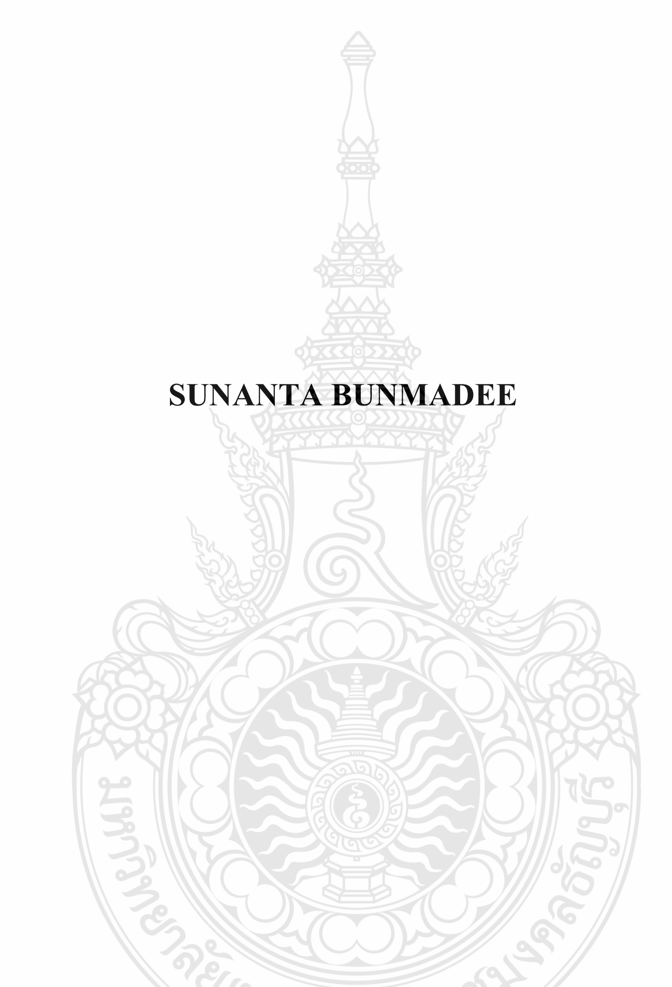
PRODUCTION OF LIPASE-PRODUCING INOCULUM FOR USING IN FAT WASTEWATER TREATMENT SYSTEM



SUNANTA BUNMADEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN APPLIED BIOLOGY

FACULTY OF SCIENCE AND TECHNOLOGY

RAJAMANGALA UNIVERSITY OF TECHNOLOGY THANYABURI

ACADEMIC YEAR 2022

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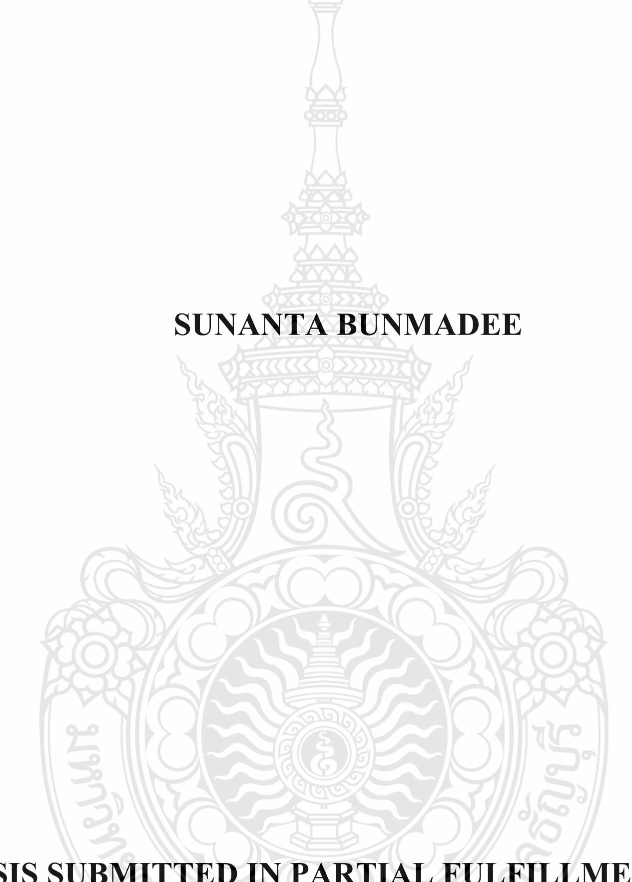

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Program	Applied Biology
Thesis Advisor	Assistant Professor Atsadawut Areesirisuk, Ph.D.
Academic Year	2022

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ABSTRACT

The objectives of this research were to: 1) screen, isolate, and identify the potential lipase-producing microorganisms from fat wastewater treatment pond at a poultry processing plant, 2) investigate the optimum condition of microbial culture for lipase production, and 3) examine the efficiency of lipase-producing inoculum for fat hydrolysis in wastewater.

First, the solid and liquid samples were collected from a wastewater treatment pond to primary screen lipase-producing microorganisms for culture in palm oil medium (PM) and Tween 80 agar. Then, titration and spectrophotometric methods were carried out for the quantitative screening of isolated lipase-producing strains. Their biosurfactant production was examined by the hemolytic method on sheep blood agar. The lipase-producing strains were identified by a molecular technique. Subsequently, Plackett-Burman Design (PBD) and Box-Behnken Design (BBD) were conducted to investigate the optimum condition of microbial culture for lipase production. Then, scaling up lipase production was performed in a 5-L bioreactor and examined the stability of lipase at different pH and temperatures. Finally, the efficiency of fat hydrolysis in wastewater through lipase-producing inoculum was evaluated in lab-scale and pilot-scale studies. In the lab-scale study, 1-5 % of *A. baumannii* RMUTT3S8-2 inoculum (liquid or powder form) was added to 10 L fat wastewater in 20 L tank and treated by a batch process. In the pilot-scale study, 5 % of *A. baumannii* RMUTT3S8-2 inoculum (powder in a gauze bag) was added to 150 L fat wastewater in 200 L tank and conducted by a continuous treatment process. Volatile fatty acid (VFA) obtained from fat hydrolysis was investigated by gas chromatography-mass spectrometry (GC-MS).

The study found that thirty-one isolated strains produced lipase in PM and Tween 80 agar. The top five isolated strains of lipase production were quantitatively examined by spectrophotometric analysis. The result showed that isolate no. RMUTT3S8-2 provided the significantly highest lipase activity of 97.43 ± 4.29 U/mL, followed by RMUTT2S3-2, RMUTT2S4-2, RMUTT3S8-3, and RMUTT3S5-1, and the biosurfactant production was not found in isolate no. RMUTT3S8-2. Furthermore, the isolate no. RMUTT3S8-2 was molecularly identified as *Acinetobacter baumannii* and chosen to study lipase production. The optimum lipase-producing condition of *A. baumannii* RMUTT3S8-2 was peptone 24.49 g/L, yeast extract 33.82 g/L, and NaCl 6.21 g/L. Under optimum conditions, the lipase activity of *A. baumannii* RMUTT3S8-2 was 216.23 ± 3.69 U/mL, which was higher than in unoptimized conditions by 2.2 times. Moreover, lipase production in the bioreactor was 16 % higher than in the flask scale. Lipase stability was above 95 % in pH value ranging from 5.0 to 9.0 at 30 °C. The efficiency of fat hydrolysis using lipase-producing inoculum was examined. It was found that the highest fat hydrolysis was 41.94 ± 4.98 % at 12 days with 5 % of the powder inoculum under a batch experiment, and the highest fat hydrolysis was 79 % at 22 h conducted by a continuous process. When the VFAs were investigated by GC-MS, it was found that acetic acid was the main product of VFA in the batch process, while acetic and propionic acids were the main products of VFA found in a continuous process. In addition, VFAs were the highest acids in the continuous process of fat hydrolysis using lipase-producing inoculum in a gauze bag.

Keywords: *Acinetobacter baumannii*, lipase, enzyme stability, fat wastewater, poultry processing factory

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List of Abbreviations

16S rDNA	16S Ribosomal Deoxyribonucleic Acid
16S rRNA	16S Ribosomal Ribonucleic Acid
3D	Three-Dimensional
ATP	Adenosine Triphosphate
BBD	Box-Benkekn Design
C	Carbon Atom
CCD	Central Composite Design
CCR	Carbon Catabolite Repression
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
ERAD	ER-Associated Protein Degradation
FA	Fatty Acid
FFA	Free Fatty Acid
FOG	Fat, Oil, and Grease
G3P	Glycerol-3-phosphate
GC-MS	Gas Chromatography-Mass Spectrometer
HRT	Hydraulic Retention Time
LI	Liquid Inoculum
MFP	Membrane Fusion Protein
NA	Nutrient Agar
NCBI	National Center for Biotechnology Information
PBD	Plackett-Burman Design
PCR	Polymerase Chain Reaction
PGBI	Powder in a Gauze Bag
PI	Powder Inoculum
R^2	Coefficient of Determination
RBC	Red Blood Cell

List of Abbreviations (Continued)

Sec	Secretory Pathway
SmF	Submerged Fermentation
SSF	Solid-state Fermentation
T1SS	Type I Secretion System
T2SS	Type II Secretion System
TAG	Triacylglycerol
Tat	Twin-Arginine Translocation Pathway
TVC	Total Viable Cell Count
VFA	Volatile Fatty Acid
YMA	Yeast Malt Agar
%	Percentage
°C	Degree Celsius
°C/min	Degree Celsius per Minute
g or RCF	Relative Centrifugal Force
g	Gram
g/bag	Gram per Bag
g/L	Gram per Liter
h	Hour
L	Liter
L/day	Liter per Day
LogCFU/g	Logarithm of Colony Forming Unit per Gram
LogCFU/mL	Logarithm of Colony Forming Unit per Milliliter
M	Molar
min	Minute
mL	Milliliter
mL/min	Milliliter per Minute
mV/sec	Millivolt per Second
N	Normal
rpm	Revolutions per Minute

List of Abbreviations (Continued)

U/g	Unit per Gram
U/mg	Unit per Milligram
U/mL	Unit per Milliliter
v/v	Volume per Volume
vvm	Vessel Volume per Minute
w/v	Weight per Volume
μm	Micrometer
μmol	Micromole
μmol/mL	Micromole per Milliliter

CHAPTER 1
INTRODUCTION

1.1 Background and Statement of the Problems

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a group of esterases that catalyze the hydrolysis of triglycerides (TGs), releasing fatty acids (FAs) and glycerol [1, 2]. Lipases trigger reactions that generate esters from glycerol and long-chain FAs, such as esterification, interesterification, transesterification, and alcoholysis [3]. Lipases have recently become very attractive for various industrial applications due to their diverse enzymatic properties in detergents, textiles, food additives, pharmaceuticals, agrochemicals, oil biodegradation, and waste treatment [4, 5].

Lipases are naturally produced in plants, animals, and microorganisms [6]. In contrast, microbial lipases are most in demand due to their broad environmental stability, cheap production cost, ease of large-scale production, and activation under mild conditions. Moreover, microbial lipase production is seasonally independent compared with lipase production from animals and plants [2, 7]. For these reasons, microbial lipases are now widely used in various industrial applications such as biofuels, cosmetics, detergents, leather, pharmaceuticals, and antifungals in agricultural products [7, 8].

Fats, oils, and grease (FOG) are relevant components in domestic and food industry wastewater, resulting in severe environmental impacts. They form a natural floating layer or surface film, blocking oxygen diffusion from the air into water, thereby reducing the effectiveness of aerobic reactions in biological treatment processes and impacting the growth of many aquatic plants and organisms [9]. Floating lipid waste is usually removed from wastewater for sanitary disposal. However, the remaining lipid residue negatively impacts oxygen transfer rates and blocks water drainage pipelines of the wastewater treatment system. Lipid waste disposal management and treatment have become urgent practical challenges.

Fat waste accumulates within the grease trap pond of a poultry processing factory (CPF (Thailand) Public Co., Ltd., Saraburi Province), causing clogging of the

fat waste in the wastewater treatment system. The capacity of the wastewater treatment pond and the efficiency of the wastewater treatment system are reduced. The oxygen transfer into the system is diminished, and fat waste decomposes in an anaerobic condition, releasing an unpleasant smell. The fat waste must be excavated from the system every 3 to 6 months, which causes at least an average cost of 0.5-1 million baht/year approximately, to decrease this problem.

Although previous studies have reported that several lipase-producing species, such as *Thalassospira permensis* M35-15, *Bacillus aryabhattai* SE3-PB, *Burkholderia ubonensis* SL-4, *Pseudomonas formosensis* TB5, and *Acinetobacter* sp. AU07 can be used for oil degradation and related substances [1, 5, 10–12]. However, the applications of lipase-producing inoculum are necessary to consider its growth and lipase performance. The inoculum must be able to grow in cultured systems without acclimatization. Furthermore, it should provide high lipase activity and stability in a realistic environment. Thus, considering these statements, the effective lipase-producing strains present in oily wastewater treatment systems of a poultry processing factory were isolated and molecularly identified in this research. Then, the lipase-producing condition was optimized using response surface methodology (RSM) and lipase stability was investigated. Additionally, the effectiveness of the lipase-producing inoculum for oil hydrolysis in actual wastewater was examined.

1.2 Purpose of the Study

In this thesis, the purpose of the study is to extend the concept of the previous works and to generalize new concepts which are:

1.2.1 To screen and isolate the high potential lipase-producing microorganisms from the oily industrial wastewater treatment system of a poultry processing factory.

1.2.2 To explore the optimum condition for lipase-producing microorganism cultivation and its stability.

1.2.3 To investigate the efficiency of lipase-producing microorganisms for oil hydrolysis in the oily industrial wastewater from a poultry processing factory.

1.3 Scope of Thesis

The scope of this research is to examine the screening and isolation of potential lipase-producing strains from oily wastewater treatment using qualitative and quantitative methods. Then, the potential lipase-producing strains are molecularly identified and create the phylogenetic tree. The media compositions for lipase production are investigated and optimized by Plackett-Burman design (PBD) and Box-Benkekn design (BBD). The optimal media compositions obtained from RSM are validated and carried out to produce lipase in a 5-L bioreactor. Subsequently, the lipase stability is examined at different pH and temperatures. The effect of lipase-producing inoculum types and quantity on oil degradation in the oily industrial wastewater from a poultry processing factory is examined in a 20-L batch process with a 10-L working volume. Finally, on a pilot scale, the oil hydrolysis in the oily industrial wastewater using a continuous fermentation process in the 150-L working volume in the 200-L plastic tank.

1.4 Expectation of Thesis

This thesis, I have the scope and limitations of studying which are concerned to the previous works which are:

1.4.1 Obtained the high potential lipase-producing microorganisms from the oily industrial wastewater treatment system of a poultry processing factory.

1.4.2 Known the optimum condition for lipase-producing microorganism cultivation and its stability.

1.4.3 Known the efficiency of lipase-producing microorganisms for oil hydrolysis in oily industrial wastewater system from a poultry processing factory.

CHAPTER 2
LITERATURE REVIEWS

2.1 Lipases

Lipases (triacylglycerol hydrolases EC 3.1.1.3) hydrolyze ester bonds of triacylglycerols (TAGs) to monoglycerides, diglycerides, glycerol, and FAs at the oil-water interface or aqueous conditions as shown in Figure 2.1. In non-aqueous medium conditions, they can synthesize esters from glycerol and long-chain FAs (reverse reaction), including esterification and transesterification reactions (Figure 2.2). Therefore, lipases perform the hydrolysis and synthesis of the TAG [13, 14].

$$\text{Triglyceride} + 3\,H_2O \xrightleftharpoons{\text{Catalyst}} \text{Fatty Acids} + \text{Glycerol}$$

Figure 2.1 Lipase hydrolysis of TAG to glycerol and FAs.
Source: Gunawan et al. 2019 [15]

1. Hydrolysis

$$R_1COOH_2 + H_2O \xrightarrow{\text{Hydrolysis}} R_1COOH + R_2OH$$

2. Esterification

$$R_1COOH + R_2OH \xrightarrow{\text{Esterification}} R_1COOH_2 + H_2O$$

3. Transesterification

$$R_1COOH_2 + R_3COOR_4 \xrightarrow{\text{Interesterification}} R_3COOH_2 + R_1COOR_4 \quad \text{(a)}$$

$$R_1COOR_2 + R_3COOH \xrightarrow{\text{Acidolysis}} R_3COOR_2 + R_1COOH \quad \text{(b)}$$

$$R_1COOR_2 + R_3OH \xrightarrow{\text{Alcoholysis}} R_1COOR_3 + R_2OH \quad \text{(c)}$$

$$R_1COOR_2 + R_3NH_2 \xrightarrow{\text{Aminolysis}} R_1CONHR_3 + R_2OH \quad \text{(d)}$$

Figure 2.2 Types of reactions catalyzed hydrolysis and synthesis esters by lipase.
Source: modified from Szymczak et al. 2021 [16]

2.2 Classification of Lipase

2.2.1 Based on sources

Lipases are produced by several plants, animals, and microorganisms (Figure 2.3). Among various lipases, microbial lipases are considerably more attention than lipases from plants and animals because of their diversity in catalytic activity, seasonal changes independent production, high yield, and low-cost production, as well as the relative ease of genetic manipulation [17, 18]. Generally, most lipase-producing microbes are found in the environment, especially oil-containing water and soil (Table 2.1). Bacteria, fungi, and yeast are microorganisms recognized as the potential synthesis of lipases. Microorganism lipase has been found in extracellular and

intracellular lipase depending on strains and nutrition medium of the cultivation [19]. However, extracellular lipase was used commercially and in several industries.

2.2.1.1 Fungi lipases

Fungal strains are known to be potential lipase producers with remarkable unique catalytic properties, which are very important to various commercial applications [20]. Most commercially and industrially important lipase-producing fungi belong to the genera of *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., and *Mucor* sp. [20–22]. Lipase production by each fungus differs according to the strain and composition of the growth medium, such as carbon and nitrogen sources [20]. Lipase-producing fungal strains have been found in different habitats, such as dairy waste-contaminated soil, jatropha press cake, dairy effluent, soil contaminated with diesel oil, and oil [23–26].

2.2.1.2 Yeast lipases

Lipase produced from yeast has unique applications in chemical, pharmaceutical, and biodiesel-producing industries [27]. Some of the essential lipase-producing yeasts are recognized as belonging to the genera of *Candida utilis*, *Candida rugosa*, *Rhodotorula* sp., *Yerrowia* sp., and *Pichia* sp. are the best and primary lipase producers [28–31]. According to the literature, *Candida* sp. is the most efficient lipase producer among other yeast strains. Their biochemical, structural, and catalytic features have been extensively characterized [32]. Lipase-producing yeast strains have been found in various environments, such as the dairy industry, oil-contaminated soil, spoilt coconuts, nuts, and vegetables, the vegetable oil refining industry, the cheese factory, palm oil mill effluent, and olive mill wastewater [33–38].

2.2.1.3 Bacteria lipases

Several extracellular lipase-producing bacteria are well-known, *Pseudomonas aeruginosa,* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Serratia marcescens*, *Acinetobacter* sp., *Achromobacter* sp., *Burkholderia multivorans*, *Burkholderia cepacia*, and *Staphylococcus caseolyticus* [13, 39, 40]. Both Gram-positive and Gram-negative bacterial strains have been discovered to produce lipase [41]. They have been exhibited in several habitats, including tannery effluent,

waste cooking oil, saline environments, wastewater treatment plants, paper industries, and oil-contaminated soil [28, 41–46].

2.2.2 Based on the specificity

The lipase has been identified based on specificity and divided into three groups, substrate-specific, regioselective, and enantioselective (Figure 2.3).

2.2.2.1 Substrate-selective lipases

These lipases promote the synthesis of the desired product by selectively catalyzing on only one substrate in a mixture of crude raw materials. FAs and alcohols are substrates that activate these lipase activities during biodiesel synthesis. [16, 47].

2.2.2.2 Regioselective lipases (regiospecific lipase)

a) Non-specific lipases; the position of the lipase on TG molecules is non-specific. These non-specific lipases can completely break down TAG molecules into glycerol and free fatty acids (FFAs) (Figure 2.4A). However, during the reaction may be established monoglycerides and diglycerides compound [48]. *C. rugosa* is a yeast that produces lipase in this group [49].

b) 1,3-specific lipases (*sn*-1, 3 positions) hydrolyze ester bonds at C-1 and C-3 of TAGs releasing fatty acids, 2 monoacylglycerols, and 1,3 or 2,3 diacylglycerols (Figure 2.4B). However, the carbon position of FAs shift occurs while the reaction is unstable; theirs are switched from the C-2 position to 1,3-diacylglycerol and 1,3-monoacylglycerols. Thus, the hydrolysis at the second ester bond was not found in this group. Compared to nonspecific lipases, 1,3-specific lipases increase the hydrolysis of TG molecules into monoglycerides more quickly [47]. 1,3-specific lipase has been reported in *Streptomyces violascens* ATCC 27968, *Rhizopus arrhizus*, and *Rhizomucor miehei* [50, 51].

c) 2-specific lipases (*sn*-2 positions) catalyze the selective hydrolyze of FAs at the C-2 position of the glycerol backbone of a TAGs molecule (Figure 2.4C). The 2-specific lipase is relatively scarce in lipase-producing microorganisms. However, lipase produced from *Geotrichum candidum* could hydrolyze oleic and linoleic acids at the C-2 position of the TAGs [47].

d) Fatty acid-specific lipases; these lipases exhibit FA selectivity; there are hydrolyze of esters at cis-double bonds position between C-9 and C-10 in long-chain FAs [18]. Even though most lipase-producing microorganisms entirely lack this characteristic, *G. candidum*, *Penicillium citrinum*, *A. niger*, and *Aspergillus oryzae* are demonstrated to catalyze the hydrolysis of FA-specific lipase [47].

2.2.2.3 Enantioselective lipases (enantiospecific lipase)

These lipases can hydrolyze one of the isomers of a racemate and differentiate enantiomers in a racemic mixture. In addition, the enantio-specificity lipases may differ depending on the substrate and related to the ester chemical characteristics [52]. Recently, lipase from *Enterobacter cloacae* has exhibited an enantioselective preference for S-enantiomer in drugs for pharmaceutical applications [19].

Table 2.1 Sources of microbial lipases.

Microbial sources	Samples/Place of habitats	Lipase activity	References
Fungal species			
Aspergillus aculeatus	Dairy waste-contaminated soil samples	9.51 U/mL	[25]
Aspergillus niger	Oil sample from vegetable oil distils factory	19.84 U/g	[53]
Aspergillus flavus strain O-8	Dairy effluent and diesel oil-contaminated soil	3.04 U/mL	[54]
Yeast species			
Magnusiomyces capitatus	Olive mill wastewater	3.96 U/mL	[33]
Meyerozyma guilliermondii	Slaughterhouse fridge effluent and oil mill effluent	285.82 U/mL	[37]
Rhodotorula glutinis HL25	Soil samples	54.40 U/L	[55]
Bacteria species			
Pseudomonas beteli	Soil samples from different oil rich environments	6.24 U/mL	[56]
Acinetobacter sp. UBT1	Soil samples from Petrol pump at Anand, Gujarat, India	291.29 U/mL	[57]
Pseudomonas helmanticensis HS6	Soil samples (altitude ranging from 2500-4272 m above sea level), Sikkim, India	179.30 U/mg	[58]

Table 2.1 Sources of microbial lipases (Cont.).

Microbial sources	Samples/Place of habitats	Lipase activity	References
P. aeuriginosa	Soil sample from a mechanic's workshop	528.54 U/L	[59]
Nocardiopsis sp. NRC/WN5	High salinity and alkalinity environments	50.11 U/mL	[60]
Burkholderia sp.	Soil sample from the disposal area of waste vegetal oil	18.7 U/mL	[61]
Xanthomonas oryzae pv. oryzae	Soil samples from different location of Hubei, China	373.9 U/mg	[62]

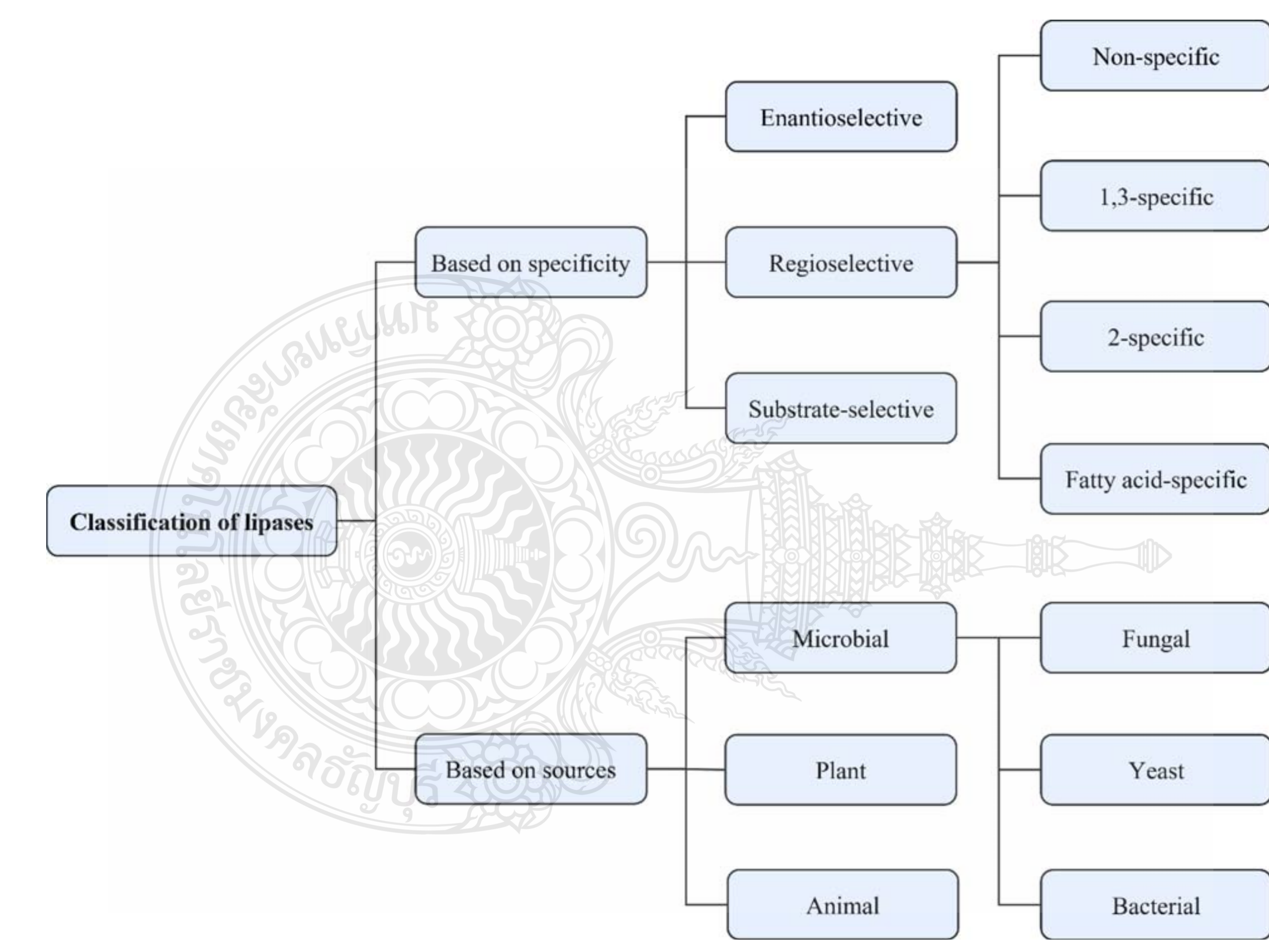

Figure 2.3 Classification of lipases.

Source: modified from Lopez Fernandez et al. 2020 [63]

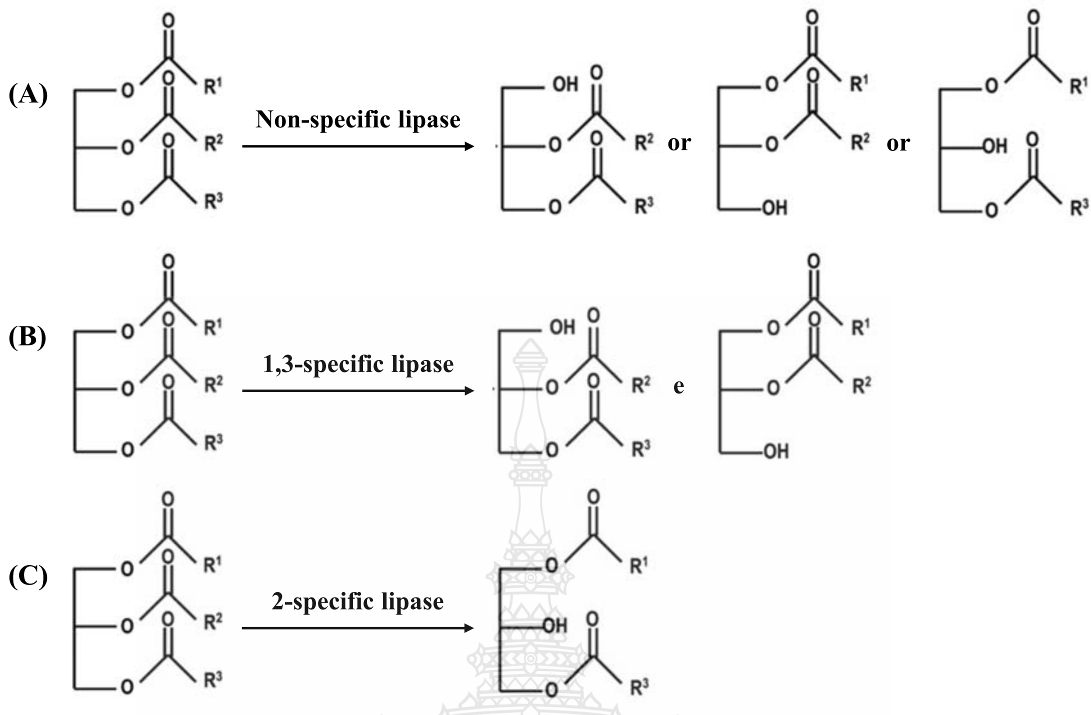

Figure 2.4 Regioselective lipases: (A) non-specific, (B) 1,3-specific, and (C) 2-specific lipases catalyze the hydrolysis of triglycerides in different manners.
Source: modified from Albayati et al. 2020 and Barros et al. 2010 [52, 64]

2.3 Mechanism of Microbial Lipase Producers

Lipases have a process referred to as interfacial activation, a unique catalytic mechanism as they characterize the presence of a mobile sub-domain as a polypeptide chain called a lid or flap, which helps the lipase stay in equilibrium in a homogeneous medium [65, 66]. The lid has two functions open and close. The lipase structure can shift between an open (active site) or closed (inactive site) conformation depending on the lid movement, as shown in Figure 2.5. The substrate molecule can enter the enzyme active site (catalytic center) in the open lid form, which is present with the oil substrate. In the case of aqueous substrates, the closed lid form blocks the substrate molecule from entering the active site of the enzyme. Even though the conformational change allows activation at an oil-water interface, this process does not apply to all lipases [67, 68].

