



# Bioconversion of Oil Palm Trunk Residues Hydrolyzed by Enzymes from Newly Isolated Fungi and Use for Ethanol and Acetic Acid Production Under Two-Stage and Simultaneous Fermentation

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## Abstract

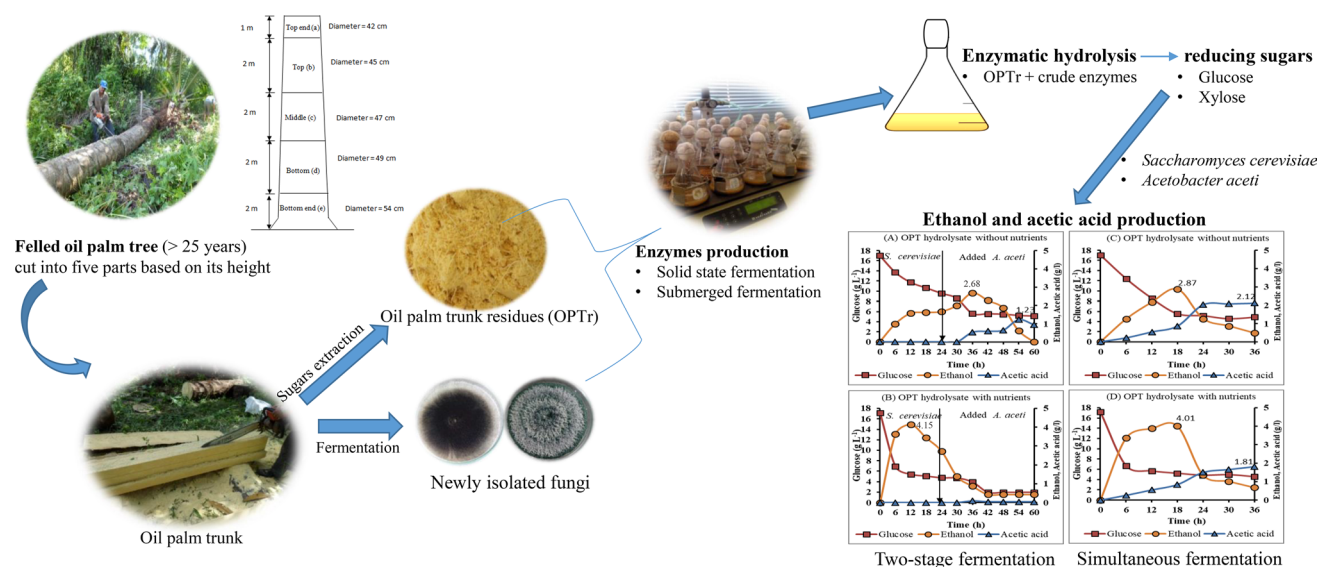
Oil palm trunk (OPT) is one of the richest biomass in Southern Thailand. The OPT residues (OPTr), obtained after separation of the sap, was hydrolyzed by the crude enzymes of the selected fungal isolates under solid-state fermentation (SSF) and submerged fermentation (SmF). From screening and identification studies, *Ceratocystis paradoxa* TT1 produced the highest CMCase activity (18.16 Unit gds<sup>-1</sup>) from SmF while *Trichoderma koningiopsis* TM3 exhibited the highest xylanase and FPase activities (56.46 and 2.13 Unit gds<sup>-1</sup>, respectively) from SSF. Enzymatic hydrolysis of OPTr using the crude enzymes of *T. koningiopsis* TM3 (25 Unit gds<sup>-1</sup>) at 50 °C for 15 h revealed the maximum reducing sugars of 11.92 g L<sup>-1</sup> with the yield of 0.48 gg<sup>-1</sup>. Bioconversion of the OPTr hydrolysate to ethanol by *Saccharomyces cerevisiae* TISTR5055 could be increased (2.13-fold) by supplementation of YM nutrients. The effects of co-cultures (*S. cerevisiae* TISTR5055 and *Acetobacter aceti*) for production of co-products (ethanol and acetic acid) from the OPTr hydrolysate with and without YM nutrients addition was conducted under two-stage fermentation and simultaneous fermentation. Both fermentation types gave similar ethanol production (4.15 and 4.01 g L<sup>-1</sup>) but the ethanol productivity (0.34 g L<sup>-1</sup> h<sup>-1</sup>) from two-stage fermentation was 1.55-fold higher than that from simultaneous fermentation. On the other hand, the maximum acetic acid production (2.12 g L<sup>-1</sup>) and productivity (0.09 g L<sup>-1</sup> h<sup>-1</sup>) were achieved from the simultaneous fermentation of the co-cultures without nutrient addition. These two values were 1.7-fold higher acetic acid concentration and 4.4-folds higher productivity than the two-stage fermentation.

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## Graphical Abstract



**Keywords** Oil palm trunk residues · Fungal enzymes · Two-stage fermentation · Simultaneous fermentation · Ethanol · Acetic acid

## Statement of Novelty

Felled oil palm trunks (OPT) were plantation waste never been utilized before. This research work initiated the valorization of felled OPT to produce ethanol and acetic acid. No previous knowledge of suitable fermentation process of substrate derived from OPT. This work compares the two-stage and simultaneous fermentation. The results showed that the two-stage process is superior to the simultaneous process in ethanol production ( $4.15 \text{ g L}^{-1}$ ), but not for acetic acid ( $2.12 \text{ g L}^{-1}$  for simultaneous process). Combination of the naturally fermentation of the original OPT sap could increase the total acetic acid to  $5.12 \text{ g L}^{-1}$ . This concentration level of acetic acid was reported to be effective for treatment of chronic wounds based on antibiofilm properties

## Introduction

Lignocellulosic biomass is reported to be one of the alternative source for fossil fuels [1]. Utilization of biomass can address the environmental issues such as global warming, extreme climate change, acid rain and human health deterioration [2]. Thermochemical process is commonly used for hydrolysis of cellulosic biomass to produce sugars [3, 4] but it causes further conversion of the released sugars to other by-products such as furfural and

5-hydroxymethylfurfural (5-HMF) that were reported to be inhibitors and interfered with microbial fermentation [5, 6]. Enzymatic hydrolysis of lignocelluloses has been discussed extensively in the literature [4, 7–9]. It is preferred to chemical hydrolysis because enzymes display the high substrate and reaction specificity, operate under mild conditions and do not generate by-products [10, 11]. The bio-conversion of lignocellulose to fermentable sugars requires the synergistic action of complete cellulase system which act randomly on soluble and insoluble cellulose chains [12–15] and also for hemicellulose degradation [16]. Considering the potential for biotechnological applications of cellulases and xylanases, the prospect of new microbial source is important, especially regarding filamentous fungi that are excellent enzymes producers [17]. The successful strategy to produce lignocellulolytic enzymes can be achieved through microorganism selection and improved fermentation process conditions. These include screening for effective lignocellulolytic enzyme-producing microbes and developing pre-treatments that alter the cellulose lattice structure and increase enzyme accessibility. It is therefore necessary to search for microorganisms that have a high rate of lignocellulolytic enzymes production [18]. In this sense, fungi are a promising group considering that most genera have been little explored as hydrolases producers and they most likely produce enzymes with interesting characteristics in terms of access and attack to the polysaccharides of plant cell walls, since they have to

invade and colonize plant tissues [19]. Most of the studies regarding the production of plant degrading enzymes by fungi involve cultivation on solid media and qualitative evaluation of substrates hydrolysis [20]. Quantitative analysis by submerged fermentation (SmF) is also cited [21]. However, solid-state fermentation (SSF) is advantageous in some aspects including higher yields in a shorter time and the use of lignocellulosic residues as substrates [22].

