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Screening of Lipolytic producing microbes from microbial consortium for treatment of palm oil contaminated wastewater

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Abstract

FOGs (fat, oil, and grease) pollution in domestic wastewaters has been a significant environmental issue worldwide. This study was designed to determine the potential of bacterial strains isolated from wastewater microbial consortiums to biodegrade the FOGs. The isolated bacteria were characterized by standard Gram staining method and lipid degradation assays. Gram staining showed that four of the selected isolates, (M9P8, MRS-1A.3W, MRS-1B-1W, and MRS-1B-1.C) were Gram-positive, rod-shaped bacteria. One isolate PCA-3A.1.Y, was a Gram-positive coccus. All five bacteria displayed lipase activity as demonstrated by tributyrin hydrolysis when tested using the drop plate method on tributyrin agar. Lipase activity was found to be highest in MRS-1B-1. W strain, at 5.89±2.16 U lipase/mg protein. Further analysis was conducted to determine the potential for palm oil reduction. The results showed that, in 0.5× Plate Count Broth (PCB) with 1% (v/v) palm oil, the five bacterial isolates had a 7.29% to 32.37% reduction of palm oil. It is concluded that this approach has resulted in collection of promising bacterial isolates that may be useful for direct applications in biodegradation of lipid-rich wastewaters.

Keyword: FOGs, Microbial consortium, Lipolytic activity, Palm oil degradation

1. Introduction

Wastewater contaminated with fat, oil, and grease (FOGs) is a prevalent problem in developing and industrialized countries [1]. FOGs are major organic matters present in wastewater from food processing and services. The presence of FOGs in wastewater often results in significant problems in biological treatment systems and cause destruction of the environment and ecosystem in the water bodies [2]. Several conventional techniques such as skimming tanks and traps for oil and grease are used for FOGs removal during wastewater treatment [2]. In addition, chemically or physically stabilized lipid/water emulsions should be managed in an appropriate manner to maintain water quality and to minimize environmental impact. This is necessary because lipids that pass through physicochemical treatment processes negatively affect water quality by contributing to the levels of biological oxygen demand (BOD) and chemical oxygen demand (COD) [1]. Generally, partial recovery of lipid residues was made by air floatation. Floated lipid wastes are commonly discarded into sanitary landfill dumping, which also pollutes the environment [3]. Therefore, research for alternative and cost-effective treatment is urgent.

Lipase (E.C.3.1.1.3) belongs to the class of hydrolase enzyme that catalyze the hydrolysis reactions. Under oil-water interface, lipases hydrolyze long-chain triglycerides to fatty acids, glycerol, mono and diacylglycerols. They are ubiquitous in nature and are produced by different types of plants, animals, and bacteria. Due to the availability of variations in catalytic activity, lower production costs and higher production yields, microbial lipases are more economically feasible and thus more widely employed in biotechnological applications [4-5]. A lipase-producing microbe is one of the most crucial factors for the biological treatment of lipids. Commonly, lipases are produced by a wide variety of microorganisms including bacteria, yeast, and fungi. There are a few examples of microbes, such as *Bacillus* spp., *Pseudomonas aeruginosa*., and *Streptomyces* spp., which have been

employed for wastewater treatment or for bioremediation in numerous industries [6]. In a previous study [7], seven selected strains of lipase producing microbes were identified as *Corynebacterium* spp., *Aeromonas* spp., and *Pseudomonas* spp.

In the past decade, the microbial consortiums have been used to reduce FOGs problem in the household effluent systems in several countries in Asia including Thailand. The local-produced microbial consortiums is considered as one of the sources of target microbes with concomitant enzymatic activity for the effective degradation of FOGs waste [8]. Thus, the main aim of this study is to screen for efficient lipase producing microorganisms isolated from the microbial consortiums associated with environmental FOGs and to determine potential capability of these microbes to be used for enhancing FOGs biodegradation during wastewater treatment.

2. Materials and methods

2.1 Recovering and primary screening of microbe strains for lipase production from freezer stock (-80°C)

Twenty microbial strains previously isolated from microbial consortiums [7] were recovered and tested for lipase activity by streak plate technique on plate count agar (PCA) and tributyrin agar (TBA) for 24 h under room temperature (approximately 30°C) [6]. The microbes were characterized by standard Gram staining technique. The diameter (cm) of the clear zone, presented due to the degradation of tributyrin and indicating the production of lipase enzyme, surrounding the growth of lipase-producing microorganisms was measured using Vernier caliper. The results of the clearness (transparency) on tributyrin agar were compared among the twenty bacterial strains. Five strains with large clear zone (higher lipase production) were selected for further testing.

