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Horticultural Waste as the Substrate for Cellulase and Hemicellulase Production by *Trichoderma reesei* Under Solid-State Fermentation

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Abstract Horticultural waste in wood chips form collected from a landscape company in Singapore was utilized as the substrate for the production of cellulase and hemicellulase under solid-state fermentation by *Trichoderma reesei* RUT-C30. The effects of substrate pretreatment methods, substrate particle size, incubation temperature and time, initial medium pH value, and moisture content on cellulase and hemicellulase production were investigated. Enzyme complex was obtained at the optimal conditions. This enzyme mixture contained FPase (15.0 U/g substrate dry matter, SDM), CMCase (90.5 U/g SDM), β-glucosidase (61.6 U/g SDM), xylanase (52.1 U/g SDM), and β-xylosidase (10.4 U/g SDM). The soluble protein concentration in the enzyme complex was 26.1 mg/g SDM. The potential of the crude enzyme complex produced was demonstrated by the hydrolysis of wood chips, wood dust, palm oil fiber, and waste newspaper. The performance of the crude enzyme complex was better than the commercial enzyme blend.

Keywords Horticultural waste · *Trichoderma reesei* · Cellulase · Hemicellulase · Solid-state fermentation · Saccharification

Introduction

In the past few years around 246,200 tonnes of wood waste and 224,600 tonnes of horticultural waste were generated per year in Singapore. Wood waste includes pallets, crates, boxes, furniture, and wood planks used in construction. Horticultural waste refers to tree trunks and branches, plant parts, and trimmings generated during the maintenance and pruning of trees and plants all over Singapore [1, 2]. Wood waste is usually grinded into wood dust and horticultural waste is usually grinded into wood chips. Cellulose and hemicellulose from wood waste and horticultural waste are the potential feedstock for fuel ethanol production. Successful conversion of such waste biomass to fuel ethanol will

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substantially reduce the amount of wastes that would otherwise exert pressure on municipal landfills [2, 3].

Solid-state fermentation (SSF) is characterized by a fermentation process on a solid support. It has low moisture content and occurs at a natural state, sometimes can be under non-aseptic conditions [4]. SSF has enormous economical and practical advantages over submerged fermentation. These include low capital cost, low energy expenditure, less water usage and therefore lower wastewater output, potential higher concentration of the products, and lesser fermentation space [5].

Cellulase and hemicellulase are industrially important hydrolytic enzymes and are of great significance in current biotechnology. They are primarily employed in the saccharification process. The production economics of bioethanol is largely dependent on the cost of cellulase [6]. Substrate costs account for a major fraction of the costs of cellulase production, and the use of cheap biomass resources as the substrate can help to reduce cellulase prices [7]. The search for low-cost substrates to produce cellulase enzymes has led to wide investigations in the utilization of different types of biomass, such as: water hyacinth [8], dairy manure [7, 9], sugar cane bagasse [10], corn cob residues [11], wheat straw [12], oil palm biomass, etc. [13]. Approximately, 90% of the wood chips are stored in the form of cellulose, hemicellulose, pectin, and lignin. While the recycling rate is only 71% for wood waste and 41% for horticultural waste in Singapore [2], horticultural waste could be a very important biomass resource suitable for cellulase and hemicellulase production. In the present investigation, an attempt was made to develop a suitable process for the production of cellulolytic and hemicellulolytic enzymes, using horticultural waste as the solid substrate and employing *Trichoderma reesei*, which has been extensively studied for cellulase production [14, 15]. This study offers the optimization process of SSF for cellulase and hemicellulase production by *T. reesei* grown on horticultural waste and their hydrolytic potentials towards different lignocellulosic biomasses. Such information would be useful for the development of a cost-effective process for cellulase and hemicellulase production and the subsequent enzymatic hydrolysis of lignocellulosic biomass.

Materials and Methods

Microorganism

The cellulase hyper-producing fungus *T. reesei* RUT-C30 (American Type Culture Collection (ATCC) 56765) was obtained from the ATCC. The strain was routinely maintained and sporulated on potato dextrose agar plate for 2 weeks till good spore crop was developed. Four milliliters of sterile 0.05% Tween 80 solution was added to the plate and swirled to gently release the spores. In general, 1 mL of the spore suspension consisting of 10^6–10^7 spores was used as the inoculum.