In Thailand, oil palm is one of the most important oil crops, the area of oil palm plantations was 882,389 ha, mainly in the South (767,677 ha) accounted for 87% of the total area [23]. Plantation area also generates large amounts of solid wastes such as oil palm trunk (OPT) and oil palm fronds (OPF) and they are cut and discarded or burnt at the plantation site [24]. To utilize the felled OPT for biofuel production, there are two sources of the substrates; the OPT sap and the OPT residues. Large quantities of OPT sap contains many simple sugars particularly xylose and glucose [24, 25]. Furthermore, the estimated cellulosic residue remaining after pressing of the trunks was approximately 30% (ww<sup>-1</sup>) of the trunk without bark [26, 27]. Three main lignocellulosic components of OPT contain 29–46% cellulose, 12–26% hemicelluloses and 10–24% lignin [28–30]. In term of its high content of holocellulose (41–72%), OPT is a potential source for production of lignocellulolytic enzymes [30] and fermentable sugar for production of biofuels. Besides ethanol, acetic acid is another important intermediate compound for industrial production of many chemicals such as vinyl acetate polymer, cellulose acetate, terephthalic acid, dimethyl terephthalate, acetic acid esters/acetic anhydride and calcium magnesium acetate. All these products are produced from petroleum-derived acetic acid [31]. The biological route via bacterial fermentation to produce acetic acid for food industry (i.e. vinegar) remains important as it can be used as flavoring agent and food preservation [32]. The production of acetic acid from sustainable biomass or biomass-based platforms has gained attention and extensively studied. Bioconversion of lignocellulosic sugars in hydrolysates from wheat straw, forest residues, switch grass and sugarcane straw to acetic acid by *Moorella thermoacetica* revealed that sugarcane straw hydrolysate gave the highest acetic acid yield (17 g L<sup>-1</sup> from 24 g L<sup>-1</sup> total sugars) [33] or 0.71 g g<sup>-1</sup> total sugar. Typical acetic acid production in a batch fermentation using purified glucose (20 g L<sup>-1</sup>) is around 13 g L<sup>-1</sup> [34] or 0.65 g g<sup>-1</sup> glucose. To the best of our knowledge, the studies on conversion of OPT into ethanol, lactic acid, hydrogen and butanol have recently been reported [24, 25, 35, 36], there is no any report available for bioconversion of OPT to acetic acid.

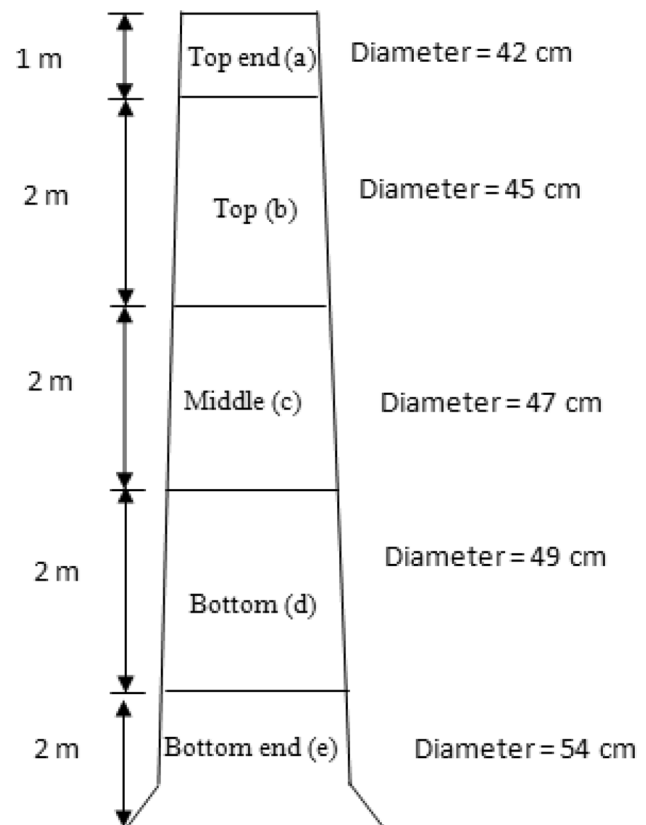
The objectives of this study are (i) to screen the high enzymes producing fungi from the natural fermented felled oil palm trunk (OPT) through SSF and SmF, (ii) to study the enzymatic hydrolysis of OPT residues (OPTr) (iii) to

investigate on the effects of fermentation type (two-stage fermentation vs simultaneous fermentation) and nutrient supplementation for production of co-products (ethanol and acetic acid), with acetic acid as main product, from the OPT hydrolysate using the co-cultures of *S. cerevisiae* TISTR5055 and *Acetobacter aceti*.

## Materials and Methods

### Chemical Composition of Oil Palm Trunk (OPT) and Preparation of OPT Residues (OPTr)

Oil palm tree, approximately 25 years old, was obtained from local oil palm plantation at Khao Phanom District, Krabi Province, Thailand. The old or felled OPT (9 m long) was cut into five parts based on its height; top end (or tip) (a), top (b), middle (c), bottom (d), and bottom end (e), as shown in Fig. 1. The strong outer part of the OPT (bark) was removed and only the soft inner part of each part of OPT except the bottom end part was ground (using a grinder) and used for determination of OPT composition (as shown in Table 1).



**Fig. 1** Diagrams of oil palm trunk (OPT) dimension by height for determination of its composition

**Table 1** Chemical composition of ground oil palm trunk (OPT) and OPT sap

Composition	Top end (a)	Top (b)	Middle (c)	Bottom (d)
OPT				
Moisture (%)	52.58 ± 0.33	53.84 ± 0.52	56.04 ± 1.02	64.92 ± 1.47
Cellulose (%)	12.98 ± 0.92	23.41 ± 1.40	36.60 ± 1.17	55.04 ± 0.50
Hemicellulose (%)	11.87 ± 0.95	12.25 ± 1.76	16.80 ± 1.00	19.67 ± 1.42
Lignin (%)	1.30 ± 0.43	1.50 ± 0.62	5.70 ± 0.66	13.00 ± 0.09
Starch (%)	3.79	2.78	Trace	Trace
OPT sap				
Cellobiose (g L <sup>-1</sup> )	0.27	0.28	0.95	1.33
Glucose (g L <sup>-1</sup> )	4.96	3.91	12.74	20.13
Fructose (g L <sup>-1</sup> )	3.04	4.63	5.06	6.02
Arabinose (g L <sup>-1</sup> )	0.84	0.43	0.00	0.00
Acetic acid (g L <sup>-1</sup> )	1.55	1.49	0.58	1.14

The OPT<sub>r</sub> was prepared by grinding the selected part of OPT (top (b) and middle part (c); mixed in the ratio 1:1 (wv<sup>-1</sup>)) and soaking in distilled water in the ratio of 1:1 (wv<sup>-1</sup>) for 1 h, then pressed through a screw press to obtain OPT sap (or OPS) and OPT residues (OPT<sub>r</sub>) [28]. The OPT<sub>r</sub> were sun-dried for 1 day, then dried in a hot air oven (68 °C) for 3 days until obtain constant weight. They were kept as feedstock in sealed plastic containers at room temperature (30 ± 2 °C) until used.

### Isolation, Selection and Identification of the High Enzyme Producing Fungi

Ground samples from each part of OPT and surface scrapped residues of OPT plank (1.5 inch thick × 4 inch wide × 2 m long) were naturally fermented (without inoculum added) at room temperature (30 ± 2 °C) for about 3 days or until growth of microorganisms appeared. All samples were added with 0.1% Tween 80 (10 mL g<sup>-1</sup> of fermented substrate) and the mixture was shaken (150 rpm) for 30 min [37], then made dilution to 10<sup>-6</sup>–10<sup>-7</sup>. The diluted culture suspension (0.1 mL) was inoculated onto PDA plates using spread-plate technique and incubated at room temperature (30 ± 2 °C). The fungal isolates were purified through repeated streaking on fresh PDA plates and pure cultures were maintained on PDA slant.

