BIODEGRADATION OF WASTE COOKING OIL AND WASTE ENGINE OIL BY LOCALLY ISOLATED MICROFUNGUS, *Aspergillus* spp. USM-AR1

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by

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LIST OF ABBREVIATIONS

g/L	gram per litre
g/g	gram per gram
w/v	weight per volume
v/v	volume per volume
g	gram
%	percent
rpm	rotation per minute
vvm	volume per volume per minute
kg	kilogram
L	litre
mL	millilitre
μL	microlitre
μm	micrometre
°C	Degree Celsius
MSM	Minimal salt medium
PDA	Potato dextrose agar
PDB	Potato dextrose broth
MEA	Malt extract agar
M	Molar
TBA	Tributyrin agar
ROA	Rhodamine olive oil agar
OST	Oil spreading technique
E_{24}	Emulsification index
SEM	Scanning electron microscope
OD	Optical density
GCMS	Gas chromatography mass spectrometry
FAME	Fatty acid methyl ester
h	hour
STR	Stirred tank bioreactor
DO	dissolved oxygen

kPa	kiloPascal
NaCl	sodium chloride
NaOH	sodium hydroxide
H_2SO_4	sulfuric acid
psi	per squre inch
NH_4NO_3	ammonium nitrate
NH_4Cl_3	ammonium chloride
$NH_4(SO_4)_2$	ammonium sulphate
$NaNO_3$	sodium nitrate
MATH	Microbial adhesion to hydrocarbon
PAH	Polycyclic aromatic hydrocarbon
WCO	Waste cooking oil
WEO	Waste engine oil
PUM	Phosphate urea magnesium sulphate
CSH	Cell surface hydrophobicity
spp.	species

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APPENDIX 12   Dissolved oxygen and pH profile of waste engine oil removal by *Aspergillus* sp. USM-AR1 when agitated at 400 rpm in a 3.6 L stirred tank bioreactor from IRIS software (Version 6.0)

BIODEGRADASI SISA MINYAK MASAK DAN SISA MINYAK ENJIN OLEH MIKROKULAT PENCILAN TEMPATAN, *Aspergillus* spp. USM-AR1

ABSTRAK

Sisa minyak masak dan sisa minyak enjin dari kawasan kediaman dan industri automotif, masing-masing boleh mengakibatkan kesan buruk jika tidak dilupuskan dengan betul. Kulat mempunyai toleransi yang lebih tinggi terhadap ketoksikan hidrokarbon kerana fisiologi dan penyesuaian mereka terhadap alam sekitar, dilengkapi juga dengan mekanisme penggunaan minyak dari alam sekitar. Oleh itu, bioremediasi berasaskan kulat boleh digunakan untuk pengurangan sisa minyak. Dalam kajian ini, kulat filamen dipencilkan daripada arang yang bercampur dengan lemak haiwan, dikenali sebagai *Aspergillus* sp. USM-AR1 berasaskan morfologi. Penyingkiran minyak dan biojisim kulat tertinggi dalam kelalang goncang adalah 81.93 ± 14.22 % dan 81.67 ± 11.08 g/L, masing-masing pada 144 jam dalam medium yang mengandungi 9.83% (v/v) sisa minyak masak, 1 g/L ammonium nitrat, 16.32% (v/v) NaCl, kepekatan inokulum 10% (v/v) dan pH awal 8.32 selepas kajian pengoptimuman. Bagi sisa minyak enjin pula, penyingkiran minyak (64.89 ± 13.83 %) dan biojisim kulat (16.19 ± 2.96 g/L) tertinggi dalam kelalang goncang dicapai dalam tempoh 6 hari pengkulturan dalam medium yang mengandungi 3% (v/v) sisa minyak enjin 3 g/L ammonium nitrat, 20% (v/v) NaCl, 10% (v/v) kepekatan inokulum dan pH awal 4. Penambahbaikan biodegradasi sisa minyak dicapai melalui fermentasi dalam bioreaktor tangki teraduk 3.6 L. Sisa minyak masak digunakan sepenuhnya dalam tempoh tiga hari pengkulturan dengan biojisim kulat 22.19 ± 0.86 g/L dan penyingkiran minyak 97.07 ± 0.02 % pada kadar pengadukan 400 rpm. Bagi sisa

minyak enjin pula, kadar biodegradasi adalah 56.86 ± 1.73 % dan biojisim kulat yang diperolehi ialah 5.15 ± 0.58 g/L dalam tempoh 6 hari pengkulturan pada kadar pengadukan 400 rpm. Hidrofobisiti sel permukaan tertinggi untuk kedua-dua sisa minyak masak (76.42 ± 2.72 %) dan sisa minyak enjin (80.3 ± 8.18 %) adalah dalam 3 hari pengkulturan. Biosurfaktan tertinggi (43.33 ± 3.33 mm menggunakan OST) dan aktiviti pengemulsian (44 ± 4.0%) untuk sisa minyak masak adalah selepas 1 hari pengkulturan. Aktiviti biosurfaktan dan pengemulsian tertinggi untuk sisa minyak enjin berlaku selama 2 hari pengkulturan, masing-masing ialah 30 ± 0.01 mm dan 48.3 ± 1.67%. Keputusan ini menunjukkan bahawa kadar degradasi dan pertumbuhan kulat yang cepat dan oleh itu, dapat diaplikasikan untuk biodegradasi.

