OPTIMIZATION OF FAT, OIL, AND GREASE BIODEGRADATION USING BACTERIA ISOLATED FROM PALM OIL MILL EFFLUENT

BY

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A dissertation submitted in fulfilment of the requirement for the degree of Master of Science in Biotechnology Engineering

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SEPT 2018

ABSTRACT

Biodegradation of fat, oil, and grease (FOG) plays an important role in water pollution control and wastewater management. However, many food services establishments generate FOG-containing wastewater which there is no accepted technology for its treatment. FOG is the causative agent of blockage sewer systems, and results in sanitary sewer overflows (SSO). To solve this problem, this study evaluated the feasibility of FOG-degrading microorganism on the biodegradation of palm oil. Six strains capable of degrading FOG were isolated from palm oil mill effluent (POME). The potential bacterial strains were selected based on Tween-80-degrading ability. *Micrococcus lylae* strain DSM 20315 showed the highest growth compared to the other strains. Hence, it was selected for FOG degradation test. The biodegradability was performed as a function of pH (6, 7, 8), initial oil concentration (1, 3, 5% v/v), and bacterial inoculum concentration (2, 6, 10% v/v). Optimization of these parameters of palm oil degradation was studied. A 2 level factorial design was used to investigate the influence of these three parameters. The maximum oil degradation was 68% obtained at pH 6, initial oil concentration 1 mL, and bacterial inoculum concentration of 10 mL. The lowest oil degradation obtained was 22%. The initial oil concentration followed by bacterial inoculum concentration enhanced the removal efficiency of FOG, but the pH level did not significantly promote the degradation rate. As a result, the optimum process conditions for maximizing oil degradation (removal) were recognized as follows: pH 6, initial oil concentration 1 mL and bacterial inoculum concentration of 10 mL. The result indicated that the use if isolated *Micrococcus lylae* strain DSM 20315 in bio-augmenting grease trap or other process might possibly be sufficient to acclimate biological processes for FOG degrading.

خلاصة البحث

للتحلل الحيوي للدهون والزيوت والشحوم (FOG) مكانة مهمة في مكافحة تلوُّث المياه وإدارة مياه الصرف الصحي، ذلك أن كثيرًا من مؤسسات الخدمات الغذائية تُنتج مياهًا عادمة تحتوي على FOG من غير تقنية مُرْضِيَةٍ لمعالجتها؛ إذ يُعدُّ FOG سببًا في انسداد نُظُم الصرف الصحي وفيضان المياه العادمة، ولحل هذه المشكلة؛ سعت هذه الدراسة إلى بيان جدوى استخدام الكائنات الدقيقة التي تعمل على تحلل زيت النخيل حيوياً. عُزلت ستُّ سُلالات بكتيرية من المياه العادمة الناتجة من استخلاص زيت النخيل POME؛ اختيرت هذه السُّلالات البكتيرية على أساس قدرتها على أن تحلُّل زيت Tween-80، وأظهرت السلالة Micrococcus lylae DSM 20315 أعلى نمو مقارنة بالسلالات الأخرى؛ لذا اختيرت لدراسة تحلل الزيت حيوياً وفق العوامل الآتية: التركيز الهيدروجيني (٦،٧،٨)، وتركيز الزيت (١،٣،٥) مل، وتركيز اللقاح البكتيري (٢،٦،١٠) مل، ثم دُرست كيفية تحسين عملية التحلل الحيوي لزيت النخيل وفق تلك الظروف؛ باستخدام level 2factorial design لدراسة تأثير العوامل الثلاثة السابقة، وكانت أعلى نسبة تحلل حيوي 68% عندما كان: التركيز الهيدروجيني 6، وتركيز الزيت الأولي 1 مل، وتركيز اللقاح البكتيري 10 مل، في حين كانت أدنى نسبة تحلل حيوي 22%. عزَّز تركيزُ الزيت الأولي وتركيزُ العزلة البكتيرية من التحلل الحيوي، أما التركيز الهيدروجيني فلم يُبد تأثيرًا ملحوظًا فيه، وعليه؛ تبيَّن أن الظروف المثالية للتحلل الحيوي تتحقق وفق العوامل الآتية: التركيز الهيدروجيني 6، وتركيز الزيت الأولي 1 مل، وتركيز اللقاح البكتيري 10 مل.إن نتائج هذه الدراسة تشير إلى أن إدراج السلالة البكتيرية Micrococcus lylae strain DSM 20315 في منظومة عمل grease trap أو في عمليات التخلص من الزيوت الأخرى؛ قد يؤثر تأثيرًا واضحًا في التخلص من الدهون والزيوت والشحوم FOG.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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ACKNOWLEDGEMENTS

Firstly, it is my utmost pleasure to dedicate this work to my dear parents and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and patience.