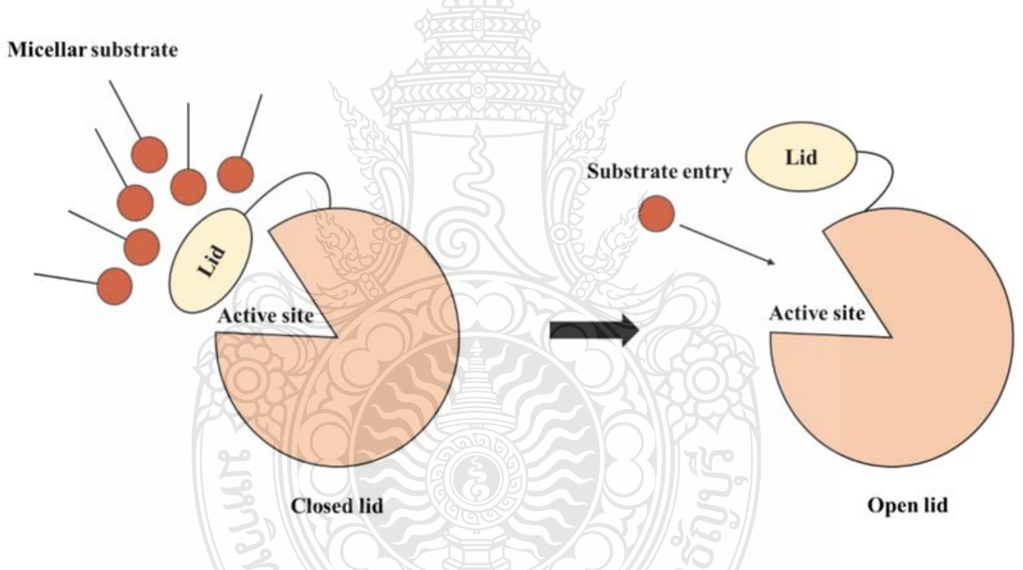

Figure 2.5 Lid structure form of lipase
Source: De Luca and Mandrich, 2020 [67]

2.4 Secretion System of Microbial Lipases

2.4.1 Lipases secretion system of Gram-negative bacteria

2.4.1.1 Type I secretion system (T1SS)

T1SS is the mechanism of the transporter to external. The protein-exporter to extracellular of T1SS consists of three envelope protein subunits.

ATP-binding cassette (ABC) protein is an inner membrane-bound, provides energy to transport the lipases, and interacts with the membrane fusion protein (MFP). The MFP in the periplasm functions in conjunction with membrane transporters (inner and outer membranes). Outer membrane protein (OMP) is anchored in the outer membrane and creates channels in the outer membrane to assist lipases (unfold state) across to extracellular. Most subfamilies I.3 of actual lipase have used this secretion pathway [69, 70].

2.4.1.2 Type II secretion system (T2SS)

T2SS is a general secretory pathway (GSP). These excreted channels are only found in the outer membrane. The secretion has two stages. First, lipases are secreted by transportation via the inner membrane using the secretory pathway (*Sec*) or the twin-arginine translocation pathway (*Tat*). The N-terminal signal sequence separates the secretory pathway between *Sec* and *Tat* genes. The unfolded lipases show in the Sec pathway. In contrast, the Tat pathway exhibited folded lipases. Finally, lipases are folded in the periplasmic space before export through the outer membrane by T2SS. The secretion system is often employed by the subfamilies I.1 and I.2 of actual lipase bacteria (Gram-negative) for transporting the lipase through the cell membrane, such as *P. aeruginosa*, *Aeromonas hydrophila*, and *Burholderia thailandensis* [69–72].

2.4.2 Lipases secretion system of Gram-positive bacteria

The lipases secreted by Gram-positive bacteria are performed by passive diffusion via the peptidoglycan layer to the extracellular environment with the Tat and Sec pathways. These transports (*Tat* and *Sec*) are inadequate to deliver lipases to extracellular because lipases secreted stay entrenched in the cell wall. However, more needs to be reported on the lipase secretion system in Gram-positive bacteria [73].

2.4.3 Lipases secretion system of yeast and fungal

The lipase secretion pathway in yeast and fungi involves three main steps (Figure 2.6). First, the polypeptides are transported from the ribosome to the endoplasmic reticulum (ER) via co-translocation or post-translational translocation. In this step, the lipases are folded and modified in ER, which requires the assistance of signal sequence processing, disulfide bond formation, N-glycosylation, degradation, and

sorting. Subsequently, the properly folded lipases are directed to the Golgi apparatus for further modification. Finally, lipases are secreted to the extracellular environment, vacuoles, or other related organelles. On the other hand, the misfolded lipases are led to the cytosol and destructed by the ER-associated protein degradation (ERAD). The ERAD proteolysis probably occurs from the partially misfolded lipases that activate the unfolded protein response (UPR) [74].

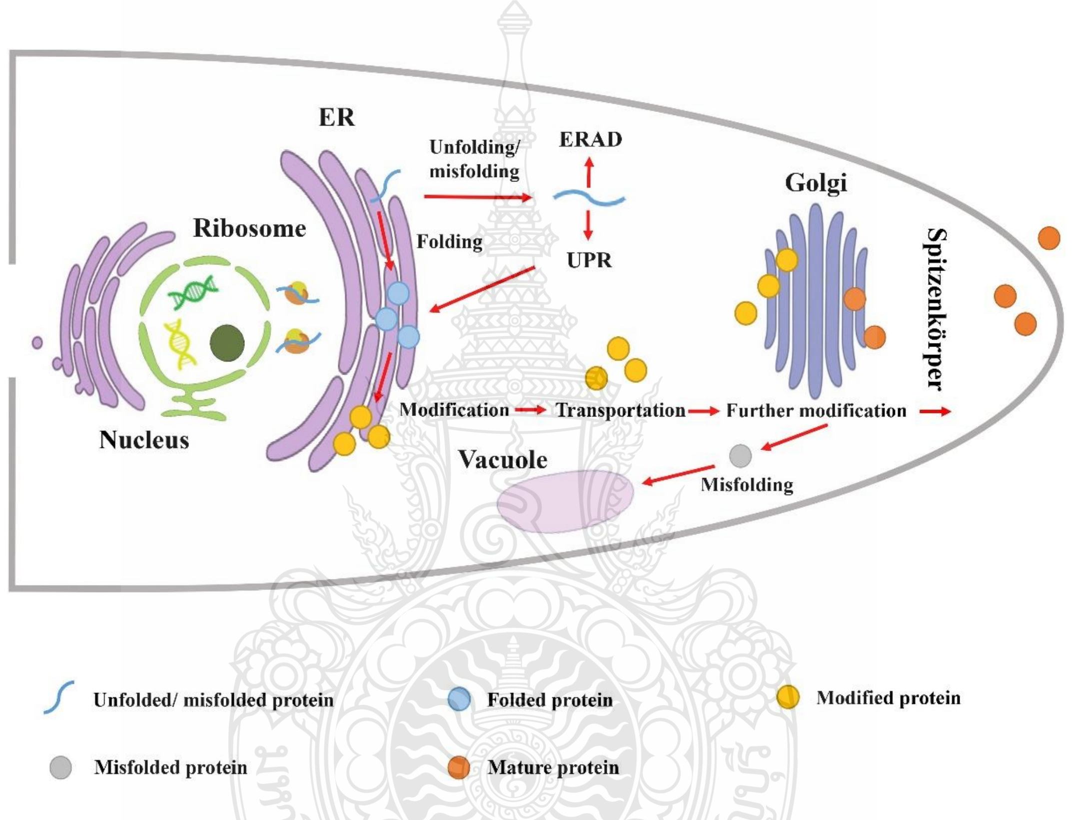

Figure 2.6 Protein secretion pathway in yeast and fungi
Source: Wang et al. 2020 [75]

2.5 Mechanism of TAG Degradation by Microbial Lipase

Microbial lipases are excreted to extracellular and catalytic the hydrolysis of the ester bond of TAG between the glycerol hydroxyl group and the carboxyl group of FAs. However, TAG degradation may occur in 2 cases: TAG degradation releases glycerol and FAs, completely exhibiting hydrolysis. On the other hand, in the case of incomplete cleavage, FAs, and monoglycerides or diglycerides were obtained.

2.5.1 Glycerol degradation in bacteria

Glycerol is transported into the cell by a transporter protein called glycerol facilitator protein (*GlpF*), which activates diffusion across the inner membrane of bacteria. The glycerol metabolism is divided into two dissimilation pathways: phosphorylation and dehydrogenation (Figure 2.7). a) Phosphorylation pathway: glycerol is phosphorylated to sn-glycerol-3-phosphate (G3P) by glycerol kinase (*GlpK*). Then G3P-oxidizing enzymes, namely aerobic glycerol-3-phosphate dehydrogenase (*GlpD*) and anaerobic glycerol-3-phosphate dehydrogenase (*GlpABC*) oxidized G3P and dihydroxyacetone phosphate (DHAP) is formed. b) Dehydrogenation pathway: glycerol dehydrogenase (*GldA*) oxidized glycerol to dihydroxyacetone (DHA). Subsequently, DHA is phosphorylated to DHAP by dihydroxyacetone kinase (*DhaKLM*) employs phosphoenolpyruvate as the phosphoryl donor. However, both phosphorylation and dehydrogenation pathways provide DHAP as the final product. Afterward, DHAP is metabolized in the glycolysis pathway is the next step (downstream metabolism) [76, 77].

2.5.2 FA degradation in bacteria

The mechanism of fatty acids degradation in bacteria following: extracellular long-chain fatty acids (carbon source) bind to the transporter protein (*FadL*), causing a conformational shift that allows fatty acid diffusion into the periplasm. Then the delivery from the periplasm to the cytosol by *FadD* and the employ of the ATP for the acyl-CoA ester formation are simultaneous. Intracellular fatty acid degradation occurs through the four steps of the β-oxidation pathway (Figure 2.8). In the first step, the acyl-CoA dehydrogenase (*FadE*) conversed acyl-CoA to enoyl-CoA, and $FADH_2$ is the product obtained. Then enoyl-CoA is changed into β-hydroxyacyl-CoA through adds H_2O at the double bond by enoyl-CoA hydratase (*FadB*). Subsequently, β-hydroxyacyl-CoA is oxidized to β-ketoacyl-CoA via β-hydroxyacyl-CoA dehydrogenase (*FadB*). In the last step, β-ketothiolase (acetyl-CoA acyltransferase) (*FadA*) is catalyzed to cleave β-ketoacyl-CoA, and acetyl-CoA and an acyl-CoA (reduced by two carbon atoms) are formed. The shortened fatty acyl-CoA is transferred to the β-oxidation pathway and repeats these four steps until all the carbons in the initial fatty acyl-CoA are converted to acetyl-CoA. On the other hand, the

degradation of unsaturated fatty acids, 2,4-dienoyl-CoA reductase (*FadH*), transforms 2,4-dienolyl-CoA derived from unsaturated fatty acids into enoyl-CoA. Then it entered the cycle at the stage of the hydration [78, 79].

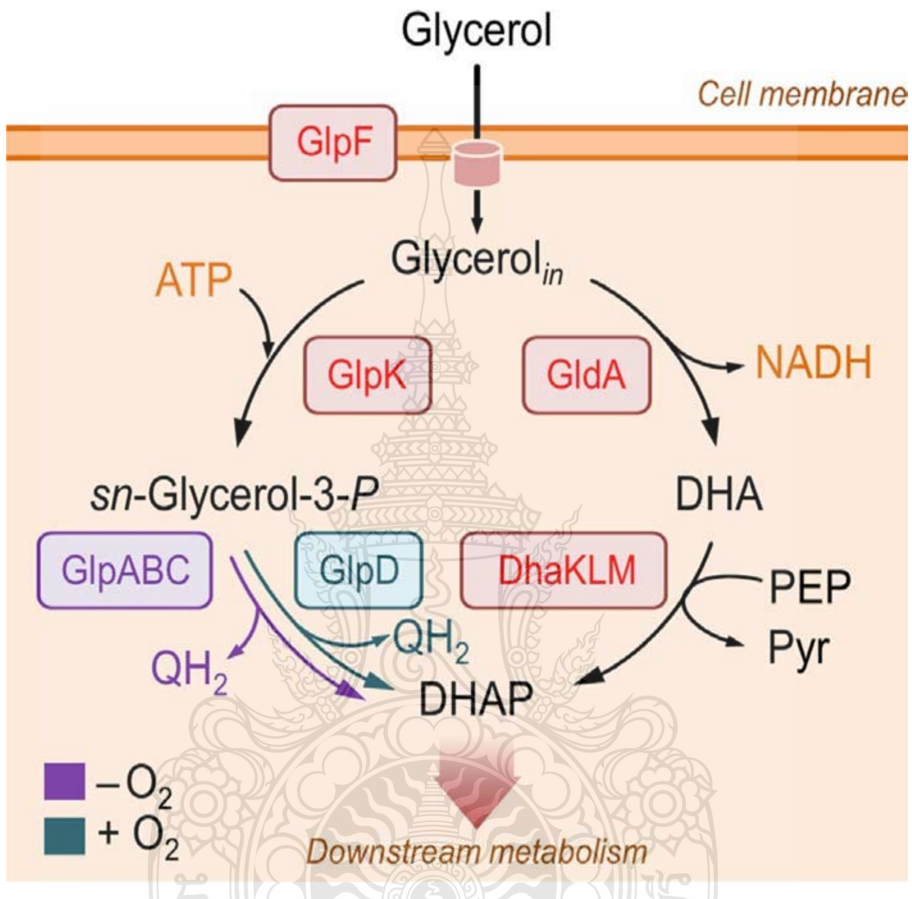

Figure 2.7 Glycerol degradation pathways and their associated enzymes.
Source: Poblete-Castro et al. 2020 [76]

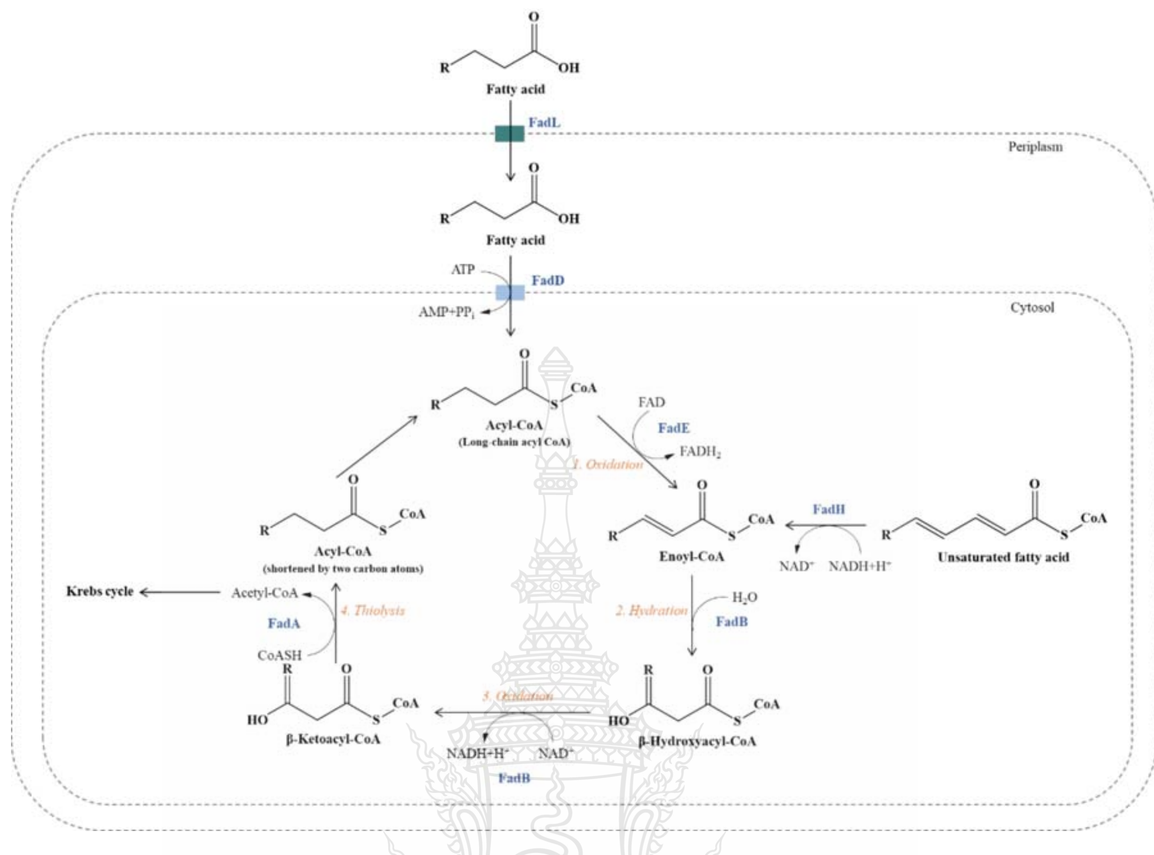

Figure 2.8 Schematic representation of the fatty acid degradation pathway.

Source: Modified from Fujita et al. 2007 and Janßen and Steinbüchel 2014 [78, 79]

2.6 Biosurfactant

Biosurfactants are amphipathic compounds formed by a hydrophilic (positive, negative, or amphoteric charged ions) and hydrophobic (long-chain FAs) component, as shown in Figure 2.9 [80]. These characteristics can reduce the surface tension and promote the emulsion of immiscible liquids, such as reducing the interfacial tension at the oil–water interface [81]. The microbial surfactants also offer low toxicity and high biodegradability, stability, and functionality under various extreme conditions of pH, temperature, and salinity [82, 83].

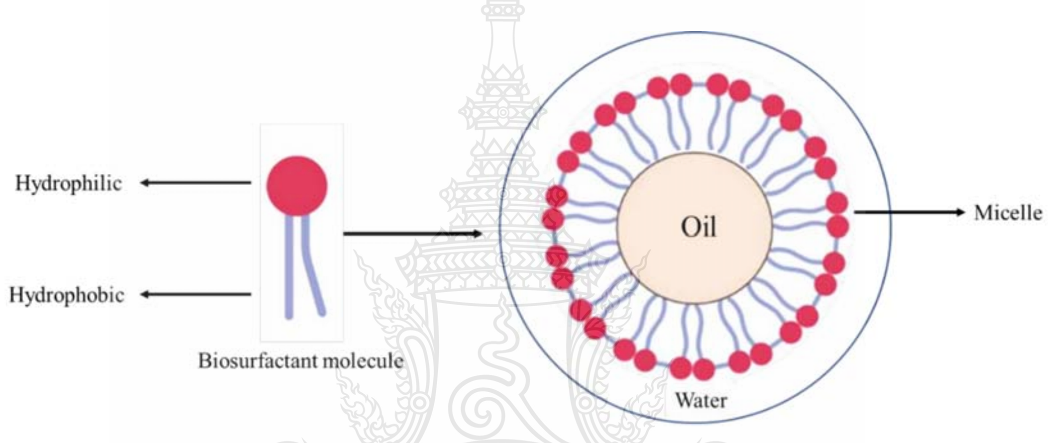

Figure 2.9 Biosurfactant molecules and the actions of the biosurfactant molecule at the water-oil interface result in the creation of micelles.
Source: Modified from Sharma et al. 2022 [84].

Generally, several microorganisms can synthesize biosurfactants, including bacteria, yeasts, and filamentous fungi [85]. The biosurfactant molecules can either be secreted into the environment or attached to the cell surface [86]. The main classifications of biosurfactants include their microbial origin, chemical composition, critical micelle concentration, mode of action, and molecular weight. According to their molecular weight, biosurfactants can be divided into two groups: (a) low molecular weight, such as glycolipids, phospholipids, FAs, neutral lipids, and lipopeptides; and (b) high molecular weight, such as polysaccharides, lipopolysaccharides, and a complex mixture of biopolymers [85, 87, 88]. When microorganisms need to metabolize the

insoluble compounds in water, they synthesize lipases and biosurfactants. Thus, lipase and biosurfactant secretion can increase the oil uptake of microorganisms [89, 90]. Numerous microorganisms which produce the lipases and biosurfactants have been reported, including *Ochrobactrum intermedium* strain MZV101, *Serratia* sp. ZS6, and *Burkholderia* sp. [61, 90, 91].

2.7 Factors Affecting Growth and Lipase Production

Several factors which significantly impact the synthesis of lipase have been reported, especially carbon sources, nitrogen sources, inducers, pH, temperature, minerals, aeration, and agitation.

2.7.1 Carbon sources and inducers

Carbon sources are important substrates for the energy production of microbes. Oils are an essential carbon source and inducer for lipase synthesis [92]. Olive oil, vegetable oil, palm oil, corn oil, coconut oil, cotton seed oil, and other oils have been used as carbon sources and inducers for microbial growth and lipase production to obtain a high-yield [41, 93]. Furthermore, glucose, fructose, sucrose, malt extract, starch, molasses, and glucose syrup were used as carbon sources for lipase production [94].

2.7.2 Nitrogen sources

Nitrogen sources supply amino acids, vitamins, cofactors, and trace metals and encourage the growth and metabolism of bacteria which increases bacterial cells and resulted in lipase production [95, 96]. Among organic nitrogen sources, yeast extract was the most suitable substrate for maximum lipase production [97]. However, peptone, tryptone, beef extract, soya peptone, and casein peptone were also used as organic nitrogen sources for lipase production [97, 98]. While several inorganic nitrogen sources have been used for lipase production, including NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, KNO_3, and N_2NO_3 [95, 98].

2.7.3 pH

Generally, the pH of cultural medium influences the growth and product formation of microorganisms. Neutral and alkaline pH conditions increase lipase synthesis in bacteria and yeast. In contrast, the acidic pH conditions enhance lipase

synthesis in lipase-producing fungi [41]. However, microorganisms grow at different pHs depending on the species. They possess stability over a wide range from pH 4.0–11.0 [99]. *Acinetobacter calcoaceticus* 1–7 showed the highest lipase activity at pH 9 and stability at a wide range of pH 4–10 [100], while *Pichia* sp. strain RT presented suitable stability in pH 7.0–10.0 [101]. Whereas *Acinetobacter haemolyticus* lipase retained more than 90 % of its activity at pH 5–11 [102]. However, *A. niger* GZUF36 demonstrated maximum activity at pH 4 and a wide range of pH stability at 3–10 [103]. Thus, lipase-producing microorganisms could be stable in a wide range of pH, suitable for application in industries.

2.7.4 Temperature

The temperature necessary for lipase synthesis generally corresponds to the growth conditions and product formation [92]. The temperature ranges for lipase production of microorganisms have been reported. The optimal temperature for lipase production of microbial lipase was mainly presented at 30-40 °C (mesophilic microorganism), such as *Yarrowia lipolytica* (38 °C) and *Staphylococcus caprae* NCU S6 (40 °C) [104, 105]. According to their biological properties, proteins are usually unstable and denature when exposed to extreme conditions, such as excessively high or low temperatures [106]. However, some strains of microorganisms could also grow, synthesize lipase, and have stability at extreme temperatures. The psychrotrophic lipase *Pseudomonas* sp. CRBC1 could grow and increase lipase production at 20 °C [107]. While the optimum temperature of thermophilic lipase *P. helmanticensis* HS6 and *Bacillus atrophaeus* FSHM2 at 50 and 70 °C, respectively [58, 108].

2.7.5 Mineral sources

Minerals are among the critical elements for microbial growth and lipase synthesis. Minerals are utilized to supply the ATP metabolism for microbial growth [109]. They are related to enzyme function since their change or maintain the structure, attaching them to specific places on their surfaces [3]. The most common minerals employed for microbial lipase production are K_2HPO_4, NaCl, $MgSO_4$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $CaCl_2$, $CaCO_3$, $MnSO_4$, $ZnSO_4$, $NH_4H_2PO_4$, KCl, $MgCl_2$, $NaNO_3$, and NaH_2PO_4 [110, 111]. Previous research reported that the mineral salt NaCl could increase the growth rate of *Pseudomonas* sp. ISTPL3 [112]. Whereas the lipase

production of *Bacillus cereus* was encouraged with $MgCl_2 \cdot 6H_2O$, $CaCl_2$, NaCl, $MnCl_2 \cdot 4H_2O$, KCl, $CuCl_2$, and $FeCl_3 \cdot 6H_2O$ containing production medium [113]. Although minerals are essential for the growth and production of lipase, however, excessive amounts of minerals could decrease the lipase synthesis [114].

2.7.6 Aeration and agitation

Aeration and agitation enhanced the oxygen transfer rate and raised the effectiveness of evenly distributing nutrients in the medium [115]. Especially in bioreactors, aeration and agitation improved growth rate and lipase synthesis. The lipase activity obtained in the aerated bioreactor is higher than in the shaking flask due to sufficient oxygen supply for growth and lipase production [116]. The practical aeration and agitation speeds are operated from 0.5-2.0 vvm and 50-400 rpm, respectively [56, 117, 118]. Nevertheless, more agitation speed leads to higher shear force, which may negatively influence cell growth and lipase production [118].

2.7.7 Inoculum size

The inoculum size is associated with the density of the microbe in the initial period of cultivation, which affects lipase synthesis. However, excessive inoculum concentration refers to the large density of microorganisms resulting in rapid depletion of the nutrients available in culture media, often leading to oxygen and decreased lipase activity. However, the aforementioned behavior could not impact lipase production in all microbial strains [119, 120]. The previous study has denoted that *Geobacillus stearothermophilus* FMR12 produces the highest lipase at an inoculum concentration of 2 % v/v (from 0.25-10 %), and the over-low or high concentrations reduced lipase activity [121]. While *Pseudomonas yamanorum* LP2 exhibited the most excellent lipase synthesis for 3 % at 1-5 % of inoculum size [122].

2.8 Experimental Designs

The statistical method is used in experimental design to determine the optimum process response and media composition for lipase synthesis by predicting the interactions between various variables and advising in reducing the number of trials [123]. The PBD is the statistical experimental design employed for screening the essential variables that affect the response variable. Types of variables of PBD; (a)

independent variables are actual and dummy variables, including media composition, which is carried out at low and high levels; (b) dependent variables which are impacted by independent variables of the experiment. Furthermore, the RSM was one of the mathematical and statical methods used for exploring the relationships between several explanatory independent variables and one or more response variables. The experimental of RSM, such as artificial neural network (ANN), central composite design (CCD), and BBD, differs in the experimental design technique [124]. The BBD is widely used to estimate the response to the linear and quadratic model and interactive effects between the independent and dependent variables [125]. BBD consists of two or more variables. The BBD is produced at the center points of the edges of the experimental area. Each variable is determined at three different interactive levels, which are low (−), center (0), and high (+) levels (Figure 2.10) [126, 127]. Moreover, the BBD is an effective technique to optimize the important variable obtained by PBD [128]. The PBD and BBD have been applied to investigate the effect of media composition and physical condition on growth and lipase production in previous research. The lipase synthesis of *P. aeruginosa* FW_SH-1 was increased 13.7 fold when optimizing by PBD and BBD [129]. Ktata et al. [98] presented that after optimization by BBD, lipase activity *of Aeribacillus pallidus strain* VP3 increased 2.83-fold compared with the unoptimization condition.

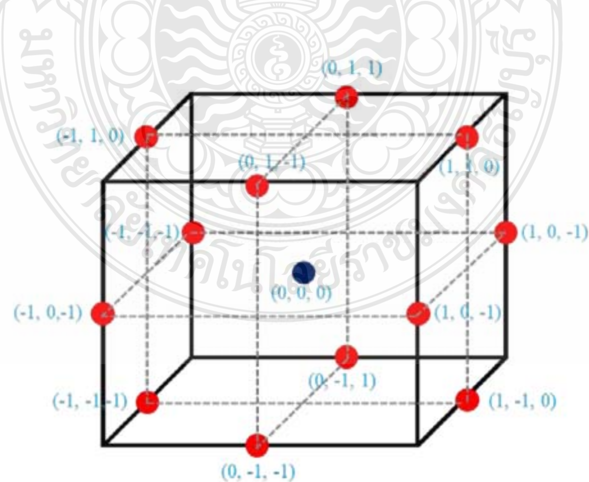

Figure 2.10 Structure of BBD for experiments.
Source: Modified from Mohammed Breig and Luti, 2021[130]

2.9 Production Process and Fermentation Technique

The common production process used for cultivating lipase-producing microorganisms was carried out in batch, repeated-batch, fed-batch, and continuous modes with solid-state (SSF) and submerged fermentation (SmF) fields [40]. SSF is the microbial cultivation process using a moisturized solid substrate under specific conditions. The general solid substrates used in SSF are renewable agricultural, including grains, soybean meal, rice husk, and wheat bran [87, 131]. In addition, they provided both surface area and a source of nutrients for microbial growth [2]. Conversely, SmF is a fermentation process in liquid media containing soluble nutrients, including organic and inorganic chemical agents [131]. However, SSF and SmF have different advantages. SSF provides low production costs, low water utilization, and reduced agricultural residues. At the same time, SmF promotes the nutrient absorption of microorganisms distributed evenly in liquid media and easy monitoring and control [132, 133].

2.10 Application of Lipases

Microbial lipases are attractive enzymes for industrial applications. They demonstrated the role of catalytic activity at extreme conditions, broad substrate specificity and stereochemistry, stability in organic solvents, ease of mass production, high yield, and affordable production [134]. Generally, microbial lipases have been extensively utilized in different industries, including biodiesel, cosmetic, detergent, food, pharmaceutical, leather, textile, paper, and wastewater treatment industries (Table 2.2) [13, 135].

2.10.1 Biodiesel industry

Lipases displayed thermostability and tolerance to the short-chain alcohol, which is acceptable for biodiesel production. Biodiesel production using lipase as the catalyze can reduce the production cost, minimize environmental pollution, decrease production waste, and possibly use non-edible vegetable oil as the raw materials. In the biodiesel industry, both free and immobilized lipases are used for biodiesel formation. The commercial lipases from *Pseudomonas fluorescens* (lipase

AsK, Amano), *Thermomyces lanuginosus* (lipase LA201, Lipopan 50BG, Novozymes), *A. niger* CALB (Novozymes) are widely used to produce the biodiesel [13].

2.10.2 Cosmetic industry

Lipase-catalyzed esterification produces wax esters, cinnamic acid, ellagic acid, ferulic acid, flavor and perfume compounds, precursors of pharmaceuticals, and additives in cosmetics and sunblock. Lipases are used as the base of supplemental food and diet supplements. In addition, lipases are coated as a part of a medicine capsule to aid in quickly assimilating the medicine from the digestive system [47].

2.10.3 Detergent industry

Lipase-containing products decrease or substitute synthetic detergents in the household laundry. In detergent industries, they reduce the use of chemicals based on phosphate in detergent compositions. The cold-active lipases lower the temperature of the wash, lower the wear on the cloth from oil and fat, and conserve energy. They are environment-friendly used. Lipases produced by *Pseudomonas alcaligenes* (named Lipomax) and *Pseudomonas mendocina* (named Lumafast) are utilized as commercial detergent enzymes [26, 136, 137].

2.10.4 Food industry

Lipases are extensively used in food processing, baking, bakery, juices, fermentation, and milk production. They serve as the catalytic hydrolysis of milk fat. Lipase is used to modify the fat and oil properties in food processing to improve product qualities such as the flavor of cream, bread, cheese, and milk aroma. Lipase contributes to producing wines, baked foods, emulsifiers, supplements, and dairy products. Moreover, the specificity and selectivity of lipase ease the synthesis of many food and bakery products [47, 136, 138].

2.10.5 Medical and pharmaceutical industry

The enantioselective properties of lipases are widely used in the medical and pharmaceutical industries. Lipase-catalyze hydrolysis and esterification of racemic esters to optically pure enantiomers (chiral compound) relate to use as a raw material in medical and pharmaceutical. *Candida antarctica* and *C. rugosa* lipases stimulated the profens production to create nonsteroidal anti-inflammatory drugs. Thus, enantioselective lipases promote the hydrolysis of racemic esters for producing pure

drug ingredients, profens, and drug intermediates. In addition, it prevents drug spread loss during flow-drug delivery [47, 136].

