Penicillium rolfsii, the potential lignocellulolytic fungus on hydrolysis of oil-palm residues from oil palm trunk as a second generation biofuel feedstock

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Abstract. Energy crisis involved the excessive consumption of fuels causing the increased in energy demands, oil price and depletion of fossil fuels. This has resulted in generation of high level of greenhouse gases emission. Therefore, in order to overcome these problems, alternative fuel has to be produced. This study was conducted to isolate potential fungal strains which can hydrolyze oil-palm residues as feedstock for bioethanol production. One hundred and sixty four fungal isolates were isolated from various sources and were screened for reducing sugars and protein production using submerged fermentation system. Out of these, 65 fungal strains were found capable to produce high specific activity relatively on oil-palm residues with the assay condition of temperature at 50°C for incubation time of 24 hours. Fungal isolate namely, *Penicillium rolfsii* was selected for subsequent study since it showed the highest capability to hydrolyze oil-palm residues comparing to other fungal strains based on time-course profile for 48 hours incubation time. Capability of *Penicillium rolfsii* on hydrolysis of oil-palm residues was evaluated by comparing to type strain of NBRC7735 and commercial enzymes (Celluclast 1.5L and Accellerase®1500). Conversion of these lignocellulosic oil-palm residues into fermentable sugars by enzymatic hydrolysis for bioethanol production has to be further investigated to move towards on alternative, renewable, sustainable, efficient and cost effective energy source.
Keywords: *Penicillium rolfsii*, oil-palm residues, feedstock, fermentable sugars

Introduction

Energy crisis is one of the most serious threats towards the sustainability of human kinds and civilization. Excessive global consumption of energy, such as fossil fuels, particularly in large urban areas with expansion of human population and increase of industrial prosperity, high levels of pollution and greenhouse gasses in the atmosphere have increased drastically during the last few decades (Sarkar *et al*., 2012). Lignocellulose is a renewable organic material and is the major structural component of all plant in which consists of three major components: cellulose, hemicellulose and lignin (Manju and Singh Chadha, 2011). The human need for efficient breakdown of lignocellulosic biomass for biofuel production and a variety of potential lignocellulolytic microorganisms have evolved to take advantage of this nutrient source.

Oil palm (Elaeis guineensis) is widely planted for its edible oil in tropical countries such as Malaysia and Indonesia (Yamada *et al*., 2010). Due to the low productivity of aging palm trees after 25 – 30 years, these old palms were cut and left in plantation site or burned to replant new seedlings. Hence, conversion of biofuels could be a productive way for disposal of these trunks by using lignocellulolytic microorganisms, such as fungi which appear to be the most active taxonomic group responsible for the breakdown of lignocellulosic biomass, forming a variety of extracellular lignocellulolytic enzymes in large quantities (Kvesitadze *et al*., 1999). Without competition of food, these old oil-palm trunks could be a compatible feedstock for generation of second-generation of biofuels.

In this research, *Penicillium rolfsii* was successfully isolated from oil-plam plantation site of northern part of Peninsular Malaysia. Its superiority of hydrolysis performance comparing to commercial enzymes such as Celluclast 1.5L and Accellerase®1500 was competitively shown in this report.

Materials and Methods

Isolation of potential fungal isolates

The soil samples were collected and serial dilution technique was conducted from 10^{-1} to 10^{-6}. The diluents were transferred to multi-plates containing autoclaved oil-palm residues in each well, respectively (Figure 1). The multi-plates were then incubated at 30^0C and the growth of fungal isolates was observed daily. The fungal isolates which were showing growth on oil-palm

residues was picked and transferred to PDA (potato dextrose agar), until to get a pure fungal isolate.

