

LIPID ACCUMULATION BY *Flavodon flavus* ATH USING PALM OIL MILL EFFLUENT AS SUBSTRATE

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ABSTRACT

Large amount of palm oil mill effluent (POME) is generated annually. The waste would be potential for production of single cell oils (SCOs). The objective of this study was to evaluate lipid accumulation by fungi using POME as substrate. Seven filamentous fungi were initially isolated from various biomes. The study results showed that out of these 7 fungi, five of them produced endoglucanase and accumulated lipid about 34.3-87.5% of their dry cell mass using POME as substrate. The five fungi were identified as *Flavodon flavus* ATH, *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp. 1 T30, and *Fusarium* sp.2 T50. The highest lipid accumulation was obtained by *Flavodon flavus* ATH. The profile of trans-esterified SCOs revealed high content of saturated and mono-unsaturated fatty acids i.e., palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids similar to conventional vegetable oils used for biodiesel production. The strain that was able to use organic substrates in POME indicated that they are promising strain for biofuel feedstock as well as for fulfilling effluent quality for wastewater discharge.

Keywords: Fatty acid methyl ester, *Flavodon flavus*, Oleaginous fungi, Palm oil mill effluent (POME), Single cell oil

INTRODUCTION

As awareness on the limited stock of fossil fuel and concern on environmental hazard of petroleum fuel is growing, research on sustainable biofuel is gaining its popularity (Richana 2010; Fujita *et al.* 2004; Wraight & Ramos 2005; Jing *et al.* 2010; Mohammadi *et al.* 2011). Microbial based biofuel (micro-diesel) has become more attractive due to its environmental incentive and renewable properties (Zheng *et al.*

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2012). The merit of micro-diesel over petroleum fuel is clear, but our understanding on microbial diversity and physiology of oleaginous microbes is still incomplete to produce economically viable micro-diesel (Zhao *et al.* 2012). Current practice of biodiesel production is through trans-esterification of vegetable oils or animal fats with short chain alcohols. Feedstock acquires more than 70% of total biofuel production cost, and this limit further expansion of biodiesel (Wei *et al.* 2013). Exploiting lipid accumulating microorganism would offer solution for feedstock generation (Wang *et al.* 2011). Some lipid accumulating fungi i.e. *Cunninghamella echinulata*, *Microspheeropsis* sp., *Mortierella isabellina*, *M. ramanniana* var. *angulispora*, *Mucor circinelloides*, *Phomopsis* sp., *Cephalosporium* sp., *Sclerocystis* sp. and *Nigrospora* sp. accumulate large amounts of lipids greater than 40% per dry cell weight (Wei *et al.* 2013; Wynn *et al.* 2001) under N-limited cultivation conditions.

Oils derived from microbes have many advantages over plant oil due to having short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up (Wang *et al.* 2013). These oleaginous characters would place microbial oils as potential feedstocks for biodiesel production in the future (Liu *et al.* 2012). Lignocellulose waste are abundant in tropical region. Several microbes use lignocelluloses hydrolysate for biofuel production (Tsigie *et al.* 2011).

POME contains high strength organic substances with total chemical oxygen demand (COD) about 30,000-40,000 ppm and 3% lipid (Wu *et al.* 2010). Utilizing these wastes for biofuel production offer manifold benefits through reducing total organic in effluent, and generate biofuel feedstock via bioconversion palm oil waste into triacylglycerol rich microbial cell (Coleman 2004). Fungi would be good candidate for biofuel feedstock, since some fungi produce extracellular cellulase which break down complex polysaccharide into fermentable sugar (Goyal *et al.* 1991), consume it for lipid synthesis and finally accumulate it into lipid bodies which primarily consist of triacylglycerol (Rossi *et al.* 2010). This study aims to evaluate the lipid accumulation by fungi using POME as substrate.

MATERIALS AND METHODS

Fungi Species Used

This study initially isolated 7 filamentous fungi from various biomes as listed in Table 1.