2.10.6 Wastewater treatment industry

Lipase is one of the important enzymes for industrial oily wastewater treatment. Numerous industries, including the dairy industry, the oil mill industry, the tannery industry, the automobile industry, and the wastewater from restaurants, currently employ lipases to treat wastewater. It is used to hydrolyze the TAG in those wastewaters. They are employed in both aerobic and anaerobic wastewater treatment processes [47]. This topic is the main of this research, it should be explained more. Adding how to use, applications in various wastewater treatment, etc.

Table 2.2 Microbial lipase for industrial application.

Applications	Functions	Microbial sources	References
1. Biofuel	Lipase performs transesterification of TG and esterification of FFA for biodiesel production.	*C. antarctica*	[139]
	Lipase immobilized magnetic nanoparticle for biodiesel production.	*A. niger*	[140]
2. Cosmetic	Lipase-mediated transesterification for the synthesis of unsaturated fatty acid ergosterol esters from plants oil.	*Proteus vulgaris* K80	[141]
3. Detergent	Ingredient of the detergent formulation and facilitating cold washing.	*Penicilium canesense* *Pseudogymnoascus roseus*	[26]
	Purified lipase for a laundry additive product.	*Bacillus methylotrophicus* PS3	[142]
4. Food	The biocatalyst for flavor and wax ester synthesis.	*Y. lipolytica* IMUFRJ 50682	[104]
	Encourage the dough structure, increase bread volume and reduced the residual water activity, attenuating the hardening process of the breadcrumb.	*Fusarium oxysporum*	[143]
	Development flavor and promote proteolysis and lipolysis in Chinese dry-fermented sausages.	*Lactobacillus plantarum* *Staphylococcus xylosus*	[144]

Table 2.2 Microbial lipase for industrial application (Cont.).

Applications	Functions	Microbial sources	References
5. Medical and Pharmaceutical	The resolution of racemic alcohols and drug intermediates.	*P. beteli*	[56]
6. Wastewater treatment	Pretreatment of industrial effluents contaminated with oil and fat.	*Bacillus* sp. VITL8	[145]
	The $CaCO_3$-immobilized lipase biocatalyst for degradation of oil in wastewater treatment.	*Bacillus stearothermophilus*	[146]
	Reducing the oil loading in textile industry effluent.	*Nocardiopsis alba*	[147]

2.11 Reviews of the Literature

Verma et al. [148] used the statistical approach design to optimize media composition for the bioremediation of crude oil by *P. aeruginosa* SL-72. PBD experiments showed Tween-80, $(NH_4)_2HPO_4$, and $MgSO_4 \cdot 7H_2O$ were the most parameters that affect lipase synthesis, cell growth, and microbial activity. The three ingredients were optimized using CCD. The optimization of media composition for lipase synthesis was 0.5 % Tween-80, 1.0 % $(NH_4)_2HPO_4$, and 0.1 % $MgSO_4 \cdot 7H_2O$. The optimization using the statistical design promotes a lipase production increase of 3.68-fold (1,376.60 U/mL) compared with unoptimized. Furthermore, *P. aeruginosa* SL-72 exhibited crude oil hydrolysis of 82.83 % in 7 days.

Zarinviarsagh et al. [91] examined the lipase and biosurfactant synthesis of bacteria isolated from washing powder. The isolated no. MZV101 showed the highest lipase activity and biosurfactant production. It was identified using 16S rDNA gene sequencing and named *O. intermedium* strain MZV101. The result demonstrated that the lipase and biosurfactant were stable in a wide range of pH and temperature. Both activities have maintained at 60 – 80 %, respectively. Furthermore, it was found that the lipase activity exhibited high stability at pH 10 – 13 and a temperature of 70 – 90 °C. The biosurfactant showed high stability at pH 9 – 13 and thermostability in a broad range of 10 – 90 °C. Combining detergent and buffer, lipase, and biosurfactant of *O. intermedium* strain MZV101 could reduce oil removal from white cotton by 82.33 % compared with the without biosurfactant.

Hu et al. [149] investigated the characterization of alkaline lipase produced by *P. aeruginosa* HFE733, which was isolated from samples of domestic waste. Then, its lipase was used for oil degradation in food wastewater treatment. The results demonstrated that the lipase exhibited optimum activity at 40 °C and pH 8.5. The enzyme stability remained higher than 70 % of its activity at pH 7.0 – 8.5 after incubation for 4 h. In addition, *P. aeruginosa* HFE733 lipase could remove the oil in food wastewater for 95.44 % in 6 days.

Balaji et al. [145] investigated the optimization of lipase production and application in the pretreatment of food industry effluent of halotolerant *Bacillus* sp. VITL8. RSM was conducted to optimization of the chemical and physical variables on

lipase synthesis were olive oil (6 % v/v), peptone (0.7 %), Tween 80 (0.9 %), and incubation time (25 h), respectively. *Bacillus* sp. VITL8 demonstrated the highest lipase synthesis of 325.0 ± 1.4 U/mL and increased 2.2-fold under optimization. They also could degrade initial oil and fat in food industry effluent, namely dairy, bakery, and poultry more than 50 % on a laboratory scale.

Ilesanmi et al. [59] studied the screening of lipase-producing bacteria from oil-contaminated soil and optimizing lipase production. The highest effective strain was screened from the soil sample and identified as *Pseudomonas aeuriginosa*. The *P. aeuriginosa* provided lipase activity of 99.69 U/L by primary screening on agar plate method and qualitative secondary by spectrophotometer method, respectively. Under optimum conditions, lipase activity was increased to 528.54 U/L, which was higher than the unoptimized condition at 5.3-fold.

Patel et al. [7] presented the optimization of lipase synthesis from *P. aeruginosa* UKHL1 for application in oily waste-water treatment. *P. aeruginosa* UKHL1 strain was screened from the oil mill dumping site. One factor at a time approach (OFAT) was used to investigate the effect of parameters on lipase production. The BBD was further carried out to optimize lipase production via RSM. Statistical design of experiments exhibited that the lipase secretion of *P. aeruginosa* UKHL1 could be enhanced 3.91-fold under optimized conditions. In addition, the *P. aeruginosa* UKHL1 showed that the oil hydrolysis in oily synthetic waste was 37 % within 72 h.

Phukon et al. [58] have screened the lipase-producing strain from soil samples in the Sikkim area for use in the detergent industry. The result informed that the highest lipase activity was provided for 66 U/mL by isolate no. HS6. The strain was identified by 16S rRNA as *P. helmanticensis* HS6. After optimization, the result showed that the lipase activity was enhanced from 2.3 to 179.3 U/mg. *P. helmanticensis* HS6 also exhibited the maximum lipase production at pH 7 and 50 °C. Moreover, the lipase activity remained for 40 – 80 % in commercial detergents at 5 °C and 30 °C.

Rmili et al. [150] explored the characterization and optimization parameters for lipase secretion of *Staphylococcus capitis* SH6 using a statistical experimental design. Among all variables, tryptone, malt extract, NaCl, and pH exhibited positive signals in PBD. The BBD was then carried out to optimize the critical variables. The

optimum lipase production was tryptone 25 g/L without adding the soy peptone and K_2HPO_4. Under optimum conditions, the maximum lipase secretion of *S. capitis* SH6 was exhibited for 42 U/mL. Furthermore, the lipase provided the maximum activity at pH 9.5.

Banoth et al. [56] have studied the isolation and identification of lipase-producing microorganisms for drug intermediate. The result represented that only one in 16 lipase-producing strains exhibited selectively converted three racemic alcohols. The isolated strain was identified by 16S rRNA gene sequencing as *Pseudomonas beteli*. Furthermore, the maximum lipase activity was obtained at 30 °C and pH 6, whereas the optimal time course for cell growth and lipase production were 72 and 96 h, respectively. Moreover, the lipase production in the bioreactor scale demonstrated that cell growth and lipase synthesis were increased by 2.75 and 3.4-fold compared to the shaking flask scale.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Instruments

3.1.1.1 Air pump (HS-120, Submersible pump, Japan)

3.1.1.2 Air pump (PS-1530, PUMA, Taiwan)

3.1.1.3 Air stone

3.1.1.4 Alcohol Burner (NP CHEM, Thailand)

3.1.1.5 Amber reagent Bottle (Pyrex, Germany)

3.1.1.6 Autoclave (NB-1080, N-BIOTEK, Korea)

3.1.1.7 Beaker (Pyrex, Germany)

3.1.1.8 Bioreactor (10 Liter) (FS-07, Winpact, Taiwan (ROC))

3.1.1.9 Bioreactor (5 Liter) (MS-F1, Major Science, Taiwan (ROC))

3.1.1.10 Biosafety cabinet (Labculture Class II Type A2, Esco, Singapore)

3.1.1.11 Burette glass class A PTFE stopcock (Qualicolor, Thailand)

3.1.1.12 Centrifuge (3-18KS, Sigma, UK)

3.1.1.13 Centrifuge tube (Biologix, Thailand)

3.1.1.14 Cylinder (Isolab, Germany)

3.1.1.15 Digital balance (SARTORIUS, Generation BP 2105, Germany)

3.1.1.16 Digital dry cabinet (WEIFO, Thailand)

3.1.1.17 Digital pH meter (HI-98127, Hanna, Italy)

3.1.1.18 Erlenmeyer flask (Pyrex, Germany)

3.1.1.19 Freezer box

3.1.1.20 Fume hood (FLEXLAB, Thailand)

3.1.1.21 GC-MS (QP2010SE/AOC-20I, Shimadzu, Japan)

3.1.1.22 Hot air oven (FD240, Binder, Germany)

3.1.1.23 Laboratory bottle (Ilmabor, Germany)

3.1.1.24 Loop

3.1.1.25 Magnetic stirrer bar

3.1.1.26 Magnetic stirrer with hot plate (MA-1827F, Thermo Scientific, Thailand)

3.1.1.27 Micropipette, Size 1,000 mL, 5,000 mL (Pipette man, Gilson, France)

3.1.1.28 Microplate reader (EZ Read 2000, Biochrom, UK)

3.1.1.29 Microscope (Olympus, Japan)

3.1.1.30 Microscope slide and glass cover slips (Sail, Thailand)

3.1.1.31 Microwave (iwave, LG, Thailand)

3.1.1.32 pH meter (ST3100-F, OHAUS, USA)

3.1.1.33 Pipette tips (Gilson Pipetman, France)

3.1.1.34 Plastic tank size 200-L

3.1.1.35 Plastic tank size 20-L

3.1.1.36 Precision balance 2 digits (SPX2202, OHAUS, USA)

3.1.1.37 Precision balance 4 digits (OHAUS, USA)

3.1.1.38 Shaker and incubator (NB-205VL, N-BIOTEK, South Korea)

3.1.1.39 Shaker and incubator (NB-205VQ, N-BIOTEK, South Korea)

3.1.1.40 Shaker and incubator (WIS-10R, WiseCube, Germany)

3.1.1.41 Silicone tube

3.1.1.42 Spreader glass (S4647, Thailand)

3.1.1.43 Sterilized petri dish plastic (Hycon, Thailand)

3.1.1.44 Syringe (Nipro, Thailand)

3.1.1.45 Syringe filters (SY1345NN, National Scientific, Chaina)

3.1.1.46 Tank (Superstar, Thailand)

3.1.1.47 Test tube with lid (Pyrex, Germany)

3.1.1.48 Vial tube (C4013-1, National Scientific, Chaina)

3.1.1.49 Volumetric flask (Pyrex, Germany)

3.1.1.50 Vortex mixture (Scientific Industries, Generation G560E)

3.1.1.51 Water bath (WNB14, Memmert, Germany)

3.1.1.52 Well plate (96 well) (Nunclon delta surface)

3.1.1.53 White filter cloth

3.1.2 Chemical reagents and culture media

3.1.2.1 Acetone (C_3H_6O) (Merck)

3.1.2.2 Agar (SRL)

3.1.2.3 Ammonium sulfate ($(NH_4)_2SO_4$) (UNIVAR)

3.1.2.4 Beef extract (Himedia)

3.1.2.5 Blood agar base (Himedia)

3.1.2.6 Bromocresol purple ($C_{12}H_{16}Br_2O_5S$) (Labchem)

3.1.2.7 Calcium chloride dehydrate ($CaC_{l2}\cdot 2H_2O$) (UNIVAR)

3.1.2.8 Chloroform ($CHCl_3$) (RCI Labscan)

3.1.2.9 Citric acid ($C_6H_{10}O_8\cdot H_2O$) (KEMAUS)

3.1.2.10 Crystal violet ($C_{25}H_{30}C_lN_3$) (Panreac)

3.1.2.11 dibasic sodium phosphate ($Na_2HPO_4.2H_2O$) (KEMAUS)

3.1.2.12 di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4\cdot 12H_2O$) (BDH)

3.1.2.13 Ethanol (C_2H_5OH) (CHEMI)

3.1.2.14 Ferrous Sulfate Heptahydrate ($FeSO_4\cdot 7H_2O$) (RANKEM)

3.1.2.15 Glucose ($C_6H_{12}O_6$) (SRL)

3.1.2.16 Glycerol ($C_3H_8O_3$) (UNIVAR)

3.1.2.17 Hydrochloric acid (HCl) (QRëC)

3.1.2.18 Iodine (I_2) (Ajax)

3.1.2.19 Magnesium sulfate heptahydrate ($MgSO_4\cdot 7H_2O$) (UNIVAR)

3.1.2.20 Malt extract (SRL)

3.1.2.21 Methanol (CH_3OH) (RCI Labscan)

3.1.2.22 Olive oil (Bertolli, Thailand)

3.1.2.23 Palm oil (Morakot, Thailand)

3.1.2.24 Peptone (SRL)

3.1.2.25 Phenolphthalein ($C_{20}H_{14}O_4$) (BDH)

3.1.2.26 Plate count agar (Himedia)

3.1.2.27 *p*-Nitrophenol (*p*-NP) ($C_6H_5NO_3$) (Alfa Aesar)

3.1.2.28 *p*-Nitrophenyl palmitate (*p*-NPP) ($C_{22}H_{35}NO_4$)

3.1.2.29 Polyvinyl alcohol $[CH_2CH(OH)]_n$ (Chem-supply)

3.1.2.30 Potassium dihydrogen orthophosphate (K_2HPO_4) (UNIVAR)

3.1.2.31 Potassium dihydrogen phthalate ($C_6H_4COOHCOOK$) (KEMAUS)

3.1.2.32 Safranin O ($C_{20}H_{19}ClN_4$) (Panreac)

3.1.2.33 Sodium carbonate (Na_2CO_3) (KEMAUS)

3.1.2.34 Sodium chloride (NaCl) (SRL)

3.1.2.35 Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) (Ajax)

3.1.2.36 Sodium hydroxide (NaOH) (UNIVAR)

3.1.2.37 Soybean meal

3.1.2.38 Tributyrin ($C_{15}H_{26}O_6$) (Acros)

3.1.2.39 Tris (hydroxymethyl) aminomethane hydrochloride ($C_4H_{11}NO_3.HCl$) (SRL)

3.1.2.40 Tris hydroxymethyl methylamine ($C_4H_{11}NO_3$) (UNIVAR)

3.1.2.41 Tryptone (Himedia)

3.1.2.42 Tween-80 (Polysorbate 80) (Chem-supply)

3.1.2.43 Yeast extract (IYEAST)

3.2 Methods

3.2.1 Sample collection and area

Wastewater and sludge from oil-separating treatment ponds in the poultry processing industry (CPF (Thailand) PLC., Saraburi Chicken Processing Industry, Thailand) were aseptically collected and kept in sterilized bottles (Figure 3.1). The samples were preserved at 4 °C until the isolation of lipase-producing bacteria was operated.

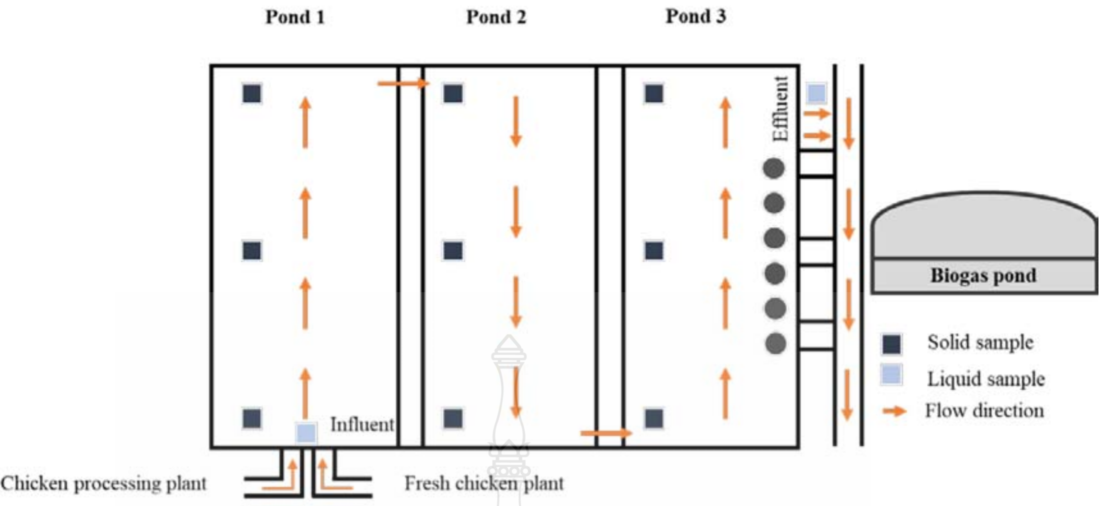

Figure 3.1 Sample collection and sampling area of fat wastewater in the grease trap tank.

3.3.2 Primary screening

3.3.2.1 Screening of qualitative lipase-producing microorganisms on an agar plate

Screening lipase producers was a modified method from Chigusa et al. [151]. Briefly, 5.0 % of sample from oil-separating treatment ponds was transferred to 100 mL of palm oil (PM) broth medium for enrichment. The cultivation was incubated at 35 °C, 200 rpm for 120 h in a shaking incubator (WIS-10R, WiseCube, Germany). The enriched culture was ten-fold serially diluted with sterile 0.85 % w/v of NaCl solution and spread on Tween 80 and PM agar [59, 151], respectively. The agar plates were incubated at 35 °C for 2 and 5-7 days, respectively. The observed colony, either a turbid zone surrounding the colony on the Tween 80 agar plates or changed the color of the medium from purple to yellow-orange on PM agar plates, was selected as a positive strain and examined the lipase production by titration technique.

3.3.2.2 Morphological characterization of lipase-producing microorganisms

All the positive strain colonies were observed morphology characteristics on the nutrient agar (NA) or yeast malt agar (YMA) plate after being cultured at 35 °C for 24-48 h. The Gram staining technique was used to identify the

bacterial cell wall structure. The bacteria and yeast cell shapes were observed under a light microscope (Olympus, Japan).

3.3.2.3 The master stock of microorganisms lipase producers

The positive strains were sub-cultured in NA or YMA by the streak plate technique to purify the strains. The purified strain was kept as master stock. One loopful of each pure strain was then transferred to nutrient broth (NB) or yeast malt broth (YMB) (for bacteria and yeast, respectively) and incubated at 35 °C, 200 rpm for 24 h. Subsequently, 0.8 mL of culture was added with 0.2 mL 75 % v/v sterilized glycerol, mixed, and kept at -80 °C as a master stock.

3.3.3 Secondary screening

3.3.3.1 Screening of quantitative lipase-producing

The titration method was used to determine the screening of preliminary lipase activity. Thirty-one lipase-producing strains from master stock were activated in NB or YMB and incubated at 35 °C, 200 rpm, for 24 h in a shaking incubator. A 10 mL cultured strain was transferred to 100 mL lipase production medium (pH 6.5) and incubated at the previous condition for 120 h. The culture was collected and centrifuged (3-18KS, Sigma, UK) at 10,000 rpm (11068 × *g*), for 10 min, at 4 °C to remove the cells, with cell-free supernatant used as crude lipase. After that, the crude lipase was examined for lipase activity by titration [152].

3.3.3.2 Selection of lipase-producing bacteria

The top five lipase-producing bacteria from the preliminary determination were investigated for lipase production by spectrophotometry, according to Gurkok and Ozdal [3]. The strain from the master stock was cultured in NB and incubated at 35 °C, 200 rpm, for 24 h in a shaking incubator. Then, 10 mL of activated culture was transferred to 100 mL of lipase production medium and incubated to the above condition for 120 h [153]. The culture was collected and centrifuged (2-16PK, Sigma, UK) at 8,500 rpm (7,673 × *g*), for 10 min, at 4 °C. The supernatant was used to determine lipase activity by a spectrophotometer. The lipase-producing strain that produced the highest lipase activity was selected for further experiments.

3.3.4 Biosurfactant production

The qualitative biosurfactant production was investigated using a hemolytic activity assay. The lipase-producing strain was activated in NB and incubated at 35 °C for 24 h. The activated strain was streaked on blood agar plates (M&P IMPEX, Thailand) containing 5 % v/v fresh sheep blood and incubated at 35 °C for 48-72 h. The hemolytic activity was denoted by observing the clear zones around the colony, indicating a positive strain [154].

3.3.5 Molecular identification and phylogenetic tree

The lipase-producing bacterium was molecularly identified using 16S rRNA gene sequencing. The genomic DNA of the isolated bacterium was extracted by the commercially available kit InstaGene Matrix (Bio-Rad, USA). The obtained genomic DNA was used as a template for amplifying the 16S rRNA gene by polymerase chain reaction (PCR) using primers 27F 5′ (AGA GTT TGA TCM TGG CTC AG) 3′ and 1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3′. The PCR reaction and DNA sequencing followed Mehmood et al. [155] method. Evolutionary analysis of the isolated lipase-producing bacterium was explored using a phylogenetic tree constructed by MEGA X [156].

3.3.6 Optimization of lipase production

3.3.6.1 Screening of important variables by PBD

Eight chemical substances in the culture medium were employed with low (−1) and high (+1) experimental levels to screen the effective variables (Table 3.1). Twelve runs were conducted in triplicate according to PBD using the statistical software package Design Expert 13.0 (Stat Ease Inc., Minneapolis, USA), as presented in Table 3.2. Each designed medium (150 mL) was operated in a 500 mL Erlenmeyer flask and inoculated with 10 % of the starter. The culture was incubated at 35 °C at 200 rpm for 3 days in a shaking incubator before collection to determine the total viable cell count (TVC). A portion of the sample was centrifuged, and the supernatant was collected to determine lipase activity by spectrophotometry. The importance of each variable was standardized and optimal levels of the three main parameters were further determined using the BBD.

Table 3.1 The chemical substances code and their levels for experiments using PBD.

Chemical substances (g/L)	Code	Experimental levels	
		Low level (-1)	High level (+1)
Glucose	*A*	0	3
Olive oil	*B*	10	30
Peptone	*C*	1	10
Yeast extract	*D*	1	10
Dummy 1	*E*	-	-
$MgSO_4{\cdot}7H_2O$	*F*	0.1	1
K_2HPO_4	*G*	0.5	5
$FeSO_4{\cdot}7H_2O$	*H*	0.1	0.5
NaCl	*J*	1	10
Dummy 2	*K*	-	-
Dummy 3	*L*	-	-

Table 3.2 Experimental design to screen the influential chemical substances variables for lipase production using PBD.

Run no.	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*
1	3 (1)	30 (1)	1 (-1)	10 (1)	(1)	1 (1)	0.5 (-1)	0.1 (-1)	1 (-1)	(1)	(-1)
2	0 (-1)	30 (1)	10 (1)	1 (-1)	(1)	1 (1)	5 (1)	0.1 (-1)	1 (-1)	(-1)	(1)
3	3 (1)	10 (-1)	10 (1)	10 (1)	(-1)	1 (1)	5 (1)	0.5 (1)	1 (-1)	(-1)	(-1)
4	0 (-1)	30 (1)	1 (-1)	10 (1)	(1)	0.1 (-1)	5 (1)	0.5 (1)	10 (1)	(-1)	(-1)
5	0 (-1)	10 (-1)	10 (1)	1 (-1)	(1)	1 (1)	0.5 (-1)	0.5 (1)	10 (1)	(1)	(-1)
6	0 (-1)	10 (-1)	1 (-1)	10 (1)	(-1)	1 (1)	5 (1)	0.1 (-1)	10 (1)	(1)	(1)
7	3 (1)	10 (-1)	1 (-1)	1 (-1)	(1)	0.1 (-1)	5 (1)	0.5 (1)	1 (-1)	(1)	(1)
8	3 (1)	30 (1)	1 (-1)	1 (-1)	(-1)	1 (1)	0.5 (-1)	0.5 (1)	10 (1)	(-1)	(1)
9	3 (1)	30 (1)	10 (1)	1 (-1)	(-1)	0.1 (-1)	5 (1)	0.1 (-1)	10 (1)	(1)	(-1)
10	0 (-1)	30 (1)	10 (1)	10 (1)	(-1)	0.1 (-1)	0.5 (-1)	0.5 (1)	1 (-1)	(1)	(1)
11	3 (1)	10 (-1)	10 (1)	10 (1)	(1)	0.1 (-1)	0.5 (-1)	0.1 (-1)	10 (1)	(-1)	(1)
12	0 (-1)	10 (-1)	1 (-1)	1 (-1)	(-1)	0.1 (-1)	0.5 (-1)	0.1 (-1)	1 (-1)	(-1)	(-1)

3.3.6.2 Optimal condition for *A. baumannii* growth and lipase production

The RSM was employed to optimize medium composition for growth and lipase production of the newly isolated strain, *A. baumannii* RMUTT3S8-2. Effective variables chosen for the experiment were peptone, yeast extract, and NaCl (Table 3.3). *A. baumannii* RMUTT3S8-2 was aseptically grown in 150 mL of basal medium (composition per liter: 1.5 g glucose, 30 g olive oil, 1.0 g $MgSO_4 \cdot 7H_2O$, 5.0 g K_2HPO_4, and 0.3 g $FeSO_4 \cdot 7H_2O$) containing peptone, yeast extract, and NaCl following BBD, as presented in Table 3.4. Data analysis was conducted using Design-Expert 13.0. A linear two-factor interaction (2FI) and quadratic regression equations were used to create the model based on high coefficients of TVC, lipase activity, and significance (p-value ≤ .05) from the model and non-significant lack of fit. The regression equations were employed to describe the mathematical relationship between a set of experimental variables (x) and the response (y) following Eq. (1).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j>1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 \quad (1)$$

where y was the predicted response value; x_i and x_j were the value of the independent variables (i, j = 1, 2, 3,..., k). The parameter β_0 was the model constant; β_i was the linear coefficient; β_{ii} was the second-order coefficient, and β_{ij} was the interaction coefficient [157]. The validity of the regression model was confirmed via the predicted optimal condition.

Table 3.3 The medium composition at different concentration and their experimental levels for BBD.

Variables (g/L)	Code	Experimental levels		
		Low level (-1)	Medium level (0)	High level (+1)
Peptone	*C*	5	20	35
Yeast extract	*D*	5	20	35
NaCl	*J*	1	8	15

Table 3.4 Experimental design to optimization of medium composition for growth and lipase production using BBD.

Run no.	*C*	*D*	*J*
1	5 (-1)	5 (-1)	8 (0)
2	35 (+1)	5 (-1)	8 (0)
3	5 (-1)	35 (+1)	8 (0)
4	35 (+1)	35 (+1)	8 (0)
5	5 (-1)	20 (0)	1 (-1)
6	35 (+1)	20 (0)	1 (-1)
7	5 (-1)	20 (0)	15 (+1)
8	35 (+1)	20 (0)	15 (+1)
9	20 (0)	5 (-1)	1 (-1)
10	20 (0)	35 (+1)	1 (-1)
11	20 (0)	5 (-1)	15 (+1)
12	20 (0)	35 (+1)	15 (+1)
13	20 (0)	20 (0)	8 (0)
14	20 (0)	20 (0)	8 (0)
15	20 (0)	20 (0)	8 (0)
16	20 (0)	20 (0)	8 (0)
17	20 (0)	20 (0)	8 (0)

3.3.7 Evaluation of lipase stability

A. baumannii RMUTT3S8-2 was activated in 30 mL of NB and incubated at 35 °C, 200 rpm in a shaking incubator until 24 h. Ten milliliters of culture were transferred to 30 mL of starter medium (NB) and cultivated in the same condition mentioned above. Then, a volume of 10 mL *A. baumannii* RMUTT3S8-2 was grown in 150 mL of the basal medium under optimization and incubated at 35 °C, 200 rpm, for 6 days. For evaluation of lipase stability following, A 5 mL aliquot of cell-free crude lipase was added to 5 mL of buffer solution (mixing ratio 1:1, v/v) at pH 5.0 (0.05 M citric acid-sodium citrate), pH 7.0 (0.05 M dibasic sodium phosphate-monobasic

sodium phosphate) and pH 9.0 (0.05 M Tris (hydroxymethyl) aminomethane-HCl). The mixed solution was incubated in a water bath (WNB14, Memmert, Germany) at experimental temperatures (30, 50, and 70 °C) for 1 h. Subsequently, lipase activity in the solution was analyzed by spectrophotometry, as mentioned below. The experiment was performed in triplicate. The lipase stability was expressed as relative lipase activity following Eq. (2).

$$\text{Relative lipase activity (\%)} = \frac{\text{Lipase activity (U / mL) at 1 h of incubation}}{\text{Lipase activity (U / mL) at the initial time}} \times 100 \qquad (2)$$

3.3.8 Production of lipase in 5-L bioreactor

Production of lipase in a 5-L bioreactor enlarged scale lipase production was performed in a 5-L bioreactor (MS-F1, Major Science, Taiwan (ROC)). A volume of 300 mL *A. baumannii* RMUTT3S8-2 starter was inoculated into 2,700 mL of optimized medium and cultured at 35 °C, 1.0 vvm of aeration at 200 rpm. The culture was analyzed to determine TVC and lipase activity throughout the cultivation period.

3.3.9 Hydrolysis of oil in poultry processing factory wastewater

3.3.9.1 Preparation of inoculum *A. baumannii* RMUTT3S8-2

The lipase-producing bacteria *A. baumannii* RMUTT3S8-2 was performed in the 10-L bioreactor (FS-07, Winpact, Thailand). The 1,000 mL of *A. baumannii* RMUTT3S8-2 starter was inoculated into 9,000 mL of optimal medium and cultivated at 35 °C, 1.0 vvm of aeration, and 200 rpm of agitation for 144 h. The culture of lipase-producing bacteria *A. baumannii* RMUTT3S8-2 from the abovementioned was used as a liquid inoculum (LI). For the powder inoculum (PI), sterilized soybean meal (SBM) and the cultured lipase-producing bacteria *A. baumannii* RMUTT3S8-2 were mixed in a 1:1 ratio. The mixture was dried at 40 °C in a hot air oven (FD240, Binder, Germany) until moisture content was < 10 % and gathered sampling for analysis of lipase activity and TVC.

3.3.9.2 Hydrolysis of oil in wastewater

The efficiency of oil hydrolysis in wastewater from a poultry processing factory was investigated by varying the quantity of PI or LI (1-5 % w/v) and

without inoculum as a control. Ten liters of aerated wastewater from the poultry processing factory in a 20 L plastic tank were inoculated with the above inoculum. The experiment was performed in triplicate under aerobic conditions at ambient temperature. Samples were collected periodically to analyze the degree of oil hydrolysis and volatile fatty acids (VFAs).

