THE USE OF ISOLATED LIPASE PRODUCTION MICROBES FROM LIQUID MICROBIAL CONSORTIUM FOR WASTEWATER TREATMENT

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MR. WEI JU LIAO

ID. 5918030

A special project submitted to the Faculty of Biotechnology, Assumption University in part fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology

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Title : The use of isolated lipase production microbes from liquid microbial consortium for wastewater treatment

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Department : Food Technology

Faculty : Biotechnology

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Abstract

Over the past few years, issues relating to fat, oil and grease (FOG) present in sewer systems have intensified. FOG is a problem not only to wastewater municipalities but also to operators of solid-waste facilities. FOG enters the sewer system from Food Service Establishments (FSEs), residences and industries or other food production facilities. Untreated wastewater containing FOG waste can damage the water bodies as well as the ecosystem by forming oil film on water surface, resulting in death of aquatic lives. FOG blockages cause by accumulation of FOG waste could lead to sanitary sewer overflows, property flooding and contamination of water bodies with sewage. Microbial consortium can be referred as "Bio-extract" is a solution composed of diverse variety of microbes coexisting together, which is one of the sources to target concomitant enzymatic activity such as lipase, for the effective degradation of FOG waste. Lipases, triacylglycerol hydrolases, are produced by microorganisms from bacteria, yeast to fungi, which could be employed for wastewater treatment and bioremediation in numerous of industries. Furthermore, 7 lipases producing microbe strains from microbial consortium of fermented agricultural waste were obtained. After lipase test on tributyrin agar, two yeasts, and one bacterium strain found to have the highest lipase production among the 7 lipases producing microbe strains, which were strain M9P8, M9P15 and PCP17. In addition to the 3 highest lipase production strains, the doubling time of the growth rate was estimated. The results of the doubling time were 2 hour and 30 minutes for both M9P8 and M9P15, for PCP17 the doubling time was 1 hour and 30 minutes.

Keywords: Fat, oil and grease, microbial consortium, lipase, lipases producing microbes.

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INTRODUCTION

Water is an essential composition of all living organisms. Understandably, water is necessary for consumption as well as daily application for human. This aqueous solution is often used in household, agricultural, industrial and manufacturing purposes. Therefore, huge amount of wastewater was generated worldwide. In the case of wastewater, houses, industries including department stores, hotels and hospitals are required to install wastewater treatment system, in order to reduce the toxicity and dirtiness of the wastewater before discharge into water sources.

Wastewater is commonly composing of both organic and inorganic compounds in which varies depending on the sources. Fat, oil and grease (FOG) are important organic contaminant of domestic wastewater. FOGs compositions can easily inhibit the diffusion of oxygen from air to water by forming an oil films on the water surfaces. These oil films are resulting in, many deaths of different forms of aquatic life. Furthermore, aggregation of oil droplets presents with other solid or liquid substances causing blockage of water drainage lines, can lead to serious flooding.

Several techniques can be applied for FOGs removal during wastewater treatment. However, chemically or physically stabilized lipid/water emulsions should be managed in an appropriate manner. This is necessary because lipids that pass through physicochemical treatment processes contribute to the levels of biological oxygen demand (BOD) and chemical oxygen demand (COD) in the effluents. (Chipasa, 2006).

Lipase-producing microbe is one of the most crucial factors for biological treatment of lipids, since lipases or known as triacylglycerol hydrolases is an enzyme that catalyze the hydrolysis and the synthesis of esters formed from triacylglycerol to glycerol and fatty acids (Patel *et al.*, 2018). Commonly, lipase is produced by microorganisms from bacteria, yeast to fungi, these include *Bacillus spp.*, *Pseudomonas aeruginosa.*, and *Streptomyces spp.,* which could be employed for wastewater treatment as well as bioremediation in numerous of industries.

In the past decade, microbial consortium has been used as inoculum to resolve FOGs problem in household in country like Thailand. It is considered as one of the sources that target microbes with concomitant enzymatic activity for the effective degradation of FOG waste. Therefore, microbial consortium could be used for isolation of efficient lipase producing microbes.

Thus, main aim of this study is to determine the efficiency of FOG biodegradation of the three microbial strains isolated from microbial consortium.

OBJECTIVES

1. To determine the efficiency of two yeasts, and one bacterium strain isolated from microbial consortium for degradation of lipid and vegetable oil.
2. To review on the microbial treatment of used FOGs in domestic wastewater.

LITERATURE REVIEW

Wastewater

Wastewater refers to all effluent from domestic, agricultural, commercial and industrial processes. Effluent in this case refers to a liquid waste that is contaminated or polluted by biological, chemical, or radioactive sources. Environment as well as health of human population can be negatively impacted if the wastewater is untreated. Unmanaged wastewater can be a source of pollutant. Therefore, wastewater requires appropriate treatment to remove pollutants prior to discharge to the environment.

The quantity and quality of wastewater is determined by countless factors. Humans and industries do not produce the same amount of waste. The different amount and type of waste produced in households is influenced by the behavior, lifestyle, and standard of living of the residents as well as the surrounded environment.

The design of the drainage system affects the wastewater composition significantly. Furthermore, often separate sewer systems are used by most of the developing countries. On the flip side, combined sewer systems are used in old urban areas where different types of wastewater are mixed (Table 1). In combined systems a part (small or big) of the total wastewater is discharged to local water bodies, often without any treatment. (Henze *et al.*, 2008)

Table 1: Wastewater source types (Henze *et al.*, 2008)

Wastewater from society	Wastewater generated internally in treatment plants
Domestic wastewater	Thickener supernatant
Wastewater from institutions	Digester supernatant
Industrial wastewater	Reject water from sludge dewatering
Infiltration into sewers	Drainage water from sludge drying beds
Stormwater	Filter wash water
Leachate	Equipment cleaning water
Septic tank wastewater	

Type of domestic wastewater

Wastewater can be separated into two categories: sewage and non-sewage. Sewage is wastewater generated from domestic activities such as, houses, restaurant, hotels, schools and hospitals. Large quantity of wastewater is being produced from these sources, which usually contain human waste. On the other hand, non-sewage is all other types of wastewater like rainwater, stormwater, or water from commercial activities for instance: laundry and water from industrial plants.

