

# การแยกและการศึกษาปัจจัยที่มีผลต่อการสร้างเอนไซม์ไลเปส โดยแบคทีเรียจากดินที่ปนเปื้อนน้ำมัน

## Isolation and Factors Affecting Lipase Production by Bacteria from Oil-Contaminated Soils

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### บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อแยกแบคทีเรียที่สามารถผลิตเอนไซม์ไลเปสจากตัวอย่างดินที่ปนเปื้อนน้ำมันพืช หรือน้ำมันเชื้อเพลิง และศึกษาปัจจัยที่มีผลต่อการสร้างเอนไซม์ไลเปส โดยคัดเลือกแบคทีเรียที่เจริญบนอาหารเลี้ยงเชื้อ Tributyrin Agar ที่มีน้ำมันปาล์มร้อยละ 1 และสร้างโซนใสได้กว้าง แล้วนำมาทดสอบกิจกรรมของเอนไซม์ไลเปส พบว่า แบคทีเรียไอโซเลต PKRU-9 มีกิจกรรมของเอนไซม์ไลเปสมากที่สุด โดยแบคทีเรียไอโซเลต PKRU-9 สามารถสร้างเอนไซม์ไลเปสได้ดีที่สุดเมื่อเลี้ยงในอาหารเลี้ยงเชื้อ Basal Medium ที่มี Yeast Extract ร้อยละ 0.5 เป็นแหล่งไนโตรเจน และมีน้ำมันถั่วเหลืองร้อยละ 1.0 เป็นแหล่งคาร์บอน โดยการเลี้ยงเชื้อที่อุณหภูมิ 35°C และความเร็วรอบ 200 รอบต่อนาที และมีการสร้างเอนไซม์ไลเปสมากที่สุดเมื่อเลี้ยงเชื้อเป็นเวลา 54 ชั่วโมง การศึกษาการบำบัดน้ำเสียปนเปื้อนไขมันของเชื้อแบคทีเรีย PKRU-9 โดยการนำไปเลี้ยงในน้ำเสียสังเคราะห์ที่เติมน้ำมันปาล์มร้อยละ 1 และในน้ำเสียจากบ่อบำบัดที่ปนเปื้อนไขมัน พบว่า เชื้อแบคทีเรีย PKRU-9 สามารถลดค่า BOD<sub>5</sub> ได้สูงสุด ร้อยละ 74 และ 90 ตามลำดับ โดยค่า BOD ลดลงมากที่สุดในวันที่ 3 ของการเลี้ยงเชื้อ แสดงว่าเชื้อแบคทีเรีย PKRU-9 มีศักยภาพในการนำไปใช้บำบัดน้ำเสียชุมชนที่มีการปนเปื้อนของไขมันได้

**คำสำคัญ:** แบคทีเรียที่สร้างเอนไซม์ไลเปส กิจกรรมของเอนไซม์ไลเปส การบำบัดน้ำเสียชุมชน

### Abstract

This research aimed to isolate lipase-producing bacteria from soil samples contaminated with vegetable oil or petrol, and study factors affecting its lipase production. The bacteria that could be grown on tributyrin agar supplemented with 1% palm oil and produced a wide clear zone were selected. These selected bacterial isolates were tested for lipase activity; among them, the isolate PKRU-9 had the highest lipase activity. The maximum lipase production was obtained when PKRU-9 was cultured in a basal

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medium containing 0.5% yeast extract as a nitrogen source and 0.5% soybean oil as a carbon source at 35°C, 200 rpm. The highest lipase production was obtained at 54h of incubation. Treatment of wastewater contaminated with the lipid of PKRU-9 was studied by culturing the bacterium in a synthetic wastewater medium containing 1% palm oil, and in wastewater contaminated with fat. The PKRU-9 was able to decrease the value of BOD<sub>5</sub> by 74% and 90%, respectively. The highest reduction of BOD<sub>5</sub> was found on day 3 of cultivation. These results suggested that the PKRU-9 has the potential to be used for treating municipal wastewater contaminated with fat.

