

Treatment of Palm Oil Mill Effluent by Thermotolerant Cellulase and Xylanase - Producing Fungi

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ABSTRACT

Fungi strain, CD, HD, GD, RH, RA, RS and PD, which produce cellulase and xylanase for palm oil mill effluent treatment were isolated from dung and agriculture waste. Studies on the properties of thermotolerant fungi at room temperature to 60°C were evaluated. The result showed that all isolated strains were found to be thermotolerant fungi that have a growth temperature at 50°C and require optimum temperature as 45°C. Strain CD1, HD1, RH1, RA2, RS1 and PD1 were classified as *Aspergillus* sp. while GD1 was classified as *Scytalidium* sp. The enzyme activity of thermotolerant fungi revealed that PD1 produced the highest activities of carboxymethyl cellulase (CMCase) (678.14 U/ml) and xylanase (2369.12 U/ml). The activities of carboxymethyl cellulase (CMCase) and xylanase exhibited 80.14 and 244.54 U/ml of protein, respectively. This study also demonstrated the effect of treatment efficiency in palm oil mill effluent. The result showed that *Aspergillus* sp. PD1 is the best strain that has the ability to reduce BOD and COD value of 31.02 % and 49.05 % after the treatment, respectively. Therefore, the thermotolerant enzyme-producing fungi are potentially useful to remove organic in palm oil mill effluent treatment.

Keywords

Thermotolerant Fungi, Cellulase, Xylanase, Organic Removal, Palm Oil Mill Effluent

INTRODUCTION

The world's markets of oil palm over the past 4 years has grown by 5.1 % and are used as raw materials for foodstuffs and energy (biodiesel) industry and has mainly been cultivated in Indonesia, Malaysia and Thailand [1]. In Thailand, 85 % of oil palm plantations and crude palm oil extraction mills are in the south of the country. The dramatic increase in demand for the plantation area was around 10 % with an average annual growth rate of 11 % from 1981 to 2000 and 9 % from 2001 to 2010 [2]. Nowadays, Thai government has set out the Oil Palm and Oil Palm Industries Development Strategy 2015-2026 to expand the oil palm plantation areas by another 3 million rai which increase yields per rai from 3.2 tonnes to 3.5 tonnes and raises the proportion of oil in the fresh palm to 20 % in Thailand including three southern border provinces (Pattani, Yala and Narathiwat) [1]. Standard or wet process is commonly employed for palm oil extraction in the palm oil mill that resulted in the generation of large quantity of a liquid waste, average 2.5 tonnes of palm oil mill effluent (POME) are generated for every ton of crude palm oil production [3]. POME had acidic pH (pH 4.2-4.5) and contained high organic matter (45.2-143.9 g/L COD), solids (17-71.5 g/L total solids (TS)), 8.5-34.2 g/L suspended solids (SS), oil & grease (2.3-10.6 g/L) but low nitrogen (0.083-0.92 g/L) [4,5,6,7]. These characteristics of POME are very high of organic matter and cause serious environmental problems. POME has generally been treated by chemical, physical and biological. Chemical method using coagulation, precipitation and ion exchange, which was found more effective than the biological method using bacteria, yeast and fungi. Biological treatment of POME to reduce the wastewater strength is one potential method that can be adopted to alleviate the pollution problem faced by both the palm oil industry and eco-friendly. Recently many researchers have resorted to the use of microorganisms to treat POME, especially, fungi. The fungi have the ability to convert dissolved and

suspended organic matter into a mycelium that is high in protein content which can be readily recovered by simple filtration or screening [8]. There are many fungi, such as *Trichoderma viride*, *T. harzianum*, *T. virens*, *Myceliophthora thermophila*, *Geotrichum candidum*, *Penicillium digitatum*, *P. italicum*, *Aspergillus* sp., *A. niger*, *A. terreus*, *Mucor racemosus*, *Rhizopus stolonifer* were used for treatment of POME [6,8,9,10]. In addition, to mitigate the environmental problem caused by POME, the organic matter would be better utilized for the production of some valuable product such as enzyme cellulase and xylanase [11]. For instance, *Phanerochaete chrysosporium*, *Aspergillus* sp., *A. terreus*, *T. reesei*, *Penicillium* sp. and *Rhizopus* sp. presence cellulase activity [12,13]. Otherwise, in a wet process, fresh fruit bunch was sterilized (at 120-130°C, 40 lb/in² for 45 min) [14] and the freshly discharged POME from the factory has a temperature of 85-90°C [15]. For this reason, this study focuses on the fungi that able to survival and growth in wide and varied extreme environments. The fungi that grow only within the temperature range of a minimum below 20°C to maximum near 50°C are considered to be thermotolerant fungi [16]. This work aims to investigate the aerobic treatment of the POME using the thermotolerant fungi for production of cellulase and xylanase to remove organic matter.