Generally, domestic wastewater is classified into 3 different types, blackwater, greywater and yellow water.

Blackwater originates from toilet fixtures, dishwashers, and food preparation sinks. This type of water is made up of human feces and urine, cleaning agents and toilet paper and wipes. Thus, blackwater is highly contaminated with dissolved chemicals, particulate matter and is very pathogenic.

Greywater is the type of wastewater that originates from non-toilet and food fixtures. These include bathtub, toilet sink and laundry machines. Generally, greywater is a sewage water that does not contain feces and urine, which is why the wastewater management for blackwater, and graywater is very different. Normally, greywater is reusable since graywater is not pathogenic.

Yellow water is basically urine collected with specific channel that is not contaminated by blackwater and graywater. ("What Are the Different Types of Wastewater?", 2020)

Constituents of domestic wastewater

The component of wastewater can be varied depending on the sources of the wastewater. Below is table 2 that shows the constituents of domestic water.

Table 2: Components that may be found in domestic wastewater (Henze & Ledin, 2001)

Component	**Of special interest**	**Environmental effect**
Microorganisms	Pathogenic bacteria, virus, and worms' eggs	Risk when bathing and eating shellfish
Biodegradable organic materials	Oxygen depletion in rivers and lakes	Fish death, odours

Other organic materials	Detergents, pesticides, fat, oil and grease, colouring, solvents, phenols, cyanide	Toxic effect, aesthetic inconveniences, bioaccumulation in the food chain
Nutrients	Nitrogen, phosphorus, ammonium	Eutrophication, oxygen depletion, toxic effect
Metals	Hg, Pb, Cd, Cr, Cu, Ni	Toxic effect, bioaccumulation
Other inorganic materials	Acids, for example hydrogen sulfide, bases	Corrosion, toxic effect
Thermal effects	Hot water	Changing living conditions for flora and fauna
Odour (and taste)	Hydrogen sulfide	Aesthetic inconveniences, toxic effect
Radioactivity		Toxic effect, accumulation

Important contaminants in domestic wastewater

Water pollutions in domestic wastewater causes by various contamination due to domestic waste generated from several sources with variable human activities, food processing waste, pollutants from livestock operations, insecticide and herbicides, volatile organic compounds (VOCs), heavy metal, chemical waste and others, such as nutrients and pathogens (Krishna & Manickam, 2017). These components generally have bio-accumulative, persistent, and synergistic characteristics jeopardizing ecosystem health and function, food production, human health, and undermining human security. (Corcoran, 2010)

Organic chemicals of anthropogenic sources are generally found in drinking water supplies. Numerous of them are carcinogenic or mutagenic (shown in table 3) (Shy, 1985).

Table 3: Some recognized and suspected carcinogens identified in U.S. drinking water, 1976 (Shy, 1985).

Chemical	**Concentration, µg/L**
Aldrin	5.4

Benzene	50.0
Benzopyrene	0.002
Bis(2-chloroethyl ether)	0.4
Lindane	-
Carbon tetrachloride	3.0
Chlordane	0.1
Chloroform	20-300
1,2-Dibromoethane	-
Dieldrin	8.0
DDT	-
DDE	0.05
1,4-Dioxane	1.0
Endrin	0.004
Heptachlor	-
Trichloroethane	-
Vinyl chloride	10.0

Nutrient pollution (nitrogen, phosphates, etc.) usually causes overgrowth of toxic algae, which will be consumed by other aquatic animals, leading to death of aquatic lives, also cause outbreaks of fish diseases. Oil pollution can affect development of marine organisms negatively, increases susceptibility to disease, and affect reproductive processes; also cause gastrointestinal irritation, and damage to liver, kidney as well as to the nervous system (Krishna & Manickam, 2017).

Waterborne diseases can be caused by consumption of disease-causing microbes or pathogens contaminated in water. The wide variety of microbes recognized as waterborne disease agents, including *Cryptosporidium, Cyclospora, Escherichia coli* O157:H7, *Legionella, Helicobacter pylori*, hepatitis E virus, and others (Hunter *et al.,* 2001). Waterborne illnesses can cause variety of symptoms. Most common symptoms include diarrhea and vomiting, other symptoms comprise skin, ear, respiratory or eye problems ("Causes and Symptoms of Waterborne Illness - Minnesota Dept. of Health", n.d.)

Microorganisms in domestic wastewater

Wastewater from some sources can be infectious, especially the source that is contaminated with pathogens. The microorganisms in domestic wastewater come mainly from human's excreta, as well as food industries. Table 3 gives an idea of the concentration of microorganisms in domestic wastewater.

Table 4: Concentrations of microorganisms in wastewater (number of microorganisms per 100 ml) (Henze & Ledin, 2001)

Microorganisms	High	Low
E. coli	$5 - 10^8$	10^6
Coliforms	10^{13}	10^{11}
Cl. perfringens	$5 - 10^4$	10^3
Fecal Streptococcae	10^8	10^6
Salmonella	300	50
Campylobacter	10^5	$5 - 10^3$
Listeria	10^4	$5 - 10^2$
Staphylococcus aureus	10^5	$5 - 10^3$
Coliphages	$5 - 10^5$	10^4
Giardia	10^3	10^2
Roundworm	20	5
Enterovirus	10^4	10^3
Rotavirus	100	20

Higher concentration of microorganisms may create a severe health risk when raw wastewater is discharged to the water source (Henze & Ledin, 2001).

Water analysis

The major aim of wastewater treatment is to remove as much of the suspended solids as possible before the remaining water is discharged back to the environment. Therefore, it is necessary to analyze the water quality parameters which are very essential to determine the chemical, physical and biological properties of water, also the composition of water which is expressed in the concentration of the compounds.

Total solid is a measurement that includes the combination of total dissolved solids and total suspended solids in a liquid. It is a measurement that is often used in the water treatment industry. A higher total solids level indicates that there is a high level of solid material in a liquid sample.