**Keywords:** Lipase Producing Bacteria, Lipase Activity, Municipal Wastewater Treatment

## Introduction

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are a group of hydrolase enzymes that catalyze the hydrolysis of ester bonds in triacylglycerols to release fatty acids and glycerol. Hydrolytic reactions of lipase occurred at the lipid-water interface due to the poor solubility of its natural substrates in water [1]. Lipases are found universally in plants, animals, and microorganisms. Among these, bacterial lipases are commercially significant due to the simplicity of their cultivation and advancement in bulk production. Lipases have diverse potentials applications in the detergent, food, and pharmaceutical industry, and the production of biodiesel [2-3]. Lipase-producing microorganisms have been discovered in various sources such as agro-industrial wastes, vegetable oil processing wastes, dairy product industries, and oil-contaminated soils [2,4-5]. Bacterial lipases are mainly inducible enzymes, typically produced in the presence of a lipid source such as oils, triacylglycerols, fatty acids, tweens, bile salts, and glycerol [1]. Some natural oils reported improving the production of lipase are olive oil, coconut oil, vegetable oil, and petroleum oil [2].

Wastewater is one of the major environmental problems in Thailand, primarily due to the disposal of untreated wastewater from cities and industrial plants to water sources. Lipid is an important component in household waste that causes significant environmental contamination. It can form oil films on water surfaces, prevents the absorption of oxygen into water, and contributes to the death of many aquatic lives [6]. Moreover, fat in lipid-rich wastewater will decrease the performance of the wastewater treatment system by interrupting the activity of aerobic bacteria in the wastewater treatment process. Hydrolysis of fat-contaminated wastewater using bacteria is one of the alternative strategies for treating lipid-rich wastewater before being discharged into freshwater. Therefore, in this study, the production of lipase from bacterial isolate PKRU-9, which was isolated from oil-contaminated soil was reported. Then, factors affecting lipase production were demonstrated and the treatment of lipid-containing wastewater was also evaluated.

## Research Methodology

### 1. Soil Samples

Soil samples contaminated with cooking oil or petrol were collected from 11 different sites. These samples were collected from dishwashing areas at Phuket Rajabhat University and a restaurant in Phuket (2 samples, S1-S2), mangrove sediment soils contaminated with cooking oil and petrol-spilled in Phuket (4 samples, S3-S6), and soil samples contaminated with petrol-spilled from garages located in Phuket and Surat Thani provinces (5 samples, S7-S11). These soil samples were collected at 10 cm-depth and stored at 4 °C for further screening of lipolytic bacteria.

### 2. Screening of Lipase Producing Bacteria

Five grams of each soil sample was enriched twice in 50 mL of screening medium (1.5%  $(\text{NH}_4)_2\text{SO}_4$ , 1% peptone, and 0.5% NaCl) [7] supplemented with 1% palm oil as a sole carbon source and cultured at 35°C, 220 rpm for 3 days. The appropriate dilution of enrichment culture from each soil sample was plated on tributyrin agar (0.5% peptic digest of animal tissue, 0.3% yeast extract, and 1.5% agar) supplemented with 1% palm oil, and then incubated at 35°C for 2 days. Lipase production isolates were detected by the presence of clear zones around colonies. The diameters of the clear zone and the colony were measured by Vernier caliper. The enzyme activity index was calculated based on a diameter ratio of the clear zone to the colony. Bacterial isolates showing high enzyme activity index were chosen for lipase activity assay.

