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**ORIGINAL ARTICLE**

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# **Screening of thermotolerant microorganisms and application for oil separation from palm oil mill wastewater**

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## **Abstract**

**Laohaprapanon, T., Prasertsan, P. and H-Kittikun, A.**

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The characteristics of palm oil mill wastewater (POMW) were brown color, pH 3.8-4.3, temperature 48-55°C, total solids 68.2-82.1 g/l, suspended solids 26.2-65.6 g/l, oil and grease 19.1-25.1 g/l, COD 49.9-160.7 g/l and BOD 32.5-75.3 g/l. After centrifugation (3,184 xg) of 50 ml POMW for 10 min, the POMW was separated into 3 layers: top (oil), middle (supernatant) and bottom layer (sediment). The sediment contained dry weight 1.19 g and oil and grease 1.07 g. In order to release oil and grease trapped in palm fiber debris in the POMW, cellulase- and/or xylanase-enzyme-producing and thermotolerant microorganisms were isolated. The isolates SO1 and SO2 were isolated from soil near the first anaerobic pond of the palm oil mill. They were aerobic, Gram positive, rod shaped, thermotolerant microorganisms and produced cellulase 12.11

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U/ml (3 days) and 7.2 U/ml (4 days), and xylanase 50.98 U/ml (4 days) and 20.42 U/ml (4 days), respectively in synthetic medium containing carboxymethylcellulose as a carbon source. When these 2 isolates were added into the sterilized POMW under shaking condition for 7 days, after centrifugation at 3,184 xg the isolate SO1 gave the better % reduction of dry weight (64.66 %) and of oil and grease in the bottom layer (85.32 %) of the POMW.

**Key words :** Palm oil mill wastewater (POMW), oil separation, thermotolerant microorganisms, cellulase, xylanase

### บทคัดย่อ

ศรีทิพพา เลาหประภานนท์<sup>1</sup> พุนสุข ประเสริฐ์สรรพ์<sup>2</sup> และ อรัญ หันพงศ์กิตติกุล<sup>2</sup>  
การคัดเลือกจุลินทรีย์ที่นร้อนเพื่อใช้แยกน้ำมันจากน้ำเสียโรงงานน้ำมันปาล์ม<sup>2</sup>  
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จากการศึกษาลักษณะน้ำเสียจากโรงงานน้ำมันปาล์ม พบว่า น้ำเสียมีสีน้ำตาลเข้ม พีเอช 3.8-4.3 อุณหภูมิ 48-55°C ปริมาณของแข็งทั้งหมด 68.2-82.1 กรัม/ลิตร ของแข็งแขวนลอย 26.2-65.6 กรัม/ลิตร น้ำมันและไขมัน 19.1-25.1 กรัม/ลิตร ค่าซีไอดี 49.9-160.7 กรัม/ลิตร และค่าบีไอดี 32.5-75.3 กรัม/ลิตร น้ำมันน้ำเสียบริรวม 50 มล. หมุนเหวี่ยงที่ความเร็วรอบ 3,184 xg เป็นเวลา 10 นาที พบว่า น้ำเสียแยกออกเป็น 3 ชั้น คือ ชั้นของน้ำมันและไขมันที่ลอยอยู่ด้านบน ชั้นกลางเป็นของเหลวใส และชั้นล่างสุดของน้ำเสียเป็นตะกอนของแข็ง นำตะกอนมาหาค่าน้ำหนัก แห้งได้ 1.19 กรัม และมีปริมาณน้ำมันและไขมันอยู่ 1.07 กรัม ซึ่งเป็นน้ำมันและไขมันที่หลงเหลืออยู่ในตะกอนน้ำเสีย เนื่องจากน้ำมันและไขมันเหล่านี้ถูกจับอยู่ในชั้นล่างส่วนล่างของปาล์มในน้ำเสีย ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ ที่จะแยกเครื่องจุลินทรีย์ที่นร้อนที่สามารถผลิตเอนไซม์เซลลูเลสและไซลานส์ เพื่อทำการย่อยสลายเส้นใยของปาล์ม และปลดปล่อยน้ำมันและไขมันที่ถูกตรึงอยู่ จุลินทรีย์ที่นร้อนสายพันธุ์ SO1 และ SO2 ที่คัดเลือกได้จากตัวอย่างดิน ใกล้กับบ่อบันด่าน้ำเสียจากโรงงานน้ำมันปาล์ม เป็นจุลินทรีย์ที่ต้องการอากาศ แกรมบวก รูปแท่ง ทนอุณหภูมิสูง และถูกเลี้ยงในอาหารสังเคราะห์ที่มีการเติมคาร์บอนออกซิมิลิกิลเซลลูโลส โดยจุลินทรีย์สายพันธุ์ SO1 ให้ค่ากิจกรรมเอนไซม์เซลลูเลสและไซลานส์สูงสุด 12.11 ยูนิต/มล. และ 50.98 ยูนิต/มล. ตามลำดับ เมื่อผ่านการบ่ม 4 วัน สายพันธุ์ SO2 สามารถผลิตเอนไซม์เซลลูเลสและไซลานส์ สูงสุด 7.2 ยูนิต/มล. และ 20.42 ยูนิต/มล. ตามลำดับ เมื่อนำเข้าห้อง 2 สายพันธุ์มาเลี้ยงในน้ำเสียโรงงานสักดันน้ำมันปาล์มที่ผ่านการฆ่าเชื้อแล้วภายใต้สภาวะการให้อากาศเป็นเวลา 7 วัน หลังจากน้ำมันเหลวที่ 3,184 xg พบว่า สายพันธุ์ SO1 ให้ค่าการลดลงของปริมาณน้ำมันและไขมันในชั้นล่างมากที่สุด คือ ทำให้น้ำหนักแห้งลดลงถึง 64.66% และปริมาณน้ำมันในชั้นล่างสุดลดลงถึง 85.32%