MATERIALS AND METHODS

Isolation, identification and maintenance of thermotolerant fungi

The thermotolerant fungal strains were isolated from cow dung (CD), horse dung (HD), goat dung (GD), rice husk (RH), rice husk ashes (RA), rubber sawdust (RS) and plant debris (PD) in three southern border provinces of Yala, Pattani, and Narathiwat. Yeast starch agar (YpSs). The YpSs medium containing 0.4 % Yeast extract, 0.1 % K₂HPO₄, 0.05 % MgSO₄·7H₂O, 1.5 % starch and 2% agar [16, 17, 18]. The incubation temperature of fungi for primary isolation was 45°C and monitored fungal growth for 5 days. Single colonies on the plates were isolated and purified by transferring in several times. The purity of the isolated fungus was checked by the pattern of fungal growth on the plate at optimum temperature for fungal growth and confirmed by microscopic examination of the culture at 40× magnification using light microscope. Fungi were identified on the basis of their colonial and morphological characteristics [16,18]. After ensuring the purity, fungi were subcultured on YpSs agar slants and allowed to grow for 5-7 days at 45°C and subsequently stored at 4°C as stock cultures.

Optimum fungal growth temperature

The isolated fungi were recultured on YpSs agar at room temperature (28±2), 45, 50, 55, 60 and 65°C for 7 days and measured the growth zone of colony daily.

Qualitative and quantitative assay of cellulase and xylanase

Agar plate assay

A modified substrate agar assay was used to assay carboxymethyl cellulase activity [19] and xylanase [17] qualitatively with the clear zone of Congo red dye. The purified colonies were selected and grown on CMC agar and xylan agar. The CMC agar medium was having the following compositions: carboxymethyl cellulose sodium salt, 1g/l; KH₂PO₄, 2g/l; NH₄NO₃, 2.0g/l; MgSO₄·7H₂O, 0.2g/l; yeast extract, 0.2g/l and agar, 15g/l. The xylan agar medium has the following compositions: Birchwood xylan, 2g/l; MgSO₄·7H₂O, 0.2g/l; KH₂PO₄, 0.4g/l; KCl, 0.2g/l; NH₄NO₃, 5g/l; FeSO₄·7H₂O, 0.01g/l; ZnSO₄, 0.01g/l; MnSO₄, 0.01g/l; and agar, 15g/l. After plating, plates were incubated at their optimum temperature for 5 days. Then, the plates were stained with 1 % Congo red for 10-15 min followed by destaining with 1 M NaCl solution to

remove unbound dye, and then observed of yellow zone of hydrolysis around the colonies. The cellulose hydrolytic activities were recorded by measuring zone of diameter hydrolysis in millimeters.

Carboxymethyl cellulase (CMCase) and xylanase activity

All the test thermotolerant fungi were screened for production of carboxymethyl cellulase (CMCase) and xylanase on YpSs medium at the optimum fungal growth temperature. Spore suspension of each strain was prepared by adding 10 ml of 0.1% Tween 80 onto YpSs slant of 5 days old culture of the each strains. The concentration was adjusted to reach 2.4×10^6 spore/ml in YpSs under shaking condition that were constant at 200 rpm for 5 days at their optimum temperature. Samples were taken every 24 h to determine for CMCase activity [20], xylanase activity [21] and protein measurement [22]. The isolated fungus which giving the highest CMCase and xylanase activity efficiency were selected for further studies.

Determination of palm oil mill effluent (POME) characteristics

The POME was taken from Cooperative estate bacho, in Mueang district, Narathiwat province, Thailand and kept at -20°C until used. The samples physicochemical parameters, such as color, pH, biochemical oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), suspended solids (SS), oil & grease [23], total nitrogen (by total Kjeldahl nitrogen method) and total phosphorus (by DIN 38402 A51 method) [24] were determined.

