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ORIGINAL PAPER

Saccharification of orange peel wastes with crude enzymes from new isolated *Aspergillus japonicus* PJ01

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Abstract This study investigated the saccharification of orange peel wastes with crude enzymes from *Aspergillus japonicus* PJ01. Pretreated orange peel powder was hydrolyzed by submerged fermentation (SmF) and solid-state fermentation (SSF) crude enzymes, the results showed that 4 % (w/v) of solid loading, undiluted crude enzymes, and 45 °C were suitable saccharification conditions. The hydrolysis kinetics showed that the apparent Michaelis–Menten constant $K_{m_{app}}$ and maximal reaction rate $V_{max_{app}}$ were 73.32 g/L and 0.118 g/(L min) for SmF enzyme, and 41.45 g/L and 0.116 g/(L min) for SSF enzyme, respectively. After 48 h of hydrolysis, the saccharification yields were 58.5 and 78.7 %, the reducing sugar concentrations were 14.9 and 20.1 mg/mL by SmF and SSF enzymes. Material balance showed that the SmF enzymatic hydrolysate was enriched galacturonic acid > arabinose > galactose > xylose, and the SSF enzymatic hydrolysate was enriched galacturonic acid > xylose > galactose > arabinose.

Keywords Saccharification · Orange peel · Enzyme · Kinetic parameter

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Introduction

Global orange production was 51.3 million metric tons in 2012/13, among which 22.4 million metric tons were processed into orange juice, essential oils and other by-products [1]. Orange peel is the principal solid by-product of the orange processing industry and constitutes about 50 % of the fresh fruit weight. On the other hand, waste orange peel contains a lot of fibers (pectin, lignin, cellulose and hemicellulose), flavonoids, D-limonenes, sugars, and proteins [2, 3]. Hydrolysis of the fibers for production of monosaccharides is the most important and critical step because monosaccharides serve as a base material for production of value-added products such as ethanol, vitamins and organic acids [4, 5].

Production of monomeric sugars from agricultural wastes using commercial pectinase, cellulase and beta-glucosidase has been reported [6–8]. However, separation and purification of industrial enzymes are expensive, and optimization of enzyme loadings is complicated. Recent years, fungal crude enzymes have been used for enzymatic hydrolysis of lignocellulosic biomass, such as sugar cane bagasse, rice straw, corn cob and potato pulp [9–12]. In our previous work, a new isolated strain *Aspergillus japonicus* PJ01 could produce crude enzymes under submerged fermentation (SmF) and solid-state fermentation (SSF) [13]. In this work, the crude enzymes were used for saccharification of orange peel wastes.

Furthermore, sound kinetic schemes have been studied for cellulose hydrolysis by cellulase [14, 15], however, only few literatures focused on the kinetics of pectin enzymatic hydrolysis [16–18], and there is no study about the hydrolysis kinetics of orange peel by fungal crude enzymes.

Therefore, the aims of this work are: (a) to study the enzymatic hydrolysis conditions of orange peel powder by

fungal crude enzymes from SSF and SmF; (b) to investigate the kinetic schemes of the crude enzymes; and (c) to perform a material balance on this bioconversion process.

Materials and methods

Microorganisms

Strain *A. japonicus* PJ01 (GenBank Accession number KF550286) was isolated from soil in Yuelu Mountain, Changsha City, China. Colonies showing degradation capacity for pectin, carboxymethyl cellulose (CMC), and xylan were assayed by plate screening using the Congo red overlay method, clearing zones appeared around the colony due to the hydrolysis of polysaccharides [19]. The strain was identified based on internal transcribed spacer (ITS) rDNA sequence analysis [20], the strain showed high identity (100 %) with *A. japonicus*. The approximate phylogenetic position of the strain is shown in Fig. 1.