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Palm oil mill industry is a very important agro-industry of Malaysia, Indonesia and Thailand. The main product is crude palm oil. Beside that, it generates many by-products and liquid waste as palm oil mill wastewater (POMW), which has an impact on the environment. The POMW is the wastewater from the extraction process of palm oil from palm fruits. This wastewater has high

temperature (60-80°C) and acidic pH (4.05-4.62) (Prasertsan *et al.*, 1990). The POMW contained a high BOD, COD, SS and oil and grease (O&G) (Prasertsan *et al.*, 1990; Ibrahim *et al.*, 1984; Ng *et al.*, 1985 and Kunghae, 1993) as well as short fibers, cell organelles, cellulose, hemicellulose, sugar, organic acids and nitrogenous compounds (Borja *et al.*, 1996). Separation of POMW into

various particulate fractions from the soluble constituents was achieved by high-speed centrifugation. The particulates were shown to be the colloidal rod-like particles of macrofibrils, raphide particles and plant cell debris, the last being the major component. A photomicrograph and an electron micrograph of plant cells are shown some of residue oil was entrapped in the plant cells debris. Therefore, centrifugation alone was unable to remove all the oil of the wastewater. Extraction (with hexane) of the dried suspended solids yielded as much as 50 % more oil. This represented the residual oil entrapped by the plant cells that was not removed by mechanical means (Ho and Tan., 1983). Therefore, biological treatment of POMW to separated oil is one potential method because of cellulase and xylanase- producing microorganisms. When plant cell debris is hydrolyzed the oil will separate and float up.

Many researchers reported that cellulase and xylanase enzymes could remove the oil and grease from POMW. Maneesri (1994) used partially purified enzyme from *A. niger* ATCC 6275 to separate oil and grease from decanter effluent at 40°C for 19 h. Enzyme from *A. niger* ATCC 6275 was able to remove 99.0 % of the oil and grease. Chantaphaso (1998) studied the factors affecting the separation of suspended solids and oil from the decanter effluent. In order to separate the oil from the wastewater (15,000 mg/l oil) after incubation at 40°C for 6 h. the minimum concentration of enzyme xylanase from *A. niger* ATCC 6275 and commercial xylanase were 200 and 600 U/ml, respectively. This enzymatic separation process resulted in the removal of 95% of the oil under the optimum conditions. Therefore, the biological method using microorganisms that produce enzyme cellulase and xylanase to hydrolyze the cellulose and xylose may help to release the oil from the POMW.

