cellobiose, glucose, fructose, and xylose was carried out using a Shodex column (Asahipak, NH2P-50 4E, 4.6 mm I.D. x 250 mm; Showa Denko, Japan) equilibrated at 40 °C, resulting in a retention time of 9.5, 7.8, 6.6, and 5.8 min, respectively. The mobile phase was a mixture of acetonitrile/Mini-Q water (7/3, v/v) at a flow rate of 1 mL/min at a constant concentration (isocratic elution) for 10 min. Peak areas for all sugars showed linear correlation with standard curves within the range of 1 to 4.5 mM. The relative standard deviation of three repeated injections was normally below 3%. Endoglucanase activity and total reducing sugar were determined through the DNS method (Miller 1959).

Hydrolysis reaction of GFIP, GFWP, OIP, and OWP using TR

Hydrolyses were carried out in 1 mL of sodium acetate buffer (50 mM, pH 5.0) with shaking at 1500 rpm and 55 °C by mixing 0.3 FPU of TR and 1% (w/v) GFIP, GFWP, OIP, and OWP for 2, 4, and 24 h. The hydrolysates were placed on ice for 15 min to quench enzymes' activity and centrifuged at 8,000 x g for 10 min immediately. The supernatants were monitored using the DNS method with glucose as a standard. All experiments were performed in triplicate, and the mean values are reported.

Optimal Reaction Conditions

Optimal solid to liquid ratio of hydrolytic reaction

Hydrolyses were carried out for 24 h in 1 mL of sodium acetate buffer (50 mM, pH 5.0) with shaking at 1500 rpm and 55 °C by mixing 0.3 FPU of TR and 1%, 2%, 3%, or 4% (w/v) of GFIP, GFWP, OIP, and OWP.

Enzyme loading

Hydrolytic reactions were carried out in 1 mL of sodium acetate buffer (50 mM, pH 5.0) with shaking at 1500 rpm and 55 °C for 4 and 24 h by mixing with 0, 0.075, 0.15, 0.3, and 0.6 FPU of TR and 1% (w/v) of substrates.

Protein adsorption

Protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin as the standard. The protein adsorption was obtained by subtracting the original proteins amount from the amount of remaining proteins. All experiments were performed in triplicate, and the mean values are reported.

RESULTS AND DISCUSSION

As shown in Table 1, inner peels (OIP and GFIP) had a higher cellulose content, hemicellulose content, and C/N rate than whole peel (OWP and GFWP). From the

elemental analysis, GFIP had 3.81% higher cellulose content, 3.09% higher hemicellulose content, and 43.8% higher C/N ratio than GFWP, while OIP had 3.3% higher cellulose content, 2.96% higher hemicellulose content, and 35.9% higher C/N ratio than OWP. The amount of nitrogen was significantly different between whole peels and inner peels. Most phenolic compounds and 70% of carotenoids are located in the outer layer of orange peel, suggesting the outer peel had more impurities and nitrogen, thus affecting the cellulose content and C/N ratio (Moussaid *et al.* 2000; Inci 2005). Conversion of the whole fruit peels to value added products requires pectinase and complex cellulose; their synergistic effect has been reported, with most pectin localized in the outer peels and most cellulose in the inner peels (Grohmann *et al.* 1992). Herein, a simple way to remove the outer peels as well as compare the whole peels and inner peels with digestion by cellulase was first investigated. Notably, the four biomaterials obtained with a high C/N ratio, *i.e.*, 60.7% of OWP, 96.6% of OIP, 60% of GFWP, and 103.8% of GFIP, are obviously suitable for bio-energy usage.