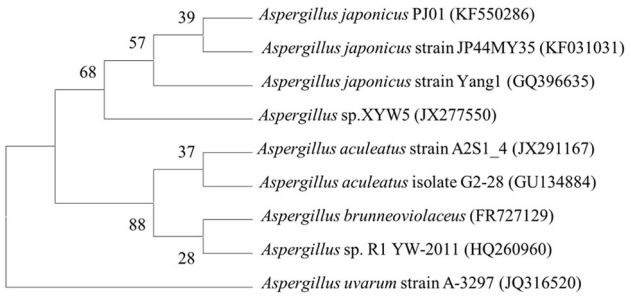

Fig. 1 Phylogenetic dendrogram for *A. japonicus* PJ01 and related strains based on the ITS rDNA sequence. *Numbers* following the names of the strains are accession numbers of published sequences

Raw materials

Orange peel was obtained locally (Changsha, China) and dried in an oven at 60 °C for 24 h, milled and sieved. Particles in size of less than 20 mesh were washed with distilled water (4 %, w/v) for 6 h at 30 °C with a rotating speed of 170 rpm, and the soluble material was separated by centrifugation. The thoroughly washed pretreated orange peel powder (POPP) was dried in an oven at 60 °C for 24 h.

Submerged fermentation (SmF) and solid-state fermentation (SSF)

SmF and SSF by *A. japonicus* PJ01 were carried out as the previous methods, orange peel and wheat bran were used as the sole substrates for crude enzymes production in SmF and SSF, respectively [13]. The cultured medium (pH were 5.0 ± 0.5) was filtered through coarse filter paper, the filtrate was centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatant was used as crude enzyme solution. After 72 h of cultivation under SmF and SSF, the maximal pectinase, carboxymethyl cellulase (CMCase), filter paper cellulase (FPase), and xylanase activities were obtained (Table 1). Commercial pectinase NCB-PE40 (Hunan Youtell Biochemical Co., Ltd. Yueyang, China) was used for comparison. Enzyme loading was 1.2 % (w/v) using 0.1 M acetate buffer (pH 5.0), and enzyme activities were also determined and shown in Table 1.

Table 1 Crude enzyme activities and enzymatic hydrolysis performances from SmF, SSF, and commercial enzyme (NCB-PE40)

	Enzyme activity (U/mL)					
	Pectinase		CMCase	FPase		Xylanase
SmF	39.2 ± 0.5		1.3 ± 0.1	0.1 ± 0.0		5.0 ± 0.2
SSF	48.3 ± 1.3		2.9 ± 0.1	0.2 ± 0.0		50.2 ± 0.1
NCB-PE40	50.2 ± 0.5		1.4 ± 0.0	0.1 ± 0.0		28.1 ± 0.3
	Reducing sugar (mg/mL)					
	Hydrolysis time (h)					
	0.5	2	6	12	24	48
SmF	2.7 ± 0.0	7.6 ± 0.1	14.9 ± 0.3	15.8 ± 0.1	15.6 ± 0.1	14.9 ± 0.1
SSF	2.5 ± 0.1	6.8 ± 0.1	15.3 ± 0.3	17.9 ± 0.2	19.4 ± 0.3	20.1 ± 0.8
NCB-PE40	3.0 ± 0.1	7.7 ± 0.1	14.9 ± 0.2	16.6 ± 0.2	18.0 ± 0.2	18.1 ± 0.1

The enzymatic hydrolysis experiments were carried out with a substrate loading 4 % (w/v) at 45 °C and 170 rpm for 48 h, values are presented as mean ± SD, $n = 3$