Total dissolved solid is a measurement of the amount of solid material that has gone into solution in a liquid sample. These solids cannot be filtered out. The liquid may be evaporated to determine the level of total dissolved solids. Potassium, sodium and magnesium are examples of these types of solids.

pH is an indicator of the acidity or basicity of water. The normal pH range for irrigation water is from 6.5 to 8.4, pH values outside this range are a good warning that the water is abnormal in quality. Normally, pH is a routine measurement in irrigation water quality assessment.

Turbidity is a measurement that can be done by naked eyes, which shows the presence of suspended solid. It is measured by the amount of light that is reflected by the particles.

Temperature has direct impact on the biological, physical and chemical characteristics of the water.

1. COD or Chemical Oxygen Demand is the total measurement of all chemicals (organics & in-organics) in the wastewater.
2. BOD is a measure of, the amount of oxygen that require for the bacteria to degrade the organic components present in wastewater.
3. Nitrogen (N) is an essential nutrient for biological development of all major organisms.
4. Phosphorus (P) is an essential nutrient for plant growth and for biological metabolism.
5. Bacteriological analysis is necessary since wastewater contain varieties of microorganisms, for example: bacteria, viruses and protozoa. (APHA, 1992)

Fat, oil and grease (FOG)

Fat, oil and grease (collectively termed FOG) is an ever-increasing environmental concern, which causes ecological damages for aquatic organisms, plant and animal (Abd El-Gawad, 2014). FOG is often produced from Food Service Establishments (FSEs) or other food

production facilities. Fat, oil and grease are usually the byproducts and wastes of variety of sources such as food preparation, food processing, domestic properties and cleanup of utensils (Wallace *et al.*, 2017). Generally, FOG comprises matter like food scraps, meat fats, lard, tallow, cooking oil, butter, margarine, sauces, gravy, dressings, deep-fried food and baked goods (Husain *et al.*, 2014).

FOG as contaminants in domestic wastewater

Contamination of FOG on water surface causes decrease of dissolved oxygen. FOG layer reduces biological activity of treatment process when the formation of oil film taken place around microorganisms in suspended matter and water. Resulting in deplete of dissolved oxygen levels in the water. This led to inhibition of oxygen molecules to be oxidative for microbial on hydrocarbon molecules and cause ecology destruction to water bodies (Abd El-Gawad, 2014). Beside contamination by forming layer on water surface, FOG blockage is also a worldwide crisis (Husain *et al.,* 2014). Accumulation of FOG can narrow sewer diameters, if worsen FOG can completely block the water drainage lines (Ashley *et al.,* 2000) leading to foul odour, flooding or sewer overflows.

Chemical composition of FOG

Lipids are a class of macromolecules that are nonpolar and hydrophobic in nature-Lipids mainly consist of hydrocarbons in the most reduced form. FOGs are a subgroup of lipid that are majorly composed of fats and oils, waxes, phospholipids, and steroids. FOGs are important structural and functional components of foods (Frankel, 2005).

1. Free Fatty Acids (FFAs)

   FFAs are the by-product of hydrolysis and oxidation reaction of fats and oils during food processing. FFAs are composed of carboxylic acid with long chain hydrocarbon side groups, hydrocarbon include exclusively of hydrogen and carbon atoms. Over 1000 identified natural fatty acids. In food science the widely investigated FFAs are only around 20, shown in table 4. Those most familiar FFAs consist of long chain of 8 to 22 carbon atoms with one or more double bonds (unsaturated centers).

2. Triacylglycerols (TAGs)

A crude oil or fat will normally contain at least 95 percent of triacylglycerols. Triacylglycerols (also called as triglycerides) are produced from the consumption of food as well as from our body generation. TAGs are non-polar and hydrophobic substances. Triglyceride consists of a molecule of glycerol combined with three molecules of esterified fatty acid side chains. Glycerol is a trihydric alcohol that can combine with up to three fatty acids. Depending on the number of hydroxyl groups that are acylated, to form monoglycerides, diglycerides, and triglycerides.

3. Waxes

Waxes are complex mixtures of high molecular weight or large carbon number alkanes (Demirbas, 2016). Wax includes various types of medium and long-chain compounds, such as hydrocarbons, alcohols, aldehydes, acids and esters. Waxes strongly hydrophobic nature allows them to function as water repellents on the leaf surfaces of some plants, as well as feathers on some aquatic birds (Thompson, 2020).

4. Phospholipids

Crude vegetable oils and animal fats usually contain phospholipids. Normally phospholipids are removed during refining of crude vegetable oil. Phospholipids are the molecules with hydrophilic phosphate heads and hydrophobic lipid tails. The amphiphilic nature of phospholipids made phospholipids valuable byproducts and are used extensively in food products, cosmetics and industrial processes.

5. Sterols and sterol esters

Sterols are present in plant and animal. Sterols can be esterified to long chain fatty acids through oxidation reactions. Most vegetable oils contain 0.1-2.2% of sterols, partly as free sterols and partly as esterified sterols. (Husain *et al.*, 2014) (Gunstone, 2004).

Table 5: Most common Free Fatty Acids in food science (Based on "Fats, Oils, and Grease (FOG) Science", n.d.)

Common Name	Systematic Name	Short-hand	Sources
Butyric acid	Butanoic acid	4:0	Butterfat
Caproic Acid	Hexanoic acid	6:0	Butterfat
Caprylic Acid	Octanoic acid	8:0	Coconut oil

Common Name	Systematic Name	Short-hand	Sources
Capric Acid	Decanoic acid	10:0	Coconut oil
Lauric Acid	Dodecanoic acid	12:0	Coconut oil
Myristic Acid	Tetradecanoic acid	14:0	Palm kernel oil
Palmitic Acid	Hexadecanoic acid	16:0	Palm oil
Palmitoleic Acid	9-Hexadecenoic acid	16:1	Animal fats
Stearic Acid	Octadecanoic acid	18:0	Animal fats
Oleic Acid	9-Octadecenoic acid	18:1	Olive oil
Vaccenic Acid	11-Octadecenoic acid	18:1	Butterfat
Linoleic Acid	9,12-Octadecadienoic acid	18:2	Grape seed oil
Alpha-Linolenic Acid	9,12,15-Octadecatrienoic acid	18:3	Flaxseed (linseed) oil
Gamma-Linolenic Acid	6,9,12-Octadecatrienoic acid	18:3	Borage oil

Table 5(cont.): Most common Free Fatty Acids in food (Based on "Fats, Oils, and Grease (FOG) Science", n.d.)