BIODEGRADATION OF WASTE COOKING OIL AND WASTE ENGINE OIL BY LOCALLY ISOLATED MICROFUNGUS, *Aspergillus* spp. USM-AR1

ABSTRACT

Large amounts of waste cooking oil and waste engine oil from residential areas and automotive industry, respectively, can impose adverse impact if not disposed off properly. Fungi have a higher tolerance to hydrocarbon toxicity due to their physiology and adaptation to the environment, subsequently equipped with the mechanism for oil utilisation. Therefore, bioremediation using fungi can be employed for waste oil reduction in the environment. In the present study, a filamentous fungus was isolated from used charcoal coated with animal fats, identified morphologically as *Aspergillus* sp. USM-AR1. The highest oil removal and fungal biomass of waste cooking oil in shake flasks obtained were $81.93 \pm 14.22\%$ and 81.67 ± 11.08 g/L, respectively at 144 h in a medium containing 9.83% (v/v) waste cooking oil, 1 g/L ammonium nitrate, 16.32% (v/v) NaCl, 10% (v/v) inoculum concentration and with the initial pH of 8.32 after optimisation studies. For waste engine oil, the highest oil removal ($64.89 \pm 13.83\%$) and fungal biomass (16.19 ± 2.96 g/L) in shake flasks were attained during 6 days of cultivation in a medium containing 3% (v/v) waste engine oil, 3 g/L ammonium nitrate, 20% (v/v) NaCl, 10% (v/v) inoculum concentration and with an initial pH of 4. Further improvements in the waste oils biodegradation were achieved by fermentation in a 3.6 L stirred tank reactor. Waste cooking oil was fully utilised within three days of cultivation with the fungal biomass of 22.19 ± 0.86 g/L and oil removal of $97.07 \pm 0.02\%$ at agitation speed of 400 rpm. For waste engine oil, the highest oil removal was $56.86 \pm 1.73\%$ and fungal biomass obtained was 5.15 ± 0.58

g/L during 6 days of cultivation with agitation at 400 rpm. The highest cell surface hydrophobicity for both waste cooking oil (76.42 ± 2.72%) and waste engine oil (80.3 ± 8.18%) were observed during 3 days of cultivation. The highest biosurfactant (43.33 ± 3.33 mm in OST) and emulsification activity (44 ± 4.0%) for waste cooking oil were observed after 1 day of cultivation. The highest biosurfactant and emulsification activity for waste engine oil occurred during 2 days of cultivation, which were 30 ± 0.0 mm and 48.3 ± 1.67%, respectively. These results show that the rates of degradation and fungal growth were rapid and consequently, applicable for oil biodegradation.

CHAPTER 1 INTRODUCTION

1.1 Research background

Fats and oils are the major wastes produced by the food processing industry. Fats and oils are typically removed from the domestic wastewater treatment plants and shipped to landfill areas without prior treatment, which causes destruction not only to the environment, but also to flora and fauna (Xia He et al., 2011). They also are often causing problems in clogging drains and damaging pumping equipment. Fats and oils are naturally occurring substances, mainly consisting of mixtures of trihydroxy alcohol or glycerol fatty acid esters (Okino-Delgado et al., 2017). Apparently, fats and oils mostly is in the form of waste cooking oil (WCO) from deep fat fryers and can also be presented in sauces, salad dressing and even in dairy based foods such as coffees and ice cream (Alias et al., 2018). These wastes are considered as fats and oils, and exist in most spectrums of food production (Wallace et al., 2017). Waste cooking oil is one of the main concerns as it is dumped into drains and other bodies of water without treatment. Generally, the term waste cooking oil (WCO) refers to vegetable oil that has been employed in food production and is not any longer viable for its intended use (Kumar et al., 2012).

Petroleum hydrocarbons (PHs) are the most important raw materials for industrial chemicals and energy resources used in daily life. However, petroleum hydrocarbons also act as major pollutants for the environment (Garapati and Mishra, 2012). With increasing energy demand, high levels of petroleum hydrocarbon from exploitation, refining, storage and transport pollute the environment, especially in the case of large-scale accidental spills (Davoodi et al., 2020). Petroleum hydrocarbons are categorised into four broad classes of chemical compounds, namely aliphatic, aromatic, resin-based and asphaltic-based hydrocarbons (Al-Hawash et al., 2019).

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Each group has different physicochemical characteristics and degradation susceptibility (Basumatary et al., 2020). The type of petroleum is extremely important in determining the extent of environmental and socio-economic damage. The expansion of the vehicle manufacturing sector stimulates the growing quantity of used oil, which depends on the global population and on economic growth (Jonusas and Miknius, 2014; Li et al., 2020). Besides, Malaysia is gradually shifting from agriculture to a large-scale factory-based industrial country. Urbanisation is expanding rapidly, in line with the national agenda to become a fully developed nation by 2020. Urbanisation and industrialisation both have environmental risks, making sustainable management of natural resources even more challenging (Hassan et al., 2014).

In treating waste oil disposals, remediation of contaminated sites using microbes, also better-known as bioremediation, has proved to be reliable and effective because of its eco-friendly features. Moreover, bioremediation process is safe, effective and can be implemented at a low cost (Ibrahim et al., 2020). To date, several good definitions of bioremediation have been given, with particular emphasis on degradation. In some cases, however, the term biodegradation is used interchangeably with bioremediation; the former is the term that applies to the process under the latter (Azubuike et al., 2016). Generally, bioremediation can be defined as a process that relies on biological mechanisms to degrade, mineralise, reduce, detoxify, or transform the concentration of pollutants to an innocuous state (Azubuike et al., 2016). In this process, the microorganisms will consume waste oils as the carbon source and the release water and carbon dioxide as waste products. These degrading organisms, including fungi, bacteria and algae, can transform toxic compounds into non-toxic compounds and from complex organic contaminants into simpler forms that can be used by other organisms (Kothari et al., 2013). In addition to the selection condition

of waste oils biodegradation efficiency, the performance condition such as nutrient and oxygen concentrations, pH, temperature, agitation speed and other abiotic factors that determine the success of bioremediation processes are also considered to be key considerations prior to applying bioremediation (Frutos et al., 2012; Smith et al., 2015).