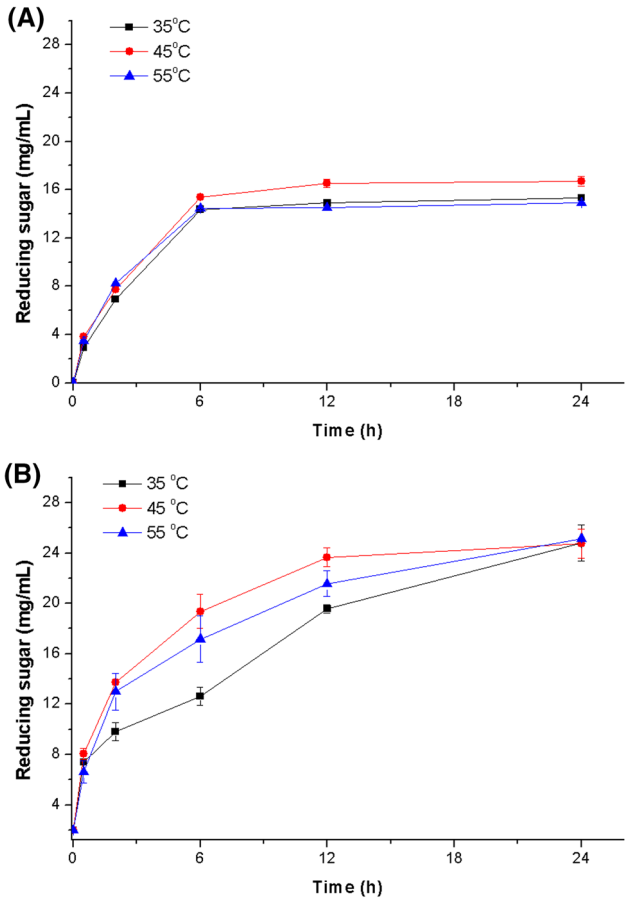

Fig. 2 Effect of temperature on saccharification of POPP [4 % (w/v)] using SmF (**a**) and SSF (**b**) crude enzymes. The experiments were carried out at 170 rpm for 24 h

Analytical methods

Pectinase activity was determined by the method of Díaz et al. [21]. Saccharifying cellulase (FPase and CMCase) assays followed the method recommended by IUPAC committee [22]. Xylanase activity assay was determined by the method of Bailey et al. [23]. One unit of pectinase, FPase, CMCase, and xylanase was defined as the amount of enzyme required to release 1 µmol of D-galacturonic acid, glucose, glucose, and xylose per minute under standard assay conditions, respectively. Reducing sugar content of the fermentation broth was determined by DNS method [24]. The carbohydrates were determined by phenol–sulfuric acid method using glucose as a standard [25].

Enzymatic hydrolysis

Enzymatic hydrolysis experiments were carried out in 100-mL Erlenmeyer flasks containing 20 mL solution. In the enzymatic hydrolysis temperature experiment, 4 % (w/v) of POPP was added to the undiluted crude enzyme solution at 35, 45, and 55 °C. In the enzyme dose experiment, 4 % (w/v) of POPP was added to diluted crude enzyme solution, which was diluted to 20–100 % (v/v) by distilled water. In the substrate loading experiment, 2–10 % (w/v) of POPP was directly added to the undiluted crude enzyme solution. All samples were incubated in an air incubator shaker at 170 rpm for different time intervals. Reaction was terminated by keeping the digests at 100 °C for 5 min. The solution was then centrifuged at 10,000×*g* for 15 min, and the supernatant was subsequently filtered through a 0.22 µm syringe filter for reducing sugar and carbohydrate analysis. Saccharification was calculated and expressed as shown in the method of Pal et al. [26].

$$\text{Saccharification}\,(\%) = \frac{\text{Reducing sugar}}{\text{Carbohydrates in substrate}} \times 0.9 \times 100$$

Carbohydrates in substrate were the sum of polysaccharides of orange peel (cellulose, hemicellulose, and pectin

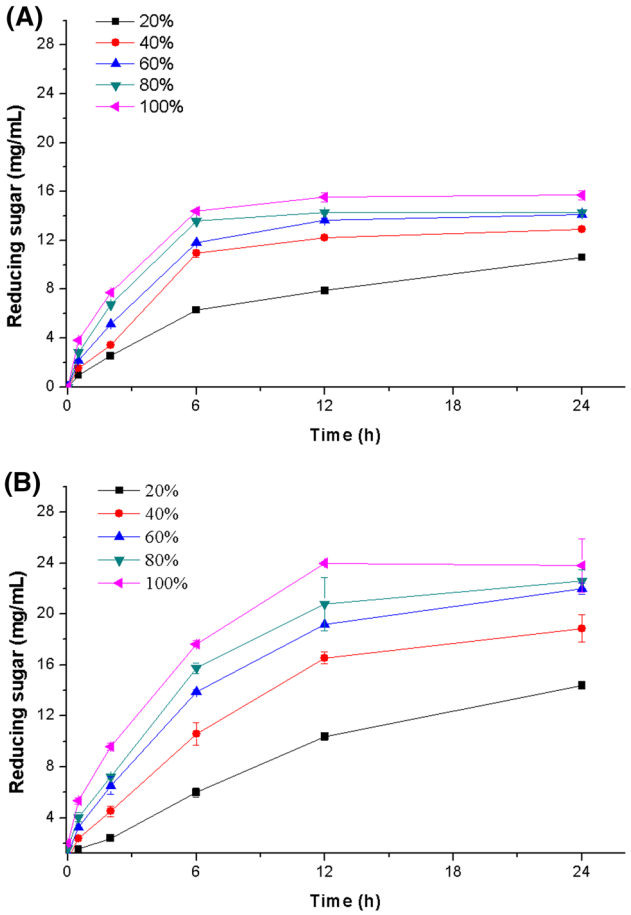

Fig. 3 Effect of enzyme dose on saccharification of POPP [4 % (w/v)] using SmF (**a**) and SSF (**b**) crude enzymes. The experiments were carried out at 45 °C and 170 rpm for 24 h