### 3. Lipase Activity Assay

The chosen bacterial isolates were cultured in liquid medium (0.5% peptone, 0.3% beef extract, 0.1%  $\text{CaCl}_2$ , 1% gum arabic, and 2% palm oil, pH 7.0) at 35°C and 220 rpm for 2 days. The culture supernatants were harvested by centrifugation at 4,724 g, 4 °C, 15 min for further use as crude lipase. The appropriate amount of crude lipase was assayed for lipase activity against 0.4 mM *p*-nitrophenyl palmitate (*p*NPP) (dissolved in propanol-2-ol) in 50 mM Tris-HCL buffer, pH 8.0 at 37°C, 15 min (adapted from Dheeman *et al.* (2011) [8]). The reaction was terminated by adding 1.0 M  $\text{Na}_2\text{CO}_3$ . The absorbance of *p*-nitrophenol (*p*NP) released from the reaction was measured at 410 nm by using a Spectrophotometer (Thermo Scientific, USA), and compared with a standard curve of *p*NP. One unit of lipase activity is defined as the amount of enzyme which released 1 μmol of *p*NP per min under the assay conditions. All enzyme assays were carried out in triplicate. Protein concentration was determined by the dye-binding assay (Protein Assay Reagent Kit; Bio-Rad, USA) [9] and compared with a standard curve of bovine serum albumin. Specific activity was calculated based on the ratio of lipase activity to protein concentration.

### 4. Factors Affecting Lipase Production

The starter culture of the selected bacterial isolate was prepared in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) at 35°C, 200 rpm, for 16 h. Effect of culture conditions on lipase production was studied in a basal medium (0.2% glucose, 0.5% tryptone, 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.1%  $\text{K}_2\text{HPO}_4$ ) and cultured at 35°C, 200 rpm for 48 h. Seven vegetable oils including palm oil, olive oil, sunflower seed oil, soybean oil, coconut oil, sesame oil, and canola oil, were chosen to study the effect of carbon source at a 2% concentration. Then, 7 nitrogen sources including peptone, tryptone, yeast extract,

glycine, urea, ammonium sulfate, and ammonium chloride were chosen to study the effect of nitrogen source at a 0.5% concentration. Afterward, the optimal concentrations of the selected carbon and nitrogen sources were studied. These optimal conditions were used to study the optimum rotational speed for lipase production. Finally, cell dry weight and lipase activity were determined periodically up to 96 h for evaluating the effect of the cultivation time. All of the above experiments were performed in triplicate.

### 5. Wastewater Treatment

Two types of lipid-containing wastewaters, which were synthetic wastewater medium (1% palm oil, 0.06% peptone, 0.04% beef extract, 0.01% urea, 0.01% Na<sub>2</sub>HPO<sub>4</sub>, 0.003% NaCl, 0.0014% CaCl<sub>2</sub>, 0.0014% KCl, and 0.001% MgSO<sub>4</sub>), and wastewater contaminated with fat from a wastewater treatment system in Phuket, were used to study bacterial ability in hydrolyzing lipid-rich wastewater. One percent of the starter culture was inoculated into these wastewaters and cultured at 35°C and 150 rpm for 120 h, adapted from Matsumiya *et al.* (2007) and Rungreang & Pattanapitpaisal (2011) [10-11]. The culture medium was taken at 0, 24, 48, 72, 96, and 120 h for the estimation of biochemical oxygen demand (BOD<sub>5</sub>) as described by Mongkolthanaruk and Dharmsthiti (2002) [6]. Each experiment was carried out in triplicate.

## Results and Discussion

### 1. Selection of Lipase Producing Bacteria

Enrichment culture of 11 soil samples (S1-S11) were screened for lipase producing bacteria on tributyrin agar supplemented with 1% palm oil. Based on clear zone production, 47 bacterial isolates were considered as lipase producing bacteria (data not shown). Among them, 9 bacterial isolates with a high enzyme activity index were chosen for lipase activity assay (Table 1).