I wish to express my appreciation and thanks to those who provided their time, effort and support for this project. To the members of my dissertation committee, thank you for sticking with me.

Finally, a special thanks to Associate Professor Dr. Maan Fahmi Al Khatib, Professor Dr.Zahangir Alam. For their continuous support, encouragement and leadership, and for that, I will be forever grateful.

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LIST OF ABBREVEATIONS

FOG	Fat, oil, and Grease
POME	Palm Oil Mill Effluent.
EC	Electro coagulation
OD	Optical density
COD	Chemical oxygen demand
DO	Dissolved oxygen
TPH	Total hydrocarbon petroleum
CCR	Carbon catabolite regulation
SSO	sanitary sewer overflow
TPS	titled Plate separator
GI	Grease interceptor
GAD	Grease abetment device
GC/MS	Gas chromatography- mass spectrometry
LCFA	Long chain fatty Acid
FFA	Free fatty acids
TAG	triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
ATP	Adenosine triphosphate
PKO	Palm Kernel oil
CPO	Crude palm oil
RPM	Round per minute
LB	Luria-Bertani
MSM	Mineral salt medium
2LDF	2 Level factorial design
CCD	Central composite design
Ca	Calcium
Mg	Magnesium
rRNA	Ribosomal ribonucleic acid
SFA	Saturated fatty acids
USFA	Unsaturated fatty acids

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

FOG accumulation in sewer system ultimately affects public health and has negative impact on the environment as it could be a causative agent of foul odor and suitable host of rodent, worms, and insects. In addition to the daily production of FOG-wastewater from private residences, FOG is generated as a result of dairy industry, slaughterhouses, food processing plants, and other industries that process fatty substances (Brooksbank et al., 2007). FOG deposits have impact on health and environment. FOG is endemic in sewer system such as pipe lines, pump stations, wet wells and downstream wastewater treatment plants. They reduce sewer diameter and can completely block the pipe lines, which often ends in sanitary sewer overflows (SSOs).

FOG is thought to contribute over 50% of total SSOs (Williams et al., 2012). In the UK for example, over 25,000 flooding events every year are due to sewer blockage. Moreover, the American environmental agency (EPA) also estimated that there are at least 10,350- 36,000 sanitary sewer overflows occurring every year in the USA, approximately 47% of which is related to FOG. Furthermore, in 2010 the wastewater municipality in Malaysia, Indah Water Konsortium (IWK) estimated that up to 70% of SSOs is thought to be contributed by FOG (Husain et al., 2014). The subsequent release of sewage increases water pollution and exposure to pathogens. FOG also can attract vermin such as rats, and sloughed deposits which can affect the operation of pumping stations and sewage treatment works (Williams et al., 2012). Environment and public health concerns mandate a comprehensive understanding of

FOG properties and effects so that FOG control can be implemented in practical applications.

Approaches in FOG control could be characterized as chemical, physical and biological. Chemical hydrolysis of fat, oil, and grease lead to bring in long chain fatty acids into wastewater which may suppress bacterial activity and might influence the diversity of living microorganisms in wastewater (Ma et al., 2015) decreasing wastewater treatment effectiveness and often unpleasant scent are produced. Physical methods exploit the FOG's low density compared to that of water which allows FOG deposit to float over wastewater surface, thus can be splitted up from wastewater with grease, dissolved air flotation devices and tilted plates.

Grease traps are one of the most common physical-based FOG pretreatment equipment applied in governmental wastewater plants, residences, and food industries. When wastewater that polluted with FOG streams into a grease trap, FOG will float on the surface while the water shall continue to flow to the wastewater collection system, then FOG layers can be take off manually.

Tilted plates (TP) are a modified grease trap, which are parallel sheet packs inclined at reverse angles, also it referred as gravity separator, that enlarge the surface area while requiring less than 10% of the volume of setting area of the classic grease trap (Willey, 2001). In dissolved air foliation, large sum of micro-bubbles adhere to suspended FOG particles causing the suspended FOG to float to the surface of the water where they can be easily removed by skimming equipments. Dissolved air flotation equipments demands high energy in order to skim the formed FOG layer, which is counted to be one of the disadvantage of this method.