Horticultural Waste and Its Pretreatment

Horticultural waste was collected from Environmental Landscape Pte Ltd, Singapore. It has been grinded into wood chips. Collected wood chips were immediately autoclaved and dried at 80 °C overnight. They were then cooled down to room temperature and stored in sealed plastic bags until use. For enzyme production, the dried wood chips were mechanically milled with a lab mill (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Germany) and sieved through standard mesh sieves using an electronic sieve shaker model

RP09 (Barcelona, Spain) to obtain a powder of 200 to 500 μm particle sizes. The dried horticultural waste powder (HWP) was pretreated with pressurized steam at 121 °C for 2 h, or with alkali by soaking them in NaOH solution of varying concentrations (1–3%; *w*/*v*) at 105 °C overnight. After alkali treatment, the HWP was washed several times in tap water to reach the neutral pH of 6.0–7.0 followed by a final rinse in the distilled water. After this they were dried in the oven at 105 °C overnight. The final pretreated HWP was cooled down at room temperature (30±2 °C) and stored in polyethylene bags for further use.

Enzyme Production

Solid-state fermentation was carried out by placing 5 g of the pretreated HWP in 500-mL cotton-plugged Erlenmeyer flasks with certain amount of mineral salt medium to attain the predesigned moisture content. The basal mineral salts solution used for the experiment had the following composition (g/L): urea, 2.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4$, 0.3; yeast extract, 0.25; and peptone, 0.75. Furthermore, the medium was supplemented with the following trace elements (g/L): $FeSO_4 \cdot 7H_2O$, 0.005; $CoCl_2$, 0.0002; $MnSO_4$, 0.0016; and $ZnSO_4$, 0.0014. The initial pH value of the medium was adjusted to 4.8. The flasks with substrate were autoclaved at 121 °C for 30 min. Each flask was inoculated with 1.5 mL spore suspension (10^6–10^7 spores/mL) at 30 °C under static conditions. The contents were mixed thoroughly and were incubated under controlled conditions of temperature and humidity.

Various process parameters affecting enzyme production under SSF were investigated. One-factor-at-a-time approach was adopted in this study. The tested process parameters were substrate pretreatment (pressurized steam or NaOH at varying concentrations of 1–3% (*w*/*v*)), incubation temperature (25–37 °C), initial pH (4.5–8.0), incubation time (1–14 days), particle size (200–500 μm), and moisture content (50–87.5%).

Enzyme Extraction

At the end of incubation period, the enzyme was recovered by extraction. Five grams (dry weight) of the fermented substrate were extracted with 100 mL 0.05 M citrate buffer (pH 4.8) containing 0.1% Tween-80, by shaking at 250 rpm for 2 h at 30 °C. The suspended materials and fungal biomass were separated by centrifugation at 12,000×*g* and 4 °C for 20 min. The supernatant was used for enzyme activity assay.

Enzyme Activity Assays

Enzyme activities were measured from the culture extract of SSF samples. Filter paper (FPase) activity and endo-glucanase (CMCase) activity were measured according to IUPAC recommendations [16]. FPase and CMCase activities were determined by measuring the reducing sugars produced from Whatman no. 1 filter paper (50 mg, 1×6 cm) and from 1% (*w*/*v*) carboxymethyl cellulose, respectively. Both reactions were carried out in 0.05 M citrate buffer at pH 4.8. The reaction mixtures were incubated at 50 °C for 1 h and for 30 min for the FPase activity assay and CMCase activity assay, respectively. Xylanase activity was measured according to Ghose [17]. The assay was carried out in the total reaction mixture of 1.5 mL containing 0.5 mL of suitably diluted enzyme and 1.0 mL of 1% (*w*/*v*) xylan solution in phosphate buffer (0.05 M, pH 6.5). This mixture was incubated at 40 °C for 10 min. The amount of reducing sugar released from FPase, CMCase, and xylanase activity assays was measured using the 3,5-dinitrosalicylic acid (DNS) method [18].

β-glucosidase activity was determined using the method described by Bailey et al. [19]. In this method, the *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (Sigma N-7006) was measured using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). The β-xylosidase activity was measured in an analogous manner by using 1 mM *p*-nitrophenyl-β-D-xylopyranoside (Sigma N2132) as the substrate. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of product from their respective substrate per minute of crude filtrate under the assay conditions. The soluble protein, an indicator of the enzyme released, was measured by the Lowry protein assay using bovine serum albumin as the standard [20].