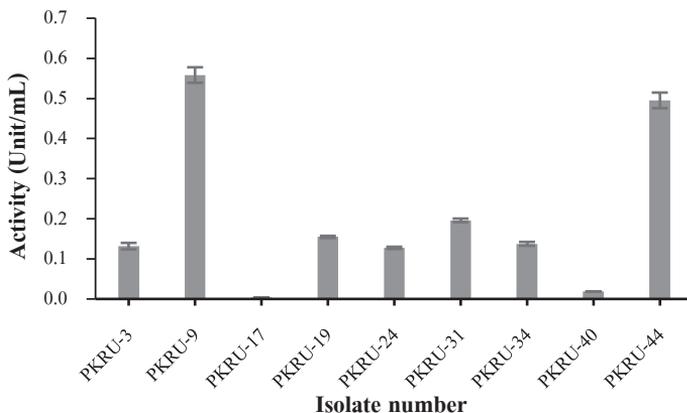
**Table 1** The chosen lipase producing bacteria from soil samples contaminated with oil or petrol-spilled

Isolate Number	Sampling site	Zone of hydrolysis (mm)	Zone of the colony (mm)	Enzyme activity index
PKRU-3	S1	3.10	1.2	2.58
PKRU-9	S1	3.20	1.3	2.46
PKRU-17	S3	3.40	1.78	1.91
PKRU-19	S4	7.10	2.01	3.53
PKRU-24	S6	3.20	1.58	2.03
PKRU-31	S8	3.78	0.9	4.20
PKRU-34	S9	7.58	2.4	3.16
PKRU-40	S10	5.60	2.1	2.67
PKRU-44	S11	2.60	0.9	2.89

Lipase producing bacteria were found in soil samples of all sampling sites with different enzyme activity index. Therefore, cooking oil and petrol-contaminated soils provide a suitable environment for inducing lipase production. The low value of the enzyme activity index obtained in this study is due to the complex fatty acid compositions of palm oil.

## 2. Lipase Activity Screening

Crude lipases from 9 chosen bacterial isolates were tested for lipase activity against the *p*NPP substrate. Among them, PKRU-9 showed the highest lipase activity (Fig. 1). Therefore, PKRU-9 was selected to study its optimal conditions for lipase production and its ability to hydrolyze wastewater containing lipid. The PKRU-9 was a gram-positive bacterium with a short rod shape. It exhibited circular form, entire margin, convex elevation, yellow color, and small size when grown on a nutrient agar plate (data not shown).



**Figure 1** Lipase activity of 9 chosen bacterial isolates after cultivated for 2 days at 35°C, 200 rpm in a liquid medium. Values are means (n = 3) and the error bars represent  $\pm$  standard deviation of the mean.

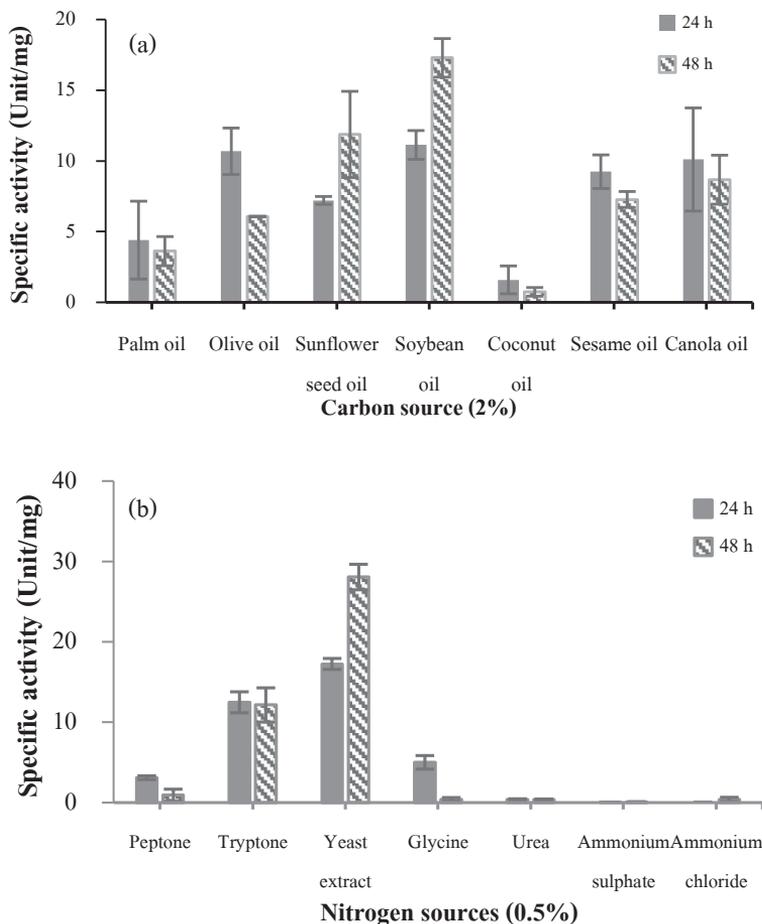
## 3. Effect of Culture Condition for Lipase Production