Treatment of thermotolerant CMCase and xylanase-producing fungi in POME

Spore suspension were prepared by adding 10 ml of 0.1% Tween 80 onto YpSs slant of 5 days old culture of the isolated fungus at the optimum fungal growth temperature. The concentration was adjusted to reach 2.4×10^6 spore/ml. Starter culture (10% v/v) of each fungal isolate was inoculated into 500 ml Erlenmeyer flasks containing 100 ml each of aseptic POME under shake-flask condition (200 rpm) for 6 days at their optimum temperature. Samples were taken every 24 h to determine the color, pH, BOD, COD, SS, TS and oil & grease.

RESULTS AND DISCUSSION

Isolation of thermotolerant fungi

Thermotolerant fungi from cow dung (CD), horse dung (HD), goat dung (GD), rice husk (RH), rice husk ashes (RA), rubber sawdust (RS) and plant debris (PD) on YpSs at 45°C for 5 days were used in this study. In this experiment, **Table 1** shows the distribution of thermotolerant fungi of different habitats. A total of 20 collected samples were thermotolerant fungi isolated from dungs and agriculture waste including strain CD (2), HD (2), GD (2), RH (3), RA (3), RS (6) and PD (2). The thermotolerant fungi have a growth temperature range of a minimum below 20°C to maximum near 50°C [16]. The various strains of thermotolerant fungi were able to grow in different habitats. The 68 thermophilic and thermotolerant fungi were isolated from various heated environments in which 15 from stored seeds, 12 from soils, 11 from compost, 10 from wheat straw, 9 from wood chips, 5 from litter, and 3 from decaying organic matter and 3 from bird nests at 45°C for 2-7 days [17]. A total of 13 and 11 fungal species were detected in the compost and air sampler at 1.5 m height in three large industrial composting facilities at 50°C for 7 days [25].

In addition, the several of environments is an outstanding habitat for growth of thermotolerant fungi such as from soil, decaying organic matter and sugarcane piles (27 of fungal strains) at 45°C [26], from composting soils (9 of fungal strains) at 50°C for 3 days

[27], from composting soils of local habitat in India (14 of fungal strains) at 45-50°C for 7-10 days [28] and from decaying vegetable and soil sample (2 of fungal strains) at 45-55°C [29].

Optimum temperature for fungal growth

Strain CD1, CD2, HD1, HD2, GD1, GD2, RH1, RH2, RH3, RA1, RA2, RA3, RS1, RS2, RS3, RS4, RS5, RS6, PD1 and PD2 were cultured on YpSs at room temperature (28±2), 45, 50, 55, 60 and 65°C for 7 days and measured growth zone of colony daily and record the qualitatively (+, -) (Table 2). The result in Table 2 showed that all strains were found to be thermotolerant fungi that have a growth temperature at 50°C and require the optimum temperature as 45°C. A total of 20 collected samples were thermotolerant fungi because able to grow at 28°C and 45°C following the definition described by Cooney and Emerson (1964) [16]. Therefore, the optimum temperature for cultivation of all strains was 45°C that this temperature was used for further studies.

Table 1 Distribution of thermotolerant fungi and qualitative assay of carboxymethyl cellulase and xylanase activity in different samples

No.	Sample	No. of fungi isolated	Strain	The average diameter of hydrolysis zone (cm)		The quantitative assay (U/mL)	
				cellulase	xylanase	CMCase	xylanase
1.	Cow dung (CD)	2	CD1	3.20±0.18 ^C	4.65±0.45 ^C	567.13±0.23 ^C	1566.43±0.13 ^C
			CD2	-	-	-	-
2.	Horse dung (HD)	2	HD1	3.85±0.15 ^B	6.05±0.13 ^B	650.43±0.56 ^B	1969.12±0.24 ^B
			HD2	-	-	-	-
3.	Goat dung (GD)	2	GD1	3.32±0.16 ^C	4.81±0.22 ^C	577.11±0.18 ^C	1697.86±0.21 ^C
			GD2	-	-	-	-
4.	Rice husk (RH)	3	RH1	3.52±0.35 ^C	5.03±0.05 ^C	597.33±0.12 ^C	1807.59±0.21 ^C
			RH2	-	-	-	-
			RH3	-	-	-	-
5.	Rice husk ashes (RA)	3	RA1	-	-	-	-
			RA2	2.45±0.03 ^D	3.55±0.17 ^D	497.56±0.13 ^D	1344.58±0.27 ^D
			RA3	-	-	-	-
6.	Rubber sawdust (RS)	6	RS1	3.45±0.52 ^C	4.93±0.02 ^C	589.27±0.23 ^C	1797.86±0.05 ^C
			RS2	-	-	-	-
			RS3	-	-	-	-
			RS4	-	-	-	-
			RS5	-	-	-	-
			RS6	-	-	-	-
7.	Plant debris (PD)	2	PD1	4.93±0.23 ^A	7.93±0.12 ^A	678.14±0.45 ^A	2369.12±0.51 ^A
			PD2	-	-	-	-