Screening was based on the ability of the fungal isolates to utilize ground OPT<sub>r</sub> (1% wv<sup>-1</sup>) as substrate for growth and enzymes production under solid-state fermentation (SSF) and submerged fermentation (SmF) using the modified CDM [38] with and without agar respectively. The fungal isolates were streaked on the agar plates and incubated at room temperature (30 ± 2 °C) for 5 days. The selected fungal isolates were subcultured on fresh PDA slant. They were cultivated under SSF using OPT<sub>r</sub> as substrate with supplementation of CDM nutrients solution (5 mL g<sup>-1</sup> dry substrate (gds)). After sterilization, spore suspension at 10<sup>6</sup> spores gds<sup>-1</sup>

was inoculated and the moisture content was adjusted to 60% [39–41], then incubated for 4 days at room temperature (30 ± 2 °C). Crude enzymes were extracted from the OPT<sub>r</sub> culture, filtered to remove the mycelium by using cheesecloth [42] and centrifuged (1789×g for 10 min) to obtain a clear supernatant [43]. Aliquots of the supernatant was diluted to appropriate concentration for determination of enzymes activity of cellulase (CMCase), xylanase and FPase.

For submerged fermentation (SmF), spore suspension (10<sup>6</sup> spores gds<sup>-1</sup>) was inoculated into the modified CDM containing 5% (wv<sup>-1</sup>) of OPT<sub>r</sub> and incubated at room temperature (30 ± 2 °C) for 4 days. Samples were taken for determination of enzymes activities (as described above). The isolates producing the highest enzymes activity were selected for further studies. The selected strains were identified by 18S rRNA gene sequence [44] and the sequences were BLAST searched against the National Center for Biotechnology Information (NCBI) database.

### Enzymes Production from the Three Selected Strains and the Mixed Cultures

Time course on enzymes production from the three selected fungal strains and the mixed-cultures cultivated in MMS medium [45] using OPT<sub>r</sub> as a carbon source was conducted under SSF and SmF at room temperature (30 ± 2 °C) for 7 days. The strain and the fermentation type (either SmF or SSF) giving the highest enzymes activity were selected for further studies.

The enzyme production from the selected strain was carried out under SSF condition (as described above). The spore suspension was inoculated to OPT<sub>r</sub> supplemented with MMS medium (ratio 1:1 wv<sup>-1</sup>) in 250 ml Erlenmeyer flasks. Samples were taken and centrifuged to remove the cells, then extracted the enzymes from the supernatant by adding cold acetone (4 °C) at the ratio of 1:4 wv<sup>-1</sup>

(supernatant: acetone) and allowed precipitation to occur at  $-20\text{ }^{\circ}\text{C}$  overnight [46]. The precipitate was dissolved in minimal amount of 50 mM citrate buffer (pH 4.8) and its enzyme activity was determined.

### Effect of Enzyme Loading During the Hydrolysis of OPTr

The 2.5% ( $\text{wv}^{-1}$ ) ground OPTr was added into the citrate buffer (50 mM, pH 4.8) and sterilized ( $121\text{ }^{\circ}\text{C}$  for 15 min). After cooling, the enzymes at various concentrations were added to obtain the enzyme loading of 0–40 Unit  $\text{g}^{-1}$  OPTr. The enzyme–substrate mixtures were incubated at  $50\text{ }^{\circ}\text{C}$  for 24 h under constant shaking (150 rpm) condition. Samples were taken at time interval and determined for sugar concentration using HPLC [24] and reducing sugar using DNS method [47].

### Two-Stage and Simultaneous Fermentation of OPTr Hydrolysate for Production of Ethanol and Acetic Acid Using *Saccharomyces cerevisiae* and *Acetobacter aceti*

The OPTr hydrolysate with and without supplementation of YM nutrients (yeast extract  $3.0\text{ g L}^{-1}$ , malt extract  $3.0\text{ g L}^{-1}$  and peptone  $5.0\text{ g L}^{-1}$ ) was used for ethanol and acetic acid production under two-stage fermentation and simultaneous fermentation using *S. cerevisiae* TISTR5055 and *A. aceti*. In two-stage process, the starter culture of *S. cerevisiae* ( $\text{OD}_{600}=0.5$ ) was inoculated ( $10\% \text{ v v}^{-1}$ ) into 250 mL Erlenmeyer flask containing the OPTr hydrolysate (total working volume of 50 mL), with and without addition of nutrients and incubated at room temperature ( $30 \pm 2\text{ }^{\circ}\text{C}$ ) for 24 h and sample (5 mL) was taken to determine for ethanol production. After that, the starter culture of *A. aceti* ( $\text{OD}_{600}=0.5$ ) was inoculated ( $10\% \text{ v v}^{-1}$ ) for acetic acid production in the second stage and incubated until 60 h. Samples were taken at time interval (every 6 h) and the supernatant was analyzed for sugars, ethanol and acetic acid concentration using HPLC [24].

For simultaneous fermentation, starter cultures of *S. cerevisiae* and *A. aceti* ( $\text{OD}_{600}=0.5$ ) were mixed in 1:1 ratio ( $\text{v v}^{-1}$ ) and inoculated ( $10\% \text{ v v}^{-1}$ ) into 250 Erlenmeyer flask containing the OPTr hydrolysate (total working volume of 50 mL), with and without addition of YM nutrients. They were incubated at room temperature ( $30 \pm 2\text{ }^{\circ}\text{C}$ ) for 36 h. During fermentation, samples were withdrawn (every 6 h) and its supernatant was analyzed for sugar, ethanol and acetic acid concentration using HPLC [24].

## Analytical Methods

### Enzyme Activity Assay

Carboxymethylcellulase (CMCase) was assayed in reaction containing 1% ( $\text{wv}^{-1}$ ) of CMC (0.5 mL) in 50 mM citrate buffer pH 4.8 and appropriate diluted enzyme (0.5 mL). After 30 min incubation at  $50\text{ }^{\circ}\text{C}$ , reducing sugar was measured by 3,5-dinitrosalicylic acid method [44] with glucose was used as standard [48, 49]. One unit (U) of CMCase activity is defined as the amount of enzymes that liberates 1  $\mu\text{mol}$  of glucose equivalents per minute.

Xylanase activity was assayed in reaction containing 1% ( $\text{wv}^{-1}$ ) of oat spelt xylan (0.5 mL) in 50 mM citrate buffer pH 4.8 and appropriate diluted enzyme (0.5 mL). After 10 min incubation at  $50\text{ }^{\circ}\text{C}$ , reducing sugar was measured by 3,5-dinitrosalicylic acid (DNS) method [44] with xylose was used as standard [48, 49]. One unit (U) of xylanase activity is defined as the amount of enzymes that liberates 1  $\mu\text{mol}$  of xylose equivalents per minute.

Exoglucanase (FPase) assay was carried out by incubating 0.5 ml suitably diluted crude enzyme with 1 mL citrate buffer (50 mM, pH 4.8) containing Whatman Filter paper (No.1) strip (1 cm  $\times$  6 cm, 50 mg) and incubation at  $50\text{ }^{\circ}\text{C}$  for 60 min. Then, reducing sugar was measured by 3,5-dinitrosalicylic acid (DNS) method [44] with glucose used as standard. One unit of FPase activity correspondent to 1  $\mu\text{mol}$  of glucose released per minute [30].