Table 1. Cellulose, Hemi-Cellulose, and Elemental Analysis of Carbon (C), Nitrogen (N), and Hydrogen (H) For OWP, OIP, GFWP, and GFIP

Samples	Cellulose (wt%)	Hemi-cellulose (wt%)	C/N Ratio	Element content (wt%)		
				C	N	H
OWP	20.06 ± 0.21	16.25 ± 0.21	60.7 ± 1.8	41.76 ± 0.08	0.69 ± 0.02	7.26 ± 0.18
OIP	23.42 ± 0.25	19.21 ± 0.14	96.6 ± 7.1	41.06 ± 0.18	0.43 ± 0.03	6.70 ± 0.18
GFWP	20.75 ± 0.08	17.34 ± 0.13	60.0 ± 0.2	39.84 ± 0.17	0.64 ± 0.04	6.60 ± 0.24
GFIP	24.56 ± 0.27	20.43 ± 0.17	103.8 ± 11.0	39.06 ± 0.01	0.38 ± 0.04	6.62 ± 0.33

In Fig. 1, GFIP, GFP, OIP, and WP display similar FTIR spectra.

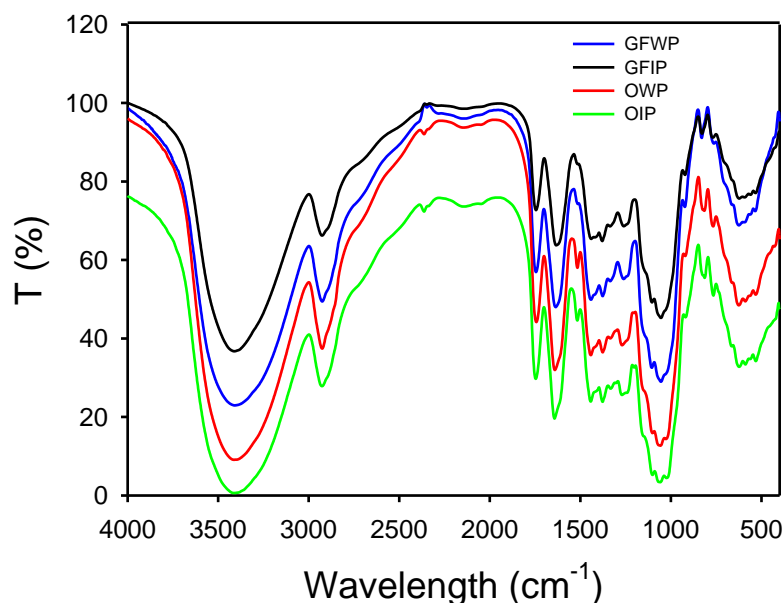


Fig. 1. FTIR analysis of GFIP, GFWP, OIP, and OWP

The broad absorption peak at $3,429\text{ cm}^{-1}$ was assigned to the stretching of the O-H group due to inter- and intra-molecular hydrogen bonding of polymeric compounds (macromolecular associations), such as alcohols and carboxylic acids, as in pectin and cellulose. In addition, the peak at $1,066\text{ cm}^{-1}$ reflects the C–OH stretching of alcohol groups and carboxylic acids. The existence of carboxyl and hydroxyl groups is consistent with the FTIR spectrum of other peels (Muhammad *et al.* 2009).

To determine different kinds of enzymatic activities of TR, the hydrolysis of CMC to reducing sugar for EG activity by DNS method, the hydrolysis of filter paper to cellobiose for CBH I and CBH II activity, and the hydrolysis of cellobiose to glucose for BGL activity by HPLC were further analyzed. As a result, 254.1 U/mL of CBH I and CBH II, 1089.1 U/mL of EG, and 44.1 U/mL of BGL in *T. reesei* cellulase complex were observed. In fact, CBH I, CBH II, and EG II are the three main components of the TR cellulase system, representing 60%, 20%, and 12% of total cellulase proteins, respectively (József *et al.* 1998). It has been reported that a supplement of BGL showed a synergistic effect between BGL and TR cellulase to accomplish the cellulosic bioconversion process (Ng *et al.* 2011). In contrast, the commercial TR cellulase in this study, including CBH, EG, and BGL (Fig. 2), is different from that used in previous research; however, it is suitable for direct hydrolysis of cellulosic materials.