Biomass Composition Characterization

Four types of biomass such as wood chips from horticultural waste, wood dust from wood waste, palm oil fiber, and waste newspaper were chosen as the hydrolysis substrates. Palm oil fiber was collected from Masai Palm Oil Mill, Johore, Malaysia. Wood dust was collected from Wah & Hua Pte Ltd, Singapore, and waste newspaper was collected domestically. All of them were merely physically milled without going through any chemical pretreatment. The amounts of hemicellulose, cellulose, and lignin in the lignocellulosic biomass were determined according to Yang et al. with modification [21].

To determine the amount of hemicellulose, 150 mL of 0.5 M NaOH solution was added to 1 g of the dried biomass and the temperature was held at 80 °C for 3.5 h. After that, the sample was washed using de-ionized water until no more Na^+ was detected (indicated by the pH value of the solution approaching 7), and then it was dried to a constant weight. The difference between the sample weight before and after this treatment is the hemicellulose content. To determine the acid soluble lignin content, 15 mL of 98% H_2SO_4 was added to the 0.5 g of hemicellulose-free biomass, followed by 2-h incubation at 30 °C. The mixture was then diluted to 4% H_2SO_4 with 352.5 mL of de-ionized water. The diluted solution was autoclaved at 121 °C for 1 h. The mixture was filtered. Aliquots of the filtrate were measured at the absorbance of 205 nm using 4% H_2SO_4 as the control. Dilutions were done to obtain optical density readings of 0.2–0.8. Acid soluble lignin content was calculated based on the sample absorbance at 205 nm [22]. The rest of the biomass residue was washed until the sulfate ion in the filtrate was undetectable (via titration of a 10% barium chloride solution); it was then dried to a constant weight. The weight of the residue is recorded as the lignin content. Finally, the content of cellulose is calculated by the difference, assuming that hemicellulose, lignin, and cellulose are the only components of the entire biomass.

Enzymatic Hydrolysis

The crude enzyme mixture of *T. reesei* RUT-C30 was produced under the optimal conditions determined for SSF and it was extracted after 7 days of growth. Enzymatic hydrolysis of the lignocellulosic biomass was carried out at a biomass loading of 2.5% (*w*/*v*) in 20 mL of 0.05 M citrate buffer (pH 4.8). The experiments were performed in duplicates at 50±1 °C in a shaking water bath (Memmert GmbH + Co. KG, Schwabach, Germany) with maximum strokes for 48 h. A commercial *T. reesei* cellulase (Celluclast 1.5 L) and a β-glucosidase preparation (Novozyme 188) were used as the control. Celluclast 1.5 L and Novozyme 188 were both kindly donated by Novozymes Malaysia sdn bhd. Celluclast 1.5 L had a FPase activity of 187 IU/mL and a β-glucosidase activity of 32 IU/mL. Novozyme 188 had a FPase activity of 4.7 IU/mL and a β-glucosidase activity

of 795 IU/mL. The enzyme activity for FPase was 10 IU/g substrate and that for β-glucosidase was 30 IU/g substrate in the control hydrolysis experiments. Enzyme loading for the crude enzyme mixture from the SSF was the same, i.e.10 IU/g substrate of FPase. At the end of the hydrolysis, samples were centrifuged at 10,000×*g* for 10 min and analyzed for glucose concentration as estimated by the DNS method [18]. The glucose yields were calculated as: reducing sugar (g)×0.9×100/initial cellulose (g) in biomass. Hydrolysis of polysaccharides involves water. For each mole of reducing sugar released, 1 mol of H_2O is required. A correction factor of 0.9 was therefore included in the calculation of the amount of polysaccharides hydrolyzed. Experiments were carried out in duplicates.