The objective of the study was to screen thermotolerant microorganisms which are capable of producing cellulase and xylanase. They were cultivated in the POMW in order to separate the oil before other biological treatment.

## Materials and Methods

### 1. Palm oil mill wastewater (POMW)

Palm oil mill wastewater (POMW) samples were collected from the combined wastewater of the first anaerobic pond of the local palm oil mill. This palm oil mill used decanter process to separate oil from the sludge. Then, all samples were examined for BOD, COD, TS, SS, O&G and pH according to the Standard Method for the Examination of Water and Wastewater (APHA, AWWA and WEF, 1998)

### 2. Isolation of cellulase and xylanase-producing microorganisms

Soil samples were collected near the first anaerobic pond of the POMW treatment system. One gram of soil sample was mixed with 9 ml of sterile 0.85 % NaCl solution. Serial dilutions were prepared and 0.1 ml of each dilution ( $10^{-2}$ - $10^{-5}$ ) was spread on the screening medium contained (g/l)  $\text{NH}_4\text{Cl}$  1.0,  $(\text{NH}_4)_2\text{SO}_4$  1.0,  $\text{KH}_2\text{PO}_4$  0.1,  $\text{CaCl}_2$  0.4,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 and carboxymethylcellulose (CMC) 10.0 in distilled water. The pH of the medium was adjusted to 7.0 with 0.1 N NaOH (Techapun *et al.*, 2001). Plates were incubated at 55°C for 7 days. Three isolates were selected.

Each colony from the screening medium was streaked on Medium A containing (g/l)  $\text{CaCl}_2$  0.3,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.3,  $\text{KH}_2\text{PO}_4$  1.0,  $\text{K}_2\text{HPO}_4$  1.0, polypeptone 2.5, yeast extract 1.0,  $\text{NH}_4\text{NO}_3$  4.4, CMC 10.0, supernatant of POMW (It was taken after centrifugation of POMW at 12,735xg, 20 min at 4°C) 10 % (v/v) and Tween 80 1 % (v/v) was added. The pH was adjusted to 7.0 with 0.1 N NaOH (Kunghae, 1993). Cultures were incubated at 55°C for 2 days and tested for clear zone by congo red (1 mg/ml) for 15 min. Then the congo red solution was poured off, and the plate was further flooded with 1 M NaCl for 15 min and colonies expressing cellulase appeared as clear zones (Teather and Wood, 1982). The morphological characteristic of the isolates was observed.

Each colony from the screening medium was streaked on Medium B containing (g/l)  $\text{NH}_4\text{Cl}$

1.0,  $(\text{NH}_4)_2\text{SO}_4$  1.0,  $\text{KH}_2\text{PO}_4$  0.1,  $\text{CaCl}_2$  0.4,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 and oat spelt xylan 10.0 in distilled water. The pH of the medium was adjusted to 7.0 (Techapun *et al.*, 2001). The plates were incubated in aerobic condition at 55°C for 2 days. Xylanase-producing microorganisms produced a clear zone around the colonies.

### 3. Cultivation

5 % inoculum of SO1 and SO2 was added into 50 ml of Medium A (without agar) and the cultivation was carried out on an incubator shaker at 55°C for 7 days. Samples were taken every 24 hrs to measure growth, pH, cellulase and xylanase activities.

### 4. Application of selected isolate to POMW for oil separation.

10 % of SO1 and SO2 inoculum was added into 100 ml sterile and nonsterile POMW with pH adjusted to 7.0 in 250 ml flasks and cultivated on an incubator shaker (200 rpm) at 55°C 7 days. Samples were collected and centrifuged at 3,184  $\times g$  for 10 min at 4°C. The culture broth was separated into 3 layers: top layer (oil layer), middle layer (supernatant) and bottom layer (sediment). Oil content was examined in each layer. In addition dry weight was determined in the bottom layer and cellulase and xylanase activities were assayed from the middle layer (supernatant) (Rajoka and Malik, 1997). The level of reducing sugars was determined in the middle layer by the Somogyi Nelson's method (Somogyi, 1951).