As often highlighted, fungi have certain advantages over bacteria in terms of biodegradation due to their resistance to petroleum hydrocarbons (Al-Hawash et al., 2019). It is established that fungi are able to produce high organic acids, oxidative enzymes and extracellular enzymes that enable them to use hydrocarbon more quickly (Ibrahim et al., 2020; Stamets, 1999). Moreover, fungi are also able to lower the pH of its environment hence, leading to a reduction state of some contaminated compounds (Ibrahim et al., 2020). Their extended mycelial networks facilitate the movement of nutrients between spatially separated regions and transport hydrophobic organic contaminants (Velez et al., 2020). In the past decade, there have been many studies on the role of fungi in petroleum products biodegradation. Among the most common fungi discovered as a biodegraders belong to *Aspergillus, Alternaria, Cephalosporium, Cladosporium, Candida, Fusarium, Geotrichum, Mucor, Penicillium, Rhodotolura, Rhizopus, Saccharomyces, Trichoderma* and *Torulopsis* (Das and Chandran, 2011; Obire and Anyanwu, 2009).

1.2   Motivation and scope of the project

Malaysia is facing an increasing problem of waste disposal, aggravated by the continuous development and population rise. Land areas available for dedicated waste handling are diminishing, forcing the adoption of more efficient waste disposal technologies. Waste oils can be treated before they are released into the environment if source segregation is done. When it comes to hydrocarbon degradation, most reports in hydrocarbon degradation suggest *in situ* bioremediation and degradation of pollution. This work, however attempts to approach the problem in a different way. Instead of applying the bioremediation agents on site where the pollutants are already distributed in the environment, the agents can be used to treat the pollutants at disposal sites. This strategy calls for bioremediation agents where in this case are fungi that require minimal nutrient supplementation to grow.

The idea of using filamentous fungi as bioremediation agents for waste oils has obtained much attention. Fungi are tolerant to higher concentrations of toxic chemicals, making them more advantageous than bacteria (Al-Hawash et al., 2019). Fungi are also remarkably aerobic despite having the ability to grow under environmentally stressed conditions such as poor nutrient status and low pH. Fungal oil degradation is advantageous because of the cost advantages, namely minimal cost and effort. It offers a robust approach as fungi are capable to grow in a wide range of environments. Mechanism of hydrocarbon degradation in fungi differ from that in bacteria. Consequently, fungi degrade oil compounds that would otherwise be left intact by bacterial oil degradation due to the latter's limited ability. Fungi degrade oil by penetrating the oil layer and increasing the surface area of contact for degradation by other microbes.

Therefore, the present study will be undertaken to explore the possibility of oil degradation using fungi. A major part of this thesis describes the optimisation of oil degradation by a filamentous fungi with the appropriate bioprocessing parameters. The study will be initiated with the screening and evaluation of locally isolated fungus for its capability of oil degradation. The fungus will be then identified via morphological characteristics. Then, appropriate medium components and environmental condition for enhanced oil degradation would be experimentally determined in shake flask. The cultivation of fungus will be pursued further in a well-controlled stirred tank bioreactor in order to improve the oil degradation. Lastly, mechanisms of oil biodegradation by a local fungal isolate such as biosurfactant and cell surface hydrophobicity would be investigated.

1.3   Problem statement

Malaysia, as with many thriving developing countries, has been plagued with diminishing areas for landfills. Limited land areas to support rapid development and population increase exacerbate the problem of waste disposal and management. One of the many mitigating strategies is waste reduction. The dire situation has led to the application of drastic measures. Waste segregation at source has been made mandatory in many states. To date, for the general public, this only applies to solid wastes. Environmental concerns dictate that waste segregation at source will soon be applied to liquid wastes as well. Two of the common liquid wastes that are generated profusely in many municipalities are waste cooking oil and waste automotive engine lubricating oil. These oils have been finding their way into the sewage system and water bodies around the country.

Waste cooking oil is a problematic waste stream that needs to be properly managed. The disposal of waste cooking oil can become challenging when it is improperly disposed of (Abdullah et al., 2013). Improper disposal of waste cooking oil leads to the discharge of waste cooking oil into the environment, which then causes environmental pollution, particularly water and land pollution. In water, the oil layer covers the surface and prevents the dissolution of oxygen through the surface, leading to suffocation and mass extinction of marine biota (Jafari, 2010). The mixture of oil and water increases the chemical oxygen demand (COD) for water and causes it to be toxic due to the presence of oil degradation by-products (Jafari, 2010). Moreover, carcinogenic compounds are absorbed by sea creatures and returned to humans through the food chain (Jafari, 2010; Kamilah et al., 2013; Kulkarni and Dalai, 2006).

In addition, approximately, up to 150 million litres of lubricating oil waste are produced annually in Malaysia due to the active transport of goods by ships and land transport, as well as the increase in traffic volumes (Idros et al., 2012). Increased numbers of automobile workshops in Malaysia has contributed markedly to the problem of soil contamination resulted from improper waste disposal. Areas within the vicinity of these workshops are often exposed to high amounts of engine oils and other hydrocarbon wastes. Most of the mechanical and chemical methods such as incineration, thermal desorption, ground confinement and controlled burning used to reduce waste oil disposals are time-consuming, expensive and mainly depend on treatments in separate areas or better treatment facilities. However, these methods do not eliminate contamination, but only simply relocate the problem. The major concern about these treatments is they can lead to secondary pollution such as the greenhouse effect. In addition to major cases of contamination by waste engine oil, most of the

contamination caused by waste engine oil has occurred in small areas and is not reported.