Table 1. Fungi used in this study

Name of species	Fungi sources
<i>Aspergillus</i> sp.	Soil of Cibinong West Java
<i>Flavodon flavus</i> ATH	Decaying wood of South East Sulawesi
<i>Paecilomyces lilacinus</i> . Comp	Insect frass
<i>Fusarium</i> sp1 T30	Sludge of wastewater treatment in West Java
sp2 T50	Sludge of wastewater treatment in West Java
<i>Trichoderma</i> sp	Soil of Cibinong West Java
<i>Mucor</i> sp.	Soil of Cibinong West Java

Culture Conditions

The fungal inoculants of the 7 filamentous fungi was prepared following Mulder *et al.* (1989) and Kimura *et al.* (2004). Briefly, fungal spores that had been incubated at 30 °C for 5 days on Potato Dextrose Agar plates (Difco Laboratories, Detroit, MI, USA) were harvested and suspended in seed culture medium. A spore suspension (500 µL) containing approximately 4×10^6 spores/mL was inoculated into 150 mL of the seed culture medium containing (g/L): glucose, 30; yeast extract, 5; KNO₃, 1; KH₂PO₄, 2.5; ZnSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.002; MnSO₄, 0.01; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.02; and CaCl₂, 0.1. The initial pH of the medium was adjusted to 5.5 by adding 10 M NaOH in a 500 mL flask and incubated at 30 °C with shaking at 125 rpm for 24 hours. Then, 10 mL of the seed culture was transferred into 250 mL of growth medium containing POME obtained from PT Perkebunan Nusantara, Malimping, West Java. Prepared medium was autoclaved for 20 minutes before use. The culture was incubated at 30 °C with shaking at 125 rpm for 24 hours.

Determination of Cellulolytic Fungi

The ability of fungi to hydrolyze cellulose was evaluated using CMC containing media following Zhou *et al.* (2004). To evaluate the effect of temperature on the endoglucanase activity, the cultures were separately incubated at 30 °C and 50 °C with shaking at 125 rpm for 5 days.

Endoglucanase Assays

Enzymatic activity was assayed following Zhou *et al.* (2004). Briefly, the culture were placed in 50 mL centrifuge tubes, then centrifuged at 2,500 g at 4 °C for 30 minutes. Supernatants were clarified by filtration through 0.45 µm nitrocellulose filters (Pall). For enzymatic activity measurements, 2% carboxymethylcellulose (CMC, Sigma) was dissolved in 50 mM citrate buffer pH 5. Enzymatic reactions contained 200 µL of supernatant, 300 µL of 50 mM citrate buffer pH 5, plus 500 µL of each substrate solution. The reaction mixtures were incubated at 50 °C for 30 minutes. Reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) assay according to Dinis *et al.*, (2009). Briefly, 50 µL aliquots were taken every 5 minutes (after adding the supernatant to the reaction mixture) up to 45 minutes, then mixed with 50 µL of a DNS solution, boiled for 5 minutes and immediately cooled on ice for 5 minutes. Finally, 500 µL of water were added and absorbance was measured at 540 nm in a spectrophotometer (UV Mini 1240 Shimadzu). One enzyme unit is defined as mMol glucose produced by 1 mL enzyme per hour.

Biomass

Biomass fungal was determined according to Ogbo (2010). Briefly, biomass was harvested by filtration, and fungal pellet were washed with 50 mL deionized water for removal of residual nutrients, and lyophilized at -50 °C in a vacuum of 1 mbar for 12 hours and weighed. Fungal biomass was expressed as grams of dry weight per liter of culture medium.

Lipid Concentration

The lipid concentration of fungi broth was determined from a standard curve obtained by plotting absorbance against the corresponding lipid concentration determined by the conventional method of acid hydrolysis followed by solvent extraction and gravimetric estimation. Forty milligrams samples were extracted with 3 mL of chloroform/methanol (1/2, v/v) by vortexing (1 minute) and centrifugating at 2,500 g for 15 minutes at room temperature. The supernatants were collected and residues were re-extracted twice with 2 mL of chloroform/methanol (1/1, v/v) by centrifugation as stated above. All the supernatants were pooled together, filtered with Whatman filter No. 1 (Whatman, USA), and washed with 2 mL of Milli-Q water, followed by centrifugation at 2,500 g for 5 minutes. The lower organic phases were collected and evaporated to dryness under nitrogen and total lipid contents were determined gravimetrically (Sitepu *et al.* 2013).

Lipid Composition Analysis

The total lipid concentration was determined by gas chromatographic analysis of the total fatty acids directly trans-methylesterified from dried cell (Liu *et al.* 2008 ; Kosa & Ragauskas 2011). One milliliter of 10% methanolic-HCl and 0.5 mL methylene chloride were added to the dried biomass and placed at 60 °C for 3 hours for direct methyl-esterification. The reaction was stopped by the addition of 2 mL saturated NaCl solution and 1 mL hexane. The resultant methyl esters recovered in the hexane layer were then applied to a gas chromatograph (GCMS-QP 2010-Ultra; Shimadzu, Kyoto, Japan) equipped with a FAMEWAX capillary column (30 m× 0.25 mm i.d., GL Science, Tokyo, Japan) under temperature programming (150-250 °C at 5 °C/minute increments). Peanut oil (Nacalai Tesque, Kyoto, Japan) was trans-methylesterified and used as the reference material.