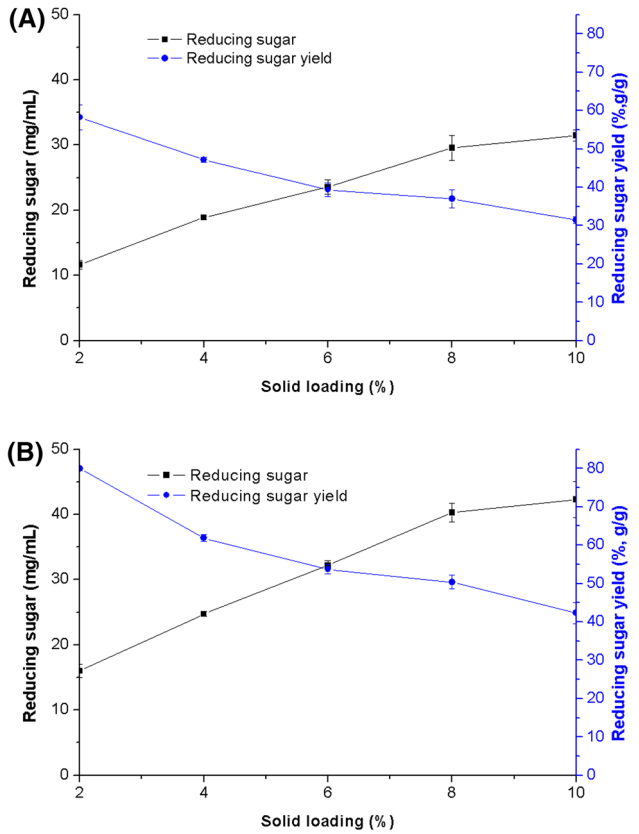

Fig. 4 Effect of substrate loading on reducing sugar concentration and yield using SmF (**a**) and SSF (**b**) crude enzymes. The experiments were carried out at 45 °C and 170 rpm for 24 h

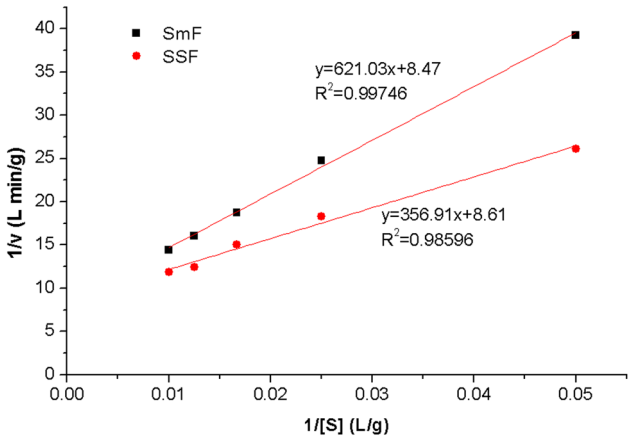

Fig. 5 Lineweaver–Burk linearization

were 14.0, 21.3, and 22.1 % (w/w), respectively), which accounted for 57.4 % of the substrate [13].

Data presented in this article show the mean of three replicates with their standard deviation (mean ± SD).