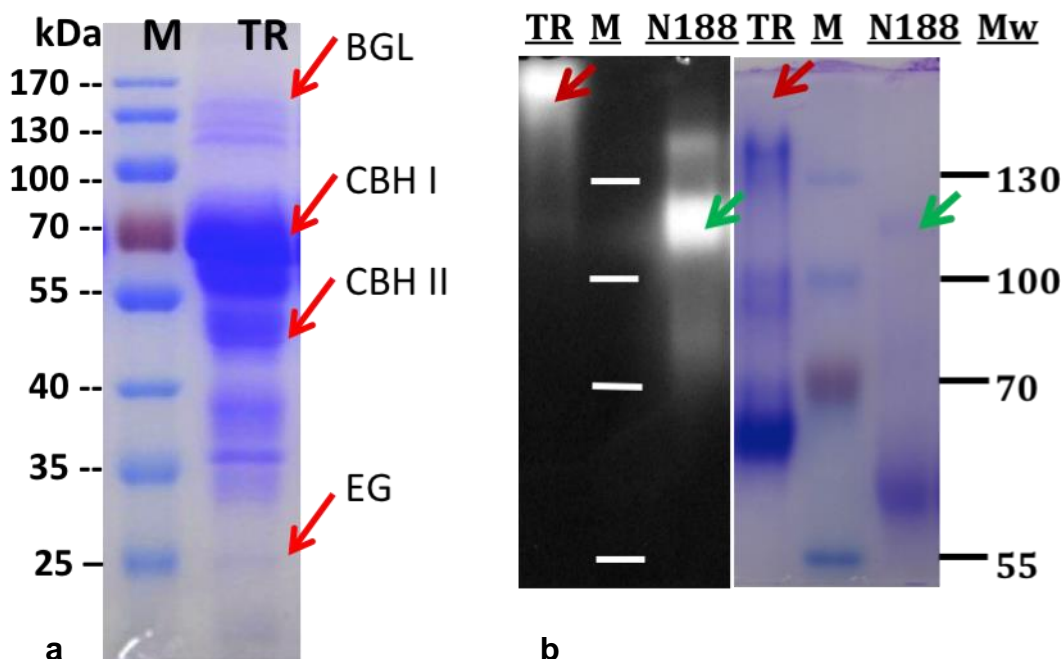


Fig. 2. (a) SDS-PAGE profiles of *Trichoderma reesei* multi-component cellulases. The proteins were extracted and subjected to 10% SDS-PAGE followed by Coomassie blue R-250 staining. The four selective proteins indicated by red arrows were further analyzed by tandem mass liquid chromatography (b) Native PAGE analysis with Coomassie blue R-250 staining (right) and MUG-zymogram (left) of *T. reesei* and Novo-188 were used to analyse the active β -glucosidase, which are indicated by red arrow for *T. reesei* and green arrow for Novo-188, respectively.

The effect of reaction time on yields of reducing sugar (RS) is shown in Table 2. The GFIP and OIP had higher yields of RS than GFWP and OWP. The self-hydrolysis of the four biomaterials by water are ranked as GFIP = OIP > GFWP > OWP, in which RS

corresponds to approximately 12 mM, 9.8 mM, and 6.8 mM within 2 h to 24 h. The hydrolytic RS was dramatically increased after addition of TR cellulase at 24 h, at which point the RS was 31.6 mM for GFIP, 24.0 mM for GFWP, 30.5 mM for OIP, and 22.6 mM for OWP. Moreover, the smaller amount of residue determined by dry weight, *i.e.*, 27.4% for GFIP and 52.5% for OIP compared to 41.0% for GFWP and 61.5% for OWP, suggested the GFIP and OIP can be digested much effectively. Thus, the hydrolytic results are consistent with the higher cellulose content, hemicellulose content, and C/N rate of inner peels in Table 1. The HPLC analysis further revealed the sugar composition by TR cellulase hydrolysis of the four biomaterials. As indicated in Table 3, the concentrations of fructose were of similar levels in all kinds of peel.

