SEQUENCE AND FUNCTION-BASED SCREENING OF GOAT RUMEN METAGENOME FOR NOVEL LIPASES

A thesis submitted in fulfilment of the requirements for the degree of Masters of Technology, in the Department of Biotechnology, Faculty of Applied and Computer Sciences, at Vaal University of Technology.

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OMICS Research Group
Vaal University of Technology
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DECLARATION

I, **Mukendi Mujinga Grace**, hereby attest that this thesis entitled "**Sequence and Function-based Screening of the Goat Rumen Metagenome for Novel Lipases**" is submitted in the fulfilment of the requirements for the degree of masters of technology in Biotechnology at Vaal University of Technology and it was composed solely by myself and the work contained herein is my own except explicitly stated otherwise. Being my original work, it has never been submitted for a degree in any other university or any professional qualification.

04 September 2019

Mukendi Mujinga Grace Date

“For I can do everything through Christ, who gives me strength."

Philippians 4:13

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Sequence and Function-Based Screening of Goat Rumen Metagenome for Novel Lipases

General Abstract

Lipases have been one of the important biocatalysts that catalyse the transformation of lipids to yield very important products that can be of beneficial in food, agriculture, pharmaceutical medicine and for the biodiesel production. In the search for novel biocatalysts, notably lipases, the conventional culture-based techniques were used but it can only address sourcing the biomolecule from 1-10% of the microbial population leaving the wealth of the biomolecules packed in 90-99% of the microbial community unaccounted for. Metagenomic technique, which is culture-independent, was developed as a comprehensive approach to address literally 100% of the microbial population thereby maximizing the chances of obtaining novel biocatalysts with superior physico-chemical and catalytic traits. In principle, any biomolecule including lipase could be sourced from any biologically-active environment, of which animal rumen is one. However, among the rumenant animals goat has diverse feeding habit, thereby laying ground for increased microbial diversity in its gastro-intestinal tract. It was thus, postulated that goat rumen could be potential source of novel lipase isoforms. Therefore, the aim of the study was to extract metagenomic DNA from goat rumen and construct a metagenomic fosmid library and screen the library for lipase isoforms. The fosmid clones were functionally screened using 1% tributyrin as a substrate and five positive clones were selected. From the five clones, two fosmids were selected for further study. Following nucleotide sequencing and *in-silico* analysis of the insert of the two selected clones, one *lipase* encoding open reading frame (Lip-VUT3 and Lip-VUT5) from each fosmid clones of approximately 212 and 248 amino acids, respectively, was identified. The amino acid sequences of the Lip-VUT3 ORF contained a classical conserved lipase GSDL sequence motif while the amino acid sequences of the Lip-VUT5 ORF contained a classical G-L-S-L-G conserved lipase/esterase motif sequence. The two genes (Lip-VUT3 and Lip-VUT5) were successfully expressed in *Escherichia coli* BL21 (DE3) and the purified enzymes exhibited respective temperature optima of 60 °C and 70 °C, and respective pH optima of 6.0 and 10.0. Further biochemical characterisation indicated that Lip-VUT3 and Lip-VUT5 lipases showed tolerance towards a wide concentration (50%-100%) of methanol. Lip-VUT3 had a K_m value of 0.287 mM while Lip-VUT5 had a K_m value of 0.556 Mm. This shows that Lip-VUT3 lipase has a higher affinity for olive oil than Lip-VUT5. Lip-VUT3 and Lip-VUT5 were characterised to be true lipases that have been recovered from the rumen environment through metagenomic approach.

Therefore, the study proved that metagenomic approach helps in recovering novel lipase isoforms with potential down stream industrial and therapeautic applications from goat rumen metagenome, a rich but untapped source.

Keywords: Functional metagenomic, Goat rumen, Lipase.

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LIST OF ABBREVIATIONS

Abbreviations	**Meaning**
%	Percentage
°C	Degrees Celsius
µmol	Micromoles
Asp	Asparagine
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EC	Enzyme Commission
EDTA	Ethylenediamine tetraacetic acid
Glu	Glutamic acid residue
HCl	Hydrochloric acid
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodaltons
LB	Luria-Bertani
M	Molar
mDNA	Metagenomic DNA
Min	Minutes
mM	Millimolar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NGS	Next-Generation Sequencing
ORF	Open Reading Frame
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
Ser	Serine residue

SOC	Super Optimal broth with Catabolite repression
TBE	Trizma-Boric acid- Ethylenediamine tetraacetic acid
TE	Trizma-Hydrochloric acid-Ethylenediamine tetraacetic acid
TEMED	Tetramethylethylenediamine
TGS	Trizma-Glycine Sodium Dodecyl Sulfate
Tris	Trisaminomethane
Tris-HCl	Trizma-Hydrochloric acid

INTRODUCTION

Enzyme catalysis or enzymatic processing among biotechnologies opts for a promising alternative, greener, cleaner, safer and sustainable process as compared to traditional or conventional (Sarmah et al., 2018). Enzymes which are also called biocatalysts, accelerate the rate of various biochemical reactions that can also be mimicked in various industries (Gurung et al., 2013). Enzymes do possess a significant advantage over the harsh chemicals used in conventional processing because of their specificity, reusability, and biodegradability. Enzymes are highly specific by only targeting some particular bonds to produce the necessary products. By doing so, the enzymatic process eliminates the generation of unwanted products. Enzymes are reputed to withstand competitive conditions. Enzymes do not leave any ecological footprints because they are readily degraded by microscopic life. The resulting decomposed materials obtained from the decomposed enzymes are always recycled back to nature (Andualema and Gessesse, 2012).

The industrial world has a preference for enzymes such as lipases, which originate from microorganisms because of their advantageous features (Ray, 2012). Lipases are useful to diverse industries such as food, cosmetics, agrochemical, biodiesel production and therapeutic (Javed et al., 2018). However, the majority of lipase producing microorganisms are not amenable to cultivation on synthetic media using the conventional laboratory techniques. To remediate to this hurdle, an alternative to the culture-dependent methods was developed. Metagenomics, the culture-independent technique, evolved as one of the molecular tools utilized to discover new lipases (Reyes-Duarte et al., 2012). The metagenomes trapped in an environmental sample are hypothesized to be opulent in genes coding for important lipases that can be useful for the bio-refinery industries (Shamim et al., 2017). Metagenomics can provide access to the metagenomes of a huge diversity of microorganisms found in both mild and extreme conditions such as the goat rumen. Hence, this study will make use of the metagenomic techniques to find potential novel lipases from the goat rumen metagenome. The following section will be discussing in detail the use of lipases, the choice of the goat rumen as the sampling element of this study, the use of metagenomic techniques and the biochemical characteristics of the discovered potential lipases.

CHAPTER 1: Literature Review

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1.1 Lipases (Lipids Degrading and Synthesis Enzyme)

Lipase also called a carboxylic ester hydrolase, is a water-soluble triacylglycerol acylhydrolase with the Enzyme Class number: EC 3.1.1.3. It is a biocatalyst, enzyme, involved in the modification of true lipids also called triglycerides in the form of fats and oils (Guncheva and Zhiryakova, 2011). The modification of triglycerides can take place in the presence of water (**Figure 1.1**), it is called hydrolysis of triglycerides and it yields di-, monoglycerides, fatty acids, and glycerol.

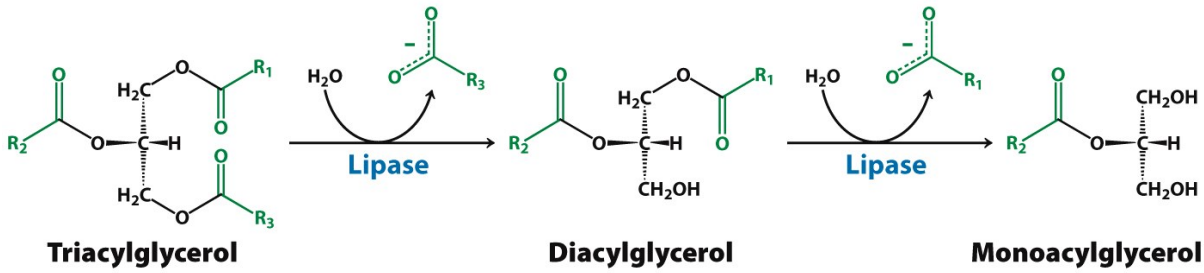

Figure 1.1: Hydrolysis of lipids through lipase as a catalyst with diacylglycerol as an intermediate (de Freitas, 2012).

The modification of triglycerides can also take place in the absence of water being replaced by any organic solvents such as methanol or superficial fluids. The process is called transesterification (**Figure 1.2**). This process yields Fatty Acid Methyl Esters (FAME).

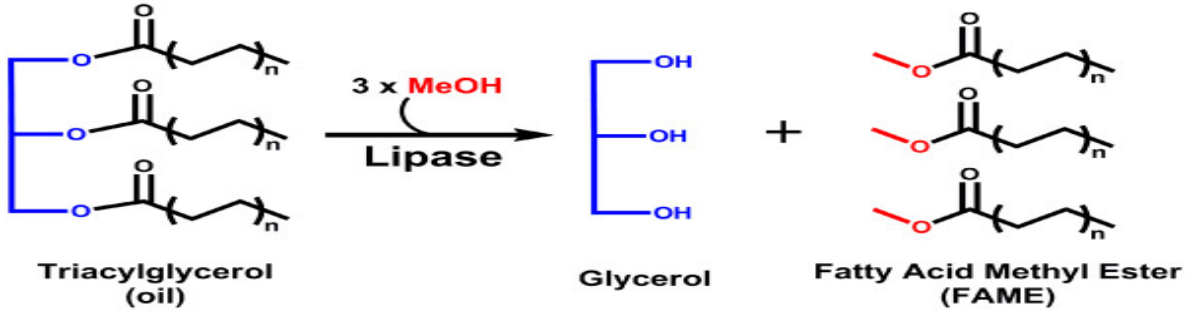

Figure 1.2: Transesterification of triglycerides using Lipases and methanol as solvent (Korman et al., 2013).

Other reactions catalyzed by lipase are alcoholysis, acidolysis, aminolysis, and interesterification (**Figure 1.3**).

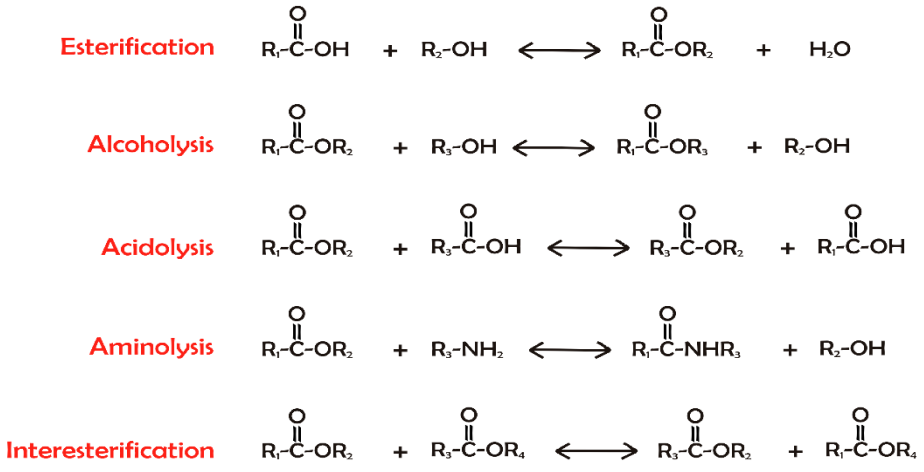

Figure 1.3: Additional reactions catalysed by isoforms of lipases (Borrelli, 2015).

1.2 The Three-Dimensional Structure of Lipases

Winkler et al. (1990) revealed that the lipases have the α/β hydrolase folding pattern. The backbone of the lipases are made up of a central β sheet with eight different β strands namely β_1, β_2, β_3, β_4, β_5, β_6, β_7 and β_8 which are in turn connected with more or less six α helices namely A, B, C, D, E, and F (**Figure 1.4**).

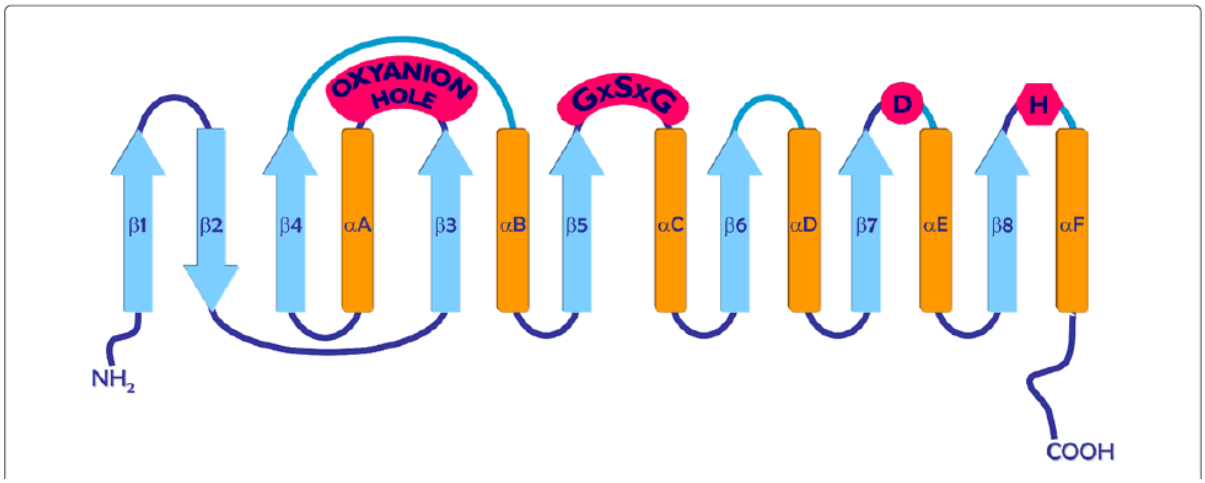

Figure 1.4: α/β hydrolase folding pattern of lipases. The central β (blue arrows) sheets which are in turn connected with the helices (yellow cylinders) (Turati et al., 2017).

During the closed conformation of the lipases, the helices and strands rearrange themselves in such a way to form a helical segment to protect the active site of the lipases. The helical segment is also referred to as “the lid”. The active site of the lipases is comprised of three amino acids or residues namely serine, aspartic or glutamic acid and histidine (Ser- (Glu) - His) (**Figure 1.5**). This active site mainly called catalytic triad is also found in the serine proteases. This is the reason why lipases ended up being classified as a serine hydrolase. Lipases have been reported to have a molecular weight ranging from 19 to 60 kDa. The difference existing between lipases and other serine hydrolases relies on the order of the residues in the active site. However, the difference occurring in various lipases lies in the order of amino acids or residue sequences inside the lipase molecules (Balan et al., 2012).

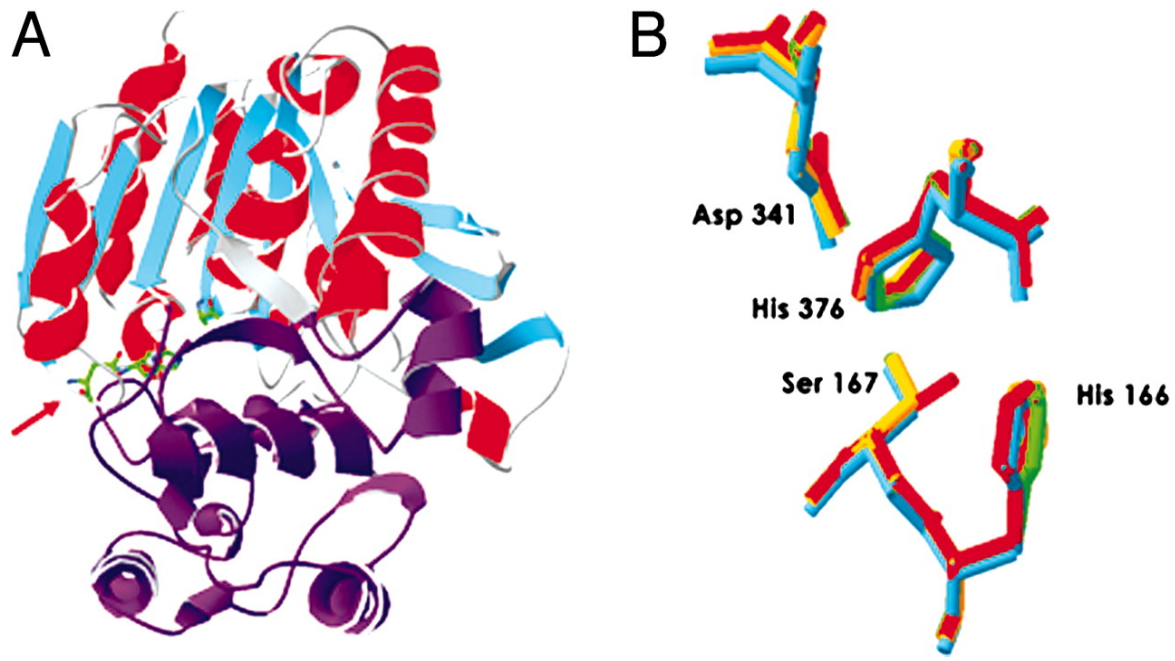

Figure 1.5: (A) The three-dimensional structure of lipase. (B) The catalytic triad of lipases. The active site commonly called catalytic triad is composed of three residues: the nucleophilic residue (Serine) which is situated at the C-terminal end of strand β_5 in a highly conserved pentapeptide GXSXG, forming a remarkable β-turn-α motif called the nucleophilic elbow. The catalytic residue (aspartate). In other lipases, aspartate is replaced by glutamate) and the histidine residue (histidine) in the respective order (Mueller et al., 2004).

1.3 Lipases Mode of Action

Lipases are reputed for their involvement at the interfacial surface during hydrolysis. Hydrolysis takes place at the water/lipid interface, a phenomenon observed during the emulsion of the lipids or triglycerides in water (Sarmah et al., 2018). During hydrolysis of the triglycerides, the interfacial formation usually activates the binding of the lipase active site to the non-polar solvent. It was established that upon the binding of the lipases to the interface, the lid that covers the active site of the enzyme during the closed conformation, was observed to shift to another position to give the enzyme the open conformation for the work to perform. The movement of the active site close to the non-polar solvent (mainly water) is said to initiate the lysis of lipids (Mala and Takeuchi, 2008). The oxygen of the uncovered nucleophilic serine residue attacks the carbonyl carbon atom of the ester bond to yield a tetrahedral intermediate. The tetrahedral intermediate is in turn stabilized by hydrogen bonding to nitrogen atoms of the His and Asp or Glu residues (from the lipase) that belong to the so-called oxyanion hole. As a result, an alcohol is produced and released from an acyl-lipase complex. The complex is finally hydrolyzed with the production of the fatty acid and regeneration of the enzyme (Lotti and Alberghina, 2007)

However, concerning the activation of the lipase by the interfacial formation or the oil-water emulsion, Verger (1997) reported that the presence or movement of the lid structure was not readily activated by the interfacial formation. The observation was made with lipases from *Pseudomonas aeruginosa*, *Burkholderia glumae*, *Candida Antarctica B*, and a coypu pancreatic lipase. All the above-cited lipases did not reveal any interfacial activation although possessing an amphiphilic lid covering their respective active sites. Therefore, it was concluded that the presence of a lid domain and interfacial activation are not the major features of a true lipase.

1.4 Origin of Lipases

1.4.1 Microbial lipases

Fungal lipases are produced extracellurlarly by fungi and yeast although some have been produced intracellularly. The location of the production is said to entirely depend on the composition of the crude enzyme production medium (Patil et al., 2011). Fungal lipases are obtained through Solid Substrate Fermentation (SSF) or Submerged Fermentation (SmF). They can catalyze a range of reactions such as hydrolysis, transesterification, etc. Fungal lipases are the widely used lipases thanks to their attributes including thermal stability, low-cost extraction and production, alcohol and other organic solvents tolerance and pH stability (Patil et al., 2011). Despite such qualities, an isoform of lipase named Lipase B from *Candida antarctica* (CALB) was noticed to be cold active although widely applied in various industries. Some of the major genera involved in the production of commercially important fungal lipases include *Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Rhizomucor* sp., *Rhizopus* sp., *Candida* sp., *Yarrowia* sp., *Pichia* sp., *Saccharomyces* sp., etc. Such genera are ubiquitous and have been isolated from diverse microbial niches such as soil contaminated with oil, hot springs, compost, industrial wastes and effluents, dairy plants and vegetable oil processing factories (Sharma et al., 2001).

Bacterial lipases are mostly produced extracellularly depending on the composition of the production medium and are deemed to be glycoproteins while some are lipoproteins. Besides, bacterial lipases can be intracellular or attached to the membrane (Javed et al., 2018). Some of the important genera reputed to produce lipases that are commercially important include *Staphylococcus* sp. *Achromobacter* sp., *Micrococcus* sp. *Burkholderia* sp., *Pseudomonas* sp., *Chromobacterium* sp. and *Bacillus* sp. the most useful lipase producer (Jaeger et al., 1994).

1.4.2 Physical properties of microbial lipases

Microbial lipases are mostly used in a wide range of industries because of many physical properties (**Table 1.1**) they do possess (Jaeger and Reetz, 1998). These properties give lipases a place of choice in the industry. The properties or features are listed in **Table 1.1**.

Table 1.1. Features and physical properties of microbial lipases

Properties	Elucidation
Specificty	• Lipases only produces desired products • Lipases eliminate unwanted by-products. • Lipases can catalyse the removal of one or several ester moieties at either specific or non-specific carbon position.
High yield	• A high concentration of products is usually obtained.
Ease of genetic manipulation	• Microorganisms genome can be altered in order to activate the production of lipases.
Regular supply due to absence of seasonal fluctuations	• Microorganisms do not depend on seasonal factors to grow. They grow anytime and anywhere depending on the availability of the nutrients.
Rapid growth of microorganisms on inexpensive media	• Use of inexpensive media to control growth of the microorganisms in the laboratory
Stability	Lipases are active: • Under optimal or ambient conditions. • In organic solvents

	• At extreme conditions such as high temperature, high or very low pH
Ease, safe and convenient mass production	• Obtention of purified lipase under safe and aseptic conditions.
No requirement of cofactors	• metal ions are not required for activation of lipases. • Calcium was revealed to increase the activity of lipase
High regiospecificity and enantioselectivity	Some lipases only act or work: • On specific bonds and • At specific position of carbon of the lipids or fats molecules

1.4.3 Extracellular and intracellular microbial lipase

Microbial lipases are usually purified and immobilised to enhance their properties and used in different forms. Lipases are either used in the intracellular form or the extracellular form (Andualema *et al*., 2012). Extracellular microbial lipases are produced using the submerged or solid state fermentation approach. Following the fermentation approach, the obtained enzyme is purified and immobilised to conserve the activity of the enzyme. Nevertheless, it was documented (Andualema *et al*. 2012) that the purification process is more expensive than the production process. A phenomenon which affects the pricing of immobilised extracellular lipases. Examples of extracellular lipases are Novozym 435, Lipozyme RM IM. To remediate to the cost effectiveness of the extracellular lipase production, an alternative Intracellular microbial lipases was developed. Intracellular microbial lipases are those trapped in the whole cells of the microbes. The cost of intracellular lipases were revealed to be lower than the extracellular lipases and they are also advantageous in the bulk production of biodiesel (Severina, 1984).

1.4.4 Classification of lipases

Among the factors that are usually taken into consideration for further classification of enzymes, the substrate specificity and amino acid sequence alignments are mainly selected. For the substrate specificity parameter, the enzymes that are the object of study or comparison, are assessed against similar or least related substrates under uniform reaction conditions. Nevertheless, this strategy is said to be unreliable for the identification or classification of an enzyme with potential for a synthetic issue. Consequently, there has been no formal classification of lipases as far as literature is concerned because variant classifications have been made based on different properties such as their sources, substrate specificity and region-specificity (Sarmah et al., 2018). However, with the development of the genomic era, the public databases were populated with information in sequences form on biological systems. These readily available sequences can align for study purposes. The comparison of amino acid sequences of enzymes to be studied is said to give an insight into the evolutionary relationship between enzymes from diverse origin (Arpigny and Jaeger, 1999). However, it was discovered that high sequence homology was not necessarily equivalent to similar enzyme properties such as substrate specificity, stereoselectivity, pH optima, etc. among the compared enzymes. Therefore, for the classification of bacterial lipases, it was decided to look at the combined substrate specificity and amino acids sequence consensus homology aspects. The bacterial lipases are were first classified into eight (I-VIII) families (Arpigny and Jaeger 1999). The family I also called True bacterial Lipases, is constituted of 11 (I.1-I.11) subfamilies as portrayed in **Table 1.2** (Jaeger and Eggert, 2002; Messaoudi et al., 2010).

Table 1.2. Classification of true bacterial lipase. *

Enzyme		Family	Subfamilies	Subfamily Name
True	Bacterial	I	I.1	Pseudomonas Lipase subfamily
Lipase			I.2	Pseudomonas Lipase subfamily
			I.3	Pseudomonas Lipase subfamily
			I.4	Bacillus Lipase Subfamily
			I.5	Staphylococcal Lipase Subfamily
			I.6	GSDL Lipase Subfamily
			I.7	GGGX Lipase Subfamily
			I.8	Other Lipase Subfamily
			I.9	Other Lipase Subfamily
			I.10	Pseudomonas Lipase subfamily
			1.11	Marine Bacterial Lipase Subfamily

* Source: Jaeger and Eggert (2002).