The data in columns was presented as mean values with standard deviations, with not significance threshold $p>0.05$

- = no activity of enzyme

Table 2 Thermotolerant fungi and optimum growth temperature of 20 fungal strains on YpSs agar plates incubated for 7 days.

No.	Strains	Temperature (°C)					
		Room temperature (28±2)	45	50	55	60	65
1.	CD1	++	+++	+	-	-	-
2.	CD2	++	+++	+	-	-	-
3.	HD1	++	+++	+	-	-	-
4.	HD2	++	+++	+	-	-	-
5.	GD1	++	+++	+	-	-	-
6.	GD2	++	+++	+	-	-	-
7.	RH1	++	+++	+	-	-	-
8.	RH2	++	+++	+	-	-	-
9.	RH3	++	+++	+	-	-	-
10.	RA1	++	+++	+	-	-	-
11.	RA2	++	++	+	-	-	-
12.	RA3	++	+++	+	-	-	-
13.	RS1	++	+++	+	-	-	-
14.	RS2	++	+++	+	-	-	-
15.	RS3	++	+++	+	-	-	-
16.	RS4	++	+++	+	-	-	-
17.	RS5	++	+++	+	-	-	-
18.	RS6	++	+++	+	-	-	-
19.	PD1	++	+++	+	-	-	-
20.	PD2	++	+++	+	-	-	-
Remarks		+++ = very good growth (> 4 cm)	++ = medium growth (2.1-4 cm)				
		+ = little growth (0.5-2 cm)	- = no growth				

Production of CMCase and xylanase by the isolated fungal strains

The aim of this study is to test CMCase and xylanase produced by thermotolerant fungi, subsequently characterize to find the interesting properties for potential application of palm oil mill effluent (POME) treatment in further experiment. Since the thermotolerant fungi may be used as viable the organic matter (75.2-96.2g/L COD) in POME that could cause environmental problems [4]. Lignocellulosic biomass which is produced from the oil palm industries includes oil palm trunks (OPT), oil palm fronds (OPF), palm shells, palm pressed fibers (PPF), empty fruit bunches (EFB) and POME. Lignocellulosic biomass refers to plant material that is mainly composed of 45-55 % cellulose, 25-35% hemicellulose and 20-30% lignin [30]. All strains of thermotolerant fungi were analyzed the qualitative and quantitative of extracellular cellulase and xylanase using enzyme diffusion technique. The modified substrate agar plate assay was used to determine the qualitative of enzyme activity by observing clear zone of Congo red dye. **Table 1** shows the diameter of hydrolysis zone on enzyme production from purified colonies of CD1, CD2, HD1, HD2, GD1, GD2, RH1, RH2, RH3, RA1, RA2, RA3, RS1, RS2, RS3, RS4, RS5, RS6, PD1 and PD2 fungal strains. Strains CD1, HD1, GD1, RH1, RA2, RS1 and PD1 showed the

activity of cellulase and xylanase, these productions being observed zones of enzymatic hydrolysis. The maximum diameter of the zones was 4.93 ± 0.23 cm on CMC agar plates, and 7.93 ± 0.12 cm on xylan agar plates (PD1) (**Figure 1A**) while 2.45 ± 0.03 mm and 3.55 ± 0.17 mm were minimum diameter of the zones presented on CMC agar, and xylan agar plates (RA2), respectively. In contrast, strains CD2, HD2, GD2, RH2, RH3, RA1, RA3, RS2, RS3, RS4, RS5, RS6 and PD2 had no hydrolysis zone of cellulase and xylanase (**Figure 1B**). Out of these 22 test fungi from 68 isolated thermotolerant fungi that collected samples of soils, stored seeds, and decomposing organic matter in India showed the xylanase activity by forming the cleared zone (4-29 mm) [17].