3.3.9.3 Hydrolysis of oil in poultry processing factory wastewater in a pilot scale

The efficiency of oil hydrolysis in wastewater from a poultry processing factory was performed with different inoculum platforms, including 5 % w/v PI, gauze bag-contained powder inoculum (50 g/bag), and control (no inoculum addition). The PI and GBPI contained TVC and lipase activity of 8.62 ± 0.20 LogCFU/g and 291.17 ± 21.47 U/g, respectively. A continuous process examined the oil hydrolysis in 150 L of aerated wastewater from the poultry processing factory contained in a 200 L plastic tank. The process was operated under aerobic conditions at ambient temperature. The hydraulic retention time (HRT) and flow rate of wastewater of continuous process were 8 h and 450 L/day, respectively. The samples were collected periodically to analyze the degree of oil hydrolysis and VFAs.

3.3.10 Analytical method

3.3.10.1 Total viable cell count

The sample was ten-fold serial diluted in NaCl-peptone solution containing 1 % Tween 80 in an aseptic condition [158]. TVC was determined by plate count agar (PCA) using the pour plate technique. The agar plate was incubated at 35 °C for 48 h. The colonies were counted and expressed as the logarithm of colony forming unit per milliliter (LogCFU/mL).

3.3.10.2 Lipase activity

1) Lipase activity determination for preliminary screening of lipase-producing bacteria was conducted by modifying the titrimetric method of Kanlayakrit and Boonpan [159]. The substrate was prepared by combining 45 mL of 2.0 % polyvinyl alcohol (PVA), 45 mL of distilled water and 10 mL of palm oil with gentle mixing. The reaction mixture was added with 4.0 mL of cell-free supernatant, 2.0 mL of 0.2 M phosphate buffer pH 6.5 and 2.0 mL of the substrate and mixed for 5 min before incubating at 35 °C, 200 rpm for 1 h in a rotary shaker (NB-205VL, N-BIOTEK, South Korea). After incubation, the reaction was stopped by adding 20 mL of 1:1 acetone and ethanol (v/v) mixing solution. The amount of FFAs was estimated by titrating with 0.1 N NaOH using 0.1 % phenolphthalein as an indicator. One unit of lipase activity was defined as the volume of the enzyme that released 1 μmol of fatty acids from palm oil per mL per hour.

2) Lipase activity was measured spectrophotometrically method using *p*-nitrophenyl palmitate (*p*-NPP) as substrate following the adjusted method of Gurkok and Ozdal. [3]. Briefly, the culture was centrifuged (3-18KS, Sigma, UK) at 10,000 rpm (11,068 g) for 10 min at 4 °C to remove the bacterial cells, with cell-free supernatant used as crude lipase. Then, 0.25 mL of the crude enzyme was mixed with 0.25 mL of 4 mM *p*-NPP and 0.5 mL of 50 mM Tris-HCl before incubation at 25 °C for 10 min. Then, 2.0 mL of 0.5 N Na_2CO_3 was added to terminate the enzymatic reaction. Finally, the absorbance of released p-nitrophenol (*p*-NP) as the reaction product was measured at 410 nm by a spectrophotometer (EZ Read 2000, Biochrom, UK). Lipase activity was expressed according to the linear equation of a standard graphical plot prepared in *p*-NP 0–3000 μmol/mL range. One unit of lipase activity was defined as the quantity of enzyme that released 1 μmol *p*-NP in 1 min.

3.3.10.3 The weight fraction of oil and degree of oil hydrolysis

1) The weight fraction of oil in poultry wastewater was performed by modifying the oil extraction method of Bligh and Dyer. [160]. Briefly, the poultry wastewater was centrifuged at 3,800 rpm (2,946 × *g*) for 10 min at 4 °C to separate the supernatant from the sludge pellet. The supernatant was mixed with a methanol-chloroform mixing solution (1:1) at a mixing ratio of 1:1. The mixed solution

was centrifuged at 3,800 rpm for 5 min at 4 °C to separate the oil-rich chloroform layer from the aqueous layer. The oil-rich chloroform layer was transferred to the test tube. Subsequently, the supernatant was re-extracted with chloroform, centrifuged, and pooled in the same test tube. The chloroform was evaporated at 80 °C in water bath. Afterward, the oil-contained test tube was dried in a hot air oven at 80 °C for 24 h. Finally, the tube was kept in desiccator at room temperature and weighed. The weight fraction of oil was calculated following Eq. (3):

$$\text{The weight fraction of oil (\% w/v)} = \frac{(W_2 - W_1)}{V} \times 100 \tag{3}$$

where W_1 is the weight of test tube (g); W_2 is weight of the test tube with extracted oil (g); V is volume of sample (mL).

2) Oil hydrolysis was determined as the degree of released FAs by the titration method according to de Almeida et al. [161]. A 5 mL sample aliquot was mixed with 15 mL acetone:ethanol mixing solution (ratio 1:1, v/v) to stop the hydrolysis reaction. Released FAs in the sample were titrated with NaOH solution using 0.1 % phenolphthalein as an indicator. The degree of oil hydrolysis was calculated following Eq. (4):

$$X = \frac{W(V - V_0)M}{10mf_0} \tag{4}$$

where X is the degree of oil hydrolysis (%), W is the mean molecular weight of the FAs, V is the volume (mL) of NaOH solution used for titration of the sample, V_0 is the volume (mL) of NaOH solution used for titration of the control; M is the molarity of NaOH solution, m is the volume of the sample (mL), and f_0 is the initial weight fraction of oil in poultry wastewater.

3.3.10.4 VFAs

The VFAs compositions of the poultry fat wastewater samples were analyzed using a gas chromatography-mass spectrometer (GC-MS). The sample

was centrifuged (VARISPIN 12R, CRYSTAL, South Korea) at 3,800 rpm (2,946 *g*) for 5 min at 4 °C. The supernatant was filtered through a 0.45 µm nylon syringe filter (SY1345NN, National Scientific, Thailand). The FAs compositions were analyzed by GC-MS (QP2010SE/AOC-20I, Shimadzu, Japan), which capillary column (InertCap WAX, 30 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as carrier gas flowing at 1.0 mL/min. The temperature program of GC was operated following the condition: injection temperature at 250 °C, column temperature at 110 °C, held time at 18 min, and then increased to 250 °C with a rate of 10 °C/min, and maintained for 5 min. Each peak area determined the VFAs profile using NIST 14 standard library.

3.3.11 Statistical analysis

SPSS software version 15.0 (SPSS Inc., NY, USA) was employed for the statistical analysis. Statistical analysis of the data was carried out by one-way analyses of variance (ANOVA). Duncan's multiple range tests at 95 % confidence interval were used to determine the difference in means of each experimental condition. All data were expressed as mean ± standard deviation (SD). The SD values were calculated using Eq. (5):

$$\mathrm{SD} = \sqrt{\frac{\sum_{i=1}^{n}(C_{\mathrm{i}} - \bar{C})^2}{(n-1)}} \tag{5}$$

where n is the number of data. C_{i} and $\bar{C}$ are each data value (i = 1, 2, 3, …, n) and mean of data value, respectively.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Primary Screening of Lipase-producing Microorganisms

4.1.1 Qualitative screening on agar plate

Thirty-one extracellular lipase-producing isolates were obtained from oily wastewater treatment at the poultry processing factory. Six isolates showed a change in the color of bromocresol purple in PM agar as a positive result (Figure 4.1A). It was indicated that the oil was digested and released FAs [151]. Moreover, 15 isolates showed a positive result on Tween 80 agar (Figure 4.1B). The turbid zone demonstrated that the calcium salt in Tween 80 was released by digesting lipase [162], and 10 isolates were indicated both positive on PM and Tween 80 agar.

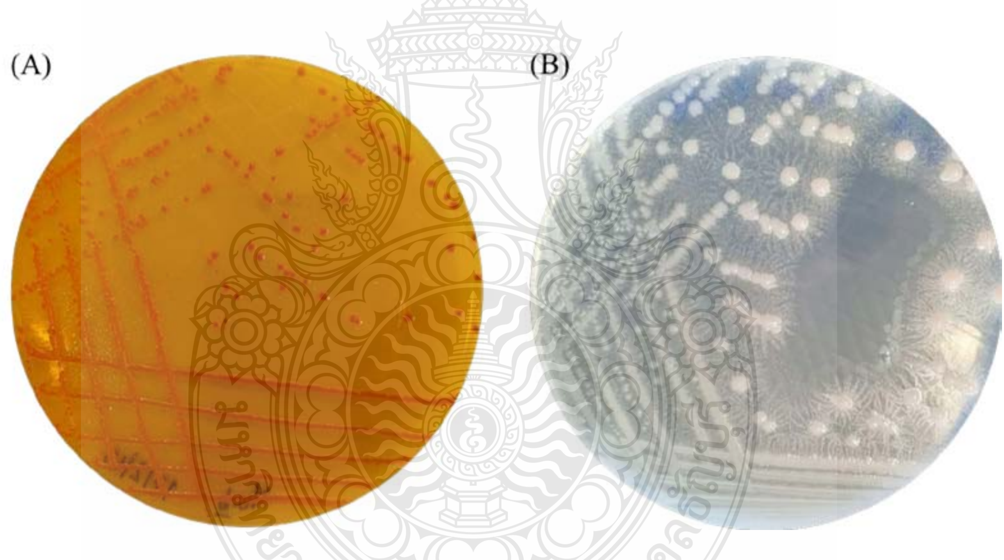

Figure 4.1 Example of lipase-producing microorganisms (A) RMUTT2W1-3 on PM agar and (B) RMUTT2S3-3 on Tween 80 agar.

Briefly, palm oil and Tween 80 were used as the elements (carbon source) of PM and Tween 80 agar, respectively. Well-known palmitic and oleic acids are one of the components of palm oil. Even though Tween 80 is known as a non-ionic surfactant, it is composed of oleic acid. Therefore, it is commonly used to test lipase activity. The palmitic (16:0) and oleic acids (18:1) are common long-chain FAs of TAG molecules. Palmitic acid is a saturated fatty acid (no double bond), while the unsaturated fatty acid double bond in the chain carbon of oleic acid (cis 9-10 position) [163, 164]. Therefore, some strains were positive on both agar types, and others were not. It may involve the positional specificity lipases (regiospecificity). Some strains demonstrated positive signals in both media. They could hydrolyze FA from any position of the palm oil and Tween 80, non-specific lipase on TAG molecules, as shown in Figure 2.4A. While some strains exhibit the only positive in PM or Tween 80 agar following: the positive strains on only PM agar were presented as lipase producers of the 1, 3-specific lipases group. It has degraded palm oil molecule at the C-1 and C-3 positions (Figure 2.4B). The FA specificity lipases hydrolyze at a double bond between C-9 and C-10 (cis-form) positions of Tween 80 [18, 47]. However, the agar plate method is only preliminary used for screening, inadequate to identify the specificity. Thus, it needs further examination.

Table 4.1 Lipase producers isolated on PM and Tween 80 agar.

Isolate no.	Lipase producing test		Isolate no.	Lipase producing test	
	PM agar[a]	Tween 80 agar[b]		PM agar[a]	Tween 80 agar[b]
RMUTT2W1-1	+	-	RMUTT3S4-2	-	+
RMUTT2W1-3	+	-	RMUTT3S5-1	+	+
RMUTT2W2-3	+	-	RMUTT3S5-3	-	+
RMUTT2W2-4	+	-	RMUTT3S6-10	-	+
RMUTT2S2-1	+	-	RMUTT3S6-12	-	+
RMUTT2S2-2	+	-	RMUTT3S6-13	-	+
RMUTT2S3-2	-	+	RMUTT3S6-14	-	+
RMUTT2S3-3	+	+	RMUTT3S6-15	+	+
RMUTT2S4-2	+	+	RMUTT3S6-18	-	+
RMUTT2S5-2	+	+	RMUTT3S8-1	-	+
RMUTT2S6-2	+	+	RMUTT3S8-2	+	+
RMUTT2S7-1	-	+	RMUTT3S8-3	+	+
RMUTT2S8-2	+	+	RMUTT3S8-6	-	+
RMUTT3S1-3	-	+	RMUTT3S9-1	+	+
RMUTT3S1-4	-	+	RMUTT3S9-3	-	+
RMUTT3S4-1	-	+			

[a]test result of + indicates a yellow-orange area around the colony and [b]test result of + indicates a turbid zone around the colony.

4.1.2 Morphological characterization of lipase-producing microorganisms

The colony morphologies of lipase-producing isolates were presented in Table 4.2. The colonies were found primarily on circle shapes, entire edges, smooth surfaces, flat elevation, and white-glossy color. It is common knowledge that microorganisms producing lipase can be found in yeast, bacteria, and fungi, and the shape also depends on the type of microorganism [165]. In this study, the morphology of all yeast strains was round. Twenty-five bacteria strains presented coccus, rod, or bacillus shapes with Gram-negative and Gram-positive cell wall structures, as shown in Table 4.3 and Figures 4.2-4.3. Previous studies reported that several lipase-producing strains, such as *Acinetobacter* sp. EH28 and *Geobacillus* sp. 12AMOR1 were Gram-negative bacteria [10, 166]. However, Gram-positive strains *Enterococcus faecium* MTCC 5695 and *Pediococcus acidilactici* MTCC 11361 were also discovered as lipase producers [167].

Table 4.2 Colony morphology characteristics.

Isolate no.	Colony morphology				
	Shape	Edge	Surface	Elevation	Color
RMUTT2W1-1	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2W1-3	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2W2-3	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2W2-4	Circular	Entire	Smooth	Convex	White and Glossy
RMUTT2S2-1	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2S2-2	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2S3-2	Circular	Entire	Smooth	Flat	Yellow and Glossy
RMUTT2S3-3	Circular	Entire	Smooth	Convex	Red and Glossy
RMUTT2S4-2	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2S5-2	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2S6-2	Circular	Undulate	Rough	Raised	Cream and Glossy
RMUTT2S7-1	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT2S8-2	Circular	Undulate	Smooth	Convex	White and Glossy
RMUTT3S1-3	Circular	Entire	Rough	Flat	Cream and Glossy
RMUTT3S1-4	Circular	Entire	Rough	Flat	Cream-Yellow and Glossy

Table 4.2 Colony morphology characteristics (Cont.)

Isolate no.	Colony morphology				
	Shape	Edge	Shape	Elevation	Shape
RMUTT3S4-1	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT3S4-2	Circular	Undulate	Smooth	Flat	Cream and Glossy
RMUTT3S5-1	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT3S5-3	Circular	Entire	Smooth	Flat	Cream-Yellow and Glossy
RMUTT3S6-10	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT3S6-12	Circular	Entire	Smooth	Flat	Cream-Yellow and Glossy
RMUTT3S6-13	Irregular	Undulate	Smooth	Flat	White and Glossy
RMUTT3S6-14	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT3S6-15	Circular	Entire	Smooth	Flat	Cream-Brown and Glossy
RMUTT3S6-18	Circular	Entire	Smooth	Flat	Cream and Glossy
RMUTT3S8-1	Circular	Entire	Smooth	Flat	Cream and Glossy
RMUTT3S8-2	Circular	Entire	Smooth	Flat	Cream and Glossy
RMUTT3S8-3	Circular	Entire	Smooth	Flat	Cream and Glossy
RMUTT3S8-6	Circular	Entire	Smooth	Flat	White and Glossy
RMUTT3S9-1	Circular	Entire	Smooth	Flat	Pink and Glossy
RMUTT3S9-3	Circular	Entire	Smooth	Flat	White-Cream and Glossy

Table 4.3 Morphology characteristics and Gram's staining of lipase producing strain under the light microscope.

Isolate no.	Gram's staining[a]	Shapes (100x)	Strains	Isolate no.	Gram's staining[a]	Shapes (100x)	Strains
RMUTT2W1-1	ND[b]	Round	Yeast	RMUTT3S4-2	+	Bacillus	Bacteria
RMUTT2W1-3	ND	Round	Yeast	RMUTT3S5-1	-	Rod	Bacteria
RMUTT2W2-3	ND	Round	Yeast	RMUTT3S5-3	-	Bacillus	Bacteria
RMUTT2W2-4	ND	Round	Yeast	RMUTT3S6-10	+	Bacillus	Bacteria
RMUTT2S2-1	ND	Round	Yeast	RMUTT3S6-12	-	Bacillus	Bacteria
RMUTT2S2-2	ND	Round	Yeast	RMUTT3S6-13	-	Bacillus	Bacteria
RMUTT2S3-2	-	Rod	Bacteria	RMUTT3S6-14	+	Bacillus	Bacteria
RMUTT2S3-3	+	Bacillus	Bacteria	RMUTT3S6-15	-	Bacillus	Bacteria
RMUTT2S4-2	-	Bacillus	Bacteria	RMUTT3S6-18	+	Bacillus	Bacteria
RMUTT2S5-2	-	Coccus	Bacteria	RMUTT3S8-1	+	Bacillus	Bacteria
RMUTT2S6-2	+	Bacillus	Bacteria	RMUTT3S8-2	-	Rod	Bacteria
RMUTT2S7-1	-	Bacillus	Bacteria	RMUTT3S8-3	-	Bacillus	Bacteria
RMUTT2S8-2	-	Coccus	Bacteria	RMUTT3S8-6	+	Bacillus	Bacteria
RMUTT3S1-3	-	Bacillus	Bacteria	RMUTT3S9-1	-	Bacillus	Bacteria
RMUTT3S1-4	-	Bacillus	Bacteria	RMUTT3S9-3	-	Bacillus	Bacteria
RMUTT3S4-1	-	Bacillus	Bacteria				

[a]test result of (+) indicates a Gram-positive, and (-) indicates a Gram-negative. [b]ND not detected for Gram's staining.

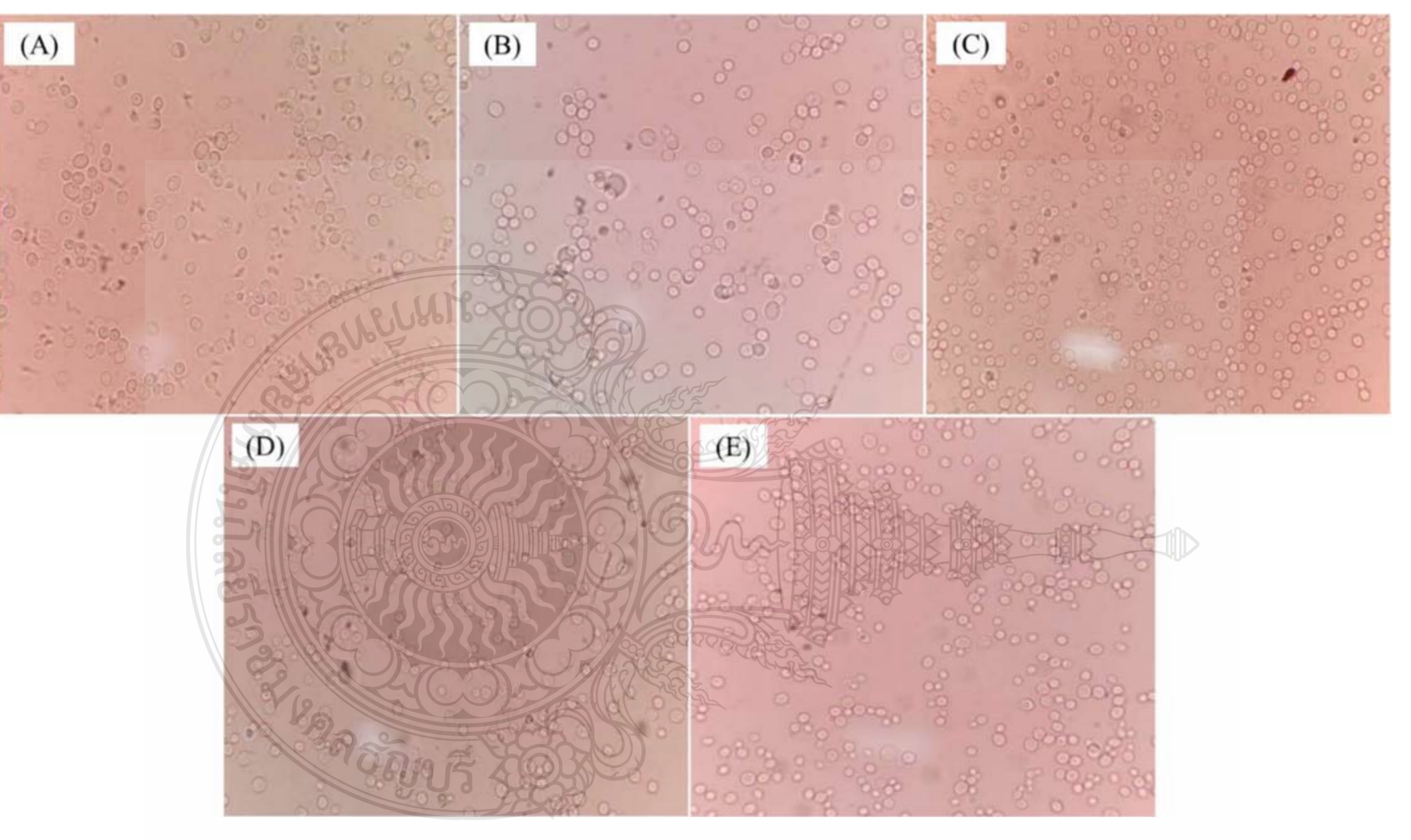

Figure 4.2 Morphology of different lipase-producing yeast strains (A) RMUTT2W1-1, (B) RMUTT2W1-3, (C) RMUTT2W2-3, (D) RMUTT2S2-1, and (E) RMUTT2S2-2 (magnification: 100x).

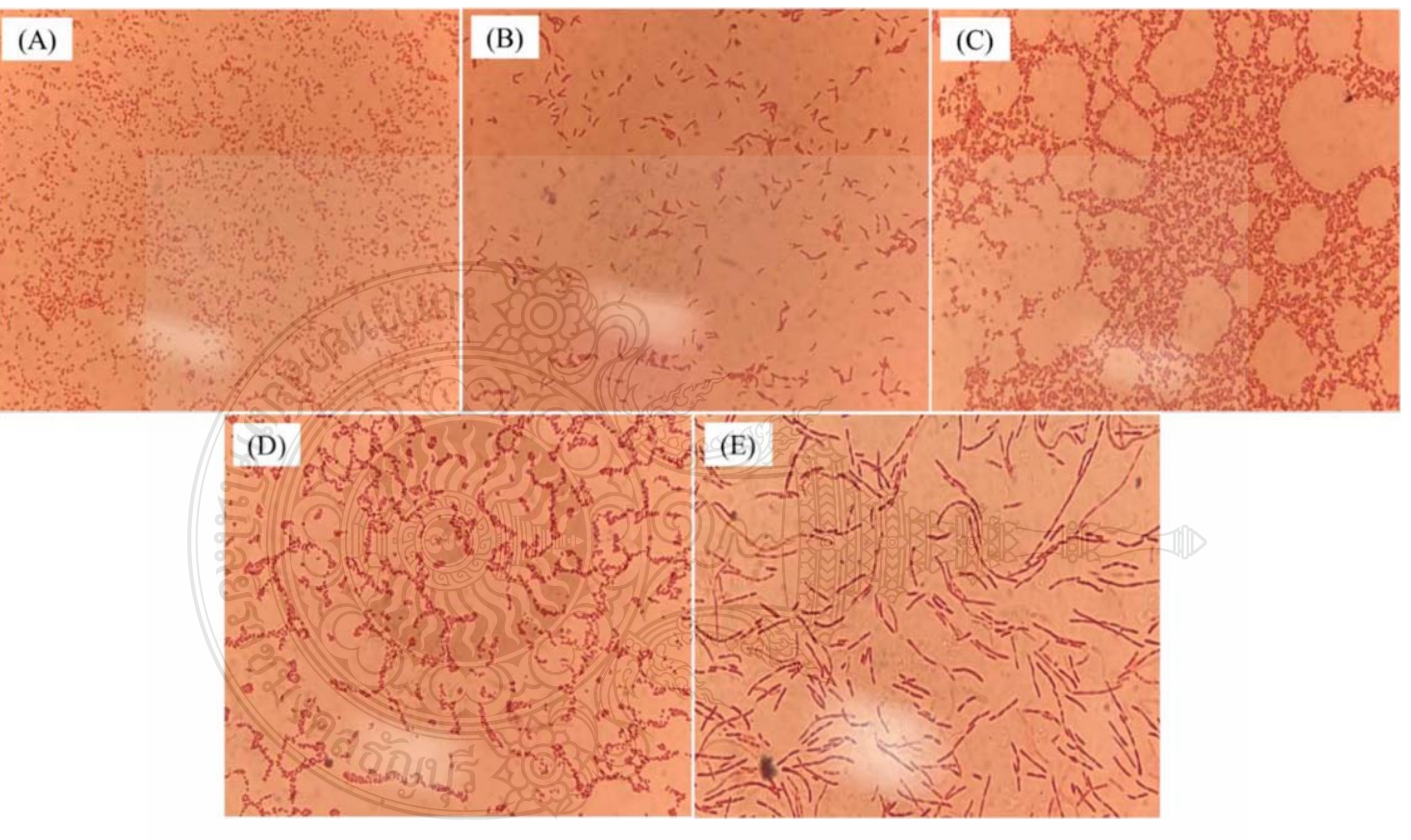

Figure 4.3 Morphology of different lipase-producing bacteria strains (A) RMUTT2S3-2, (B) RMUTT2S4-2, (C) RMUTT3S5-1, (D) RMUTT3S8-2, and (E) RMUTT3S8-3 (magnification: 100x).

4.2 Secondary Screening

4.2.1 Screening of quantitative lipase-producing

The results showed that all isolates could produce extracellular lipase. Five of the thirty-one isolated strains which demonstrated a significant (*p*-value ≤ .05) highest lipase activity were isolate RMUTT3S8-3 (7.18 ± 0.35 U/mL) following RMUTT3S5-1 (5.22 ± 0.31 U/mL), RMUTT3S8-2 (4.87 ± 0.16 U/mL), RMUTT2S3-2 (4.63 ± 0.66 U/mL), and RMUTT2S4-2 (4.63 ± 0.00 U/mL), respectively. While isolate RMUTT3S9-3 exhibited the least lipase activity was 0.05 ± 0.02 U/mL, as shown in Table 4.4 and Figure 4.4. Lipase activity in this study was approximated to *Streptomyces exfoliates* LP10 (6.9 U/mL) and *Pseudomonas mendocina* (8.5 U/mL) [152, 168].

4.2.2 Selection of lipase-producing bacteria

The quantitative lipase activity of five isolates was confirmed by spectrophotometric assay. Isolate RMUTT3S8-2 exhibited significantly highest lipase activity of 97.43 ± 4.29 U/mL (*p*-value ≤ .05), followed by RMUTT2S3-2 (76.67 ± 8.95 U/mL), RMUTT2S4-2 (56.46 ± 1.94 U/mL), RMUTT3S8-3 (50.86 ± 0.83 U/mL), and RMUTT3S5-1 (42.65 ± 1.90 U/mL) (Figure 4.5). Priji et al. [169] reported that the *Pseudomonas* sp. strain BUP6 production of lipase of 96.15 U/mL, close to the isolate RMUTT3S8-2 in this study. However, lipase activity quantity depends on the strain of microorganisms, such as *Acinetobacter* sp. UBT1 demonstrated lipase activity of 42 U/mL [170], while *Natrialba asiatica* was 3.39 U/mL [171]. Thus, the RMUTT2S3-2, RMUTT2S4-2, RMUTT3S5-1, RMUTT3S8-2, and RMUTT3S8-3 strains were chosen to identify the species by molecular method.

Table 4.4 Lipase activity of isolated strains from poultry oil wastewater treatment system analyzed by titrimetric.

Isolate no.	Lipase activity (U/mL/h)	Isolate no.	Lipase activity (U/mL/h)
RMUTT2W1-1	1.38 ± 0.43^{no}	RMUTT3S4-2	3.41 ± 0.25^{gh}
RMUTT2W1-3	3.56 ± 0.09^{fg}	RMUTT3S5-1	5.22 ± 0.31^{b}
RMUTT2W2-3	2.92 ± 0.14^{ij}	RMUTT3S5-3	3.71 ± 0.25^{fg}
RMUTT2W2-4	3.87 ± 0.31^{ef}	RMUTT3S6-10	2.51 ± 0.12^{jk}
RMUTT2S2-1	4.02 ± 0.17^{ef}	RMUTT3S6-12	3.01 ± 0.31^{hi}
RMUTT2S2-2	4.22 ± 0.00^{de}	RMUTT3S6-13	2.96 ± 0.16^{i}
RMUTT2S3-2	4.63 ± 0.66^{cd}	RMUTT3S6-14	0.60 ± 0.41^{qr}
RMUTT2S3-3	2.88 ± 0.25^{ij}	RMUTT3S6-15	4.02 ± 0.12^{ef}
RMUTT2S4-2	4.63 ± 0.00^{cd}	RMUTT3S6-18	0.85 ± 0.19^{pq}
RMUTT2S5-2	1.63 ± 0.43^{mn}	RMUTT3S8-1	2.01 ± 0.16^{lm}
RMUTT2S6-2	1.46 ± 0.29^{no}	RMUTT3S8-2	4.87 ± 0.16^{bc}
RMUTT2S7-1	0.25 ± 0.22^{rs}	RMUTT3S8-3	7.18 ± 0.35^{a}
RMUTT2S8-2	0.25 ± 0.33^{rs}	RMUTT3S8-6	1.15 ± 0.30^{op}
RMUTT3S1-3	2.41 ± 0.23^{kl}	RMUTT3S9-1	3.87 ± 0.23^{ef}
RMUTT3S1-4	3.06 ± 0.25^{hi}	RMUTT3S9-3	0.05 ± 0.02^{s}
RMUTT3S4-1	2.16 ± 0.16^{kl}		

The bar represents the mean ± SD. Different superscript letters presented significant differences at p-value $\leq .05$.

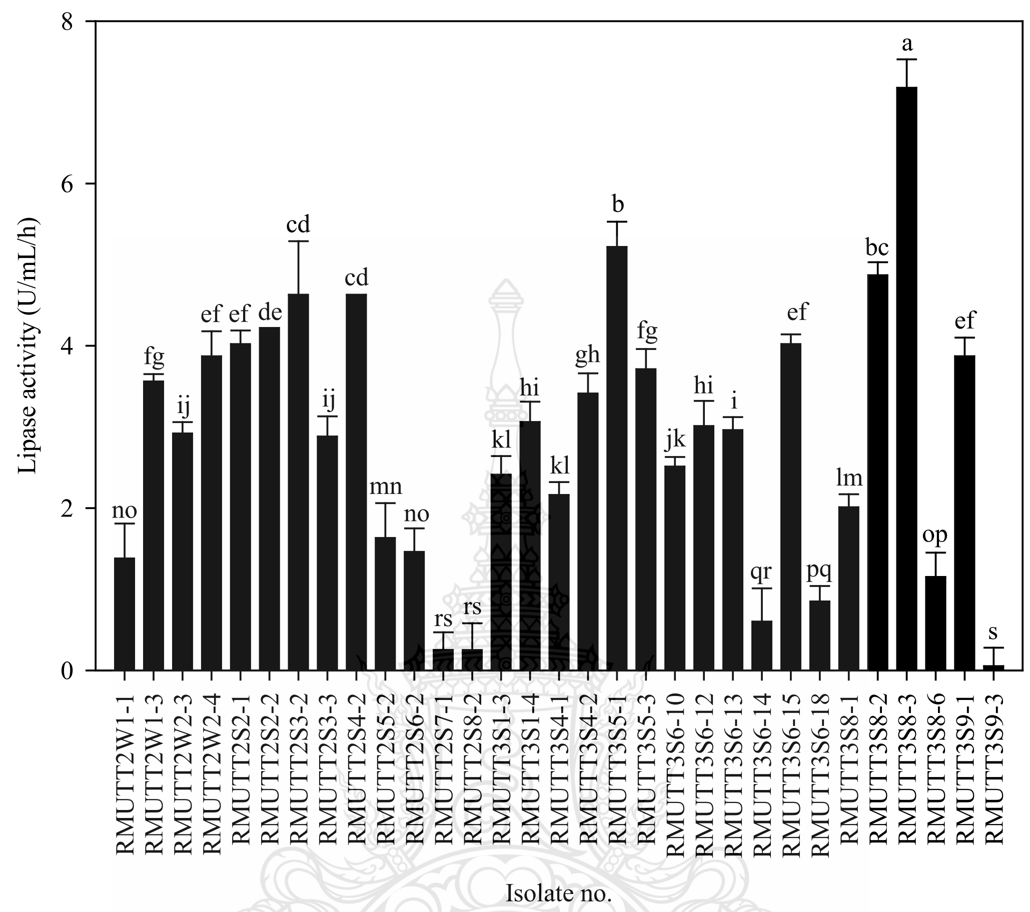

Figure 4.4 Lipase activity of isolated strains from poultry oil wastewater treatment pond analyzed by titrimetric. The bar represents the mean ± SD. Different letters presented significant differences at *p*-value ≤ .05.