Fungal isolates are known to degrade oil effectively, nonetheless most require a long time and economically prohibitive nutrient supplementation to grow and utilise the contaminating oil. Hence, further experimentation will help determine the specific growth requirement to expedite and enhance the rate of growth even further. Moreover, it is evident from the literature review that much of the work reported are focused on bacterial biosurfactant production and its relation to hydrocarbon degradation. On contrary to the study on the mechanisms of oil degradation using filamentous fungi, it is limited since relatively few studies on the subject have been published.

1.4   Research hypothesis

Fungus is hypothesised to have the ability to utilise waste oils with biosurfactants and modify its cell surface hydrophobicity. Also, several factors including nitrogen and carbon concentration, pH, salinity and agitation speed are hypothesised to affect the efficiency of oil degradation.

1.5   Research objectives

i.   To screen and identify oil-utilising fungi from used charcoal coated with animal fats;

ii.   To perform biodegradation experiments on waste cooking oil and waste engine oil using the isolated fungus;

iii. To develop medium formulation of the selected fungus for the highest oil removal using one factor at one time and response surface methodology study approach;

iv. To determine the oil uptake by fungi via cell surface hydrophobicity and biosurfactant production.

CHAPTER 2 LITERATURE REVIEW

2.1 Fats and oils

Fats and oils, is an ever-growing environmental concern. It can be referred to as plant and animal-based fats and oils. It is a by-product produced from food preparation facilities, for instance; restaurants, food stalls and residents or from any other food service establishments including deep-fried food, sauces, cheeses gravy and baked goods (Aziz, 2010; Husain et al., 2014). Fats and oils may be a solid or a liquid and they are usually insoluble in water but soluble in organic solvents such as ether, chloroform and hexane (Aziz et al., 2011). Fats and oils are basically esters (or triglycerides) consisting of straight chain fatty acids that attached to glycerol. The composition of edible fats and oils in fatty acids differ substantially. They can differ in terms of the number of carbon atoms, chain length and of being saturated or unsaturated (Biermann et al., 2011).

Moreover, fats and oils have high viscosity that varies based on the presence of double bonds and fatty acid composition where the more double bonds present in the carbon chain, the lower the viscosity of fats and oils due to the more loosely packed structure (Sincero and Sincero, 2003). Fats and oils usually will form emulsions in an aqueous media with the presence of an emulsifying agent or soap (Husain et al., 2014). The presence of a large amount of free fatty acids in fats and oils, which are generally formed by the oxidation reactions and hydrolysis of oils during deep frying of food, results in a characteristically low acid content of fats and oils (Sharoba and Ramadan, 2012; Tan et al., 2010). Also, oils and fats are a subdivision of lipids that are composed of triacylglycerols, fatty acids and lipid- soluble hydrocarbons (Aziz, 2010).

2.1.1   Composition of fats and oils

2.1.1(a)   Free fatty acids (FFAs)

Free fatty acids are carboxylic acids with long hydrocarbon side chains. There are over 1000 identified natural fatty acids that can be characterised by configuration, saturation and chain length. 20 of these are commonly found in food sciences (Husain et al., 2014). Figure 2.1 illustrates the chemical structurearachidonic acid that adopted from Voet et al., (2012). Fatty acids with less than 2-6 carbons are referred to as short chain fatty acids (SCFA), whereas, those with 8-10 carbons are known as medium chain fatty acids (MCFA). Long chain fatty acids (LCFA) refers to those with 12-24 carbons. The vast majority of FFAs are fatty acids with straight chains (aliphatic) of 8-22 carbon atoms. They frequently consist one or more unsaturated centres, indicated by the presence of double bonds (Voet et al., 2012).
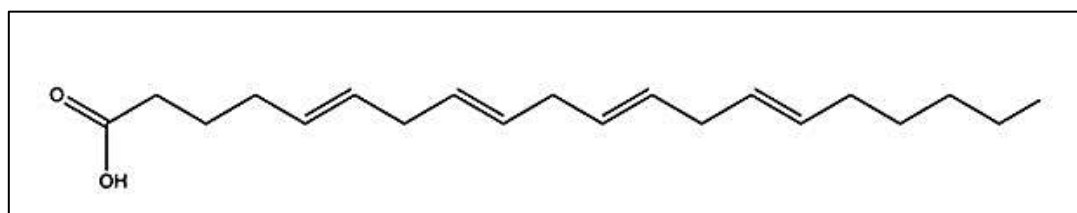
Figure 2.1   Arachidonic acid (Voet et al., 2012)

The degree of which the fatty acid is saturated or unsaturated can be referred to by the number of the double bonds. Saturated fatty acids are fatty acid chains that have no double bonds whereas monounsaturated fatty acids are those with one double-bond. Similarly, polyunsaturated fatty acids are fatty acids with more than one double-bond. In addition, unsaturated fatty acids take the cis configuration primarily where

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two identical groups are on the same side of the double bond (Voet et al., 2012). The chain length and the degree of unsaturation determine the properties of the fatty acids (Biermann et al., 2011). Table 2.1 depicts a list of the most common fatty acids (Voet et al., 2012).