### Determination of Sugars, Ethanol and Acetic Acid Concentration

The concentrations of hexose (glucose), pentose (xylose, arabinose), ethanol and acetic acid were determined using high performance liquid chromatography (HPLC) (Agilent 1200) equipped with a HPX-87H (300 mm  $\times$  7.8 mm) column (Bio-Rad, Hercules, CA) and a refractive index (RI) detector. The sample was diluted with deionized water, filtered through 0.22  $\mu\text{m}$ , 13 mm Nylon membrane filter (Sartorius, Goettingen, Germany) and then injected in the chromatograph under the following conditions: column temperature at  $65\text{ }^{\circ}\text{C}$ , 5 mM sulfuric acid as mobile phase at a flow rate of  $0.6\text{ mL min}^{-1}$ , and an injection volume of 20  $\mu\text{L}$ . The concentration of these compounds was calculated using calibration curves obtained from standard solutions [24]. Data shown were the average of three replicated assessments.

## Statistical Analysis

The data presented were analyzed using SPSS (SPSS Inc., version 15.0). One-way analysis of variance (ANOVA) was carried out to compare the means of different treatment where significant  $F$  value was obtained. Differences between individual means were tested using Duncan's Multiple Range Test (DMRT) at 0.05 significant levels. The data was performed in triplicate.

## Results and Discussion

### Chemical Composition of OPT and Preparation of OPT<sub>r</sub>

The chemical composition of cross section of OPT was previously reported [1, 25, 28] but not yet the composition along its length. In this study, the chemical composition of 9 m long OPT (weight of 1786 kg) divided into five parts (see Fig. 1) and its four parts were analyzed (Table 1). The OPT was found to accumulate starch with the highest quantity at the top end (tip) part (1 m long, diameter 42 cm) (3.79%  $w w^{-1}$ ) followed by the top part (2 m long, diameter 45 cm) (2.78%  $w w^{-1}$ ). The values were similar to the average starch content of the middle part of the whole log (2.5 m long and 36–41 cm in diameter) (3.5% of dried solid OPT disc) [50]. The moisture content of OPT decreased along its height with the highest value at the bottom part (about 65%) was lower than that reported by Noparat et al. [28] (about 75%). In addition, the total amount of lignocelluloses of OPT was also highest at the bottom part (87.71%) and decreased along its height (59.10, 37.16 and 26.15%, respectively). The holocellulose content of OPT (13–55% cellulose, 12–20% hemicellulose) was comparable with that reported by Ang et al. [30] (45.81% cellulose, 17.74% hemicellulose) but the lignin content was much lower (1.30–13.00% compared to 24.49%). Based on the composition of high holocellulose with low lignin content, the top and middle parts of felled OPT were selected as feedstock for enzyme production.

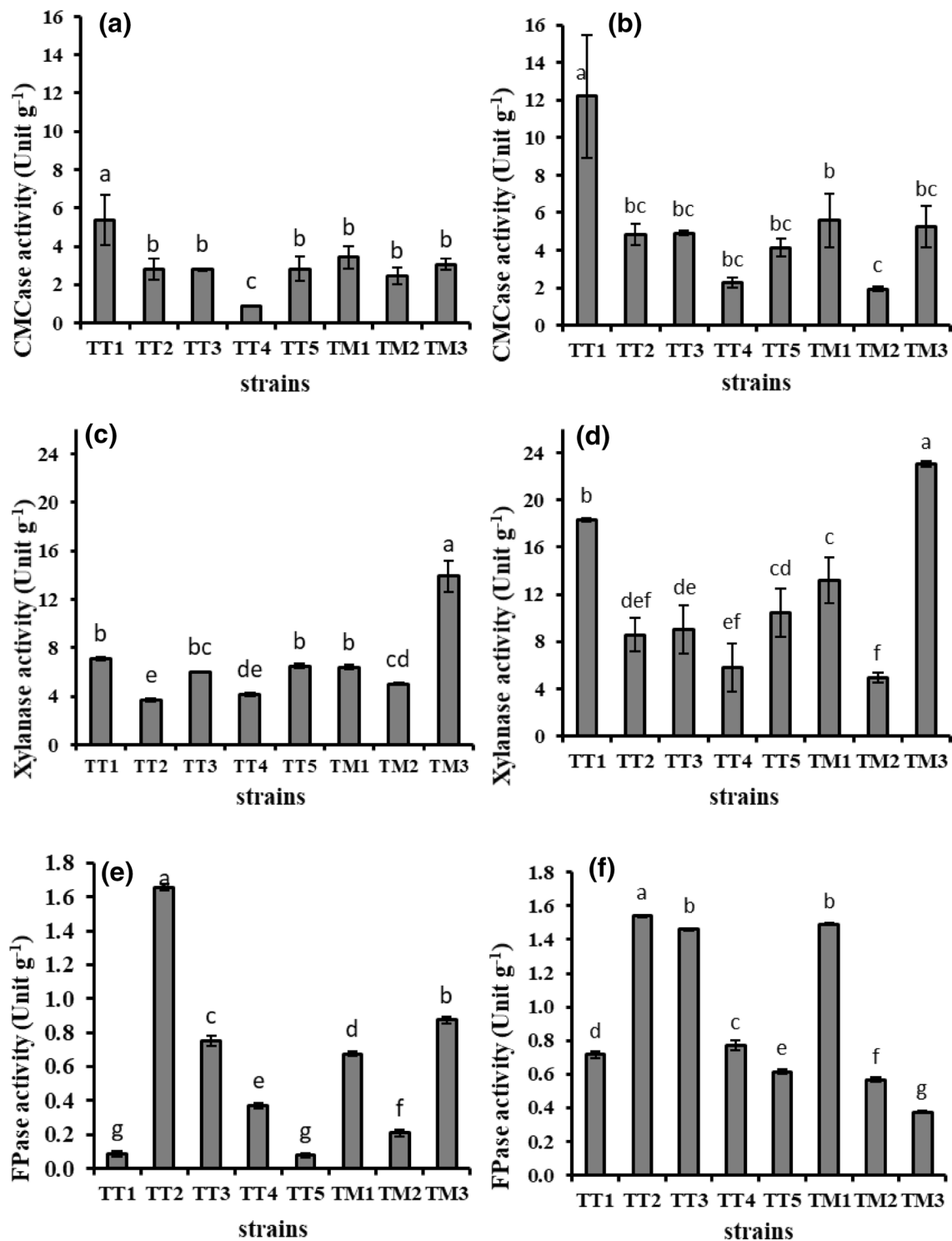
The OPT sap contained glucose as the dominant sugar (4.96–20.13  $g L^{-1}$ ) followed by fructose (3.04–6.02  $g L^{-1}$ ) (Table 1). The highest values of glucose (20.13  $g L^{-1}$ ) was 2.5 times lower while fructose (6.02  $g L^{-1}$ ) was two times higher than the values of Malaysian oil palm sap (50.17  $g L^{-1}$  glucose and 3.07  $g L^{-1}$  fructose, respectively) [25]. The amount of glucose in this study was about 1.3 times higher than that reported by Noparat et al. [28] (15.72  $g L^{-1}$ ). Arabinose was present only in trace amount (0.43–0.84  $g L^{-1}$ ) in the top end and top part of OPT. In contrast to OPT sap, the sap of *Raphia* palm (*Raphia*

*hookeri*) contained sucrose as the dominant sugar [51]. The discrepancy may be due to the difference in varieties, species and/or cultivating conditions.

