ORIGINAL ARTICLE

Screening and application of thermotolerant microorganisms and their flocculant for treatment of palm oil mill effluent

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Abstract

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Screening and application of thermotolerant microorganisms and their flocculant for treatment of palm oil mill effluent

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Among fifteen thermotolerant polymer-producing isolates, three strains SM 29, WD 90, and SM 38 produced polymer posessing very high flocculating activities (24.81, 14.63 and 10.84, respectively) and flocculation rates (94.29, 90.69 and 87.84, respectively). These three strains were identified to be *Bacillus subtilis* WD90, *Bacillus subtilis* SM 29, and *Enterobacter agglomerans* SM 38. Treatment of palm oil mill effluent (POME) by these three selected strains under aerobic condition at 45ºC for 48 h revealed that neither oil separation nor flocculation of solids was observed. However, all three strains were able to decolorize the POME from dark brown to very light yellow. Flocculant produced from the three selected isolates could not separate the suspended solids and oil from the POME.

Key words : bioflocculant, screening, decolorization, palm oil mill effluen (POME), thermotolerant bacteria

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การคัดแยกและการประยุกต์ใช้จุลินทรีย์ทนร้อนและสารตกตะกอนชีวภาพสำหรับน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์ม
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ในบรรดาจุลินทรีย์ทนร้อนที่ผลิตพอลิเมอร์ที่แยกได้จำนวน 15 สายพันธุ์ มีเพียง 3 สายพันธุ์คือ สายพันธุ์ SM29 WD90 และ SM8 ที่สามารถผลิตพอลิเมอร์ที่มีกิจกรรมการตกตะกอนสูง (24.81, 14.63 และ 10.84 ตามลำดับ) และอัตราการตกตะกอบสูง (94.29, 90.69 และ 87.84 ตามลำดับ) จากการจำแนกชนิดของจุลินทรีย์ทั้ง 3 สายพันธุ์ พบว่าเป็น ***Bacillus subtilis*** **WD29** ***Bacillus subtilis*** **SM29** และ ***Enterobacter agglomerams*** **SM38** เมื่อนำเชื้อ 3 สายพันธุ์ไปบำบัดน้ำทิ้งโรงงานสกัดน้ำมันปาล์มภายใต้สภาวะไร้อากาศที่อุณหภูมิ 45°C เป็นเวลา 48 ชม. พบว่าไม่สามารถแยกน้ำมันและตกตะกอนของแข็งได้ แต่สามารถลดสีของน้ำทิ้งโรงงานสกัดน้ำมันปาล์มจากสีน้ำตาลเข้มเป็นสีเหลืองอ่อน นอกจากนี้สารตกตะกอนที่ผลิตจากสามสายพันธุ์นี้ไม่สามารถแยกของแข็งแขวนลอยและน้ำมันออกจากน้ำทิ้งโรงงานสกัดน้ำมันปาล์มได้

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Flocculants are generally divided into three major groups: inorganic flocculants, organic synthetic high polymer and naturally occurring flocculants such as microbial flocculant. These flocculants have been used for various purposes according to properties and toxicity (Lee, *et al*., 1995) including water and wastewater treatment, dredging, downstream technique, fermentation process, and food industries (Kwon, *et al*., 1996). Of these flocculants the organic synthetic high polymer flocculants have been used most frequently since they are both very cost-effective and strong agents. However, some of them especially polyacryla-mides, are not easily degraded in nature and some of monomers are both neurotoxic and strong human carcinogens.

Biopolymer from microbial source has been received much attention recently due to the awareness of environmental problem which inevitably affects the health of human being (Kurane, *et at*., 1994), but they show weak flocculating activities. The development of a new biological flocculant, which has a strong activity and also can be biodegraded by the nature, is required especially for the fermentation and for food industries (Kwon, *et al*., 1996). In this paper, selection and identification of thermotolerant polymer-producing bacteria with high flocculating activity and their application to treat wastewater from palm oil mill are investigated.

Materials and Methods

Microorganisms

Fifteen thermotolerant bacterial strains were kindly provided by Madla (2001) and Dermlim (1999) who isolated them from activated sludge, anaerobic pond, aeration tank, hot spring, etc. These cultures were maintained in their isolated media at 45ºC for 48 h before keeping in a refrigerator.

Medium

Composition of the medium for screening of polyglutamic acid polymer-producing bacteria (PGA medium) was as follows: 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 . 7H_2O$. The pH was adjusted to 7.0 (Yokoi, *et al*., 1995).

Palm oil mill effluent (POME)

POME from decanter was taken from Pure Oil Company Limited, Thumbon Banphu, Amphur Hat Yai, Songkhla Province. The POME were kept at -20ºC until used.

