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Anaerobic Bacteria and Enzyme System for Efficiency Oil-Palm Empty Fruit Bunch Degradation

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ABSTRACT

Oil palm empty fruit bunch (OPEFB) is a potential and sustainable feedstock for bio-refinery production due to its high cellulosic content and availability in Thailand. OPEFB consists of mainly cellulose, hemicellulose and lignin that are forming a complex structure. Thus, it is difficult to hydrolyze and requires the synergistic action of many enzymes. This study aimed to screen thermophilic anaerobic bacteria that showed high-performance OPEFB degrading ability and utilization. Soil samples were collected from Krabi province, and inoculated directly into a basal medium (BM) pH 7.0 containing 1% (w/v) OPEFB as the sole carbon source at 60°C under anaerobic conditions. Three isolates KB9, KB17 and KB30 showed 45.49%, 28.52% and 32.10% of OPEFB degradation respectively, after 5 days incubation. The results exhibited that KB9 possessed greater OPEFB degrading ability into 1.17 times than *C. thermocellum* ATCC27405 (38.85 %). The protein pattern and zymogram of KB9 and *C. thermocellum* ATCC27405 were compared and showed quite a difference. The crude enzyme pattern of KB9 has indicated 18 visual protein bands (19-215 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Zymogram analysis revealed that 14 xylanases, 8 cellulases, and 9

mannanases were produced by isolate KB9, leading active OPEFB degradation. Furthermore, the isolate KB9 promoted high xylanase activity of 578.47 $U.g^{-1}$ protein and 185.46 $U.g^{-1}$ protein of cellulase activity. Thus, the isolate KB9 would indicate the high biodegradable potential in utilizing OPEFB and might apply to the OPEFB bio-refinery industry.

Keywords: Anaerobic bacteria, Bio-refinery production, Oil palm empty fruit bunch, OPEFB degrading ability

INTRODUCTION

Oil Palm (*Elaeis guineensis*) is one of the most versatile crops in the tropical world. Oil palm mill plant also generates a large amount of solids waste such as empty fruit bunches (23%), mesocarp fiber (12%) and shell (5%) for every ton of fresh fruit bunches (O-Thong et al., 2012). One of the most challenging problems is the management of OPEFB that is the other problematic by-product of palm oil mills (Nieves et al., 2011). OPEFB constitutes nearly 50 % of these solid wastes and composed of cellulose, hemicellulose, lignin and other extractive materials. Mostly, OPEFB has excellent potential to be processed as bioenergy, fertilizer and other products with higher economic value. Because of the high content of lignin up to 21 %, OPEFB carries similar hardness as wood (Kumar et al., 2009). However, high moisture content of 60-70% and the high content of organic matter has the potential to be used for biogas production and bio-refinery industry (Kim et al., 2013).

Degradation of lignocellulosic biomass, with high lignin concentration, requires a pretreatment process. That can be performed through a physical, chemical, mechanical, as well as a biological method using microbes or enzymes (Atlas and Bartha, 1986). The purpose of pretreatment is to remove or break down the lignin structure and increase the digestibility of the cellulose fraction. The natural decomposition process of lignocellulosic biomass, instead of the physical and chemical process, is performed by a biological pretreatment. Anaerobic bacteria can degrade cellulosic biomass and produce ethanol, hydrogen and organic acids as products. Therefore, many researches aimed at obtaining new microorganisms producing cellulolytic enzymes with greater degradation efficiency. Bacteria, which has a high growth rate as compared to fungi, has good potential to be used in cellulose enzyme production (Ariffin et al., 2008). Especially, thermostable cellulase can increase the rate of reaction, decrease the amount of enzyme needed and

longer half-life. It also reduces the possibility of microbial contamination (Ibrahim and El-diwany, 2007). This study aimed to screen and isolate thermophilic anaerobic bacteria that showed high-performance OPEFB degrading ability and utilization.

MATERIAL AND METHODS

Materials

The OPEFB was collected from palm oil mill plant, in Krabi province, Thailand. OPEFB was prepared by cutting with scissors to small sizes (approximately 1 cm in length) and soaked in water (50 g/L suspension) overnight at room temperature. The wet grinding operation was performed using Super Mass Colloider (MKCA6-2, Masuko Sangyo Co., Ltd, Japan) at 1200 rpm. The soaked OPEFB was directly thrown into the machine with the gap between the disks adjusted at 500 µm for rough grinding. This process was more repeated for 3 times for fine grinding using the gap between the discs of 100 µm. The ground OPEFB was washed, dried at 50 °C until constant weight and used as a substrate for the culture. The chemical composition of raw OPEFB was shown in Table 1.