RESULTS AND DISCUSSION

Overall, all fungi studied showed CMC-ase activity (Fig.1), but the strains of *Mucor* sp., and *Paecilomyces lilacinus* Comp grew slowly. Therefore, we omitted these strains for further study. *Flavodon flavus* ATH produced the highest CMC-ase at 96 hours incubation. Other strains having slightly lower activity were *Aspergillus* sp. and *Paecilomyces lilacinus* Comp. Maximum activity attained at 96 hours, and decreased at 144 hours. The ability of cultures to produce CMC-ase indicated these fungi produced endoglucanases, EC 3.2.1.4, and played important role in cellulose hydrolyses (Hasper *et al.* 2002). Kitcha and Cheirsilp (2014) observed newly isolated fungi *Aspergillus tubingensis* as being able to hydrolyze cellulose of palm by products. To gain cellulolytic character of fungi in this study, we further evaluated their ability to hydrolyze POME at 30 °C and 50 °C.

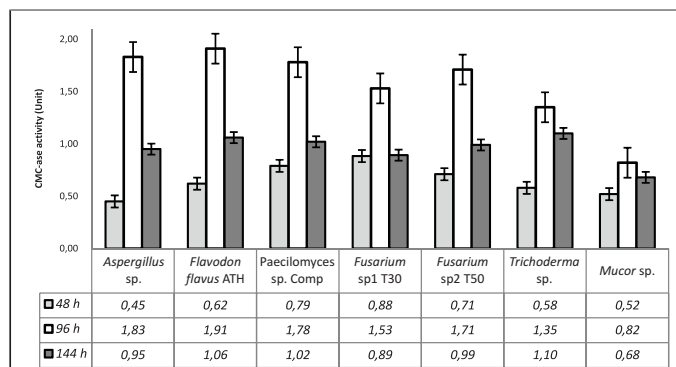


Figure 1. CMC-ase (endoglucanase) activity of fungi grown on CMC-medium

Endoglucanase Activity at 30 °C

Overall, endoglucanase activity of fungi grown in POME is summarized in Figure 2. The highest endoglucanase activity was obtained by *Flavodon flavus* ATH and *Aspergillus* sp. grown at 30 °C after 96 hours cultivation. Slightly lower activity was observed on *Fusarium* sp.2 St50. Generally lower CMC-ase was observed at 144 hours fermentation.

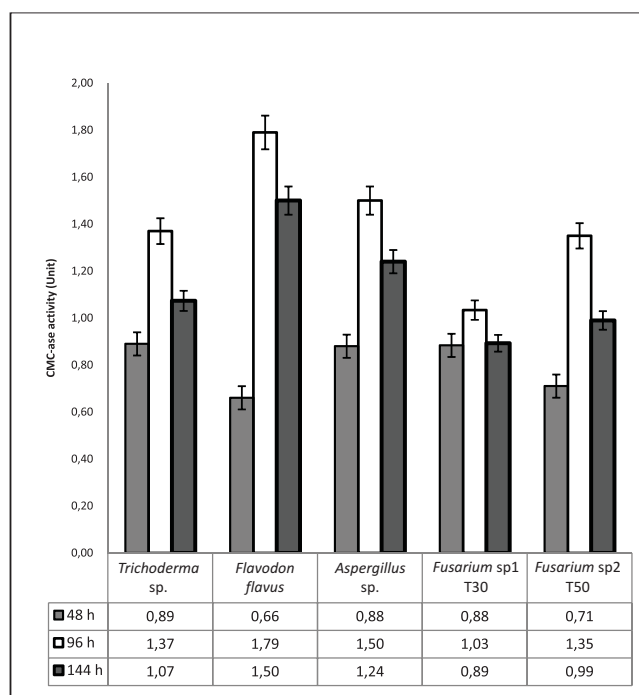


Figure 2. Endoglucanase activity of selected isolates grown on POME at 30 °C

Endoglucanase Activity at 50 °C

Endoglucanase activity of selected fungi grown on POME at 50 °C varies among fungal species depending on incubation period (Fig.3). Maximum activity was showed by *Flavodon flavus* ATH at 96 hours incubation. Lower activity was obtained after 144 hours cultivation.