1.4.4.1. *Pseudomonas* lipase subfamilies

The *Pseudomonas* family **(Table 1.3)** is constituted of diverse subfamilies. Based on their amino acid sequence homology, *Pseudomonas* lipases were grouped in different subfamilies ranging from three to six. Allan et al. firstly reported in 1995 that *Pseudomonas* lipases were to be grouped into five subfamilies. On the other hand, Arpigny and Jaeger (1999) grouped the *Pseudomonas* lipases into six subfamilies. However, Jaeger and Eggert (2002) reported four subfamilies: I.1, I.2, I.3, and I.10 of *Pseudomonas* lipases. The classification was done according to the biochemical properties and automatic sequence alignment.

Table 1.3: Three of the four groups or subfamilies of the *Pseudomonas* Lipase Subfamily I. *

Enzyme	Family	Subfamilies	Organisms	Secretion type and similarities
Pseudomonas lipases	I	I.1	*Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Pseudomonas mendocina*, *Vibrio vulnificus*, *Proteus* sp. and *Yersinia enterocolitica*	Relatively high amino-acid sequence similarity with I.2. Type II secretion system
		I.2	Burkholderia genus, a previously part of Pseudomonas genus	Type II secretion system
		I.3	*Pseudomonas* sp., *Pseudomonas fluorescens*, *Acinetobacter* sp., *Psychrobacter* sp. and *Parvularcula bermudensis*	Type I secretion system

* Source: Jaeger and Eggert (2002)

1.4.4.2. *Bacillus* lipase subfamily

The *Bacillus* lipase subfamily is either designated as subfamily I.4 or I.5 of the true bacterial lipase Family 1. The difference between the Bacillus lipase and other lipase lies around the sequence consensus motif G-X-S-X-G around the lipase active site serine residue. The first Glycine residue is changed into Alanine (G-X-S-X-G becomes A-X-S-X-G) in the *Bacillus* lipase subfamily. The other difference is in size of the enzyme. *Bacillus* lipase has a molecular mass of approximately 19 kDa. Thus, *Bacillus* lipase is much smaller than another lipase with known structures (Arpigny and Jaeger, 1999, Messaoudi et al., 2010).

1.4.4.3. *Staphylococcal* lipase subfamily

The subfamily I.5 is made of *Staphylococcus* lipases. The uniqueness of lipases belonging to this subfamily is based on their origin. The *Clostridium* genus, and *Bacillus cereus* species are the main producers. Some of this lipase are said to have sequence similarities with thermos-

alkalophilic lipases from *Bacillus stearothermophilus* (Jeong et al., 2002, Messaoudi et al., 2010).

1.4.4.4 GDSL lipase subfamily

The GDSL lipases, the subfamily I.6, hold various functionalities. They are specific to a broad substrate and do possess a GDSL sequence motif instead of the GXSXG motif, present in many lipases. The GDSL family lipases are characterized by the presence of the important five blocks of highly conserved homology (Upton and Buckley, 1995). The GDSL hydrolases are said to own a flexible active site which functions like the induced fit mechanism of Koshland: a mechanism occurring when the active site usually changes conformation with the presence and binding of the different substrates, much like the induced fit mechanism proposed by Koshland (Akoh et al., 2004).

1.4.4.5. GGGX lipase subfamily

The GGGX lipases, the subfamily I.7, is further subdivided into 4 super-families of known protein structures having the oxyanion hole-forming residue positioned in a well-conserved GGG pattern situated next to a conserved hydrophobic amino acid (Pleiss et al., 2000 2006).

1.4.4.6 Marine bacteria lipase subfamily

Marine bacteria Lipases, a constituent of the subfamily I.11, owe the name to their producers which are mainly inhabitants of the sea. This classification was recently suggested by Lee and his c0-workers after discovering a LipG gene from a Korean tidal flat metagenomic library, which coded for a lipolytic enzyme and six other putative bacterial lipases that had never been previously classified (Lee et al., 2006).

1.4.4.7. Other lipase subfamilies

The lipase subfamily I.8 is comprised of lipases produced by *Mycobacterium* genus namely *Mycobacterium ulcerans*, *Mycobacterium tuberculosis* and *Mycobacterium segmatis species.* Their particularity comes from the sequences of the consensus catalytic serine (Gly-X-Ser-X-Gly-). All the I.8 subfamily lipase are characterized by the catalytic serine Glycine-Aspartate-Serine-Alanine-Glycine (Gly-Asp-Ser-Ala-Gly/ G-A-S-A-G).

The lipase subfamily I.9 is made of lipase from *Aeromonas hydrophyla*, and *Serratia proteomaculans*. After aligning their amino sequences, no homology nor significant conserved motif were observed. The reason why the members of the subfamily I.9 is yet to be further researched on is to establish some clear criteria based on which this class should be documented (Messaoudi et al., 2010).

1.4.5 Industrial applications of lipases

Generally, enzymes are preferred for the turnover of the earth's biomass into useful products because of the advantages coming along with the use of enzymes or biocatalysts. Enzymes are more specific. Thus, the choice of the right enzyme lead to the production of the desired products and elimination of side reactions which yield unwanted products. Enzymes are also cost effective and energy saving because of the reduction in waste generation and lower capital operation. Specifically, the hydrolysis and synthesis of triglycerides and ester-derived compounds that the lipase catalyzes render lipase a versatile enzyme. The versatility of lipases is one of the features that attract the industrial world to the extent of being applied in a wide range of industries from the food to the oleo-chemistry

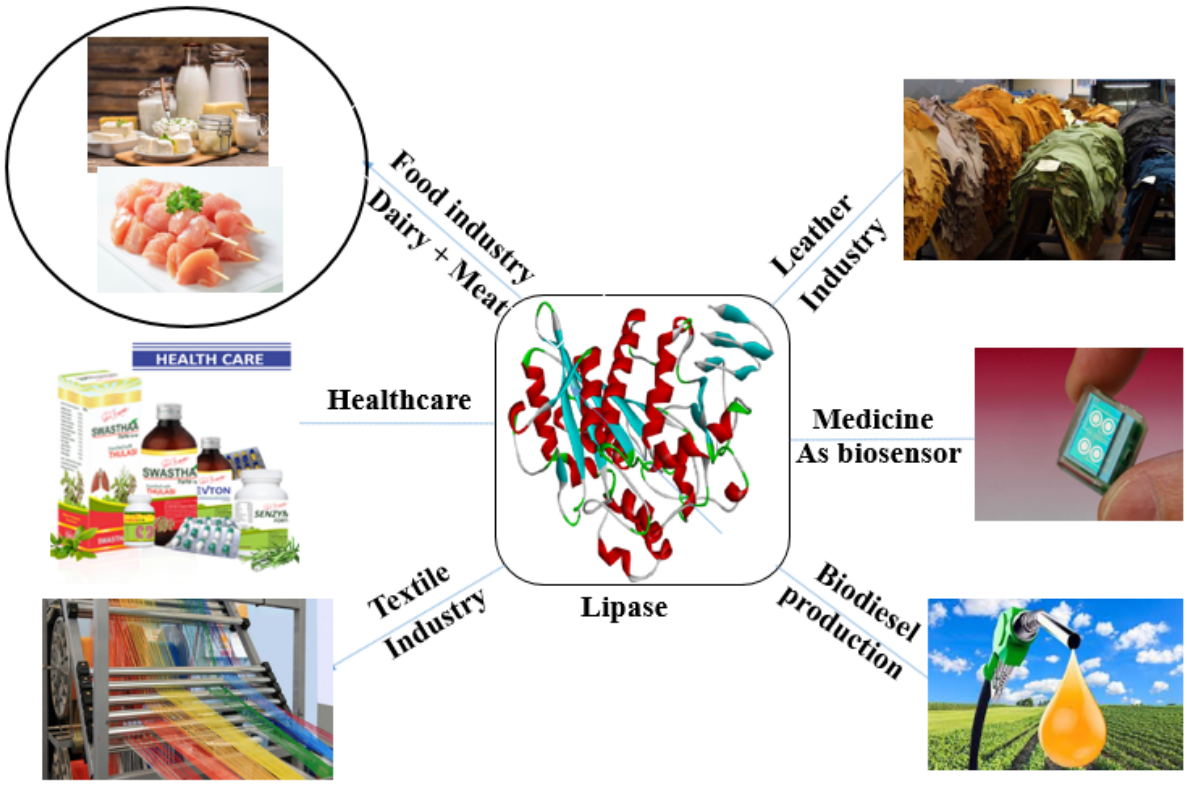

Figure 1.6: Applications of lipases in the food, leather, textile industries and for biodiesel production.

Therefore, lipase is considered as the third largest group of applied biocatalyst after proteases and carbohydrates.

1.4.5.1. Food industry

In the food industry, lipase found many applications:

- In the dairy industry, lipase is firstly used for flavor modification by synthesizing esters of short chain fatty acids moiety plus alcohols. The resulting esters are compounds of developed flavor for dairy products and fragrance. Secondly, lipase can be used for flavor development in dairy products. Lipase hydrolyzes fat triglycerides to yield free fatty acids moiety. The resultant fatty acids moiety can act as flavor compounds or flavor precursors in cheese making of milk production (Andualema and Gessesse, 2012).
- In the meat processing industry, lipase is used to remove the fat from the meat products in order to obtain lean meat. Lipase is also used to remove fat from fatty fish products through the bio-lipolysis process (Andualema and Gessesse, 2012).

1.4.5.2. Leather industry

The leather is a tough material produced from hides and skins of animals such as bovine. These hides and skins that are used to be transformed into leather through tanning, do possess proteins and fats that pose a major problem in the tanning. To remediate to the formerly mentioned problem, lipase is applied in the leather industry to decrease and remove fat from the skin prior initiation of a smooth tanning process (Andualema and Gessesse, 2012).

1.4.5.3. Textile industry

While the textile industry consists of sizing and improving features of clothing materials, lipase is regarded as one of the tools utilized to do so. Lipase is mainly used in the textile industry to render the polyester fabric capable of taking up chemical compounds namely cationic compounds, fabric finishing compositions, dyes, anti-static compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds and/or deodorant compounds (Andualema and Gessesse, 2012).

1.4.5.4. Healthcare

Lipases that are generally isolated from the wax moth (*Galleria mellonella*) do possess a bacteriocidal action on *Mycobacterium tuberculosis* (MBT) H37Rv. Therefore, lipases were deemed suitable for health care. Lipases are also incorporated in the products enhancing digestion, malignant tumors treatment, skin infection treatment, etc. (Andualema and Gessesse, 2012).

1.4.5.5. Medicine

In medicine, lipases are used in many forms:
As biosensors, lipases are used in the immobilized form in combination with other enzymes to determine the concentration of lipids/ triglycerides or cholesterol in the blood.
Lipases can also be used as a diagnostic tool because it is one of the enzyme markers in medicine. The presence of lipase at a certain concentration can indicate the initiation of an infection or disease. For further illustration, the presence and quantity of lipase in the blood serum prognosticate (Andualema and Gessesse, 2012)

1.4.5.6. Biodiesel production

Biodiesel has been seen as one of the alternatives to fuel since the raising of the environmental conservation issue. Biodiesel is a liquid produced from the transesterification of lipids with methanol or ethanol, short chain alcohol (Vicente et al., 2004). The industrial production is said to produce less amount of greenhouse gases (sulphur oxide) as compared to the burning of fossil fuels for the production of diesel or petrol. The biodiesel production is additionally seen as a replacement to the fossil fuel because of the depletion of the resources of fossil fuels. The transesterification process requires lipase as a catalyst because the use of lipase render the production of biodiesel less harmful to the environment (Andualema and Gessesse, 2012)

1.5 Strategies for Discovery of Novel Microbial Lipases

The Nature with its constituents is reputed to be abundant in microscopic living organisms. These microorganisms are even able to adapt to relatively severe conditions through their metabolic aptitude thanks to their genome (Handelsman, 2004). Therefore, it is assumed that such extreme habitats could be explored for their richness in novel genes, which in turn would be tapped for novel lipases to be used in industries for synthesis of synthetic organic compounds while other bio-molecules may be involved in the biotransformation of some pollutants (Daniel et al., 2011). However, the richness and diversity of genes in such microbial habitats cannot be addressed unless methods of exploitation of such habitats, are developed. It would be advantageous for such methods to not only reveal the identity of microorganisms present in the domain but also reveal their functionality in the consortium. Currently, two techniques are being used to discover novel lipases from various microbial habitats: the culture-dependent techniques and the culture-independent techniques.

1.5.1 Culture-dependent technique

The culture-dependent technique is a process that tries to mimic as much as possible the natural microbial environment of microorganisms to be cultured, in order to sustain their viability in the laboratory for further studies. The culture-dependent or conventional technique relies on the isolation and maintenance of microorganisms using synthetic medium composed of basic

nutrients such as carbon source, nitrogen source and trace elements (Basu et al., 2015). Upon growth, the desired microorganisms are identified and characterised based on the traits they manifest (**Figure 1.7**). For selection purpose, the medium is further supplied with a synthetic indicator like tributyrin in the case of the cultivation of microorganisms producing lipase. The criterion for selection of positive lipase producing microorganisms is the formation of a clear zone around the colony, a result of the digestion of tributyrin or lipid ester bond (Sirisha et al., 2010).

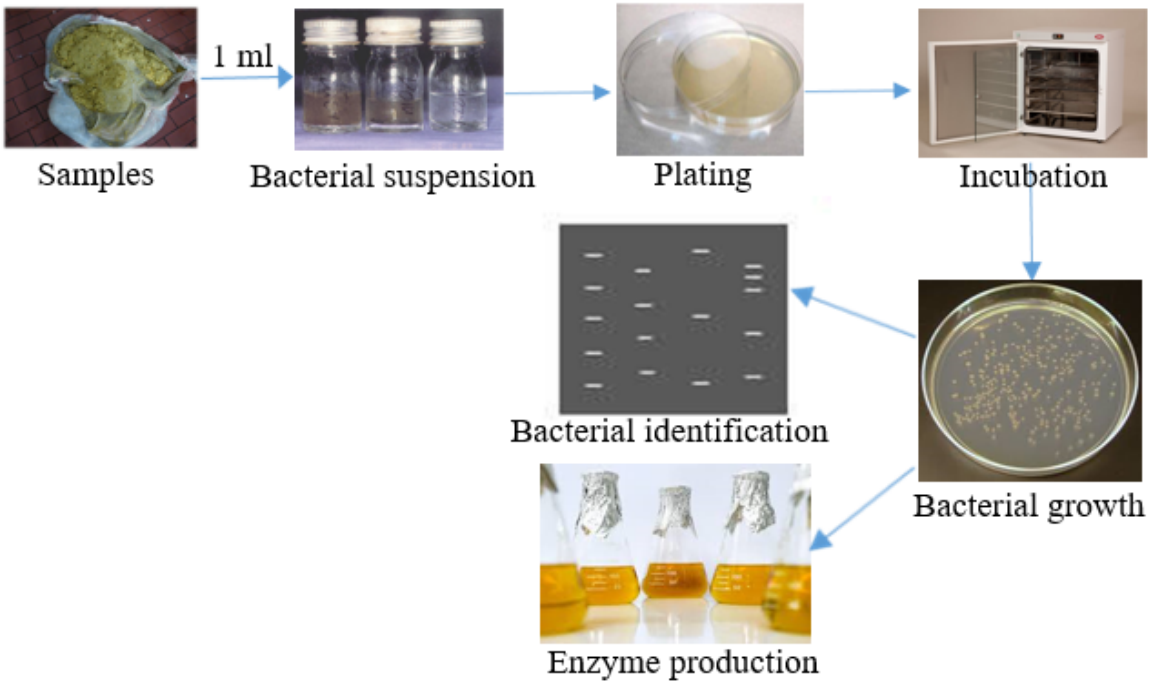

Figure 1.7: The conventional (culture-dependent) methods used to isolate microorganisms in order to discover novel enzymes.

However, the approach of the use of pure culture was deemed irrelevant through the "total plate count method". Such problems occurred when Brock and his colleagues came across Yellowstone inhabitants that were uncultivable because the temperature required for them to survive was greater than the temperature of the solid media used and others did not display the activities that they usually display *in situ* (Handelsman, 2004). Based on the above mentioned findings, it was hypothesized that there could be an ignorance towards microorganisms that were not able to be cultured using synthetic media. The problem remained a question mark in the mind of persistent scientists who started suggesting that novel approaches should be used since culturing could not trap the full or a wide range of diverse microorganisms in an environment (Handelsman, 2004). Despite the much efforts furnished to advance and improve

the conventional or culture-dependent techniques to accommodate the recalcitrant to grow microorganisms on synthetic media, the number of uncultivable microorganisms still remained high and the culture-independent technique was deemed a better alternative.

1.5.2 Culture-independent technique

Many techniques were developed until the application of PCR (Polymerase Chain Reaction) came onto the scene and provided correction to misinterpreted results from the culture approach; information that uncultured microorganisms were different from each other. Polymerase Chain Reaction also revealed that uncultured organisms were larger than the actual cultured ones (Rappé and Giovannoni, 2003). Polymerase Chain Reaction together with phylogenetic analysis helped scientists to conclude that the genomes of the majority of uncultured microorganisms coded for large numbers of unexploited biomolecules and other metabolic potential. In order to exploit this potential, new methods were developed to have access to the genetic materials of uncultured microorganisms and one of these approaches is metagenomics (Daniel et al., 2011).

1.5.2.1. Metagenomics

Metagenomics is the analysis of the pooled genome of the total microbial population present in a certain environment (**Figure 1.8**). Metagenomics circumvents the step of isolation and culturing of organisms and is based on the direct isolation of high quality genetic material from a sample. Metagenomics firstly relies on the construction of genomic or plasmid or fosmid libraries, a step which comprises the ligation of the isolated or extracted DNA to a vector of choice. The isolated DNA should be a representation of all the microorganisms present in a given environmental sample. The isolated DNA should be of good quality meaning free of contaminants that can hinder enzymatic reactions of cloning. Lane *et al.* (1985) were the first to propose the direct cloning of environmental DNA from picoplankton. They used a phage vector to ligate the DNA in order to detect 16 S rRNA gene by sequence-based analysis (Handelsman, 2004).

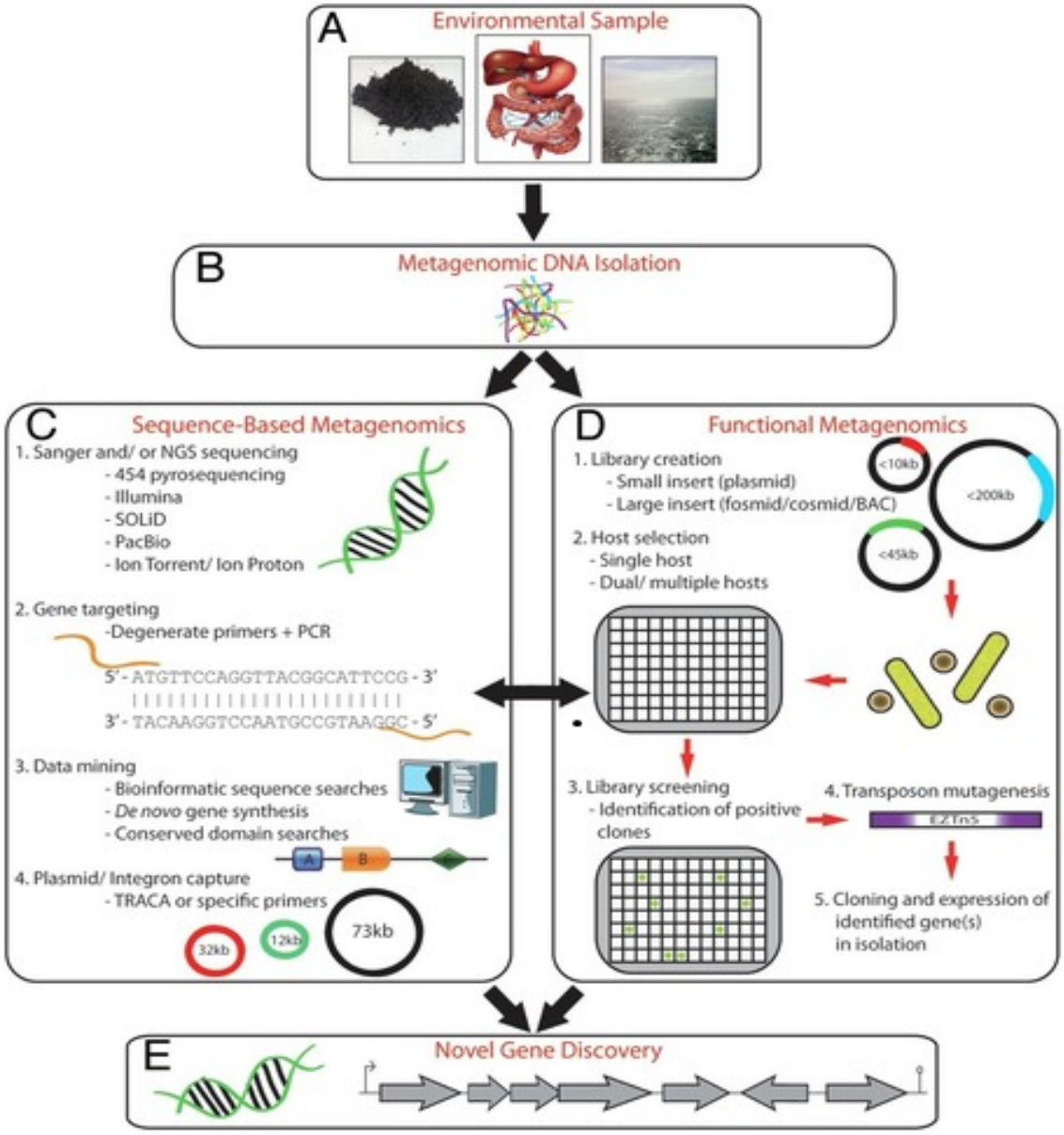

Figure 1.8: The culture-independent (metagenomics) techniques used for the discovery of novel lipases (Culligan et al., 2014)

The features that should be considered for the first step while applying the metagenomics approach are: the DNA extraction protocol and the choice of the cloning vector. These two features may constitute a challenge if not followed properly. Firstly, the DNA isolation step should be adjusted according to the physical properties of its original environmental sample. For example, the protocol developed and used for DNA isolation from mesophilic environments should not be totally followed when extracting DNA from thermophilic environments to avoid genetic material denaturation or release of stable nucleases during cell

lysis. Many scientific papers provided newly developed protocols for DNA extraction from diverse environments such as soil, groundwater, hot springs, surface river waters and buffalo rumens (Liu et al., 2009b). Secondly, the choice of the vector should depend on the size or length of the DNA, targeted genes as well as the screening strategy. Generally, small insert vectors such as plasmid are used for insert with a size up to 15 kb, cosmid and fosmid vectors are used for insert with a size up to 40 kb. Inserts of 40 kb are generally desired while the study is conducted to identify novel biomolecules such as enzymes encoded by a gene or an operon. Bacterial Artificial Chromosome vectors are used to ligate an insert of approximately a size of up to 40 kb. Such inserts are important to study complex pathways (Daniel, 2005). After the extraction and the size identification of the desired DNA, a metagenomics library is constructed and can be further processed for sequencing using Next Generation Sequencing through the MiSeq Illumina System (**Figure 1.9**) or phenotype-based screening (**Figure 1.10**) using appropriate substrates depending on the nature of the study.

1.5.2.1.1. Gene-based metagenomics

The unselective gene-sequence metagenomics is mainly characterised by the complete sequencing of metagenomic DNA.

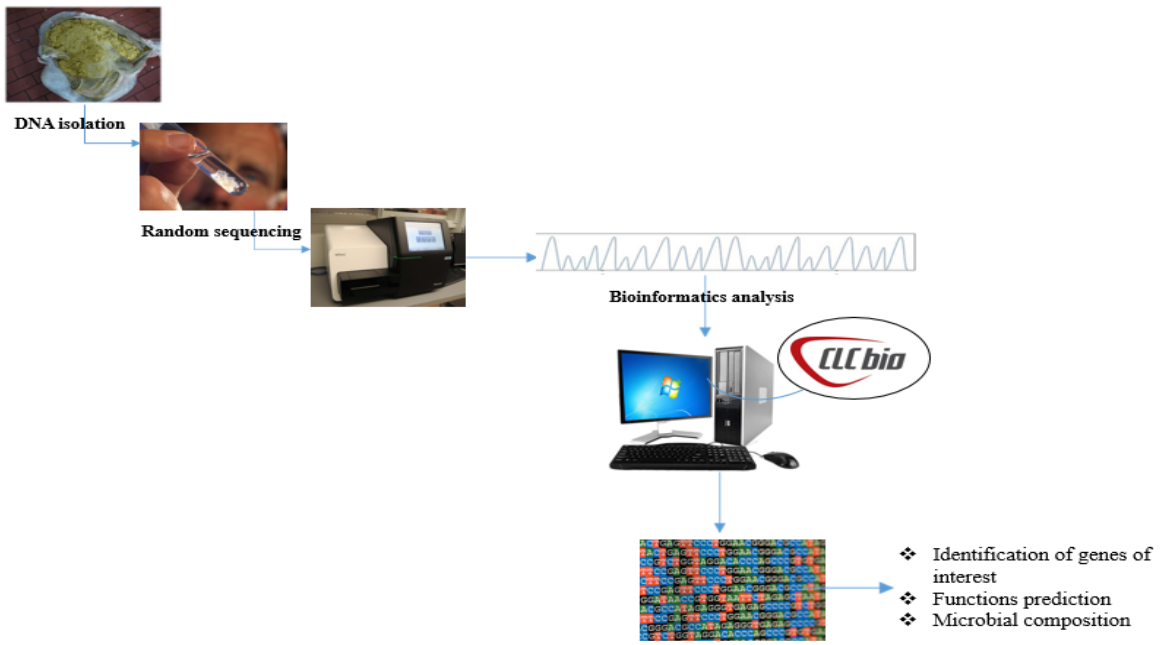

Figure 1.9: The sequence-based metagenomics for discovery of genes coding for lipases and other enzymes/biomolecules.