Results and Discussion

Effect of Substrate Pretreatment Methods

HWP with the particle size of 200–500 μm was used in this experiment. It was pretreated by steam alone or by dilute NaOH solution. Steam pretreatment was conducted at 121 °C for 2 h and dilute NaOH pretreatment was carried out at varying concentrations at 105 °C over night. Room-temperature-dried HWP was used as the control. The enzyme activities results are displayed in Fig. 1. As can be seen, at the end of 7-day incubation, pretreatment using dilute NaOH solutions showed no obvious advantages in the enzyme activities and soluble protein concentration. On the other hand, the highest enzymes activities were obtained when the steam-pretreated HWP was used. Pretreatment with both steam and NaOH did not contribute much to the improvement in the enzyme activities compared to that with steam alone. It was observed that pretreatment with increased concentrations of NaOH, e.g. 1% to 3%, did not show significant increase in the yield of any enzymes. These observations indicate that pretreatment of HWP with steam is optimal for its use as the substrate for enzyme production by *T. reesei*. Pretreatment is usually carried out to reduce lignocellulosic biomass crystallinity, render cellulose accessibility, and remove lignin [23]. Palonen et al. reported the adsorption of cellulase on lignin [24]. Lignin is known to inhibit cellulase activity [25]. Steam explosion can cause hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis [26]. By the solvolysis of hot compressed liquid water, between 40% and 60% of the total biomass is dissolved in the process, with 4–22% of the cellulose, 35–60% of the lignin, and all of the hemicelluloses being removed [27]. The insignificance of alkaline pretreatment on the enzyme yield might be due to the adsorption of enzymes on lignin or be due to the inhibitory substances produced during the pretreatment process.

Effect of Particle Size

Particle size of the substrate is related to substrate characterization and system capacity to interchange with microbial growth and heat and mass transfer during SSF process [4]. Smaller particle size could provide larger surface area for microbial action and it is also advantageous for heat transfer and exchange of oxygen and carbon dioxide between the air and the solid surface. However, too small particles may result in substrate agglomeration, which may interfere with microbial respiration and thus result in the poor cell growth [28].

HWP with varying particle size was used in this experiment and results are displayed in Table 1. It was found that FPase activity with HWP of 200–500 μm particle size was higher than those obtained with HWP of other particle diameters. It was over twofold higher than

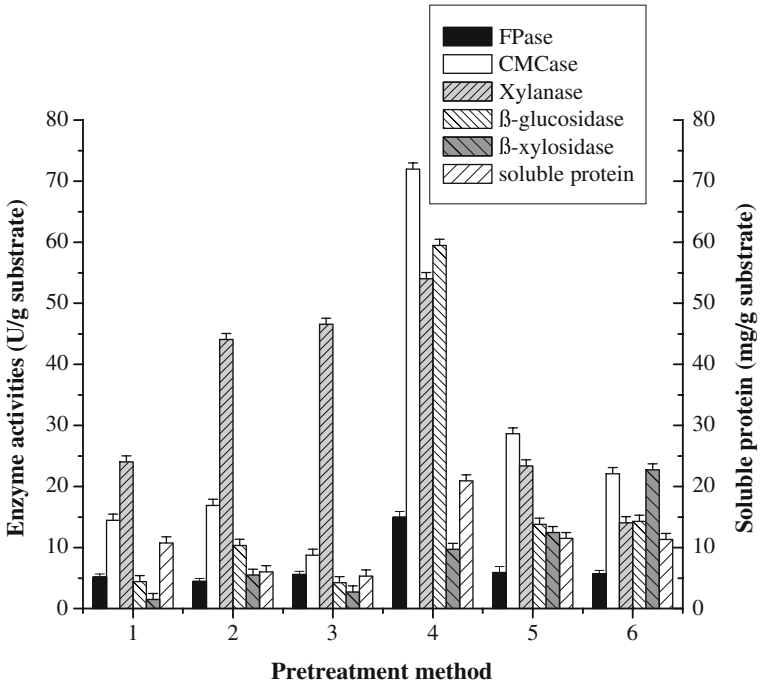

Fig. 1 Effect of different pretreatment methods on cellulolytic and hemicellulolytic enzyme activities and soluble protein concentration. 1. room-temperature-dried, 2. 1% NaOH, 3. 3% NaOH, 4. Pressurized stream, 5. 1% NaOH- and steam, 6. 3% NaOH- and steampretreated

that obtained with HWP with particles size greater than 500 μm. In addition, HWP with particle size of 200–500 μm favored the soluble protein production. This suggests that fungi grow best on HWP of 200–500 μm under SSF. It is worth noting that higher xylanase, β-glucosidase, and β-xylosidase activities were obtained on HWP with bigger particle size (>500 μm). This reveals that the substrate particle size has significant effect on the cellulolytic and hemicellulolytic enzyme production by *T. reesei* under SSF.