Table 2. Comparison of Reducing Sugar between GFIP, GFWP, OIP, and OWP by Water Extraction and *T. reesei* Cellulase Hydrolysis at 4 h and 24 h

Conditions	GFIP		GFWP	
	RS (mM)	Residue (wt%)	RS (mM)	Residue (wt%)
Water-4h	12.6 ± 1.2	67.1 ± 2.8	9.3 ± 0.6	73.5 ± 1.7
Water-24h	12.0 ± 0.3	68.4 ± 2.9	9.8 ± 0.2	73.9 ± 1.5
TR-4h	23.8 ± 0.4	39.5 ± 1.5	17.8 ± 0.4	53.3 ± 2.1
TR-24h	31.6 ± 0.8	27.4 ± 2.1	24.0 ± 1.5	41.0 ± 1.4
	OIP		OWP	
	RS (mM)	Residue (wt%)	RS (mM)	Residue (wt%)
Water-4h	12.1 ± 0.4	84.2 ± 1.4	6.8 ± 0.2	90.1 ± 1.3
Water-24h	12.5 ± 0.3	85.2 ± 1.1	6.7 ± 0.4	91.2 ± 1.0
TR-4h	20.1 ± 0.8	70.3 ± 1.6	13.4 ± 1.4	78.2 ± 1.4
TR-24h	30.5 ± 0.5	52.5 ± 1.9	22.6 ± 2.1	61.5 ± 1.8

Table 3. Comparison of Sugar Composition with HPLC Analysis between GFIP, GFWP, OIP, and OWP by Water Extraction and *T. reesei* cellulase Hydrolysis at 4 h and 24 h

Reaction	GFIP				GFWP			
	Fructose (mM)	Glucose (mM)	Cellobiose (mM)	Total (mM)	Fructose (mM)	Glucose (mM)	Cellobiose (mM)	Total (mM)
Water	4.25 ± 0.07	5.05 ± 0.07	8.36 ± 0.06	17.67 ± 0.14	3.17 ± 0.02	3.34 ± 0.11	9.40 ± 0.17	15.91 ± 0.31
TR-4h	10.75 ± 0.14	10.08 ± 0.19	8.28 ± 0.14	29.12 ± 0.46	9.37 ± 0.12	7.00 ± 0.05	9.17 ± 0.12	25.54 ± 0.45
TR-24h	11.21 ± 0.04	12.93 ± 0.19	8.05 ± 0.12	32.20 ± 0.36	9.84 ± 0.15	9.05 ± 0.06	8.84 ± 0.27	27.73 ± 0.34
	OIP				OWP			
	Fructose (mM)	Glucose (mM)	Cellobiose (mM)	Total (mM)	Fructose (mM)	Glucose (mM)	Cellobiose (mM)	Total (mM)
Water	4.28 ± 0.04	3.95 ± 0.07	2.76 ± 0.05	10.99 ± 0.16	4.24 ± 0.04	3.79 ± 0.08	3.13 ± 0.05	11.17 ± 0.16
TR-4h	10.47 ± 0.03	8.36 ± 0.13	2.74 ± 0.06	21.57 ± 0.22	10.25 ± 0.13	7.07 ± 0.13	3.09 ± 0.07	20.42 ± 0.33
TR-24h	11.37 ± 0.13	12.77 ± 0.14	2.65 ± 0.07	26.79 ± 0.28	10.91 ± 0.09	9.27 ± 0.08	3.06 ± 0.05	23.24 ± 0.22

The concentrations of glucose increased with time, while cellobiose slightly decreased. This was caused by the synergism between BGL and EG or CBH in TR. Furthermore, the BGL activity was inhibited by cellobiose; thus, the hydrolytic glucose was trend to equilibrium at the range of 9.05 mM to 12.93 mM as in Table 3, and was similar to our previously results that BGL activity would be inhibited by cellobiose (Ng *et al.* 2011). Consequently, GFIP was judged to be the best substrate for sugar production among grapefruit and orange peels. The four biomaterials could be hydrolyzed *via* TR to obtain more fructose, glucose, and cellobiose.