Screening of potential fungal Isolates by submerge fermentation

The selected fungal isolates from isolate works were grown on 1% of oil-palm trunk residues with basal minimum salts medium (MSM) consisted of $NaNO_3$, 1.0 g; KH_2PO_4, 1,0 g; $MgSO_4 \bullet 7H_2O$, 0.5 g; yeast extract, 0.5 g which were dissolved in 1 L of distilled water. The fungal isolates were grown in MSM with agitation rate of 200 rpm at 30°C for 7 days. Comparison of hydrolysis efficiency on oil-palm residues among the selected fungal isolates was conducted by using their crude enzymes. The hydrolysis of oil-palm residues was evaluated by production of reducing during the incubation at the interval time of 0, 6, 12, 24 and 48 hours. The concentration of crude enzymes for all fungal isolates was adjusted to a concentration of 100 µg/mL. From the total volume reaction of 200 µl (100 µl enzyme sample + 100 µl oil-palm residues) in an 1.5 ml eppendorf tube , 10 µl of sample is taken out each interval time for determination of reducing sugar content by using modified Somogyi-Nelson method (Somogyi, 1945).

Identification of fungus by means of 18S rRNA analysis

DNA was extracted from each fungal isolate's mycelia grown for 1 week, using the Dneasy plant mini kit (Qiagen) according to the manufacturer's instruction. Portion of nuclear rDNA (ITS), comprised between the 18S and 28rDNA genes were amplified using primer ITS1F (5' - TCC GTA GGT GAA CCT GCG G- 3') and ITS4R (5' –TCC TCC GCT TAT TGA TAT GC- 3'). The PCR products were sent for sequencing at 1st BASE Laboratory, Ltd. (Malaysia). The 18S rDNA sequences of fungal strains were compared to all fungal sequences available from GenBank database by using BLAST program (National Center for Biotechnology Information).

Enzyme preparation

After one week fermentation, the culture broth was collected by filtration for removing fungal mycelium and oil-palm solid wastes. The resulting supernatant was precipitated with 80% saturated ammonium sulfate and then dissolved in 50 mM sodium acetate buffer (pH 5.0). Excessive salt was removed with Econo-Pac 10 DG desalting column (Bio-Rad). Sample preparation steps by desalting column were adopted according to the protocol of Bio-Rad manufacturer.

Enzyme assay & Protein determination

Hydrolysis on oil-palm residues, avicel, arabinan, carboxymethylcellulose, chitin, laminarin, barley β-glucans, and xylan from birchwood activities were determined by measuring the amount of reducing sugars liberated. Liberated reducing sugar from all the enzymatic assay with different substrates was determined by using Somogyi-Nelson method, except xylose liberated by determination of 3,5-Dinitrosalicylic acid (Nelson, 1944). One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 µmol/min of reducing sugar equivalent to glucose, arabinose or xylose under the specified assay conditions. The protein concentration in the sample was determined by measuring the absorbance at 750 nm with standard Lowry method using bovine serum albumin as standard (Lowry *et al*., 1951).

Results and Discussions

Fungi have been widely reported as plausible biomass degraders for large-scale applications due to their ability to produce large amount of extracellular lignocellulolytic enzymes. Isolation of potential fungal isolates had been successfully conducted to select the fungi which capable on utilization of oil-palm trunk residues as substrate. The growth of the fungus in the well of multi-plate containing the solid substrate can be observed as shown in Figure 1. From this isolation work, we have isolated 164 fungal isolates which showing promising growth on oil-palm trunk residues.

A total 65 out of 164 fungal isolates were selected from screening work regarding on the time-course profiles basis within 48 hours evaluated by sugar production. A fungal isolate namely *Penicillium rolfsii* was identified by molecular level of 18S rDNA which was showing a superior performance of hydrolysis on oil-palm trunk residues compared to other fungal isolates (Figure 2). Characterization of enzyme produced by *P. rolfsii* was conducted by using different substrates compared to commercial enzymes and enzyme from type strain of NBRC7735 (Table 1). We

found that oil-palm trunk residues-hydrolyzing specific activity of the culture supernatant from *P. rolfsii* was superior to that commercial enzymes Celluclast 1.5L and Acellerase® 1500 (Genencor) which was exhibited 2 to 3-fold and 3 to 4-fold higher activity, relatively. Oil-palm trunk residues activity was compared with type strain NBRC7735 as well and result showed that higher activity of 1 to 2-fold of isolated *P. rolfsii*. We also investigated that *P. rolfsii* exhibited a greater xylan, arabinan and laminarin-hydrolyzing activities than those commercial enzymes. The superior performance of cellulase preparations produced by *Penicillium* species over *Trichoderma* enzymes has been well reported by other research findings (Castellanos *et al*., 1995, De Castro *et al*., 2010, Gusakov *et al*., 2007).