The results of quantitative evaluation of CMCase and xylanase activity on YpSs medium at the optimum fungal growth temperature are showed in **Table 1**. The results exhibited that seven thermotolerant fungal can produce CMCase and xylanase including CD1, HD1, GD1, RH1, RA2, RS1 and PD1. CMCase and xylanase productions ranging from 497.56 to 678.14 and 1344.38 to 2369.12 U/mL, respectively, were observed. The strain PD1 produced the highest CMCase and xylanase activity (678.14 and 2369.12 U/mL) and specific CMCase activity (80.14 and 244.54 U/mg protein) at 6 days cultivation (**Figure 2**). In addition, HD1 produced the high CMCase and xylanase activity with 650.43 and 1969.12 U/mL and specific CMCase activity with 72.14 and 226.63 U/mg protein at 6 days cultivation. Conversely, strains RA2 that isolated from rubber ashes produced the lower CMCase and xylanase activity (497.56 and 1344.38 U/mL and specific CMCase activity with 61.17 and 172.58 U/mg protein) at 6 days cultivation. Since the temperature relationship affects the growth of fungal isolates [17]. As a result, the cultivation at room temperature (28 ± 2) and 45°C may be unviable optimum growth for enzyme production (**Table 2**). Therefore, the strain PD1 and HD1 cultivation at 45°C was the optimum condition for production of CMCase and xylanase and it also used for further studies.

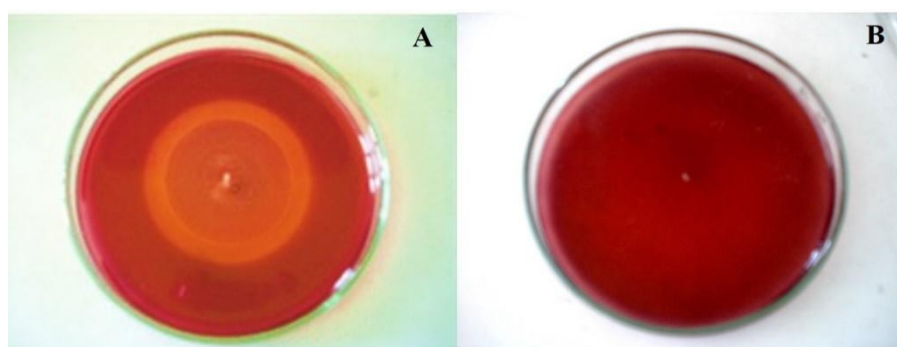


Figure 1 Plate presence hydrolysis zone of crude enzyme xylanase in substrate-agar: plate (A) presence the highest xylan hydrolysis of PD1 isolated from Plant debris and (B) absence the xylan hydrolysis of PD2 isolated from Plant debris.

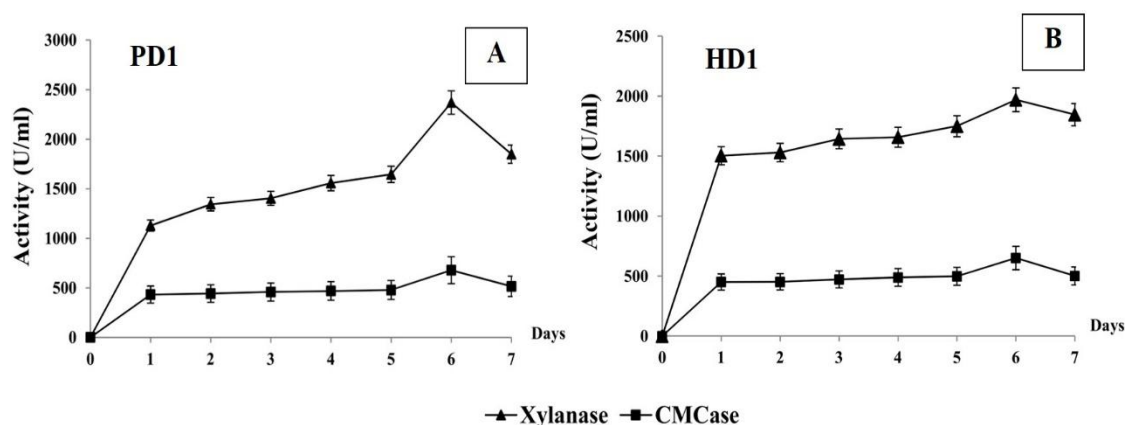


Figure 2 Comparison of enzymes production in YpSs medium by strain PD1 (A) and HD1 (B).