### Isolation, Selection and Identification of the High Enzyme Producing Fungi

A total of 20 fungal strains were isolated from the natural fermented OPT and the plank samples. Eight out of the 20 fungal isolates were able to grow on CDM containing ground OPT<sub>r</sub> as carbon source and encoded as the isolate TT1, TT2, TT3, TT4, TT5, TM1, TM2 and TM3. They were screened for their ability to produce cellulase and xylanase under SSF and SmF (Fig. 2). The isolate TT1 exhibited the highest CMCase activity under SmF (12.20 Unit  $g ds^{-1}$  with 2.3 times higher than its highest value under SSF (5.37 Unit  $g ds^{-1}$ ). The isolate TM3 gave the highest xylanase activity under SmF (23.06 Unit  $g ds^{-1}$ ) which was 1.7 times higher than under SSF (13.91 Unit  $g ds^{-1}$ ). The isolates TT2 gave the highest FPase activities both under SSF (1.66 Unit  $g ds^{-1}$ ) and SmF (1.54 Unit  $g ds^{-1}$ ). Based on these results, the isolates TT1, TM3 and TT2 were selected as the producer of CMCase (12.20 Unit  $g ds^{-1}$  from SmF), xylanase (23.06 Unit  $g ds^{-1}$  from SmF) and FPase (1.66 Unit  $g ds^{-1}$  from SSF), respectively.

The three isolates possessing high enzyme activity were identified using 18S rRNA gene sequence and NCBI blast search (Fig. 3). It showed a 100% sequence identity of the isolate TT1 with *Ceratocystis paradoxa* (KJ881375) that was reported as a sugarcane phytopathogen [52] causing black seed rot disease in oil palm sprouted seeds [53] as well as bud and trunk rots affecting almost all species of palm [54, 55]. The isolate TM3 exhibited 98% sequence identity with *Trichoderma koningiopsis* T-404 (JQ278019). Strains of *Trichoderma* can accumulate high activities of endo- and exo-glucanases, but are poor in  $\beta$ -glucosidases [56]. Additionally, *T. koningiopsis* Th003 was able to induce the activity of  $\beta$ -1,3-glucanase and endo-chitinases to control different pathogens including the system tomato-*Fusarium oxysporum* and stimulate growth in many crops [57]. The growth of some phytopathogenic fungi could be inhibited by the chemicals (volatile substances such as alkanes, monoterpenes and arenes, heterocycles, and aldehydes) produced by *T. koningiopsis* YIM PH30002 [58]. These chemical compounds possessed the antifungal activity, nitric oxide inhibition and anticoagulant activity [59]. The isolate TT2 exhibited 95% sequence identity with *Hypocrea nigricans* NBRC 30611 (JN941681). *H. nigricans* is an anamorph of *Trichoderma* sp., by nature a wood decaying fungus and a common fungal species of moist forests [60]. In addition, *Hypocrea* species was used in the biological control of plant pathogenic fungi; many species have the ability to break down cellulosic materials. This ability has led to

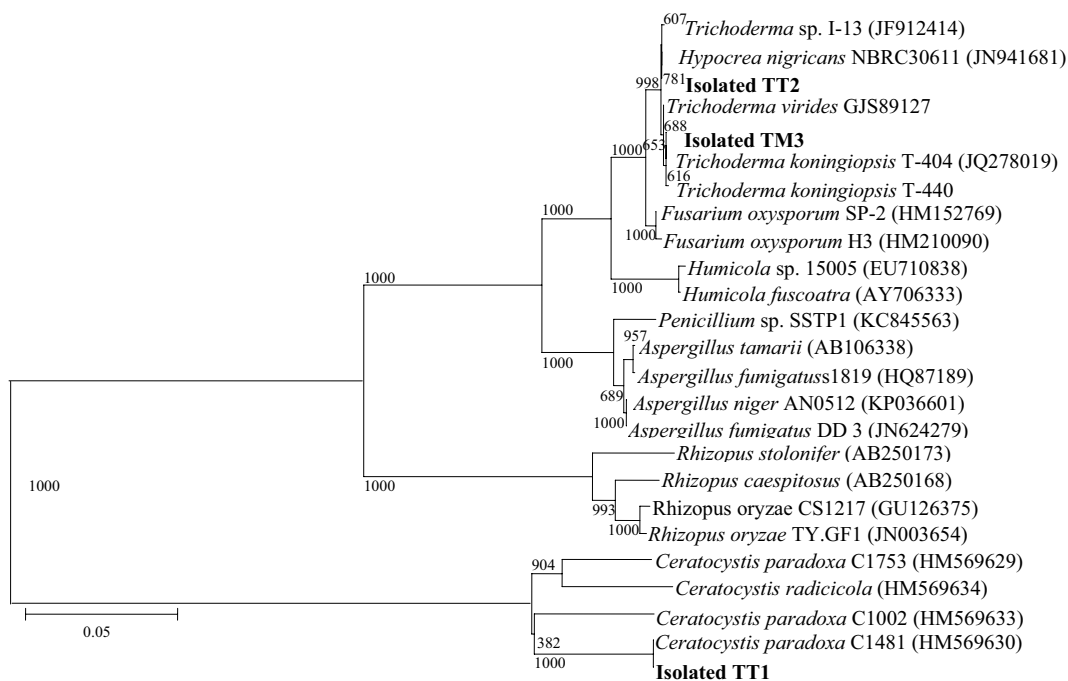


**Fig. 2** Comparison on enzymes production from the eight selected fungal strains cultivation in CDM medium containing oil palm trunk residues (OPT<sub>r</sub>) as a carbon source under solid-state fermentation

(SSF) (a, c, e) and submerged fermentation (SmF) (b, d, f) after cultivation at room temperature ( $30 \pm 2$  °C) for 4 days

the commercial exploitation of some *Hypocrea* and *Trichoderma* species in production of cellulolytic enzymes used in manufacture of denims, animal feed and bio fuels [60, 61].

Under the optimum condition, *H. nigricans* produced higher endo- and exo- $\beta$ -1,4-D-glucanases (from 3.9 to 11.89 U g<sup>-1</sup> and 2.98 to 11.89 U g<sup>-1</sup>, respectively) [60]. Besides the three



**Fig. 3** Neighbor-joining tree showing the phylogenetic position of isolated strain based on the 18S rRNA sequences. The numbers at the nodes indicate the levels of bootstrap support percentages based on the neighbor-joining of 1000 replicates. Bar 0.005 nucleotide substitutions per site

selected isolates (TT1, TM3 and TT2), the other five isolates were identified; TT3, TT5 and TM1 were belonged to the genus *T. asperellum* and the isolate TM2 and TT4 were with the genus *Aspergillus niger* and *A. tubingensis*, respectively.

### Enzymes Production from the Selected Strains and the Mixed Cultures

Improvement in lignocellulolytic enzymes production could be achieved via the mixture of different fungi. Mixed-culture is beneficial in lignocellulolytic enzymes production via SSF as the fungi are normally co-existed symbiotically on solid substrates in nature [62, 63]. Besides, mixed-culture also offers advantages such as higher productivity, adaptability

**Table 2** The maximum enzymes activity of three selected strains and their mixed cultures (TT1:TM3:TT2, 1:1:1 ratio) cultivated under solid-state fermentation (SSF) and submerged fermentation (SmF) using oil palm trunk residues (OPT<sub>r</sub>) as a carbon source