It involves the use of DNA probes, primers or adaptors, designed from conserved regions of studied and known genes pertaining to an organism (in taxonomy) or encoding a family of proteins (novel biomolecules). Next-Generation Sequencing technologies are the most used nowadays for this type of screening. The gene of interest is identified after sequencing, assemblage of sequences and annotation based on the phylogenetic anchors. Lane et al. (1985) were the first to use the gene-sequence metagenomics to analyse the picoplankton DNA for the presence of 16 S rRNA gene and concluded that this method was only suitable for the detection of novel genes from organisms having their genomes already sequenced, or novel genes encoding biomolecules with sequence analogues or similar to the previously studied ones. Consequently, gene sequence-based metagenomics could not be used to recover novel biomolecules encoded by completely novel genes (López-López et al., 2014).

1.5.2.1.2 Function-based metagenomics

In contrast to gene sequence-based approach, functional metagenomics is mainly characterised by the exhibition of a metabolic activity by clones or transformants on a solid or liquid medium containing a specific substrate for metabolic activities.

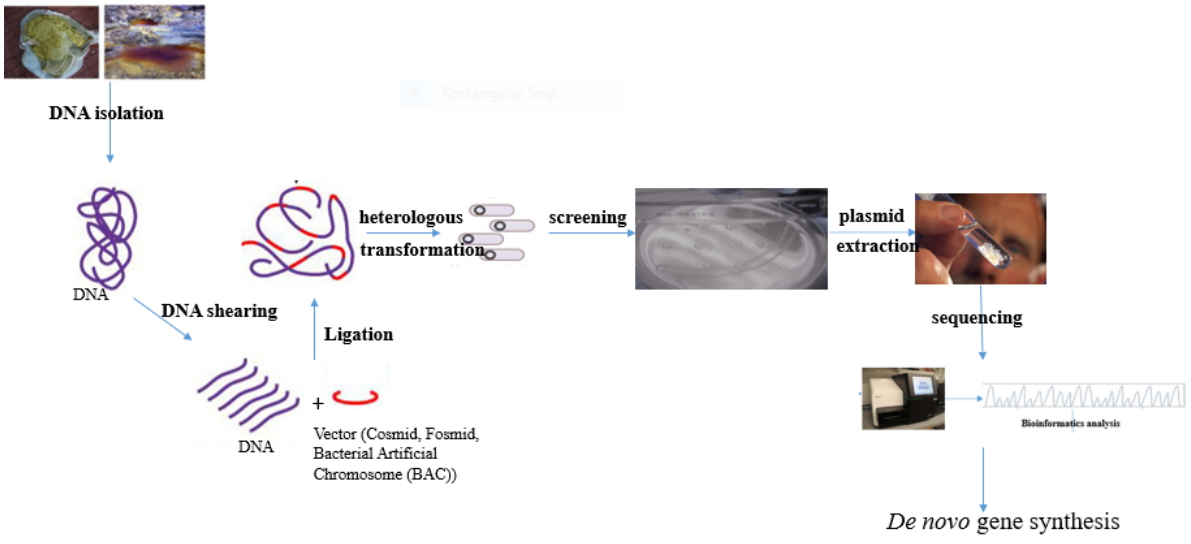

Figure 1.10: Function-based metagenomics for the discovery of novel enzymes/ lipases and other biomolecules.

Generally, genes encoding novel or known biomolecules are isolated via the aforementioned screening method (**Figure. 1.10**). There is no need for sequence information in such approach. Function-driven metagenomics is strictly based on phenotype and biochemical characteristics and is the most suitable strategy, having the capacity to identify entirely novel genes coding for known or novel metabolic functions, the main advantage of this method.

Function-based screening is subdivided in three classes. There is the phenotypical detection of the desired activity which makes use of chemical dyes, insoluble or chromophore-bearing enzyme derivatives substrates, incorporated into the synthetic medium to favour the observation of the specific metabolic activity of individual clones (Coughlan et al., 2015). The second class of function-based metagenomics is the heterologous complementation of host strains which makes use of selective conditions for growth of strains harbouring targeted genes and is required for simple and fast screening of metagenomic library constituted of millions of clones. It is highly sensitive and selective for targeted genes (Coughlan et al., 2015). The third class of function-based metagenomics is substrate-induced gene expression screening, which is used to recover genes involved in the catabolism of organic compounds because these genes require activation through induction of specific substrates (Uchiyama et al., 2005).

The challenging step in the function-based metagenomics is the expression of the genes in the heterologous organism. The transcription and translation of the genes in the host should be a success to deem this approach relevant. Gabor and colleagues (2004) reported that 40% of enzymes are recovered by functional screening with *E.coli* as the heterologous organism. Healy and collegaues (1995) were the first to conduct successful functional screening of metagenomic libraries which were termed “zoolibraries”. They reported the isolation of genes coding for cellulases from the microbial diversity inhabiting thermophilic, anaerobic digesters which were maintained on lignocellulose. After this successful experiment, many scientists used the same function-driven metagenomics to discover new genes encoding new biocatalysts and other biomolecules. The DNA polymerases, chitinases, cellulases and others are examples of enzymes that were recovered through function-driven metagenomics. To date, genes encoding lipases are the most frequently discovered genes from metagenomics resulting in the discovery of novel lipases, which is the interest of this study. Furthermore, Nagarajan (2012) stated that many new families of lipases have been discovered through the functional metagenomic-based techniques.

1.6 Rationale

The conversion of lipids into useful products has always been carried out using chemical catalysts until the statistics on the industrial revolution revealed that the process was contributing to the 40% increase in the concentration of greenhouse emission gases and the accumulation of undesirable by-products. This issue gave rise to a major concern on the preservation of the environment leading to the development of procedures that would comply with the concepts of green technology. On account of this, the use of conventional chemical processes with chemical catalysts as servomotor was subdued by the use of biocatalysts or enzymes to reduce the footprint left by the chemical technologies in the environment, to save energy and time. Therefore, the concepts of green technology with the use of enzymes such as lipase for rapid and large industrial applications, was deemed a suitable alternative to conventional chemical procedures. With the use of lipases, renewable sources like lipids or triglycerides can be converted into feed additives or biodiesel (monoalkyl esters) under mild conditions with less to no undesired side products. Such enzyme-based process of conversion is more eco-friendly than the use of harsh chemicals as catalysts.

1.7 Problem Statement

There is always a need to discover novel enzymes with superior catalytic activity and stability under different physicochemical condition, besides the ambient ones. Therefore, recent culture-independent approach like metagenomics can help us discover lipases with desired features such as thermostability and stability under alkaline conditions or high concentration of inhibitors such as alcohol, metal ions and surfactants. Therefore, the study was conducted with the goal of realizing the following aim and objectives.

1.8 Aim and Objectives

1.8.1 Aim

The aim of this research was to construct a fosmid library from the goat rumen metagenome and perform function-based screening to identify novel lipases.

1.8.2 Objectives

The following were the objectives of the study:

1. To sample five goats and extract ingesta from rumen, reticulum, omasum and abomasum from each goat and pool.
2. To extract metagenomic DNA from the pooled rumen samples.
3. To construct fosmid library from the extracted metagenome using CopyControlTM HTP Fosmid Library Production Kit with pCC2FOSTM Vector.
4. To functionally screen the library for potential lipases using Tributyrin and olive oil as substrates.
5. To sequence the insert in the positive clones using MiSeq from Illumina.
6. To *in-silico* optimise the codon of the ORF of interest, synthesisze and clone into pET 30 (a) + expression Vector.
7. To transform the chemically-competent *E. coli* BL 21(DE3) pLysS cells with the recombinant plasmid and express the cloned gene and purify the recombinant protein using AKTA-Start FPLC.
8. To determine the enzyme activity of the purified enzyme based on biochemical characteristics like pH, temperature and methanol tolerance; and the kinetic parameters of the enzyme and analyse using GraphPad Prism software.

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CHAPTER 2:

A book chapter submitted for publication

A Review on *Bacillus* spp. of Ruminant Origin as a Major Potential Source of Diverse Lipases for industrial and Therapeutic Applications

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Abstract

Bacillus, composed of many species, are one of the major sources of useful compounds that contribute to the industrial sectors. *Bacillus* genera are recognized as one of the safe organisms being able to produce useful compounds such as enzymes that are useful for biotechnology. *Bacillus* Lipases have been reported as one of the most useful biocatalysts in the food, detergent, pulp and paper, cosmetics, agrochemical, waste management, for therapeutic applications and biodiesel production. *Bacillus* are characterized by their capability to survive in extreme ecosystems such as the soil, hot spring, marine sediments, volcanic water, industrial effluents, human gut and the rumen. *Bacillus* spp. being one of the predominant microorganisms of ruminant origin, have been widely exploited for their thermostable and alkalophilic biomolecules. Although *Bacillus* have been reported as one of the predominant microbes of the ruminant microbiome, *Bacillus* of rumen origin has been less exploited for biocatalysts of industrial, environmental and therapeutic importance. Consequently, no comprehensive review has been reported on *Bacillus* lipases of ruminant origin. Therefore, the chapter aims to provide more insights into the benefit for in-depth analysis of the microbiome of ruminant origin with a particular emphasis on *Bacillus* spp as a source of diverse lipase-isoforms with potential industrial and therapeutic applications.

Keywords: *Bacillus* spp., Biocatalyst, Lipase, Microbiome, Ruminant, Rumen**.**

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2.1. Introduction

The *Bacillus* genus with its species was drawn attention to by the industrial world because of its rapid growth rates and its capacity of producing extracellular biochemical compounds directly to the fermentation medium (Schallmey et al. 2004). Additionally, *Bacillus* further attracted the industrial world because of their ability to inhabit drastic ecosystems. Their survival under such extreme conditions is entirely dependent on their biomolecules notably enzymes such as lipases, having exceptional properties (Guncheva and Zhiryakova, 2011).

Enzymes are proteins that are being produced by all the living organisms such as humans, animals, plants, and microorganisms for their sustainability. Although the enzymes of all the organisms have been reported to be important, the microbial enzymes have attracted the industrial world and are used extensively in this sector. Microbial enzymes have mostly been preferred because of their ease in handling, ease storage, stability, reusability through immobilization, broad substrate specificity and their bulk production is time efficient through fermentation methods (Thakur, 2012; Mienda et al., 2014).

Lipases, among many other enzymes, are one of the most applied enzymes in the biotechnology field because of its catalytic relevancy in the bioprocess of food, detergent, pulp and paper, leather, textile, cosmetics, agrochemical, waste, therapeutic and biodiesel. Therefore, there is an increase in demand for lipases with enhanced properties because of its versatility. *Bacillus* lipases constitute a large family of diversified enzymes. *Bacillus* lipases are suitable for a wide range of industries because of some interesting features such as stability at alkaline pH and high temperature, versatility, substrate specificity, solvents; salt and detergents-tolerance (catalytic properties). Based on the features listed, some recent studies reported the production of extracellular lipases by *B. pumilus* that was isolated from tannery wastes. The produced *Bacillus* lipase was found to be thermostable and potential to the industrial application (Guncheva and Zhiryakova, 2011)

Since the use of enzymes has been an adequate alternative to the use of chemicals, the demand for lipases has been increasing over time and since the application of lipases depends entirely

on the features or properties of enzymes, the search for novel enzymes particularly lipases with enhanced properties such as thermos-stability and pH alkaline tolerance, led to the exploration of new complex microbial resources such as the rumen.

As the rumen has been less explored for novel biomolecules with enhanced properties while being rich in sources of potential industrial biocatalysts, the current chapter aim at providing more insights on the benefit for in-depth analysis of the microbiome of ruminant origin with particular emphasis on *Bacillus* spp. as source of diverse lipase-isoforms with potential industrial and therapeutic applications. The following section will discuss the diversification of lipases, their structure, and mechanism of action, their sourcing methods and their various applications.

2.2. Lipases

The development in the use of enzymes for the sake of the environmental protection as mentioned in the previous section has been remarkable over time due to its input in the reduction of pollution and energy consumption. Among all the enzymes, lipases are said to be the third largest group of enzymes regarding their usage in the industries. This position is owed to their versatile nature (Ülker et al., 2011). Lipases were first discovered in 1901 in *Bacillus prodigiosus, Bacillus pyocyaneus* and *Bacillus fluorescens* that have been renamed as *Serratia marescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (EIJKMAN, 1903). Lipases are serine hydrolases **(Figure 2.1)** that catalyze the cleavage of ester bonds between the glycerol group and fatty acids moiety to yield free fatty acids or acyl-glycerides and alcohol. Lipases have an affinity for water-insoluble long-chain acyl triglycerides notably more than twelve carbons. Lipases are always activated by the occurrence of water and lipid interphase for lipids hydrolysis (Ramnath et al., 2017). In the case of limited water content, lipases can catalyze the reverse reaction designated as esterification. Moreover, under deficient water activity, lipases are still capable of catalyzing diverse transesterification reactions such as alcoholysis, acidolysis, aminolysis, and inter-esterification. Therefore, based on the diverse reactions **(Table 2.1)** that lipases catalyzed, they are always regarded as one of the versatile enzymes found in nature (Borrelli and Trono, 2015) and have versatile applications in

biotechnology. Lipases are diverse and are classified **(Figure 2.2)** based on the substrate specificity, region-selectivity or organisms of origin (sources).

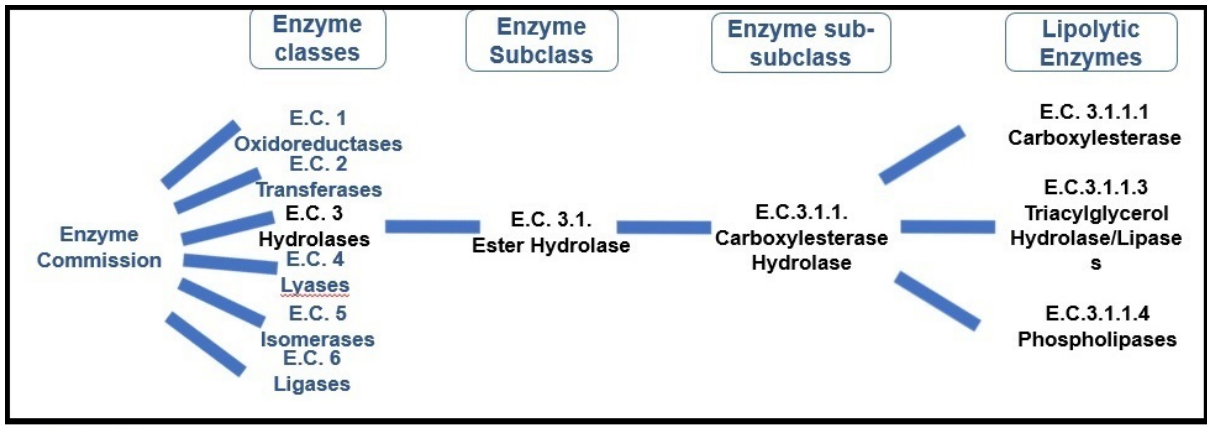

Figure 2.1: The numerical classification of lipases by the Enzyme Commission number (EC). This classification is based on the chemical reactions the enzymes catalyze. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme.. Lipases belong to the third class of hydrolases (3), the first subclass of Ester Hydrolase (1), first sub-subclass of carboxylesterase hydrolase (1) and the third family of Lipolytic enzymes (3).

Therefore, the following sub-section will focus on *Bacillus* spp., *Bacillus* lipases, their structural variations, mechanism of action, biological sources with emphasis on the rumen, mining techniques of bacterial lipases and their applications in industrial and therapeutic sectors.

Table 2.1: List of Reactions Catalyzed by microbial lipases.

Type of reaction	**Industrial application**	**Source of enzyme**	**Reference**
Hydrolysis	Bioorganic synthesis, Food industry Detergent industry	*Bacillus pumilus*	(Laachari et al., 2015, Litantra et al., 2013)
	Oil and fat industry	*Bacillus thermoleovorans*	(Patil et al., 2011)
Esterification	Food processing industry	*Bacillus coagulans*	(Thakur, 2012)
	Medical and healthcare	*Bacillus licheniformis*	(Sharma et al., 2015)
Transesterification	Biodiesel	*Bacillus subtilis*	(Treichel et al., 2010, Bajaj et al., 2010)

	Incorporation of different acids	*Bacillus stearothermophilus*	(Thakur, 2012)

2.2.1. *Bacillus* spp. as sources of lipases

As previously elaborated in the preceding section, lipases differ among themselves in the order of amino acids or residue sequences making them up although catalyzing the same range of lipolytic reactions. There has been no formal classification of lipases as far as literature is concerned because variant classifications have been made based on different properties **(Figure 2.2)** such as their sources, substrate specificity and region-specificity (Sarmah et al., 2018). In the current review, the attention will be given to *Bacillus* lipases class obtained from the classification based on sources or organisms of origin.

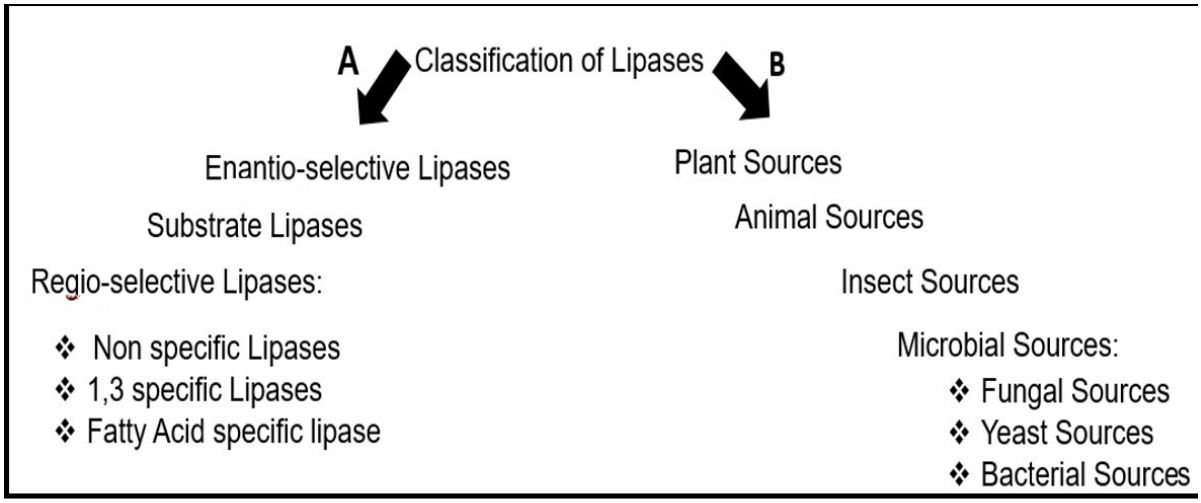

Figure 2.2: Classification of lipases based on (A) their substrate specificity, region-selectivity and (B) organism of origin (source).

Bacillus lipases are considered more suitable for industrial applications because of their ability to withstand the harsh conditions imposed by the industrial environment. Therefore, *Bacillus* lipases are preferred for industrial applications because they are thermostable, alkaline pH stable, organic solvent tolerant, substrate nonspecific and abundant **(Table 2.2)**.

Table 2.2: Biochemical properties of *Bacillus* and other bacterial lipases

Enzymes	Microorganisms	Sources	pH	Temp. (°C)	Tolerance	References
Lipases	*Bacillus alcalophillus*	Mangrove detritus	10-11	60	7.5% NaCl tolerance	(Ghanem et al., 2000)
	Bacillus sp. A30-1	Hot spring	9.5	60	H_2O_2, alkaline protease tolerant	(Wang et al., 1995)
Monoacylglycerol Lipase	*Bacillus sp. H-257*	Soil	6.0 - 8.0	75	-	(Kitaura et al., 2001; Ülker et al., 2011)
Lipase	*Geobacillus sp. T1*	-	9.0	70	Ions tolerant	(Leow et al., 2007)
	Geobacillus thermocatenulatus BTL2	-	8.0 - 9.0	65-75	-	(Eggert et al., 2003)
	Acinetobacter sp.	-	10.0	50	-	(Ahmed et al., 2010)
	Ralstonia sp.	-	8.0 - 9.5	50-55	Methanol tolerant	(Chouhan and Sarma, 2011)

Bacillus is identified as a much safer bacterium to use for industrial production, and it is generally regarded as safe. The genus *Bacillus* belongs to the *Bacillaceae* family. *Bacillus* and some allied genera of the *Bacillaceae* family such as *Geobacillus* are the most predominant lipase-producing bacteria in the family. *Bacillus* is composed of many species that are gram-

positive, rod-shaped, motile, aerobic and facultative anaerobic and gram-variable endospore-forming bacteria. *Bacillus* genera are recognized as one of the lipases producing bacteria that are ubiquitous. They are characterized by their capability to survive in extreme ecosystems such as the soil, Hot Spring, marine sediments, volcanic water, industrial effluents, Human gut, the Rumen, etc. (Turnbull et al., 1991; Duc et al., 2003; La Ragione and Woodward, 2003).

The survival capability in adverse conditions that *Bacillus* does possess is mainly owed to the extraordinary biomolecules that this genus produces. These special biomolecules are believed to protect the host from autolysis or unforeseen death that can be caused by the unfriendly conditions. Therefore, such biomolecules can be applied in industries having processes running under unfriendly conditions because it is believed that such biomolecules would withstand the harsh conditions. Taking into considerations that *Bacillus* is opulent in genes of interests, scientists started diverting their attention to *Bacillus* spp. until *Bacillus* became one of the best choices opted for the production of enzymes with potential applications in industries. Hence, some studies recently reported that *Bacillus* spp. enzymes make up 50% of enzymes in the global market (Schallmey et al., 2004).

As industrial processes take place at highly alkaline and thermophilic conditions, *Bacillus* enzymes have been opted as one of the biochemical compounds of choice to meet the industrial application requirements (Horikoshi, 1999; Madigan and Martinko, 2006). Moreover, the high demand for alkali-thermostable enzymes encouraged the development of the isolation of *Bacillus* spp. Hence, Lee and colleagues (1999) isolated a thermophilic *Bacillus thermoleovorans* ID-1 from hot springs in Indonesia. The thermophilic *Bacillus thermoleovorans* ID-1 showed extracellular lipase activity of maximum value of 520 U and high growth rates (2.50 h) on Olive oil (1.5%) substrates at an elevated temperature of 65°C. The isolate also grew on soybean oil, mineral oil, triolein, Tributyrin and emulsifiers (Tween 20, 40). The purified lipase produced by the isolate had optimal activity at 70–75°C and pH 7.5. Additionally, Shariff and co-workers isolated a thermophilic bacterium *Bacillus* sp. strain L2 from a hot spring in Perak, Malaysia. (Shariff et al., 2007). Another *Bacillus* sp. was cultivated in the optimal medium at pH 6 and 30 °C for 64 h was and extracellular and intracellular lipases were produced and had activities of 15 and 168 U/ml, respectively (Ertuğrul et al., 2007). Consequently as noticed from the above-listed experiments, *Bacillus*

was subjected to the production of lipases for further biochemical characterization. For long-term production and usage of enzymes, the biochemical characterization of enzymes should be joined by the study of the structures of enzymes to understand their mechanism of action as well as their folding pattern.

2.2.2. Bacillus lipases structure

As mentioned on the section above, lipases are serine hydrolases. Lipases are generally characterized by the α/β folding signature. *Bacillus* lipases as all microbial lipases have a backbone that is made up of eight different strands constituting the central β sheet.

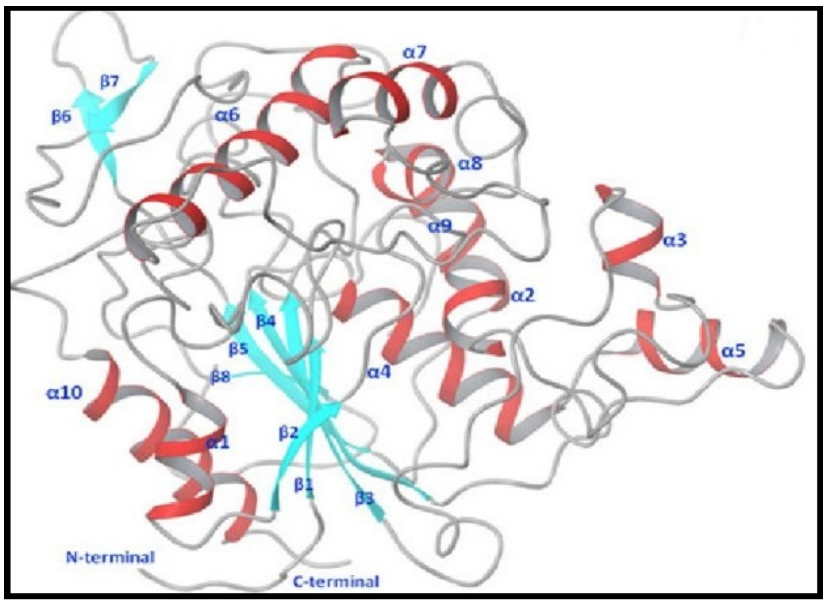

Figure 2.3: The 3D Structure of *Bacillus* lipase showing the α/β folding signature of the lipase formed of eight different strands (β_1, β_2, β_3, β_4, β_5, β_6, β_7 and β_8) in blue that are connected the six α helices (α1, α2, α3, α4, α5 and α6) in red (Poddar and Das, 2018).