### 5. Reduction of dry weight in the bottom layer

% reduction of dry weight in the bottom layer =

$$\left( \frac{D_i - D_t}{D_i} \right) \times 100$$

Where:

$D_i$  = Dry weight of the bottom layer at day 0 (g)

$D_t$  = Dry weight of the bottom layer at day t (g)

### 6. Reduction of oil in the bottom layer

% reduction of oil in the bottom layer =

$$\left( \frac{X_i - X_t}{X_i} \right) \times 100$$

Where:

$X_i$  = amount of oil in the bottom layer at day 0 (g/l)

$X_t$  = amount of oil in the bottom layer at day t (g/l)

### 7. Dry weight of bottom layer

Bottom layer after centrifugation was dried at 103°C for 3 h in hot air oven until a constant weight was obtained.

## Results and Discussion

### 1. Composition of palm oil mill wastewater

The POMW from this local palm oil mill factory was collected for 7 times in 7 months for analysis. The characteristics of POMW were brown color,  $\text{pH } 4.18 \pm 0.18$ , temperature  $72.33^\circ\text{C} \pm 11.24^\circ\text{C}$ , total solid  $71.55\text{ g/l} \pm 7.95\text{ g/l}$ , suspended solid  $47.27\text{ g/l} \pm 13.95\text{ g/l}$ , oil & grease (O&G)  $28.41\text{ g/l} \pm 28.48\text{ g/l}$  and COD  $120.08\text{ g/l} \pm 94.64\text{ g/l}$ . Prasertsan *et al.* (1990) studied palm oil mill that used a decanter. It generated a BOD of 50.00-60.00 g/l, COD 80.50-115.00 g/l; SS 18.50-52.00 g/l; O&G 1.6-2.50 g/l and pH 4.05-4.62. The mill that used either separator or separator and decanter had lower values of these parameters. On average, wastewater from a palm oil mill contained COD 70.65 g/l, BOD 35.16 g/l, SS 17.50 g/l and O&G 11.10 g/l (Kullavanij *et al.*, 1988).

### 2. Screening of thermotolerant microorganisms and cultivation

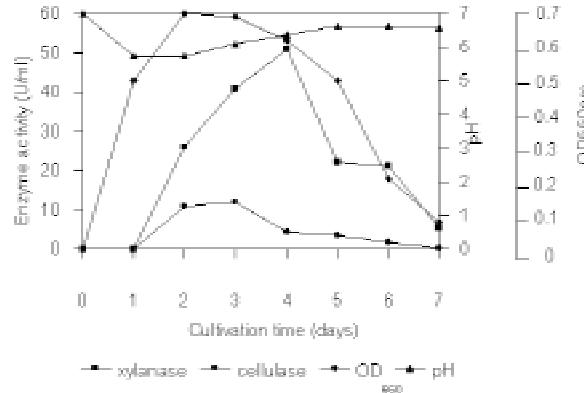
Three microorganisms (SO1, SO2 and SO3) were isolated from the soil samples near the first anaerobic pond of the wastewater treatment system of the local palm oil mill. The 3 isolates cultivated on the medium A and medium B were tested for cellulase and xylanase activities. The results

showed that isolated SO1 and SO2 had both cellulase and xylanase enzymes. Meanwhile, isolate SO3 could not produce both enzymes. The morphological characteristic of SO1 was filament shaped and the isolated SO2 was rod shaped. Both isolates were aerobic, Gram positive and thermo-tolerant microorganisms. When the isolate SO1 was grown on the screening medium at 55°C for 48 h, the isolate formed flat, white and opaque colonies. Whereas the isolate SO2 formed rounded, convex and light brown colonies.