Strain	Condition	Time (days)	Enzymes activity (U gds <sup>-1</sup> )*		
			CMCase	Xylanase	FPase
<i>Ceratocystis paradoxa</i> TT1	SSF	5	6.49 ± 0.29	12.01 ± 0.88	1.69 ± 0.31
	SmF	4	18.16 ± 2.73 (0.83 ± 0.13)**	36.99 ± 0.43 (1.68 ± 0.35)**	1.64 ± 0.27 (0.075 ± 0.013)**
<i>Trichoderma koningiopsis</i> TM3	SSF	3	7.12 ± 0.13	56.45 ± 0.60	2.12 ± 0.29
	SmF	4	4.82 ± 0.15 (0.22 ± 0.01)**	12.16 ± 0.22 (0.55 ± 0.01)**	1.24 ± 0.04 (0.06 ± 0.00)**
<i>Hypocrea nigricans</i> TT2	SSF	3	3.55 ± 0.13	11.95 ± 0.92	1.13 ± 0.05
	SmF	2	4.24 ± 0.88 (0.19 ± 0.04)**	10.27 ± 0.98 (0.47 ± 0.05)**	1.50 ± 0.04 (0.07 ± 0.00)**
Mixed culture TT1:TM3:TT2 (1:1:1)	SSF	3	6.06 ± 0.31	20.24 ± 0.52	1.26 ± 0.05
	SmF	3	4.15 ± 0.17 (0.19 ± 0.01)**	11.58 ± 1.97 (0.53 ± 0.09)**	1.25 ± 0.05 (0.06 ± 0.01)**

\*Ugds<sup>-1</sup> = units per gram of dry fermented substrate; \*\*U ml<sup>-1</sup> = unit per mL



and substrate utilization compared to pure and monoculture [64]. In this study, time course on enzymes production from the three selected fungal strains and the mixed-cultures (TT1:TT2:TM3 in the ratio of 1:1:1) cultivated in MMS medium using OPTr as a carbon source under SSF and SmF at room temperature ( $30 \pm 2$  °C) for 7 days was conducted. The maximum enzymes activities of all three strains cultivated under SSF and SmF were summarized in Table 2. *C. paradoxa* TT1 showed the highest CMC<sub>ase</sub>, xylanase and FPase of 18.16, 36.99 and 1.64 Unit gds<sup>-1</sup> at 4 days cultivation under SmF. These were 1.5, 1.6 and 2.3 times higher than those cultivated in CDM medium. *C. paradoxa* was reported to show the highest xylanase activity when grown on wheat bran (12,728 IU mL<sup>-1</sup>) and β-glucosidases when grown on steam-treated bagasse (1068 IU mL<sup>-1</sup>) [49]. *T. koningiopsis* TM3 gave the highest xylanase and FPase (56.46 and 2.13 Unit gds<sup>-1</sup> at 3 days cultivation). Therefore, *C. paradoxa* TT1 was cellulase producer while *T. koningiopsis* TM3 was xylanase (and FPase) producer. The lower enzymes activities of the mixed cultures than those from the monoculture may be due to the undesirable competition between the fungal strains [65]. *Trichoderma* species were reported to produce both volatile and nonvolatile metabolites that adversely affect growth of different fungi such as the fungistatic effects on the growth of *C. paradoxa* [53]. In

addition, the inoculation sequence of different fungal strains might play a significant role in stimulating the enzyme production [66] or imposing any significant negative effect on the growth of each other [67]. Furthermore, the inoculating time of the fungal strains also had an impact on enzyme production. For examples, the co-cultivation of *T. reesei* RUT-30 and *Phanerochaete chrysosporium* exhibited the maximum cellulase activity when inoculation time was delayed for 1.5 day which correlated to the higher saccharide yields than those from monoculture [68]. Similarly, the maximum cellulase activity (3.2 IU g<sup>-1</sup>) was obtained when *A. oryzae* was co-cultured on soybean fiber with *T. reesei* and *P. chrysosporium* after 36 h incubation [69].

Comparison on enzymes production from this study to the other fungal strains is given in Table 3. It should be mentioned that the different values of enzyme activity were partly due to the difference in enzyme activity assay. Considering among oil palm wastes, *C. paradoxa* TT1 produced 2.23-fold higher CMC<sub>ase</sub> activities than that of *Botryosphaeria* sp. (8.13 Unit g<sup>-1</sup>) [43] but lower than that of *A. turingensis* TSIP9 (26.10 Unit g<sup>-1</sup>) [63]. *T. koningiopsis* TM3 produced 1.60-fold higher xylanase (56.46 Unit gds<sup>-1</sup>) than *Aspergillus niger* USM A1 1 (35 Unit gds<sup>-1</sup>) [70] and slightly lower than *A. turingensis* TSIP9 (59.3 Unit gds<sup>-1</sup>) [71]. In this present study, *T. koningiopsis* TM3 was the

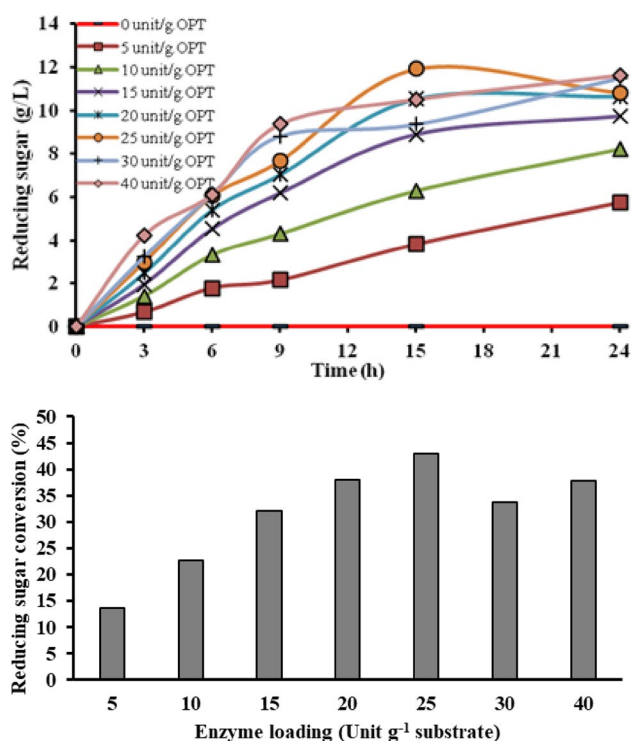
**Table 3** Comparison on enzymes production from the three isolated fungal strains and their mixed culture with the other fungal strains

Enzymes	Strain	Activity (unit g <sup>-1</sup> )	Carbon source	References
CMCase	<i>Fomitopsis RCK2010</i> sp.	71.70	Wheat bran	[14]
	<i>Aspergillus fumigatus</i>	16.90	Wheat straw	[71]
	<i>Botryosphaeria</i> sp.	8.13	Empty fruit bunch	[43]
	<i>A. turingensis</i> TSIP9	26.10	Empty fruit bunch	[63]
	<i>Ceratocystis paradoxa</i> TT1	18.16	Oil palm trunk	Present work
	<i>Hypocreanigricans</i> TT2	6.10	Oil palm trunk	Present work
	<i>T.koningiopsis</i> TM3	7.13	Oil palm trunk	Present work
	Mixed TT1:TM3:TT2	6.06	Oil palm trunk	Present work
Xylanase	<i>A.niger</i> USM A1 1	35.00	Palm kernel cake	[70]
	<i>Aspergillus fumigatus</i>	56.40	Wheat straw	[71]
	<i>A.turingensis</i> TSIP9	59.30	Empty fruit bunch	[63]
	<i>Ceratocystis paradoxa</i> TT1	40.00	Oil palm trunk	Present work
	<i>Hypocreanigricans</i> TT2	21.75	Oil palm trunk	Present work
	<i>T.koningiopsis</i> TM3	56.46	Oil palm trunk	Present work
	Mixed TT1:TM3:TT2	20.24	Oil palm trunk	Present work
FPase	<i>Thermoascusauraticus</i>	4.40	Wheat straw	[72]
	<i>Fomitopsis</i> sp. RCK2010	3.50	Wheat bran	[14]
	<i>Aspergillus fumigates</i>	0.98	Wheat straw	[71]
	<i>Botryosphaeria</i> sp.	3.30	Empty fruit bunch	[43]
	<i>Ceratocystis paradoxa</i> TT1	1.64	Oil palm trunk	Present work
	<i>Hypocreanigricans</i> TT2	1.70	Oil palm trunk	Present work
	<i>T.koningiopsis</i> TM3	2.13	Oil palm trunk	Present work
Mixed TT1:TM3:TT2	1.26	Oil palm trunk	Present work	

most suitable for lignocellulase enzymes production from the OPTr with higher enzymes production (7.13 Unit g<sup>-1</sup> of CMCase, 56.46 Unit g<sup>-1</sup> of xylanase and 2.13 Unit g<sup>-1</sup> of FPase activity) than those selected strains.