Flocculating activity and flocculation rate

Flocculating activity of the culture broth was measured from the turbidity of a kaolin clay suspension after aggregation. The procedures were modified from the methods as described by Kurane, *et al*. (1986) and Suh, *et al*. (1997). In a 50 ml beaker, 4.5 ml of $CaCl_2$ solution (1% w/v) was added into 45 ml kaolin clay suspension (5 g/l) and stirred with a glass rod (or magnetic bar) for 20 seconds. After stirring, 0.5 ml of culture broth or polymer solution was added. The pH of the mixture was adjusted to 7.0 with 0.5 M HCl or 6 M NaOH solution and stirred again. After standing for 5 min at room temperature, the sample was taken from the middle part of the container to measure for the absorbance at 550 nm using a spectrophotometer. The control was prepared using the same method but replacing the sample with deionized water. The flocculating activity and flocculation rate were calculated according to the equations:

$$\text{Flocculating activity} = [1/(OD_{550})_s]-[1/(OD_{550})_c]$$

$$\text{Flocculation rate (\%)} = \frac{[(OD_{550})_c-(OD_{550})_s] \times 100}{(OD_{550})_c}$$

$(OD_{550})_s$ = absorbance of the sample
$(OD_{550})_c$ = absorbance of the control

Dry cell weight and polymer yield

Dry cell weight and polymer yield determined followed the methods as described by Dermlim *et al*. (1999).

Screening and identification of thermotolerant polymer-producing bacteria with high flocculating activity

One loop of 48 h old culture slant of fifteen thermotolerant polymer-producing isolates were inoculated into 50 ml PGA medium. Cultivation was carried out on a shaker (200 rpm) at 45ºC for 48 h, then their culture broth were taken to measure for growth (as dry cell weight), polymer yield, flocculating activity and flocculation rate. Three strains producing polymer with very high flocculating activity and flocculation rate were selected and identified using the api 20 E system (bioMerieux Company) and for biochemical tests according to the procedure as described by Slepecky and Ernest (1992).

Treatment of POME by the three selected isolates

Characteristics of palm oil mill effluent (POME)

Decanter effluent was measured for pH and analyzed for chemical oxygen demand (COD), biochemical oxygen demand (BOD), total solids (TS), suspended solids (SS), oil & grease, using the methods as described in the Standard Method for the Examination of Water and Wastewater (APHA, AWWA and WPCF., 1985) and total Kjeldahl nitrogen (A.O.A.C., 1990).

Treatment of POME by the three selected isolates

Ten ml of 18 h starter culture of the three selected isolates was inoculated into 250 ml flasks containing 100 ml POME (pH adjusted to 7.0). They were cultivated on a shaker (150 rpm) at 45ºC for 48 h. One flask of each culture was taken from the shaker every 12 h and poured into 100 ml cylinder before let it setting for 1-3 h to observe for oil separation and flocculation. The samples were taken from the middle layer the cylinder to measure for TS, SS, and COD.

Treatment of POME by the flocculant of the three selected isolates

Preparation of the bioflocculant

The bioflocculant was prepared by cultivating the three selected isolates in PGA medium on a shaker (200 rpm) at 45ºC for 48 h. After that, the culture broth was centrifuged at 7,600x*g* (10,000 rpm) for 15 min at 5ºC to remove cells. The polymer was extracted from the supernatant by precipitation with 4 volumes of cold (-20ºC) 95%

ethanol, left overnight and centrifuged at 7,600 x *g* (10,000 rpm) for 15 min at 5ºC (Dermlim, 1999). The moisture content of the polymer was also determined in order to calculate the amount of dried polymer needed for flocculation. The polymer was freezed-dried.

Treatment of POME by the bioflocculant

Each polymer obtained was added into 100 ml POME at concentrations in the range of 0-50% (wet weight/volume of POME) in the presence of 1.0% $CaCl_2$. The mixture was mixed well and let it standing for 24 h at room temperature to observe for flocculation. Solution from the upper layer was taken to measure for SS and oil & grease, then calculated for the removal efficiencies.

Results and Discussion

Screening and identification of thermotolerant polymer-producing bacteria with high flocculating activity

After 48 h cultivation, the culture broth of the fifteen thermotolerant polymer-producing isolates were taken to measure for growth, polymer yield, flocculating activity and flocculation rate. Results (Table 1) indicated that these isolates gave the dry cell weight in the range of 0.26-2.51 g/l and polymer yield in the range of 31.69-58.08 g/l. Their flocculating activities were in the range of 0.13-24.81 while the flocculation rates were in the range of 33.48-94.29%. Among them, the isolates SM 29, WD 90, and SM 38 produced polymer possessing very high flocculation rates (94.29, 90.69 and 87.84% respectively) and flocculating activities (24.81, 14.63 and 10.84, respectively). They gave the dry cell weights of 1.90, 2.36 and 0.48 g/l, respectively, and the polymer yields of 54.45, 55.27 and 49.27 g/l, respectively. It should be noted that the highest polymer yield was achieved from the isolate WD 90 while the highest flocculating activity and flocculation rate were belong to the isolate SM 29.