### 3. Time course of cellulase and xylanase production

The time course on growth, cellulase and xylanase activities of the isolate SO1 cultivated in Medium A at 55°C is shown in Figure 1. It grew rapidly within 24 hrs. The maximum growth was at 48 hrs. The isolate SO1 produced both cellulase and xylanase enzymes. The highest cellulase activity was obtained at 48 hrs (12.11 U/ml) and the highest activity of xylanase at 96 h of cultivation (50.98 U/ml).

The time course of the isolate SO2 cultivated in Medium A at 55°C is shown in Figure 2. It grew rapidly within 24 hrs. The highest cellulase and xylanase activities were obtained at 96 hrs of cultivation with the activities of 7.2 and 20.45 U/ml, respectively.



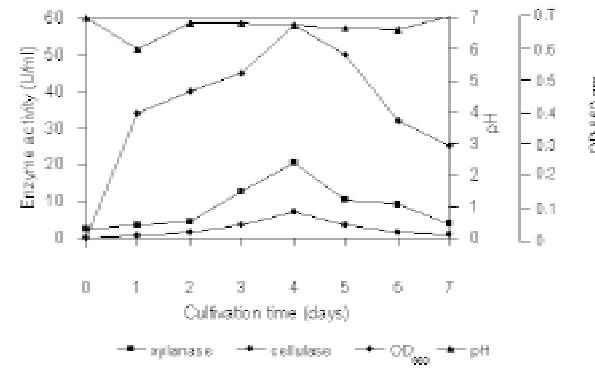
**Figure 1.** Time course of growth, xylanase and cellulase activities of the isolate SO1 cultivated in Medium A at 200 rpm, 55°C.

It has been reported that *Trichoderma reesei* was used to produce a mixture of both cellulase and xylanase from agricultural material including wheat bran, rice bran, peanut hulls and sugarcane bagasses as carbon sources. Wheat bran gave the highest xylanase activity (23.931 U/ml) plus 0.226 U/ml of cellulase at 12 days cultivation (Punnapayak and Kunpratum, 2001).

### 4. Application of the isolate SO1 and SO2 for oil separation

Isolates SO1 and SO2 were cultivated in the sterile POMW for 7 days at 55°C. The results are shown in Table 1. The isolate SO1 gave more oil in the top layer than the isolate SO2. It was noted that when the cultivation time increased the oil in the top layer increased. At the same time, oil in the bottom layer decreased.

The reason that the oil in the top layer increased was that the microorganisms produced cellulase and xylanase to hydrolyze plant cell debris tissues. Then, the oil was released from the fibers in the suspensions and floated to the top. After 7 days, the isolate SO1 gave the best result of % dry weight reduction and % oil reduction in the bottom layer, 79.8 % and 90.7 %, respectively. The isolate SO1 had more oil and dry weight reduction efficiency because it produced high activities of cellulase (9.72 U/ml) and xylanase (16.92 U/ml)



**Figure 2.** Time course of growth, cellulase and xylanase activities of the isolate SO2 cultivated in Medium A at 200 rpm, 55°C.

**Table 1.** Oil separation from sterile palm oil mill effluent after cultivation of the microorganism isolates SO1 and SO2.

Cultivation time (days)	Micro-organism	Quantity of oil (g) in the		% Reduction of oil in the bottom layer	Dry weight of bottom layer (g)	% Reduction of dry weight in the bottom layer
		Top layer	Bottom layer			
0	Control	0.13	1.07	0.00	1.19	0.00
1	Isolate SO1	0.24	0.73	31.78	1.15	3.36
	Isolate SO2	0.12	0.92	14.02	1.16	2.52
3	Isolate SO1	0.64	0.31	71.03	0.43	63.87
	Isolate SO2	0.15	0.87	18.69	1.08	9.24
5	Isolate SO1	0.84	0.11	89.72	0.33	72.27
	Isolate SO2	0.19	0.72	32.71	1.00	16.00
7	Isolate SO1	0.95	0.10	90.65	0.24	79.83
	Isolate SO2	0.22	0.65	39.25	0.91	23.53
	Control	0.13	0.93	13.08	1.16	2.52

while the isolate SO2 showed lower activities (Figure 3).