Table 2.1　　List of common fatty acids (Voet et al., 2012)

Name	Systematic name	Short hand	Type of fatty acids	Melting point (°C)
Acetic acid	Ethanoic acid	2:0	Short chain fatty acids	16.6
Butyric	Butanoic	4:0	Short chain fatty acids	-7.9
Caproic	Hexnoic	6:0	Short chain fatty acids	-3.2
Caprylic	Octanoic	8:0	Medium chain fatty acids	16.2
Capric	Decanoic	10:0	Medium chain fatty acids	31.6
Lauric	Dodecanoic	12:0	Long chain fatty acids	44.8
Myristic	Tetradecanoic	14:0	Long chain fatty acids	54.4
Palmitic	Hexadecenoic	16:0	Long chain fatty acids	62.9
Stearic	Octadecanoic	18:0	Long chain fatty acids	70.1
Oleic	Octadecenoic	18:1	Long chain fatty acids	16.2
Vaccenic	Octadecenoic	18:1	Long chain fatty acids	44.1
Linoleic	Octadecadienoic	18:2	Long chain fatty acids	-5
Linolenic	Octadecatrienoic	18:3	Long chain fatty acids	-11
Arachidonic	Eicosatetraenoic	20:4	Long chain fatty acids	-49
EPA	Eicosapentaenoic	20:5	Long chain fatty acids	-
DHA	Dodosahexaenoic	22:6	Long chain fatty acids	-

2.1.1(b)   Triacylglycerols

Triacylglycerols (TAGs), also called triglycerides, are fully acylated derivatives of glycerol. Triacylglycerols are considered as a class of abundant lipids, water-insoluble substances and nonpolar. They are synthesized in living cells by the interaction of an intermediate of carbohydrate metabolism called glycerol phosphate, formed by a complete fatty acid residue coupled to coenzyme A and by the reduction of dihydroxyacetone phosphate (Gunstone, 2009). They are also fatty acid esters of the trihydric alcohol glycerol that contain three acyl chains, each carrying a hydroxyl group ($HOCH_2CH$ (OH) CH_2OH). Depending on the number of hydroxyl groups that are acylated, the glycerol esters are called monoacylglycerols (MAG), diacylglycerols (DAG) or triacylglycerols (TAG) (Gunstone, 2009; Husain et al., 2014). Fats and oils are predominantly triacylglycerols, with fatty acid composition varies based on the organism of origin. Typically, plant oils are richer in unsaturated fatty acids residues than animal fats, as determined by the lower melting points of the former. Therefore, plant oils are usually liquids whereas animal fats are solid at room temperature (Khan et al., 2004).

2.1.1(c)   Phospholipids

Phospholipids are one of the important raw materials in pharmaceuticals, cosmetics and foods as they possess amphiphilic nature which imparts unique properties to them (Gunstone, 2009; Husain et al., 2014). The major components of phospholipids are phosphatidylinositides (PI), phosphatidylethanolamines (PE) and phosphatidylcholines (PC), supplemented by other smaller proportions of

phospholipids. Sunflower seed oil, rapeseed oil and soybean oil contain 1.5%, 2.5% and 3.2% of total phospholipids, respectively and are the main sources of commercial lecithin. In contrast, palm oil contains little or no phospholipids (Gunstone, 2004).

2.1.1(d)   Sterols and sterol esters

Most of vegetable oils typically consist of 1000 – 5000 mg/kg of sterols, partly as free sterols and partly as esterified sterols with free fatty acids. The ratio of esterified to free sterols varies, where the free sterols (41 – 83%) are generally predominant. Although sterols are not lipids, they occur in many fats and oils and display certain similar physical properties. Table 2.2 shows the sterols and sterol esters content of common plant oils (Gunstone, 2009).

Table 2.2   The content of sterols and sterol esters (mg/100 g) in several plant oils (Gunstone, 2009)

Oil	Esterified sterols (%)	Free sterols (%)	Total sterols
Palm (crude)	16 (25)	49 (75)	65
Corn (crude)	423 (47)	485 (53)	908
Coconut (crude)	26 (39)	41 (61)	67
Palm olein (crude)	23 (29)	56 (71)	79
Rapeseed (crude)	475 (59)	336 (41)	811
Soybean (crude)	79 (25)	239 (75)	318

2.1.1(e) Ester waxes

Waxes usually consist of long-chain hydrocarbons (TCH$_3$), acids (RCOOH), alcohols (RCH$_2$OH) and diols, ketones, aldehydes along with the wax esters (RCOOR'). Figure 2.2 shows the chemical structure of ester waxes which adopted from Gunstone (2009). Generally, the latter are monoesters of long-chain acids with long-chain alcohols that comprise 40 carbon atoms. Typically, they are produced by plants (jojoba, carnauba, candelilla) and by animals (wool wax and beeswax or lanolin) (Gunstone, 2009).
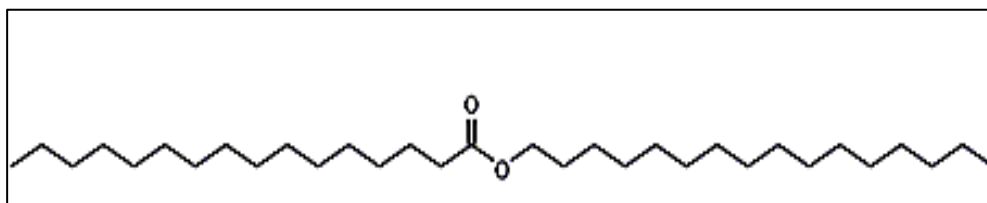
Figure 2.2　　The chemical structure of ester waxes (Gunstone, 2009)