Effect of Growth Temperature on Endoglucanase Activity

In general, CMC-ase activity was higher in cultures incubated at higher temperature (Fig.4). Almost 50% increase of CMC-ase activity was achieved by *Flavodon flavus* ATH. Fungi have been intensively explored for hydrolyses of lignocellulose materials. *Flavodon flavus* ATH is wood decayed fungi isolated from South East Sulawesi, Indonesia. *Flavodon flavus* appears to be widely distributed lignocellulolytic fungi isolated from decaying sea grass from a coral lagoon off the west coast of India (Raghukumar *et al.* 1999; Mtui & Nakamura 2008) and produces extracellular lignin-modifying enzymes (LMEs): manganese-dependent peroxidase (MNP), lignin peroxidase (LIP), and laccase when grown in N-limited media (Mtui & Nakamura 2008). These enzyme characters would be advantageous for producing fermentable substances for triacylglycerol synthesis using lignin containing waste such as POME (Duarte 2013; Lam & Lee 2011).

We also noticed that a culture of *Flavodon flavus* ATH having high CMC-ase or endoglucanase activity indicated that hydrolyses and product hydrolyses consumption affected enzyme synthesis (Vlasenko *et al.* 2010).

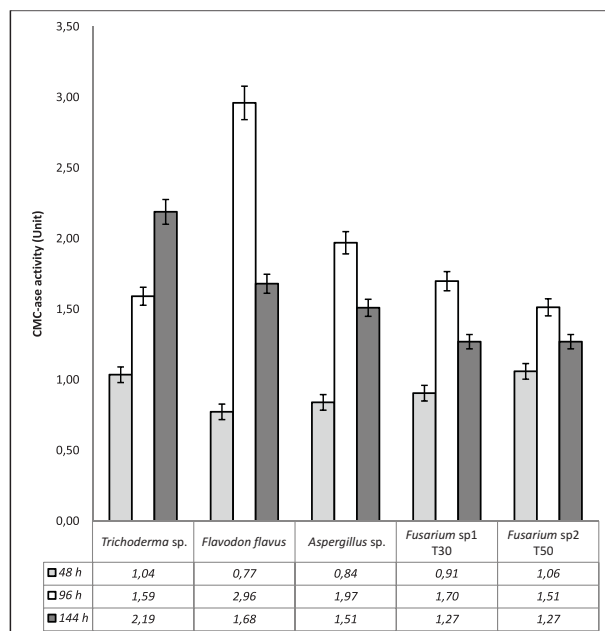


Figure 3. Endoglucanase activity of selected isolates grown on POME at 50 °C

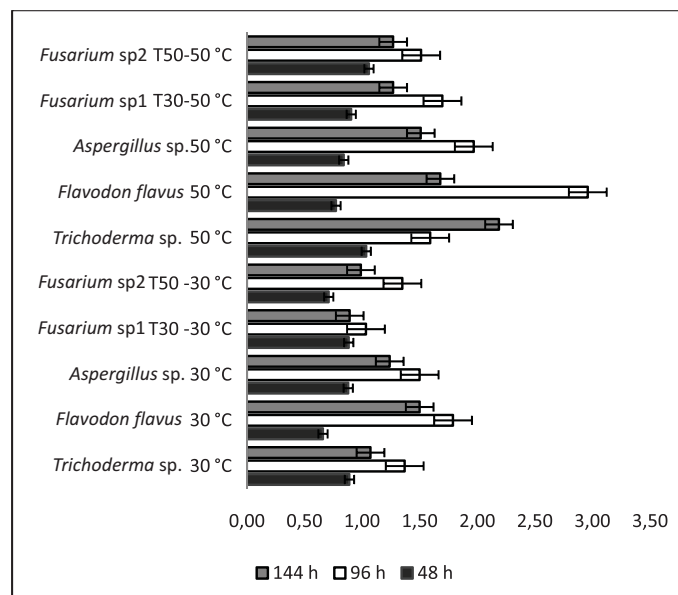


Figure 4. Comparison of endoglucanase activity of selected isolates grown on POME at 30 °C and 50 °C

Lipid Accumulation

POME is good substrate for growing lipid accumulating fungi (Fig.5), as shown by the lipid production (75% lipid per cell dry-weight). The highest lipid accumulation was attained by *Flavodon flavus* ATH when grown at 30 °C, but less when the culture was grown at 50 °C. Incubation temperature affected lipogenesis of fungi (Fig.5).