The factors influencing on the separation of oil from POMW by enzyme have been studied. Chantaphaso (1998) reported that the main factor

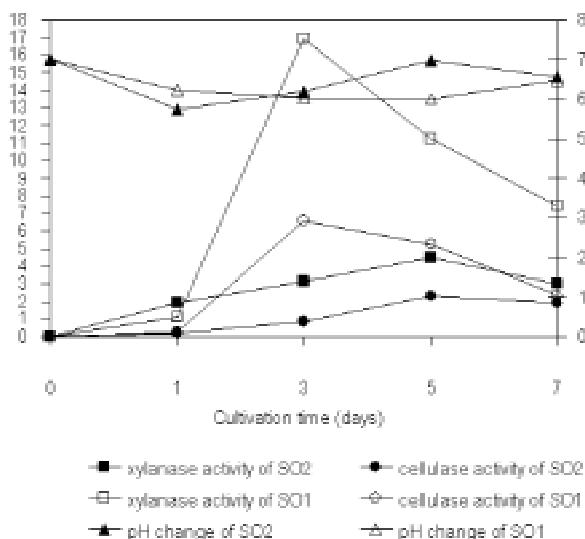
was the quantity of oil must be not less than 15.00 g/l. The bulking solid occurred from the reaction of the enzyme (with xylanase activity of 200 U/ml) from *Aspergillus niger* ATCC 6275. This enzymatic separation process resulted in the removal of 95% of the oil under optimal conditions.

##### 5. Separation of oil from non-sterile and sterile POMW in shaking and static conditions by isolate SO1

The isolate SO1 was cultivated to separate oil from non-sterile and sterile POMW under static and shaking conditions. For shaking condition, the isolate SO1 gave better reduction of dry weight and oil in bottom layer in sterile POMW than in non-sterile POMW. The result showed the % dry weight reduction of 69.7% and gave 85.3% reduction of the oil in the bottom layer. For static condition, the isolate SO1 also gave better reduction of dry weight (18.1%) and oil (36.7%) in the bottom layer in the sterile POMW after 7 days cultivation.

Therefore, the cultivation of isolate SO1 in shaking condition with sterile POMW was the best condition. The non-sterile POMW was not suitable because it contained natural microorganisms.

**Figure 3.** Changes of pH, cellulase and xylanase in sterile palm oil mill wastewater (adjust pH to 7.0) cultivated with isolates SO1 and SO2 at 200 rpm, 55°C.



**Table 2. Oil separation from POMW by cultivate of isolate SO1 in non-sterile and sterile POMW.**

Cultivate time (days)	Type of POMW	Condition	Quantity of oil in the		% reduction of oil in the bottom layer	Dry weight of the bottom layer (g)	% Reduction of dry weight in the bottom layer
			Top layer (g)	Bottom layer (g)			
0	Non-steriled (without SO1)	Control	0.14	1.09	0.0	1.16	0.0
	Steriled (without SO1)	Control	0.14	1.09	0.0	1.16	0.0
7	Non-steriled (without SO1)	Shaking	0.08	1.00	8.26	1.12	3.45
		Static	0.01	0.89	18.35	1.18	1.72
	Non-steriled (with SO1)	Shaking	0.31	0.41	62.39	1.04	10.34
		Static	0.24	0.55	49.54	1.14	2.00
	Steriled (without SO1)	Shaking	0.12	1.04	4.59	1.12	3.45
		Static	0.01	1.09	0.0	1.15	0.86
	Steriled (with SO1)	Shaking	0.84	0.16	85.32	0.41	64.66
		Static	0.53	0.69	36.70	0.95	18.10

Although these microorganisms could grow in the POMW they might not digest plant cell debris efficiently and also produce lipase to reduce the yield of oil.

### Conclusion

The POMW is highly polluted wastewater which contains plant cell debris and oil. This study show that it is possible to use the microorganism that could grow at high temperature and produce cellulase and xylanase to pretreat this wastewater by hydrolyzing cellulose and xylose of the cell debris. The trapped oil will be separated and float up. When this oil is skimmed off the wastewater will be less polluted and can easily be treated.

### Acknowledgement

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