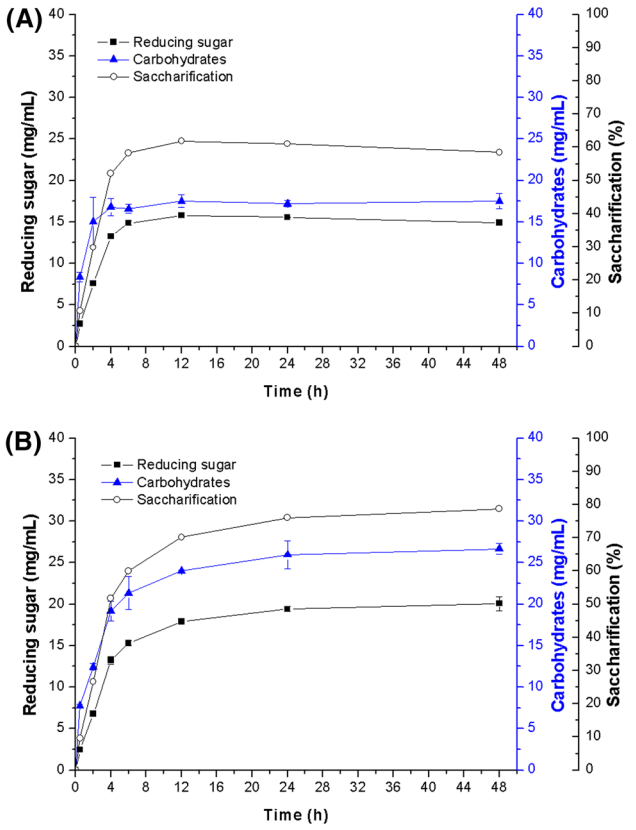

Fig. 6 Time course for yield of reducing sugars, carbohydrates, and saccharification from POPP [4 % (w/v)] using SmF (**a**) and SSF (**b**) crude enzymes. The experiments were carried out at 45 °C and 170 rpm for 48 h

Preparation of standard solution and 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization

PMP derivatization of monosaccharides was carried out as previous description with proper modification [27]. Briefly, 8 standard monosaccharides or hydrolyzed samples (100 µL) were dissolved in 0.3 M aqueous NaOH (100 µL) and a 0.5 M methanol solution (100 µL) of PMP was added to each. Fucose as an internal standard was added to each sample before the derivatization. Each mixture was allowed to react for 120 min at 70 °C, then cooled to room temperature and neutralized with 100 µL of 0.3 M HCl. The resulting solution was mixed with chloroform (1 mL), fully shook and allowed to stand for a while, then organic phase was removed and the extraction process was carried out three times. The aqueous layer was centrifuged at 10,000×g for 2 min and filtered through a 0.45 µm membrane for HPLC analysis.

HPLC equipment and conditions

The analysis of PMP-labeled monosaccharide was carried out on a Shimadzu LC-2010A HPLC system, a diode array detector (DAD), and LabSolutions chromatography workstation. The analytical column was a Thermo Hypersil C18 column (4.6 mm × 150 mm, 5 μm). The wavelength for DAD detection was 250 nm. Elution was carried out at a flow rate of 1.0 mL/min. The mobile phase A consisted of 40 mM KH_2PO_4 buffer (pH 7.0) and the mobile phase B was acetonitrile, A:B was 83:17, and the injection volume was 20 μL.

Results and discussion

Enzymatic hydrolysis of pretreated orange peel powder (POPP)

Effect of temperature and enzyme dose on the enzymatic hydrolysis of POPP

Figure 2 shows the effect of temperature on enzymatic hydrolysis of POPP [4 % (w/v)] using SmF and SSF crude enzymes, which indicates that 45 °C was the most suitable temperature for both crude enzymes. So, 45 °C was fixed in the following studies. Figure 3 shows the enzymatic hydrolysis of POPP under different dose of crude enzymes in the range of 20–100 % (v/v). At the same dose of the crude enzymes, the reducing sugar concentrations in SSF hydrolysates were higher than SmF hydrolysates after 24 h of incubation. For SmF enzyme, the reducing sugar concentration increased rapidly before 6 h, afterwards the growth was insignificant. As for SSF enzyme, the reducing sugar concentration increased rapidly until 12 h. Figure 3 also shows that reducing sugar concentration increased with enzyme dose. To obtain the maximum saccharification, undiluted crude enzymes were used in the following experiments. It is noteworthy that both SmF and SSF enzymes exhibited xylanase activity, which might contribute to the hydrolysis of hemicellulose. Previous studies demonstrated the hydrolysis of orange peel using the mixtures of pectinase and cellulase, but hydrolysis of orange peel by xylanase has not been reported [6, 28].