### 3.1 Effect of Carbon and Nitrogen Sources

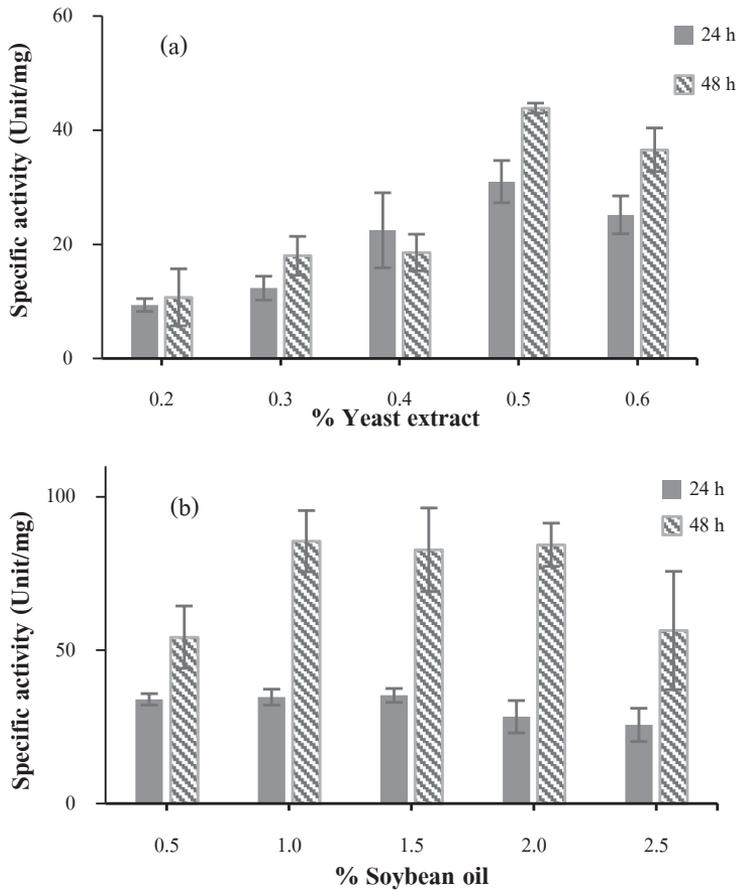
Seven vegetable oils were used as carbon sources in basal medium to determine the maximum lipase activity from PKRU-9 isolate. The specific activity values revealed soybean oil as the best carbon source for lipase production from PKRU-9 (Fig.2a). Olive oil has been reported as one of the best inducers for bacterial lipase production [2]. In this study, olive oil induced lipase production during the first 24h as a high amount as soybean oil. However, after 24h, lipase production in culture medium supplemented with olive oil decreased, while it increased in the medium supplemented with soybean oil. As a result, soybean oil was selected to study the effect of nitrogen source. Among the 7 nitrogen sources tested, PKRU-9 utilized 0.5% yeast extract as the best nitrogen source for lipase production (Fig. 2b). The organic nitrogen sources such as yeast extract, tryptone, and peptone were better used to induce lipase production than the inorganic nitrogen sources. Moreover, yeast extract and peptone have generally been used for lipase production. Consequently, yeast extract was selected as a nitrogen source for the following experiments.