2.1.2 Fats and oils problems in sewage system

Fats and oils are typically produced at residences, slaughterhouses and food service establishments as reported by Husain et al., (2014). Ashley et al., (2000) discovered that fats and oils can reduce the diameter of sewers, eventually causing blockage of pipes leading to sewer overflows and flooding. Keener et al., (2008) suggested that fats and oils can accumulate between 50 m and 200 m downstream from the source of fats and oils. Wallace et al., (2017) reported that the sewer diameter, gradient and age can contribute to the location of fats and oils blockages. Whitney (2014) found that the oldest city intact sewer dates back to 1852 in Dublin. The UK

has one of the oldest sewer systems in the world with 26% built between 1914 and 1945 and about 24% built prior to this period (Williams et al., 2012). Arthur et al., (2008) stated that in the UK, over 25,000 flooding events per annum were due to sewer blockages. Keener et al., (2008) suggested that fats and oils contributed to over 50% of such incidents. Similarly, in Malaysia, fats and oils have been attributed to up to 70% of sanitary sewer overflows (SSOs). Within the last ten years, Indah Water Consortium (IWK), Malaysia's national sewerage company, received a total of 22,184 blockage enquiries (Indah Water Consortium, 2010). SSOs cause very unpleasant odours and come with rat and insect infestations. The sewage may make its way into water sources, polluting surface and ground water (Suratman et al., 2011). It is detrimental to sewage treatment works (Williams et al., 2012).

Usually, fats and oils components are reportedly introduced into sewer system either by escaping from grease traps (GTs) installed in restaurants or typically by direct dumping into sewer. Fats and oils may melt and emulsify within the wastewater phase if high temperature water is used for dish washing. Consequently, it may escape the GT, only to solidify and form deposits on the pipe surfaces upon the subsequent flow into the sewer pipes, causing obstruction to wastewater flow (He et al., 2013; He et al., 2011; Husain et al., 2014; Keener et al., 2008). The efficiency of the GT are strongly dependent on its frequency of maintenance (Wong et al., 2007).

In Thailand, Stoll and Gupta (1997) reported that high concentrations of fats and oils in wastewater ranging from 730 to 1100 mg/L were attributed to discharge from Asian restaurants. Over the past two decades, the number of fast-food outlets has continued to increase, subsequently leading to increased improper discharges of fats and oils into the sewer system. Inevitably, this causes continuous fats and oils

deposition on pipe and channel surfaces, eventually causing blockage of sewer system (Metcalf and Eddy, 2013).

2.1.3   Methods to control fats and oils deposition

Currently, the approach of overcoming fats and oils deposition is via installing grease interceptor (GI) in the restaurants (Husain et al., 2014). Grease trapping systems (GTSs) are also known as grease interceptors, grease separators, grease abatement systems or grease recovery units. The purpose of GTSs is to separate fats and oils and fine food waste from wastewater through gravitational separation (Wallace et al., 2017). Nowadays, many efforts have been made to enforce good management practices at food service establishments and restaurants to ensure proper fats and oils disposal (He et al., 2013). In some cases, waste cooking oil from restaurants is collected and used to produce biodiesel (Sahar et al., 2018).

Most sewerage companies used high-pressure water jets to clear blocked sewer pipes (Stoll and Gupta 1997). Fats and oils deposits were collected manually using an arm net and a shovel. The collected fats and oils deposits were then disposed as solid waste in landfills (Alade et al., 2011). However, this subsequently raises another environmental concern, because fats and oils highly reduced the hydroconductivity of soil (Al-Hawash et al., 2018). Dumping fats and oils in landfills leads to the biological oxidation of high concentrations of fats and oils, releasing carbon dioxide and methane into the atmosphere (Husain et al., 2014).

2.1.4 Treatment of fats and oils

Lemus and Lau (2002) conducted a series of experiments evaluating the biodegradability of organic wastes rich in fats and oils via composting. They found that the lipid content was reduced to about 70% over after a processing period of 10 days. In addition, the volatile solid content also was reduced by 20%.

In a study by Bhumibhamon et al., (2002), 87.7% of fats and oils removal was achieved via biodegradation using lipase-producing microorganisms that were isolated from the wastewater of the palm oil and bakery industry. In addition, El-Masry et al., (2004) found four bacterial isolates, namely *Pseudomonas* sp. (L1) and *P. diminuta* (L2), *P. pseudoalcaligenes* (L3), and *Escherichia sp.* (L5), that could degrade oil and grease. These were isolated from vegetable grease and oil-contaminated industrial wastewater. An optimisation study for degradation of oil and grease by El-Bestawy et al., (2005) has been done using these bacterial isolates, showing that all isolates were able to completely degrade palm oil and utilise the free fatty acids (FFA) as a carbon source.

In another study, Wakelin et al., (1997) reported using a mixed bacterial culture isolated from a grease trap, for the biological treatment of fats and oils rich wastewater, achieving up to 90% removal. Witharana et al., (2017) claimed up to 50% fats and oils degradation using a lipolytic fungi after 72 h of incubation via solid state fermentation in a tray-type reactor, a novel approach of bioaugmentation.

2.1.5 Waste cooking oil (WCO)

Large quantities of waste edible oils originate from the food processing sectors and domestic consumption. Waste cooking oil is the by-product of frying foods in processed animal or plant oils. Cooking oil is a glycerol ester that consists of different types of essential fatty acids and are soluble in organic solvents (Alias et al., 2018). Typically, cooking oil is plant-based lipids, such as olive oil, palm oil, canola oil, coconut oil or animal-based lipids (Yaakob et al., 2013).