Common Name	Systematic Name	Short-hand	Sources
Gamma-Linolenic Acid	6,9,12-Octadecatrienoic acid	18:3	Borage oil
Arachidic Acid	Eicosanoic acid	20:0	Peanut oil, Fish oil
Gadoleic Acid	9-Eicosenoic acid	20:1	Fish oil
Arachidonic Acid	5,8,11,14-Eicosatetraenoic acid	20:4	Liver fat
EPA	5,8,11,14,17-Eicosapentaenoic acid	20:5	Fish oil
Behenic acid	Docosanoic acid	22:0	Rapeseed oil
Erucic acid	13-Docosenoic acid	22:1	Rapeseed oil

Common Name	Systematic Name	Short-hand	Sources
DHA	4,7,10,13,16,19-Docosahexaenoic acid	22:6	Fish oil
Lignoceric acid	Tetracosanoic acid	24:0	Small amounts in most fats

Type of FOGs

A well-known lipid is triglyceride. Triglycerides are the main constituents of vegetable oils and animal fats. Normally, fats are categorized into 4 types, saturated fats, monounsaturated fats, polyunsaturated fats and trans fats (Hu, Manson, & Willett, 2001). Saturated fats are typically solids and are derived from animals, while monounsaturated fats and polyunsaturated fats are liquids and usually extracted from plants or fishes. Moreover, trans fats are a byproduct of a process called hydrogenation that is used to turn healthy oils into solids and to prevent them from becoming rancid ("The truth about fats: the good, the bad, and the in-between - Harvard Health", 2015). Generally, the viscosity of lipid varies based on the fatty acid composition and presence of double bonds. The more double bonds present in the carbon chain, the lower the viscosity because of the more loosely packed structure (Husain *et al.*, 2014). Both vegetable oils and animal fats are commonly used in the food industry and hence become part of the domestic waste.

Palm oil

Palm oil is extracted from the ripened fruits of oil palm tree (*Elaeis guineensis*). Top five leading palm oil producing countries are Indonesia, Malaysia, Thailand, Colombia and Nigeria. Palm oil consumption is forecast to grow in Thailand due to increased usage in biodiesel and in food. Domestic consumption of palm oil in Thailand was approximately of 2.720 million metric tons by 2019, which makes Thailand the top ten leading palm oil consumption country ("Thailand Palm Oil Domestic Consumption by Year (1000 MT)", n.d.). Palm oil has a unique profile of fatty acid (FA) and triacylglycerol (TAG), therefore suitable for various food applications. The major fatty acid composition of palm oil is palmitic acids (43.99%), followed by oleic acid (39.24%) (Yanty, Marikkar & Miskandar, 2012). Palm oil is the only vegetable oil with almost 50–50 composition of saturated and unsaturated fatty acids. Moreover, palm oil has high smoke point of

about 230°C due to the unique fatty acid composition, which makes palm oil top prime among frying oils (Mba, Dumont, & Ngadi, 2015).

Lard

Lard is pork fat from back and kidneys and have long been used as fat ingredients in food applications. China remained largest volume of lard consumption (2.6M tons), accounting for 40% of total consumption ("World Lard Industry Analysis 2007-2025 - Global Market to Grow by 1.6% a Year through 2025, Fuelled by Rising Demand in China", 2020). Although, Thailand is not in the leading countries that produce lard, but lard is still frequently used in Thailand. Lard is not as high in saturated fatty acids as once thought (Marcus, 2013). The major fatty acid composition of lard is oleic acids (38.24%), followed by palmitic acids (22.68%) and linoleic acids (20.39%), which means lard found to have more unsaturated fatty acids (USFA) than saturated fatty acids (SFA) (Yanty, Marikkar & Miskandar, 2012). Lard has high smoking point of about 205°C, so lard is usually used for quick frying.

Wastewater treatment for removal of FOGs

Presently, conventional techniques for FOGs removal are using Grease trapping systems (GTSs), also referred to as grease abatement systems and grease interceptors (GIS) to control FOGs at the source of generation. Grease trapping systems are generally multi-compartment tanks where the aqueous grease containing flow is retained long enough consequently that grease can rise to the water surface and solids can settle to the bottom and treated water can be discharged to the sewer (Ragauskas, Pu, & Ragauskas, 2013). GTSs are usually the term used to classify kitchen grease separation devices waste quantities less than 200 L while GIS are the term used to denominate larger outdoor devices with a minimum quantity of 3000 L (Radin *et al.*, 2018). The commercial FOG trap is based on the gravity separation, chemical enhanced separation process, as well as conventional filtration and ultrafiltration processes. Various FOG trap has different effectiveness. Therefore, the efficiency of the GT depends strongly on the frequency of its maintenance (Husain et al., 2014). The remaining oil causes clogging of pipes in treatment units that need cleaning and sometimes replacement of pipes. This led to increase maintenance and inspection cost.

Electrocoagulation is a technology based on the application of electric field between metal anode and cathode with specific material, mostly iron or aluminum, to provide active metal cations

that react to pollutants such as oil, resulting in coagulation and flocculation (Kabdaşlı *et al.*, 2012). Electrocoagulation is one of the most efficient technologies for the treatment of domestic wastewater, oil shale wastewater, and oil–water emulsion. Electrocoagulation process is characterized a fast rate of pollutant removal, simplicity in the operation, compact size of the equipment, as well as low capital and operating costs and has achieved over 94% of FOGs removal from the wastewaters tested (Chen, Chen & Yue, 2000).