The eight strands are designated as followed: β_1, β_2, β_3, β_4, β_5, β_6, β_7, and β_8. Studies performed on the three-dimensional structure of lipases from various sources except for the lipases from the pancreas revealed the presence of the eight β sheets that are sequentially connected to about six α helices. The helices are named α1, α2, α3, α4, α5 and α6 (**Figure 2.3**) (Gupta et al., 2015). The backbone holds the active site of the lipases. The active site is made of some amino acids that are involved in the hydrolysis mechanism. The three most essential amino acids called the

catalytic triad, of the active site, are similar to all the microbial lipases. However, *Bacillus* lipases do possess some variations at the backbone of the active sites, and such variations are responsible for the enhanced properties of the *Bacillus* lipases. The following section will give insights on structural variations in the *Bacillus* lipases. *Bacillus* lipases differ from other lipases group despite their diversified features or properties as demonstrated by the analysis of a large number of *Bacillus* lipases (**Table 2.1**) that have been isolated to date.

Bacillus lipases with their fascinating feature (**Table 2.2**) which is thermo-stability, are considered to be potential lipase producers for biotechnological applications (Rashid et al., 2013). The pentapeptide conserved motif of *Bacillus* lipases has their amino sequences as follow: Ala-His-Ser-(Met or Gln)-Gly. Alanine residue substituted the Glycine residue present in the canonical pentapeptide conserved motif of other microbial lipases. Such substitution conveys the increased thermos-stability to the *Bacillus* lipases (Jeong et al., 2002). *Bacillus* lipases are divided into two groups: I.4 subfamily and I.5 subfamily *Bacillus* lipases. The I.4 subfamily *Bacillus* lipases are produced by *B. subtilis, B. pumilus* and *B. licheniformis* and have a low molecular weight of 19-20 kDa. The I.4 subfamily *Bacillus* lipases are extreme alkaline tolerant because they do conserve their maximum activities over a wide range of alkaline pH 9.5-12. However, their thermo-stability is reduced to 45 ºC. Thus, they are said to be less thermo-stable than the other *Bacillus* lipases sub-family. The I.4 subfamily *Bacillus* lipases do not possess a lid to protect the active site. Therefore, their active Serine residue is opened to the

organic solvent. Thus, it facilitates the catalysis of the esterification reaction (Van Pouderoyen et al., 2001; Guncheva and Zhiryakova, 2011)

The I.5 subfamily of *Bacillus* lipases are synthesized by *Geobacillus thermocatenulatus* and have a molecular weight doubled the size of the I.4 subfamily *Bacillus* lipases (40-45 kDa). They are neutral to moderate alkaline pH (8.0-10.0) stable and are highly thermo-stable. They possess a lid to cover the active site. *Bacillus* lipases are mostly produced by submerged culture fermentation (Guncheva and Zhiryakova, 2011)

2.2.2.1. *Bacillus* lipase mechanism of action

Bacillus lipases like other microbial lipases do possess the nucleophilic elbow containing the Serine residue as one of the many residues that form the pentapeptide motif. Additionally, some of the *Bacillus* lipases possess a lid covering the active site. For the *Bacillus* lipases possessing a lid, they are generally known to have a closed conformation in their inactive state. The closed conformation means that the lid covers the active site when the lipases do not perform their catalytic activity. But, during activation the lipases have the lid removed from the active site so that the nucleophilic residue, Serine, can have access to the carbonyl carbon atom of the substrate that can be fats or lipids. Triglycerides of long acyl-chain carbon are insoluble in water as stated in the above section. During the hydrolysis of triglycerides in the presence of lipases, triglycerides do not completely dissolve in water. Therefore, there is an observation of the formation of a water-oil emulsion which, in fact, activates the lipases by having their lid moved away from the active site leaving it open for catalysis. Therefore, one of the characteristics of the lipases is the activation by the water-lipids interphase (Sharma et al., 2001; Jaeger et al., 1999).

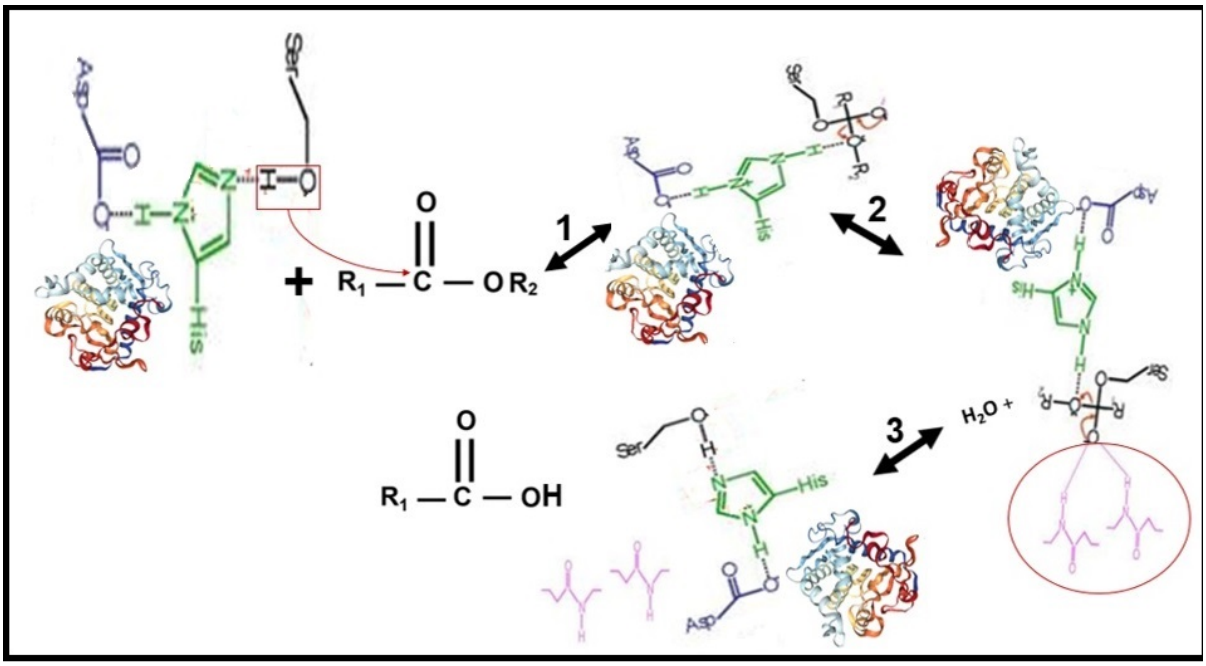

Figure 2.4: The three steps of the lipids or triglycerides hydrolysis. The first reaction (1) is the result of the covalent bonding of the hydroxyl group from the Serine residue and the triglyceride. The tetrahedral intermediate (enzyme-acyl complex) is formed. (2) The resultant tetrahedral intermediate is stabilized by having its carbonyl Oxygen atom bonding to the two hydrogens from the nitrogen atoms

of the amino acid residues of the oxyanion hole. (3) The water molecule hydrolyzes the covalent bond between the enzyme and acyl product to release the alcohol and stabilized enzyme in a closed conformation and the acyl that can be the diacylglycerol (Borrelli, 2015) .

The hydrolysis of triglycerides by lipases is a three steps mechanism **(Figure 2.4).** When the active site of the lipases is opened, the hydroxyl group (-OH) of the serine residue binds to the carbonyl carbon of the substrate **(Figure 2.4, reaction 1).** The tetrahedral intermediate (enzyme-acyl complex) is formed. The resultant tetrahedral intermediate is in turn stabilized by two hydrogens bonding between the negatively charged carbonyl oxygen atoms of the intermediate to nitrogen atoms of the amino acid residues of the oxyanion hole **(Figure 2.4, reaction 2).** Then, the alcohol product is released. The water molecule hydrolyzes the covalent bond between the enzyme and acyl product to release the stabilized enzyme in a closed conformation and the acyl that can be the diacylglycerol **(Figure 2.4, reaction 3).** After hydrolysis, the lipases reclaims the closed conformation by having their helices and strands rearranging themselves in such a way to form a helical segment, the lid, to protect the active site of the lipases (Vaquero et al., 2016). The active site of the lipases with its catalytic triad is also found in the serine proteases. Consequently, lipases are classified as a serine hydrolase.

2.2.3. Rumen as a source of *Bacillus* spp.

The above section highlighted the fact that *Bacillus* is reputed for their survival status in unfriendly ecology. The rumen **(Figure 2.5)** being one of those unfriendly ecosystems will be elaborated on this section.

The rumen is the four-chambered stomach of the ruminants. Ruminants are herbivorous animals digesting their food through rumination. The rumination consists of firstly eating the raw material, regurgitating a semi-digested form known as cud and secondly chewing the resulting cud (Oyeleke and Okusanmi, 2008). Examples of ruminants are sheep, cattle, cow, goat etc. Ruminants differ from each other based on many characteristics such as physical composition, heights, feeding habits, etc. Of all the ruminants, the goat (*Capra aegagrus hircus*) is one of the outstanding ruminants reputed for adapting in harsh environments such as arid zones. The Boer goat, for example, a breed that predominates the drier areas of the Eastern

Cape in South African adapted to this region of low rates of energy and water turnover. Thus, this domesticated animal is considered as one of the animals distributed within the largest ecology (Erasmus, 2000). The adaptation of goats to arid zones is mainly due to their diverse feeding habits. Goats can eat materials that are not regarded as foods by fellow ruminants, and the diverse habits are hypothesized to influence the rumen microbiome of the goats.

The four chambers of the stomach include the rumen, reticulum, omasum, and abomasum **(Figure 2.5).** The rumen which is also qualified as the unique digestive vessel is inhabited by a group of strictly to facultative anaerobic microbes namely protozoa, archaea, fungi and bacteria such as *Bacillus* which work in a symbiotic relationship. This group of rumen microbiome plays a pivotal role in the digestion process by synthesizing enzymes to be applied in the breaking down of complex ingested feeds into simpler compounds that can be readily assimilated by the host (Miyagi et al., 1995, Hungate, 1966) Therefore, the rumen, being a very complex microbiota, can have its microbiome explored for sources of enzymes like lipases with potential in the biotechnological applications, research, and industries (Hess et al., 2011).

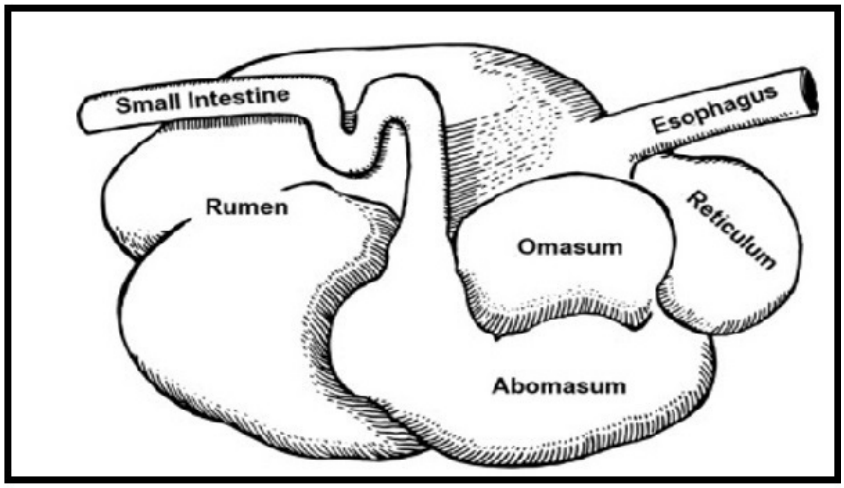

Figure 2.5: The rumen, four-chambered stomach of ruminants, where microorganisms work in symbiotic relationship to convert the ingested foods into useful organic compounds (DePeters and George, 2014).

That's why, scientists started exploring the rumen to give in-depth analysis on the microbiome inhabiting this ecosystem, and they concluded that bacteria would make 50-75% of the rumen microbiome (Choudhury et al., 2015). Ishaq et al. (2015) performed further analysis by isolating 31 bacteria from the rumen of North American Moose (*Alces alces*) and reporting that

the majority of bacteria (26) were identified as *Bacillus* spp. The report confirmed that the genus *Bacillus* is one of the predominant bacteria genera inside the rumen. Similarly, Oyeleke et al. (2008) isolated microorganisms from the rumen of the cow, sheep, and goat and discovered that *Bacillus spp.* represented 37.8% of the bacterial population of the rumen microbiome.

As the rumen is classified among the harsh environments because it is prevailed by a strict anaerobic condition, a physiological pH range between 5.5 to 6.0 and a temperature of about 40 ºC, the bacteria notably *Bacillus* genus surviving in such a complex environment, their biocatalysts especially lipases are believed to demonstrate exceptional qualities. However, getting access to them is difficult to accomplish because it is quite difficult to mimic such conditions in the laboratory. That being so, the rumen though considered as a rich niche is less explored. Development of techniques for mining sources of industrial enzymes such as *Bacillus* and its genetic material became of great interest. The newly developed techniques will be discussed in the following section.

2.3. Mining Techniques of *Bacillus* Lipases

Despite the diversity, specificity, uniqueness versatility and the ease of bulk production of microbial lipases, the quest for lipases with improved and novel catalytic and stability under competitive conditions is still intense. Biological resources such as rumen are one of the ecosystems to be explored for sources of enhanced lipases such as *Bacillus spp.* To facilitate the hunt for novel lipases, different approaches of access to enzymes sources **(Table 2.3)** have been used to answer the above-stated issue easily.

2.3.1. Conventional method

The conventional approach **(Figure 2.6)** which consists of cultivating and isolating the potential lipases producing strains has been used so long to get access to the lipases of interest. The traditional methods or culture-dependent methods consists of growing the organism of

interest on an appropriate substrate, usually the emulsified synthetic oil (Tributyrin) under laboratory conditions similar to the natural habitat of the organisms to be studied.

Table 2.3: List of approaches used to mine *Bacillus* and other microbial lipases

Enzymes	Sources	Methods of mining	References
Moderately thermostable (and thermally activated) lipase	Soil	Metagenomics	(Faoro et al., 2012)
Protease insensitive feruloyl esterase	Cow Rumen	Metagenomics	(Cheng et al., 2012)
Two Lipases	Cattle rumen	Metagenomics	(Liu et al., 2009a)
Two Esterases	Sheep rumen	Metagenomics	(Bayer et al., 2010)
Two esterases	Soil and water	Metagenomics	(Ouyang et al., 2013)
Three carboxylic ester hydrolases	Soil and drinking water	Metagenomics	(Elend et al., 2006)
Thermostable esterase	Mud-Sediment rich water	Metagenomics	(Rhee et al., 2005)
Lipase	Palm oil-contaminated waste	Culture-based methods	(Hasan et al., 2018)
Lipase	Degrading oil cakes	Culture-based methods	(Sarkar and Chatterji, 2018)
Lipase	-	Culture-based methods	(Navvabi et al., 2018)

The detection of positive lipases producing strains is based on the formation of a transparent or clear zone of halos around the colony. Nevertheless, it is stated that the culture-dependent methods managed to tap more or less 1% of the microbial diversity of an ecosystem leaving about 99%, the vast natural variety untapped (López-López et al., 2014). The 99% which are made of the viable but non-culturable microbes are believed to produce a novel family of lipases isoforms. Such findings implied that the exploration of sources of novel lipases could be limited and restricted.

To explore the vast intrinsic diversity, the culture-independent method called metagenomics was developed **(Figure 2.7).**

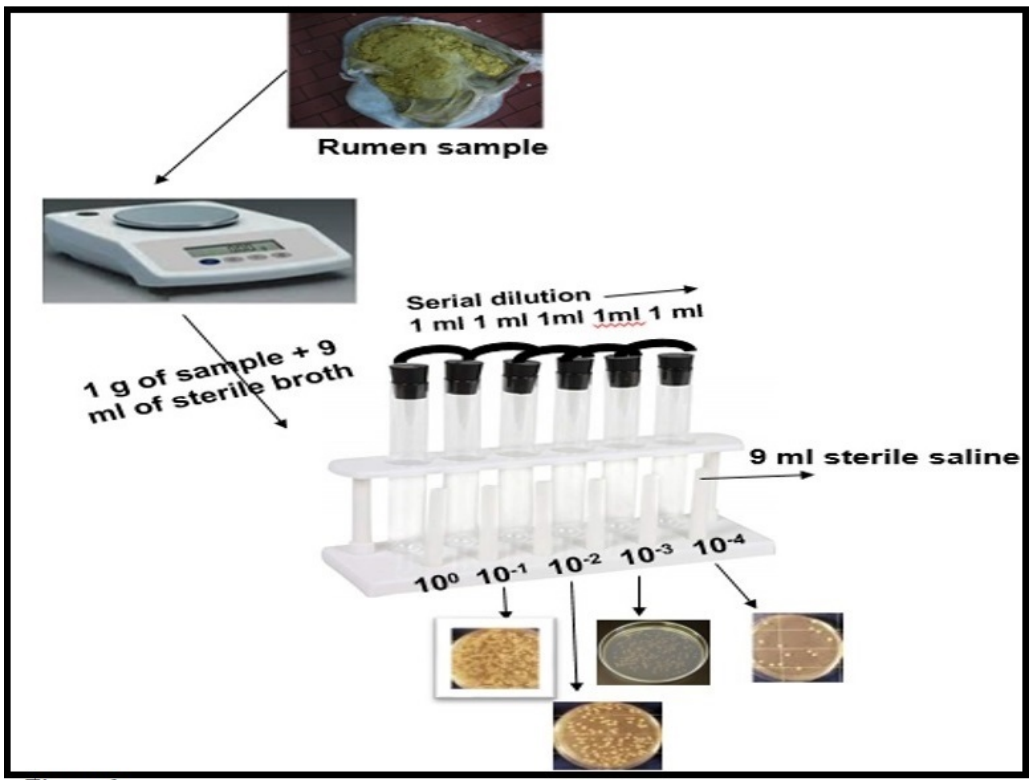

Figure 2.6: The conventional culture-based technique commonly used for the isolation of microorganisms. The technique that favors only viable culturable microorganisms.

2.3.2. Metagenomic approach

The Metagenomic technique consists of the three steps. The first step entirely relies on the extraction of the pooled genomes from an environmental microbiome, followed by the genomic librarie construction step and the sequencing step. The genomic library construction step helps in storing the genomes of microbes that are viable but non-culturable. Such genomes could be sources of genes coding for unknown or novel enzymes notably lipases possessing novel and unique properties. The progress that was made recently in the field of metagenomics favored the accessing of enzymes from unaccommodating habitats such as hot springs, compost, Antarctic soil, flat intertidal sediments, seawater, coastal environment, deep-sea marine sediments, marine sponges, mangrove sediments, fat-contaminated soils, and rumen.

(Tuffin et al., 2009) reported the isolation of novel lipolytic enzymes that had variation in protein sequence as compared to those from the commonly reported classes of enzymes. Thus, the most significant accomplishment of metagenomic approach in the discovery of novel families of biocatalysts notably lipases is the isolation of biocatalysts possessing unique protein sequences from deeply divergent lineages without exhibiting any close relativeness and representing new families. And, metagenomic studies are revealed to continuously adding enzymes to the growing number of already existing industrial biocatalysts. Then, it is hypothesized that biocatalysts discovered through metagenomics are on the verge of surpassing biocatalysts determined through conventional approach. There are two types of metagenomic approaches: the sequence-based strategy and the function or phenotype-based strategy.

2.3.2.1. Sequence-based metagenomic approach

New enzymes are discovered through the analysis of the sequences obtained via sequencing using platforms such as MiSeq, HiSeq Illumina from the Next-Generation Sequencing **(Figure 2.7)**. The study of sequences relies on the alignment and annotation of the metagenomic data against known and available sequence data for enzymes homologous to known biocatalyst such as lipases/esterases. The sequence-based metagenomics approach is not readily used because this approach only detects genes coding for enzymes that are related to previously reported families. Consequently, the new sequences are overlooked because of lack of similarity with known sequences.

2.3.2.2. Function-driven metagenomic approach

Alternatively, with the function-based metagenomic approach, metagenomic libraries are screened based on their activity such as lipolytic activity reflecting on their phenotypes. Many factors influence the success of this approach. The cloned genes should be compatible with the transcription and translation machinery of the heterologous host which is usually *Escherichia coli*. The expression of lipases depends on the requirement for specific chaperones for the correct folding of the enzyme and the toxicity of the lipases to the host cells. Using the function-

driven metagenomic approach is advantageous because it allows for the discovery of entirely new genes coding for novel biocatalysts. Currently, new families of lipases are still discovered through both traditional and metagenomic approaches. Metagenomics like the conventional approach also experiences some challenges and bottlenecks such as under-representation of genes of interest that are quickly picked through inefficient screening of metagenomic libraries because genes coding for enzymes of interest are represented in the minority in the metagenome. The above bottlenecks made the metagenomic approach to only recover 40% of desirable enzymes from a metagenomic library through functional screening (Gabor et al., 2004).

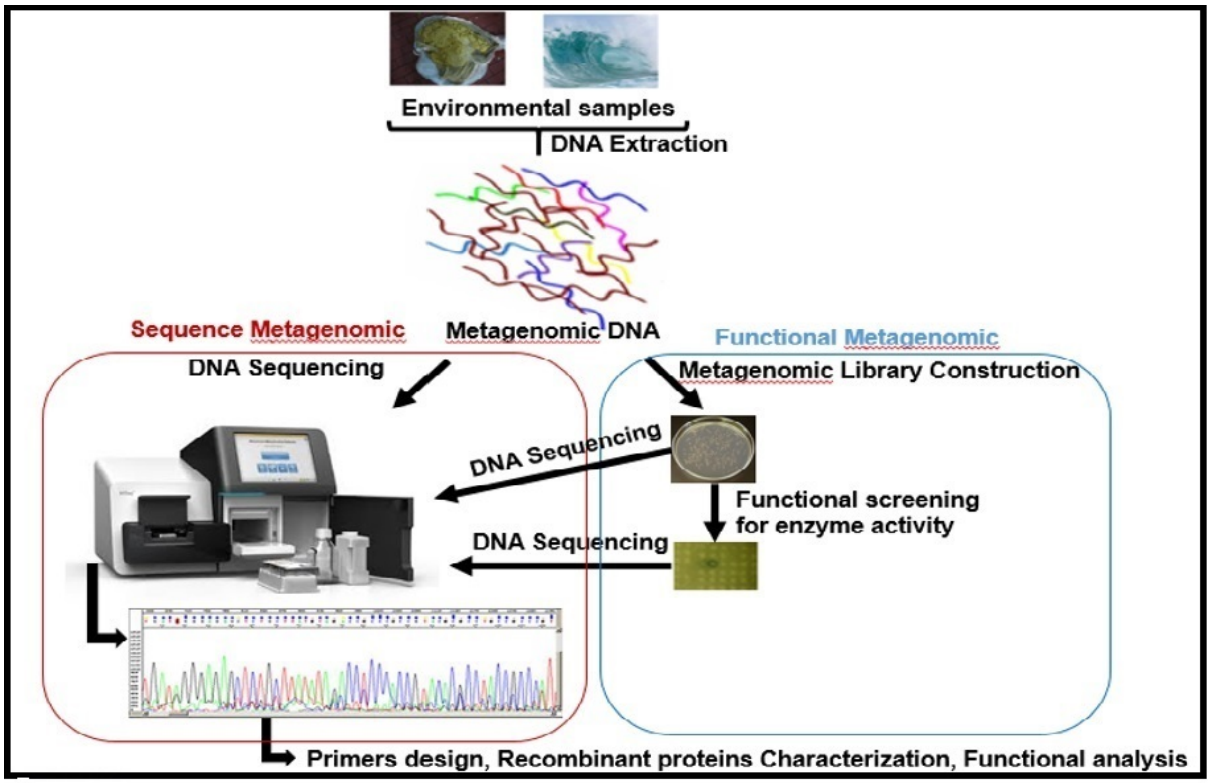

Figure 2.7: The illustration of the metagenomic approach (Sequence and Function-based screening) used to exploit the unculturable microbial world.

By 2009-2010, the functional metagenomic approach had so far only identified two lipases from the rumen of cattle (Liu et al., 2009a) and two esterases from the rumen of sheep (Bayer et al., 2010). The isolated genes were less similar to genes coding for lipases from other environments. The resulting lipases also had their function unidentified. Moreover, a study

used culture-independent metagenomic approach to isolate lipases from the complex microbiome of the sponge *Ircinia* sp. obtained from the South China Sea. The recombinant enzyme LipA was analyzed and found to be alkaline and organic solvent tolerant. Based on these characteristics, it was hypothesized that the isolated recombinant enzyme could be of great utility in the detergent industry and for the synthesis of organic compounds (Su et al., 2015).

Another study made use of functional metagenomics to isolate and characterize the bacterial lipases from the rumen of the bovine. The isolated lipases showed maximum activity against mostly short- to medium-chain substrates, a wide range of temperatures and pH. However, the function of the novel isolated lipase was not determined (Prive et al., 2015).

Based on the few examples provided, it is understood that there is a need to explore biological resources that have never been explored before to gain access to biocatalysts with enhanced properties.