Chemicals

Cellulose powder (Type 20), birchwood xylan, mannan (locust bean gum) were purchased from Sigma (St. Louis, MO, USA). The bovine serum albumin was purchased from Fluka (Buchs, Switzerland). The other chemicals and reagents were analytical grade.

Microorganisms and Medium

The consortium, KB9, KB17 and KB30 were isolated from the soil sample in Krabi province, Thailand. The fresh cultures were maintained by routinely transferring 1% (v/v) inoculum into new basal medium (BM) containing (per liter) 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of NaCl, 0.9 g of $(NH)_4\ SO_4$, 5 g of Yeast extract powder, 0.12 g of $CaCl_2.2H_2O$, 0.18 g of $MgSO_4.7H_2O$, 0.001 g of resazurin, 4 g of Na_2CO_3 and 0.5 g of cysteine hydrochloride. The pH of the media was adjusted to 7.0 and autoclaved at 121°C for 15 min. The media were prepared under anaerobic conditions, as previously described (Tachaapaikoon et al., 2012).

Screening and Isolation

The candidates were isolated using OPEFB as the sole carbon source. The cultures were incubated at 60°C under anaerobic conditions. The samples showing the most effective OPEFB degradation was carried out several times to obtain the target candidates. The isolation was carried out on BM agar medium by using the roll-tube technique (Hungate, 1969) under sticky anaerobic conditions and incubated at 60°C. A single colony was picked up by using a single colony technique with a needle and inoculated into fresh BM contained cellulose powder as the sole carbon source. Isolate strains that could grow well on cellulose powder as the sole carbon source were repeated several times on agar rolling tube technique until truly single pattern appearance. The pure isolates were kept in appropriate culture broths containing OPEFB as the sole carbon sources.

Oil Palm Empty fruit bunches Degradation

Degradation of OPEFB was calculated using the following equation:

$$\text{Degradation of OPEFB (\%)} = 100 - [\frac{Wr \times 100}{Wi}]$$

Where: W_i = Initial dry weight of OPEFB in basal medium (g)
W_r = Remaining dry weight of OPEFB after cultures were incubated at 60°C until the late exponential growth phase (5 days).

Preparation of Enzyme from Isolated Strains

The isolated strains were grown in BM medium consisted of 1% (w/v) OPEFB as the sole carbon source. The culture was incubated at 60°C until the late exponential growth phase (5 days) and harvested by centrifugation (8,000 rpm for 15 min) at 4°C. Subsequently, the supernatant was used as the crude enzyme. Vivaspin™ 20 was used to concentrate crude enzyme with a molecular weight cut-off (MWCO) of 10,000 kDa.

Protein Determination and Enzyme Assays

All experiments were conducted in triplicate. Protein concentration was measured by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951). Enzyme activities were determined using 50 µl of an enzyme (containing 100µg protein/ml) mixed with 50 µl of the substrate in 0.1 M sodium phosphate buffer, pH 7.0 and incubated at 50°C for 10 min. One percent (w/v) of birchwood xylan, cellulose powder, and mannan were used as substrates for xylanase, cellulase, and mannanase, respectively. The release

of reducing sugars was determined by the Somogyi–Nelson method using xylose or glucose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme-producing 1 µmol of reducing sugar per minute under the assay conditions (Nelson, 1944).

Gel Electrophoresis Analysis and Zymograms

All samples contained 100 µg of protein. SDS–PAGE was performed on a 10% polyacrylamide gel by the method of Laemmli (Laemmli, 1970). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (CBB). The molecular weight standards were used as a high-molecular-weight calibration kit (Bio-Rad). Xylanase, CMCase and Mannanase zymogram were prepared from polyacrylamide gels containing 0.1% (w/v) soluble xylan, 0.1% (w/v) CMC and 0.1% (w/v) locust bean gum as described respectively. Gels were regenerated by 1% triton-X for 2 times and incubated with 50 mM sodium phosphate buffer, pH 7.0 at 50ºC for 5 min and then stained with 0.1% (w/v) Congo red solution.

Chemical Composition Analysis and Structural Characterization

The polysaccharide and lignin contents were determined according to National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2008).