Morphological characterization of thermotolerant cellulase and xylanase-producing fungi

The CD1, HD1, GD1, RH1, RA2, RS1 and PD1 isolated thermotolerant fungi that produced cellulase and xylanase were identified on the basis of their colonial and morphological characteristics. [16,17,31,32]. The result shows the CD1, HD1, RH1, RA2, RS1 and PD1 were identified as *Aspergillus* sp. (Figure 3) while GD1 was *Scytalidium* sp. There are several thermotolerant *Aspergillus* sp. in the different habitats are grown in the range of 26-48°C such as *A. carneus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *A. ficuum* and *A. versicolor* [31,33,34,35,36,37]. Moreover, some strains of *Aspergillus* sp. were found to produce xylanase in wheat bran contain *A. fumigatus* and *A. terreus* at 45°C for 7 days [17]. The thermotolerant *A. terreus* was cultivated on wheat bran, solka floc and pectin showed the xylanase activity of 171, 33 and 0.2, respectively [38]. The *Scytalidium thermophilum* which refer to a synonym of *Humicola insolens* collected from the dung of different herbivore animal; cow and sheep dung were reported to associate with 20% of frequency and 3.2% abundance of *Scytalidium* sp. in this sample [36]. *Scytalidium* sp. GD1 in this study was obtained from dung that similar result with previous studies [36]. In addition, this strain was able to grow and produce xylanase at 45°C for 7 days in wheat bran as a carbon source [17].

In this case, *Aspergillus* sp. PD1 and *Aspergillus* sp. HD1 gave the high CMCase and xylanase production at 45°C. Thus, the strains *Aspergillus* sp. PD1 and *Aspergillus* sp. HD1 were chosen for the next experiments.

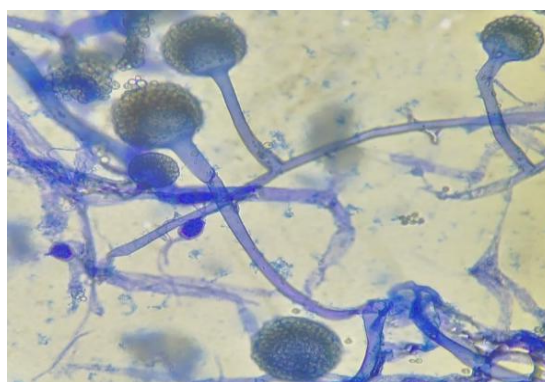


Figure 3 Microscopic image showing morphological of thermotolerant fungi: *Aspergillus* sp. PD1

Determination of POME Characteristics

The characteristics of the POME taken from a palm oil mill were determined (**Table 3**). pH value of POME was 4.52 which is the typical acidic pH for POME. The POME contains the average values of 14.35g/l BOD, 42.33g/l COD, 25.4g/l total solids, 10.6g/l suspended solids, 4.7g/l oil & grease, 0.52g/l total nitrogen and 0.15g/l total phosphorus. These values were similar to those from other remarks as reported in **Table 3** [4,6,7]. The difference in POME characteristics in various studies was due to the difference in the quality of raw material, the extraction process, the efficiency of the process and the sampling time [39,40].

Table 3 Characteristics of palm oil mill effluent from Cooperative estate bacho in comparison to other palm oil mills.

Parameter	This study	1	2	3
Color	Brown	Brown	-	Brown
pH	4.52	4.2-4.5	4.2	4.5
BOD (g/l)	14.35	-	16.3	-
COD (g/l)	42.33	75.2-96.3	105	143.9
Total solids (g/l)	25.4	35-42	18.7	71.5
Suspended Solids (g/l)	10.6	8.5-12	-	34.2
Oil & grease (g/l)	4.7	8.3-10.6	7.5	10
Total nitrogen (g/l)	0.52	0.8-0.9	1.04	1.2
Total phosphorus (g/l)	0.15	0.09-0.12	-	0.5

Remark; 1: O-Thong *et al.* [4], 2: Njoku *et al.* [6], 3: Prasertsan and Binmaeil [7]