Effect of Initial pH Values

Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi have reasonably good growth over a broad range of pH, 2–9, with an optimal range of 3.8 to 6.0 [29]. To evaluate the effect of initial pH value under SSF on cellulase and hemicellulase production, the initial pH values in the

Table 1 Effect of particle size on cellulase and hemicellulase activities and soluble protein concentration.

Particle size	Enzyme activities (U g^{-1} substrate)					Soluble protein (mg g^{-1} substrate)
	FPase	CMCase	Xylanase	β-glucosidase	β-xylosidase	
>500 μm	6.6±0.3	40.9±1.1	64.8±0.6	211.9±3.1	44.2±1.5	18.7±0.5
200–500 μm	14.9±1.4	69.8±0.8	54.0±2.1	59.5±0.9	9.7±0.8	23.6±1.1
<200 μm	12.8±0.7	69.5±1.2	49.0±1.5	32.3±1.1	9.1±0.7	18.3±0.6

medium were adjusted by the addition of 1 M HCl or 1 M NaOH to 3.5, 4.0, 4.5, 5.0, 5.6, 6.0, 7.0, and 8.0. The results of seven batches of solid-state fermentation in flasks with varied initial substrate pH values are shown in Fig. 2. The cultivation period for each batch was 7 days. Generally speaking, lower pH (4.5–5.5) favored the production of all the five enzymes investigated with the optimal initial pH value of 5.0 for CMCase, xylanase, and β-xylosidase activities. When pH value was greater than 6.0, increase of the initial pH value resulted in the decrease of all the enzyme activities and the soluble protein concentration except β-xylosidase activity, which was almost constant. This indicates that β-xylosidase activity was less sensitive to the medium pH values.

Effect of Initial Moisture Content

Fungi prefer a moist environment for their growth. Moisture content is a critical factor on SSF processes because this variable has influences on both cell growth and the biosynthesis and secretion of enzymes [29]. Lower moisture content causes the reduction in the solubility of substrate nutrients, low degree of swelling, and high water tension [30, 31]. On the other hand, higher moisture levels can cause a reduction in the enzyme yield due to its steric hindrance to cell growth of the enzyme producing strain resulted from the reduction in the solid matrix porosity (interparticle spaces). Less porosity interferes with oxygen transfer and in turn influences the cell growth [29]. As oxygen transfer affects both the growth and the metabolism of fungi, the solid substrate should contain suitable amount of water to enhance mass transfer.

The effect of initial moisture content on enzyme activities by *T. reesei* grown under SSF is depicted in Fig. 3. The culture temperature was kept at 30±1 °C for 7 days, and the initial pH

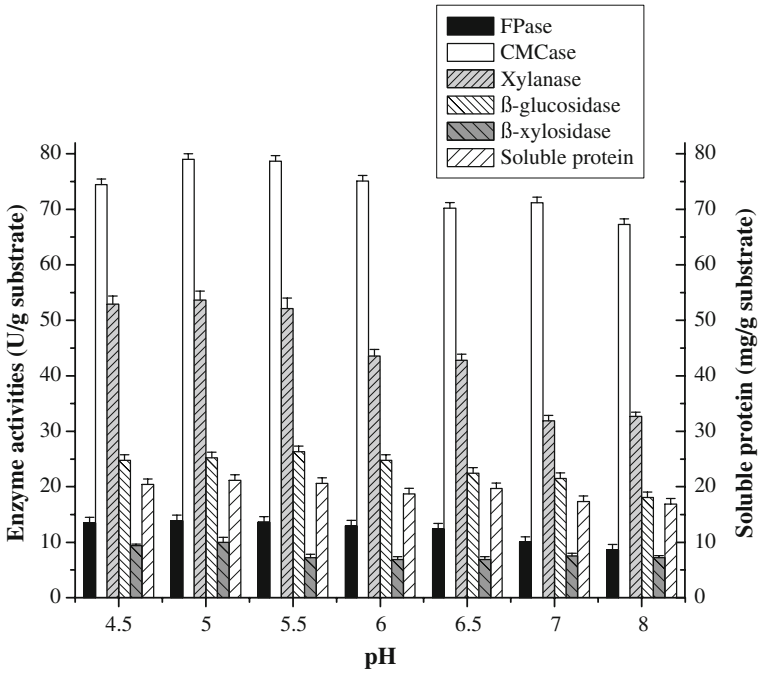

Fig. 2 Effect of initial pH values on cellulolytic and hemicellulolytic enzyme activities and soluble protein concentration