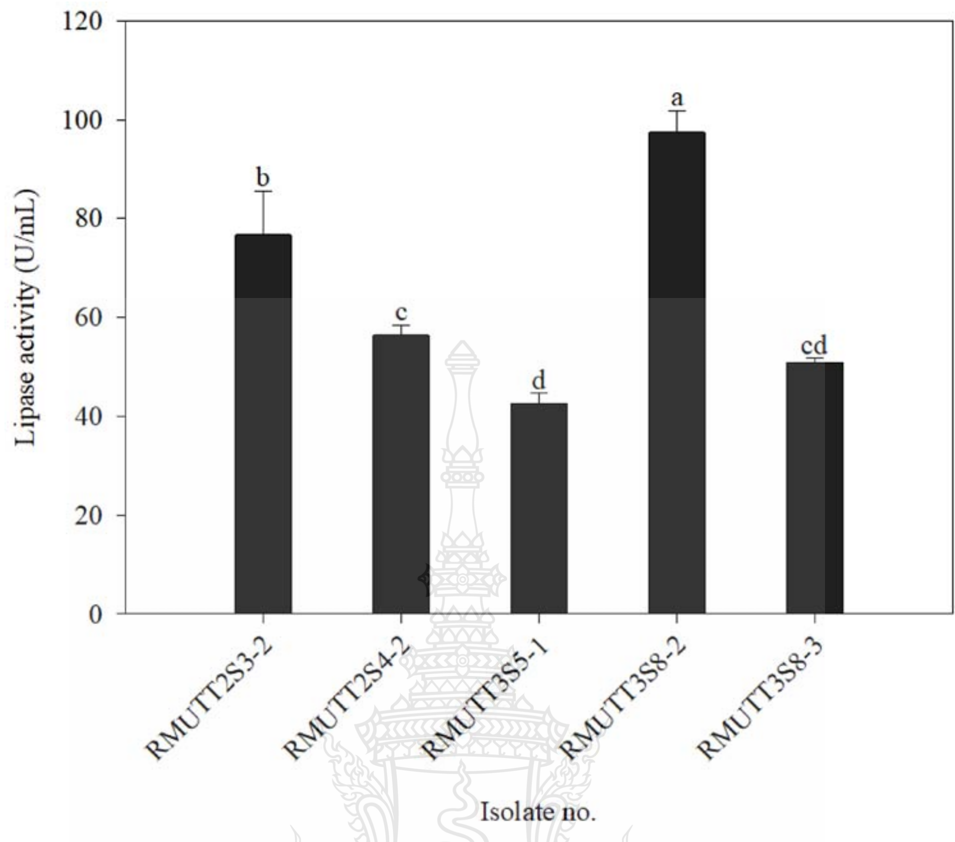

Figure 4.5 Lipase activity of isolated strains from poultry oil wastewater treatment pond analyzed by spectrophotometry. The bar represents the mean ± SD. Different letters present significant differences at *p*-value ≤ .05.

4.3 Biosurfactant Production

The biosurfactant production of five effective bacteria strains was investigated on a blood agar plate. Isolate RMUTT2S3-2, RMUTT2S4-2, RMUTT3S5-1, and RMUTT3S8-3 could lysis the red blood cell (RBC) and hemoglobin in blood agar, compared with *Staphylococcus aureus* as a positive control (Figure 4.6). Isolate RMUTT3S8-3 exhibited complete lysis RBC and hemoglobin (or beta hemolysis). The beta hemolysis destroyed the blood in blood agar and made the clearance surround the colonies [154, 172]. In comparison, isolate RMUTT2 S3 -2 , RMUTT2 S4 -2 , and RMUTT3 S5 -1 exhibited partial destruction of RBC as alpha hemolysis. The alpha hemolysis showed greenish-grey or brownish discoloration of agar medium around the colonies [173]. Adversely, isolate RMUTT3 S8 -2 showed no destruction of RBC in

blood agar. The gamma hemolysis or no lysis of RBC showed no color change in the medium [172]. Thus, the isolate RMUTT3S8-3 could produce the biosurfactant during its growth. Additionally, microorganisms produce lipases and biosurfactants simultaneously, which could aid in metabolizing insoluble substances in water [91]. However, the relationship between the production of lipases and biosurfactants needs to be better established. Kanjan and Sakpetch [173] demonstrated that not all bacteria produce lipase concurrently with biosurfactant production.

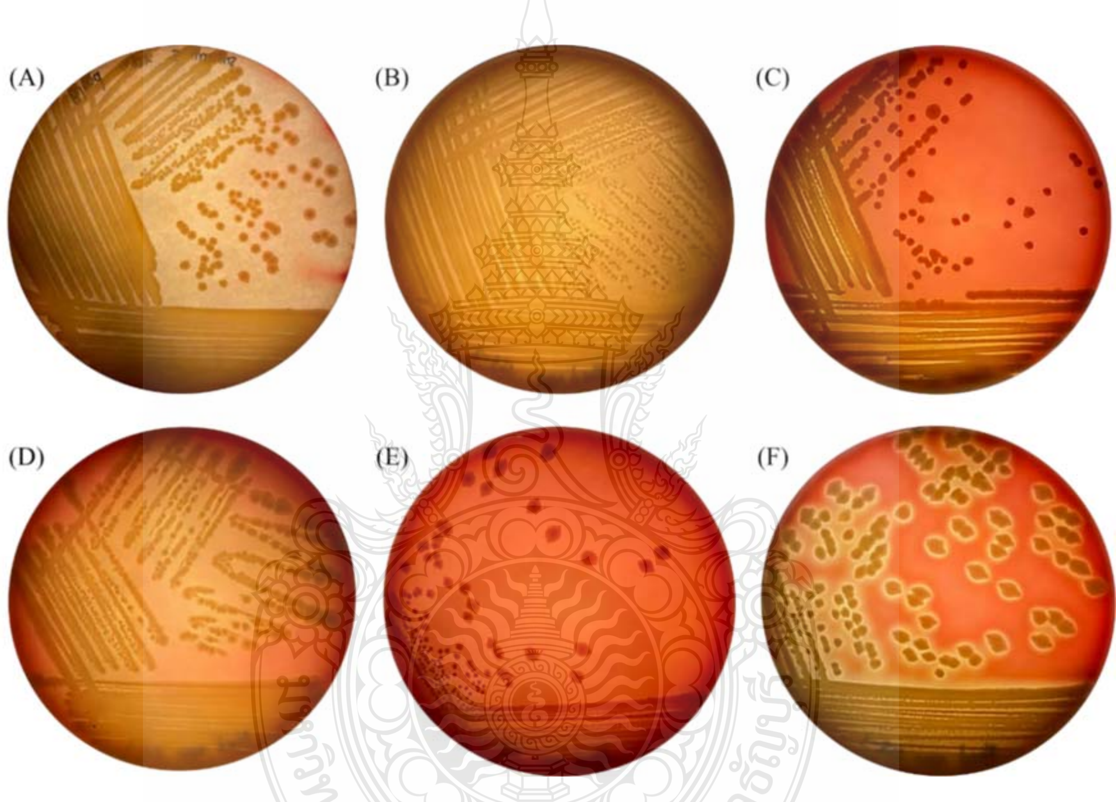

Figure 4.6 The color change of lipase-producing isolate in biosurfactant-producing test (A) *S. aureus*, (B) RMUTT2 S3-2, (C) RMUTT2 S4-2, (D) RMUTT3S5-1, (E) RMUTT3S8-2, and (F) RMUTT3S8-3.

4.4 Molecular Identification and Phylogenetic Tree

Five lipase-producing bacterial strains were molecularly identified based on 16S rRNA sequence analysis. Nucleotide BLAST analysis of the obtained sequences showed > 99 % similarity with *Sphingomonas* sp., *Burkholderia thailandensis*, *Enterobacter cloacae*, *Acinetobacter baumannii*, and *Aeromonas caviae* (Table 4.5). The 16S rRNA sequences of the five newly isolated strains were submitted to NCBI under the accession numbers, as shown in Table 4.5. The evolution of the newly isolated lipase-producing strains was compared with previous literature on lipase-producing bacteria in the phylogenetic tree (Figure 4.7). Isolates RMUTT3S5-1 and RMUTT3S8-3 were clustered together with the reference lipase-producing strain in a clade including *Yersinia enterocolitica* KM1, *E. cloacae*, *A. caviae* AU04 and *Idiomarina* sp. W33 [174–176]. Isolate RMUTT3S8-2 was assigned in the clade containing *Acinetobacter* sp. EH28, *Chromohalobacter* sp. LY7-8, *Pseudomonas* sp. BUP6, *P. aeruginosa* AAU2 and *Pseudomonas gessardii* CLRI1 [177–181]. Isolate RMUTT2S4-2 was allocated in a similar clade with *Burkholderia ubonensis* SL-4 and *Ralstonia* sp. CS274 [5, 182]. By comparison, isolate RMUTT2S3-2 was classified in the same clade as *Sphingomonas* sp. [183]. The phylogenetic tree also showed that the five isolated strains evolved differently from the genera of *Arthrobacter*, *Streptomyces*, and *Bacillus* (Figure 4.7).

Table 4.5 Molecular identification of five newly isolated lipase-producing strains from poultry oil wastewater treatment pond.

Isolate no.	Accession no.	Species	Identities (%)
RMUTT2S3-2	ON076056	*Sphingomonas* sp. (EF433552.1)	100
RMUTT2S4-2	ON076057	*Burkholderia thailandensis* (CP020391.1)	99.52
RMUTT3S5-1	ON076058	*Enterobacter cloacae* (MT138639.1)	99.52
RMUTT3S8-2	ON076059	*Acinetobacter baumannii* (KT956238.1)	99.12
RMUTT3S8-3	ON076060	*Aeromonas caviae* (CP092181.1)	99.53

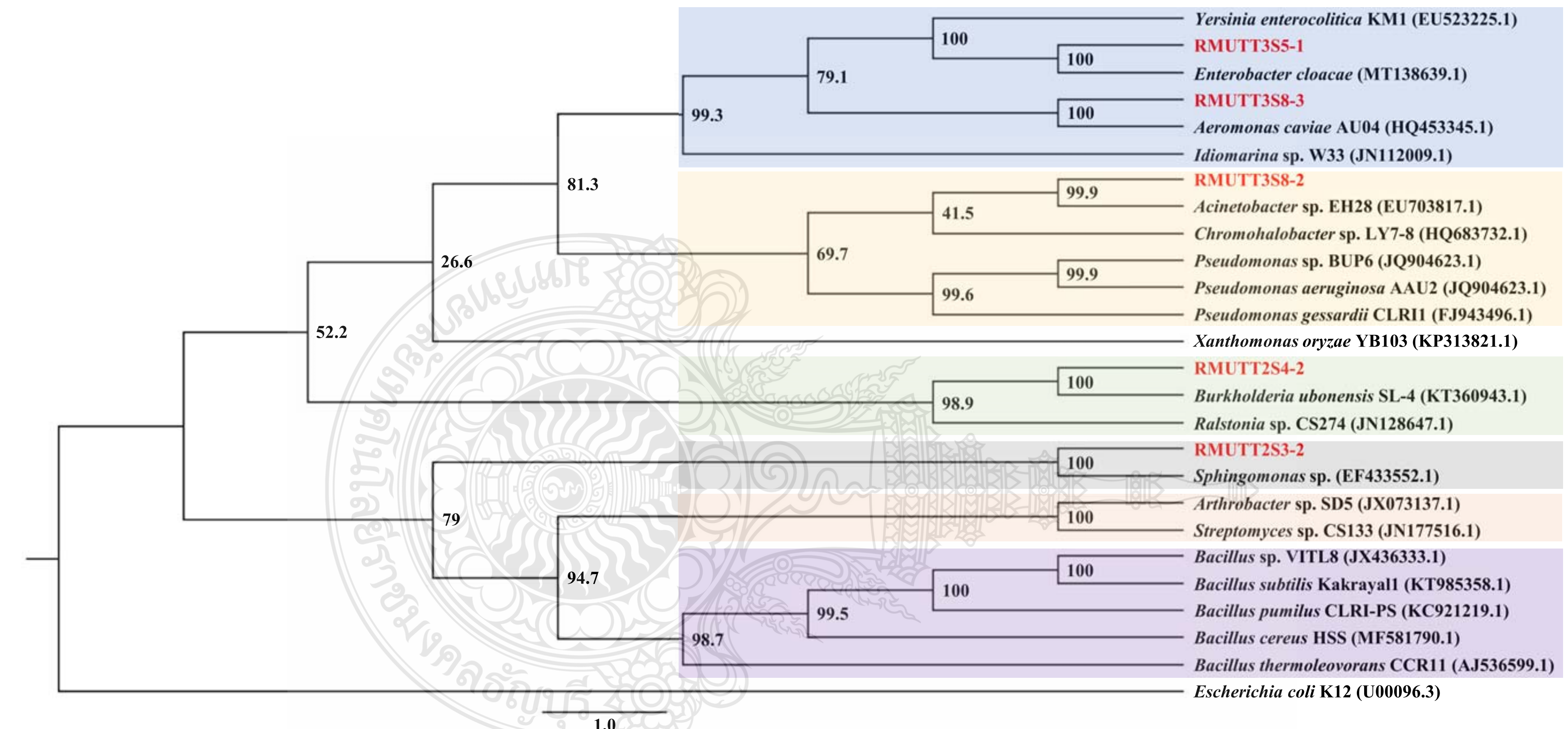

Figure 4.7 Phylogenetic tree of five newly isolated lipase-producing strains from a poultry oil wastewater treatment pond. Values at nodes indicate bootstrap percentages of 1000 replications. Lengths of the branches show relative divergence among the reference nucleotide sequences, while the scale bar represents the estimated evolution distance. GenBank accession numbers are shown in brackets after each species name.

4.5 Optimization of Lipase Production

4.5.1 Selection of important variables by PBD

PBD was carried out to select the important variables affecting TVC and lipase activity of *A. baumannii* RMUTT3S8-2 in batch cultivation. The TVC and lipase activity were used as response variables, as shown in Table 4.6. The effects of independent variables on TVC and lipase activity were standardized to compare the impact of each variable (Figure 4.8). Results exhibited that peptone presented the highest positive effect on TVC, followed by yeast extract, NaCl, $MgSO_4{\cdot}7H_2O$, K_2HPO_4, and olive oil (Figure 4.8A). By contrast, glucose and $FeSO_4{\cdot}7H_2O$ showed adverse impacts on TVC. The negative effect of glucose on growth of *A. baumannii* RMUTT3S8-2 in this study concurred with Ktata et al. [98]. It was suggested that glucose negatively impacted microbial growth due to carbon catabolite repression (CCR) as the paradigm of cellular regulation. For bacterial cultivation, one carbon source will be utilized more quickly than two or more carbon sources, resulting in CCR [184]. In this research, olive oil positively affected the growth of *A. baumannii* RMUTT3S8-2, conforming with the cultivation of *Burkholderia* sp. C20 for lipase production, olive oil could increase lipase production in both small-scale and upscale experiments [185]. In this experiment, nitrogen sources (yeast extract and peptone) were essential for cell growth and lipase formation. The influence of yeast extract on growth of lipase-producing *S. aureus* ALA1 has been previously investigated. The result denoted that yeast extract can promote the growth of *S. aureus* ALA1 more effectively than tryptone and other inorganic nitrogen sources [186].

Minerals are important components of a microbial cultural medium. In this study, mineral content encouraged positive signals on the growth of *A. baumannii* RMUTT3S8-2. Previous literature mentioned that K_2HPO_4 and $MgSO_4{\cdot}7H_2O$ increased ATP metabolism during microbial growth [109]. Musa et al. [115] indicated that *Marinobacter litoralis* SW-45 grew more readily when exposed to $MgSO_4$, NaCl, K_2HPO_4, and $FeSO_4{\cdot}7H_2O$. By contrast, $FeSO_4{\cdot}7H_2O$ showed signals against growth in this investigation. Microbial growth will be ceased and eventually die under inappropriate metal concentration. The excessive concentration of metal ions damages

the DNA, denatures the protein, and inhibits cell division and transcription. As a result, an adequate amount of metal ions for microbial cultivation depended on the species [114].

Glucose presented positive signals as a carbon source for lipase activity in our study (Figure 4.8B). Similar to a prior study, glucose was the best carbon source and encouraged lipase synthesis [145]; however, excessive glucose concentrations inhibited enzyme synthesis [187]. On the other hand, lipase production by *C. rugosa* was suppressed by glucose due to the CCR phenomenon, which is frequently generated by multiple mechanisms impacting the synthesis of catabolic enzymes [184, 188]. This result also demonstrated that olive oil provided a positive impact on lipase production. As the substrate for microbial enzyme synthesis, it was an appropriate lipid carbon source for inducing lipase synthesis [145].

Peptone had a detrimental effect on *A. baumannii* RMUTT3S8-2 lipase activity in our investigation same as *B. subtilis* strain Kakrayal 1 (Figure 4.8B) [189]. However, this result contradicted previous reports that peptone was a key inducer and organic nitrogen source for lipase synthesis [94]. The influence of peptone on *A. baumannii* RMUTT3S8-2 lipase production requires further detailed study.

This result also found that $FeSO_4 \cdot 7H_2O$ exhibited the most positive effect on lipase production, followed by yeast extract, K_2HPO_4, glucose, olive oil, $MgSO_4 \cdot 7H_2O$, and NaCl (Figure 4.8B). Minerals have the potential to influence microbial growth through altering or maintaining the structure and function of enzymes by attaching to a specific site on their surfaces. Minerals were also identified as an important cofactor influencing enzyme activity [3]. Previous studies documented that NaCl, $MgSO_4$, KCl and $FeSO_4$ in a culture medium positively impacted lipase production in *Bacillus* sp. [190]. However, $FeSO_4$ presented a negative effect on lipase production by *B. cereus* ASSCRC-P1 [191]. A few studies showed that K_2HPO_4 had a similar positive effect on the lipase synthesis of *Streptomyces sclerotialus* [192]. By contrast, lipase production of *Pseudomonas guariconesis* decreased with increasing K_2HPO_4 concentration [193]. Magnesium ions influenced the solubility and behavior of ionized FAs at interfaces, as well as changes in the catalytic properties of the enzyme. Furthermore, magnesium was a necessary mineral ion for the activity and stability of

halophilic proteins [194], while NaCl positively impacted lipase production of *M. litoralis* SW-45 and *P. aeruginosa* UKHL1 [7, 115].

The variables *C* (peptone), *D* (yeast extract), and *J* (NaCl) were the main desired independent variables that impacted both TCV and lipase activity. Thus, these three variables were chosen for further optimization by the RSM method, with the other variables kept constant at the following fixed values: *A*, 1.5 g/L; *B*, 30 g/L; *F*, 1 g/L; *G*, 5 g/L; *H*, 0.3 g/L for the next experiments.

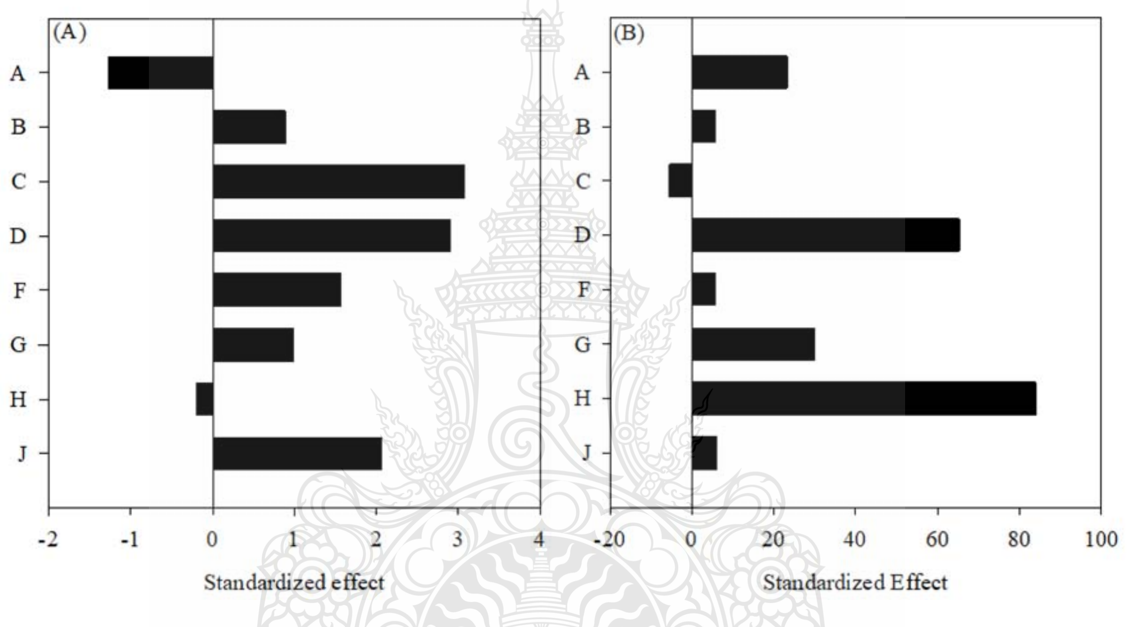

Figure 4.8 Standardized effect of (A) TVC and (B) lipase activity of *A. baumannii* RMUTT3S8-2. A: glucose, B: olive oil, C: peptone, D: yeast extract, F: $MgSO_4 \cdot 7H_2O$, G: K_2HPO_4, H: $FeSO_4 \cdot 7H_2O$, J: NaCl.

Table 4.6 PBD for eight variables with actual values, total viable cell count and lipase activity of *A. baumannii* RMUTT3S8-2.

Run no.	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*	TVC (LogCFU/mL)	Lipase activity (U/mL)
1	3 (1)	30 (1)	1 (-1)	10 (1)	(1)	1 (1)	0.5 (-1)	0.1 (-1)	1 (-1)	(1)	(-1)	7.62 ± 0.01	120.00 ± 4.45
2	0 (-1)	30 (1)	10 (1)	1 (-1)	(1)	1 (1)	5 (1)	0.1 (-1)	1 (-1)	(-1)	(1)	9.82 ± 0.02	22.62 ± 6.60
3	3 (1)	10 (-1)	10 (1)	10 (1)	(-1)	1 (1)	5 (1)	0.5 (1)	1 (-1)	(-1)	(-1)	10.21 ± 0.03	229.94 ± 0.87
4	0 (-1)	30 (1)	1 (-1)	10 (1)	(1)	0.1 (-1)	5 (1)	0.5 (1)	10 (1)	(-1)	(-1)	10.23 ± 0.01	199.34 ± 11.05
5	0 (-1)	10 (-1)	10 (1)	1 (-1)	(1)	1 (1)	0.5 (-1)	0.5 (1)	10 (1)	(1)	(-1)	10.01 ± 0.05	110.15 ± 1.27
6	0 (-1)	10 (-1)	1 (-1)	10 (1)	(-1)	1 (1)	5 (1)	0.1 (-1)	10 (1)	(1)	(1)	10.30 ± 0.05	124.19 ± 12.12
7	3 (1)	10 (-1)	1 (-1)	1 (-1)	(1)	0.1 (-1)	5 (1)	0.5 (1)	1 (-1)	(1)	(1)	2.77 ± 0.08	135.42 ± 6.60
8	3 (1)	30 (1)	1 (-1)	1 (-1)	(-1)	1 (1)	0.5 (-1)	0.5 (1)	10 (1)	(-1)	(1)	5.90 ± 0.09	130.59 ± 1.89
9	3 (1)	30 (1)	10 (1)	1 (-1)	(-1)	0.1 (-1)	5 (1)	0.1 (-1)	10 (1)	(1)	(-1)	8.81 ± 0.03	98.45 ± 5.00
10	0 (-1)	30 (1)	10 (1)	10 (1)	(-1)	0.1 (-1)	0.5 (-1)	0.5 (1)	1 (-1)	(1)	(1)	9.45 ± 0.03	166.94 ± 4.74
11	3 (1)	10 (-1)	10 (1)	10 (1)	(1)	0.1 (-1)	0.5 (-1)	0.1 (-1)	10 (1)	(-1)	(1)	10.07 ± 0.07	75.94 ± 1.25
12	0 (-1)	10 (-1)	1 (-1)	1 (-1)	(-1)	0.1 (-1)	0.5 (-1)	0.1 (-1)	1 (-1)	(-1)	(-1)	3.15 ± 0.00	27.34 ± 7.05

Note: (-1) and (+1) represent low and high levels, respectively. *A*: glucose (g/L), *B*: olive oil (g/L), *C*: peptone (g/L), *D*: yeast extract (g/L), *E*: dummy 1, *F*: $MgSO_4 \cdot 7H_2O$ (g/L), *G*: K_2HPO_4 (g/L), *H*: $FeSO_4 \cdot 7H_2O$ (g/L), *J*: NaCl (g/L), *K*: dummy 2, *L*: dummy 3.

4.5.2 Optimization by BBD

The most effective variables for TVC and lipase activity were chosen from PBD as *C*, *D* and *J*. Optimization of growth and lipase-producing condition of *A. baumannii* RMUTT3S8-2 in flask scale were investigated by BBD (Table 3.3). TVC and lipase activity retrieved from the experiments were 9.73 ± 0.02 to 10.40 ± 0.04 LogCFU/mL and 81.68 ± 2.64 to 214.35 ± 1.26 U/mL, respectively (Table 4.7). ANOVA was used to determine the response surface quadratic model (Table 4.8). The TVC model was not significant (p-value > .05). Actual values of TVC for all treatments were close to 10.0 LogCFU/mL, indicating that the growth of *A. baumannii* was not significantly different under the range of experimental variables. The TVC model was not further focused on defining the optimal condition.

By contrast, the lipase activity model values were significantly different (p-value ≤ .05), indicating that lipase production was well described by this model (Table 4.8). The F-value (19.78) demonstrated that the model was suitable for simulating lipase production with any combination of the variables tested. High F-values and very low p-values indicated that the model accurately predicted lipase production [1].

The coefficient of determination (R^2) of TVC was 0.7937, implying that the model explained the responses at 79.37 % variability. Betiku and Taiwo [195] recommended that the correlation coefficient should be at least 0.80. A high R^2 value indicates good agreement between the predicted and observed results within the range of the experiment. While the R^2 of lipase activity was 0.9622, showing a relatively high correlation between the actual and predicted values. This result demonstrated that the lipase activity model described 96.22 % variability of the response with 3.78 % of the total variation unexplained. The F-value for lack of fit for both answer variables indicated that the lack of fit was insignificant compared to the pure error. Non-significant lack of fit is suitable for a model to be fit [1].

The quadratic equations of TVC (Y_1, LogCFU/mL) and lipase activity (Y_2, U/mL) were fitted to the actual values and further generated as Eqs. (6) and (7):

$$Y_1 = -9.81 - 0.0184C + 0.0318D + 0.0097J - 0.0004CD - 0.0002CJ - 0.0011DJ - 0.0007C^2 - 0.0002D^2 + 0.0014J^2 \quad (6)$$

$$Y_2 = -19.74 + 6.35C + 7.63D + 6.29J - 0.0665CD - 0.1334CJ - 0.1742DJ - 0.0611C^2 - 0.0627D^2 + 0.0693J^2 \quad (7)$$

where C, D, and J are the concentration (g/L) of peptone, yeast extract, and NaCl, respectively.

In this study, three-dimensional (3D) response surface plots were created by Design Expert 13.0 software to examine the optimal concentration for all possible combinations. The 3D response surface plots successfully illustrated the effects of variable levels and their interaction on TVC and lipase production (Figure 4.9-4.14). The interactive effect of independent variables on TVC and lipase production was analyzed by changing the levels of two variables while keeping the other variable constant at the center point [1, 7].

Concentrations of C and J in this range were not significant for the growth of *A. baumannii* RMUTT3S8-2 (p-value > .05) (Table 4.7 and Figures 4.9 and 4.10). By contrast, only D significantly affected TVC (p-value $\leq$ .05) and TVC increased when yeast extract concentration increased (Figure 4.9 and 4.11). Yeast extract has been described as one of the most crucial sources of nitrogen, rich in amino acids, minerals and vitamins for the metabolism and proliferation of microorganisms, and also necessary for the production of RNA, DNA, and enzymes [94].

The 3D surface plots were illustrated to describe the effects of variable levels and their interactions on lipase production (Figure 4.12-4.14). The result demonstrated that increasing C, D, and J concentrations could significantly enhance lipase activity. The interaction plot between C and D (Figure 4.12) demonstrated that lipase activity increased with rising variables C and D, resulting in lipase production of >200 U/mL. Similar to the interaction of C and D, enzyme activity reached 200 U/mL when C and J were interacted (Figure 4.13). Concentrations of C and J were optimal at 29-35 and 13-15 g/L, respectively. Surface plots of D and J for lipase production (Figure 4.14) showed that maximum lipase production was obtained at 24-35 g/L of D.

Peptone and yeast extract supplied amino acids, vitamins, cofactors, and trace metals, which encouraged the growth and metabolism of bacteria. Therefore, increased bacterial cells resulted in lipase production [96]. This result also indicated that

A. baumannii RMUTT3S8-2 could grow and produce lipase at 13.0-15.0 g/L NaCl, similar to *Staphylococcus capitis* SH6 [150]. However, ranges of NaCl suitable for lipase production differed depending on microorganism species.

The desired point prediction of RSM was analyzed as the optimal condition of lipase production. The optimized concentration of peptone (24.49 g/L) with yeast extract (33.82 g/L) and NaCl (6.21 g/L) provided maximum lipase activity of 215.23 U/mL. The actual experiment was performed to validate the predicted optimal condition, with the results of lipase activity at 216.23 ± 3.69 U/mL, and not significantly different from the predicted value at α = 0.05. Moreover, TVC reached 10.36 ± 0.01 LogCFU/mL at 72 h of cultivation under this condition. This result suggested that the model was accurate and reliable for predicting lipase production by *A. baumannii* RMUTT3S8-2. The optimization procedure also increased lipase activity higher than the non-optimized condition by 2.2 times.