Bioaugmentation is a biological technology that introduces specialized and actively growing microbial strains into a microbial community in an effort to enhance the degradation of FOGs by producing hydrolysis enzyme such as lipase. Recently, alternative use of bioaugmentation have potentially gained more attention due to the environmentally desirable application for reducing the entry of high levels of FOG levels. However, the main problems of most bioaugmentation process is retention time since high volume of wastewater is being generated by FSEs and inappropriate process time for completion of FOGs degradation (Alves, 2013).

Lipase produced by microorganisms

Lipase or triacylglycerol hydrolases is an enzyme that catalyze the hydrolysis and the synthesis of esters formed from triacylglycerol to glycerol and fatty acids. Lipases appear widely in nature; nonetheless only microbial lipases are commercially significant. Numerous applications of lipase include syntheses and hydrolysis of FOGs, modification of fats, detergents and flavor enhancement in food processing (Patel *et al.*, 2018).

Lipases are ubiquitous in nature and are produced by different types of plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most broadly used class of enzymes in biotechnological applications. The common bacterial lipase producers are as follow: are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas.* Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications (Jaeger *et al.*, 1994).

In addition to lipase producing bacteria, yeast strains isolated from seawater, sediments, mud of salterns, guts of the marine fish and marine algae grown in olive oil medium could produce lipase, which could actively hydrolyse different oil for example lard, peanut oil, olive oil and soybean oil. The 9 yeast strains were identified by performing routine identification and molecular

methods, the results shown that the strains belonged to *Candida intermedia* YA01a, *Pichia guilliermondii* N12c, *Candida parapsilosis* 3eA2, *Lodderomyces elongisporus* YF12c, *Candida quercitrusa* JHSb, *Candia rugosa* wl8, *Yarrowia lipolytica* N9a, *Rhodotorula mucilaginosa* L10-2 *and Aureobasidium pullulans* HN2.3, respectively (Wang *et al.,* 2007).

Microbial consortium

Microbial consortium can be referred as Bio-fermented solution or "Bio-extract" (Kunathigan, & Wiratthikowit, 2016). The widely known bio-extract is EM*, effective microorganisms, developed by Professor Teruo Higa in 1982 where various type of microbes isolated from natural decomposition system ("Learn how to live sustainably using EM microbial technology on agriculture and environment", 2016). In the case of this project, the Microbial consortium was produced from fermented agricultural waste.

Microbial consortia have the potential for two or more organisms to share chemical compounds usually for their mutual benefits. Presently, Microbial consortium can be applied into different field, wastewater treatment is one on the popular usage (Nisuwat, 2013). Normally, it is much more efficiently when they are intimately associated versus if they are not. Therefore, microbial consortium can have high impact on wastewater treatment.

Previous Study

Ever since microbial consortium or known as "Effective Microorganisms" was discovered by Dr. Teruo Higa, there were numerous of studies conducted to determine the efficiency on various application. Example of research conducted are as follow:

The previous studies conducted on the "The Study of the Biodiversity in Local Bio-Extract and the Treatment of Community Wastewater at Laboratory Scale: Wastewater from Restaurants" by Supatchayaporn Nitsuwat. The study aimed to determine the biodiversity as well as the efficiency of the local bio-extract in restaurant wastewater treatment. From this project, bio-extract was examined for the presence of mold, yeast, lactic acid bacteria, actinomycetes, purple non-sulfur bacteria, *bacillus spp.* and total viable cells using RBA (Rose-Bengal Agar), YM (Yeast Mold) agar, MRS (de Man, Rogosa and Sharpe) agar, GYEA (Glycerol-Yeast Extract Agar), GM broth and PCA (Plate Count agar) respectively . Viable cell counts on selective and non-selective enrichment mediums were performed to determine the biodiversity of the bio-extract. After the

viable cell counts was performed, the biodiversity of the bio-extract was consisting of *Bacillus spp.* 3.00×10^3 CFU/ml, mold 3.63×10^3 CFU/ml, lactic acid bacteria 4.35×10^4 CFU/ml, Actinomycetes 1.27×10^5 CFU/ml, and yeast 1.35×10^5 CFU/ml. Moreover, treatment of wastewater using bio-extract was able to significantly reduce of TS and FOG at 53.07% and 69.89% respectively. The study also concluded that the most suitable condition for restaurant wastewater treatment was 0.25 ml of bio-extract per liter of wastewater with the absence of oxygen and light for 48 hours. (Nitsuwat *et al.,* 2012)

Another previous study conducted on the "The Study of Lipase Producing Microorganisms from Bio-Extract for Fat and Lipid Treatment in Wastewater at Laboratory Scale" by Aunchisa Charoenpanich. The two strains, M9P16Y and PCP16Y, isolated from bio-extract can degrade used palm oil, 48.20% and 50.32% respectively after 48 hours. However, the effectiveness was not as good as the bio-extract, 65.48%. Moreover, the reduction of total solid by using bio-extract, M9P16Y and PCP9Y were 60.173%, 28.432% and 5.999% respectively. (Charoenpanich *et al.*, 2016)

Thus, in this project the additional strains of microbes isolated from microbial consortium was studied to determine their ability to produce lipase enzymes to be candidates for degradation of wastewater contaminated with used cooking oil.

MATERIALS AND METHODS

1. Lipase test of microbial strains isolated from microbial consortium on tributyrin agar

Lipase producing microorganisms isolated from microbial consortium were reactivated and tested for lipase using the tributyrin agar test. Seven isolates were transferred from plate count agar on to tributyrin agar, namely, M9P5, M9P8, M9P10, M9P15, M9P16, PCP16, PCP17, respectively. The radius of the clear zone surrounding the growth of lipid producing microbes were measured using ruler 24 hours and 48 hours after streaking on tributyrin agar under room temperature (Approximately 30℃). The results of the clearness (transparency) and clean zone radius (mm.) were compared between the strains.

2. Confirmation test of the three selected strains on tributyrin agar incubated for 48 hours

Three selected strains were then tested for the lipase production again on tributyrin agar under room temperature, namely M9P8, M9P15 and PCP 17 respectively. The radius of the clear zone was measured using Vernier caliper 48 hours after streaking on tributyrin agar plate. The results of the clearness (transparency) and clean zone diameter (mm.) were compared between the three selected strains.