was 5.0. It can be seen that the optimal moisture content in the solid-state substrate for FPase and CMCase activities appears to be 80%. In addition, fungus *T. reesei* secreted more soluble protein at this moisture content. A positive relationship between the activities of FPase and CMCase and the moisture content was observed when it was lower than 80%. However, further increase in moisture content influenced the activities of these two enzymes negatively. On the other hand, higher moisture contents (85–87.5%) were necessary for maximal xylanase, β-glucosidase and β-xylosidase production. The difference of the optimum moisture content for each individual enzyme under SSF might be due to their dependence upon the water binding properties of the substrate as well as the microorganism used [4].

Effect of Incubation Temperature and Time

Probably the most important factor among all the physical variables affecting the SSF performance is the incubation temperature, because both cell growth and the production of enzymes and metabolites are usually sensitive to temperature [4]. Tao et al. [32] found that maximal growth and cellulase production by *Trichoderma* sp. were at 25–35 °C.

The cellulolytic and hemicellulolytic activities secreted by *T. reesei* cultivated in SSF with steam-pretreated HWP on day 7 at varied incubation temperature from 26 °C to 37 °C are shown in Fig. 4. The optimal FPase, CMCase, β-xylosidase activities, and soluble protein concentration were obtained at 30 °C, whereas the optimal xylanase and β-glucosidase activities were obtained at 37 °C and 26 °C, respectively. This proves that the optimum temperature for cell growth could be different from that for product formation [4].

Varying incubation time was also investigated and the results are displayed in Fig. 5. The optimal incubation time for the production of all the enzymes investigated is 7 or 8 days, but further culture resulted in the reduced enzyme activities. The decrease of the enzyme activity may be due to the denaturation of the enzymes, resulted from the variation of pH value and the cellular metabolism during fermentation.

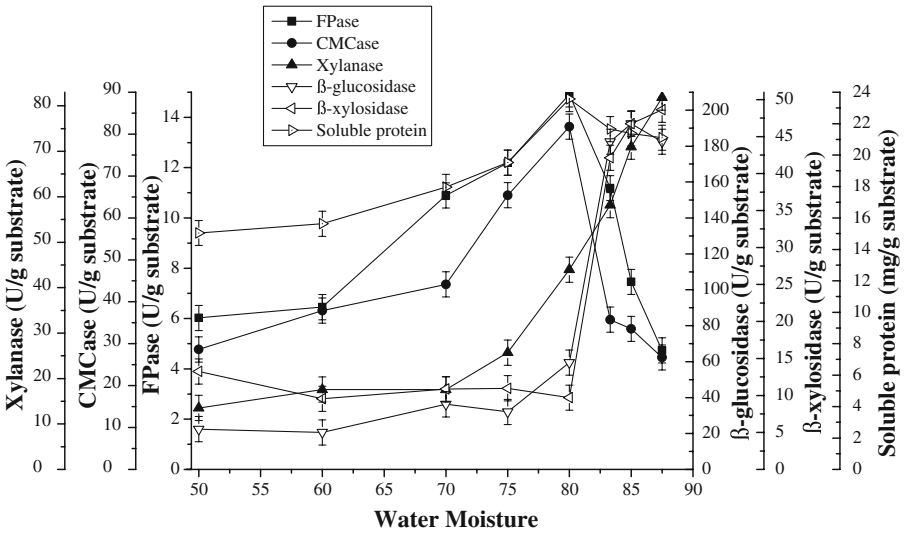

Fig. 3 Effect of water moisture content on cellulolytic and hemicellulolytic enzyme activities and soluble protein concentration