2.2 Confirmation test for lipase production on tributyrin agar and chromogenic plate

Five selected bacterial strains from 2.1 were tested for lipid degradation ability on tributyrin agar and phenol red chromogenic plates with tributyrin or used palm oil (obtained from deep frying shop nearby Assumption University, Hua Mak Campus) as lipid substrates [9]. Measurements were made using the drop plate method at room temperature. The inoculum for the drop plate was prepared by inoculating a loop of overnight culture from PCA slant to plate count broth (PCB) and incubated at room temperature for 24 h. The absorbance of OD600 nm was adjusted to approximately 0.1. The diluted inoculum (0.01 mL) was inoculated as a drop on both tributyrin agar and chromogenic plates with lipid substrates. Upon 24-h and 48-h incubations the diameter (cm) of the clear zone was measured using Vernier caliper. The colour zone was observed visually on chromogenic plates after 24-h of incubation.

2.3 Quantitative screening of lipase activity using titrimetric method and determination of protein concentration

A loop of five selected bacterial strains that were grown overnight on PCA slants were transferred into 30 mL of 0.5X PCB media containing 1% (v/v) palm oil as substrate; flasks were incubated in shaker incubator with 120 rpm at 30°C for 48 h. Lipase assayed was modified from Esmaeili et al. [7]. Bacterial supernatants were centrifuged at 3,500 rpm for 10 min. To a 50 mL flask were added 1 mL of 0.1M Tris-HCI buffer (pH 7.5), 1 mL of 50 mM KCl, 200 µl Tween 80, 1 mL of bacterial supernatant and 1 mL of palm oil as substrate. The flask was then incubated in a shaker at 162 rpm at 37°C for 90 min. Following incubation, the pH was adjusted to the end point of phenolphthalein by titrating with 0.01M NaOH. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 µmol of free fatty acids per min. Specific activity was expressed as units of lipase activity per milligram protein. Blank and positive control were performed with the same procedure, with the enzyme aliquot being substituted with water and commercial lipase from porcine pancreas Type II 100-650 units/mg (Purchased from SIGMA), respectively. Total protein concentration of the bacterial supernatant was determined using the Bradford method [10]. A standard curve was constructed using BSA concentration between 0.1 mg/mL to 1 mg/mL, which was used to calculate enzyme specific activity.

2.4 Determination of palm oil reduction

A loop of five selected bacterial strains that were grown overnight on PCA slant were transferred into 30 ml of 0.5X PCB media containing 1% (v/v) tributyrin oil without glucose and 0.5X PCB media solutions containing 1% (v/v) palm oil with 0.05% (w/v) glucose and without glucose respectively. Flasks were then incubated in a shaker incubator at 120 rpm at 30°C for 48 h. The oil and grease measurement were performed using a partition-gravimetric method [11].

2.5 Statistical analysis

The results were statistically analyzed using RCBD (Randomized Complete Block Design) by constructing an ANOVA table. Duncan's multiple comparison tests were performed in SAS 9.4 program and were used to test the significant difference of each comparison.

3. Results

3.1 Preliminary verification and primary screening of microbe strains for lipase production

Primary screening of the twenty microbial isolates on TBA showed five selected bacterial isolates, M9P8, PCA-3A.1.Y, MRS-1A.3.W, MRS-1B-1.C and MRS-1B-1.W, had average clear zone diameters of 0.54±0.05, 0.58±0.04, 0.62±0.04, 0.68±0.04 to 0.82±0.09 cm, respectively. Gram staining of the five selected isolates indicated that four of the strains (M9P8, MRS-1A.3.W, MRS-1B-1.C and MRS-1B-1.W) were Gram-positive rod shape bacteria, and PCA-3A.1.Y was Gram-positive coccus (Figure 1).