Physical approaches require human assistance to get rid of the FOG stratum that accumulate. The need for human resources maximizes the cost to municipalities which spend lots of money each year to implement FOG removal and maintain a correlated infrastructure. Moreover, these techniques are inefficient in minimizing emulsified and dissolved fats and oil which might limit the oxygen transfer range, thus deteriorate biological treatment (Brooksbank et al., 2007).

In comparison, chemical/physical techniques with regard to fat, oil, and grease control, their minimum cost, maximum FOG removal potency, and easier to maintain over biological techniques have rose up their popularity. Biological treatment degrades FOG or accelerates its break down using competent bacterial strains, lipase enzyme, commercial supplements, and biosurfactants. Research on biological techniques focus on isolating eligible bacteria strains, finding optimum operating conditions, designing a suitable refining carrier for FOG plucking out improvement and merging biological techniques with physical processes.

Microorganism bioaugmentation is additionally another technique employed by choosing microbes that shall assist in the efficient biodegradation of particular molecules found in wastewater with particular compositions. A study evaluated the effectiveness of (FOG) biodegradability using lipase enzyme secreted by *Pseudomonas* sp. Strain D2D3. The FOG removal capability of this strain was at range of 94.5% and 94.4 % for olive oil and animal fat respectively, however sunflower oil was the minimum at 62% (Shon et al., 2002). Another study employed bacterial consortium culture for treatment of bakery wastewater with maximum FOG content. Throughout a 7-days treatment interval by involving a bacterial culture of single strain , the removal efficiency was about 73 – 88% (Bhumibhamon et al., 2002). Moreover, it was founded that maximum degradation efficiencies were attained by involving bacterial cultures with single strain. The potential strain of this study was known as members of the genera *Pseudomonas*, *Acinetobacter, and Bacillus* as well

(Bhumibhamon et al., 2002). Another study showed that a bacterial consortium of choosing microbes, that had been acclimatized to the conditions of wastewater, had an FOG degrading potential of more than 90% (Wakelin & Forster, 1997).

The idea of this research was to investigate the biodegradability of palm oil (as it is considered as one of the main oils that cause FOG depositions, and because the fatty acids compositions of palm oil is similar to that one in FOG). This may be started by firstly isolating bacterial isolates from palm oil mill effluent and screening the isolates for lipolytic activity on Tween 20. Secondly, to subject the palm oil to the bacterial strains that show positive results on Tween 20 degradation in order to choose the bacterial strain with the highest growth. Lastly, optimize the growth conditions in order to have the highest level of degradation which could assist in FOG removal.

1.2 PROBLEM STATEMENT

Despite the physical and mechanical ways of fat oil and grease (FOG) removal available, it is still a serious issue to address. FOG is not completely removed by the above methods, and there is a need to treat the grease waste before being transported to wastewater treatment plants.

FOG is singled out for particular attention because of its low solubility in water, therefore, they tend to be separated from water. When warm FOG is washed down the sink into sewer system, it does not look harmful, but as it cools, hardens, and sticks inside the sewer pipes. And by time, it gradually accumulates inside the pipe lines, sewers, and manholes. This growing accumulation leads to restrict water flow into the pipes system, and reduces the capacity of the pipes, results in clogged pipeline, which ultimately causes sanitary sewer overflows (SSOs), by which the raw sewage back-ups into homes and business, and overflows discharges into street. Moreover, it interferes with sewage treatment processes at wastewater treatment plants. FOG build up in sewer system has many harmful effects upon human kind as well as environment. Sewer backups onto homes create a health hazard because such conditions are preferable for disease causing organisms. Moreover, it causes unpleasant mess and foul smell. Furthermore, that clogging in sewer system requires that the piping system be cleaned more often, and some pipes to be replaced sooner than otherwise expected, thus an increase in cleaning operations and maintenance costs will be inflicted. Few methods have been used to reduce the accumulation of FOG into sewer system. Mechanical and physical separation methods are involved. Grease trap is one of the physical methods that has been used to treat FOG in wastewater. Grease trap separates FOG from wastewater based on the physical characteristics of FOG which tend to float on wastewater surface. However, not all FOG can be isolated from wastewater and some of FOG can pass to wastewater treatments plants. Electrocoagulation method is another physiochemical method that is used to separate FOG, which aims to coagulate of the colloidal particles in wastewater. However, this method depends on electricity which might be costly, and an impermeable oxide layer may be formed which reduces the efficiency of electrocoagulation process. Biodegradation is an alternative method that could be used to treat FOG problem based on the microbe activity. In this study, bacteria will be isolated from POME because it is expected to isolate potential FOG-degrading bacterial isolates from it. And therefore the oil-capable-degrading isolates will be applied to treat FOG.