- : Not Determined

Comparison on treatment efficiency of thermotolerant CMCase and xylanase - producing fungi in POME

The POME was treated by *Aspergillus* sp. PD1 and *Aspergillus* sp. HD1 at their optimum temperature of 45°C under shaken flask condition (200 rpm) for 6 days. *Aspergillus* sp. PD1 gave the highest BOD reduction (31.02%), COD reduction (49.05%), total solids reduction (28.13%) with less amount of sediment after 6 days of cultivation (**Figure 4**). In addition, *Aspergillus* sp. PD1 also produced the highest activities of carboxymethyl cellulase (CMCase) (623.25U/ml) and xylanase (2103.15U/ml) while *Aspergillus* sp. HD1 gave lower activity with 580.19U/ml and 1723.86U/ml, respectively. Consequently, the *Aspergillus* sp. PD1 was thermotolerant fungi producing CMCase and xylanase property could be used to treat POME. A higher amount of the mycelial growth, enzyme and biopolymer production by *R. oryzae* ST29 cultivated in POME, complemented with 0.025% fertilizer and the initial pH of 4.5 at 45°C for 4 days were found. This result also showed treatment efficiency of 80% COD removal and 54.1 mg polymer/g biomass (9.77g/L) in POME [7]. To improve the treatment efficiency in POME by *Aspergillus* sp. PD1 should be optimized conditions such as POME concentration on grow, nitrogen source and initial pH on growth.

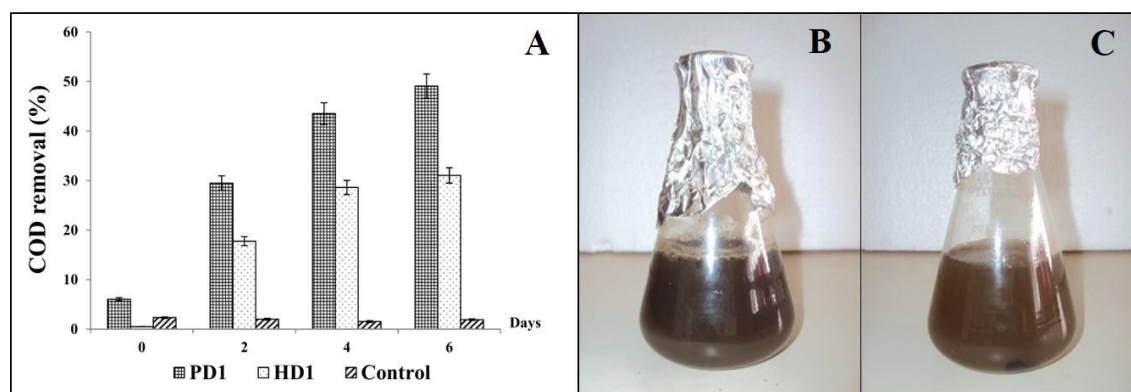


Figure 4 Comparison of (A) COD removal from *Aspergillus* sp. PD1 and *Aspergillus* sp. HD1 and comparison of (B) before treatment and (C) after treatment from enzyme-producing fungi *Aspergillus* sp. PD1 cultivation in palm oil mill effluent on a shaker (200 rpm) at 45°C for 6 days.

CONCLUSIONS

The twenty thermotolerant fungal strains were isolated from cow dung (CD), horse dung (HD), goat dung (GD), rice husk (RH), rice husk ashes (RA), rubber sawdust (RS) and plant debris (PD). All isolated strains have the optimum temperature at 45°C and maximum growth at 50°C. The isolates of CD1, HD1, RH1, RA2, RS1 and PD1 were classified as *Aspergillus* sp. while GD1 was classified as *Scytalidium* sp. The CD1, HD1, GD1, RH1, RA2, RS1 and PD1 were tested for their enzyme activities. *Aspergillus* sp. PD1 showed the activities of carboxymethylcellulase (CMCase) (6.78.14U/ml) and xylanase (2369.12U/ml) outstanding at 45°C followed by *Aspergillus* sp. HD1. Therefore, the strains *Aspergillus* sp. PD1 and *Aspergillus* sp. HD1 cultivation at 45°C was the condition of CMCase and xylanase production and used for POME treatment studies. The optimum temperatures used for treatment of POME and CMCase/xylanase produced by the *Aspergillus* sp. PD1 found 49.05% COD removal was achieved.

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