Effect of substrate loading on the enzymatic hydrolysis of POPP

Figure 4 shows the effect of substrate loading on reducing sugar concentration and yield under SmF and SSF enzymes, respectively. Reducing sugar concentration increased with solid loading, but the relationship between the reaction rate and the solid loading was not proportional when the solid loading was more than 8 % (w/v). The results imply that product inhibition might occur during the reaction. High reducing sugar concentration was usually found to be one of the main factors reducing hydrolysis rates and impeding high fermentable sugar yield [15]. Figure 4 shows a significant reduction in hydrolysis yield with the increase of solid loading. After 24 h, hydrolysis yields by SmF and SSF enzymes decreased from 58.3 to 80.0 % (2 % solid loading) to 31.5 and 42.3 % (10 % solid loading), respectively. In this work, end-product inhibition was significant, which was not the same with enzymatic

Table 2 Effect of hydrolysis time on monosaccharides production by crude enzyme from SmF and SSF

Time (h)	Production of monosaccharides[a] (mg/mL)							
	Man	Rhm	GlcUA	GalUA	Xyl	Glc	Gal	Ara
SmF								
0.5 h	0.03 ± 0.00	0.15 ± 0.02	0.04 ± 0.00	2.56 ± 0.10	0.25 ± 0.02	0.32 ± 0.00	0.31 ± 0.00	1.03 ± 0.05
2 h	0.03 ± 0.00	0.26 ± 0.02	0.04 ± 0.00	5.66 ± 0.15	0.40 ± 0.02	0.73 ± 0.05	0.71 ± 0.01	2.04 ± 0.03
6 h	0.05 ± 0.00	0.48 ± 0.04	0.03 ± 0.00	7.65 ± 0.15	0.75 ± 0.04	0.97 ± 0.07	1.63 ± 0.09	3.41 ± 0.11
12 h	0.08 ± 0.01	0.74 ± 0.05	0.04 ± 0.02	8.75 ± 0.20	1.17 ± 0.10	1.07 ± 0.09	2.22 ± 0.09	3.86 ± 0.08
24 h	0.08 ± 0.01	1.05 ± 0.07	0.03 ± 0.00	8.35 ± 0.16	1.58 ± 0.09	1.08 ± 0.08	2.30 ± 0.10	3.90 ± 0.20
SSF								
0.5	0.07 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.87 ± 0.09	0.89 ± 0.02	0.33 ± 0.03	0.37 ± 0.00	0.37 ± 0.04
2	0.11 ± 0.01	0.05 ± 0.00	0.06 ± 0.02	2.36 ± 0.13	1.46 ± 0.02	0.68 ± 0.02	0.74 ± 0.03	0.83 ± 0.02
6	0.29 ± 0.03	0.18 ± 0.01	0.09 ± 0.02	4.73 ± 0.17	2.69 ± 0.05	1.18 ± 0.11	1.93 ± 0.09	1.48 ± 0.12
12	0.41 ± 0.02	0.23 ± 0.01	0.11 ± 0.01	5.40 ± 0.10	3.46 ± 0.11	1.24 ± 0.07	2.60 ± 0.11	1.63 ± 0.07
24	0.59 ± 0.04	0.34 ± 0.03	0.14 ± 0.01	6.31 ± 0.16	4.39 ± 0.07	1.35 ± 0.07	3.29 ± 0.12	1.94 ± 0.24

[a] *Man* mannose, *Rhm* rhamnose, *GalUA* galacturonic acid, *GlcUA* glucuronic acid, *Xyl* xylose, *Glc* glucose, *Gal* galactose, *Ara* arabinose