1.3 RESEARCH OBJECTIVES

The main objective of this study is to enhance FOG removal, and the specific objectives are:

1- To isolate and screen bacterial strains that are capable of oil biodegrading.

2- To identify the strains that are capable of biodegrading FOG.

3- To optimize the physiological parameters (pH, oil concentration, bacterial inoculum concentration)

1.4 RESEARCH SCOPE

In this study, the potential bacteria are isolated from POME. The experiments are conducted on small scale. The incubation temperature is set to 37°C and 150 rpm. The study involves optimization of the degradation using three variables (pH, oil concentration and bacterial concentration). FOG removal is simulated by palm oil.

1.5 DISSERTATION ORGANIZATION

The thesis is organized into five chapters, where chapter one is an introduction/ background of research work, while chapter two presents review of related literature. The material and methodology of the research are covered in chapter three, giving a detailed description of materials, and the experimental procedures used in the study. The findings of the research work are thoroughly discussed in chapter four. Finally, chapter five concludes the findings together with some recommendations on how to improve further research.

CHAPTER TWO

LITERATURE REVIEW

2.1 FAT OIL AND GREASE

Lipids; categorized as fats, oils, greases and long-chain fatty acids, are important ingredients of foods, many synthetics molecules and emulsions (Chipasa & Mędrzycka, 2006). FOG is significantly characterized to possess both hydrophobic and hydrophilic properties. These characteristics are due to specific components called fatty acids, which are straight hydrocarbon chain combined with a carboxylic acids. Lipid consists of fatty acids bounded to glycerol molecule via esters (Bharathi et al., 2012).

The simple lipids are referred as triglycerides or Triacylglycerols because 3 fatty acids are connected to glycerol molecule as shown in Figure (2.1). However, the triglyceride composition is unique for any plant oil (Bharathi et al., 2012). Therefore, due to different triglyceride composition of edible oil, different properties are also noticed. Triacylglycerides inclined to be the most common lipid category in natural oil and edible fats. The component fatty acids of edible fats and oil are very considerable and have a diversity of types and length. They differ in carbon-atom-chain length which varies as minimum as 2 to as maximum as 22 carbon atoms, saturated or unsaturated, and whether it contains even number or odd of carbon atoms (Berg et al., 2002a).

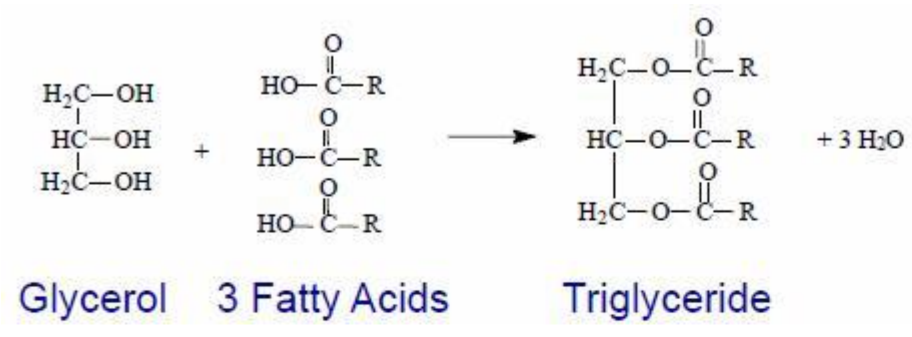

Figure 2.1 Chemical formula for triglyceride synthesis (Berg et al., 2002).

Saturated term refers to hydrogen which means that the hydrogen chain has as many atoms of hydrogen as possible, i.e. the last atom of carbon linked to three hydrogen atoms ($-CH_3$) and every atom of carbon within the tail is bounded to two atoms of hydrogen ($-CH_2-$). However, unsaturated fatty acids unsimilar with saturated ones by existence of 1 or 2 double bond (s) within the chain, and 1 alkenyl (-CH: CH-) group replaces the carbon atoms with single bond ($-CH_2-$ CH_2-) (Berg et al., 2002) as shown in figure 2.2.