### Effect of Enzyme Loading During the Hydrolysis of OPTr

The crude enzymes from the supernatant of *T. koningiopsis* TM3 was concentrated using acetone which was reported to be better than ammonium sulphate [46]. The concentrated enzyme TM3 possessed CMCase and



**Fig. 4** The efficacy of crude enzymes (CMCase and xylanase) from *Trichoderma koningiopsis* TM3 in hydrolyzing the OPT residues to produce reducing sugar

xylanase activities of 3.22 and 54.14 Unit mL<sup>-1</sup>, respectively, with the yields of 60.04% and 68.74%, respectively (data not shown).

The effect of enzymes TM3 concentrations (0–40 Unit g<sup>-1</sup>OPTr) and reaction times (0–24 h) for hydrolysis of OPTr was studied. Figure 4 shows the optimum enzyme concentration at 25 Unit g<sup>-1</sup> OPTr and 15 h reaction time, giving the highest reducing sugars concentration of 11.92 g L<sup>-1</sup> with the yield of 476.8 mg g<sup>-1</sup> OPTr. The increase of enzymes concentration from 5 to 25 Unit g<sup>-1</sup> substrate resulted in the increase of reducing sugar conversion from 13.7% to 42.9% and dropped to 33.7% and 37.8% conversion at 30 and 40 Unit g<sup>-1</sup> OPTr, respectively. Similar pattern was reported by Zulkefli et al. [1] that the hydrolysis of OPT did not improve the conversion further when loading the high amount of enzymes. The maximum concentration of this study (11.92 g L<sup>-1</sup>) was about 4.5 times higher than that from hydrolysis of pretreated empty fruit bunch (EFB) (2.4 g L<sup>-1</sup> after 24 h reaction time) using the ratio of cellulase to  $\beta$ 1-4 glucosidase of 5:1 while the productivity was times lower when higher  $\beta$  1-4 glucosidase has been added into the reaction mixture (ratio 1:5) [73]. Higher enzyme loading giving lower reducing sugar conversion may be caused by the decrease of adsorption efficiency due to the saturation of enzyme on cellulose surface [74]. However, some studies showed the increase of sugar production at higher enzyme loading [75, 76]. Therefore, the enzyme hydrolysis reaction should be performed under the optimum condition (enzyme concentration and reaction time) and based on the cost. The enzymes from this strain exhibited higher saccharification efficiency on the OPTr than those from many brown rots fungi reported earlier (Table 4). This result showed that the OPTr was a better substrate than the other agricultural wastes (rice straw, wheat straw, etc.) as it gave the highest yield of reducing sugar without addition of the commercial enzyme (i.e. Novozyme). The sugar yield of this strain was almost 15 times higher than that of *Fomitopsis palustris* acting on Avicel (32 mg g<sup>-1</sup> after 43 h) [77].

**Table 4** Comparison of enzymes hydrolysis of different substrates by cellulase from different fungi and commercial preparations

Source of enzymes	Substrate	Reducing sugar*	References
<i>Laetiporus sulphureus</i>	<i>Pinus densiflora</i>	70.9	[78]
<i>Trichoderma reesei</i> and novozyme 188	Corn cob	826.2	[79]
<i>Trichoderma reesei</i> and novozyme 188	Prosopis	838.9	[79]
<i>Trichoderma reesei</i> and novozyme 188	Lantana	777.6	[79]
<i>Fomitopsis spenicola</i>	<i>Pinus densiflora</i>	3.53	[78]
<i>Fomitopsis palustris</i>	Avicel	32	[77]
<i>Fomitopsis</i> sp. RCK2010	Rice straw	157.2	[14]
<i>Fomitopsis</i> sp. RCK2010	Wheat straw	214.1	[14]
<i>Trichoderma koningiopsis</i> TM3	OPT residues	476.8	Present work

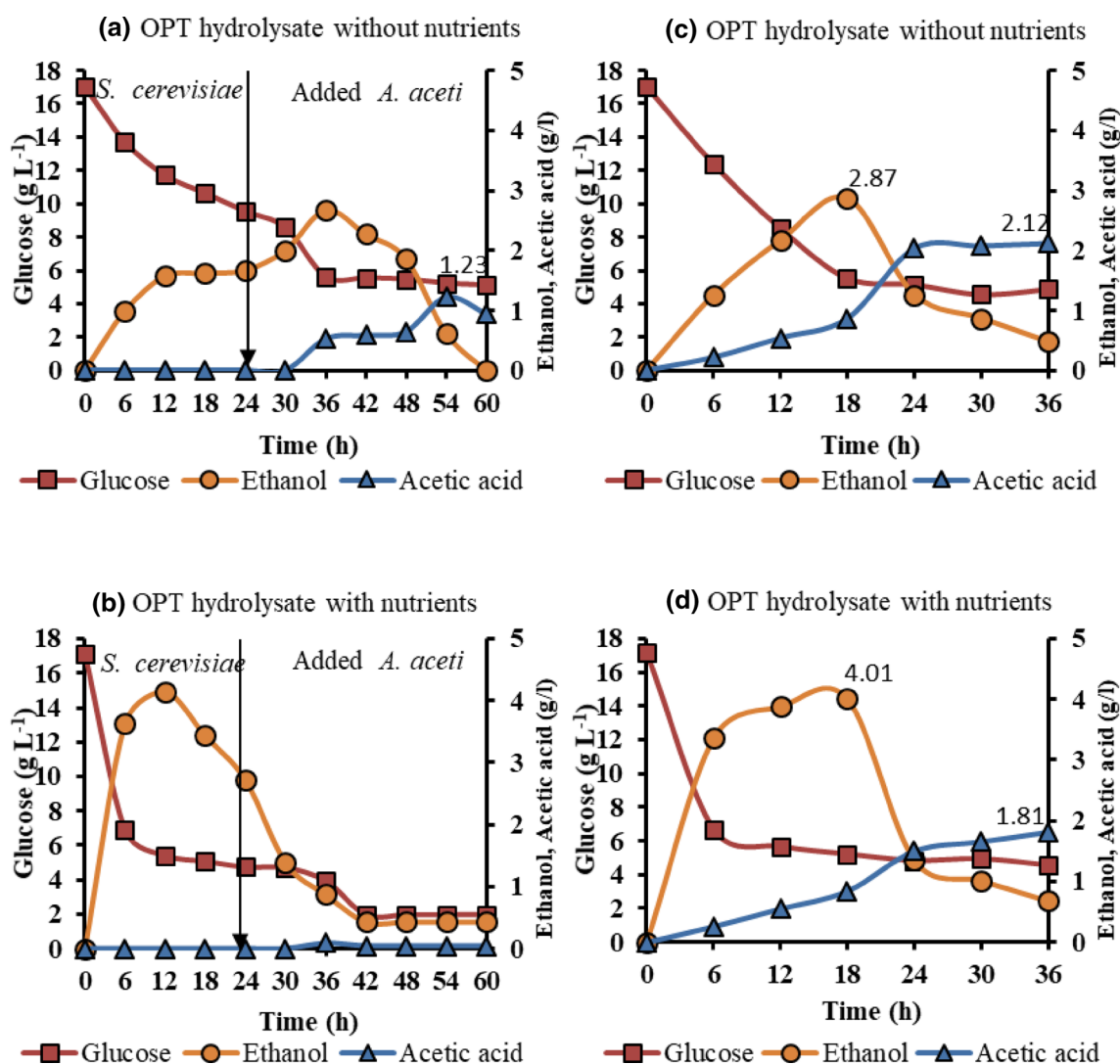
\*mg g<sup>-1</sup> of substrate

## Two-Stage and Simultaneous Fermentation of OPT<sub>r</sub> Hydrolysate to Ethanol and Acetic Acid Using *S. cerevisiae* and *A. aceti*