RESULTS

Screening of Anaerobic Bacteria for OPEFB Degradation

Thermophilic anaerobic bacteria which high-performance OPEFB degrading ability was isolated and their chemical composition were measured. The soil sample was collected from Krabi province, Thailand and placed in first-screen Hungate tubes (40 ml) containing 10 ml of BM medium and 10 $g.l^{-1}$ of EFB as a carbon source. The tubes were incubated at 60°C under anaerobic conditions. The cultures were transferred to fresh medium more than three times to ensure that the OPEFB degrading microorganisms were predominant compared with *C. thermocellum* ATCC27405. Using agar roll tube containing cellulose powder, many kinds of colonies characteristics appeared on agar rolling tube. However, a single colony was picked up by using a single colony technique with a needle and inoculated into fresh BM contained cellulose powder as the sole carbon source. Isolate strains namely KB9, KB17 and KB30 could grow well on cellulose powder as the sole carbon source, and it was repeated several times on agar rolling tube until there was

single pattern appearance. The pure isolates KB9, KB17 and KB30, were kept in appropriate culture broths containing OPEFB as the sole carbon sources. A colony that could degrade cellulose was selected, and the tube was re-rolls more than three times to obtain a pure culture. The isolated, namely KB9, KB17 and KB30, could grow well on OPEFB as the sole carbon source and were effective OPEFB degradation shown in Table 1.

The results of degradation for OPEFB shows isolate KB9 possessed greater OPEFB degrading ability (45.49%) into 1.17 times higher than *C. thermocellum* ATCC27405 (38.85 %), while degrading ability of isolate KB17 and KB30 were 28.52 and 32.10 %degradation, respectively. Therefore, isolate KB9 is an exciting option for further study because of its high efficiency of OPEFB degradation.

Table 1. The degradation from isolated strains and *C. thermocellum* ATCC27405 and chemical composition of OPEFB after cultures were incubated at 60°C, 5 days.

Isolates	Degradation (%)	Chemical composition in remaining residue (%)		
		Cellulose	Hemicellulose	Lignin
Control (Raw OPEFB)	N/D	46.27	32.38	21.03
C. thermocellum ATCC27405	38.85	32.69	33.40	28.53
Isolate KB9	45.49	32.31	25.02	30.51
Isolate KB17	28.52	36.94	29.22	27.24
Isolate KB30	32.10	36.66	29.42	28.68

Note: *N/D = not determined

Chemical Composition Analysis

The composition of cellulose, hemicellulose and lignin content of the OPEFB after incubated at 60°C were investigation. The composition of OPEFB after cultures by isolate KB9 for 5 days consisted of 32.31% cellulose, 25.02% hemicellulose and 30.51% lignin. While, the composition of OPEFB after cultures by strain KB17 and KB30 consisted of 36.94% cellulose, 29.22% hemicellulose and 27.24% lignin and 36.66% cellulose, 29.42%

hemicellulose and 28.68% lignin, respectively (Figure 1 and Table 1). The cellulose content was decreased towards the end of the culture process by isolate KB9 and *C. thermocellum* ATCC27405 with 61.94% and 56.78% of reduction, respectively. KB9 showed active degradation of cellulose more than *C. thermocellum* ATCC27405, KB30 and KB17, respectively. At the same time, hemicellulose content decreased by KB9 more than *C.thermocellum* ATCC27405 with the decrement of 57.87% and 36.93% of reduction, respectively. The lignin content was reduced slightly towards the end of the culture process by isolate KB9 and *C. thermocellum* ATCC27405 with 20.92% and 17.07% of reduction, respectively. Therefore, isolate KB9 is interesting to study their enzymatic system.

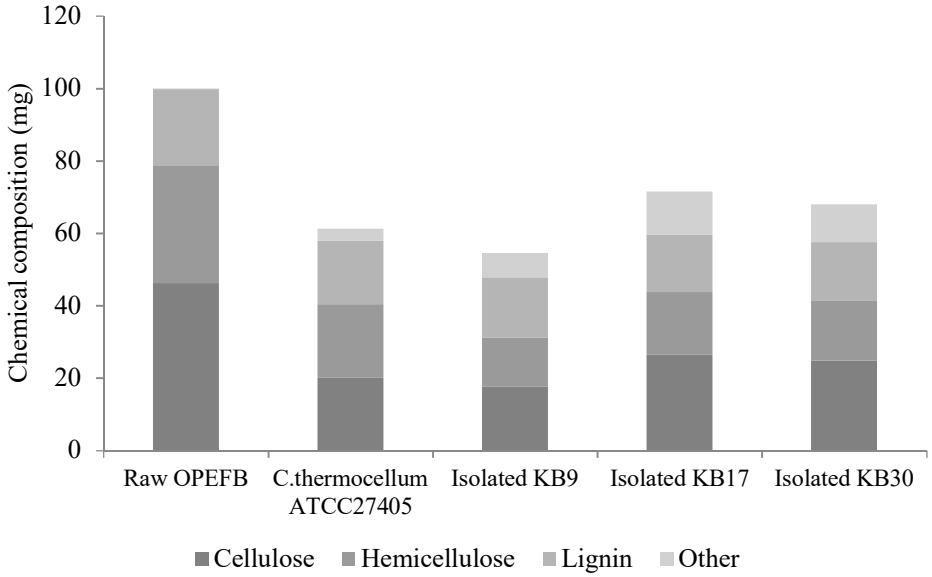

Figure 1. Chemical composition in remaining residue OPEFB after cultures by isolated KB9, KB17, KB30 and *C.thermocellum* ATCC27405 at 60°C for 5 days