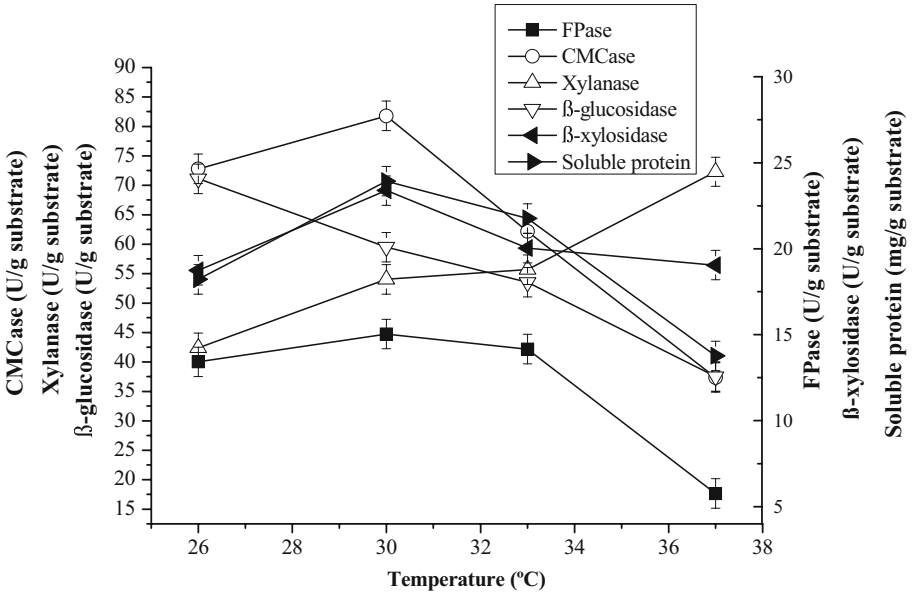

Fig. 4 Effect of incubation temperature on cellulolytic and hemicellulolytic enzyme activities and soluble protein concentration

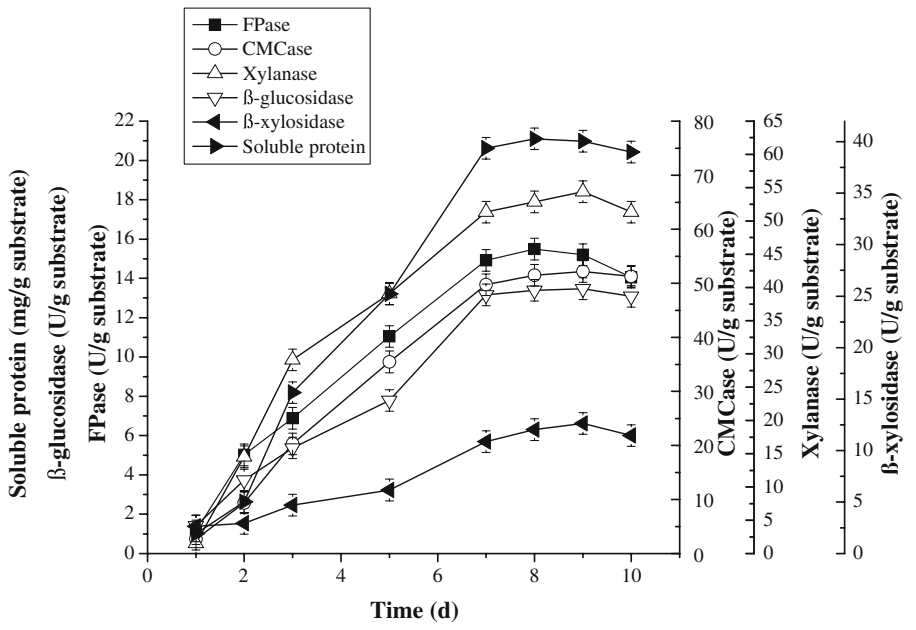

Fig. 5 Effect of incubation time on cellulolytic and hemicellulolytic enzyme activities and soluble protein concentration

Table 2 Chemical composition of different types of lignocellulosic biomass.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Wood chips	24.7	37.2	34.9
Wood dust	33.0	27.5	36.6
Palm oil fiber	30.2	39.2	29.5
Newspaper	53.3	26.3	11.2

Results were based on the mass of dry matter

Hydrolytic Potential of the Crude Enzyme Complex

The enzymatic hydrolysis is more dependent on the characteristics of the substrate rather than on measured standard enzyme activities [33]. In order to test the hydrolytic potential of the enzyme complex produced by *T. reesei* RUT-C30 under SSF using HWP, crude enzyme mixture was prepared at 30 °C, initial pH value of 5.0, and 80% moisture content using the steam-pretreated HWP of 200–500 μm particle size. Enzyme was extracted on day 7. This enzyme mixture contained FPase (15.0 U/g SDM), CMCase (90.5 U/g SDM), β-glucosidase (61.6 U/g SDM), xylanase (52.1 U/g SDM), and β-xylosidase (10.4 U/g SDM). Four types of untreated lignocellulosic biomass, i.e. wood chips, wood dust, palm oil fiber, and waste newspaper, were examined. The compositions of the untreated biomass investigated are displayed in Table 2. The low lignin content is advantageous, since inhibition in both enzyme production and activity has been reported for lignin-rich materials [25, 34]. While the wood dust and wood chips both have higher lignin contents (36.6% and 34.9%), newspaper has the lowest lignin content (11.2%).