Ethanol production from plant biomass has received considerable attention because of the expectation that bioethanol will alleviate demands for petroleum-based fuels [80]. The hydrolysis of lignocellulosic biomass liberates sugars, primarily glucose and xylose, which are subsequently converted to ethanol by microbial fermentation [81]. *S. cerevisiae* has been used in the industrial bioethanol production due to its robustness and high ethanol productivity. However, this yeast strain cannot naturally ferment xylose [82] while *Candida shehatae* can utilize both glucose and xylose as substrates for ethanol fermentation [83]. However, our

preliminary studies on ethanol production from OPT<sub>r</sub> hydrolysate using the co-culture of *S. cerevisiae* TISTR5055 and *C. shehatae* TISTR5843 produced lower ethanol concentration than the mono-culture (data not shown). As a consequence, only *S. cerevisiae* TISTR5055 was employed for ethanol production.

In this study, ethanol and acetic acid were produced by two-stage fermentation and simultaneous fermentation of the OPT<sub>r</sub> hydrolysate, with and without addition of nutrients in YM medium (Fig. 5). In two-stage process, ethanol production by *S. cerevisiae* increased and rather stable during 12–24 h, then *A. aceti* was inoculated. The ethanol production was continued and reached the highest value of 2.87 g L<sup>-1</sup> at 36 h. *A. aceti* assimilated ethanol and started to produce acetic acid after 30 h and reached the highest



**Fig. 5** Time course of ethanol and acetic acid production by *Saccharomyces cerevisiae* TISTR5055 and *Acetobacter aceti* under two-stage (a, b) and simultaneous (c, d) fermentation from oil palm trunk

hydrolysate, with (a, c) and without addition of nutrients (b, d) in shake-flask (150 rpm) culture at room temperature (30 ± 2 °C)

value ( $1.23 \text{ g L}^{-1}$ ) at 54 h with simultaneous depletion of ethanol (Fig. 5a). With nutrients supplementation (Fig. 5b), ethanol production increased sharply and reached the maximum value ( $4.15 \text{ g L}^{-1}$ ) at 12 h fermentation with the yield of  $0.35 \text{ g ethanol g}^{-1}$  sugar consumed. Hence, the ethanol production increased by 1.43-folds while its productivity increased by 4.27-folds. This was due to the influence of some nutrients that were essential for cell growth and metabolite production [84]. Higher ethanol production was reported from using empty fruit bunch (EFB)-derived sugar by *S. cerevisiae* ( $12.13 \text{ g L}^{-1}$ ) [85] and from crude glycerol supplemented with yeast extract by *Kluyvera cryocrescens* (about  $11 \text{ g L}^{-1}$ ) [86]. In addition, the ethanol yield from this study ( $0.35 \text{ g g}^{-1}$ ) was slightly lower than those produced from EFB hydrolysate (via dilute-acid hydrolysis) fermented by *S. cerevisiae* ( $0.46 \text{ g g}^{-1}$ ) and *Mucor indicus* ( $0.45 \text{ g g}^{-1}$ ) [87]. In two-stage process, there was no production of acetic acid which may be due to the growth inhibition of *A. aceti* by too high concentration of ethanol.

For simultaneous fermentation of OPTr, the maximum values of ethanol and acetic acid were  $2.87 \text{ g L}^{-1}$  at 18 h and  $2.12 \text{ g L}^{-1}$  at 24 h, respectively, with the productivity of  $0.16$  and  $0.09 \text{ g L}^{-1}\text{h}^{-1}$  (Fig. 5c). The maximum acetic acid concentration and productivity were 1.7-fold and 4.0-fold higher than those from the two-stage fermentation. Addition of nutrients to the OPTr hydrolysate also exhibited strong influence on ethanol production ( $4.01 \text{ g L}^{-1}$  at 18 h) and acetic acid production ( $1.81 \text{ g L}^{-1}$  at 36 h) (Fig. 5d). Therefore, co-culture of *S. cerevisiae* and *A. aceti* in the OPTr hydrolysate without nutrients supplementation could increase the ethanol production from monoculture of *S. cerevisiae* by only 7% (from  $2.68$  to  $2.87 \text{ g L}^{-1}$ ) but gave 2.15-fold increase in ethanol productivity ( $0.074$  to  $0.159 \text{ g L}^{-1} \text{ h}^{-1}$ ). The production efficiency (productivity) was considered from the cultivation time required to reach the maximum values (co-culture reached within 18 h while the monoculture required 36 h). Nutrient supplementation to the monoculture could further enhance by 1.4-fold both the ethanol concentration (from  $2.87$  to  $4.01 \text{ g L}^{-1}$ ) and ethanol productivity (from  $0.159$  to  $0.223 \text{ g L}^{-1} \text{ h}^{-1}$ ). However, the nutrients supplementation had an adverse effect on acetic acid production as it decreased the acetic acid concentration by 1.2-fold (from  $2.12$  to  $1.81 \text{ g L}^{-1}$ ) and also the productivity (from  $0.09$  to  $0.05 \text{ g L}^{-1} \text{ h}^{-1}$ ). Acetic acid production, on the other hand, reduced ethanol production as the ethanol was oxidized to acetic acid. However, the decrease of acetic acid production may be due to the sharp increase of ethanol within the first 6 h (corresponding to the rapid consumption of glucose) and also the higher concentration of ethanol. These factors may affect the growth of acetic acid bacteria. Therefore, simultaneous fermentation using co-culture of *S. cerevisiae* and *A. aceti* could increase the ethanol productivity as well as acetic acid productivity by supplementation of

nutrients to the system. The co-culture was more efficient in conversion of reducing sugars to ethanol. Furthermore, for efficient bioconversion of biomass-derived sugars to acetic acid, it is crucial to have proper balance of sugars in the hydrolysate [33].

## Conclusion

The OPTr is a potential feedstock for enzymes, ethanol and acetic acid production. OPTr was used as substrate for production of lignocellulolytic enzymes by various isolated fungal strains through SSF and SmF. *C. paradoxa* TT1 and *T. koningiopsis* TM3 produced the highest CMCase from SmF and xylanase from SSF, respectively. Then the OPTr was hydrolyzed using the crude enzyme TM3 ( $25 \text{ Unit g}^{-1}$ ) and incubated at  $50 \text{ }^{\circ}\text{C}$  for 15 h. The OPTr hydrolysate with nutrients supplementation showed the maximum ethanol production ( $4.1 \text{ g L}^{-1}$ ) under the two-stage fermentation. The maximum acetic acid concentration ( $2.12 \text{ g L}^{-1}$ ) and productivity ( $0.09 \text{ g L}^{-1}\text{h}^{-1}$ ) was achieved from the simultaneous fermentation without nutrient addition. The integration of OPTr utilization for production of both enzymes and biofuel will not only reduce the enzyme cost but also generate ethanol and acetic acid as its additional valuable products.

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