Waste cooking oil mainly comprised vegetable fats but can also contain small amounts of animal fats such as that in butter. The source of oils dictates its composition. Triglycerides account for about 95- 96% of the edible oils and they are accompanied by small amounts of mono- and diglycerides, phospholipids, free fatty acids, hydrocarbons and sterols (Marchetti et al., 2020). The difference in composition between the oils of origin and WCOs are attributed to the changes that occur during the cooking, with some compounds originating from the food. Cooking or frying produces some pleasant and desirable substances, which adds to the aroma and flavour of foods. However, it degrades some components and increases the viscosity, acidity and fat saturation of the oils (Knothe and Steidley, 2009; Marchetti et al., 2020; Thürer and Granvogl, 2016). During the frying process, oxidation, polymerisation and hydrolysis occur along with the formation of carcinogenic and toxic molecules, such as acrolein, polycyclic aromatic hydrocarbons and malonaldehyde (Wang et al., 2016).

Waste cooking oil quantities differ according to the quantity of edible oil consumed. Presently, about 200 million tons of vegetable oil are consumed worldwide. Teixeira et al. (2018) estimated that 32 percent of the edible oil consumed ended up as waste. A total of 64 million tons of WCO were estimated to be available worldwide.

Europe produces about 700,000-1,000,000 tons of WCO per annum. Approximately 40,000 tons of WCO were produced annually in Asian countries such as China, Malaysia, Indonesia, Thailand, Hong Kong and India (Kamilah et al., 2013).

There are several methods that are used for treating waste cooking oil including using high pressure water jet and bioremediation. One of the established methods is via biodegradation of waste cooking oil using microorganisms. Sharma et al. (2019) studied *Pseudomonas aeruginosa* MTCC7815 for biosurfactant production using waste cooking oil (WCO) as the sole carbon source. Culture conditions have been optimized on the basis of surface tension reduction and biomass density. Optimal cultivation conditions were found to be similar to those of natural domestic sewage, such as a pH value of 10, a temperature of 25 °C and a very high WCO concentration of 40 gL^{-1} with a C/N ratio of 40:1. Biosurfactant yield was 11 ± 0.2 gL^{-1} when approximately 90 per cent of WCO was used within 5 days of incubation. These results suggest the possibility of *Pseudomonas aeruginosa* for commercial biosurfactant production along with economical waste removal.

A study by Teng et al. (2019) investigated the influence of *Aspergillus niger* laccase on oxidation indices on polycyclic aromatic hydrocarbon (PAH) and oxygenated PAH (OPAH) content in waste cooking oil after frying nugget, pumpkin pie and youtiao. They reported that phenanthrene, 9-fluorenone and anthracene-9,10-dione concentrations were removed 55.52–62.57, 15.82–23.24 and 14.99–27.50 μg / kg, respectively. These were removed by a maximum of 18.31%, 26.92% and 19.41% after 24 h and 29.24%, 70.02% and 62.88% after 48 h. Removal was higher with time. These results also suggest that laccase could be used for the green removal of specified OPAH and PAH in waste cooking oil.

2.2 Petroleum hydrocarbon

Crude oil is the main source of all petroleum hydrocarbons. It is believed to be the fossilised remains of prehistoric zooplankton and algae which have undergone catagenesis to generate hydrocarbons (Kothari et al., 2013). It is basically a complex mixture of hydrocarbons and, as the name implies, its composition is predominantly carbon and hydrogen. Alongside these two elements, there are also nitrogen, oxygen and sulphur, as well as trace metals. Crude oil is not commercially useable prior to refining. The refining process, via fractional distillation which is the breaking down of enormous hydrocarbons process, is employed to form smaller, more commercially valuable, hydrocarbons (Davoodi et al., 2020).

2.2.1 Composition of petroleum hydrocarbon

Generally, hydrocarbons in crude petroleum can be classified into four classes namely; the saturates, the aromatics, the asphaltenes (fatty acids, ketone, porphyrins, esters and phenols) and the resins (quinolines, sulfoxides, amides, carbazoles and pyridines) (Davoodi et al., 2020). Hydrocarbons may vary in their susceptibility to microbial attack. They have been ranked in the following order of decreasing susceptibility, which are n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Perry, 1984). However, some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PHAs), may not be susceptible to microbial degradation at all (Das and Chandran, 2011).

2.2.1(a)   Saturated hydrocarbons

Saturated hydrocarbons are hydrocarbons in which all the carbon-carbon bonds are single bonds. Each carbon atom is bonded to four other atoms, resulting in no multiple bonds in these compounds (Kothari et al., 2013).

The term 'saturated hydrocarbon' refers to alkanes-acyclic hydrocarbons, containing only sp3 hybridised carbon atoms, having a general formula of C_nH_{2n+2} (Deborah and Thatheyus, 2019). Among the petroleum hydrocarbons, alkanes are the most biodegradable. Usually, alkanes that in the range of C5 to C10 are inhibitory to most hydrocarbon-degraders at higher concentrations as the alkanes disrupt lipid membranes (Deborah and Thatheyus, 2019). Alkanes in range of C20 to C40, are referred to as waxes and are less amenable to biodegradation as they occur in hydrophobic solids, with low solubility in water (Kothari et al., 2013). Table 2.3 depicts two examples of the names and formula of the simplest alkanes. Upon degradation, alkanes are converted to alcohol by the action of oxygenase enzymes that attack the terminal methyl group. The alcohol is further oxidised to aldehyde and then to fatty acids, which are subjected to β-oxidisation (Eglinton and Hamilton, 1963).

Table 2.3   Name and formula of alkanes (Eglinton and Hamilton, 1963)

Alkane	Molecular Formula	Structural Formula	Condensed Formula	Line Formula
Propane	C_3H_8	(structural formula of propane)	$CH_3CH_2CH_3$	(line formula)
Butane	C_4H_{10}	(structural formula of butane)	$CH_3CH_2CH_2CH_3$	(line formula)

Generally, cycloalkanes, also known as alicyclic hydrocarbons, are less degradable than alkanes (Eglinton and Hamilton, 1963). They have higher melting and boiling points than the alkanes. Biodegradability decreases with the increasing number of ring structure. Cycloalkanes are degraded to cyclic alcohol by via the action of oxidases which then dehydrogenated to ketone. The main products of metabolism of cycloalkanes are cycloalkane-carboxylic acids and cycloketones (Kothari et al., 2013).