The flocculating activity of the polymer in this study was determined using kaolin clay suspension as a flocculation test material. This criteria was previously employed by many researchers (Kurane, *et al*., 1994, Lee, *et al*.,1995, Kwon, *et al*., 1996, Yokoi, *et al*., 1997). Other inorganic

Table 1. Flocculating activity and flocculation rate of culture broth from the fifteen thermotolerant polymer-producing bacterial isolates after 48 h cultivation at 45ºC

Isolate	Dry cell weight (g/l)	Polymer yield (g/l)	Flocculating activity	Flocculation rate (%)
SM 1	1.55	48.06	1.85	55.23
SM 7	1.35	45.68	0.13	67.72
SM 9	2.51	47.63	2.88	65.72
SM 11	1.54	43.69	0.33	47.00
SM 12	0.97	31.69	0.89	37.09
SM 13	1.94	58.08	4.42	74.63
SM 16	1.10	46.16	0.6	33.48
SM 21	1.65	43.09	4.63	75.53
SM 22	0.36	42.50	7.78	83.83
SM 25	1.37	52.39	1.53	50.45
SM 29	1.90	54.45	24.81	94.29
SM 34	0.26	53.22	2.44	61.86
SM 38	0.48	49.27	10.84	87.84
WD 79	0.94	47.66	2.65	63.81
WD 90	2.36	55.27	14.63	90.69

suspended solids such as active carbon, calcium or magnesium compound, organic suspension such as cellulose and yeast suspension were used to test the flocculating activity of the crude and the purified flocculant produced by *Enterobacter* (Yokoi, *et al*., 1997). The flocculant from *Archuadendron sp*. could flocuclate all the materials tested, including microorganisms, silica gel, coal ash, river bottom sediment, cellulose powder, active carbon, aluminium oxide, and flour (Lee, *et al*., 1995). This indicated that the flocculant from microorganisms had the flocculating activity for a wide range of both organic and inorganic materials. As a consequence, it could be widely used in many area of industry including wastewater treatment. Flocculation of suspended solids in the palm oil mill effluent by the flocculant from the three selected strains therefore would be investigated.

The three strains; SM 29, WD 90 and SM 38 were selected for identification. The isolates SM 29 and WD 90 were found to be Gram positive bacteria while the isolate SM 38 was Gram negative bacteria. All of them were aerobic bacteria with rod shape and motile. According to their morphological and biochemical characteristics (Table 2), the isolates SM 29 and WD 90 were

Table 2. Taxonomical and biochemical characteristics of the three selected thermotolerant polymer-producing bacyrtia

Test	SM 29	WD 90	SM 38
Gram strain	Positive	Positive	Negative
O_2 requirement	+	+	+
Cell morphology	rod	rod	rod
Motility	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Indole production	-	-	-
Voges-Proskauer	+	+	+
Citrate utilization	-	-	+
H_2S production	-	-	-
Urea hydrolysis	+	+	-
Gelatin hydrolysis	+	+	-
Starch hydrolysis	+	+	-
Casein hydrolysis	+	+	-
Nitrate reduction	-	-	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Oxidation-Fermentation			
Glucose	+	+	+
Arabinose	-	-	+
Rhamnose	-	-	+
Mannitol	+	+	+
Sorbitol	-	-	+
Inositol	-	-	+
Sucrose	+	+	+
Identified as	*Bacillus subtilis* SM 29	*Bacillus subtilis* WD 90	*Enterobacter agglomerans* SM 38

Note: - : negative result ; + : positive result ; Identified with the api 20 E

both identified to be *Bacillus subtilis*, whereas the isolate SM 38 was identified to be *Enterobacter agglomerans*.