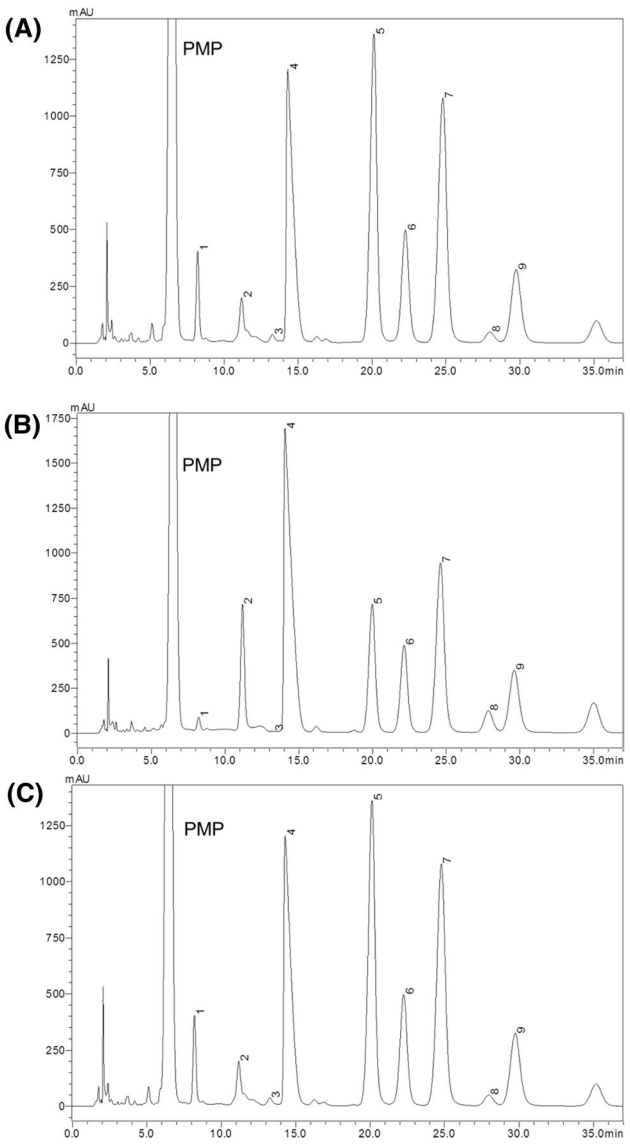

Fig. 7 The HPLC chromatograms of mixed standard sample of monosaccharides (**a**); and hydrolysates of POPP by SmF (**b**) and SSF (**c**) crude enzymes. Enzymatic hydrolysis experiments were carried out at 45 °C and 170 rpm with substrate loading 4 % (w/v) for 24 h. Peaks: *1* mannose, *2* rhamnose, *3* glucuronic acid, *4* galacturonic acid, *5* xylose, *6* glucose, *7* galactose, *8* arabinose, *9* fucose (internal standard)

hydrolysis of sugar beet pulp [29]. Therefore, 4 % (w/v) of POPP loading was used for reducing sugars production in further experiments, because relatively high reducing sugar concentration and yield were obtained at this substrate loading.

Figure 5 shows a Lineweaver–Burk plot of experimental data for the crude enzymes. The apparent Michaelis–Menten constants of SmF and SSF crude enzymes were $K_{m_{app}} = 73.32$ and 41.45 g/L, and maximal reaction rates were $V_{max_{app}} = 0.118$ and 0.116 g/(L·min), respectively. SSF enzyme had a smaller Km_{app} than SmF enzyme, indicating the enzyme required only a small amount of substrate to become saturated, the maximum velocity was obtained at lower substrate concentrations than SmF crude enzyme.

Effect of time on the enzymatic hydrolysis of POPP

Table 1 shows the enzyme activities and enzymatic hydrolysis performances from SmF, SSF, and commercial enzyme. Pectinase activities were not significant among these enzyme, however, SSF crude enzyme showed higher cellulase and xylanase activities. The higher enzyme activities led to higher hydrolysis efficiency. After 48 h of incubation, the reducing sugar concentrations were 14.9, 20.1, and 18.1 mg/mL for SmF, SSF, and commercial enzymes, respectively. Figure 6 depicted the reducing sugar and carbohydrates production, and saccharification yields of POPP with SmF and SSF crude enzymes. Grohmann et al. [28] observed high level of conversion (>70 %) to monomeric sugars from orange peel after treatment with pectinase enzyme. Wilkins et al. [6] found grapefruit peel waste could be hydrolyzed by pectinase, and the yield of total sugars reached 63.8 %. In this work, saccharification yields of POPP using SmF and SSF enzymes were 58.5 and 78.7 %, respectively, which indicated that SSF crude enzyme showed higher hydrolysis efficiency than commercial enzymes.

Carbohydrates concentration was higher than reducing sugar concentration, which indicated that some oligosaccharides were produced during enzymatic hydrolysis [30, 31]. The difference in concentrations between carbohydrates and reducing sugars by SSF enzyme was more obvious than SmF enzyme (Fig. 6). The results indicate that more oligosaccharides were produced by SSF enzyme.