### 3.2 Effect of Carbon and Nitrogen Source Concentration

The concentration of yeast extract was varied from 0.2 to 0.6% in a basal medium containing 2% soybean oil. It was found that 0.5% yeast extract was the optimal concentration for lipase production (Fig. 3a). Therefore, 0.5% yeast extract was selected to examine the optimal concentration of soybean oil between 0.5 and 2.5%. There was almost no difference in specific activity when 1.0% to 2.0% soybean oil were used (Fig. 3b). However, the value seemed to decrease when the concentrations of soybean oil were 2.0% and 2.5%. As a result, 1.0% soybean oil was selected as the optimal concentration for lipase production of PKRU-9. Previously, the optimal condition of lipase production by *Pseudomonas* sp. HCU2-1 was reported as the basal medium containing 2% wastewater from palm oil and 0.5% peptone [3]. However, in this study, yeast extract induced lipase production from PKRU-9 better than peptone.



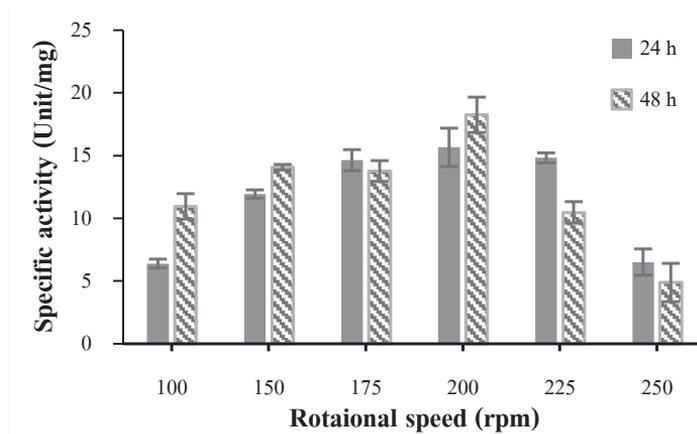
**Figure 2** Effect of carbon (a) and nitrogen (b) sources on lipase production by PKRU-9 in a basal medium at 35°C, 200 rpm for 24 and 48h. Values are means (n = 3) and the error bars represent ± standard deviation of the mean.



**Figure 3** Effect of yeast extract (a) and soybean oil (b) concentrations on lipase production by PKRU-9 in a basal medium at 35°C, 200 rpm for 24 and 48h. Values are means (n = 3) and the error bars represent ± standard deviation of the mean.

### 3.3 Effect of Rotational Speed

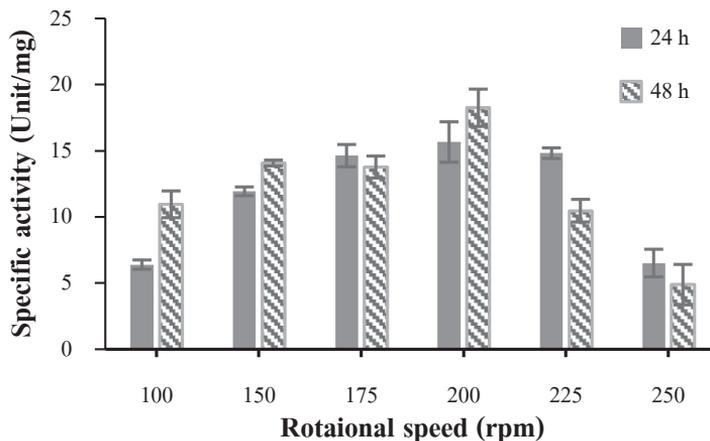
PKRU-9 was cultured in a basal medium containing 0.5% yeast extract and 1.0% soybean oil at 35°C with varying rotational speed from 100 to 250 rpm. The optimal rotational speed for lipase production was 200 rpm (Fig. 4). Speed higher than 200 rpm resulted in a decrease production of lipase since higher rotational speed increases shear forces leading to a negative impact on cell growth and lipase production [12].



**Figure 4** Effect of rotational speed on lipase production by PKRU-9 in a basal medium containing 0.5% yeast extract and 1.0% soybean oil at 35°C, 200 rpm for 24 and 48h. Values are means (n = 3) and the error bars represent ± standard deviation of the mean.