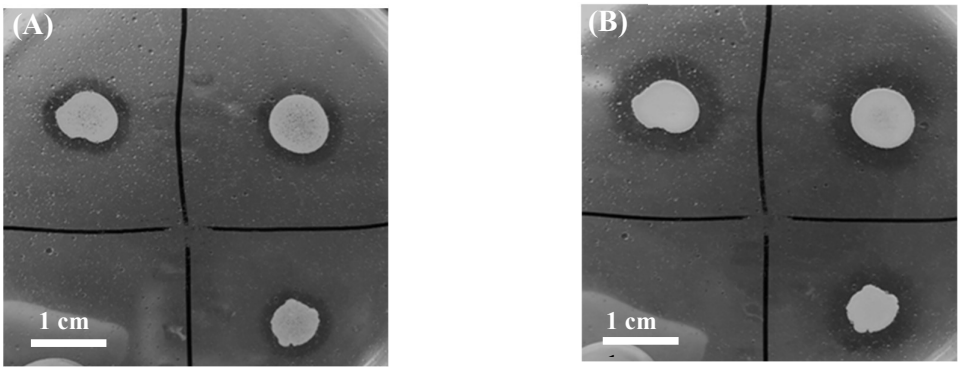

Figure 1 (A) Clear zone on TBA after 24 h incubation for isolate M9P8, and (B) clear zone on TBA after 48 h incubation for isolate M9P8.

Table 1 Gram staining, average clear zone diameter on tributyrin agar plate in cm and clear zone transparency of the twenty microbial strains.

Strain	Gram stain	Average clear zone (cm)	Transparency
MRS-1B-1.W	Gram + Long Rod	0.82 ± 0.09^{A}	+++
MRS-1B-1.C	Gram + Long Rod	0.68 ± 0.04^{B}	+++
MRS-1A.3.W	Gram + Long Rod	0.62 ± 0.04^{BC}	+++
PCA-3A.1.Y	Gram + Coccus	0.58 ± 0.04^{BCD}	+++
M9P8	Gram + Long Rod	0.54 ± 0.05^{CDE}	+++
PCA-2E.1	Gram - Filamentous	0.48 ± 0.03^{DEF}	++
GYE-3B	Gram + Rod shape	0.44 ± 0.03^{EFG}	++
M9P4	Yeast	0.40 ± 0.00^{FG}	++
PCP12	Gram + Long Rod	0.34 ± 0.05^{G}	++
M9P3	Gram + Rod shape	0.20 ± 0.00^{H}	+
M9P15	Yeast	0.20 ± 0.00^{H}	++
M9P20	Yeast	0.20 ± 0.00^{H}	+
PCP6	Yeast	0.20 ± 0.00^{H}	+
PCA-2G.1	Gram + Rod shape	0.20 ± 0.00^{H}	++
GYE-3D	Gram + Rod shape	N/G	+
PCP17	Gram + Rod shape	N/G	-
M9P5	Gram + Rod shape	N/G	-
PCA-2D	N/G	N/G	-
MRS-0B	N/G	N/G	-
MRS-1E-1	N/G	N/G	-

N/G indicates no growth. Transparency of clear zone, High (+++), Medium (++) and Low (+).
^{A,B,C,D,E,F,G,H} represent significant difference ($p > 0.05$).

3.2 Confirmation test of lipase production on tributyrin agar and chromogenic plate using the drop plate method

Tributyrin and phenol red chromogenic agar directly provided qualitative and quantitative results for the degradation of lipid substrates. The average number of bacteria inoculated was 1.72x 10^5 CFU/drop (0.01 mL). After 24 h, the highest lipase production was observed from MRS-1B-1.C strain with clear zone diameter of 1.35±0.03 cm. However, after 48 h significantly higher tributyrin hydrolysis was observed from isolate M9P8 with clear zone diameter of 1.66±0.04 cm (Table 2).

Table 2 Diameter of the clear zone after 24 and 48 h on tributyrin agar using the drop plate method with average cell number at 1.72 x 10^5 CFU/drop for the 5 selected strains.

Strain	24 h Clear zone diameter (cm)	48 h Clear zone diameter (cm)
M9P8	1.33±0.03[a]	1.66±0.04[a]
PCA-3A.1.Y	1.10±0.04[b]	1.29±0.07[c]
MRS-1A.3.W	1.33±0.03[a]	1.58±0.03[b]
MRS-1B-1.W	1.31±0.03[a]	1.55±0.03[b]
MRS-1B-1.C	1.35±0.03[a]	1.59±0.05[b]

[a,b,c] represent significant difference ($p > 0.05$).