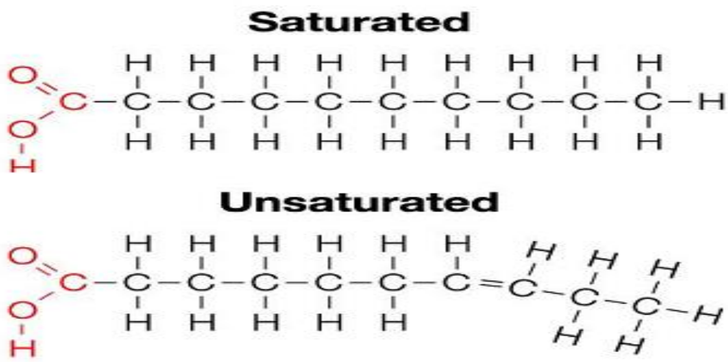

Figure 2.2 Structure of saturated and unsaturated fatty acids (Berg et al., 2002)

Based on fatty acids length, there are three major categories of fatty acids. Carboxylic acids as short as butyric acid which consist of four to five carbon atoms, which are termed as short chain fatty acids. Fatty acids that containing carbon atoms ranging from six to twelve atoms are usually called to as medium chain fatty acids, whereas the ones with more than twelve carbon atoms are counted to be long chain fatty acids (Table 2.1). Most of naturally originated fatty acids contain an even number of carbon atoms due to their biosynthesis encompasses acetyl-CoA.

Table 2.1 List of the most common free fatty acids (Berg et al., 2002)

C-Atoms: Double Bonds	Common Name	Systematic Name	Abbrev.	Structural Formula
4:0	Butyric	Butanoic	C4:0	$CH_3(CH_2)_2COOH$
5:0	Valeric	Pentanoic	C5:0	$CH_3(CH_2)_3COOH$
6:0	Caproic	Hexanoic	C6:0	$CH_3(CH_2)_4COOH$
7:0	Enanthic	Heptanoic	C7:0	$CH_3(CH_2)_5COOH$
8:0	Caprylic	Octanoic	C8:0	$CH_3(CH_2)_6COOH$
9:0	Pelargonic	Nonanoic	C9:0	$CH_3(CH_2)_7COOH$
10:0	Capric	Decanoic	C10:0	$CH_3(CH_2)_8COOH$
12:0	Lauric	Dodecanoic	C12:0	$CH_3(CH_2)_{10}COOH$
14:0	Myristic	Tetradecanoic	C14:0	$CH_3(CH_2)_{12}COOH$
15:0	Valerenic	Pentadecanoic	C15:0	$CH_3(CH_2)_{13}COOH$
16:0	Palmitic	Hexadecanoic	C16:0	$CH_3(CH_2)_{14}COOH$
16:1	Palmitoleic	*cis*-9-hexadecenoic	C16:1	$CH_3(CH_2)_5CH{:}CH(CH_2)_7COOH$
17:0	Margaric	Heptadecanoic	C17:0	$CH_3(CH_2)_{15}COOH$
18:0	Stearic	Octadecenoic	C18:0	$CH_3(CH_2)_{16}COOH$
18:1	Oleic	*cis*-9-octadecenoic	C18:1	$CH_3(CH_2)_7CH{:}CH(CH_2)_7COOH$
18:2	Linoleic	*cis*-9,12-octadecadienoic	C18:2	$CH_3(CH_2)_4(CH{:}CHCH_2)_2(CH_2)_6COOH$

The fatty acids components of microorganisms and the edible oil are often very different. Generally, bacterial lipid tends to have considerable amount of C_{14} to C_{18} of saturated straight-chain and monoenoic fatty acids. In addition, bacterial lipids might have branched chain, odd-chain, hydroxyl and cycloalkane fatty acids which rare to be synthesized by plants and animals.

2.2 FATTY ACIDS COMPOSITION OF FOG

Saturated fatty acids are the main component of FOG, and monounsaturated fatty acids are the second major component. For saturated fatty acid palmitic acid is the main fatty acid, while oleic acid is the major monounsaturated fatty acid. In addition, linoleic is the primary polyunsaturated fatty acid in FOG deposit samples (He et al., 2011). According to Williams et al. (2012b), the predominant fatty acids in FOG are long-chain fatty acids (LCFA) that have the range of 14-18 carbon atoms. Moreover, linoleic acid (C18:2) and stearic acid (C18:0) have also been reported to be commonly present in FOG (Cyril, Dominic er al., 2013; He et al., 2011; Keener et al., 2008).