2.4. Applications of Lipases

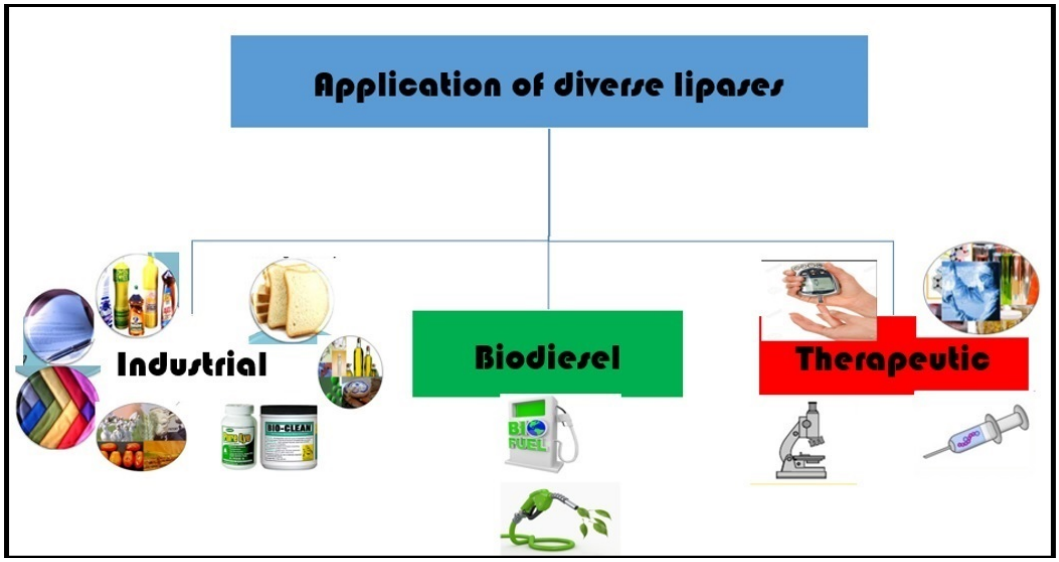

Figure 2.8: Different industrial applications of diverse lipases in term of white, green and red biotechnology.

The high demand for resources contributing to the sustainability of the population encouraged the expansion of industries. Enzymes being one of the pivotal elements in the industries are being developed to sustain the industrial sectors. As a result, The Global Enzymes Market was estimated at a value of $7,082 million in 2017, nature notably *Bacillus* is constantly being

exploited for the production of lipases which constitute one of the groups of biocatalysts in high demand. In virtue of their versatility, stability, regiospecificity, enantioselectivity, ease of production, catalytic properties, microbial notably *Bacillus* lipases constitute one of the critical group of enzymes valuable to the biotechnology and therapeutic applications **(Table 2.1; Figure 2.8)**. Therefore, this section will elaborate on the use of lipases in different industrial sectors.

2.4.1. Industrial applications

2.4.1.1. Food industry

In the food industry, lipases used for many applications:

2.4.1.1.1. Fat and Oil industry

The value of triglycerides depends on its structure. Therefore, triglycerides of low cost can be transformed into valuable triglycerides using lipase. In the fat or oil industry, *Bacillus* lipase is used for the modification of lipids by either changing the position of one fatty acid moiety from one carbon of the glycerol backbone to another or replacing one or more fatty acid moieties with new ones (Pabai et al., 1995; Undurraga et al., 2001). The newly produced lipids usually have the form of vegetable oils with nutritional importance, low calories and oleic acid enriched. An example of the use of lipase in the oil industry is the use of lipase for the inter-esterification of the palm (cheap) oil to produce cocoa butter equivalents. The palmitic acid of the palm oil at the second carbon position is replaced with oleic acid, and the cocoa butter is then used in chocolate manufacturing (Nakajima et al., 2000). *G. stearothermophilus* SB-1 and *B. atrophaeus* SB-2 produce lipases that could hydrolyze jasmine and rose oils and could be applied for the manufacture of personal care products through interesterification reactions (Bradoo et al., 1999).

2.4.1.1.2. Bakery industry

In the bakery industry, lipase helps in the extension of bread shelf-life. Lipase also enhances and controls non-enzymatic browning while increasing the loaf volume and improving the crumb structure. An example of such is the lipase from *Bacillus subtilis* (Sangeetha et al., 2011; Ray, 2012).

2.4.1.2. Detergent industry

Detergent is one of the cleaning agents used, worldwide, for household purposes. It is used for either automatic or hand-wash laundry, dishwashing, household cleaning, etc. The addition of lipase in the detergent industry constitutes one of the most commercially essential applications of lipase. Lipase has been used in combination with other enzymes such as amylase and protease to increase the performance of the detergent (Ito et al., 1998). However, during the improvement of the detergent, some factors are said to be taken into consideration. The detergent lipase should have water solubility, a low substrate specificity in order to remove either the smallest or biggest fatty or greasy stains, tolerance to harsh washing conditions by being able to perform at high temperature and alkaline pH, should operate in the presence of inhibitors or inactivators such as surfactants or detergent proteases (Chauhan et al., 2013). Lipases with the above features mentioned are suitable to be incorporated in washing powders and liquid detergents. Lipase is incorporated in the detergent to help in the removal of fatty stains like fried oil, sauce oil, lipstick, etc. (Jaeger and Reetz, 1998). Some of the lipase used in the detergent industry are sourced from *Staphylococcus arlettae*, *Burkholderia cepacia*, *P. fluorescens*, and *Candida sp* (Ahmed et al., 2010; Phuah et al., 2015, Su et al., 2014; Su et al., 2015). Generally, the chelating agents in commercial detergents inactivate the incorporated enzyme, but the lipase from *B. licheniformis* had its activity restored through the addition of calcium chloride to the enzyme-detergent complex (Romdhane et al., 2010). Similarly, one of the recent studies on anionic surfactants reported the retention of lipase stability and activity in the presence of sodium lauryl ether sulfate with two ethylene oxide units (Magalhaes et al., 2016).

2.4.1.3. Pulp and Paper industry

Lipase is mainly used in the paper industry as one of the solutions provided to the problem of pitch formation. Pitch is mostly made of hydrophobic triglycerides or waxes coming from the wood or lignocellulosic biomass components (Jaeger and Reetz, 1998). Pitch makes its way through the paper manufacture process and is deposited in the paper mill machines ending up making holes and spots in the final paper. Pitch control in the pulp and paper industry should be implemented to enhance the quality of the paper to be produced, prolong the equipment shelf life, to save energy and reduce the level of pollution caused by the chemical process. Lipase does solve the problem by being applied to the wood to hydrolyze 90% of the pitch into monoglycerides and fatty acids. The hydrolysis renders the pitch less sticky thus easily washable (Jaeger and Reetz, 1998). Therefore, a hyper-thermostable lipase was isolated from *G. stearothermophilus* SB-1 with an additional broad substrate selectivity feature and was said to be a suitable candidate in the application the processing of paper and pulp, for removal of pitch (Bradoo et al., 1999).

2.4.1.4. Cosmetics

In the cosmetic industry, lipase is used to yield esters such as isopropyl myristate, isopropyl palmitate, and 2-Ethylhexyl palmitate to be applied as a moisturizer or soothing ingredients in personal skin care products. Examples include skin and sun-tan creams, bath oils, etc. It was discovered that the replacement of conventional acid catalyst with lipase generates much higher quality products than those obtained from the traditional procedure. The downstream refining process was also minimized (Andualema and Gessesse, 2012). Another example is the use of lipases from *B. atrophaeus* SB-1 and *B. licheniformis* SB-3 for the hydrolysis of short chain fatty acids for the manufacture of cosmetic products (Bradoo et al., 1999)

2.4.1.5 Agrochemical industry

Lipase is used in the agrochemical industry to synthesize organic compounds to use as herbicides or pesticides to kill insects which damage crops (Barbosa et al., 2011). An example is the production of insecticides and fungicides by transesterification using *Pseudomonas* lipase as a catalyst. Notably, one of the herbicides (S)-indanofan, which was produced using lipases as a catalyst has proven successful and useful against wild grass and weeds (Sangeetha et al., 2011). Lipases from *B. subtilis* was employed for the synthesis of optically active forms of pesticides from a racemic mixture of alcohol and carboxylic esters

2.4.1.6. Waste management

Lipase, a fat-splitting molecule, is incorporated in chemicals that are widely used to continuously remove thin layers of fats formed on the surface of aerated tanks used for aerobic waste processes of many industries (Sarmah et al., 2018). Lipase from organisms like *Bacillus, Pseudomonas, Candida,* etc. is also used to treat oily effluent from abattoirs, poultry waste processing, food processing, restaurants, and domestic sewage. Lipases from *Bacillus subtilis* COM-B6 has been used as one of the effective decontaminants of wastes from lipid-processing factories. Lipase was also found to be useful in the degradation of diesel oil in freshly contaminated, unfertilized and fertilized soils.

2.4.2. Therapeutic applications

Lipases constitute the primary enzyme involved in the metabolism of lipids in the body. In virtue of its hydrophobicity, lipids are not assimilated by the body because they are insoluble in the blood which is primarily made of water. Therefore, lipase needs to break down lipids in to small fatty acids to be absorbed by the body. Therefore, lipases are used as digestive aids to stimulate the digestion process of ingested lipids. Bacterial lipases are currently used to treat cystic fibrosis and pancreatitis. They replaced pancreatic lipases for such treatment (Ye et al., 2011). Bacterial lipases are also used in the treatment of malignant tumors by activating the

tumor necrosis factor. Moreover, monoglyceride lipase is a promising drug target for cancer, neurodegenerative and inflammatory diseases (Grabner et al., 2017).

Lipases can also be used in the treatment of skin scalp disease and hair loss while *Bacillus* lipase is used for the synthesis of enantio-pure compounds to be used as pharmaceutical products.

2.4.3. Biodiesel production

Biodiesel has been seen as one of the alternatives to fuel since the raising of the environmental conservation issue. Biodiesel is a liquid produced from the transesterification of lipids with methanol or ethanol, short chain alcohol. The industrial production is said to provide less amount of greenhouse gases (sulfur oxide) as compared to the burning of fossil fuels for the production of diesel or petrol. The biodiesel production is additionally seen as a replacement to the fossil fuel because of the depletion of the resources of fossil fuels. The transesterification process requires lipase as a catalyst because the use of lipase render the production of biodiesel less harmful to the environment (Lukovic et al., 2011). Another advantage that the enzymatic transesterification has over the chemical one is the easy recovery of the purified glycerol which is a byproduct of the transesterification reaction (Lukovic et al., 2011). The enzymatic production of biofuel consumes less energy and produces waste in a reduced quantity as compared to the chemical process. Examples of raw materials used in the production of biodiesel are non-edible oils. The use of non-edible oils does not affect food production (Ahmia et al., 2014). Furthermore, the edible oils can also be used for the production of biodiesel. As stated that the properties and features of lipases determine its relevancy in the industry, (Aguieiras et al., 2015) reported on the current status and perspectives of the use of fungal lipases for biodiesel production. The lipase activity and stability was tested against various raw materials, alcohols, the concentration of solvents, etc. The type of lipase to be used for the transesterification of lipids/fats with alcohol is highly dependent on the variety of raw materials. For example, lipase from *M. miehi* (Lipozyme IM-20) was deemed more effective on animal fat, while the lipase from *Geotrichum sp.* worked best for the transesterification of waste cooking oil. Whereas, lipase from *P. fluorescens* was suitable for the transesterification of waste sunflower oil. Further studies were also conducted to develop strategies to reduce the cost of biodiesel production (Ghaly et al., 2010).

2.5. Conclusion and Future Prospects

The development of green technology brought a different perspective on microorganisms. Microorganisms are no longer considered as strictly harmful organisms. Microorganisms have been found to be essential in the sustainability of humans. Research proved that *Bacillus* is opulent in genes coding for useful biomolecules such as lipases. Therefore, it can be concluded that *Bacillus* should be exploited in green technology for its potential biochemical compounds. Since industrial processes require biocatalysts that are stable under extreme conditions, *Bacillus* is regarded as one of the potential candidates to offer such biocatalysts because it can withstand conditions similar to those of the industry. *Bacillus* lipases, for instance, have been proved to be attractive for the industrial sectors because of their versatile nature in virtue of their catalytic activities towards a wide range of reactions.

Moreover, lipases with improved features such as selectivity, region-specificity, enantio-selectivity, thermo-stability, pH stability, organic solvent tolerance are highly necessary. Therefore, it is recommended to explore drastic ecosystems such as rumen notably for *Bacillus* and other sources of potential biocatalysts with enhanced features listed above, for biorefinery applications and other industrial applications. The goat rumen would be ideal to be looked at because of its diverse feeding habit which is assumed to impact the rumen microbiome. The recently developed technique "metagenomics" would be of great help and a step closer for mining novel *Bacillus* and other microbial lipases.

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CHAPTER 3:

Metagenomic DNA Extraction from The Goat Rumen

Abstract

Metagenomics is a developed approach that came to the rescue of researchers for the study of unexploited niches that were left out by the traditional culturing techniques. One of the crucial steps in metagenomics is the isolation of pure high-molecular weight DNA that can represent most of the microorganisms present in a certain environment. Therefore, the aim of this study was to develop an effective and time-efficient extraction protocol, that would recover high-molecular-weight and cloneable metagenomic DNA (mDNA) from goat rumen. The CTAB method was optimized and adapted to the rumen ingesta type of sample. Using this protocol, high molecular weight DNA of about 30-40 kb was obtained. The $A_{260/280}$ and $A_{260/230}$ of the DNA were 1.7 and 1.0, respectively demonstrating that the DNA was pure. The purity of mDNA was further tested by subjecting the DNA to 16S rDNA PCR amplification. The agarose gel showed PCR amplicons of about 1 kb in size. Therefore, it could be concluded that the optimized method succeeded in isolating DNA of high-molecular weight from rumen samples.

Keywords: Goat, Rumen, Metagenomic DNA, High-Molecular weight

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3.1. Introduction

The goat is a ruminant, grazing herbivorous animal that usually regurgitates the partially ingested feed for re-chewing and re-swallowing whenever they are resting. The goat is characterized by its diverse feeding habit (**Figure 3.1**) because they can feed on materials that are not regarded as foods by other ruminants (Solanki, 1994).

Figure 3.1. Goats feeding on plastics and some other materials other than herbs (http://www.picsbud.com).

The goat does possess and rely on the rumen, the digestive machinery, for complete digestion of feed.

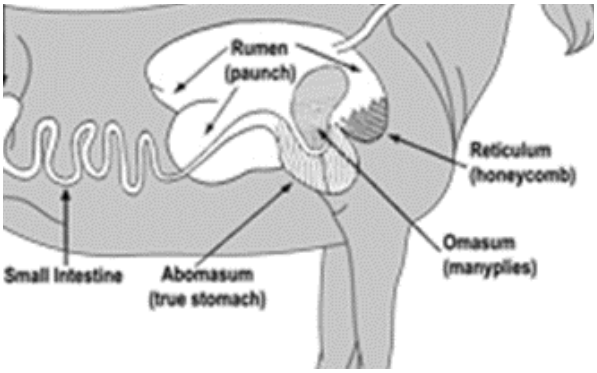

Figure 3.2. The four compartments stomach of the goat called the rumen, owing the name to the first compartment which is also called the paunch. It is the largest chamber of all and the place of the initiation of the true digestion. The second compartment is called the reticulum, followed by the

omasum the third and lastly the abomasum which is also known as the true stomach (adopted from https://thekebun.wordpress.com/2008/09/07/the-digestive-system-of-the-goat/ accessed on the 2nd of February 2019).

The rumen (**Figure 3.2**), digestive machinery, is a highly complex niche because it is populated by many different species and genera of bacteria, archaea, fungi, ciliate protozoa and viruses (Fouts et al., 2012; Berg Miller et al., 2012 & Kim et al., 2011). These microorganisms produce several enzymes to catalyze the conversion of the ingested complex organic compounds into smaller organic compounds. Although these enzymes play a pivotal role in providing the host (the goat) with sufficient nutrients and source of energy (Hungate, 1966), they can be applied in biotechnological processes (Sharma et al., 2014).

However, the accessibility of the enzymes of many different species inhabiting the rumen, was restricted due to the inability of some microorganisms to grow on synthetic media in the laboratory. With the conventional culture-dependent techniques, only about 1% of the total microbial population was recovered leaving out about 99% (Bashir et al., 2015). Therefore, recent developments in molecular biology led to the conceptualization of metagenomics as a study of the pool of DNA to access even the un-culturable microorganisms and to overcome the limitations of the culture-dependent methods (Handelsman et al., 1998).

The success of the metagenomics approach depends on many factors. One of the crucial steps in a metagenomic study is the extraction of the metagenomic DNA. The metagenomic DNA extracted from an eco-niche should be a representative of the entire community of such environment (Handelsman et al., 1998; Rondon et al., 1999; Nordgård et al. 2005). The metagenomic DNA should be of high quality, suitable for downstream analyses such as sequencing, PCR and construction of genomic libraries. The recovered metagenome is rich in genes coding for biomolecules that are useful in biotechnology. However, the recovery of metagenomic DNA for further process like metagenomic library construction has diverse challenges such as the shearing of DNA during mechanical lysis of cells or the co-extraction of contaminants along with DNA that can interfere with downstream analyses (Bag et al., 2016). The protocol used for extraction of metagenomic DNA should be optimized to avoid shearing of DNA to conserve the full length of genes and to remove all contaminants that can

possibly interfere with downstream analyses such as sequencing (Bag et al., 2016). The recovery of a sufficient quantity of pure metagenomic DNA representing all the genomes of a particular community is very crucial for metagenomics studies (Martin-Laurent et al., 2001; Lorenz and Schleper, 2002; Bertrand et al., 2005). Therefore, the aim of this part of the study was to optimize and come up with a DNA extraction protocol to recover pure, non-sheared high-molecular-weight metagenomic DNA that can represent the goat rumen microbial community that can be sequenced and cloned.

3.2. Materials and Methods

3.2.1. Sampling

Five goats were purchased from different locations in South Africa, precisely in the Gauteng and Limpopo provinces. The two location were chosen because the environment is more natural with green flora and possess nutrients available for animals. Such feed could have an impact on the feeding habits of the animals like goats. Due to richness in flora, it was assumed that the goats selected from the above-mentioned regions, would have rumen highly diversified in microbiomes.The purchased 5 goats were brought to Bob's farm located in Vereeniging, Gauteng in South Africa in order to be slaughtered following the animal ethics guidelines. After slaughtering, the ingested materials and fluids from rumen, reticulum, omasum and abomasum of each goat were collected in triplicate and aseptically to avoid cross contamination of microbial populations among different compartments. Upon arrival at the OMICS laboratory, the samples were kept at -20° C until DNA extraction was performed.

3.2.2. High-molecular weight metagenomic DNA (mDNA) Extraction

A representative sample of the rumen digesta was collected aseptically from four different compartments of the stomach (reticulum, rumen, omasum and abomasum) and homogenized in a 15ml sterile Falcon tube containing cetyltrimethylammonium bromide (CTAB) buffer.

Cetyltrimetylammonium bromide (CTAB) buffer was prepared by mixing 20 g CTAB in 100 ml 1 M Tris-Hydrogen Chloride [pH=8], 40 ml of 0.5 M EDTA and 280 ml of 5 M NaCl and volume adjusted to one litre of ddH_2O.

Then, 0.1g of the homogenized sample was weighed into a 2 ml microcentrifuge tube and suspended in 500 µl of pre-warmed (60°C) CTAB extraction buffer [(2% (w/v), 100mM tris-HCl (pH 8), 20 mM EDTA and 1.4 M NaCl] already containing 2.5 µl of proteinase K and 1.5 µl β-mercaptoethanol which were added immediately after prewarming the buffer. The suspension was incubated at 60 °C for 10 minutes. After 10 minutes of incubation, 500 µl of chloroform: isoamyl alcohol (24:1) solution was added to the tube under the fume hood. Thereafter, the mixture was gently mixed by inverting the tube and then centrifuged for 15 min at maximum speed (13500 rpm) at 4 °C. The aqueous layer was carefully transferred to a new sterile 2 ml microcentrifuge tube. To precipitate the DNA, equal volume of ice-cold isopropanol was added and the tube was gently inverted to ensure complete mixing of the solution. The solution was then stored in the freezer (-20 °C) for 25 minutes, then centrifuged for 5 minutes (13500 xg) at 4 °C to pellet the DNA. The supernatant was carefully removed and then the pellet was washed twice with 70% ethanol and afterwards dried by leaving the tube open. The dried pellet was re-suspended in 15 µl low TE (pH 8.0) and then stored at -80 °C for further analysis (Worden, 2009).

A few aliquot of DNA sample was further purified using DNA Clean & Concentrator™-25 kit from Zymo Research. A 1.5 ml microcentrifuge tube was used to add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample. The mixture was briefly mixed by vortexing. The mixture was transferred to a provided Zymo-Spin™ Column2 in a Collection Tube the centrifuged for 30 seconds at 1,000 - 16,000 x g. The flow-through was discarded and 200 µl DNA Wash Buffer was added to the column. It was then centrifuged at 1,000-1,600 x g for 30 seconds. The wash step was repeated and ≥ 25 µl DNA Elution Buffer was added directly to the column matrix and incubated at room temperature for one minute. The column was transferred to a 1.5 ml microcentrifuge tube and centrifuged at for 30 seconds to elute the DNA. The Ultra-pure DNA was now ready for further processing.

3.2.3. mDNA Size, Integrity, quantification and purity determination

The size and integrity of the isolated DNA were analyzed by electrophoresis in agarose (0.8%, w/v) gel prepared in 1× TBE buffer (prepared by diluting 10× TBE with an adequate volume of distilled water. Ten times TBE was prepared by mixing 108 g Tris base, 55 g boric acid, 7.45 g EDTA to a final volume of 1 L with dH2O containing 5 µl of 0.5 µg/mL ethidium bromide (Sambrook et al., 2001). Five microliter of the extracted DNA was mixed with 2 µL of 6X DNA loading dye (KAPA Biosystems, South Africa) and the mixture was loaded into the gel and run at 100 volts (V) for 45 minutes using the PerfectBlueTM Horizontal Mini Gel System (PeqLab, UK). KAPA Universal DNA Ladder was used as the molecular weight marker to determine the size of the DNA. Upon completion of the electrophoresis session, the gel was visualized and photographed using a digital imaging system UV-transilluminator (SYNGENE G- Box, United Kingdom).

The concentration, purity and intergrity of the DNA was assessed using the Thermo Scientific™ NanoDrop™ One^{C}Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and fluorometrically using Qubit® 3.0 Fluorometer (Qubit™ dsDNA BR Assay Kit, Invitrogen, Carlsbad, CA, USA). The purity was obtained from the A260 nm to A230 nm and A260 nm to A280 nm ratios in order to indicate the possible contamination of the DNA with either organic solvents or components of buffers used for DNA extraction, tannins and proteins, respectively.

3.2.4. Assessment of the Suitability of mDNA for PCR

The purity of mDNA for downstream analysis was assessed by amplification of 16S rDNA using the universal primers. The PCR amplification was done using a 25 µl reaction mixture containing 0.2 mM each dNTP (Fermentas), 10 µmole of each forward and reverse primer, ≈100 ng mDNA template and 1 µl Taq 2X Master Mix with standard buffer. Amplification was performed with a thermal cycler (Applied Biosystems,) by using the following program: 95 ºC for 3 min; 30 cycles consisting of 95ºC for 30 sec; 55 ºC for 30 sec; 72 ºC for 1 min; and a

final extension step consisting of 72°C for 5 min. The amplification was assessed by electrophoresis of the reaction products in a 0.8% agarose gel.

3.3. Results and Discussions

The aim of the current study was to recover an intact, high-molecular-weight metagenomic DNA of high purity from the goat rumen digesta (Table 3.1). In order to achieve this aim, the CTAB protocol used was optimized according to the type of sample (rumen ingesta) dealt with. The CTAB protocol used was the one making use of 2% CTAB chemical. However, some parameters were changed. The CTAB protocol used was initially used to extract DNA from *Micromonas* RCC299 and *Micromonas* RCC472 for complete genome sequencing project. Therefore, the DNA was supposed to be of genome sequencing quality (i.e. unsheared) and be used for large insert libraries. Since the current study wanted to make use of DNA of such quality, the same protocol was used and adapted to the type of sample used (rumen ingesta). The optimisation was done by removing the cell harvesting step, by increasing the concentration of β-mercaptoethanol and increasing the concentration of proteinase K to break open open the cells and recover maximum DNA as possible, having minimal proteins contaminant. The time for lysis of the cells was also shorten to avoid having the DNA sheared.

Table 3.1 Quantitative and qualitative results of extracted DNA from the goat rumen

Instrument used for quantification	**Concentration**	**DNA total mass**	**A $_{260/280}$**	**A $_{260/230}$**
Nanodrop	215ng/µl (150 µl)	9 µg	1.71	1.0
Qubit3	60 ng/µl (150 µl)	32 µg	N/A	N/A

Metagenomic DNA was extracted using the optimized CTAB protocol. The CTAB protocol was used because it is made up of a chemical constituent which is an important surfactant in the DNA extraction buffer system. The CTAB method facilitates the separation/disruption of

polysaccharides or membrane lipids which hold the cell membrane intact during the extraction process. The disruption of membrane lipids results in the lysis of the cell. The quantification using the Qubit3 revealed that a total of 9 µg of metagenomic DNA was extracted from one gram of goat rumen starting material while the Nanodrop estimated the quantity of metagenomic DNA to be of 32 µg (**Table 3.1**). The difference is mainly due to the fact that Qubit3 only quantifies double-stranded DNA with the help of dyes provided in the kit. The dyes only bind to double-stranded DNA and the quantification is done. However, the nanodrop quantifies DNA by absorption. It quantifies DNA by the ability of DNA to absord the light at wavelength 260 nm. All the substances that are able to absorb the light are quantified as DNA while in the DNA mixture, there could be some chemicals present absorbing the light at the same wavelength. There lies the discrepancy between quantifying the DNA by absorption and by usage of the dye. With the yield being high, it is assumed that the extracted metagenomic DNA represented the metagenome of almost all the present microbes in the rumen niche. The metagenomic DNA extracted was a High-Molecular-Weight DNA having a size of more than 10 kb as compared to the fosmid control DNA (**Figure 3.3**). The extracted DNA was pure because it had an $A_{260/280}$ ratio of 1.7 (Table 3.1). Such ratios provide the evidence that the extracted DNA was less contaminated by the proteins.