Table 4.7 BBD of the variables and experimental results regarding *A. baumannii* RMUTT3S8-2 growth and lipase activity.

Run	*C*	*D*	*J*	TVC (LogCFU/mL)		Lipase activity (U/mL)	
				Actual value	Predicted value	Actual value	Predicted value
1	5 (-1)	5 (-1)	8 (0)	10.01 ± 0.08	10.00	81.68 ± 2.64	87.82
2	35 (+1)	5 (-1)	8 (0)	10.24 ± 0.03	10.20	165.04 ± 0.42	162.97
3	5 (-1)	35 (+1)	8 (0)	10.33 ± 0.00	10.36	187.67 ± 2.96	189.73
4	35 (+1)	35 (+1)	8 (0)	10.21 ± 0.05	10.23	211.21 ± 0.40	205.07
5	5 (-1)	20 (0)	1 (-1)	10.33 ± 0.04	10.24	145.37 ± 6.11	133.55
6	35 (+1)	20 (0)	1 (-1)	10.40 ± 0.04	10.32	210.43 ± 5.56	208.61
7	5 (-1)	20 (0)	15 (+1)	10.28 ± 0.02	10.36	175.38 ± 0.61	179.00
8	35 (+1)	20 (0)	15 (+1)	10.25 ± 0.03	10.35	184.41 ± 1.92	196.23
9	20 (0)	5 (-1)	1 (-1)	9.73 ± 0.02	9.85	109.85 ± 5.20	115.53
10	20 (0)	35 (+1)	1 (-1)	10.22 ± 0.04	10.28	214.35 ± 1.26	224.10
11	20 (0)	5 (-1)	15 (+1)	10.23 ± 0.02	10.17	179.29 ± 4.04	169.53
12	20 (0)	35 (+1)	15 (+1)	10.24 ± 0.02	10.12	210.64 ± 3.00	204.96
13	20 (0)	20 (0)	8 (0)	10.08 ± 0.00	10.08	193.38 ± 11.72	189.24
14	20 (0)	20 (0)	8 (0)	10.04 ± 0.00	10.08	194.19 ± 6.54	189.24
15	20 (0)	20 (0)	8 (0)	10.18 ± 0.00	10.08	192.76 ± 2.94	189.24
16	20 (0)	20 (0)	8 (0)	10.03 ± 0.00	10.08	183.85 ± 8.17	189.24
17	20 (0)	20 (0)	8 (0)	10.08 ± 0.00	10.08	182.02 ± 8.27	189.24

C: peptone (g/L), *D*: yeast extract (g/L) and *J*: NaCl (g/L)

Table 4.8 ANOVA of experimental results.

Source	Degree of freedom	TVC		Lipase activity	
		F-value	*p*-value	*F*-value	*p*-value
Model	9	2.9900	0.0812	19.7800	0.0004*
C- Peptone	1	0.1990	0.6690	36.7600	0.0005
D-Yeast extract	1	6.5100	0.0380	93.1000	< 0.0001
J-NaCl	1	1.0300	0.3432	5.4600	0.0522^{ns}
CD	1	2.4200	0.1634	8.0300	0.0253
CJ	1	0.1684	0.6938	7.0500	0.0327
DJ	1	4.7500	0.0656	12.0100	0.0105
C^2	1	9.2200	0.0189	7.1300	0.0320
D^2	1	0.8027	4.0000	7.5200	0.0288
J^2	1	1.7400	0.2292	0.4356	0.5304
Residual	7				
Lack of Fit	3	6.5600	0.0503	6.3600	0.0529
Pure Error	4				
Cor Total	16				

R^2 of TVC = 0.7937 and R^2 of lipase activity = 0.9622.

* Significant *p*-value at < .05, and ^{ns} not significant *p*-value at > .05

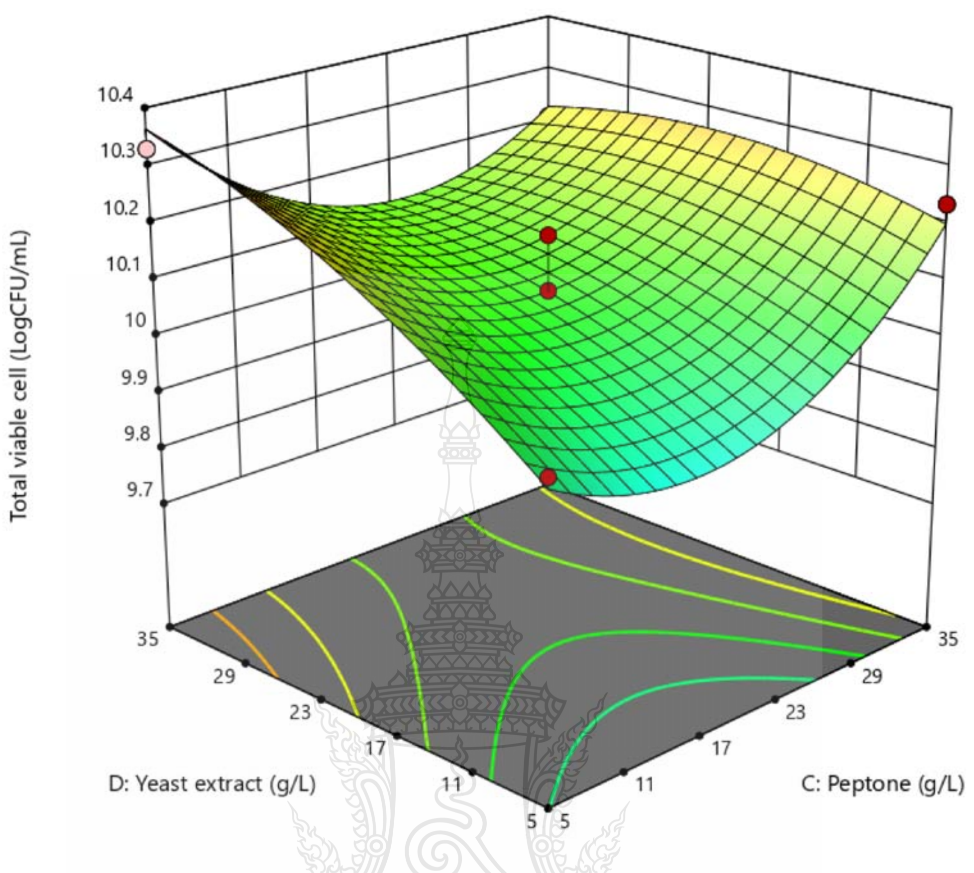

Figure 4.9 3D response surface plots and their interaction between peptone and yeast extract variables for TVC of *A. baumannii* RMUTT3S8-2.

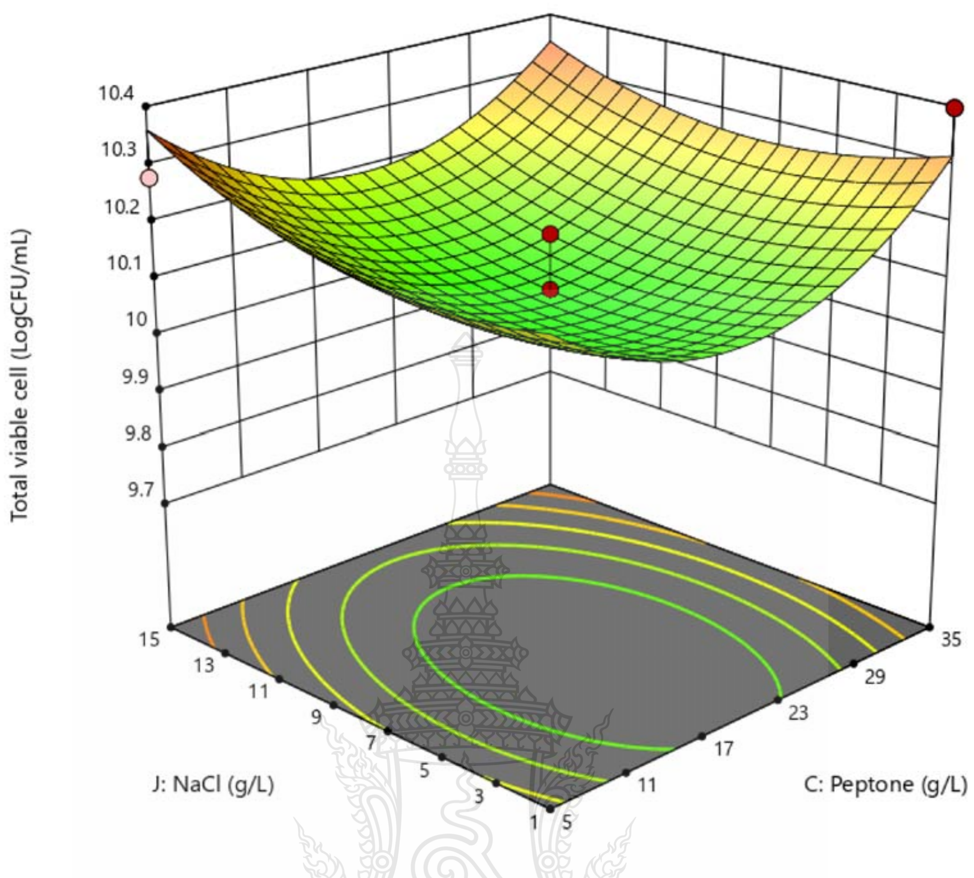

Figure 4.10 3D response surface plots and their interaction between peptone and NaCl variables for TVC of *A. baumannii* RMUTT3S8-2.

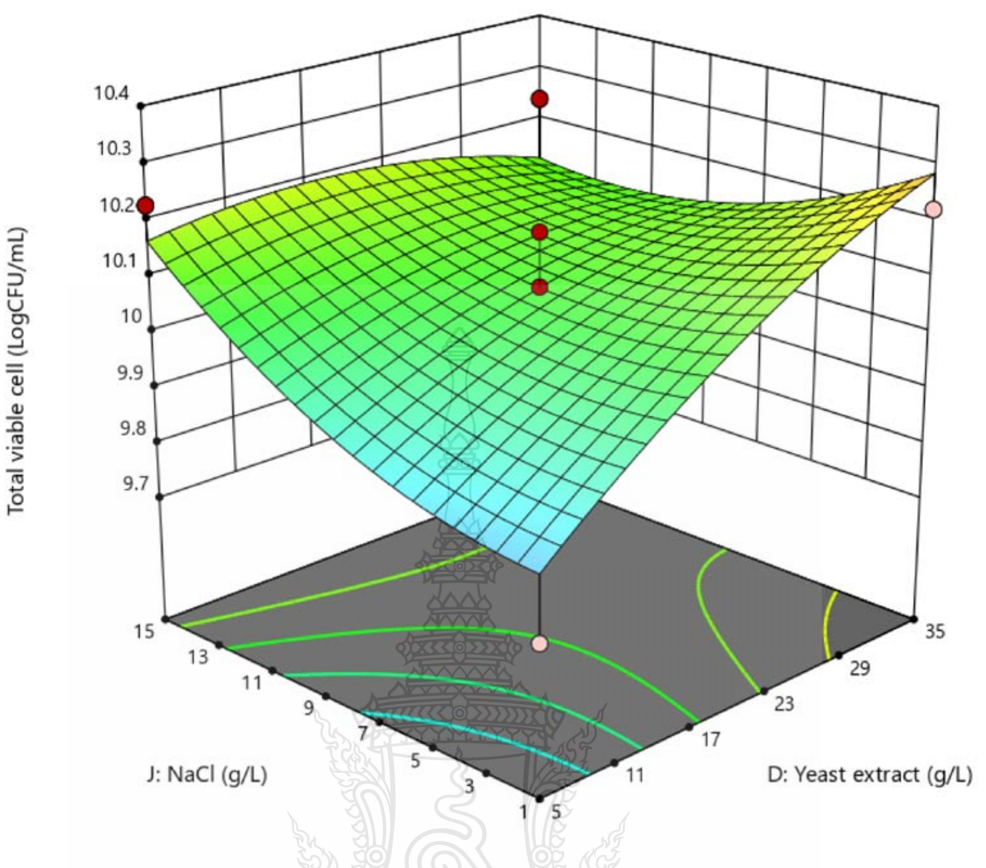

Figure 4.11 3D response surface plots and their interaction between yeast extract and NaCl variables for TVC of *A. baumannii* RMUTT3S8-2.

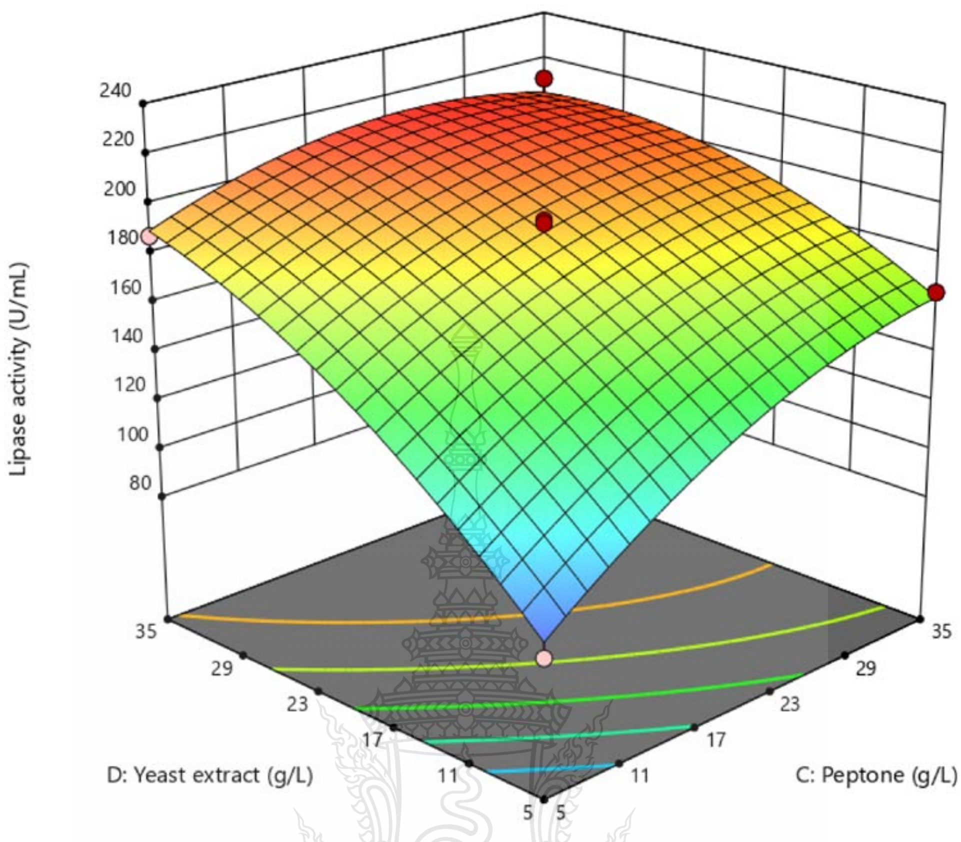

Figure 4.12 3D response surface plots and their interaction between peptone and yeast extract variables for lipase activity of *A. baumannii* RMUTT3S8-2.

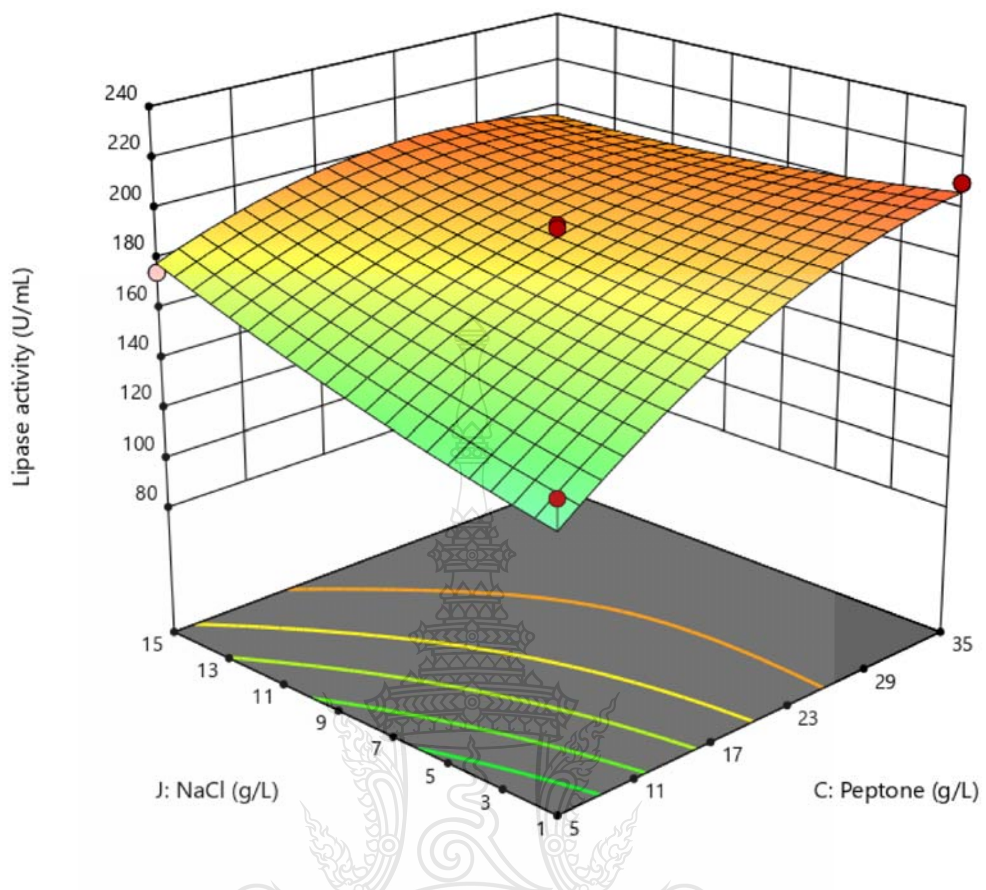

Figure 4.13 3D response surface plots and their interaction between peptone and NaCl variables for lipase activity of *A. baumannii* RMUTT3S8-2.

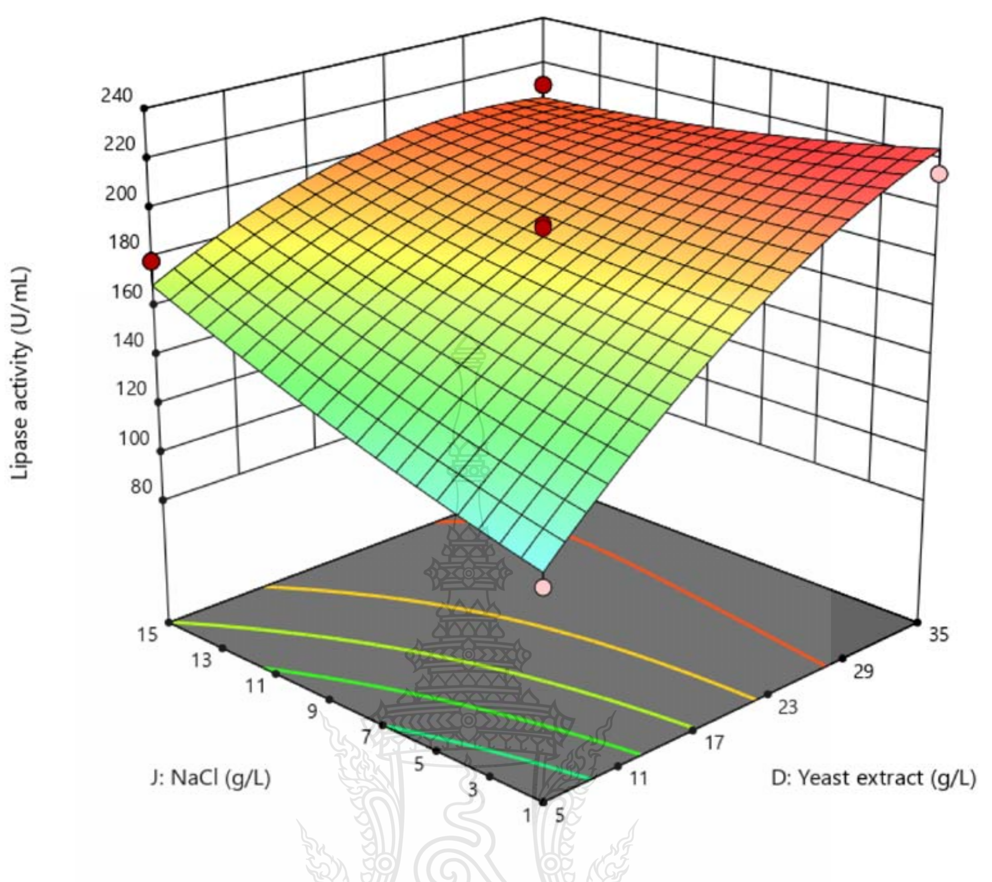

Figure 4.14 3D response surface plots and their interaction between yeast extract and NaCl variables for lipase activity of *A. baumannii* RMUTT3S8-2.

4.6 Evaluation of Lipase Stability

The effects of pH and temperature on lipase stability were investigated at different pH and temperatures (Figure 4.15). Relative lipase activity was used to evaluate the remaining enzyme activity as lipase stability under experimental conditions. The effect of pH on lipase stability was determined from 5.0 to 9.0. Relative lipase activity was not significantly different (p-value ≤ .05) under this experimental pH level at the same temperature, demonstrating that crude lipase of *A. baumannii* RMUTT3S8-2 was still active over a range of pH levels from 5.0 to 9.0. The stability of lipase at wide-ranging pH is a valuable property for use in different pH oily wastewaters, especially poultry processing factories.

Activity and stability were essential properties of the enzyme when used at the proper pH level. pH level influenced the stability of the enzyme. Most enzymes show reduced activity at either extremely high or low pH values. Previous reports indicated that the lipase of *Acinetobacter* sp. provided stability over a broad pH range from 6.0 to 12.0 [196], while the lipase of *A. calcoaceticus* 1-7 remained stable in acid condition at pH 4.0 [197]. Results demonstrated that lipase obtained from the *Acinetobacter* genus presented high stability at wide-ranging pH levels. Lipase from this strain could be used in several industries, such as bioenergy, organic compound synthesis, pharmaceutical, and detergent.

The effects of temperature on lipase stability were explored at 30 °C to 70 °C in this study (Figure 4.15). Results revealed that the highest relative lipase activity was observed at 30 °C for all experimental pH levels with > 95 % residual activity. Although, lipase stability decreased significantly (p-value ≤ .05) when the temperature increased to over 30 °C. However, the enzyme activity was not significantly different (p-value > .05) and retained 87.97 ± 3.11 to 91.36 ± 4.23 % of its activity at 50 °C to 70 °C after 1 h of incubation. This result was similar to Kuan et al. [198], who reported that the lipase activity from *Acinetobacter johnsonii* LP28 was > 95 % at 30 °C. Compared to the lipase produced by *Geobacillus* sp. 12AMOR1, the relative lipase activity at 50 °C was closely associated with *A. baumannii* RMUTT3S8-2 lipase. However, it decreased to nearly 50 % at 70 °C in 1 h [166]. This result demonstrated that the lipase of *A. baumannii* RMUTT3S8-2 was a thermostable enzyme.

Thermostable lipase is one of the most helpful enzymes used in biotechnology applications [199].

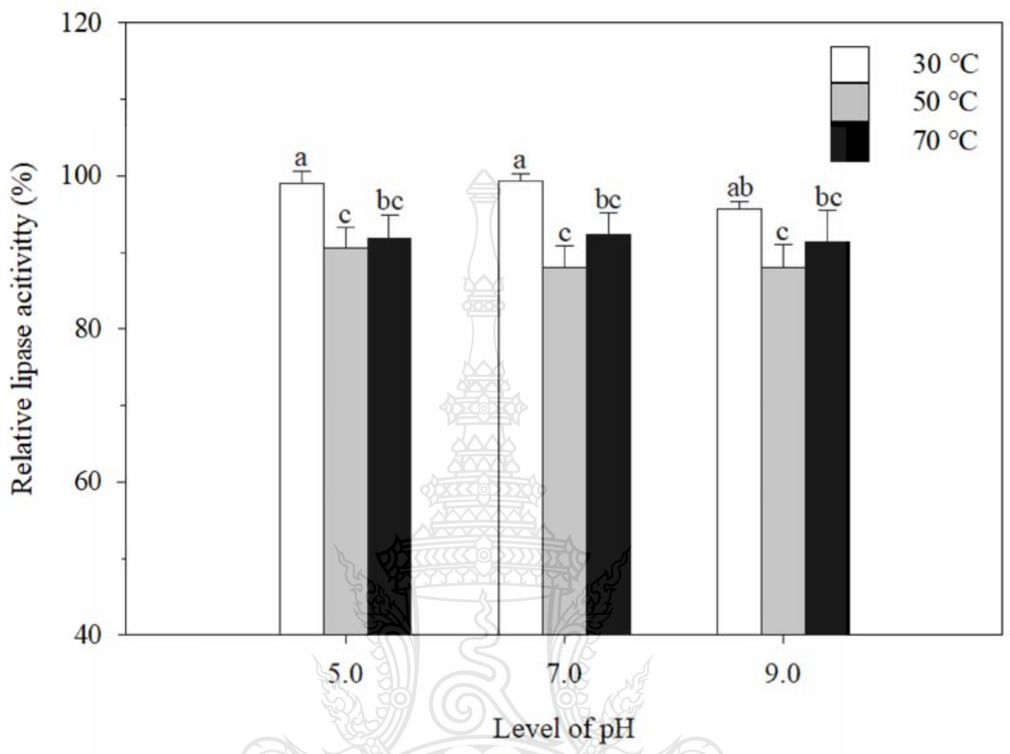

Figure 4.15 The relative lipase activity on pH level and temperature. The bar represents the mean ± SD. Different letters (a, b, c) present significant differences at p-value ≤ .05.

4.7 Lipase Production in Bioreactor

The profile of lipase production by *A. baumannii* RMUTT3S8-2 in a 5-L bioreactor is illustrated in Figure 4.16. As previously mentioned, *A. baumannii* was grown in 3 L of the optimized medium under aerobic condition. Growth of *A. baumannii* was rapid, with TVC increasing from 7.62 ± 0.61 to 10.36 ± 0.00 LogCFU/mL within 12 h of cultivation. Thereafter, the TVC value of *A. baumannii* was increased and remained between 10.36 ± 0.00 and 10.58 ± 0.20 LogCFU/mL up to 144 h. The specific growth rate of *A. baumannii* cultivation expressed by the mathematical model of Baranyi and Robert was 0.261 h^{-1} (https://browser.combase.cc/DMFit.aspx).

Simultaneously, lipase production increased, relating to the growth of *A. baumannii*. Lipase production increased quickly to 126.07 ± 44.66 U/mL in 12 h and then continuously raised up to 251.62 ± 11.86 U/mL after 144 h of production. This study produced 16 % higher lipase than the Erlenmeyer flask scale, suggesting that aeration, agitation, dissolved oxygen, and pH control affected lipase production on a bioreactor scale. Indicating that growth and lipase activity in a bioreactor differed from the Erlenmeyer flask [200]. However, there is no information about the effect of aeration and agitation on lipase production from *A. baumannii* in previous literature. Understanding these effects is useful for incrementing lipase production by this strain in bioreactors for commercial use.

Results of lipase production were compared for the effectiveness of lipase-producing strains (Table A1). Lipase was produced by various microorganisms such as *T. permensis* M35-15, *B. aryabhattai* SE3-PB, *B. ubonensis* SL-4 and *Acinetobacter* sp. AU07 [1, 5, 10, 11]. Here, a newly isolated *A. baumannii* RMUTT3S8-2 produced lipase at the same level as *B. aryabhattai* SE3-PB. In addition, *A. baumannii* RMUTT3S8-2 gave higher lipase activity than *Acinetobacter* sp. AU07, demonstrating that the performance of microbial lipase production depended on the strain and culture condition. These data indicated that RMUTT3S8-2 was an effective strain for lipase production. However, other lipase properties, such as hydrolyzed FAs, transesterification, and ester hydrolysis, of *A. baumannii* RMUTT3S8-2 should be further studied to evaluate the possible use for industry.

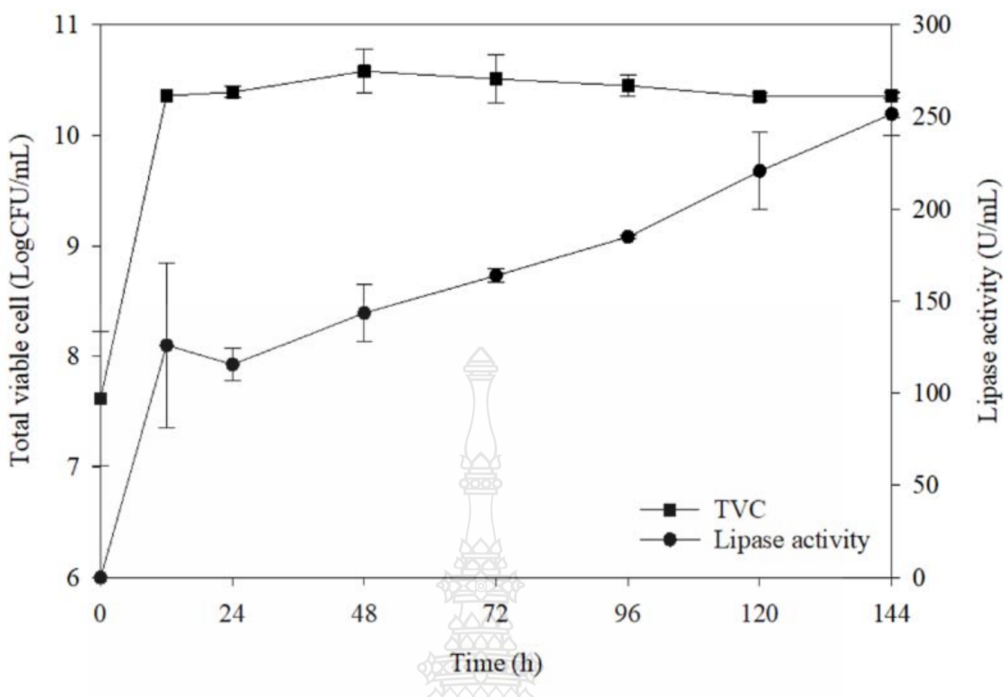

Figure 4.16 The TVC and lipase activity in the 5-L bioreactor. The data represents the mean ± SD bar.

4.8 Hydrolysis of Oil

4.8.1 Hydrolysis of oil in wastewater of poultry processing factory

The hydrolysis efficiency of *A. baumannii* RMUTT3S8-2 inoculum was investigated in a 20-L aerated plastic tank. Various quantities of inoculum were added to wastewater from a poultry processing factory. The powder inoculum (PI) contained TVC and lipase activity of 8.08 ± 0.01 LogCFU/g and 144.00 ± 0.11 U/mL, respectively, and the liquid inoculum (LI) contained TVC and lipase activity was 7.44 ± 0.01 LogCFU/mL and 204.00 ± 2.78 U/mL, respectively. Results revealed that the quantity of inoculum affected oil hydrolysis efficiency. On the 12 days of operation, the quantity of PI demonstrated the highest oil hydrolysis of 5 % w/v was 41.94 ± 4.98 %, followed by 3% w/v (26.12 ± 5.99 %) and 1% w/v (2.57 ± 0.18 %), respectively (Figure 4.17). However, the oil was slightly hydrolyzed in both LI (1-3 % w/v) and the control experiment. On the 27 days of operation, oil removal of all quantities of inoculum (1-5 % PI and LI) was decreased. According to previous reports, *A. psychrotolerans* could oil hydrolysis of 60 % in 7 days [201]. Awasthi et al. [202]

reported that the *Brevibacillus agri* and *B. cereus* reduced vegetable oil by a maximum of 45.00 % and 65.12 %, respectively. *Bacillus* strain LPB4 could also degrade the lipid content of oil mill waste and dairy waste by 53.3 % and 51.3 %, respectively [203]. *P. aeruginosa* UKHL1 strain and *P. aeruginosa* HFE733 degraded 37 % and 95.44 % oil after 3 and 6 days, respectively [7, 149]. Thus, lipid degradation depends on the microbial strains. Moreover, GC-MS was performed to analyze the VFAs compound obtained from oil hydrolysis. The result shows that *A. baumannii* RMUTT3S8-2 inoculum could be degraded of oil and release VFAs compounds, including acetic acid, propionic acid, butyric acid, and pentanoic acid. Thus, the mention of described, the 5 % PI was chosen to examine the oil hydrolysis in the fat poultry wastewater for the experiment on a pilot scale.