3.3 Quantitative screening of lipase activity and determination of protein concentration

Lipase production of the bacterial isolates directly correlated to the biodegradation efficiency towards FOGs, which increased while the nutrients given decreased [12]. MRS-1B-1.W strain demonstrated significantly higher lipase production (5.89 U/mg) after 48 h incubation at 30°C with agitation at 120 rpm (Figure 2). While supernatant collected from M9P8 strain had highest protein concentration at 0.075 mg/mL (Table 3).

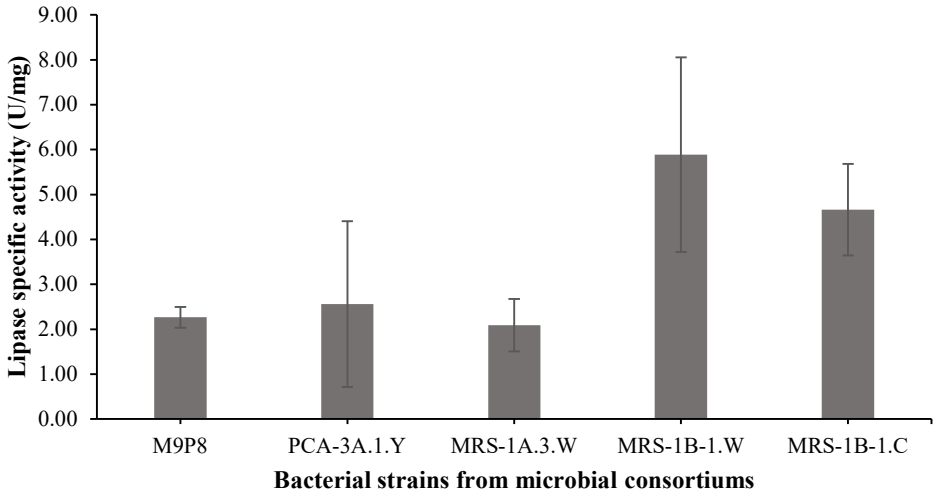

Figure 2 Enzyme specific activity after 48 h incubation using 5 selected isolates in 0.5X PCB.

Table 3 The average protein concentration of positive control with 5 selected isolates.

Strain	Average protein concentration (mg/mL)
Lipase	0.044±0.0028
M9P8	0.075±0.0043[a]
PCA-3A.1.Y	0.024±0.0042[c]
MRS-1A.3.W	0.064±0.0037[b]
MRS-1B-1.W	0.028±0.0051[c]
MRS-1B-1.C	0.031±0.0040[c]

[a,b,c] represent significant differences ($p > 0.05$).

3.4 Determination of palm oil reduction

Lipid reduction was tested using 0.5X PCB with 1% (v/v) lipid substrate. Tributyrin oil was used as reference lipid substrate for the comparison of the biodegradation efficiency in liquid medium. The highest reduction of 19.36% for tributyrin was demonstrated by isolate MRS-1B-1.C with no significant difference with the other

isolates. The highest lipid reduction (32.37%) was demonstrated by isolate PCA-3A.1.Y after 48 h incubation with 1% (v/v) palm oil without glucose at 30°C with agitation at 120 rpm. Reduction of palm in 0.5X PCB with 1% (v/v) palm oil and glucose after 48 h at 30°C with agitation at 120 rpm was found to be highest for isolate MRS-1B-1.C at 26.43% (Figure 3).