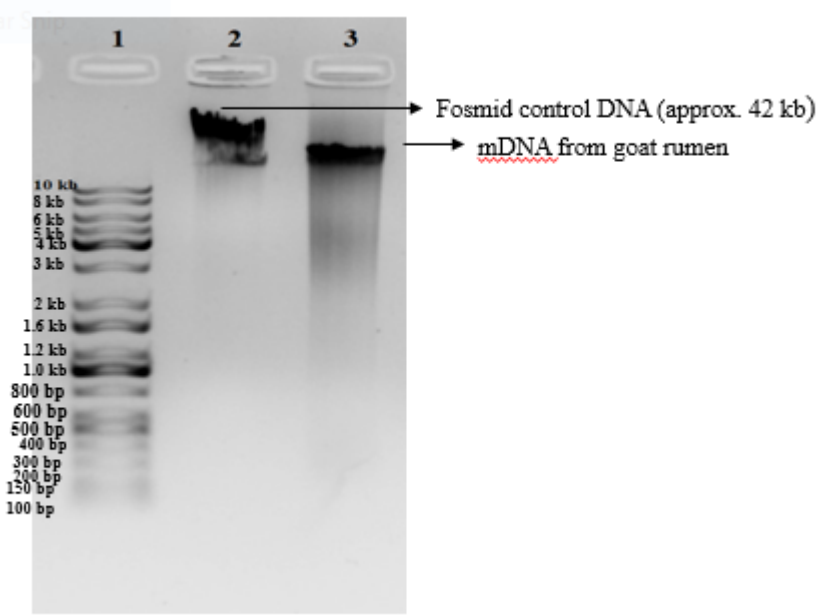

Figure 3.3: Agarose 0.8% gel electrophoresis illustrating a loading of 5 µl of the metagenomic DNA extracted from the goat rumen using the CTAB protocol. The metagenomic DNA was not exposed to UV light/ethidium bromide for the success of downstream analysis. Lane 1: 1 kb DNA ladder, lane 2: Fosmid control DNA, 42 kbp and lane 3: metagenomic DNA from goat rumen approximately 20-30 kb.

Since the DNA was isolated from the goat rumen, it was advised that the workers working with the rumen sample use the same DNA because it was of high quality and the the workers played a role in the optimization of the protocol and extraction of the DNA.

The purity of extracted DNA was further assessed by amplifying the 16S rRNA regions (**Figure 3.4**). The reults obtained showed that the DNA was free of contaminants (tannins) that could interfere with enzymatic reactions such as ligation (Henderson et al., 2013). However, the duplicates appeared to not have the same concentration mainly due to error in pipetting. It could be assumed that air was sucked during pipetting leading in difference of volumes being loaded, especially in lane 3.

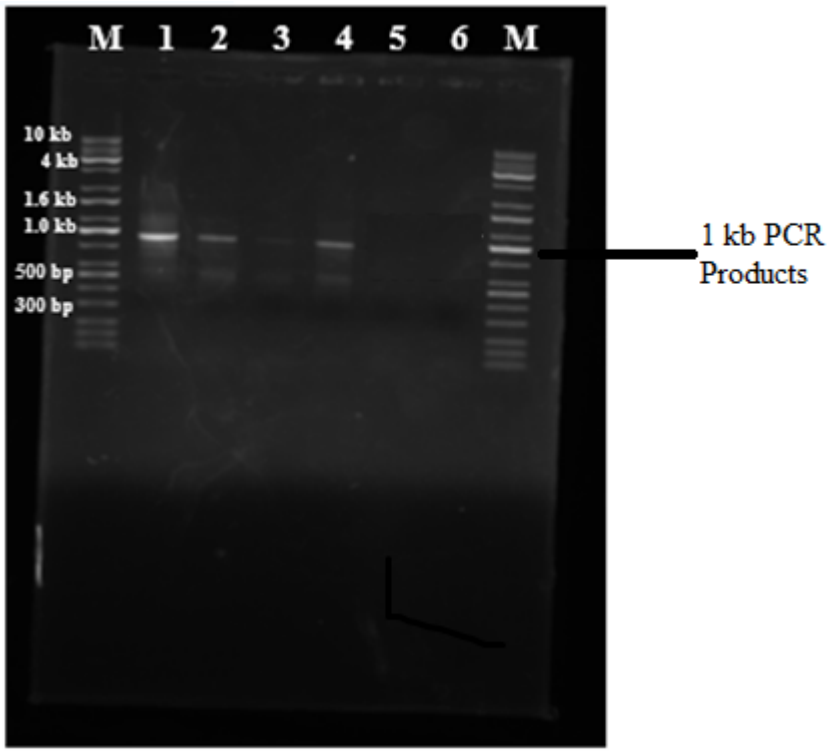

Figure 3.4: Gel electrophoresis image of the extracted DNA obtained from the goat rumen. Lane M: DNA ladder, lane 1: 16S amplification products from DNA isolated using CTAB protocol, lane 2: duplicate of lane 1, lane 3: 16S amplification products from DNA isolated using CTAB protocol and further purified using zymo kit, lane 4: duplicate of lane 3, lane 5: 16S amplification from DNA isolated using zymo kit, lane 6: duplicate of lane 5, lane M: DNA ladder.

3.4. Conclusion

The challenges faced during extraction of metagenomic DNA from different environmental samples led to the development and optimisation of many protocols for genomic DNA extraction. Optimisation depends on the nature of the samples because different types of samples are contaminated with different types of contaminants acquired from nature. Therefore, many DNA extraction protocols have been developed and published to recover metagenomic DNA of high purity and integrity from different eco-niches. However, optimization are still made nowadays to suit the type of samples dealt with. In this study, the optimized CTAB protocol that was used to extract metagenomic DNA from the goat rumen led to the successfully extraction of high-molecular-weight intact metagenomic DNA (30-40 kb) (Figure 3.3). Thus, the optimized protocol could be extrapolated for extraction of intact high-molecular-weight metagenomic DNA from other ruminants besides the goat. Hence, the extracted high quality metagenomic DNA was used for construction of Fosmid library as discussed in Chapter 4.

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CHAPTER 4:

Fosmid Library Construction, Activity-based Screening and Sequencing

Abstract

The metagenomic library of goat rumen sample was constructed using a fosmid vector (pCC2FOS). A total of 1435 fosmid clones were functionally screened using Tributyrin as a substrate and five clones were selected based on the size of the clear zone. The five clones had the soluble lipases characterized based on pH, temperature and methanol tolerance. Two clones pCC2FOS-Lip-VUT3 and pCC2FOS-Lip-VUT5 having pH optima of 3 and 10, and temperature optima of 30 ºC and 40 ºC were selected for further sequencing. Sequencing of the two clones had a depth of 50 Mb each and the insert harboured many ORFs among which one was selected per clone. Lip-VUT3 ORF comprises 636 bp and coded for a putative 212 amino acid protein having a size of 24.8 kDa correlating with the GSDL-lipase from *Bacillus* spp. while Lip-VUT5 ORF comprises 745 bp and coded for a putative 248 amino acid protein having a size of 28.4 kDa, predicted to be a lipase having amino acid sequence identical to those of lipase from the hydrolase family and alpha/hydrolase superfamily from multiple species of *Bacillus*. Therefore, this study revealed that the goat rumen metagenomic libraries are a potential source of genes coding for lipases

Keywords: Fosmid library, Functional screening, Sequencing.

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4.1 Introduction

Enzymes have been contributing to the development of eco-friendly industrial processes and the one mostly used originate from microorganisms. Therefore, microorganisms are no longer only labelled as harmful but they are now playing a major role in the development of industrial processing but providing industries with important materials like enzymes. Consequently, mining of microbial enzymes notably lipases became a major point of entry contributing to the proliferation of industries. The discovery of novel microbial enzymes became more feasible with the use of metagenomic techniques (Uchiyama and Miyazaki, 2009).

Metagenomics is a powerful approach or technique used to accelerate the mining of novel or useful genes from microorganisms, coding for important or potential industrial enzymes. Metagenomics offers the opportunity of directly investigating the functionality of microorganisms present in a certain eco-niche. As previously stated, metagenomics is further subdivided into two parts: sequence-based metagenomics and function-based metagenomics (Rabausch et al., 2013).

With the development of Next-Generation Sequencing (Sripreechasak et al. 2018) and bioinformatics, the sequence-based metagenomics became readily available to analyze a set of metagenome extracted from a certain environment. Sequence-based metagenomic relies on metagenomic DNA extraction followed by sequencing of the mDNA or cloned mDNA to the vector of choice. However, the sequence-based methods encountered some limitations. Such limitations include the identification of genes restricted to only those that are similar to those already existing in databases and the recovery of only part of the open reading frame of a gene of interest causing the expression of the product to be difficult. Additionally, the sequence-based metagenomics does not give any insights on the kinetics of the discovered putative enzymes (Daniel, 2005, Rabausch et al., 2013).

On the contrary, function-based metagenomics is said to be a straightforward approach used to discover novel genes coding for novel enzymes (Uchiyama and Miyazaki, 2009). Function-based or activity-based metagenomics is carried out by first constructing a genomic library then screening the constructed library using a solid matrix containing the substrate of interest. Sometimes, the substrate of interest is supplied with an indicator to facilitate the selection of positive clones or libraries (Coughlan et al., 2015). One of the criteria for selection of positive clones can be the appearance of the clear zone around the clones to display the presence of genes or enzymes or activity of interest like in the case of Lipolytic activity against tributyrin substrate.

The construction of genomic library **(Figure 4.1)** depends on the following steps: (i) extraction of high molecular weight quality metagenomic DNA, (Maina et al. 1988) end-repair of the isolated DNA, (Maina et al. 1988) ligation into an appropriate vector (which could be a plasmid, cosmid or fosmid) and (iv) transformation of an appropriate host strain which is usually *E.coli* for heterologous expression. For most of the functional metagenomic studies, the genomic libraries created made use of PCC2FOS^{TM} vector (fosmid) from Epicentre (www.epibio.com) because the fosmid vector is used for insert DNA of 25-40 kb.

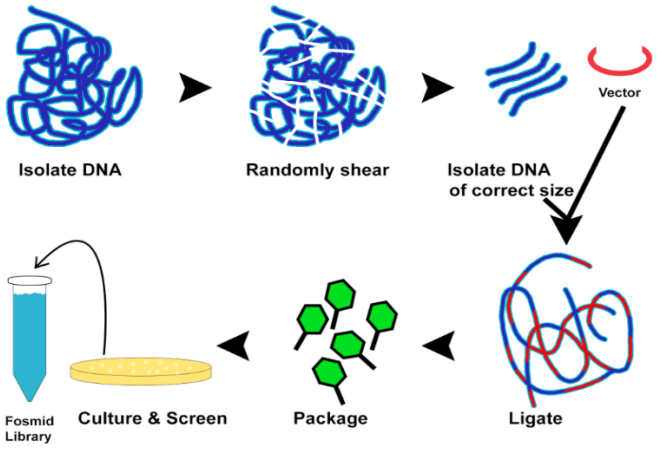

Figure 4.1: The steps involved in the Fosmid Library Construction according to epicenter illustration (https://www.cephamls.com/BAC-Fosmid-Library-Construction)

The PCC2FOS™ vector **(Figure 4.2)** is made of: (i) the use of chloramphenicol as an antibiotic selectable marker, (Maina et al. 1988) *E. coli* F factor-based partitioning and a single copy origin of replication, (Maina et al. 1988) an *oriV* high copy origin of replication, (iv) a

bacteriophage lambda *cos* site for lambda packaging or lambda terminase cleavage, (v) a bacteriophage P1 loxP site for cre-recombinase cleavage and (vi) a bacteriophage T7 RNA polymerase promoter flanking the cloning site.

The *E. coli* is used as a heterologous host or surrogate for the construction of the fosmid library has some of the genes manipulated to facilitate heterologous transcription and translation. Genes for homologous recombination (*recA*, *recBC*) or transcription and translation have been deleted to make the *E.coli* strain useful for various cloning process. Therefore, the aim of this part of the study was to create a high quality metagenomic library that would subsequently be screened for lipolytic activities using tributyrin as a substrate. The positive clones would be selected based on the size of the halos and would have its crude lipase characterized based on physio-chemical parameters. The selected clones would have their insert DNA sequenced using the MiSeq Illumina system.

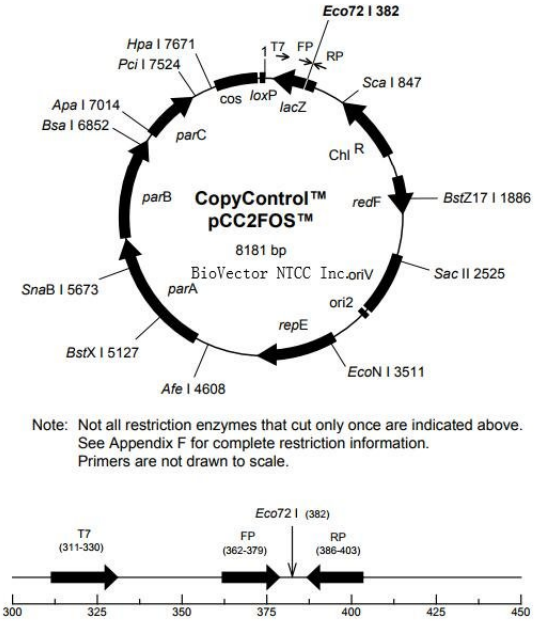

Figure 4.2: The vector map of PCC2FOS^{TM}, Fosmid vector used for construction of genomic libraries (https://currentprotocols.onlinelibrary.wiley.com/doi/pdf/10.1002/0471142905.hg0520s54)

4.2. Materials and Methods

4.2.1. Fosmid library construction

The EPI300-T1R Plating strain was supplied as a glycerol stock. Prior to the beginning, the CopyControl Fosmid Library Production procedure, the EPI300-T1R cells were streaked out on an LB plate without incorporating any antibiotic. The cells were grown at 37 °C overnight and then stored at 4 °C.

After assessing the purity and quantity of the metagenomic DNA extracted in Chapter III, the DNA was end-repaired to blunt, 5'-phosphorylated ends. The end-repair step was performed by first shearing the metagenomic DNA. The shearing step consisted on pipetting in and out several times the metagenomic DNA. Then, the sheared metagenomic DNA was mixed with sterile water, End-repair buffer, dNTPs, ATP and End-repair enzyme as described on the protocol from the EpiFOS™ Fosmid Library Production Kit (Epicentre, USA). The mixture was incubated at room temperature (25 °C) for 45 minutes thereafter, incubated at 70 °C for 10 minutes to inactivate the End-Repair Enzyme Mix. Upon inactivation, the metagenomic End-Repaired DNA was kept on the bench for 30 minutes before proceeding to the purification process using the GenJet DNA purification Kit (QIAGEN, USA) . The DNA was then purified and concentrated using the GenJet DNA purification Kit. The second process after the end-repair step was to ligate the purified blunt-ended DNA to the Cloning-Ready CopyControl pCC2FOS Vector. The ligation took place by mixing 10X Fast-Link Ligation Buffer, 10 mM ATP, CopyControl pCC2FOS Vector (0.5 µg/µl), concentrated purified end-repaired DNA (0.25 µg), Fast-Link DNA Ligase as provided on the EpiFOS™ Fosmid Library Production Kit (Epicentre, USA) manual. The mixture was incubated at room temperature for 4 hours. The total 10 µl of the resultant ligation reaction mixture was kept on the bench while thawing the MaxPlax Lambda packaging extract for the packaging reaction. The day before the Lambda Packaging reaction, 50 ml of LB broth + 10 mM $MgSO_4$ + 0.2% Maltose was inoculated with a single colony of EPI300-T1R cells and the flask was shaken overnight at 37 °C. Then, the ligation reaction mixture (10 µl) was packaged into the thawed MaxPlax Lambda extract as directed by the protocol, then they were used to infect the EPI300-T1R phage resistant strain. A volume of 55 µL infected cells were plated on LB agar supplemented with 12.5 µg/mL

chloramphenicol and incubated at 37 °C overnight to select for CopyControl fosmid clones. The number of colony forming units was determined using the following equation:

$$Titre = \frac{(\#\ of\ colonies)(dilution\ factor)}{volumephageplated(\mu l)}$$

Where:

#:number and μl volume in microliter

4.2.2 Fosmid library storage

The CopyControl fosmid clones were stored by suspending all colonies from the agar surfaces using approximately 2 mL of LB broth supplemented with 12.5 µg/mL chloramphenicol for each plate. The mixture of 1.6 ml was mixed with 0.4 ml of 100% sterile glycerol to make 20% final concentration of glycerol stock, into cryo-vial tubes. The tube was stored at -80 °C for long term storage.

4.2.3 Determination of library insert size

After constructing the fosmid library, it was decided to validate the results by confirming the presence of the inserted DNA and its size. In order to do so, two clones were randomly picked from the replicate master plate and were respectively suspended into 50 ml of LB broth + 12.5 µg/ml chloramphenicol. The culture was incubated at 37 ºC for 16 hours and shaken at a speed resolution of 150 rpm. After incubation, 5 ml of the culture was transferred to a second sterile Erlenmeyer flask containing 45 ml of the LB broth + 12.5. µg/ml chloramphenicol in order to be induced with 50 µl of the 500X auto-induction solution for high-copy number of plasmids. The mixture was shaken for 5 hours at 37 ºC at a speed resolution of 150 rpm. After the induction procedure, the plasmid (vector + insert DNA) was extracted using the GenJet Miniprep Plasmid Kit according to the manufucturers instructions.

The plasmid DNA was quantified using the Nanodrop One C and the size of the inserted DNA was assessed on an agarose gel to confirm the success of the cloning process. Thereafter,

plasmid DNA were confirmed by restriction enzymes *Kpn I*, *Pst I* and *Nco I* and the size of the insert calculated (the sum of the DNA pieces minus the size of the pCC2FOS vector).

4.2.4 Screening of the fosmid libraries for lipase

The screening of the clones for lipolysis activity was carried out using LB agar supplemented with 0.1% Tributyrin, 12.5 µg/mL chloramphenicol, 1 mM IPTG and 0.1% L-arabinose to enhance lipolysis activity, for selection of positive clones.

The screening was carried out as follows: one cryo-vial tube that was stored at -80 ºC, was opened. A sterile toothpick was used to scrub the top of the frozen glycerol stock and it was added into a sterile Erlenmeyer flask containing sterilised 50 ml LB broth supplemented with 12.5 µg/ml chloramphenicol. The mixture was incubated at 37 ºC for 16 hours and shaken at 150 rpm. After incubation, 1 ml of the culture was serially diluted to 10^{-8} using sterile saline (0.9%) and 100 µl of the diluted culture was plated on LB agar supplemented with 0.1% Tributyrin prepared as described above. The plates were incubated at 37 ºC for a period of 2-4 days.

The criteria for selection of positive clones depended on the clear zone around the colony with the lipolysis activity. The marked positive clones were selected and screened on Phenol Red Agar supplemented with 0.1% tributyrin for a second screening to confirm if the lipases produced were true lipases or not.

4.2.5 Selection of positive clones

Due to the fact that many positive clones could be identified based on the clear zones around the colony, the second criteria for the selection of clones having potential lipase genes was further based on the size of the halos around the margins of the clones and the activity of their potential lipase at different range of pH, temperature and their alcohol tolerance.

The activity-based assay was performed by sub-culturing the clones of choice into sterile 50 ml Falcon tubes containing sterile 2 ml of lipase production medium, 12.5 µg/ml chloramphenicol with 50 mM Phosphate Buffer Solution (PBS) as solvent. The contents of one Litre of the lipase production medium were: 50 mM Phosphate Buffer, 5 g/l Yeast Extract Powder, 2 g/l Peptone, 2.5 g/l NaCl, 0.4 g/l $MgSO_4.7H_2O$, 0.4 g/l $CaCl_2.2H_2O$, 1 g/l $(NH_4)H_2PO_4$, 2% Olive oil, 10 drops of Tween 80 as surfactant. The optimal growth conditions were set as followed: the incubation temperature was at 37 ºC, the time was of 15 hours, and the aeration speed was at 150 rpm. After 15 hours of incubation, 10 µl of the 15 hours culture was inoculated into a sterile Erlenmeyer flask containing 50 ml lipase production medium and 12.5 µg/ml chloramphenicol. The mixture was incubated for an additional 5 hours and shaken at 150 rpm at 37 ºC in order to reach an Optical Density at 600 nm wavelength of 0.4-0.8. After 5 hours of incubation, the cultures were removed from the incubator in order to remove 5 ml of the liquid culture and 45 µl of 1M IPTG was added to make a final concentration of 1mM. Then, the mixture was further aerated for 3 hours to let the induction perform its work. After 3 hours, the 45 ml cultures were centrifuged at 4 ºC for 20-30 minutes at maximum speed resolution of 6000 rpm. The supernatant was considered as the crude lipase to be used for the lipase assay while the pellet was stored at 4 ºC as the source of intracellular lipase. The assay was performed against a wide range of pH, temperature and methanol for methanol tolerance.

4.2.6 Enzyme characterisation studies

4.2.6.1 Effect of pH on metagenomic lipase activity

The lipase activity assay was performed in triplicate for validation purposes at pH 3-12 with a control (blank). One percent of the substrate used was freshly prepared consisting of 15 µl of the stock emulsion of olive oil and 15 ml of 50 mM PBS). The stock emulsion of the olive oil was prepared by mixing 100 ml of olive oil and 10 µl of Tween 80. The mixture was refrigerated at 4° C for 30 minutes, thereafter homogenized for 3 minutes at low speed and finally for 10 minutes at high speed. The mixture was refrigerated again for over 30 minutes and homogenized for 10 minutes at high speed, thereafter stored in the refrigerator at 4° C for several months.

The effect of pH on the crude lipase was assessed using 50m M PBS. The pH of the buffer was adjusted from 3 to 12 using 1 M NaOH to increase the pH or 1 M HCl to decrease the pH and the assay was done at temperature 30 ºC. Fifteen mls of different pH buffers were mixed with 15 µl of the stock olive oil emulsion. It was then mixed for 90 seconds by hand prior to usage. Two hundred and fifty microliter of the working olive oil emulsion was incubated and stabilised in water, thereafter, 250 µl of the crude lipase transfered. The reaction was carried out for 20 minutes thereafter, stopped using 500 µl of 6N HCl. The fatty acids were retrieved upon addition of 1000 µl of Benzene. Two hundred microliter of cupric-acetate was added to the benzene-fatty acids complex to quantify the concentration of the fatty acids obtained. The absorbance was observed at 715 nm wavelength and the readings compared against the oleic acid standard curve. The control reaction mixture (excluding the enzyme) was included to eliminate the assumption for auto hydrolysis of the substrate.

4.2.6.2 Effect of temperature on crude lipase activity

The effect of temperature on the crude lipase was assayed by using a range of temperature from 30-60 ºC with 10 ºC intervals, for the assay at corresponding pH. The reactions were carried out in 50 mM PBS buffer consisting of 0.1% stock olive oil emulsion. Two hundred and fifty microliter of the working olive oil emulsion at different pH and 250 µL of the crude lipase were incubated for 20 minutes as stated in 4.2.6.1. The protocol given in 4.2.6.1 was strictly followed for the temperature assay. The control reaction mixture (excluding the enzyme) was included to eliminate the assumption for auto hydrolysis of the substrate.

4.2.6.3 Alcohol tolerance

The effect of different concentration of methanol on the crude lipase was assayed by a different range of concentration from 1% to 30% with 5% intervals, for the assay. The reactions were carried out in 50 mM PBS buffer consisting of 0.1% stock olive oil emulsion. 250 µl of the working olive oil emulsion (pH 7) and 250 µL of the crude lipase were incubated for 20 minutes as stated in 4.2.6.1. The protocol given in 4.2.6.1 was strictly followed for the alcohol tolerance

assay. The control reaction mixture (excluding the enzyme) was included to eliminate the assumption for auto hydrolysis of the substrate.