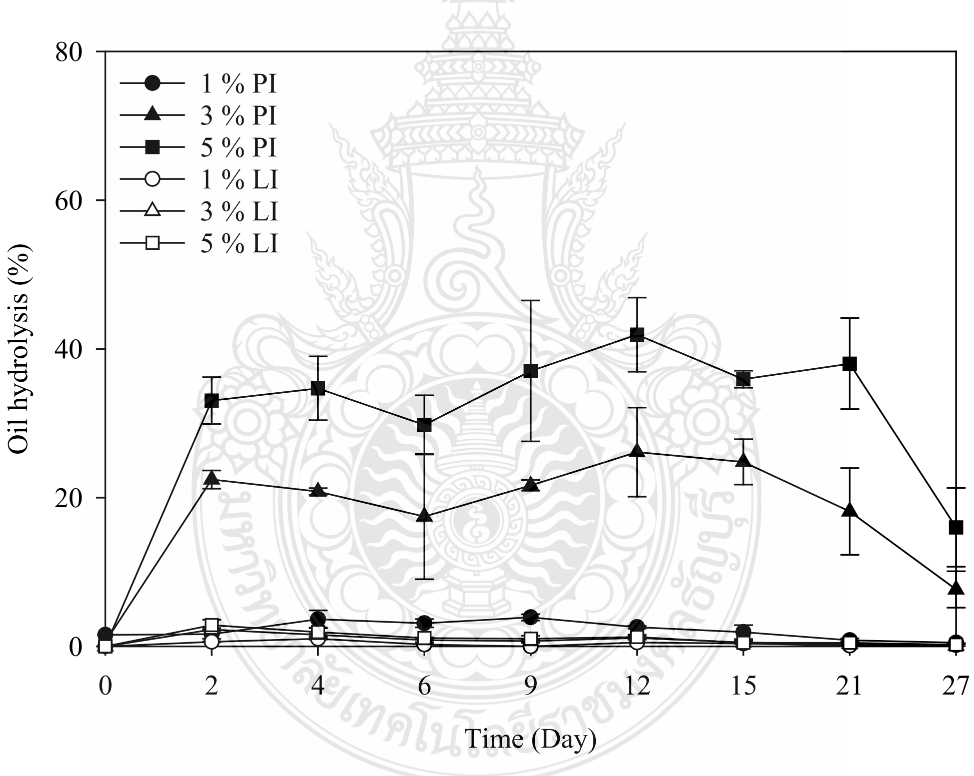

Figure 4.17 Efficiency of lipase-producing inoculum in batch operation. The data represents the mean ± SD bar.

4.8.2 Hydrolysis of oil in wastewater of poultry processing factory on a pilot scale

The hydrolysis efficiency of *A. baumannii* RMUTT3S8-2 inoculum was investigated in a 200-L plastic tank with a continuous treatment process. The powder (PI) and powder in a gauze bag (PGBI) inoculum were added to wastewater from a poultry processing factory 150-L. The PI contained TVC of 8.62 ± 0.20 LogCFU/g and lipase activity of 291.17 ± 21.47 U/mL. PI and PGBI at 5 % w/v demonstrated rapidly increased hydrolyzed of oil was 79.08 % and 51.40 %, while the oil was slightly hydrolyzed control experiment after 22 h of treatment, as shown in Figure 4.18. Our results are consistent with earlier reports by Verma et al. [148]; the oil hydrolysis from *P. aeruginosa* SL-72 can be reduced crude oil by 82.83 % from the industry of mustard-oil plant in 7 days, and *B. cereus* HSS degraded oil in wastewater treatment by 94.7 % after 72 h [93]. However, the oil hydrolysis was decreased in all conditions after 54 h of the experiment. The highest average oil hydrolysis (54 – 222 h) was obtained from a 5 % PI of 6.95 %. While the oil hydrolysis of 5% PGBI and control were 5.38 and 1.84 %, respectively. Furthermore, the result found that acetic acid, propionic acid, butyric acid, and pentanoic acid were VFAs compounds obtained during the experiments. VFAs are intermediates in the methane formation pathway. They are suitable for applying a substrate for biogas production.

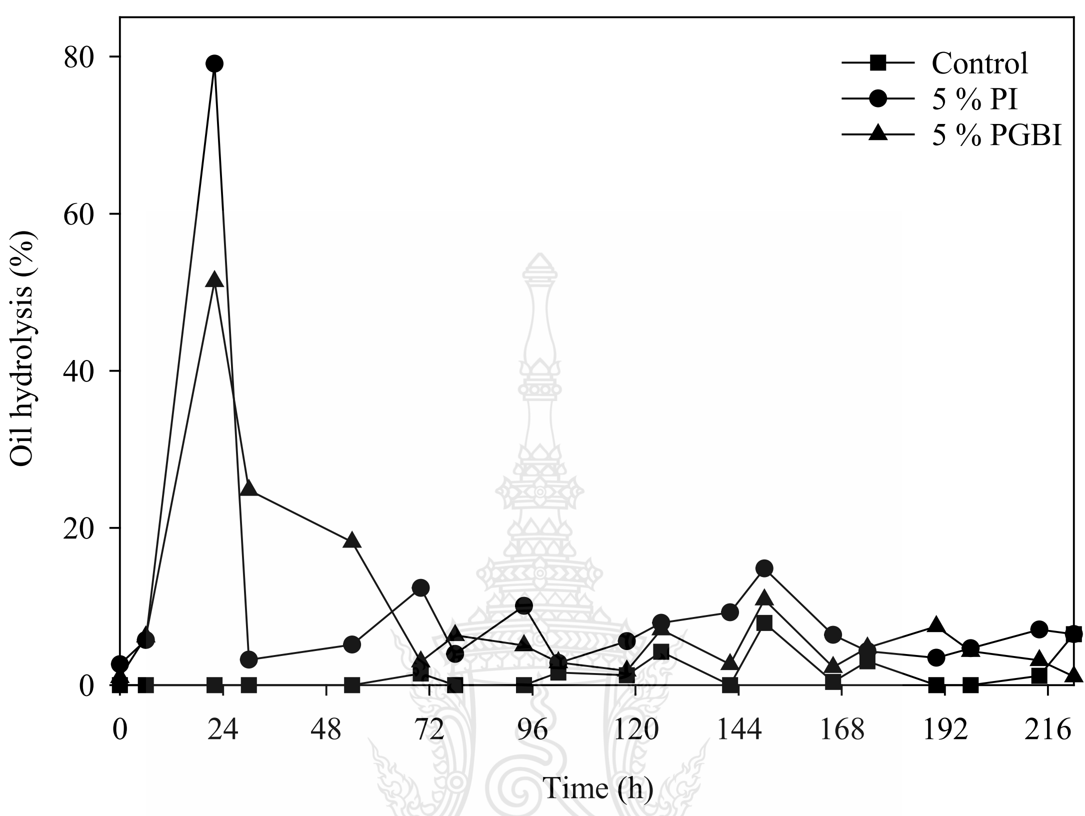

Figure 4.18 Efficiency of lipase-producing inoculum in continuous operation.

4.9 Volatile Fatty Acids

The VFAs compound derived from the hydrolysis of oil in wastewater was explored by GC-MS analysis. The contributed VFAs of the laboratory and pilot scale were presented following:

4.9.1 VFAs profile from laboratory scale-oil hydrolysis

This study investigated VFA compositions in batch experiments. The samples of control (0, 2, and 12 days), 1-5 % LI (2 days), and 1-5 % PGBI (12 days) were selected for VFA analysis. The results exhibited that the inoculum of *A. baumannii* RMUTT3S8-2 could degrade oil in wastewater into VFA compounds. The VFAs were not detected at 0, 2, and 12 days in the control experiment (Figures 4.19 and 4.20). Three percent of LI presented the highest acetic acid of $9.37\times10^4 \pm 2.16\times10^4$ mV.

However, the VFAs of 3 % LI was slightly more than the 5 % LI. Acetic acid was a dominant VFA in the LI conditions at 2 days (Figure 4.19). The 1-5 % PI could degrade oil in wastewater to short-chain FAs, and 5 % PI showed the highest VFAs at 12 days of operation. Five percent PI demonstrated the maximum acetic acid of $219.54 \times 10^4 \pm 53.73 \times 10^4$ mV, followed by propionic, butyric, and pentanoic acids (Figure 4.20). In addition, it was also observed that the quantity of VFAs related to the degree of oil hydrolysis (Figure 4.17). Sukphun et al. [204] reported that the maximum VFA production in batch mode is generally attained in 4–10 days, similar to the 5 % PBGI condition of this research. In batch fermentation, the most VFA composition was acetic, propionic, iso-valeric, and butyric acids [205, 206]. The observed VFAs in this study were also similar to the research of Jomnonkhaow et al. [207]. They employed cow manure as a substrate for VFA production in a batch process. The VFA compounds exhibited the most acetic acid, followed by propionic and butyric acid.

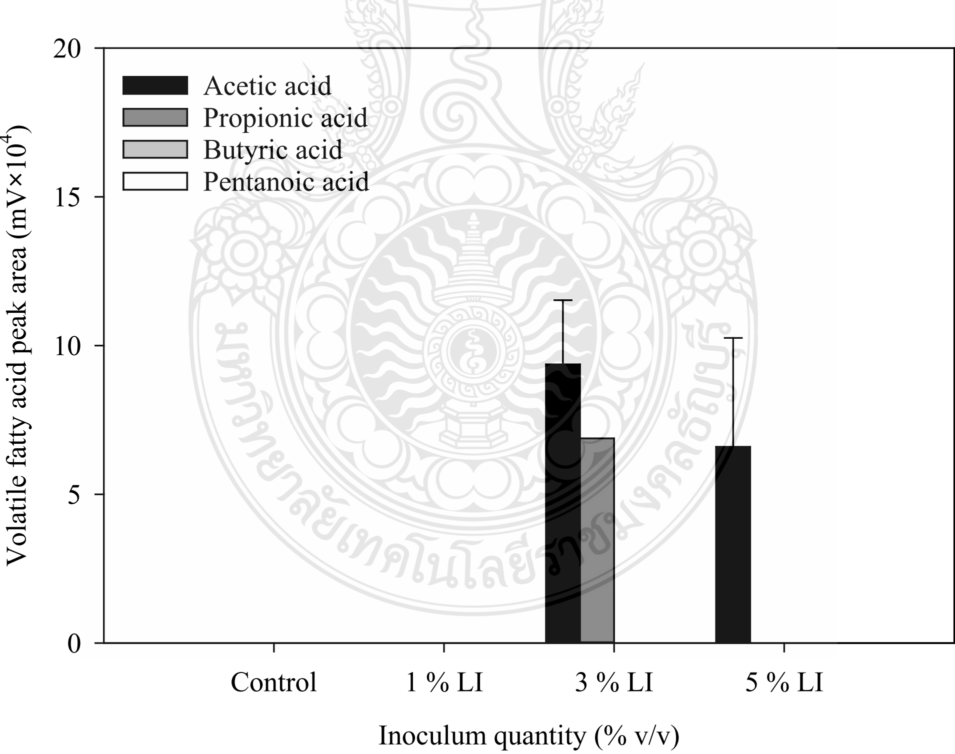

Figure 4.19 VFA compounds of LI in batch experiments. The data represents the mean ± SD bar.

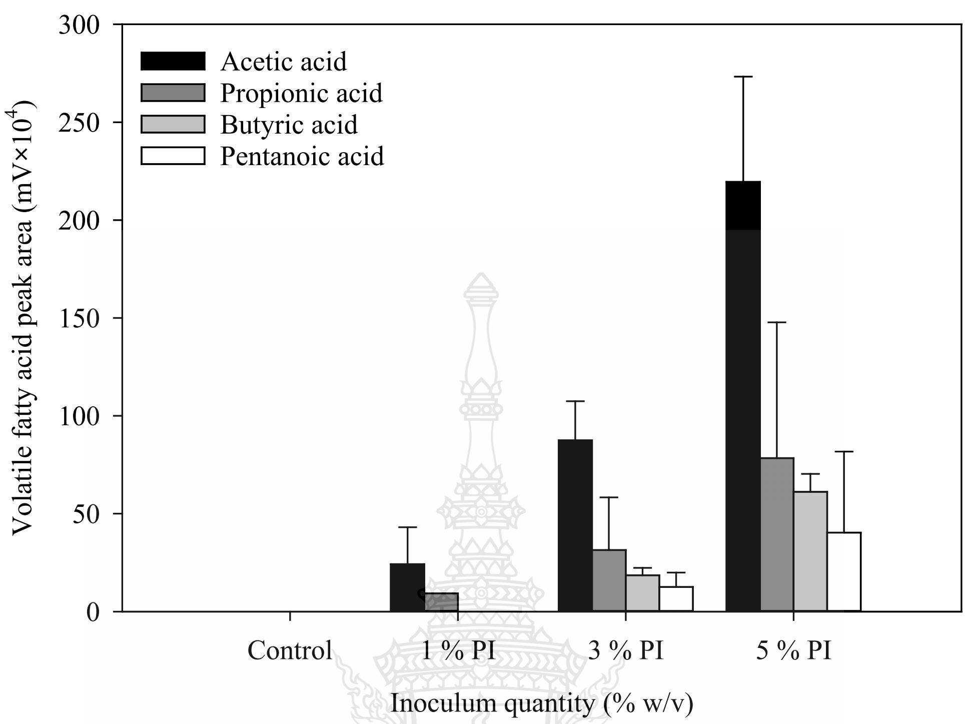

Figure 4.20 VFA compounds of PI in batch experiments. The data represents the mean ± SD bar.

4.9.2 VFAs profile from pilot scale-oil hydrolysis

This treatment used 5 % PI, 5% PGBI, and control (0 % inoculum) as the initial inoculum concentration. The VFAs compound of influent and effluent of oily wastewater in the operation tank system was investigated. Among the three experiments, 5 % PGBI presented the highest VFAs, followed by 5 % PI, control, and influent conditions, as shown in Figure 4.20-4.24.

The VFAs production in wastewater influent was lower than in other conditions throughout the experiment (Figure 4.21). Acetic and propionic acids were the dominant VFA in wastewater, followed by butyric acid. However, the highest acetic acid was found for $11.46\times10^4 \pm 2.70\times10^4$ mV at 166 h. Figure 4.22 illustrated the VFA composition in the effluence wastewater without lipase-producing inoculum. The result showed that acetic acid was the main VFA, followed by propionic and butyric acids.

Acetic and propionic acids were slightly released in early treatment and then increased rapidly were $24.03\times10^4 \pm 0.82\times10^4$ and $17.78\times10^4 \pm 4.25\times10^4$ mV, respectively, at 126 h. This result suggested that the oil in wastewater was less hydrolyzed, resulting in low VFAs. The wastewater system contains several lipase-producing strains, this may be the reason found the short-chain FAs were in the control and influent conditions. The results agree with Lukitawesa et al. and Bharathi & Rajalakshmi [41, 208] reported that the lipids or organic wastes consist of mixed microbes, including lipase-producing microbes, methanogenic bacteria, pathogenic microbes, and others.

Five percent PI could degrade the oil and release the short-chain FAs. The VFAs production increased in the first period (6-22 h), then decreased slightly. The VFAs rapidly increased may be due to the addition of lipase-producing inoculum to the system resulting in the increasing oil digestion. The oil in wastewater was digested to VFAs, which was consequently relative to oil hydrolysis (Figure 4.18). Afterward, the VFAs rapidly increased during 94-166 h (Figure 4.23). Propionic and acetic acid was the dominant VFA resulting in the highest production of $24.34\times10^4 \pm 1.87\times10^4$ and $21.56\times10^4 \pm 4.77\times10^4$ mV, respectively, at 166 h of operation. However, except for the time of operation at 126 and 166 h, neither butyric acid nor pentanoic acid was not observed.

The 5 % PGBI experiment was quite attractive for VFA production. It exhibited a great trend of VFA production when compared to all experiments, as shown in Figure 4.24. The highest VFA production was gained at 54 h. The various VFA compositions were found as acetic acid ($26.31\times10^4 \pm 1.22\times10^4$ mV), propionic acid ($29.21\times10^4 \pm 7.50\times10^4$ mV), butyric acid ($16.69\times10^4 \pm 4.22\times10^4$ mV), and pentanoic acid ($8.42\times10^4 \pm 2.03\times10^4$ mV). Especially propionic acid and acetic acid were the dominant products in this experiment.

Even though the efficiency of oil digestion in wastewater encourages VFA production, as shown in Figures 4.23 to 4.24, the high VFA compound affects the pH of wastewater. It disrupts cell metabolism and cell growth [209]. Furthermore, the decline of VFAs may relate to methanogenic bacteria in oily wastewater, which are VFA-consuming bacteria [204]. However, Andersen et al. [210] reported that the

adaptability tolerant to VFA toxicity of microorganisms enhanced the VFAs in a continuous mode similar to this research (Figure 4.23-24).

In this investigation, the concentration of VFAs in the continuous process was lower than in the batch process might be related to the HRT and oil hydrolysis efficiency of the wastewater treatment system. High HRT stimulates the growth of slow-growing methanogens. In contrast, low HRT could reduce methanogens bacteria [204]. Wang et al. [211] reported that continuous mode at a 1.5-day HRT promoted VFA generation and decreased VFA consumption. However, when compared with previous studies considered a very short HRT (8 h) in this study.

Sukphun et al. [204] reported that raising the organic loading rate (OLR) further increases the VFAs accumulation and lowers the pH, resulting in a poor VFA production rate and yield, according to the results of VFA in a continuous process in this study. Adversely, Owusu-Agyeman et al. [209] reported that VFA concentration in the batch mode was lower than in the semi-continuous mode because of the higher initial organic waste concentration during operation.

The VFA compositions depend on pH, temperature, HRT, and type of organic waste [204, 212]. Strazzera et al. [213] discovered that acetic acid was the dominant product in low HRT. Furthermore, the type and concentration of organic waste and inoculum influenced the metabolic pathways of microorganisms in the hydrolysis and acidification process for the VFAs production [214]. The distribution of the VFAs profile was altered by the increased substrate concentration [211].

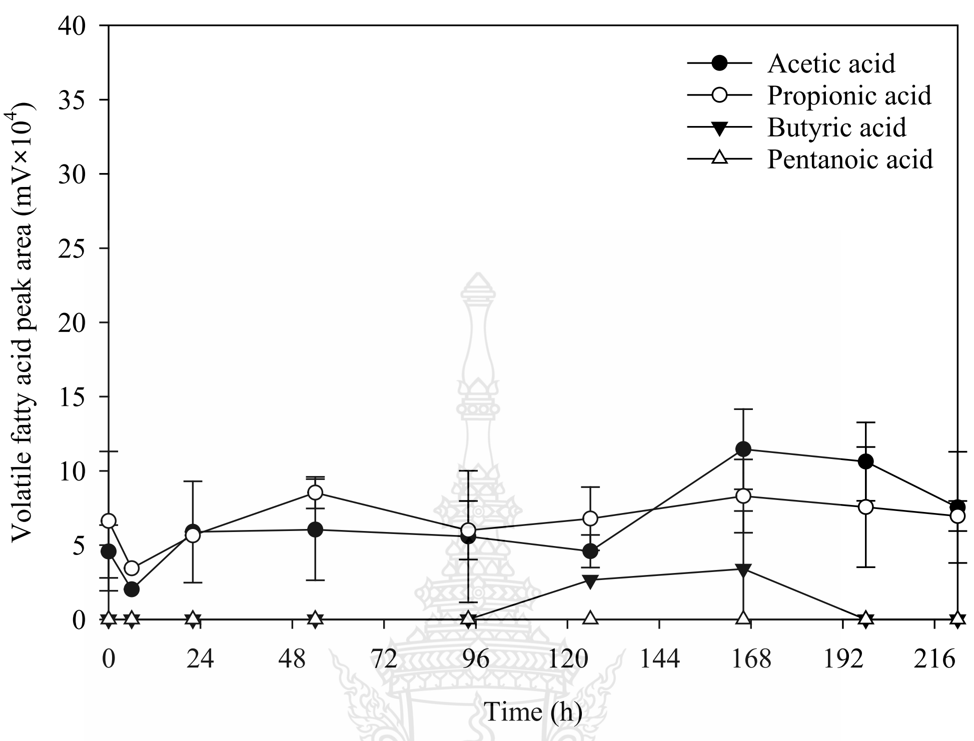

Figure 4.21 VFA compounds of influent wastewater in continuous experiment. The data represents the mean ± SD bar.

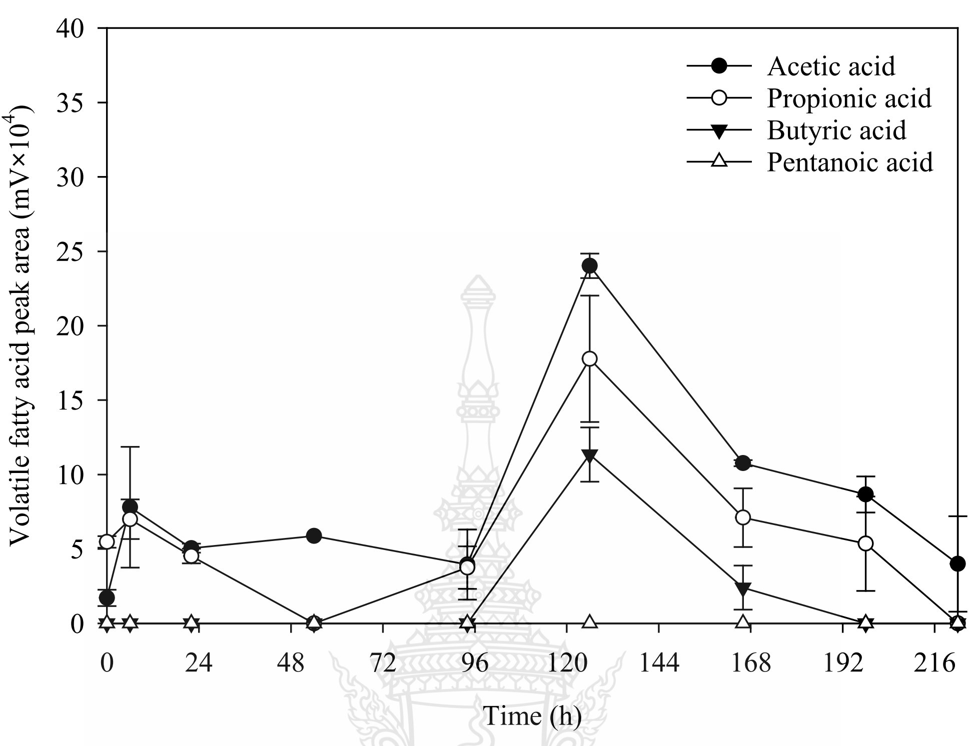

Figure 4.22 VFA compounds of effluence wastewater of control condition in continuous experiment. The data represents the mean ± SD bar.

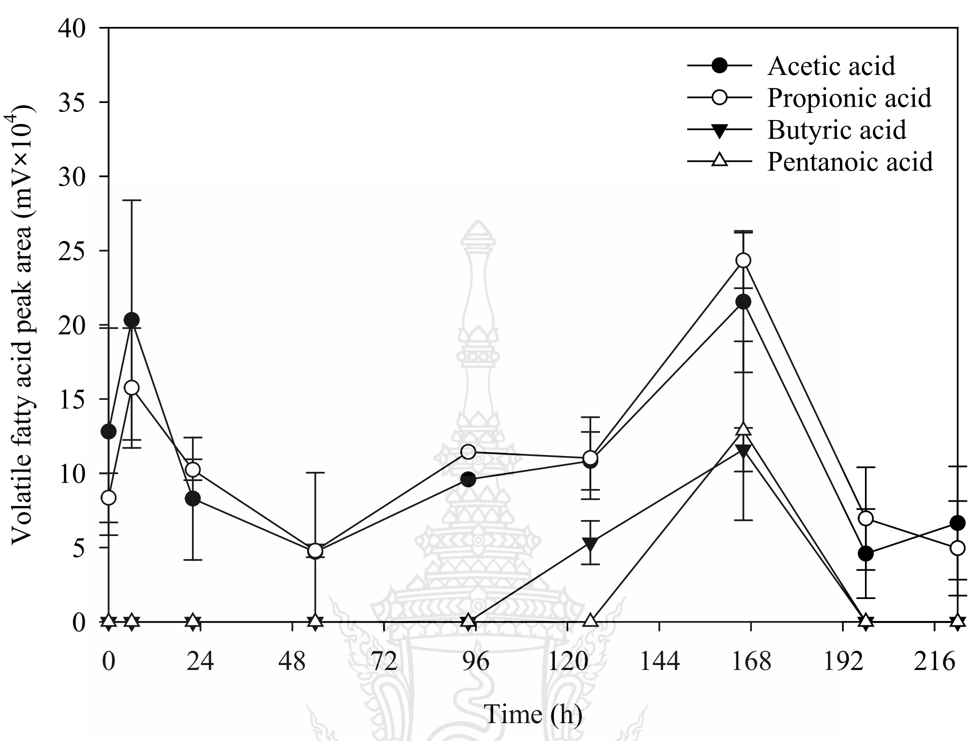

Figure 4.23 VFA compounds of effluence wastewater adding 5 % PI in continuous experiment. The data represents the mean ± SD bar.

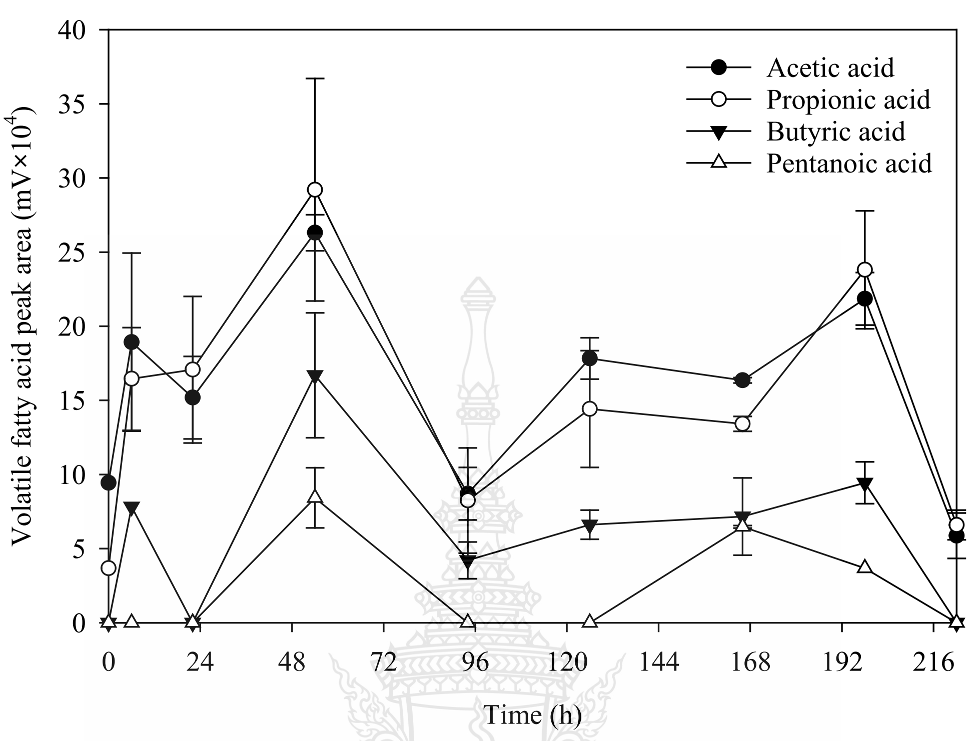

Figure 4.24 VFA compounds of effluence wastewater adding 5 % PGBI in continuous experiment. The data represents the mean ± SD bar.

CHAPTER 5
CONCLUSIONS

Thirty-one lipase-producing strains were obtained from an oily wastewater treatment pond in a poultry processing factory. The top five lipase-producing bacteria were molecularly identified as *Sphingomonas* sp. (RMUTT2S3-2), *Burkholderia thailandensis* (RMUTT2S4-2), *Enterobacter cloacae* (RMUTT3S5-1), *Acinetobacter baumannii* (RMUTT3S8-2) and *Aeromonas caviae* (RMUTT3S8-3). *A. baumannii* RMUTT3S8-2 provided the highest lipase activity of 97.43 ± 4.29 U/mL under unoptimized conditions and was chosen as lipase producers in this study. The PBD and BBD were employed to achieve the optimum cultural condition of *A. baumannii* RMUTT3S8-2. Lipase activity obtained under optimum conditions was enhanced by 2.2 times more than the unoptimized condition. The lipase stability remained in the pH range of 5.0 to 9.0 at 30 °C for > 95 %.

Subsequently, *A. baumannii* RMUTT3S8-2 was prepared as the powder inoculum for oil hydrolysis of oily wastewater treatment from a poultry processing factory. The inoculum could hydrolyze the oil in poultry wastewater to 41.94 ± 4.98 % with 5 % PI under batch mode. In addition, it exhibited oil degradation in poultry wastewater for 79.08 % at 22 h under continuous mode on a pilot scale. During the oil hydrolysis, acetic acid was obtained as the main VFA compound in the batch experiment. At the same time, acetic and propionic acids were the dominant VFA in the continuous experiment. The VFA compounds could be further utilized as intermediates for biogas production of the wastewater treatment system in a poultry processing factory. Thus, newly isolated *A. baumannii* RMUTT3S8-2 can be used as a microbial lipase producer for industrial oil hydrolysis.