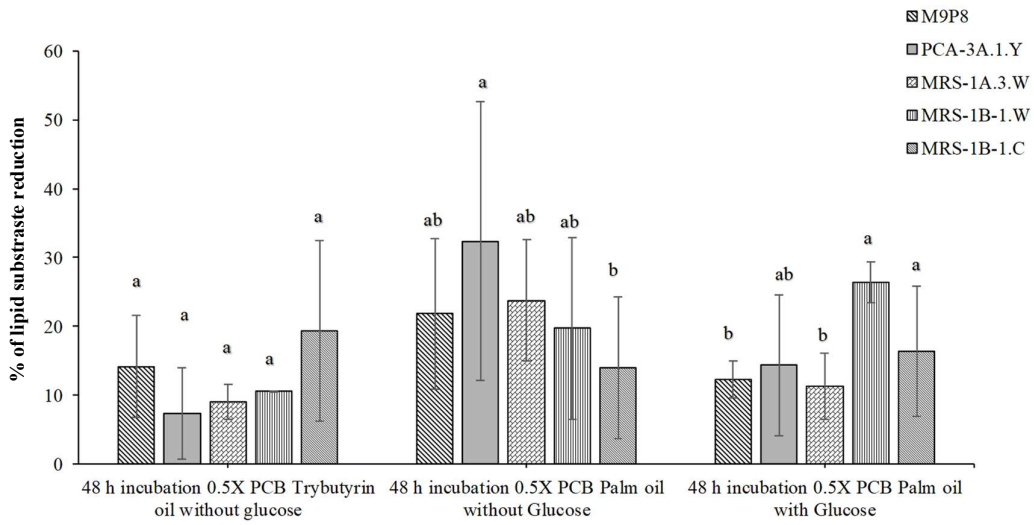

Figure 3 Percent reduction of tributyrin oil in 0.5X PCB without glucose and palm oil in 0.5X PCB with and without glucose after 48 h of incubation using 5 bacterial isolates (M9P8, PCA-3A.1.Y, MRS-1A.3.W, MRS-1B-1.C and MRS-1B-1.W) from microbial consortiums. $^{a, b, ab}$ represents significant differences ($p > 0.05$).

4. Discussion

In this study, twenty isolates of microorganisms from fermented microbial consortiums were primarily screened for their lipid hydrolysis activity on tributyrin agar plate. Five bacterial strains, namely M9P8, PCA-3A.1.Y, MRS-1A.3.W, MRS-1B-1.C and MRS-1B-1.W, had the largest clear zone on tributyrin agar and were chosen for further testing for the ability to degrade lipid substrates.

Lipolytic activity was confirmed using phenol red chromogenic plate method. Five selected bacterial isolates were able to degrade tributyrin oil and changed the color of the media to yellow. The change of colour indicated the production of free fatty acids, which lower the pH of the media [9]. In contrast, for phenol red chromogenic plate using palm oil, the five selected bacterial isolates did not show yellow coloration.

The crude enzymes from the supernatant of the five selected bacterial strains were used to determine lipases specific activity after 24 and 48 h. Strain MRS-1B-1.W, showed the highest lipases specific activity after 48 hours. Strains M9P8 and MRS-1A.3.W had higher protein concentration in the supernatant, which resulted in lower enzyme specific activities of lipases. The higher protein concentration could imply that there were other extracellular proteins that produced from these strains (Table 3). According to a previous study [7], isolate M9p5y had the best lipase activity production with 22.66 U/µg after 24 h. Bhumibhamon et al. [13], showed that the microorganisms isolated from soil and wastewater have lipase activity of 3-7 units, which is comparable with results from this study (Figure 2).

The lipid reduction experiment was conducted with individual bacterial strains M9P8, PCA-3A.1.Y, MRS-1A.3.W, MRS-1B-1.W, and MRS-1B-1.C (Figure 3). Theoretically, lipase producing strains will metabolize the most simplified substrates first. In the presence of tributyrin oil, the simplest triglyceride, the biodegradation efficiency should be greater than compared with long-chain fatty acids, such as those in palm oil [14]. However, the results of tributyrin oil in this research indicated otherwise. This contrary finding may be explained by the sinking of tributyrin oil to the bottom of the broth media (observed from the experiment). This resulted in different reaction conditions such that the lipase in the isolates instead catalyzed interesterification reactions rather than catalyzed the degradation reactions [13]. Consequently, the weight of tributyrin oil after extraction was not significantly reduced.

In the lipid reduction test, glucose was added to the palm oil (lipid substrate) to provide an additional carbon source for the bacteria. However, as reported by Gheethi [12], the presence of sugar, especially glucose, could cause catabolite repression in the medium, resulting in the inhibition of the production of some hydrolytic enzymes. In this work, without the presence of glucose in the media, strain PCA-3A.1.Y showed the highest degradation followed by MRS-1A.3.W. Previous observations of the growth in the liquid media showed that PCA-3A.1.Y

grew faster and could be producing greater amount of lipases. However, under the influence of glucose, the degradation of palm oil by strains MRS-1B.1.W and MRS-1B-1.C was increased, implying that the lipases activity of those two bacterial isolates were not repressed by glucose. The other three bacterial isolates (strains M9P8, PCA-3A.1.Y and MRS-1A.3.W) have decreased percentages in the reduction of lipid in the presence of glucose, which may have repressed the production of lipase in the medium. In previous work conducted by Charoenpanich and Kunathigan (unpublished work), the two bacterial strains (M9P16Y and PCP16Y) isolated from microbial consortiums were employed to degrade the palm oil in artificial wastewater. The result showed that strain M9P16Y and PCP16Y could reduced the amount of palm oil by 48.014% and 50.292% respectively after 48 h. In this study, the greatest percent reduction in palm oil was in without glucose and was lower than the results ontained by Charoenpanich [15]. Therefore, the experiments to test the bacterial strains used in this experiment should be further conducted in artificial wastewater to compare efficiency of these strains to reduce lipids to the efficiencies in previously published works.