4.2.7 Sequencing and sequence analyses

After the lipase activity-based assay, two positive clones were selected and cultured in sterile 50 ml LB broth supplemented with 12.5 µg/ml for plasmid isolation. The resulting purified fosmid DNA was quantified and the purity assessed using Nanodrop one C, thereafter sent for sequencing at Inqaba Biotec (Pty) Ltd in South Africa using the Illumina Next-Generation Sequencing (Sripreechasak et al. 2018) platform MiSeq AMP. The sequences were analysed using CLC Bio for assembly. The assembled sequences were annotated and the prediction of the open reading frames (ORFs) was performed within the NCBI database (https://www.ncbi.nlm.nih.gov/). The sequenced genes were compared against other proteins in the database by using the basic local alignment search tool (BLAST) for protein (Altschul et al., 1997)

4.3 Results and Discussions

4.3.1 Fosmid library construction and screening

A fosmid library from the goat rumen was constructed using a CopyControl pCC2FOS™ vector and it resulted in a library size of approximately $1.4x10^3$ or 1,435 colony forming units per ml (cfu/ml). Rabausch et al. (Rabausch et al. 2013) constructed a fosmid library of 1920 clones generated from a single *Bacillus cereus* isolate. Their findings were similar to the current results obtained in this study thus, the construction of the fosmid library was a success. The two clones that were randomly picked had the fosmid extracted for quality control. The protocol used to extract plasmids was a success because highly purified plasmids were obtained as reported on **Table 4.1**. The EPI cells were used as negative control from plasmid extraction to restriction digestion reactions.

Table 4.1 Quantification of plasmids isolated from random clones to determine the average size of the metagenomic DNA insert.

Sample	Concentration (ng/ul)	$A_{260/280}$	$A_{260/230}$
EPI 300 Cells	68.4	1.95	1.36
Clone G2	163.9	1.89	2.04
Clone G6	146.6	1.87	1.86

The restriction digestion reactions were set as indicated on **Table 4.2** for each respective restriction enzyme.

Table 4.2 Total volume of all the components used for the restriction digestion of metagenomic DNA insert extraction from randomly picked clones.

Components	G2	G6	Vector
H2O (µl)	11.8	11.8	16.3
RE 10X Buffer (µl)	2.0	2.0	2.0
BSA (µl)	0.2	0.2	0.2
DNA (µl)	5.0	5.0	0.5
Restriction Enzyme (µl)	1.0	1.0	1.0
Total volume (µl)	20	20	20

The three restriction enzymes used were selected based on the list of restriction enzymes provided in the CopyControl™ HTP Fosmid Library Production Kit and Phage T-1 Resistant EPI300™-T1R *E.coli* Plating Strain Cat. No. CCFOS059 protocol. Some restriction enzymes did cut the vector PCC2FOS into two while others did cut into three pieces (**Figures 4.3; 4.4** and **4.5**). Figure 4.3 for example displays a true example of digestion. L5 and L7 contains the the uncut plasmid. Reason why, the three layers are appearing while L6 and L8 contain cut plasmid.

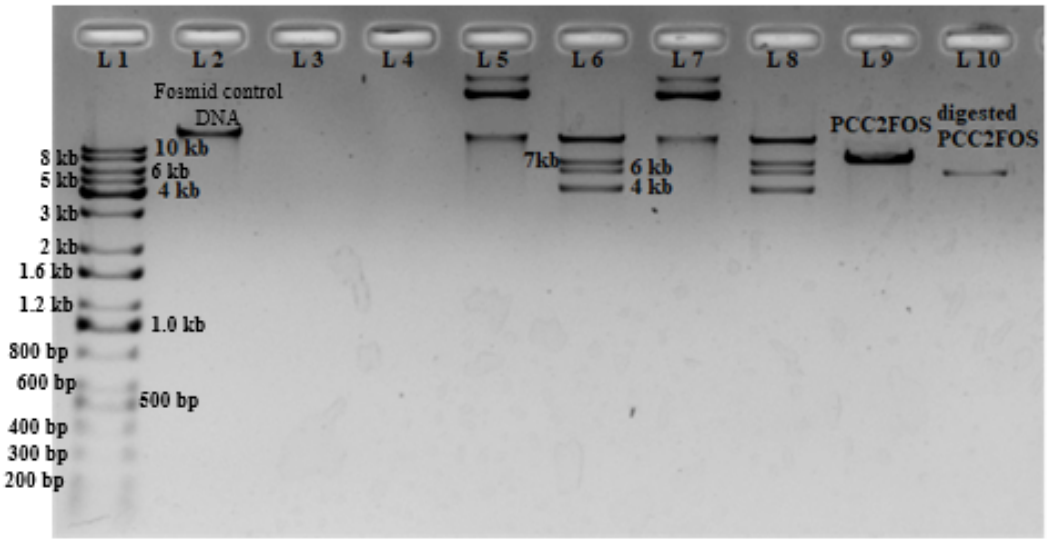

Figure 4.3: 0.8% gel electrophoresis image showing the digestion of the construct and the Fosmid vector with *Nco I*. L1: 10 kb ladder; L2: Fosmid control DNA (~42 kb); L3: plasmid extracted from EPI300 cells; L4: digested plasmid extracted from EPI300 cells; L5: construct extracted from clone 2; L6: digested construct from clone 2 with *Nco I*; L7: construct extracted from clone 6; L8: digested construct from clone 6 with *Nco I*: L9: pCC2FOS Fosmid and L10: digested pCC2FOS Fosmid with *Nco I*.

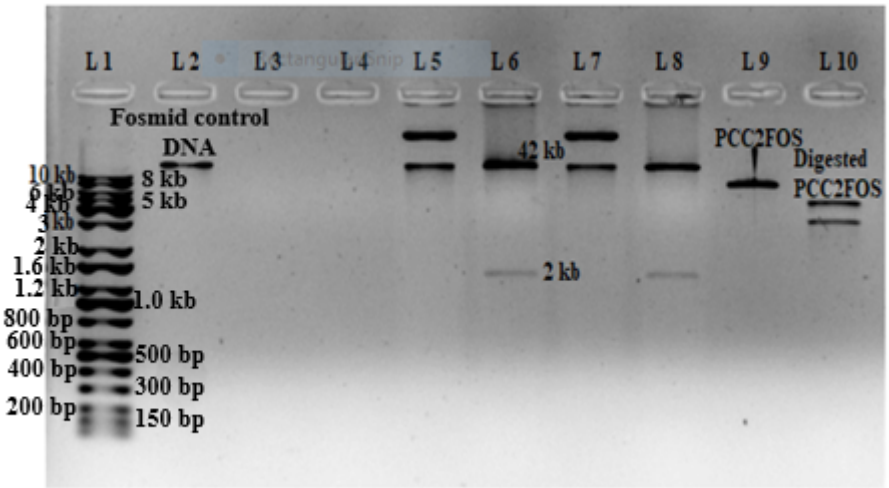

Figure 4.4: The gel indicates the digestion of the construct and the Fosmid with *Kpn I* on 0.8% agarose gel. L1: 10 kb Kapa universal ladder, L2: Fosmid control DNA (~42 kb), L3: plasmid extracted from EPI300 cells, L4: digested plasmid extracted from EPI300 cells, L5: construct extracted from clone 2, L6: digested construct from clone 2 with *Kpn I*, L7: construct extracted from clone 6, L8: digested construct from clone 6 with *Kpn I*, L9: pCC2FOS fosmid and L10: digested pCC2FOS Fosmid with *Kpn I*.

Based on the observation, it could be confirmed that the average insert size of the library was calculated to be above 25 kb as displayed (**Figures 4.3; 4.4** and **4.5)**. As *Nco I* always cut the pCC2FOS vector at 959 and 7229. At least a band of about 6 kb should be expected which was the case for this study. Two bands were at least expected.

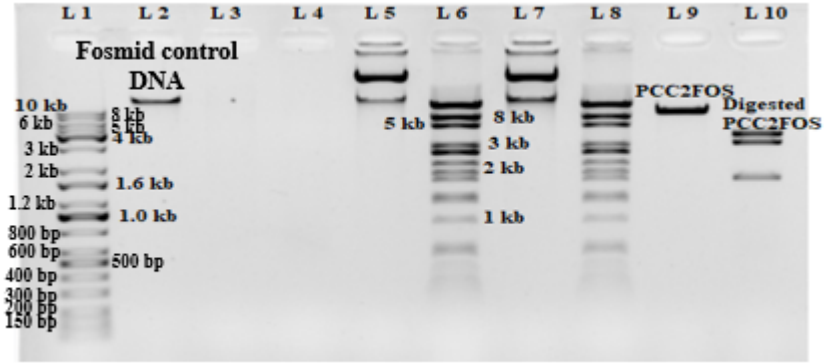

Figure 4.5: 0.8% gel electrophoresis image showing the digestion of the construct and the Fosmid with *Pst I* on 0.8% agarose gel. L1:10 kb Kapa universal ladder, L2:Fosmid control DNA (~42 kb), L3: plasmid extracted from EPI300 cells, L4: digested plasmid extracted from EPI300 cells, L5: construct extracted from clone 2, L6: digested construct from clone 2 with Pst I, L7: construct extracted from clone 6, L8: digested construct from clone 6 with Pst I, L9: pCC2FOS Fosmid and L10: digested pCC2FOS Fosmid with *Pst I*.

The size of pieces of DNA were added up to yield a total sum of 19 kb without taking into consideration the DNA bands that were above the 10 kb band of the DNA ladder. Upon confirming the presence of the insert inside the transformed cells, it was decided to proceed to the functional screening based on phenotype or activity. Functional screening consisted of subjecting the positive clones from the library to a substrate of interest which in the case of this study was tributyrin, a form of a synthetic oil. Some clones were deemed positive after observation of clear halos around the colony as shown (**Figure 4.6)**. The selected positive clones were sub-cultured on a tributyrin agar plate for further analysis and stored in glycerol stock. The presence of a clear zone around the clones simply indicates that the clones were supplied with genes coding for lipases involved in the catabolism of Tributyrin.

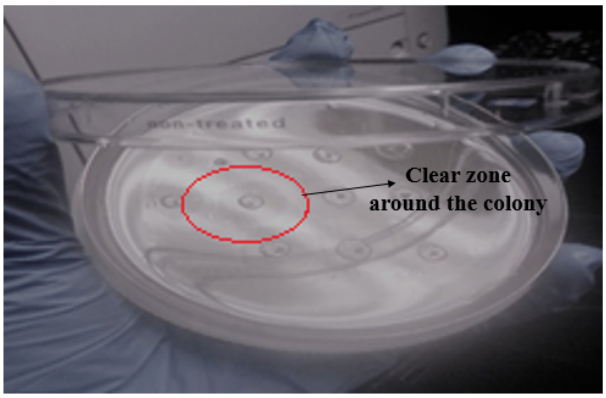

Figure 4.6: 1% Tributyrin Agar plate supplemented with 12.5 µg/ml chloramphenicol containing Putative lipases producing fosmid clones screened with zones of clearance.

4.3.2 Enzyme characterization studies

The positive clones had their lipases produced for characterization as a part of in-depth selection. Five clones were characterized but only the selected two clones (pCC2FOS-Lip-VUT3 and pCC2FOS-Lip-VUT5) for further studies, were reported. The characterization was made based on pH, temperature and alcohol tolerance as shown in the figures below (**Figure 4.7** and **4.8**). The pH affects the ionization state of the amino acids which dictates the primary and secondary structure of the enzyme and hence, pH plays a significant role in enzyme stability because it maintains an enzyme's three-dimensional structure required for its biological activity (Talley and Alexov, 2010). A change in pH mostly has a progressive effect on the structure of the protein and the enzyme activity (Fullbrook, 1996). For example, Wang et al. (Wang et al. 1995) found optimal lipase activity in the highly alkaline pH range (8.5–10.0) from *Bacillus* strain A30-1 (ATCC 53841)

Lipase from clone pCC2FOS-Lip-VUT3 showcased a maximum activity at pH 3 and the lipase maintained its activity from pH 5 to 9 while lipase from clone pCC2FOS-Lip-VUT5 had its maximum activity at pH 10 and the pH profiles could not be established because the activity fluctuated greatly (**Figure 4.7A**). Therefore, the metagenome was comprised of genes coding for lipases that could perform under both acidic and alkaline conditions.

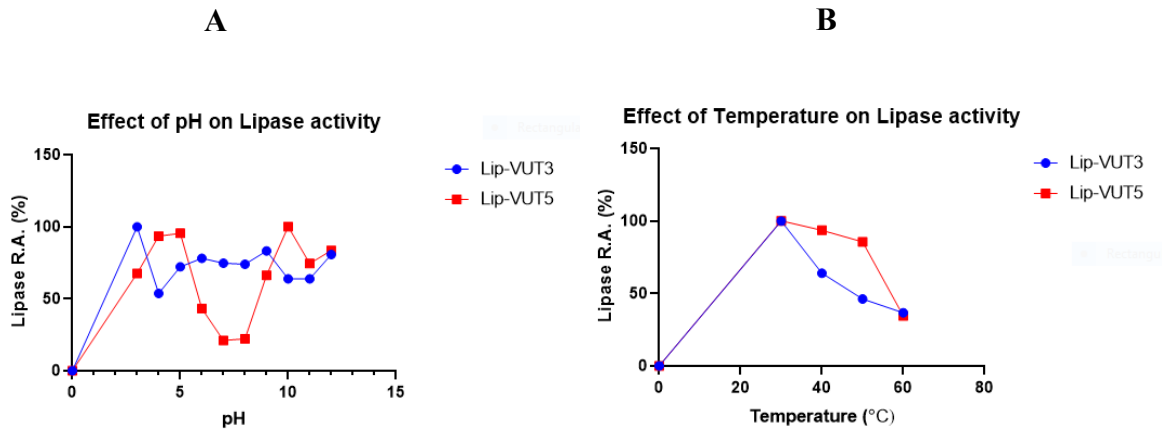

Figure 4.7 (A) Effect of pH on metagenomic Lip-VUT3 and Lip-VUT 5 lipases. Cell free culture filtrate was assayed with 0.1% olive oil in 50 mM Phosphate Buffer Solution. The assay was performed at 30 ºC in buffers at different pHs. Clones Lip-VUT 5 had highest activity at pH 10-12. (B) Effect of temperature on metagenomic Lip-VUT3 and Lip-VUT5 Lipases activity. The assay was performed at respective optimum pH (3, 10) against a wide range of temperature 30-60 ºC.

Temperature greatly affects the activity of the enzyme and the rate of action. For an enzyme-catalzed process to be successful, it should take place at the optimum temperature of that enzyme. An enzyme becomes useful in a chemical reaction only when that reaction takes place at the required temperature. Lip-VUT5 was observed to be stable at temperature 30-50 ºC but the activity greatly declined at temperature 60 °C. The optimum temperature for lipolytic activity of metagenomic Lip-VUT3 was at 30 °C (**Figure 4.7B**) and lost 50% of its activity as temperature increased. The inter-connection between the habitat of microorganisms isolated and the enzyme properties could be a possible reason for explaining the temperature stability of isolated lipases (Ahmed et al., 2010) . However, thermo-stability is a desirable characteristic for enzymes used in applications at high temperatures. It was hypothesized that the presence of other proteins could hinder the activity of the supposed lipases. The pH and temperature profiles obtained were not considered to be the definitive ones but they were just used for selection purposes.

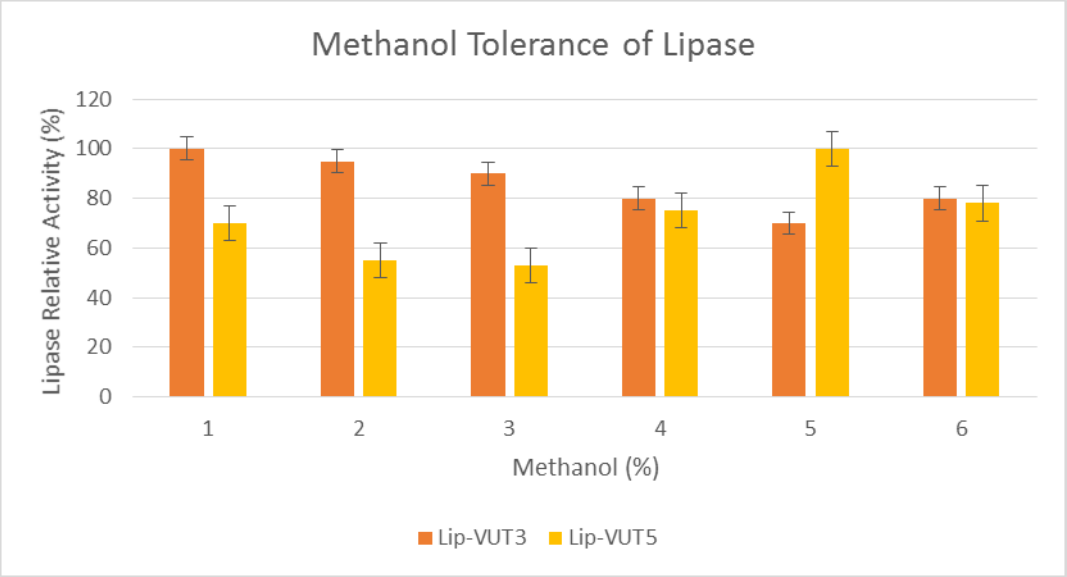

Figure 4.8 Metagenomic Lip-VUT3 and Lip-VUT5 lipases tolerance towards different concentrations (1-25%) of methanol.

The activity of Lip-VUT3 has been observed to slightly decrease when the methanol concentration is increasing. It was observed that when the concentration of alcohol was increased of 5%, the activity of the Lip-VUT3 decreased of 5-10% and only lost 20% of its relative activity overall. The alcohol did not have a huge impact on the activity of the enzyme as compared to Lip-VUT5. The activity of Lip-VUT5 has been observed to have some noisy signal showing the fluctuation of the activity of the lipase at different concentration of methanol. This could be simply caused by some other contaminants because the lipase was not thoroughly purified. The application of lipase as a biocatalyst for synthetic processes requires a stable and active lipase especially in the presence of organic solvents like methanol. Hence, for the development of lipase for industrial purposes, one should consider the stability of lipase in the presence of wide range of methanol concentrations as a parameter to consider. However, most of the microbial lipases rarely showed high stability in solvents (Doukyu and Ogino, 2010; Zhao et al., 2008). Therefore, the effect of methanol at various concentrations (1-25%) towards lipase activity was determined at respective optimum pH and temperature for the metagenomic Lip-VUT3 and Lip-VUT5 lipases. Lip-VUT3 was selected because it exhibited the highest of all activities at pH 3 and remained stable throughout pH 5-9 while Lip-VUT5 remained stable from temperature 30-50 °C.

4.3.3 *In-silico* analysis and annotation of sequences

The Fosmid DNA from clones pCC2FOS-Lip-VUT3 and pCC2FOS-Lip-VUT5 clones were sequenced to a depth of 50 MB in order to have a full coverage for the fosmid DNA. The sequences were *de novo* assembled and annotated using the algorithms for sequence analysis such as BLASTp and software like CLC Bio workbench. The ORFs of Lip-VUT3 and Lip-VUT5 had their codons optimised and were translated *in-silico*.

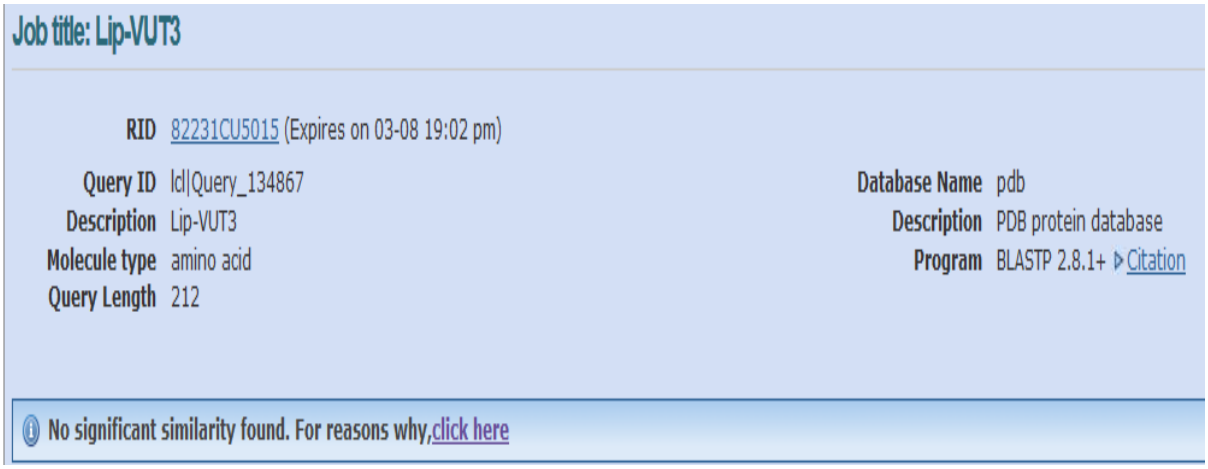

Figure 4.9: Amino acid sequences of Lip-VUT3 obtained from *in-silico* translation of the modified ORF of Lip-VUT3 revealed no similarity during BLASTp search.

For the fosmid DNA of clone pCC2FOS-Lip-VUT3, 663 contigs of average size of 367 bp were retrieved. The size of the sequenced DNA was of 24 kb (**Appendix B1**). From the 24 kb, one Open Reading Frame (ORF) of *Lipase* gene had its codon optimised and resulted in a Lip-VUT3 gene of size 636 bp. Using SnapGene software, the ORF Lip-VUT3 was translated *in-silico* and predicted a polypeptide of 212 amino acids with an estimated molecular weight of 24.8 kDa. No similarity was obtained from the BLASTp search of Lip-VUT3 (**Figure 4.9**). The absence of similarities could simply mean that the putative protein could be a novel one since it was sourced from the metagenome of the rumen or it could also be assumed that the novelty of the lipase came from the codon adjustment of the ORF. Therefore, it was decided to perform a BLASTx search from nucleotides of Lip-VUT3 ORFs to proteins using the proteins data bank (PDB) protein.

The BLASTx results reported the detection of a putative conserved domain similar to the GDSL-lipase from the SGNH hydrolase superfamily (**Figure 4.10**). This lipase possesses the motif GDSL at the active site, located at the 60-75 nucleotides region of gene (**Figure 4.11**) and possessed the oxyanion hole coding codon towards the 390 nucleotide position of the gene. The SGNH-hydrolase superfamily is a superfamily of a diverse family of lipases and esterases. The members of this superfamily fold in different manner from the enzymes of the alpha/beta hydrolase family. The members of this superfamily are very unique among all known hydrolases because their active site lacks the carboxylic acid but still resembles the Ser-His-As triad from other serine hydrolases (Lenfant et al., 2012).

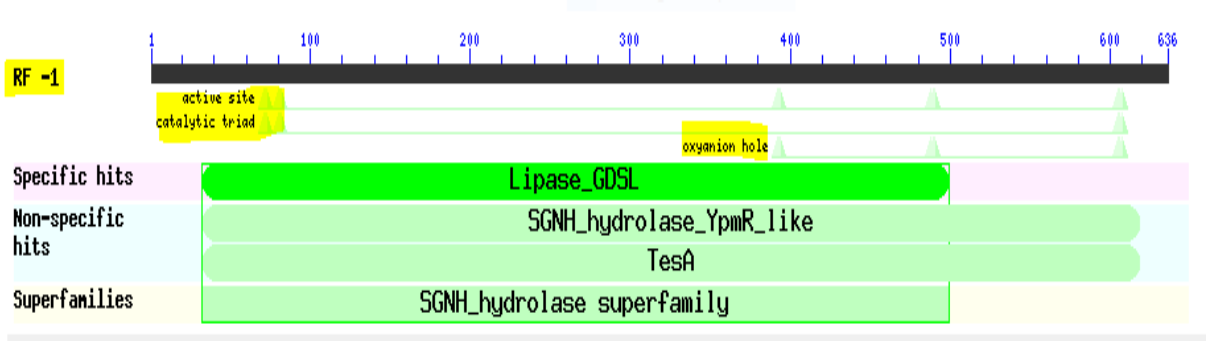

Figure 4.10 The central domain scan of Lip-VUT3 which shows that it belongs to a lipase GDSL family and SGNH-hydrolase superfamily. The highlighted yellow zones indicate the active site, catalytic triad and oxyanion hole

The amino acids sequences obtained had 32.14% similarity with the amino acids sequences of Chain A lipase of 234 amino acids sourced from *Streptomyces rimosus.* This was considered the best hit (**Appendix B2**). Additionally, the amino acids sequences were 30.95% identical to Chain A, phospholipase A1 of 236 amino acid produced by *Streptomyces albidoflavus* NA 297 and 26.97% similar to Chain A, acetyl xylan esterase of 219 a.a sourced from *Geobacillus stearothermophilus*.

Chain A, Crystal Structure Of Extracelular Lipase From Streptomyces Rimosus At 1.7a Resolution
Sequence ID: 5MAL_A Length: 234 Number of Matches: 1
▶ See 1 more title(s)

Range 1: 4 to 83 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
32.3 bits(72)	0.42	Compositional matrix adjust.	27/84(32%)	38/84(45%)	9/84(10%)	-1

```
Query  642  YTALGDSLIVGVGAGLFE--PGFVQRYKRKMEEDLNEEVSLIVF---AKSGLETSEILAM  478
            Y ALGDS + GVGAG ++   G  +R  +          +   F   A SG  T ++LA
Sbjct  4    YVALGDSYSSGVGAGSYDSSSGSCKRSTKSYPALWAASHTGTRFNFTACSGARTGDVLAK  63

Query  477  LNEPFIMEQVKKADVITITGCGND  406
               P         D+++IT  GND
Sbjct  64   QLTPVN----SGTDLVSITIGGND  83
```

Figure 4.11 The motif of the GSDL-lipase of the *in-silico* translated Lip-VUT3 ORF.

For the fosmid DNA of pCC2FOS-Lip-VUT5 clone, 465 contigs of average size of 394 bp (**Appendix B1**) were retrieved. The size of the sequenced DNA was of 18.3 kb (**Appendix B1**) from which one Open Reading Frame (ORF) of *Lipase* gene having 745 bp in size was considered for further studies. Using SnapGene software, the ORF Lip-VUT3 was translated *in-silico* and predicted a polypeptide of 248 amino acids with an estimated molecular weight of 28.4 kDa. Alignment using BLASTp was performed against the protein data bank (PDB).