We also analyzed the effect of solid to liquid ratio for TR hydrolysis of different peels. As shown in Fig. 3, the RS production increased linearly from 1% to 4% of substrates. In spite of this, GFIP and OIP are the better substrates for sugar production. Finally, the optimal solid to liquid ratio is 4% (w/v) because all substrates (*i.e.*, solid part) are occupied to 80% of reaction volume at this concentration (data not shown).

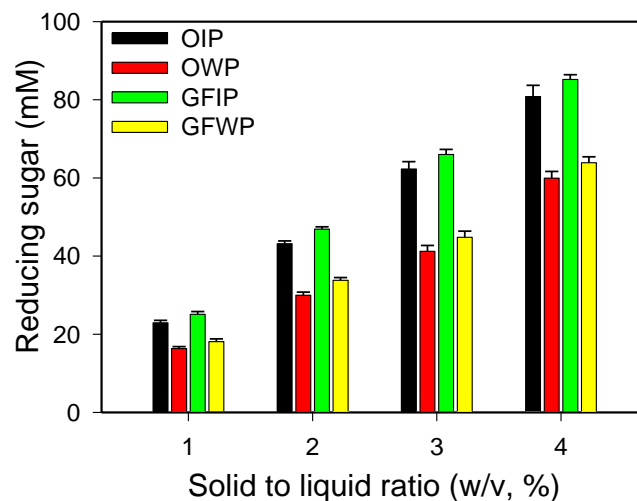


Fig 3. Effect of solid to liquid ratio of biomaterials by 0.3 FPU of *T. reesei* cellulase hydrolysis for 24 h at 55°C

Enzyme loading amount is the key factor in the hydrolysis of cellulosic substrates (Arantes and Saddler 2011). An enzyme dosage of 1%, based on substrate weight, was selected based on an assumption of linear performance over the range from 1 to 4%. As shown in Figs. 4a and 4b, RS productions were very slightly enhanced by increasing the enzyme loading amount, whether at 4 h or 24 h. The specific productivity (mM/U) decreased from 0.075 FPU to 0.6 FPU (Figs. 4c and 4d). After the analysis of protein adsorption, as in Figs. 4e and 4f, the higher protein adsorption occurred at a lower enzyme amount, *i.e.*, 0.075 U. The lower amount of enzyme facilitated a better enzymatic reaction, possibly because of the monolayer adsorption for enzyme loading (Maurer *et al.* 2012; Weiss *et al.* 2013).

As a result, better enzymatic hydrolytic performance of GFIP and OIP may also be caused by increased protein absorption in the inner peels, stimulating cellulase activities. After combining all the conditions, the optimal enzyme loading was 0.075 FPU of TR. The optimal pH of TR for hydrolysis of GFIP, GFWP, OIP, and OWP was at pH 5.0 (data not shown). In addition, the temperature effect showed that TR in hydrolysis of GFIP, GFWP, OIP, and OWP had the maximum activity at 55 °C. The temperature effects

had the same trend, consistent with other studies of cellulytic reactions using TR hydrolysis (Andreas *et al.* 1999).

Pectin hinders the hydrolysis of cellulose and hemicellulose in the grapefruit cell wall (Wilkins *et al.* 2007), which explains why the inner peels were more favorable for bioconversion by TR cellulase.