### 3.4 Effect of Cultivation Period

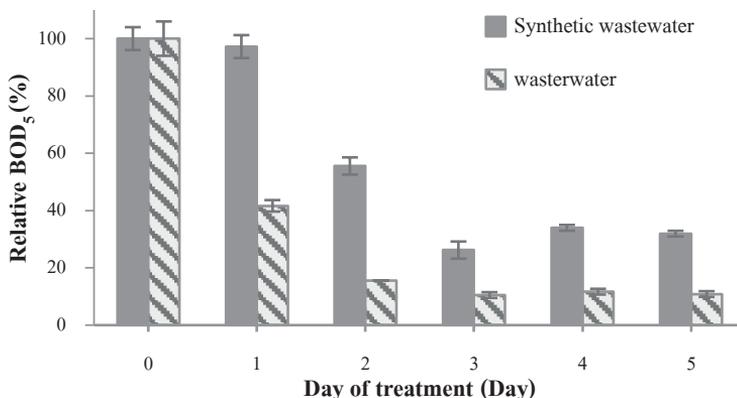
PKRU-9 was cultured in its optimal conditions for 96 h. Lipase production increased simultaneously as cell growth, and still increasing until the cell was in the stationary phase (Fig. 5). The highest lipase production was at 54h of cultivation, in which the cell was in the late stationary phase. Then, the rate of lipase production gradually decreased when the cell entering the dead phase. The results are in agreement with previous studies of bacterial lipase, which revealed maximum lipase production during the stationary phase [13-15]



**Figure 5** Effect of cultivation period on the growth (line graph) and lipase activity (bar graph) of PKRU-9 in a basal medium containing 0.5% yeast extract and 1.0% soybean oil at 35°C, 200 rpm. Values are means (n = 3) and the error bars represent ± standard deviation of the mean.

### 3.5 Wastewater Treatment

The ability of PKRU-9 in the hydrolysis of lipid-containing wastewaters was estimated by the BOD<sub>5</sub> values. PKRU-9 was able to decrease the BOD<sub>5</sub> values to 74% and 90% of its starting BOD<sub>5</sub> in synthetic wastewater medium, and wastewater contaminated with fat, respectively (Fig. 6). The lowest BOD<sub>5</sub> value was obtained on day 3 of treatment in both types of wastewaters. The BOD<sub>5</sub> value slightly increased in the synthetic wastewater medium after 3 days however, remained constant in wastewater contaminated with fat. This might be because PKRU-9 entered the death phase during days 4 and 5 of cultivation. The rate of BOD<sub>5</sub> decreasing in wastewater contaminated with fat was higher than that of synthetic wastewater due to the higher value of starting BOD<sub>5</sub> in synthetic wastewater. The application of lipase in wastewater treatment was also done with the immobilized bacterial lipases [11]. They found that the BOD<sub>5</sub> values of the immobilized lipase in synthetic wastewater and wastewater from the meat factory were decreased around 51 to 64%. However, in this study, PKRU-9 was used directly to treat the wastewater. Therefore, it can decrease the BOD<sub>5</sub> value higher than the immobilized lipase.



**Figure 6** Relative BOD<sub>5</sub> values after cultivated the PKRU-9 in synthetic wastewater medium, and wastewater for 5 days at 35°C, 200 rpm. Values are means (n = 3) and the error bars represent ± standard deviation of the mean.

### Conclusion

A lipase-producing bacterium, PKRU-9, isolated from oil-contaminated soil showed the highest lipase activity among the other selected isolates. The maximum lipase production from PKRU-9 was obtained in a basal medium containing 0.5% yeast extract and 0.5% soybean oil, and cultured at 35°C, 200 rpm. The PKRU-9 can be used to treat lipid-rich wastewater as a whole cell, which is more convenient than using its enzyme. Further studies such as engineering the lipase gene for improved enzyme function, are important to enhance the effectiveness of PKRU-9 in municipal wastewater treatment.

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