3. Growth characteristic

After the selection of the strains, growth curve of the three strains with highest lipase production were constructed, M9P8, M9P15 and PCP17, respectively. The growth curve was then constructed by inoculating a loop of colonies from PCA slant to 15ml PCB and incubated in the shaker under 30°C with 100rpm for 26 hours. Then, 3ml inoculum from 15ml PCB was transferred into new 90ml PCB, in order to adjust the absorbance of OD600nm to approximately 0.1. The absorbance was measured every hour under the spectrophotometer at wavelength 600nm until the curve became straight. The growth was done at 30°C with shaking(100rpm).

4. Viable cell count and selection of dilution under OD600nm

The number of viable cells of two yeast strains were measured by colony forming unit method, M9P15 and PCP17, respectively. The measurement was then done by inoculating a loop of colonies from PCA slant to 15ml PCB and incubated in the shaker under 30°C with 100rpm for

26 hours. Then, 3ml inoculum from 15ml PCB was transferred into new 90ml PCB, in order to adjust the absorbance of OD600nm to approximately 0.1. Followed by observing the absorbance until 0.2 and 0.5 for both strains. The sample were then diluted to 10^{-7} and 10^{-8} by 10-fold dilution, respectively. 0.1ml of dilution $10^{-5}, 10^{-6}$ and 10^{-7} for absorbance 0.2 and 0.1ml of dilution $10^{-6}, 10^{-7}$ and 10^{-8} for absorbance 0.5 were then spread uniformly on the surface of PCA plate, with the incubation time of 24 hours under room temperature. The results were expressed as cfu/ml and the dilution that obtained 30-300 colonies was used to analyze with the absorbance data from spectrophotometer at wavelength 600nm to estimate the number of cells and select the suitable dilution.

RESULTS AND DISCUSSION

Lipid degradation and microbial strain selection

Seven microorganisms previously isolated from microbial consortium and were kept at -80 °C were revived and subject to lipid degradation test. The lipid degradation test was done using tributyrin agar and the result was demonstrated in table 6. M9P8 had the highest clear zone radius after 48 hours, which was 0.4mm. Moreover, M9P5, M9P10 and PCP16 showed lowest clear zone diameter of 0.1mm.

After the preliminary experiment, tributyrin agar test was performed once again for selected strains. According to table 6, M9P8 showed the clear zone of 0.4mm with the incubation time of 48 hours, which was the highest among the other strains. Followed by M9P15, M9P16 as well as PCP17, with the radius of 0.2mm after incubated for 24 hours and 0.3 after 48 hours incubation time. Thus, M9P8, M9P15 and PCP17 were selected for construction of the growth curve. Despite that M9P16 had the same result as M9P15 and PCP17, M9P16 was not selected, due to the lower transparency of the clear zone, which indicates that the lipid degradation was not that effective.

Table 6: The radius of clearness in mm after 24 hours and 48 hours compared between different microbial strains.

Strains	Incubation Time				Transparency of clear zone
	24 hours		48 hours		
	Average (mm)	S.D	Average (mm)	S.D	
M9P5	0.1	±0.04	0.1	±0.10	++
M9P8			0.4	±0.05	+++
M9P10	0.1	±0.04	0.2	±0.00	+
M9P15	0.2	±0.05	0.3	±0.05	+++
M9P16	0.2	±0.05	0.3	±0.05	++
PCP16	0.1	±0.04	0.2	±0.00	++
PCP17	0.2	±0.04	0.3	±0.05	+++

Note: Transparency of clear zone, low (+), medium (++) and high (+++).

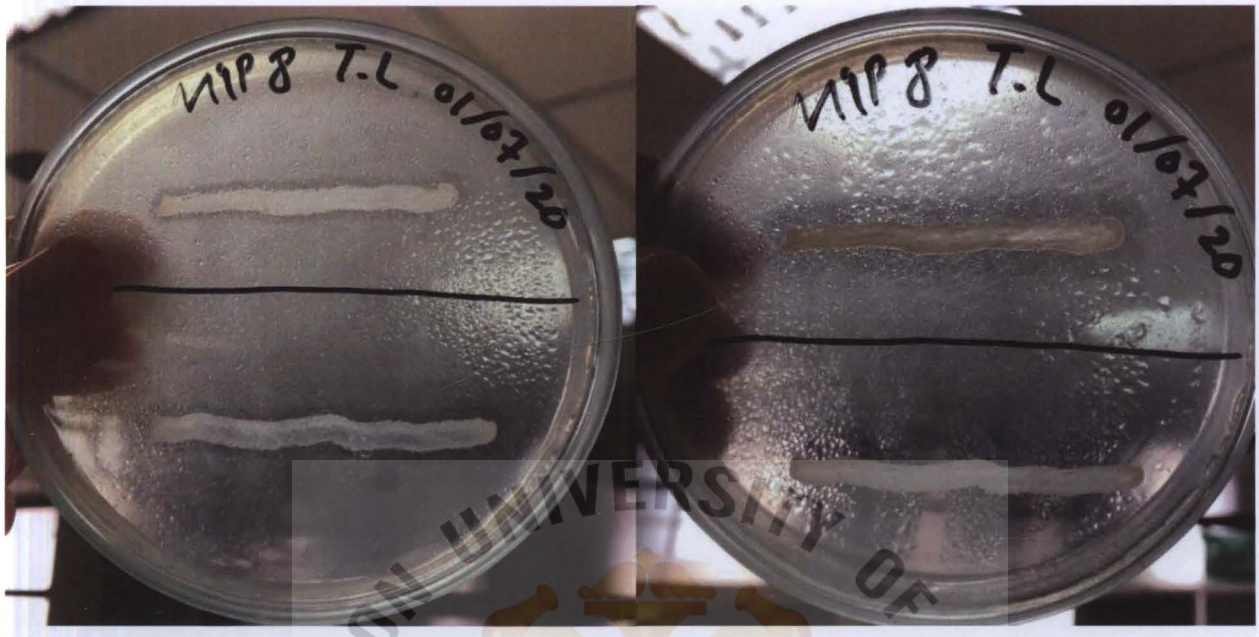

Figure 1: Lipase test of the strains M9P8 after 24 hours (Left) and 48 hours (Right) on tributyrin agar.