Monosaccharide analysis

Table 2 shows the monosaccharides production from SmF and SSF enzymatic hydrolysates. Since SSF crude enzyme provided higher CMCase, FPase, and xylanase activities (Table 1), the concentrations of glucose and xylose in SSF enzyme hydrolysate were higher than that in SmF. However, the concentrations of galacturonic acid and arabinose were higher in SmF enzyme hydrolysate, which indicated that SmF might provide higher endopolygalacturonase and arabinase activities. In the case of SmF crude enzyme hydrolysis, the monosaccharides increased significantly in the initial 12 h and then maintained constant (Table 2). As for SSF crude enzyme hydrolysis, monosaccharides kept an increased tendency during 48 h (Table 2). Presence of significant amounts of glucose and galacturonic acid in the

Table 3 Material balances (dry weight, g) of enzymatic hydrolysis

Hydrolysate[a]	Total[b]	Production of monosaccharides[c]								Total monosaccharide	Total oligomers	Solid residue[d]
		Man	Rhm	GlcUA	GalUA	Xyl	Glc	Gal	Ara			
SmF	100	0.3	3.6	0.0	23.2	5.2	3.0	6.4	11.7	53.4	9.8	36.8
SSF	100	2.0	1.3	0.3	20.0	14.3	4.1	10.1	6.7	58.8	10.8	30.4

[a] The enzymatic hydrolysis experiments were carried out with a substrate loading 4 % (w/v) at 45 °C and 170 rpm for 48 h

[b] Data based on 100 g of Oven-Dried pre-wash pretreated orange peel powder (POPP)

[c] *Man* mannose, *Rhm* rhamnose, *GlcUA*, glucuronic acid, *GalUA* galacturonic acid, *Xyl* xylose, *Glc* glucose, *Gal* galactose, *Ara* arabinose

[d] Solid residue may include acid-insoluble residue, ash, and proteins

medium might lead to catabolite repression to cellulase and pectinase [7].

Figure 7 shows the HPLC chromatograms of PMP derivatives of 8 standard monosaccharides released from orange peel enzymatic hydrolysis after 24 h. It can be concluded that the proposed method is suitable for determining the monosaccharides in orange peel hydrolysates. Compared with the gradient elution of the mobile phase [32, 33], isocratic elution in this work had a good elution profile. Refractive index detection was usually used in monitoring monosaccharide of enzymatic hydrolysates [34]. However, this method is not sensitive and/or selective enough for differentiating the trace amounts of aldoses and uronic acids [35]. PMP-labeled method could obviously improve the sensitivity and detection limit of monosaccharides, in which the aldoses and uronic acids could be simultaneously determined by HPLC method [27].

Material balance

Before enzymatic hydrolysis, the orange peel powders were washed with distilled water (see "Raw materials"). In this stage, the nonvolatile solutes accounted for 44.7 % (w/w) of oven-dry OPP, and the water-extracted solid (POPP) accounted for 55.3 % (w/w) of oven-dry OPP. Table 3 shows the material balance after enzymatic hydrolysis. After 48 h of enzymatic hydrolysis, total monosaccharides from enzymatic hydrolysis of POPP by SmF and SSF enzymes were 53.4 and 58.5 % (w/w) of oven-dry POPP, respectively. These results indicate that SSF crude enzyme provided higher enzymatic hydrolysis efficiency than SmF. SmF enzymatic hydrolysate was enriched with galacturonic acid > arabinose > galactose > xylose, and the SSF enzymatic hydrolysate was enriched with galacturonic acid > xylose > galactose > arabinose. Galacturonic acid concentrations were similar between SmF and SSF enzymatic hydrolysate, which could be attributed to the equivalent pectinase activities. Xylose concentration from SSF enzymatic hydrolysate was much more than SmF hydrolysate, which could be attributed to the higher xylanase activity.

Conclusions

Orange peel can be hydrolyzed to monomeric sugars using the crude enzymes produced by *A. japonicus* PJ01 in submerged and solid-state fermentations. Pectinase activity from SSF crude enzyme was similar to SmF crude enzyme, but FPase, CMCase and xylanase activities of the former were much higher than the latter. 4 % (w/v) of solid loading, undiluted crude enzymes, and 45 °C were suitable conditions for reducing sugars production by these two crude enzymes. After 48 h of incubation, the reducing sugars concentration using SmF and SSF enzyme was 14.9 mg/mL (saccharification 58.5 %) and 20.1 mg/mL (saccharification 78.7 %), respectively. This study demonstrated that enzymatic hydrolysis of orange peel using SSF crude enzyme showed promise for further scale-up. Compared with commercial enzymes, fungal crude enzymes would help improve the productivity and economic viability of the pectin-rich biomass hydrolysis.

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