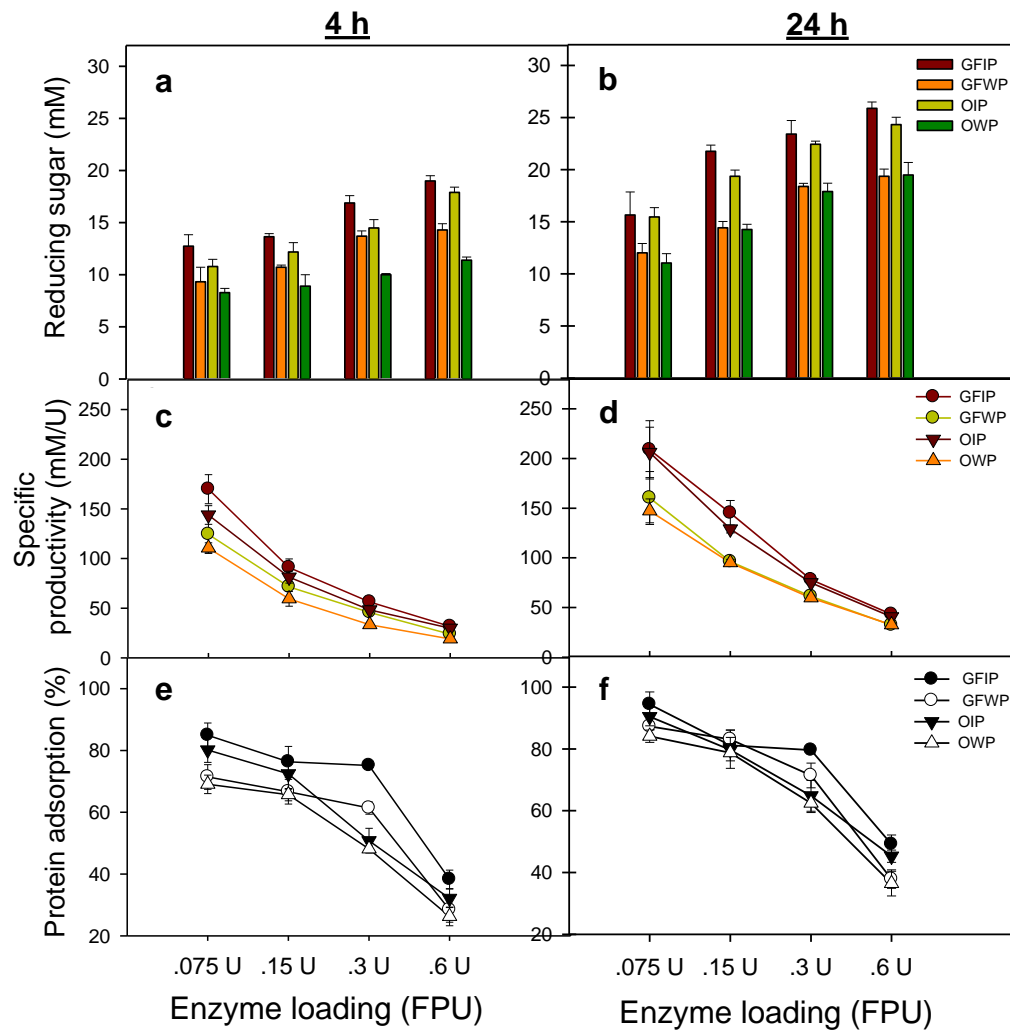


Fig. 4. *T. reesei* cellulase hydrolysis of 1% (w/v) of GFIP, GFWP, OIP, and OWP for (a and b) the reducing sugar, (c and d) specific productivity, and (e and f) proteins adsorption in different enzyme loading (i.e., 0.075, 0.15, 0.30, and 0.60 FPU) at 4 h and 24 h.

CONCLUSIONS

1. The GFIP, GFWP, OIP, and OWP can be hydrolyzed to monomeric sugars using *T. reesei* multi-component cellulases. The major components of sugars are fructose, glucose, and cellobiose, as shown by the results of HPLC.
2. The optimal conditions for these biomaterials use 0.075 U/mL of *T. reesei* cellulases in 1% (w/v) substrate at 55 °C and a pH 5.0. As a result, GFIP produced 31.7% higher RS than GFWP, while OIP produced 34.9% higher RS than OWP.

3. The elemental analyses indicated that inner peels had a higher C/N ratio; thus, GFIP and OIP are more favorable substrates in industrial bioconversion.
4. This study also provides an approach to the utilization of agricultural waste for food and energy applications.

ACKNOWLEDGMENTS

The authors are grateful to the financial support of the National Natural Science Foundation of China (21206141), the Fujian Provincial Department of Science & Technology (2012I0009), and the National High-Tech R&D Program of China (No.2014AA021701).

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Article submitted: June 13, 2014; Peer review completed: August 17, 2014; Revised version received: August 20, 2014; Accepted: August 23, 2014; Published: September 8, 2014.

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