The suitability of SCOs from oleaginous fungi was evaluated and observed *Cunninghamella echinulata* contained 92% (w/w) neutral lipids in its SCO (Wu *et al.* 2011). Other lipid producer, *Mucor circinelloides*, contained lower neutral lipid fractions (18.5%, w/w), higher amount of polar lipids (35%, w/w), and free fatty acids (32% w/w) in its SCO (Wynn *et al.* 2001).

Fatty Acid Profiles of Fungal SCOs

Fatty acid profiles compositions of selected fungi were obtained through transesterification of triacyl glycerol with methanol under alkaline condition. Fatty Acid Methyl Esters (FAME) was mainly composed of Methyl palmitate ($C_{17}H_{34}O_2$), Methyl cis-10-heptadecenoate ($C_{18}H_{34}O_2$) and Methyl oleate ($C_{19}H_{36}O_2$) (Table 2), indicating that the FAME is closely related to palm and soybean fatty acids (Ratledge & Wynn, 2002). There was slight differences in concentration of lipid species among fungi evaluated (Wynn *et al.* 2001; Khot *et al.* 2012; Chan *et al.* 2010). The variability in fatty acid composition of oleaginous organism could be due to culture technique (Chi *et al.* 2011) and species dependence (Gasmi *et al.* 2011).

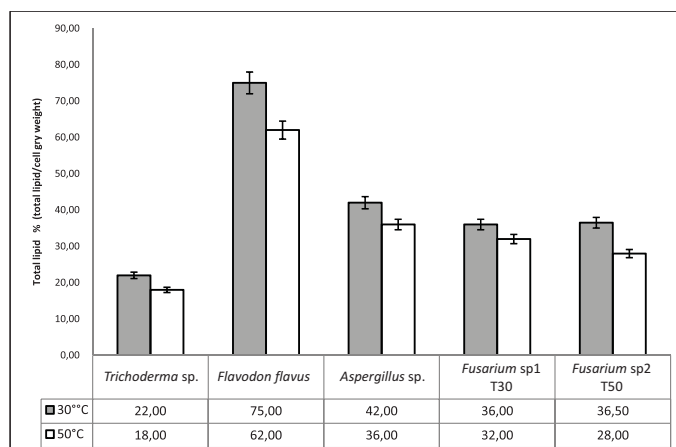


Figure 5. Lipid accumulation by fungi grown on POME at 30 °C and 50 °C after 6-day fermentation

Table 2. Lipid composition of fungi grown on POME cultured at 30 °C after 6 days fermentation

Fatty Acid Methyl Esters (FAME)	<i>Trichoderma</i> sp.	<i>Flavodon flavus</i> ATH	<i>Aspergillus</i> sp.	<i>Fusarium</i> sp1 T30	<i>Fusarium</i> sp2 T50
Methyl palmitate(C ₁₇ H ₃₄ O ₂)	31.39	27.05	23.62	21.36	20.21
Methyl palmitoleate(C ₁₇ H ₃₂ O ₂)	29.65	26.28	21.08	21.08	20.68
Methyl cis-10-heptadecenoate (C ₁₈ H ₃₄ O ₂)	3.1	5.32	4.36	5.32	4.32
Methyl stearate (C ₁₉ H ₃₈ O ₂)	4.14	4.28	5.23	4.23	3.69
Methyl linoleate (C ₁₉ H ₃₄ O ₂)	5.41	6.21	5.21	4.65	6.1
Methyl butanoate	6.72	11.2	7.06	6.1	9.2
Methyl oleate (C ₁₉ H ₃₆ O ₂)	6.06	6.21	7.32	7.36	6.35
Methyl linolenate (C ₁₉ H ₃₂ O ₂)	4.11	3.11	9.81	9.98	8.21
Methyl cis-vaccenate(C ₁₉ H ₃₂ O ₂)	2.38	2.32	6.8	8.69	6.98
Methyl cis-12-Octadecenoate	2.71	4.82	5.91	4.91	8.6
Methyl myristate(C ₁₅ H ₃₀ O ₂)	4.33	3.2	3.6	6.32	5.66

CONCLUSIONS

Fungi were able to accumulate lipid in large amount using POME as substrates, indicating that introducing fungi into POME wastewater treatment plant could be proposed for future biofuel production and pollution reduction.

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