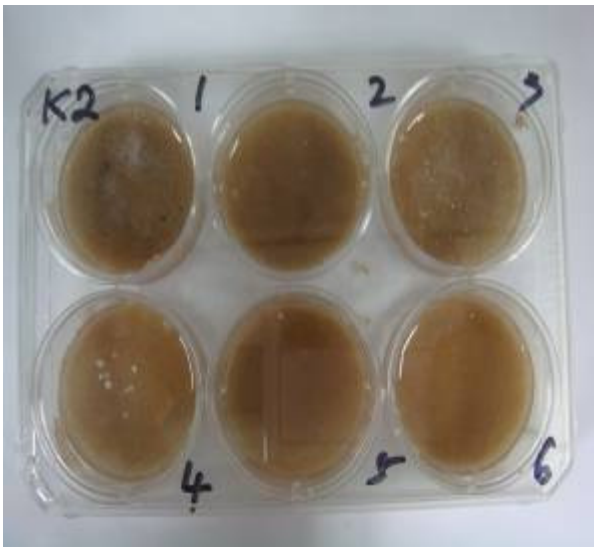

Figure 1. The multi-plate which was used for the isolation of potential oil-palm-trunk-residues-degrading fungal isolates containing autoclaved oil-palm residues.

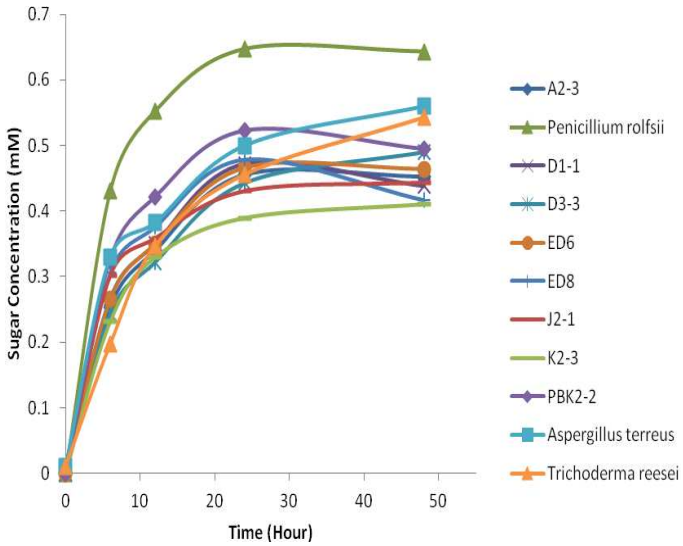

Figure 2. Time-course profiles of selected potential fungal isolates which were showing the capability on hydrolysis of oil-palm trunks residues evaluated by sugar production

Table 1. Specific activity (U/mg) of enzyme (*P. rolfsii*) comparing with commercial enzymes and type strain NBRC7735 on different kind of substrates, respectively.

Substrate	Specific Activity (U/mg)			
	P. rolfsii	Celluclast 1.5 L	Accellerase®1500	Type strain NBRC7735
Destarched-oil-palm residues	0.063	0.0234	0.018	0.031
Avicel	0.413	0.721	0.831	0.324
CMC	6.656	5.032	11.011	6.589
β-glucans	9.844	10.618	13.658	7.692
Chitin	0.013	0.008	0.002	0.002
Arabinan	0.287	0.011	ND	0.400
Xylan	12.563	3.607	2.821	9.397
Laminarin	6.904	ND	2.859	7.279

Conclusions

This study showed that *P. rolfsii* was able to secrete a wide range of lignocellulolytic enzymes activities at high titers when cultured in liquid submerged fermentation using oil-palm trunk residues as carbon source compared to commercial enzymes Celluclast 1.5L and Acellerase® 1500. High hydrolysis activity on oil-palm residues by *P. rolfsii* suggesting that it was potentially used in conversion of biomass derived from oil-palm trunks to produce fermentable sugars for the production of biofuel and other value-added chemicals.

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