Characterization of an extracellular enzyme from isolated KB9

The extracellular enzyme from the isolate KB9, grown in basal medium containing 1% (w/v) OPEFB, was characterized. The substrate specificity of the extracellular enzyme was investigated using various substrates. As shown in Table 2, the culture supernatant contained predominated xylanase activity of 578.47 $U.g^{-1}$ protein and 185.46 $U.g^{-1}$

protein of cellulase activity, whereas, slightly mannanase activity of 49.05 U.g^{-1} protein. To investigate the pattern of components of the extracellular enzyme, gel electrophoresis and zymograms were used. At least 18 proteins with molecular masses in the range of 19 to 215 kDa were observed on SDS–PAGE (Figure 2, lane 2A). The zymograms revealed 14 proteins with xylanase activity (Figure 2, lane 2B), 8 proteins with cellulase activity (Figure 2, lane 2C) and 9 proteins with mannanase activity (Figure 2, lane 2D). Seven high-molecular-mass proteins, of 215, 180, 104, 85, 80, 72 and 69 kDa showed tri-functional enzyme of xylanase, cellulase and mannanase activities. Besides, protein pattern and characteristic of the extracellular enzymes from the *C. thermocellum* ATCC27405 were different from isolate KB9. The result suggested that the strain KB9 was appropriate for increased OPEFB degradation and utilization.

Table 2. Enzymatic activities of the crude enzyme from isolate KB9 and *C.thermocellum* ATCC27405

Enzymes	Specific activities (U.g^{-1} of protein)	
	Isolate KB9	*C. thermocellum* ATCC27405
Cellulase	185.46	74.63
Xylanase	578.47	200.18
Mannanase	49.05	23.45

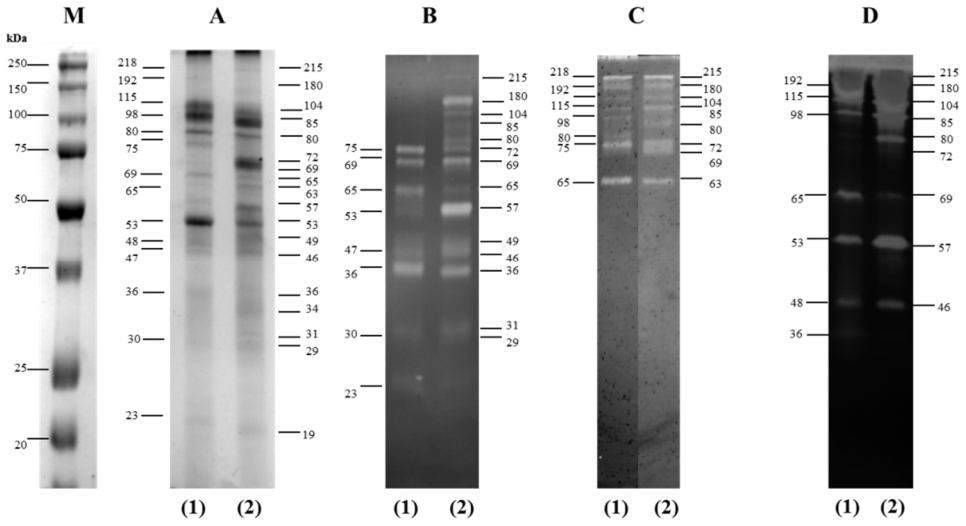

Figure 2. Patterns of proteins and enzyme activities on gel electrophoresis of extracellular enzyme, including 10% SDS-PAGE (A), standard protein (M), xylanase zymogram (B), cellulase zymogram (C) and mannanase zymogram (D) of the crude enzyme from *C.thermocellum* ATCC27405 (lanes 1) and isolate KB9 (lanes 2).

CONCLUSION

In this study, the isolate KB9 was successfully isolated from the soil sample in Krabi province, by using OPEFB as the sole carbon source. The isolate KB9 was shown high-efficiency OPEFB degrading ability. It produced several cellulases, xylanases and mannanases. It strongly indicated that the strain KB9 effective to degrading cellulose and hemicellulose higher than *C.thermocellum* ATCC27405, which different in term of xylanase and mannanase patterns. Thus, the isolate KB9 would indicate the high biodegradable potential in utilizing OPEFB and might apply to the OPEFB bio-refinery industry. Moreover, the isolate KB9 will identify using 16S rRNA gene analysis, and whole-genome sequence will be done in the near future.

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