A comparison of the effectiveness of crude enzyme samples and the commercial enzyme preparation in the saccharification of the four types of lignocellulosic biomass is shown in

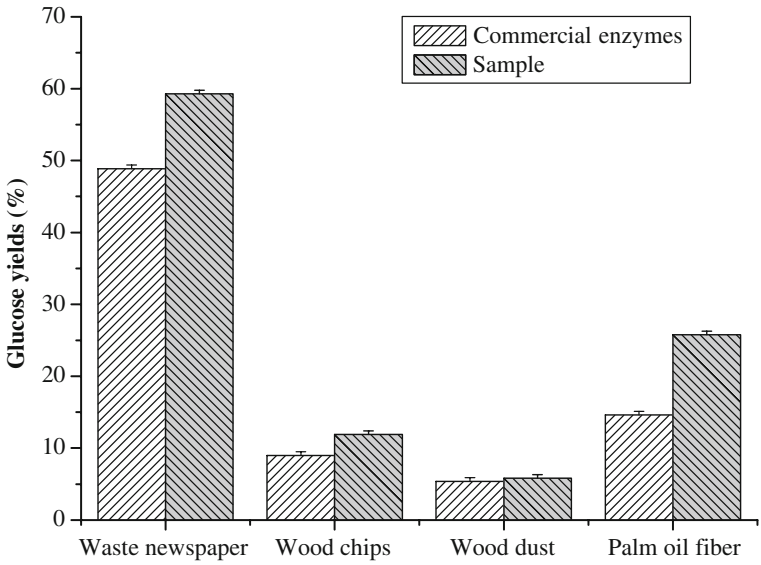

Fig. 6 Comparison of the hydrolysis potentials using the crude enzyme sample and the commercial enzyme mixture

Fig. 6. It was observed that compared with the commercial enzyme mixture, the laboratory-produced crude enzyme solution resulted in the higher glucose yield for all the biomasses investigated. This may be due to the fact that the crude enzyme solution prepared is composed of a series of enzymes such as celluases and hemicellulases, which could contribute to the higher reducing sugar concentration, whereas the commercial enzyme mixture is only composed of FPase and β-glucosidase. The diversity of the enzymes in the crude enzyme mixture has an advantage over the mixed commercial enzymes as lignocellulosic biomass hydrolysis needs the concerted action of multiple enzymes. Therefore, the above results imply that horticultural waste collected in Singapore might be a potential substrate for cellulolytic and hemicellulolytic enzyme production. Such discovery offers a more cost-effective way for horticultural waste utilization in Singapore. Instead of being directly burned or composted, horticultural waste can be effectively used to produce more value-adding products, such as lignocellulolytic enzymes. It is also interesting to find that waste newspaper demonstrated the highest sugar yield compared with other three types of biomass. This may be attributed to its less lignin content and its accessibility to the enzymes. Pretreatment is necessary for the other lignocellulosic biomasses to improve their accessibility to the lignocellulolytic enzymes and in turn to obtain a higher sugar yield.

Conclusion

The present work showed that horticultural waste collected in Singapore could be a potential substrate for cellulolytic and hemicellulolytic enzyme production by *T. reesei* under solid-state fermentation. Optimization of the enzyme production demonstrated clearly the impact of the process parameters on the gross yield of the lignocellulolytic enzymes. Furthermore, the potentials of the laboratory-produced crude enzyme mixture in the saccharification of different biomass feedstock were investigated. The enzyme mixture produced in this work can be effectively used to hydrolyze the lignocellulosic biomass to its constituent sugars. These sugars in turn are the potential substrates for the production of fuel ethanol and other valuable chemicals. Further work is necessary to enhance the enzyme hydrolytic capability by constructing an enzyme cocktail using enzyme mixtures produced by a consortium of fungal strains on horticultural waste powder under solid-state fermentation. This is ongoing in the bioethanol lab at Ngee Ann Polytechnic.

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