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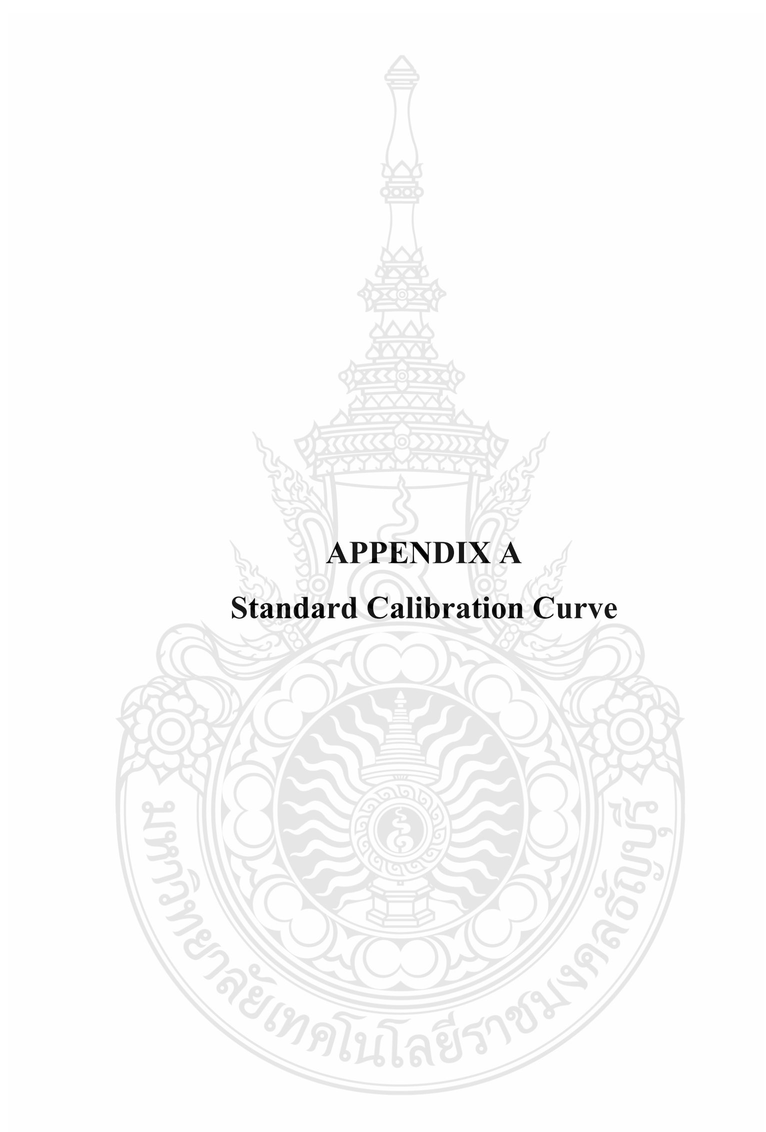


APPENDIX A
Standard Calibration Curve

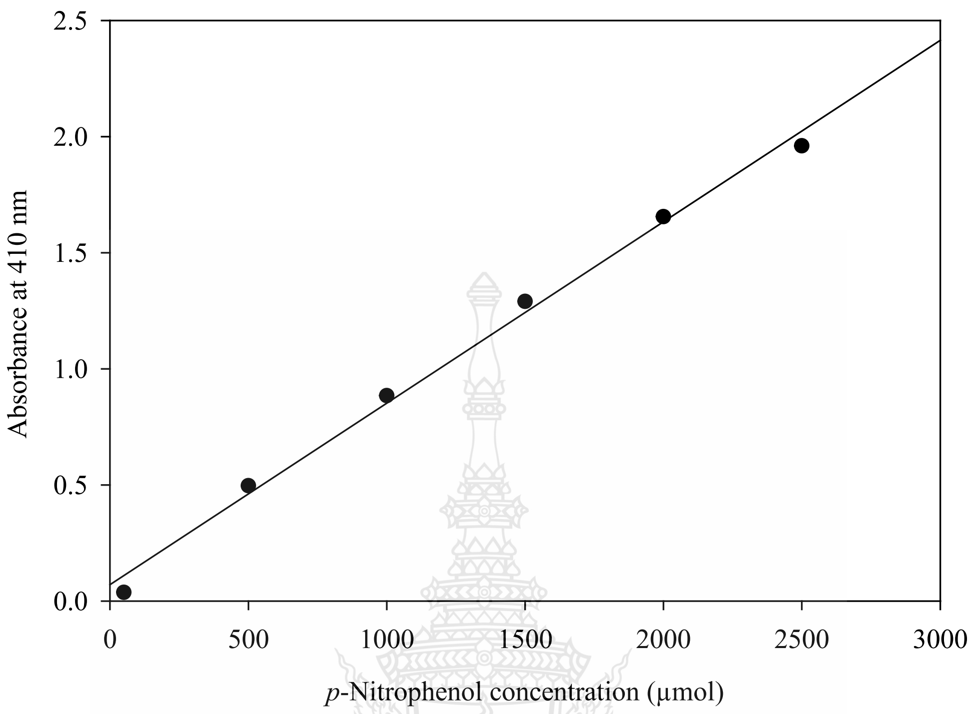
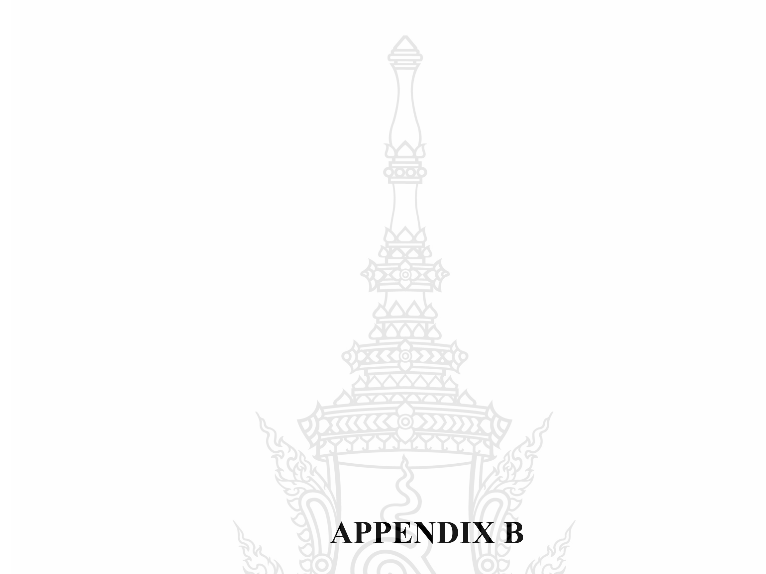


Figure A1 Standard calibration curve between *p*-Nitrophenol concentration and the absorbance at 410 nm for lipase activity analysis.

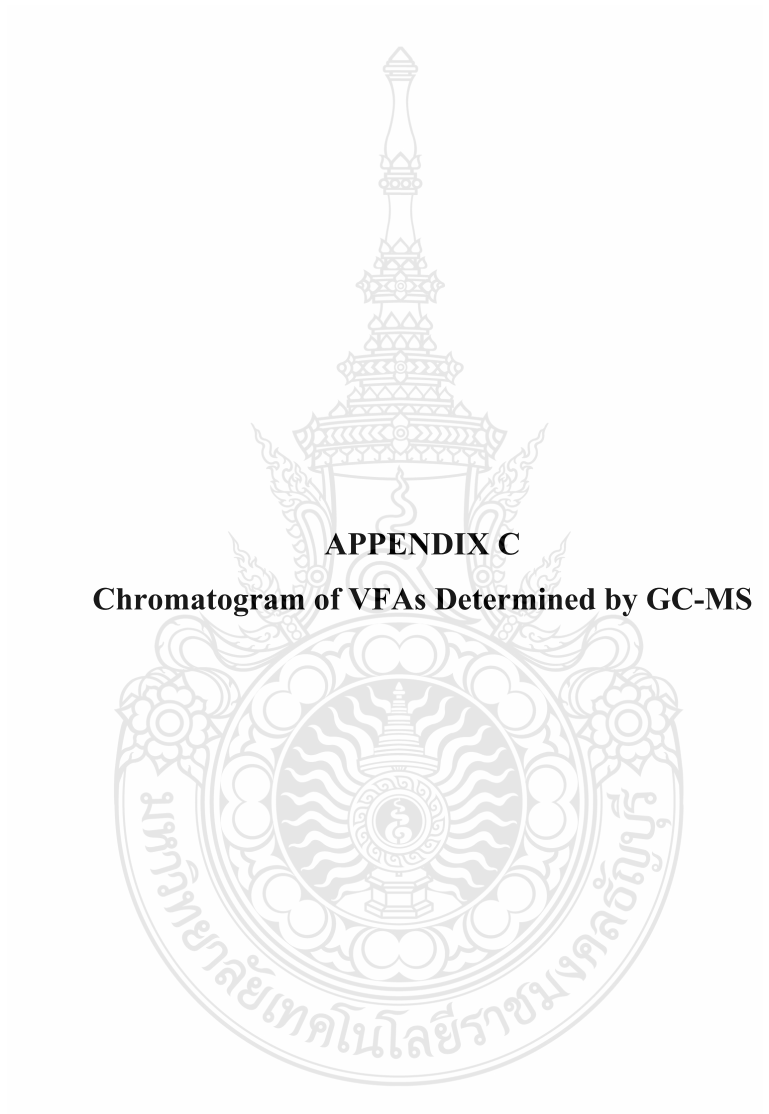


APPENDIX B

Summary of Lipase Production using Different Microorganisms

Table A1 Summary of lipase production using different microorganisms, production scale and culture conditions.

Microorganisms	Production scale	Culture conditions	Lipase activity (U/mL)	References
T. permensis M35-15	Erlenmeyer flask	28°C	11.49	[11]
B. aryabhattai SE3-PB	Erlenmeyer flask	pH 7.6, 40°C, 193 rpm, 120 h	264.02	[215]
Geobacillus thermodenitrificans AZ1	Erlenmeyer flask	55°C, 200 rpm, 24 h	593.00	[216]
B. ubonensis SL-4	Erlenmeyer flask	37°C, 200 rpm, 96 h	11.07	[5]
Acinetobacter sp. EH28	Erlenmeyer flask	37°C, 150 rpm, 48 h	3.80	[217]
Acinetobacter sp. AU07	Erlenmeyer flask	pH 7, 30°C, 150 rpm, 16 h	14.50	[10]
	3-L bioreactor	pH 7, 30°C, 150 rpm, 1.5 vvm, 16 h	48.00	[10]
A. baumannii RMUTT3S8-2	Erlenmeyer flask	35°C, 200 rpm, 72 h	216.23	This study
	5-L bioreactor	35°C, 200 rpm, 1.0 vvm, 144 h	251.62	This study



APPENDIX C

Chromatogram of VFAs Determined by GC-MS

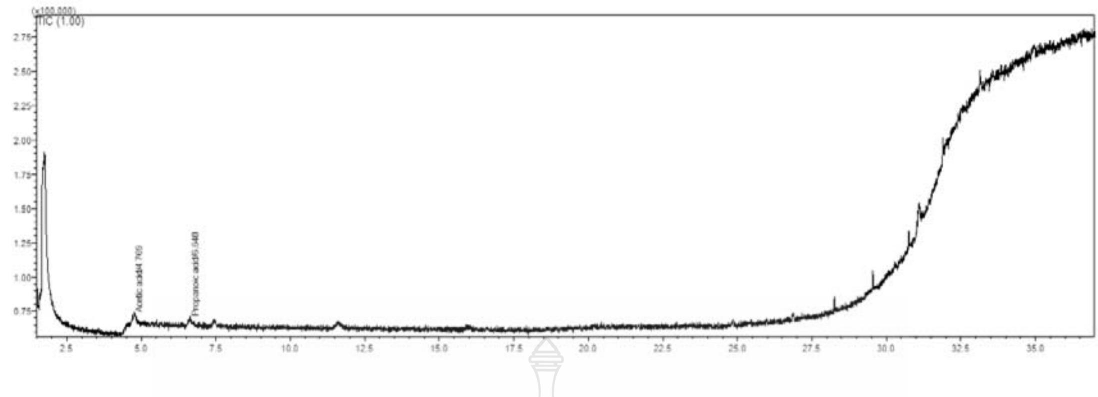


Figure A2 GC-MS chromatogram of the VFA compounds obtained from hydrolyzed oily wastewater poultry factory by 3 % LI on a 20-L plastic tank.

Table A2 The VFA compounds obtained from 3 % LI on 20-L plastic tank.

Time (day)	Peak	Area (%)	Name
2	1	53.26	Acetic acid
	2	46.74	Propanoic acid*

* Propionic acid

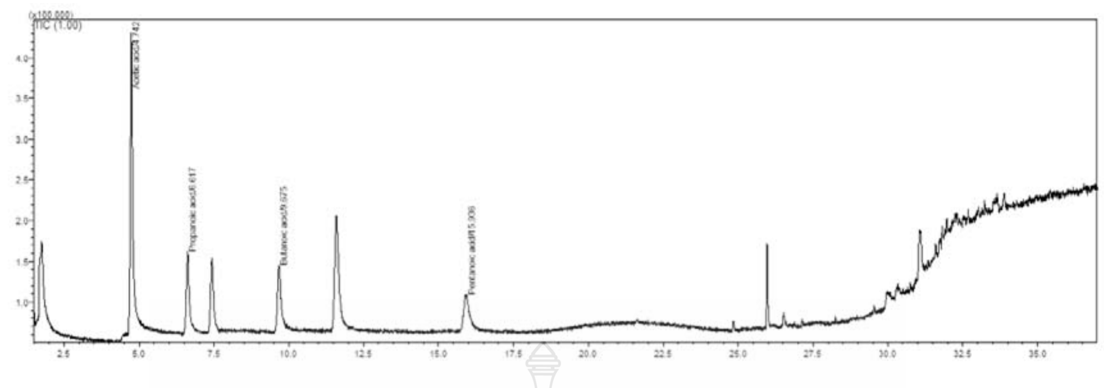


Figure A3 GC-MS chromatogram of the VFA compounds obtained from hydrolyzed oily wastewater poultry factory by 5 % PI on a 20-L plastic tank.

Table A3 The VFA compounds obtained from 5 % PI on 20-L plastic tank.

Time (day)	Peak	Area (%)	Name
12	1	60.27	Acetic acid
	2	17.40	Propanoic acid
	3	15.31	Butanoic acid**
	4	7.03	Pentanoic acid

**Butyric acid

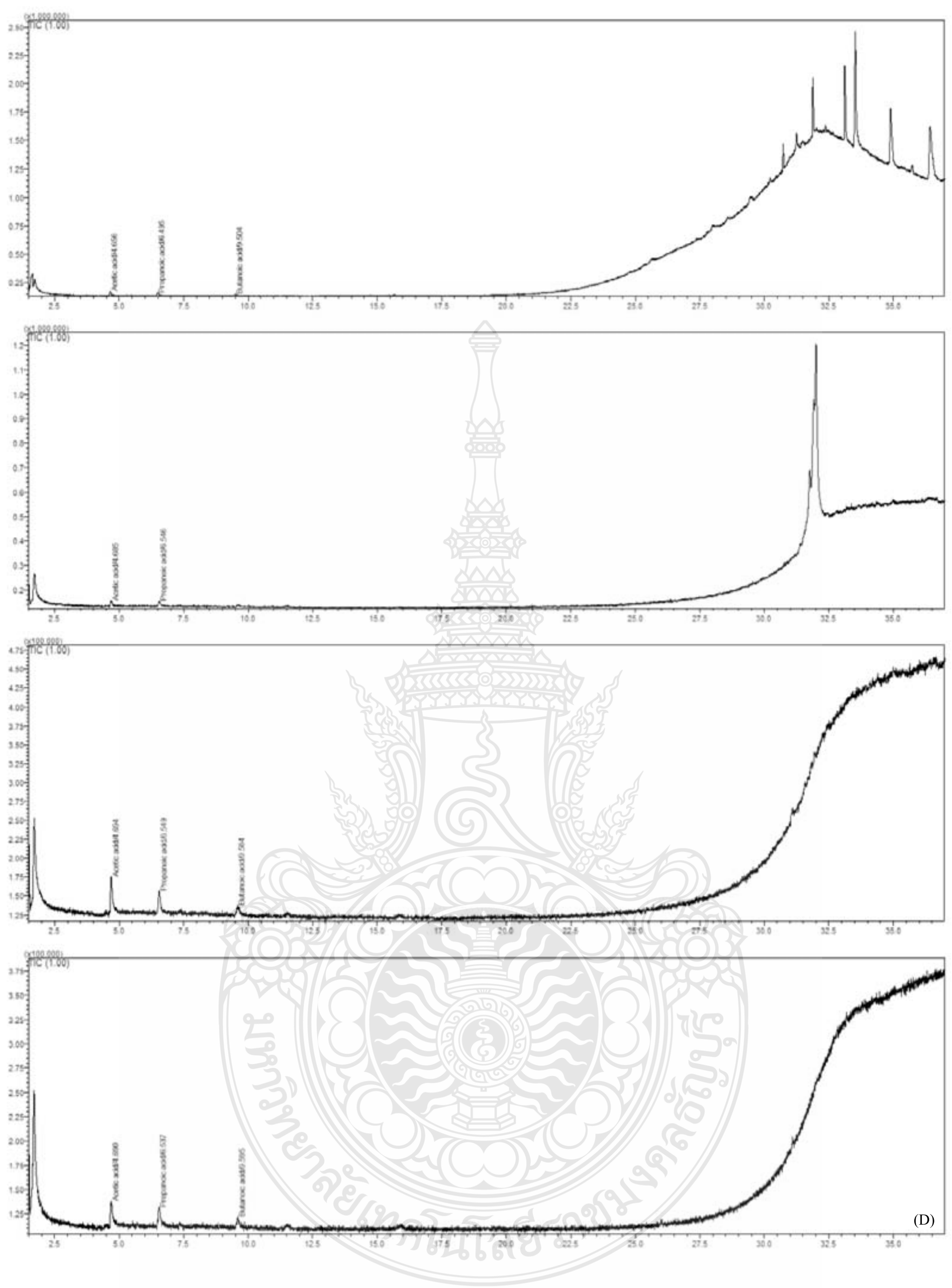


Figure A4 GC-MS chromatogram of the VFA compounds at 0 h of treatment in a 200-L plastic tank: (A); influent (B); control, (C); PI, and (D); PGBI.

Table A4 The VFA compounds at 0 h of treatment.

Conditions	Peak	Area (%)	Name
Influent	1	40.59	Acetic acid
	2	38.75	Propanoic acid*
	3	20.65	Butanoic acid**
Control	1	45.39	Acetic acid
	2	54.61	Propanoic acid
5 % PI	1	51.85	Acetic acid
	2	32.82	Propanoic acid
	3	15.33	Butanoic acid
5 % PGBI	1	45.76	Acetic acid
	2	41.13	Propanoic acid
	3	13.12	Butanoic acid

*Propionic acid **Butyric acid

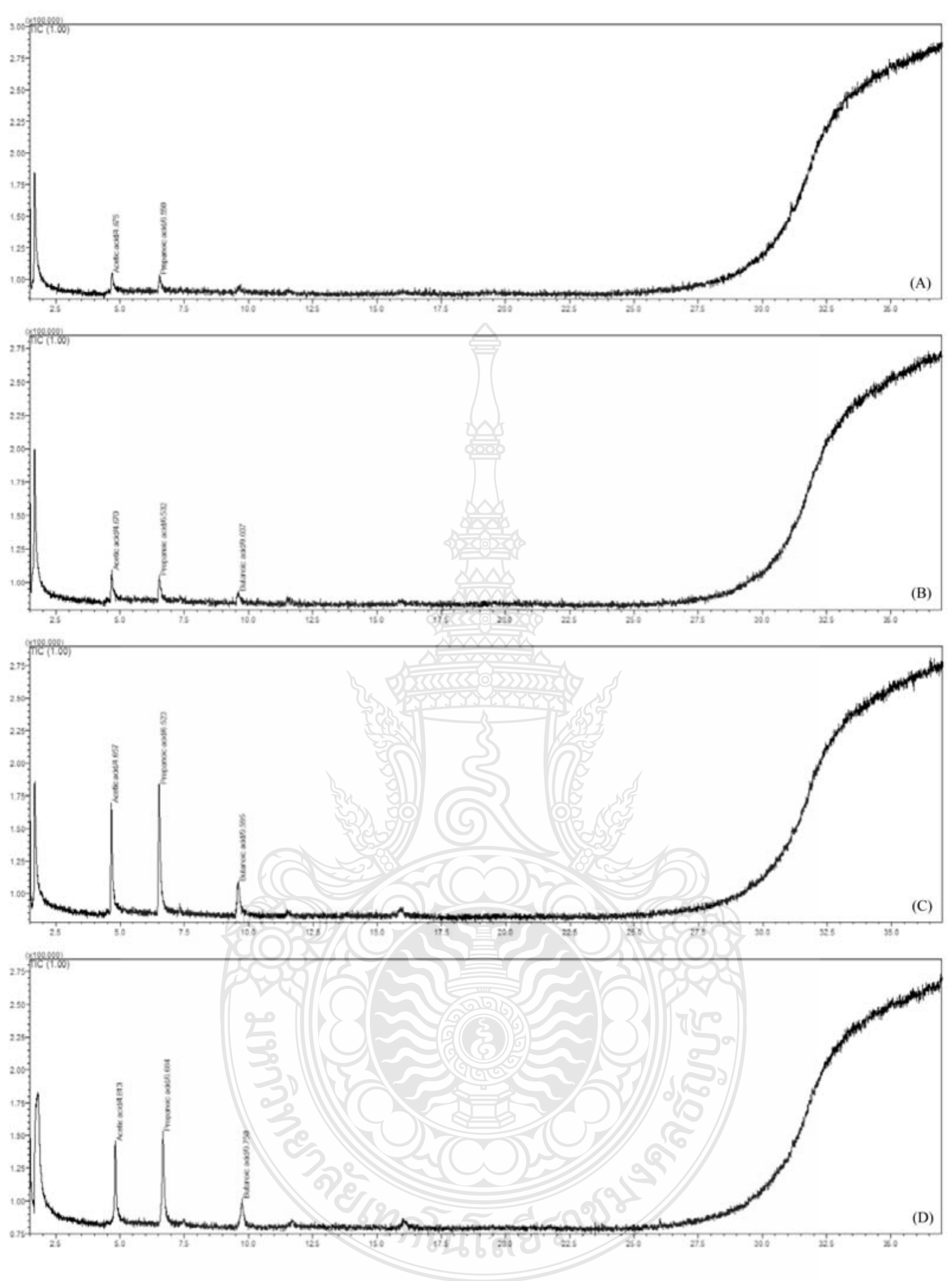


Figure A5 GC-MS chromatogram of the VFA compounds at 22 h of treatment in a 200-L plastic tank: (A); influent (B); control, (C); PI, and (D); PGBI.

Table A5 The VFA compounds at 22 h of treatment.

Conditions	Peak	Area (%)	Name
Influent	1	52.43	Acetic acid
	2	47.57	Propanoic acid*
Control	1	45.86	Acetic acid
	2	37.16	Propanoic acid
	3	16.98	Butanoic acid**
5 % PI	1	33.25	Acetic acid
	2	49.48	Propanoic acid
	3	17.28	Butanoic acid
5 % PGBI	1	35.12	Acetic acid
	2	43.34	Propanoic acid
	3	21.54	Butanoic acid

*Propionic acid **Butyric acid

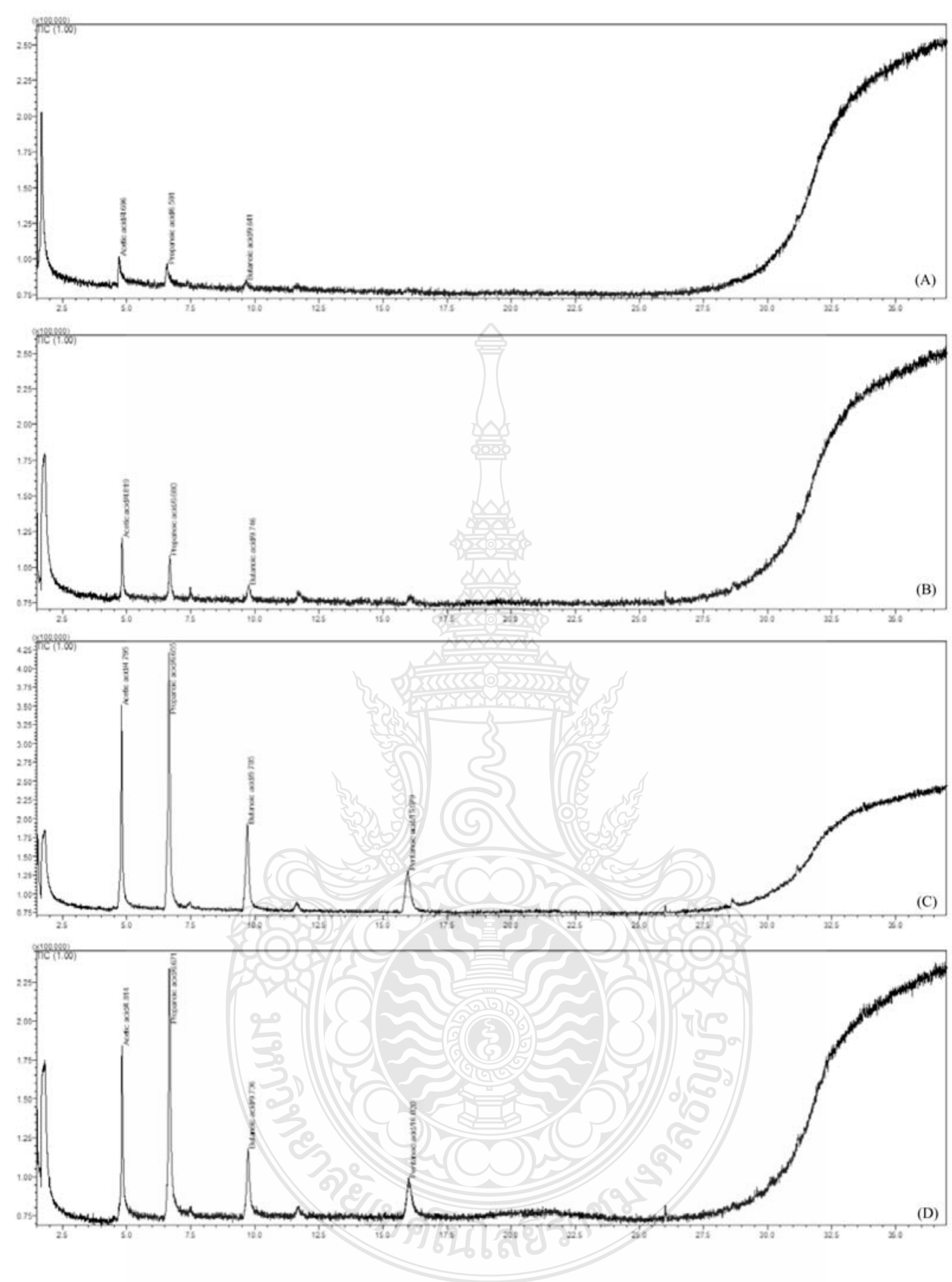


Figure A6 GC-MS chromatogram of the VFA compounds at 54 h of treatment in a 200-L plastic tank: (A); influent (B); control, (C); PI, and (D); PGBI.

Table A6 The VFA compounds at 54 h of treatment.

Conditions	Peak	Area (%)	Name
Influent	1	47.65	Acetic acid
	2	42.92	Propanoic acid*
Control	1	45.19	Acetic acid
	2	36.26	Propanoic acid
	3	18.55	Butanoic acid**
5 % PI	1	25.14	Acetic acid
	2	39.55	Propanoic acid
	3	19.55	Butanoic acid
	4	15.76	Pentanoic acid
5 % PGBI	1	24.46	Acetic acid
	2	45.93	Propanoic acid
	3	17.3	Butanoic acid
	4	12.3	Pentanoic acid

*Propionic acid **Butyric acid

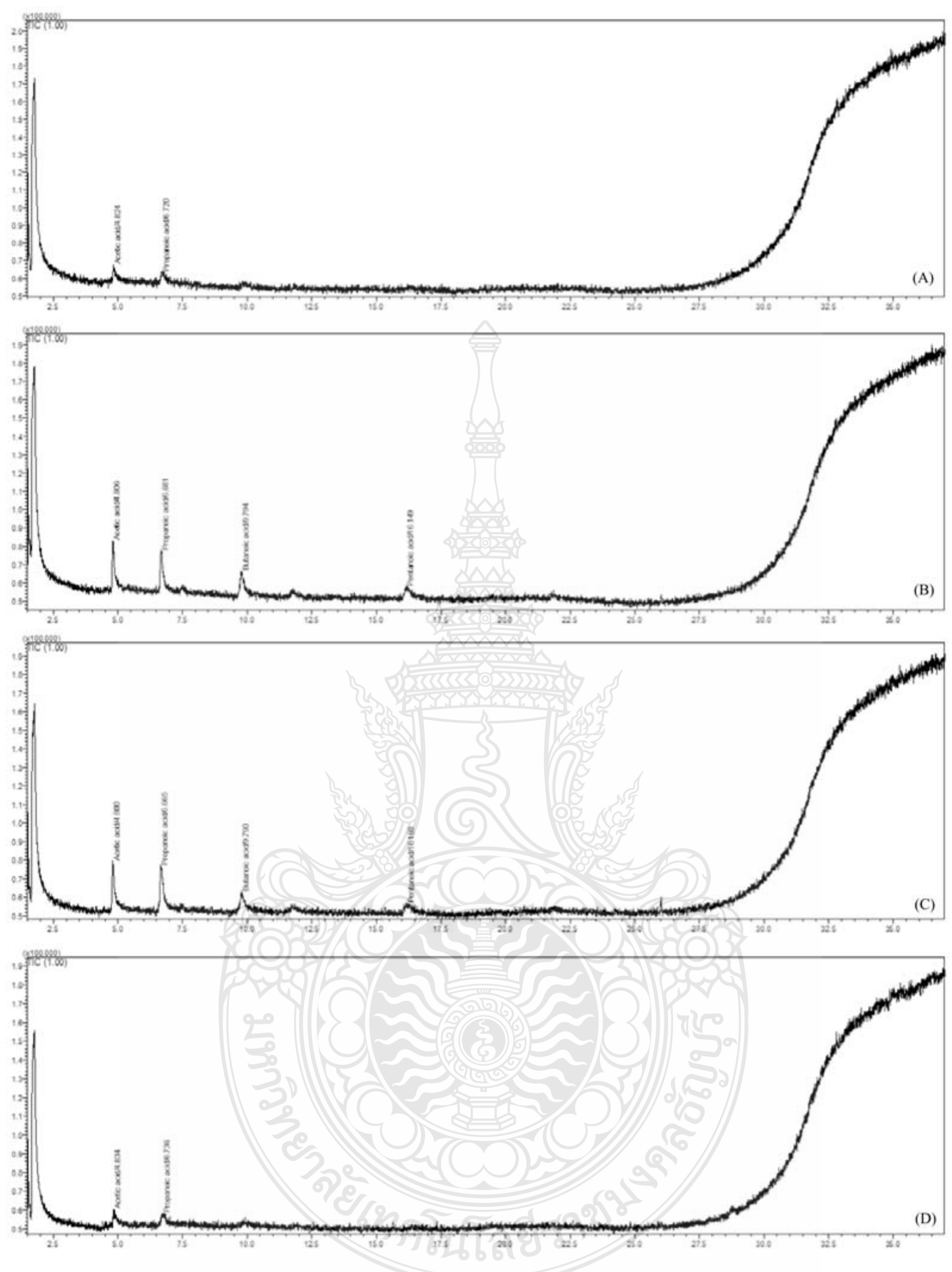


Figure A7 GC-MS chromatogram of the VFA compounds at 222 h of treatment in a 200-L plastic tank: (A); influent (B); control, (C); PI, and (D); PGBI.

Table A7 The VFA compounds at 222 h of treatment.

Conditions	Peak	Area (%)	Name
Influent	1	64.31	Acetic acid
	2	35.69	Propanoic acid*
Control	1	31.11	Acetic acid
	2	28.75	Propanoic acid**
	3	21.32	Butanoic acid
	4	18.81	Pentanoic acid
5 % PI	1	33.27	Acetic acid
	2	34.14	Propanoic acid
	3	17.89	Butanoic acid
	4	14.70	Pentanoic acid
5 % PGBI	1	47.40	Acetic acid
	2	52.60	Propanoic acid

*Propionic acid **Butyric acid

Biography

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