2.2.1(b)   Aromatic

Generally, aromatic hydrocarbons molecules consist of benzene-based structure. Aromatic compounds are more stable as compared to most other cyclic compounds due to its characteristic of sharing of delocalised electrons by pi bonds. Moreover, BTEX (benzene, toluene, ethylbenzene, xylene) compounds are more water-miscible and mobile comparatively (Gibson, 1988). Apparently, there are two major steps involved in biodegradation for aromatic molecule which are; (1) ring cleavage as shown in Figure 2.3 (2) activation of the ring (Eweis et al., 1998; Kothari et al., 2013; Ward and Singh, 2004).
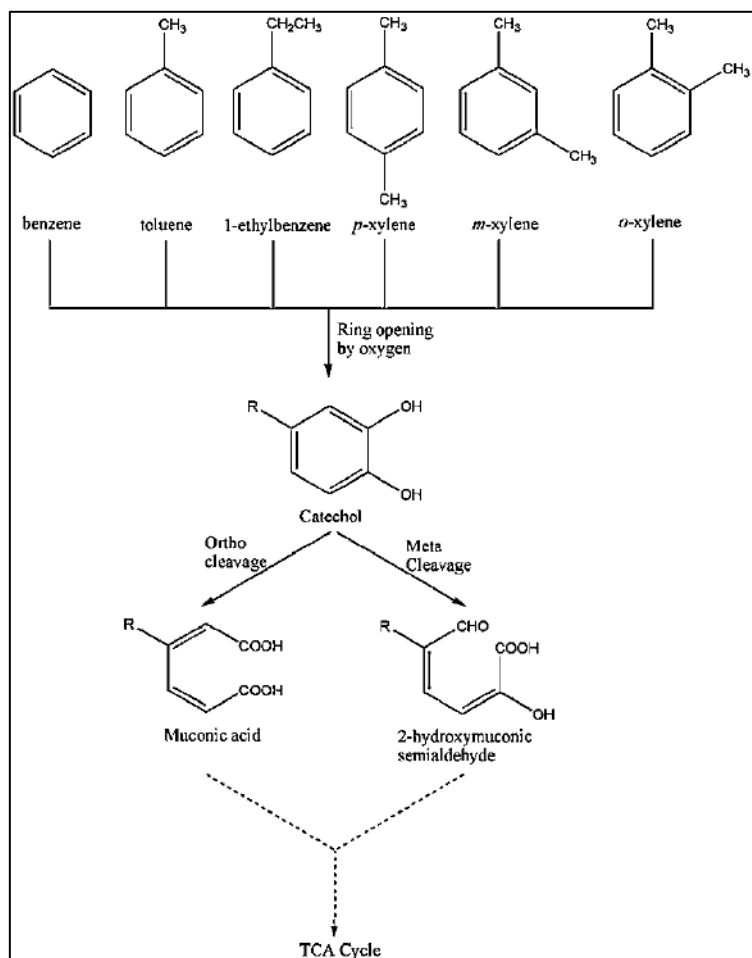
Figure 2.3   General pathway for aromatic hydrocarbon degradation (Eweis et al., 1998; Kothari et al., 2013; Ward and Singh, 2004)

Furthermore, activation is attained by the incorporation of molecular oxygen into the aromatic ring which lead to dihydroxylation of aromatic nucleus, subsequently, and the enzymes that causing this are oxygenases (Gibson, 1988; Kothari et al., 2013). Moreover, monooxygenases which are the characteristic of fungi and other eukaryotes responsible in catalysing the incorporation of a single atom of oxygen to form an epoxide, consequently, undergo hydration in order to yield transdihydrodiols (Eweis et al., 1998). Meanwhile, dioxygenase can catalyse incorporation of two atoms of oxygen simultaneously, to form a dihydrodiol. A study by Gibson (1988) has showed that these dioxygenases reactions have been appeared

to work on anthracene, xylenes, naphthalene, biphenyls, toluene, benzene, halogenated benzenes and para-chlorotoluene. In addition, these dihydrodiols are being further oxidised to catechols, which are the precursors to ring cleavage (Gibson, 1988). Next, a study by (Cerniglia, 1984) has found that catechol can be further oxidised by either via the meta-cleavage pathway which involves the cleavage of bond between a carbon atom with the adjacent carbon and a hydroxyl group to produce 2-hydroxymuconic semi aldehyde or via ortho-cleavage pathway that involves cleavage of the bond between carbon atoms of two hydroxyl groups to produce muconic acid. Lastly, these compounds are then further degraded to form organic acids which are utilised by microorganisms for their energy generation and cell synthesis, subsequently (Cerniglia, 1984).

Polycyclic aromatic hydrocarbons (PHAs) also known as polynuclear aromatic hydrocarbons (PNA) are being produced during high temperature industrial operations such as coke production, wood preservation and petroleum refining (Park et al., 1990). Moreover, this groups of compounds comprised of two of more benzene rings which involves 16 priority pollutants (Dzombak and Luthy, 1984; McEldowney et al., 1993). Furthermore, some of these compounds are believed to be carcinogens. Besides, the increase in number of ring structures of PAHs and molecular weight may reduce their votality and solubility, while increasing the capacity of absorption (Cerniglia, 1993). Another study by Cerniglia (1984) has found that fungal degradation of PHAs is environmentally important as some of products have been incriminated as toxic forms in higher life forms.