Treatment of POME by the three selected isolates

Characteristics of the palm oil mill effluent (POME)

The characteristics of decanter effluent from a palm oil mill were determined (Table 3). It was found to contain high organic matter (BOD 54.56 g/l and COD 90.40 g/l), high solids (TS 65.36 g/l, SS 45.92 g/l), oil & grease (34.31 g/l), with low nitrogen (TKN 0.83 g/l), and acidic pH (4.35). BOD, COD, TS, SS, oil & grease values were extremely high compared to those from other sources of wastewater. The BOD value was much higher than those previously reported by Ng *et al*. (1985), Chin and Wong (1983), and Ho and Tan (1983), (26.22, 28.2, and 21.50 g/l respectively). The COD value was higher than that reported by Borja and Banks (1993) (45-90 g/l). Total solids and suspended solids were also higher than those reported by Chin and Wong (1983) and Muneesri (1996) (54.0 and 53.03 g/l; 31.8 and 33.1 g/l, respectively). Oil & grease value was also higher than those reported by Muneesri (1996) and Borja and Banks (1995) (24.90 and 4-12 g/l, respectively). However, total nitrogen was similar to that reported by Ho and Tan (1983) (0.85 g/l). These results illustrate that the characteristics of palm oil mill effluent varied widely from day to day and from plant to plant, depending on the quality of the raw material (fresh fruit brunches) and the efficiency of the machines involved during the extraction process as well as the operational control. It should be noted that with these variations in characteristic, treatment efficiency of POME using different samples may not be the same.

Treatment of POME by the three selected isolates

After cultivation of *B. subtilis* SM 29, *B. subtilis* WD 90 and *E. agglomerans* SM 38 for 48 h and letting it stand for 1 h, neither oil separation nor flocculation was observed. Total solids, suspended solids and COD values of the POME after standing for 1 h fluctuated with the cultivation time. Compared to the control (with the average values of 3.95, 1.25 and 3.85 g/l), the values from *B. subtilis* WD 90 were the highest (13.15, 7.12 and 10.31 g/l) which may indicate the better growth of this strain. The other two strains gave similar results with the average values of 10.28, 4.2 and 6.96 from *B. subtilis* SM 29 and 10.38, 4.25 and 8.26 from *E. agglomerans* SM 38. These higher values than the control may be due to the growth of microorganisms and their polymers as well as the sampling procedure. Nevertheless, it was found that all three isolates were able to decolorize the POME from dark brown to very light yellow.

Table 3. Characteristics of palm oil mill effluent (POME) of this experiment compared to other sources

Parameter	This work		Ng *et al.* (1985)	Chin and Wong (1983)	Ho and Tan (1983)	Muneesri (1996)	Borja and Banks (1993)
	Range	Average					
pH	4.16-4.57	4.35	4.10	4.4	3.5-4.5	4.7	4.5-5.0
BOD	31.19-82.50	54.56	26.22	28.2	21.5	-	20-40
COD	79.92-111.29	90.40	62.93	67.4	43.0	35.50	45-90
Total solids (TS)	32.15-94.00	65.36	48.43	54.0	47.5	53.03	40-70
Suspended solids (SS)	25.32-77.26	45.92	26.46	31.8	25.3	33.10	20-40
Oil & grease	20.50-59.95	34.31	-	-	8.5	24.90	4-12
Total nitrogen	0.65-1.00	0.83	1.00	1.00	0.85	0.90	-

Unit in g/l except pH

Treatment of POME by the bioflocculant

The wet polymer of the three selected isolates were added into POME at the concentrations of 5, 10, 20, 30, 40, and 50% (wet weight/ volume of POME). The moisture content of the polymers from *B. subtilis* WD 90, *B. subtilis* SM 29, and *E. agglomerrans* SM 38 were 55.6, 53.2, and 55.0% respectively. Therefore, the concentrations of the dried polymers used in this experiment were 22.2, 44.4, 88.8, 133.2, 177.6, and 222.0 g/l for *B. subtilis* WD 90, 23.4, 46.8, 93.6, 140.4, 187.2, and 234.0 g/l for *B. subtilis* SM 29, and 22.5, 45.0, 135.0, 180.0, and 225.0 g/l for *E. agglomerrans* SM 38. After addition of the polymers into the POME and let them standing for 24 h, no flocculation was observed in all samples, although 4.5 ml of 1% (w/v) Ca^{2+} was added into the POME. It was reported that the flocculating activity of biopolymer floc-culant produced by *Bacillus subtilis* PY-90 was markedly increased by the addition of Ca^{2+}. The optimum concentration of Ca^{2+} for maximum flocculating activity was 2 to 8 mM (Yokoi, *et al*., 1995).

Conclusion

Among fifteen strains of thermotolerant polymer-producing bacteria, three isolates WD 90, SM 29 and SM 38 produced polymer with very high flocculating activities and flocculation rates. They were identified to be *Bacillus subtilis* WD 90, *B. subtilis* SM 29, and *Enterobacter agglomerans* SM 38. These three strains grew well in the decanter effluent and could decolorize the effluent from dark brown to very light yellow. Separation of oil and flocculation of solids in the POME were not successful using either one of these strains or their extracted polymers.

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