Figure 2: Lipase test of the strain M9P15 after 24 hours (Left) and 48 hours (Right) on tributyrin agar.

Figure 3: Lipase test of the strain PCP17 after 24 hours (Left) and 48 hours (Right) on tributyrin agar

Confirmation test of the three selected strains on tributyrin agar

In addition to the previous experiment, the confirmation test was performed on tributyrin agar to determine the efficiency of oil degradation of the strain M9P8, M9P15 and PCP17 by observing the clear zone using Vernier caliper. Based on table 5, M9P8 showed the highest average of 0.45mm of the clear zone radius compared to M9P15 and PCP17, which have the clear zone of 0.27mm and 0.23mm respectively.

Furthermore, gram staining of M9P8, M9P15 and PCP17 was performed. According figure 5, M9P8 found to be gram positive spore producing bacteria, which is possible to be Bacillus sp. On the other hand, after observation of M9P15 and PCP17 under microscope, both of the strains were found to be yeast.

Table 7: The radius of clearness in mm after 48 hours between three of the selected strain.

Confirmation Test	Average (mm)	S.D
M9P8	0.45	±0.04
M9P15	0.27	±0.04
PCP17	0.23	±0.05

Figure 4: Confirmation test of the strain M9P8, M9P15 and PCP17 after 48 hours on tributyrin agar.

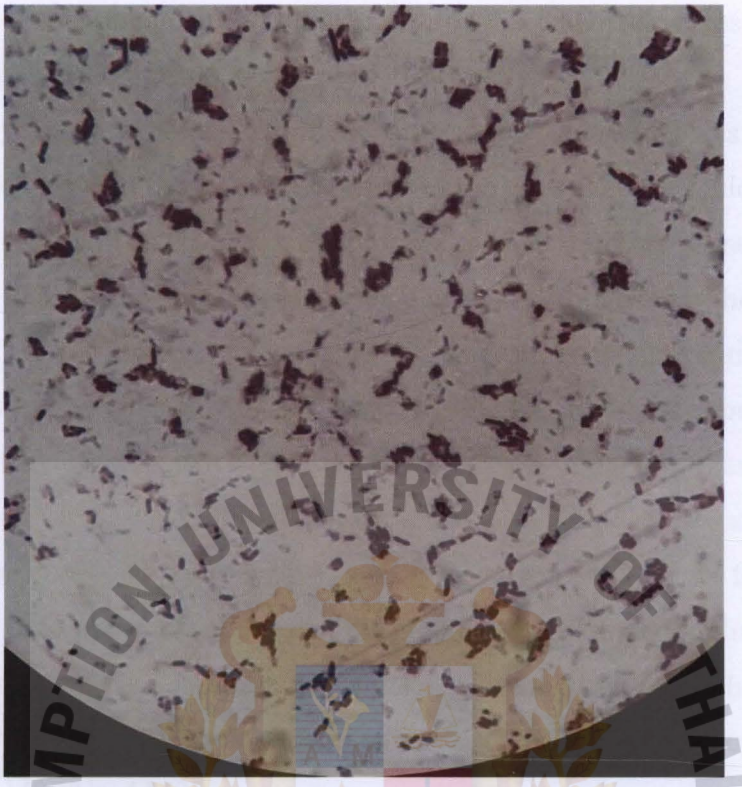

Figure 5: Gram staining of M9P8 under microscope (1000X)

Growth curve construction

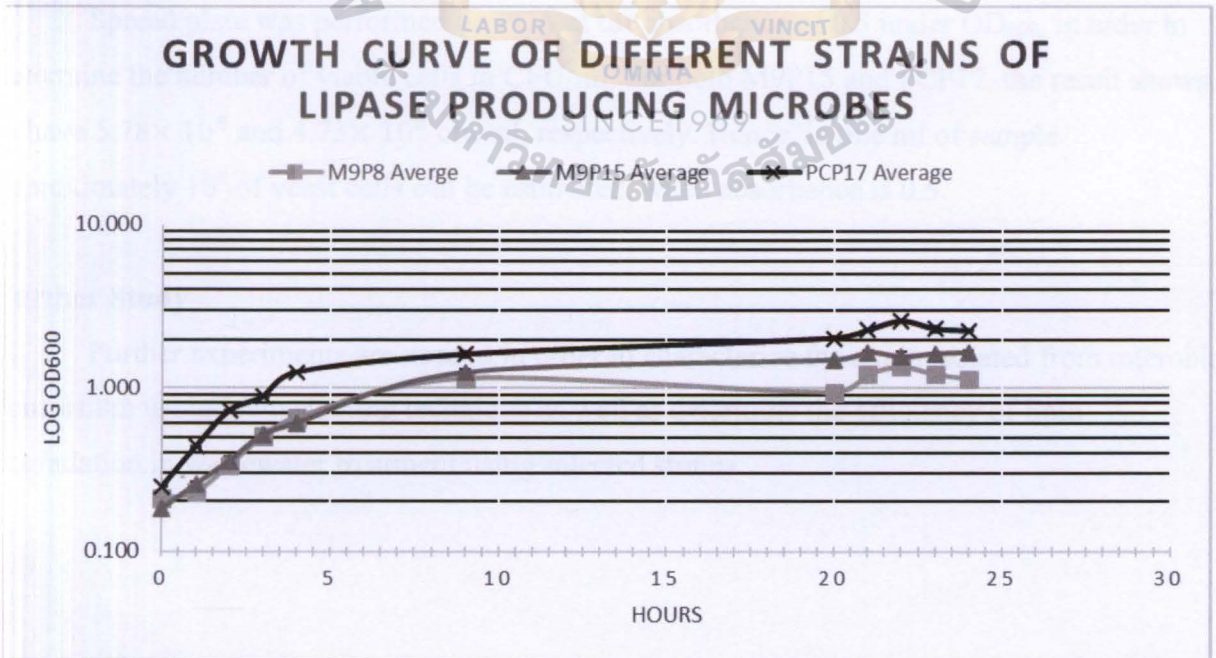

Figure 6: Growth curve of three selected strains (M9P8, M9P15 and PCP17) in PCB at 30℃ with shaking(100rpm) and was measured at OD_{600}.