It would be noted that the results of the percent lipid reduction had large variation among the replicates. The large variation might have been caused by the partition-gravimetric method which rely on the dry weight of lipid substrates that were left from degradation experiments. In addition, palm oil reduction efficiency should also be conducted using monoculture as well as the consortium of two or more bacteria to find the suitable bacteria or bacterial consortium that provide the most effective degradation of used palm oil in wastewater treatment.

5. Conclusion

Microbial lipases are lipolytic enzymes that have enormous potential in various applications. The versatility of lipases has been successfully employed in numerous biotechnology fields such as bioremediation or food and pharmaceutical industries. Results revealed that all five selected bacterial isolates (M9P8, PCA-3A.1.Y, MRS-1A.3.W, MRS-1B-1.W, and MRS-1B-1.C) out of twenty candidates from microbial consortiums were able to degrade palm oil. The highest lipase specific activity is 5.89±2.16 U/mg from isolate MRS-1B-1.W, and the lowest is 2.09±0.58 U/mg from isolate MRS-1A.3.W. The selected bacterial isolates could be used as potential candidates for the treatment of palm oil contaminated wastewater.

6. References

[1] Popoola B, Olanbiwoninu A, Fashogbon R. Bioremediation of vegetable oil contaminated soil with two microbial isolates. Adv Microbiol. 2022;12(04):218-241.

[2] Gawad HAE. Oil and grease removal from industrial wastewater using new utility approach. Adv Environ Chem. 2014;2014:1-6.

[3] Parthipan P, Preetham E, Machuca L, Rahman P, Murugan K, Rajasekar A. Biosurfactant and degradative enzymes mediated crude oil degradation by bacterium *Bacillus subtilis* A1. Front Microbiol. 2017;8:1-14.

[4] Javed S, Azeem F, Hussain S, Rasul I, Siddique M, Riaz M, et al. Bacterial lipases: a review on purification and characterization. Prog Biophys Mol Biol. 2018;132:23-34.

[5] Chandra P, Enespa, Singh R, Arora P. Microbial lipases and their industrial applications: a comprehensive review. Microb Cell Fact. 2020;19(1):169.

[6] Patel U, Chandpura J, Chauhan K, Gupte S. Screening and isolation of an organic solvent tolerant lipase producing bacteria from various oil contaminated sites. IJAM. 2018;21(01):22-36.

[7] Esmaeili N. Isolation and identification of palm oil and soybean oil degrading bacteria from bio-extract [thesis]. Samut Prakan: Assumption University; 2016.

[8] Bala J, Lalung J, Gheethi AAS, Kaizar H, Ismail N. Reduction of organic load and biodegradation of palm oil mill effluent by aerobic indigenous mixed microbial consortium isolated from palm oil mill effluent (POME). Water Conserv Sci Eng. 2018;3(3):139-156.

[9] Lanka S, Latha J. A short review on various screening methods to isolate potential lipase producers: lipases-the present and future enzymes of biotech industry. Int J Biochem. 2015;9(5):207-219.

[10] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-254.

[11] American Public Health Association (APHA). Standard methods for the examination of water and wastewater. 20th ed. Washington: APHA; 1999.

[12] Gheethi AAS. Recycling of sewage sludge as production medium for cellulase by a *Bacillus megaterium* strain. Int J Recycl Org Waste Agric. 2015;4(2):105-119.

[13] Bhumibhamon O, Koprasertsak A, Funthong S. Biotreatment of high fat and oil wastewater by lipase producing microorganisms. Agric Nat Resour. 2002;36(3):261-267.

[14] Elumalai P, Parthipan P, Huang M, Muthukumar B, Cheng L, Govarthanan M, et al. Enhanced biodegradation of hydrophobic organic pollutants by the bacterial consortium: impact of enzymes and biosurfactants. Environ Pollut. 2021;289:117956.

[15] Charoenpanich A. The study of lipase producing microorganisms from bio-extract for fat and lipid treatment in wastewater at laboratory scale. Student's report. Bangkok: Assumption University; 2016.