2.3 HYDROLYSIS OF LIPIDS

Lipid breakdown is catalysed by particular enzyme referred as lipases. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyse, under normal conditions, the breakdown of triglycerides to diacylglycerols, monoacylglycerols, fatty acids, and glycerol (Iqbal & Rehman, 2015; Kanmani et al., 2015). They also catalyse the reverse reaction (esterification) of hydrolysis where the synthesis of long-chain acylglycerols occurred in the environment that has low water content, which called a microaqueous system. Esterification reaction occurres at the interface area between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Moreover, lipases catalyse a transesterification reaction where an exchange of ester bonds that present in non-aqueous media (Kanmani et al., 2015).

Lipases are serine hydrolases which act at lipid-water interface (Figure 2.3). Lipases have catalytic triad composed of Serine-Histidine-Aspartate/Glutamate and usually have a consensus sequence (Glycine/Alanine-X-Serine-X-Glycine) at the active site serine called nucleophilic elbow(Gupta et al., 2004). Structural investigations disclosed that most lipases feature a lid which consists of an amphiphilic peptide loop which covers the active site of the enzyme in its inactive state (Figure 2.3). In the presence of hydrophobic substrate, the active site becomes accessible after conformational changes of this lid. These conformational changes are called 'interfacial activation' (Bourlieu et al., 2009)

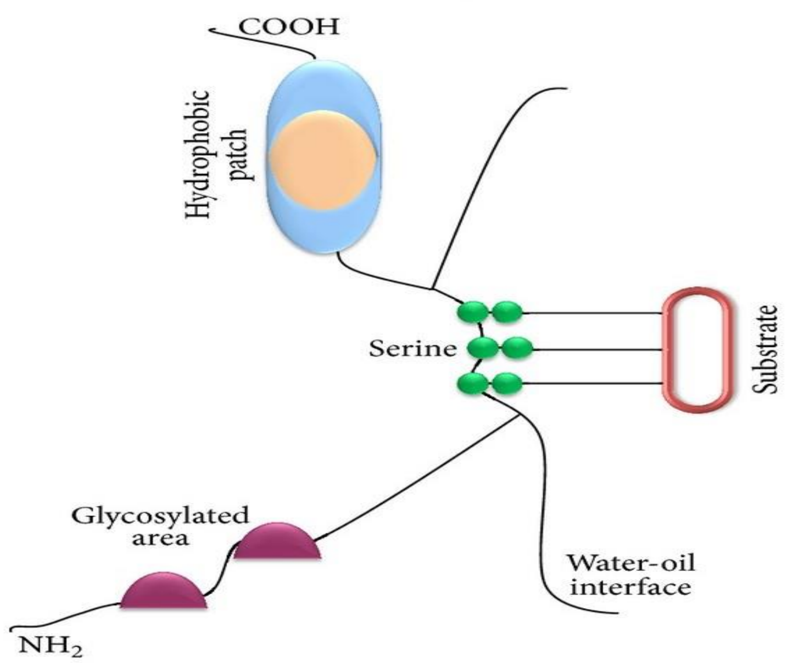

Figure 2.3 representation of a Lipase molecule showing its main features, the substrate can be ant triglycerides. Substrate interactive regions are displayed (Gopinath et al.,2013)

One characteristic of lipase enzyme that has been determined is that lipase has an adsorption affinity with oil droplets in liquid medium. Shape and size of the oil droplets play a vital role in the rate of production of the enzymes (Tamerler & Keshavarz, 2000).

Lipases display a high level of specific as chemo-, region- and anantioselectivity in nature (Kanmani et al., 2015). Lipases can be categorized according to their positional specificity into two groups: 1, 3-positional-specific and non-positional-specific. The first group contains specific or region-specific lipases, where lipases breakdown esters in the 1 and 3 positions of glycerides liberate free fatty acids and mixtures of mono- and di-glycerides. However, the 2-monoglycerides and the 1, 2- or 2, 3-diglycerides are unsteady; therefore the enzymatic breakdown is followed by spontaneous-non-enzymatic acyl migration from 2- to 1, 3-position in mono- and diacylglycerols (Pabai et al., 1995). Therefore, the increase of incubation time is anticipated to result in total breakdown of triacylglycerols. The previous reaction is spontaneous and prompted by acid, alkaline and heat. The second group is termed non-positional-specific lipases, where the enzymes do not differentiate between the three positions of glycerol esters lead to total breakdown of triacylglycerols to fatty acids and glycerol. Usually, pancreatic and fungal lipases are 1.3-positional-specific, while yeast and bacterial ones are on-positional-specific or weakly 1.3-positional-specific, figure (2.4) (Sztajer & Zboinske, 1988). Lipase may additionally display the type based on the kind of fatty acids and the length of the carbon chain.