An inoculum of microbial cells was inoculated in PCB and observed as absorbance values increased over a period of time.

As reported by figure 5 above, the growth curve of selected strains was constructed. By plotting the absorbance value at OD600, three phases of the growth curve could be identified. Based on the growth curve, all selected strains appeared to have short lag phase initiating from hour 0 to hour 2. For PCP17, the absorbance started increasing quickly after the first hour from absorbance 0.436 to 0.720, which indicated that PCP17 have entered exponential phase very quickly, the result also shown that PCP17 has somewhat higher growth rate compared to M9P8 and M9P15. On the flip side, although M9P8 and M9P15 have similar absorbance during the first four hours, but at hour 9 as shown from the curve the growth rate difference was noted, 1.144 and 1.140, respectively. Therefore, M9P15 and PCP17 were chosen for further experiment. In addition, doubling time was calculated using the graph, estimation of doubling time for M9P8 and M9P15 were approximately 2 hours and 30 minutes. On the flip side, doubling time increase more rapidly for PCP17 with only 1 hour and 30 minutes, which means the growth rate of PCP17 is faster compared to M9P8 and M9P15.

Viable cell count

Spread plate was performed on PCA at the absorbance of 0.5 under OD_{600}, in order to determine the number of viable cells in CFU/ml. For both M9P15 and PCP17, the result shown to have 5.78×10^8 and 4.73×10^8 cfu/ml, respectively. Hence, in one ml of sample approximately 10^8 of yeast cells can be estimated, when absorbance is 0.5.

Further Study

Further experiments are needed in order to characterize the strain isolated from microbial consortium using gram staining technique as well as determine the efficiency of lipid degradation in wastewater treatment using selected strains.

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Appendix

Media

- **Plate Count Agar (PCA) (1 liter)**

Tryptone	5	g.
Yeast extract	2.5	g.
Glucose	1	g.
Agar	15	g.
Distilled water	1	L.

Remark: Autoclave at 121℃ for 15 minutes

- **Tributyrin agar (1 liter)**

Peptone	5	g.
Beef extract	3	g.
Agar	15	g.
Distilled water	1	L.
Tributyrin	10	ml.

Remark: Autoclave at 121℃ for 15 minutes

- **PCB (1 liter)**

Tryptone	5	g.
Yeast extract	2.5	g.
Glucose	1	g.
Distilled water	1	L.

Remark: Autoclave at 121 ℃ for 15 minutes

- **0.1% Peptone Broth (1 liter)**

Peptone (meat)	1	g.
Distilled water	1	L.

Remark: Autoclave at 121℃ for 15 minutes

Table 8: Raw absorbance data of M9P8, M9P15 and PCP17 at OD600nm for growth curve construction. All microorganisms were grown at 30℃ under static condition.

Average/hr	0	1	2	3	4	9	20	21	22	23	24
M9P8	0.210	0.229	0.335	0.506	0.655	1.144	0.941	1.223	1.361	1.220	1.139
S.D	0.021	0.014	0.017	0.003	0.021	0.040	0.026	0.117	0.091	0.026	0.011
M9P15	0.180	0.253	0.353	0.515	0.605	1.240	1.496	1.666	1.563	1.632	1.656
S.D	0.009	0.012	0.023	0.021	0.015	0.082	0.016	0.044	0.133	0.032	0.064

PCP17	0.246	0.436	0.720	0.878	1.246	1.617	2.037	2.293	2.586	2.320	2.267
S.D	0.003	0.001	0.002	0.006	0.010	0.065	0.055	0.025	0.040	0.118	0.019

Table 9: Raw data of viable cell count for M9P15 at OD600nm with absorbance 0.2 All microorganisms were grown at 30°C under static condition.

Dilution factor	M9P15		Average	CFU/ml	Average	S.D
10^-5	2	61	31.5	3.15E+07	2.04E+08	201432319.07
10^-5	6	52	29	2.90E+07		
10^-6	96	8	52	5.20E+08		
10^-6	19	0	9.5	9.50E+07		
10^-7	0	2	1	1.00E+08		
10^-7	8	1	4.5	4.50E+08		

Table 10: Raw data of viable cell count for M9P15 at OD600nm with absorbance 0.5 All microorganisms were grown at 30°C under static condition.

Dilution factor	M9P15		Average	CFU/ml	Average	S.D
10^-6	67	69	68	6.80E+08	5.78E+08	212793914.4
10^-6	48	47	47.5	4.75E+08		
10^-7	13	0	6.5	6.50E+08		
10^-7	5	5	5	5.00E+08		
10^-8	0	2	1	1.00E+09		
10^-8	1	1	1	1.00E+09		

Table 11: Raw data of viable cell count for PCP17 at OD600nm with absorbance 0.2 All microorganisms were grown at 30°C under static condition.

Dilution factor	M9P15		Average	CFU/ml	Average	S.D
10^-5	127	128	127.5	1.28E+08	3.98E+08	218944754.6
10^-5	145	190	167.5	1.68E+08		
10^-6	65	77	71	7.10E+08		
10^-6	22	35	28.5	2.85E+08		
10^-7	3	9	6	6.00E+08		
10^-7	0	10	5	5.00E+08		

Table 12: Raw data of viable cell count for PCP17 at OD600nm with absorbance 0.5 All microorganisms were grown at 30°C under static condition.

Dilution factor	M9P15		Average	CFU/ml	Average	S.D
10^-6	63	56	59.5	5.95E+08	4.73E+08	759899938.6
10^-6	34	36	35	3.50E+08		
10^-7	8	10	9	9.00E+08		
10^-7	4	2	3	3.00E+08		
10^-8	0	1	0.5	5.00E+08		
10^-8	5	0	2.5	2.50E+09		