The BLASTp detected the putative conserved domains to be similar to the esterase/lipase from the hydrolase 4 family and the alpha/beta hydrolase (abhydrolase) superfamily (**Figure 4.12)**.

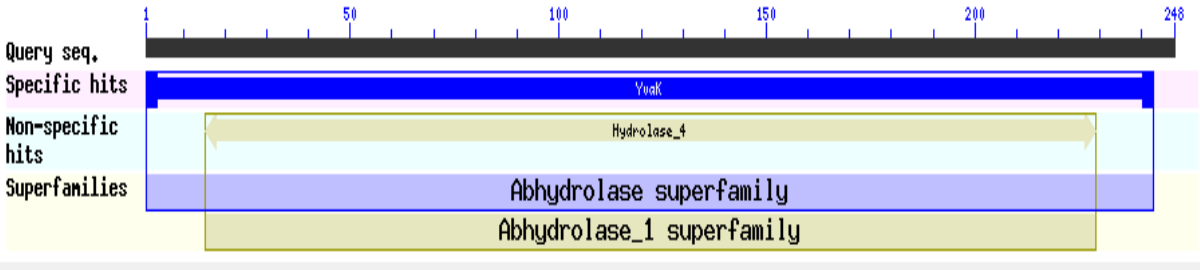

Figure 4.12 The central domain scan of Lip-VUT5 which shows that it belongs to the hydrolase 4 family, alpha/hydrolase superfamily.

The members of this superfamily possess the catalytic apparatus of three residues (catalytic triad): a Serine from the pentapeptide motif (GXSXG), a Glutamate or Aspartate and a

Histidine from the oxyanion hole. They are reputed for possessing the nucleophilic elbow which is crucial during the catalysis of the lipolytic reaction. The nucleophilic elbow attacks the carbonyl carbon atom of the substrate. The putative identified alpha/beta hydrolase (esterase/lipase) possessed a pentapeptide motif of GLSLG, the HG oxyanion hole and the aspartate to form the catalytic triad of the active site (**Figure 4.13**) (Alvarez et al., 2014).

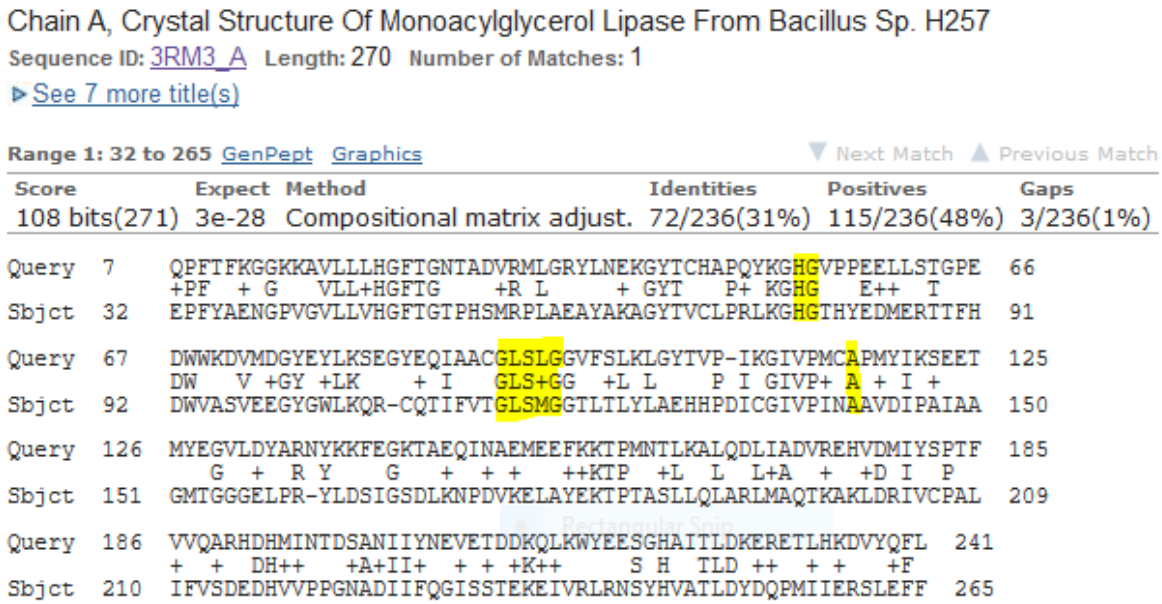

Figure 4.13 The *in-silico* translated Lip-VUT5 displaying the motif of the alpha/beta hydrolase from multiple species of *Bacillus*, with the HG oxyanion hole and the Aspartic acid as member of the catalytic triad: Ser 93, His 53 and Asp 116.

The alignment revealed a 32% similarity with the amino acids sequences of thermostable monoacylglycerol lipase of 256 amino acids sourced from *Geobacillus.* This was considered the best hit (**Appendix B2**). Additionally, the amino acid sequences were 30.51% identical to thermostable monoacylglycerol lipase of 250 amino acid produced by *Bacillus*, 30.51% identical to thermostable monoacylglycerol lipase of 270 amno acids produced by *Bacillus* sp-H257, 29.15% similar to Esterase D of 251 amino acid from *Lactobacillus rhamnosus* and 26.56% identical to lips, lipolytic enzyme of 281 amino acid from unidentified organisms.

The evolutionary relationships among the respective aligned sequences was established using clustalW (**Figure 4.14**) and it revealed that the lipases from the different microorganisms were not closely related.

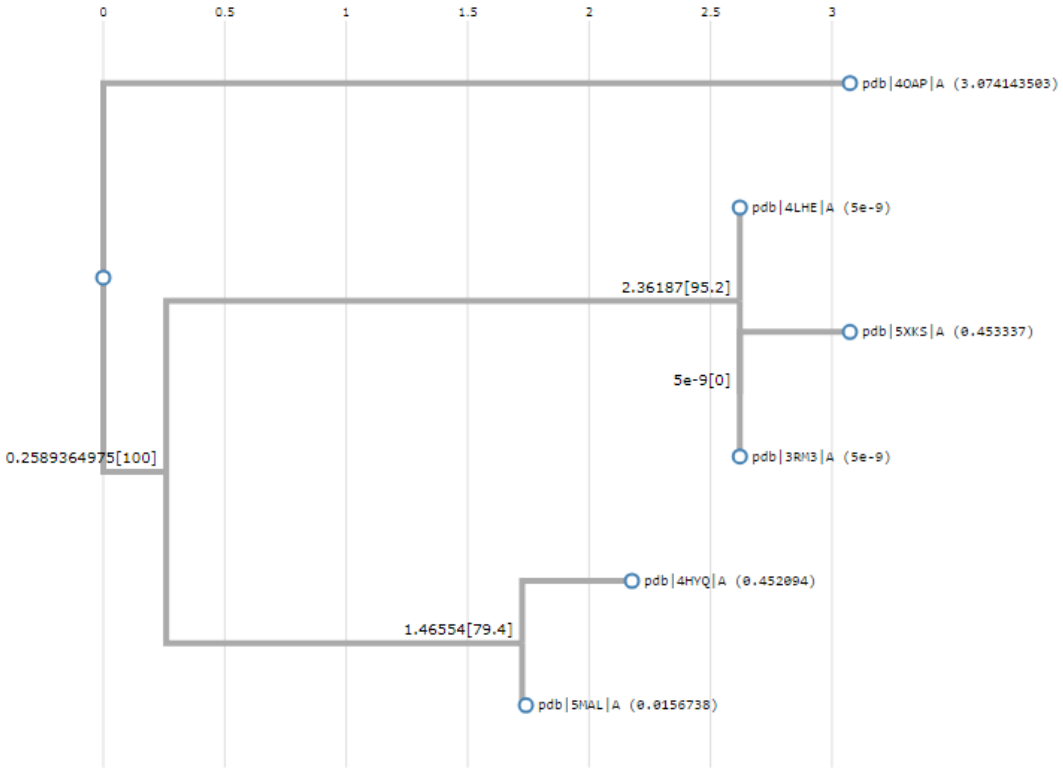

Figure 4.14 Phylogenetic tree from the alignment of different amino acid sequences of lipase isoforms constructed using ClustalW rooted phylogenetic tree UPGMA (Unweighted Pair Group Method with Arithmetic Mean**)** method. The Phylogenetic tree indicates the difference between the two ORF selected for further studies. **pdb 4HYQ_A**: Chain A, phospholipase A1 from *Streptomyces albidoflavus* strain NA 297; **pdb 5MAL_A**: Chain A, lipase from *Streptomyces rimosus*; **pdb 4LHE_A**: thermostable monoacylglycerol lipase from *Bacillus* ; **pdb 3RM3_A**: thermostable monoacylglycerol lipase from *Bacillus* sp-H-257; **pdb 5XKS_A**: thermostable monoacylglycerol lipase from *Geobacillus*; **pdb 4OAP_A**: Chain A, acetyl xylan esterase from *Geobacillus stearothermophilus*.

The data shows that all the selected lipase are different in size. The Chain A, phospholipase A1 from *Streptomyces albidoflavus* strain NA 297 has 236 amino acids, Chain A, lipase from *Streptomyces rimosus* has 234 amino acids; thermostable monoacylglycerol lipase from *Bacillus* has 250 amino acids ; thermostable monoacylglycerol lipase from *Bacillus* sp-H-257 has 270 amino acids; thermostable monoacylglycerol lipase from *Geobacillus* has 256 amino

acids and Chain A, acetyl xylan esterase from *Geobacillus stearothermophilus* has 219 amino acids..

Seeing the limitations of *in-silico* analysis to the prediction level, functional analysis was required to determine the actual functionalities and characteristics of Lip-VUT3 and Lip-VUT5. Functional analysis would either confirm or reject the prediction made by the *in-silico* analysis.

4.4 Conclusion

The success of the metagenomic library construction was confirmed by quality control experiment whereby the fosmid construct was reisolated and digested with appropriate restriction endonucleases and clearly showing the presence of the > 30 kb insert as opposed to the size of the vector, which was only ca. 8 kb. Construction and screening of fosmid libraries led to the discovery of isoforms of lipases obtained from an enormous genetic diversity of unculturable rumen microorganisms. Tributyrin revealed itself as a substrate of choice to screen lipase producing clones because the *in-silico* analysis of sequences obtained revealed the presence of a true lipase from the GDSL superfamily. The recovered crude lipases had properties like high activity over a range of alkaline pH and high temperature. The temperature and pH profiles of the selected clones demonstrated that the rumen is diverse in isoforms of lipases. The depth of sequencing helped in recovering full ORFs. However, *in-silico* analysis did not completely reveal the identity of the obtained lipases. Additionally, The low percentage in similarity gave an indication that the lipases sourced from the metagenome could be novel. Hence, the ORFs were cloned and expressed for further characterisation, as discussed in the next chapter, to confirm or refute the hypothesis made.

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CHAPTER 5:

Protein Purification, Characterisation and Kinetic Studies

Abstract

Two lipase genes were ligated to pET 30a(+) expression vector and cloned into *E.coli* BL21 (DE3) pLysS for expression studies. The recombinant lipases Lip-VUT3 and Lip-VUT5 lipases were extracted from the cell lysates and were found to be soluble. The recombinant proteins were further purified using IMAC semi-automated AKTA Protein purification system. The molecular mass of Lip-VUT3 and Lip-VUT5 lipases were estimated to be about 24.8 and 28.4 kDa respectively. The purified Lip-VUT3 and Lip-VUT5 could be considered as thermostable lipase because their maximal activity was observed at 60 ^{o}C and 70 ^{o}C, respectively. However, Lip-VUT3 was considered to prefer slightly acidic condition with the maximum activity measured at pH 6 while Lip-VUT5 was classified as an alkaline one with the maximal activity measured at pH 10. Lip-VUT3 and Lip-VUT5 lipases showed tolerance towards a wide concentration (50%-100%) of methanol. Accordingly, the kinetic study showed that Lip-VUT3 had a K_m value of 0.267 mM while Lip-VUT5 had a K_m value of 0.556 mM showing that Lip-VUT3 has higher substrate affinity as compared to the Lip-VUT5.. Therefore, it could be concluded that this study managed to isolate isoforms of lipases using metagenomic techniques.

Keywords: Proteins, Lipase, Protein expression, Protein Purification, Kinetic studies, V_{max}

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5.1 Introduction

Proteins are the building blocks of life because they play a major role in the functionality of cells (Demain and Vaishnav, 2009). They trigger important functions such as the metabolism of foods and other components in all living organisms. Proteins are large peptide molecules and are constituted of hormones, enzymes, etc. Proteins are not only useful inside the cells of all living organisms but they also play a vital role in industries. Proteins favoured the development of sectors such as nutrition, pharmaceuticals, medicine, chemistry, textile, detergent, pulp and paper, etc. Therefore, the need for more proteins led to the production of bulk proteins for commercialisation. This could be possible thanks to the development and applications of recombinant technology and protein engineering (Demain and Vaishnav, 2009).

The development of recombinant technology and protein engineering is entirely based on the principles of the central dogma which basically summarises the two steps involved in the production of proteins (Sodoyer and Legastelois, 2019). Recombinant DNA technology makes use of the fusion of two DNA molecules from different sources to produce proteins with desired properties. Such proteins are called recombinant proteins (Sodoyer and Legastelois, 2019).

One of the well-known recombinant proteins is enzymes. Enzymes are biocatalysts that accelerate the rate of chemical reactions. In the early ages around the 1970s, enzymes were only derived from plant and animals. They were obtained with great difficulties and they hampered the growth of the industrial world. At a later stage, the development of microbial enzymes brought the enzyme industry to flourish. Recombinant technology boosted the production and use of recombinant microbial enzymes. An example is the production of thermophilic lipase, used in the detergent and cosmetic industries by *Aspergillus oryzae*. In 1993, 50% of industrial enzymes were made up of recombinant enzymes (Hodgson, 1994). In short, it can be said that recombinant DNA technology really helped in the production of recombinant proteins with desired traits imposed by the users and it helped in bypassing the search for native enzymes with natural desired properties.

The production of recombinant proteins relies on (i) extraction of a gene of interest, (Maina et al. 1988) (ii) ligation to selected expression vector system, (Maina et al. 1988) (iii)

transformation of expression strain and (iv) purification of expressed product. The extraction of a gene of interest, once dependent on PCR and restriction endonuclease cleavage, is now being replaced by gene synthesis and supply of constructs ready for expression and purification (Quax et al., 2015) . During *in-silico* design of constructs, some fusion tags are added to the gene of interest to assist in the solubility, detection and purification of the protein of interest. Some of the more common fusion partners include a histidine tag, maltose-binding protein (MBP), thioredoxin, glutathione-S-transferase (GST) and pectate lyase (pelB) (Smith and Johnson, 1988; Maina et al., 1988; LaVallie and McCoy, 1995; Luo et al., 1996; Jonasson et al., 2002).

Among the heterologous host strains used for expression, *E. coli* is the mostly used and the earliest host for the production of recombinant proteins because they are easily cultured, produce high yields of soluble products, grow rapidly and are easily genetically manipulated (Terpe, 2006). However, the products obtained from the expression in *E. coli* are not always sufficient and do not always display the desired function due to improper folding. Nevertheless, *E. coli* expression systems remains the dominant and best expression system because it facilitates maximum protein recovery, soluble protein production and easy protein purification as compared to other expression systems (Jonasson et al., 2002).

Upon expression of recombinant proteins, the test of expression is done by running the proteins on SDS-PAGE or using western blot techniques. Then follows the purification step in order to study the functionality of the recombinant proteins obtained. The purification of recombinant proteins relies on the chromatography principles. Chromatography purifies recombinant proteins by separating proteins based on their electrical charge, size, polarity and structures. The most used of all the purification methods is affinity chromatography in which the proteins have affinity towards the matrix of the column. The proteins have a special structure containing the tag covalently bonded to the histidine. The histidine has affinity towards the nickel of the agarose dead (matrix). Immobilized Metal Affinity Chromatography (IMAC) is either automated or semi-automated. One of the automated IMAC is AKTA that has facilitated the purification process of recombinant proteins (Konczal and Gray, 2017).

The purified recombinant proteins can be further characterised based on pH, temperature, and solvent-tolerance, and have their kinetic parameters studied. Therefore, the aim of this chapter was to express the *in-silico* designed constructs from the previously identified putative *lipase* genes inside *E.coli* BL21 (DE3), purify and characterize the recombinant lipases.

5.2 Materials and Methods

5.2.1 *In-silico* design of constructs

The two lipase gene sequences obtained from the sequencing of the insert of the two selected clones were retrieved and had their codon optimised. The resulted genes were used to build the constructs with the expression vector pET 30a(+) *in-silico* using SnapGene. The designed constructs were sent to GenScript for chemical synthesis to avoid challenges faced during cloning of genes retrieved using PCR.

5.2.2 Transformation

The synthesized constructs pET 30a(+)-Lip-VUT3 and pET 30a(+)-Lip-VUT5 were delivered in lyophilized form in 2 ml sealed tubes and were used to transform *E.coli* BL21 pLysS. The total amount of the respective recombinant plasmids (constructs) was of 4 µg. The recombinant plasmids were diluted in 100 µl of sterile DNase, RNase free distilled water to yield a stock solution of 40 ng/µl. Since the transformation protocol required 1-10 ng of plasmid, it was decided to prepare a working solution of 10 ng/µl for each respective construct. After obtaining the suitable concentration of plasmids (10 ng/µl), the transformation was performed by first preparing the 2 ml centrifuge tubes in which transformation would occur. The sterile 2 ml centrifuge tubes were prepared by being chilled on ice for few seconds. While being chilled, the electro-competent pLysS cells were also thawed, then gently flicked 1-2 times to be evenly re-suspended. Twenty µl aliquots of cells were pipetted into the pre-chilled tubes and 1 µl of the stock solution of constructs was directly added to the cells. The tubes were incubated on ice for 5 minutes and immediately placed in a 42 °C water bath for 30 seconds without shaking.

Immediately after the incubation in the water bath, the tubes were replaced on ice for 2 more minutes. Eighty µl of SOC medium at room temperature was added to the tubes thereafter, kept on ice shortly then shaken at 37 ºC for 30 minutes at 200 rpm. After 30 minutes of incubation, 70 µl of the transformed cells were plated on SOC agar supplemented with Kanamycin (50 µg/ml) while the remaining 80 µl of the transformed cells were plated on LB agar plates supplemented with kanamycin (50 µg/ml). The plates were incubated for approximately 18 hours at 37 ºC. The plates were taken out of the incubator and the colonies were counted on both LB and SOC agar plates. A master plate of approximately 10-20 clones was produced in both the SOC and LB plates. The SOC and LB plates were rinsed with sterile LB broth supplemented with 50 µg/ml kanamycin. Based on the volume obtained, a glycerol stock of transformed *E.coli* of 10-20% was then prepared from sterile 100% glycerol as indicated in the pET Novagen Manual (Novagen, I., 2002)

5.2.3 Confirmation of ligation reaction

From the master plate, 2 clones per gene of interest were sub-cultured in 50 ml LB broth supplemented with 50 µg/ml of kanamycin. The cultures were shaken overnight at 37 ºC at a speed of 180 rpm. After overnight incubation, the cultures were centrifuged and the cells were collected for plasmid extraction. The plasmid was extracted based on the protocol provided in the GeneJet Plasmid Miniprep Kit. The extracted plasmids were quantified using the Nanodrop One C to find the suitable amount for digestion reaction set up. Two restriction enzymes *EcoRI* and *Xbal* were used for digestion and the digested plasmids were loaded and run on 0.8% agarose gel for 45 minutes at 100 Volts.

5.2.4 Expression and purification

Ten microliters from the glycerol stock was inoculated into 100 ml of LB broth supplemented with 50 µg/ ml of kanamycin in a 500 ml Erlenmeyer flask. The culture was incubated at 37°C at a speed of 180 rpm until OD600 reached roughly 0.5 (this was after about 4 hours of incubation). A considerable volume of the culture was removed for the uninduced control. To

the remainder, IPTG was added to a final concentration of 1 mM and the incubation continued for 16 hours at 25 ºC.

5.2.4.1 Extraction and analysis of protein overexpression using SDS-PAGE

After the 16 hour incubation period, the cultures were taken out of the incubator and centrifuged at 4 ºC, 5000 x g for 30 minutes to precipitate all the recombinant bacterial cells. After centrifugation, the supernatant was discarded and the pellets were processed for protein extraction. Before starting with protein extraction, the pellets were weighed and the mass was recorded to obtain the volume of the B-PER reagent to be used. Four ml of B-PER reagent was added per gram of cells pellet. The suspension was pipetted up and down until it became homogeneous. The suspension was incubated for 10-15 minutes at room temperature and centrifuged at 15000 x g for 90-120 minutes to separate the soluble proteins from insoluble proteins. The supernatant was aliquoted in a fresh sterile centrifuge tube and labelled as soluble proteins while the pellet was stored for further extraction of insoluble proteins. The soluble proteins were kept on ice during further processing. Ten µl of soluble proteins was mixed 10 µl of 2X Laemmli SDS Protein Sample buffer in order to be boiled for 5 minutes. After boiling the samples, casting the stacking and resolving gels, 10 µl of the boiled mixture was loaded on the SDS-PAGE gel. Electrophoresis was performed in 1× SDS-PAGE running buffer at 200 V for 45 minutes. Gels were stained with Coomassie Brilliant Blue staining solution and de-stained with de-staining solution.

5.2.4.2 Purification of overexpressed proteins

The HisTrap FF column (5ml) was equilibrated with five column volumes of binding buffer and 12 ml of the soluble crude fraction containing a targeted His-tagged protein was loaded. The unbound proteins were washed with ten column volumes of binding buffer followed by elution of the targeted protein with five column volumes of the elution buffer. The fractions of the recovered proteins were analysed using SDS-PAGE.

5.2.5 Characterisation and kinetic studies of recombinant proteins

The characterisation of the proteins was performed with both purified recombinant proteins provided from GenScript and un-purified proteins overexpressed in the laboratory.

For the purified recombinant lipases, the concentrations of the recombinant lipases were normalised for accuracy. Therefore, it was decided to work with approximately 100 ng/µl (~0.1 mg/ml) of recombinant lipases. The dilution of the enzymes was carried out using RNase and DNase deionised water (Lip-VUT3: 104.4 µg/ml and Lip-VUT5: 99.5 µg/ml). Ten µl of the respective diluted solution of lipases (~100 µg/ml) was used to study the effect of pH on the activity of the recombinant lipases at the ambient temperature of 32ºC, the effect of temperature on the activity of the recombinant lipases at the optimum pH, the affinity of the recombinant lipases for olive oil at different concentrations, the tolerance of the recombinant lipases for different concentrations of methanol, finally the small scale production of biodiesel using cooked vegetable oil and animal fat as raw materials or substrates for the recombinant lipase.

For the un-purified proteins, 10 µl of lipases were used to study the same effect described above. The experiment was done in triplicate for all and the negative control was treated the same way at the exception of the addition of the lipases.

5.2.5.1 Effect of pH on lipase activity

The effect of pH on the purified enzymes was determined using phosphate buffer at pH ranging from 4 to 12 at ambient temperature of 32ºC. Hundred and ninety ml of 50 mM PBS buffer (pH 4-12) containing sterilised olive oil emulsion as a substrate at a final concentration of 1%, was aliquoted in a 2 ml sterile tube. The substrate was stabilised at 32ºC for 5 minutes before proceeding with the actual reaction. Then, 10 µl of appropriately diluted solutions enzymes was added to the stabilised substrate for 20 minutes of reaction. After 20 minutes, 200 µl 6N HCl was added to stop the reaction. One ml of benzene was added to dilute the oleic acid products. The mixture was shaken for 10 min at 210 rpm. After shaking, 1 ml of the supernatant was transferred to the new tube already containing 200 µl of the cupric acetate-pyridine. The mixture was shaken for an additional 10 min at 210 rpm. The absorbance reading of the oleic

acid produced was taken at the wavelength of 715 nm using a spectrophotometer. The reaction was performed in triplicate. One unit of the enzyme activity was defined as the amount of lipase required to release 1 μmol of oleic acid/min. The control reaction mixture (excluding the enzyme) was included to correct for auto hydrolysis of the substrate.

5.2.5.2 Effect of temperature on lipase activity

The effect of temperature on the purified enzymes was determined using phosphate buffer at the optimum pH identified from the results obtained from the effect of pH. The reactions were carried at different temperatures ranging from 30-95ºC. The protocol of **5.2.5.1** was followed.

5.2.5.3 Kinetic studies (Substrate affinity of lipases)

After identifying the optimum pH and temperature, different concentration of olive oil emulsion substrate were prepared to study the substrate affinity of the different recombinant lipases. One %, 5%, 10%, 15% and 20% of the substrate were used and the reactions were carried out at the identified optimum pH and temperatures. The reaction were carried out as previously described in section **5.2.4.1**. The K_m and V_{max} were obtained using GraphPad Prism for Michaelis Menten kinetics.

5.2.5.4 Methanol tolerance of recombinant lipases

Different concentrations of methanol were used from 50-100%. The methanol was diluted with the PBS buffer at optimum pH corresponding to respective recombinant protein optimum pH. The reactions were also carried out as previously described in setion **5.2.4.1** at optimum temperature.

5.3. RESULTS AND DISCUSSIONS

5.3.1 *In-silico* designed constructs

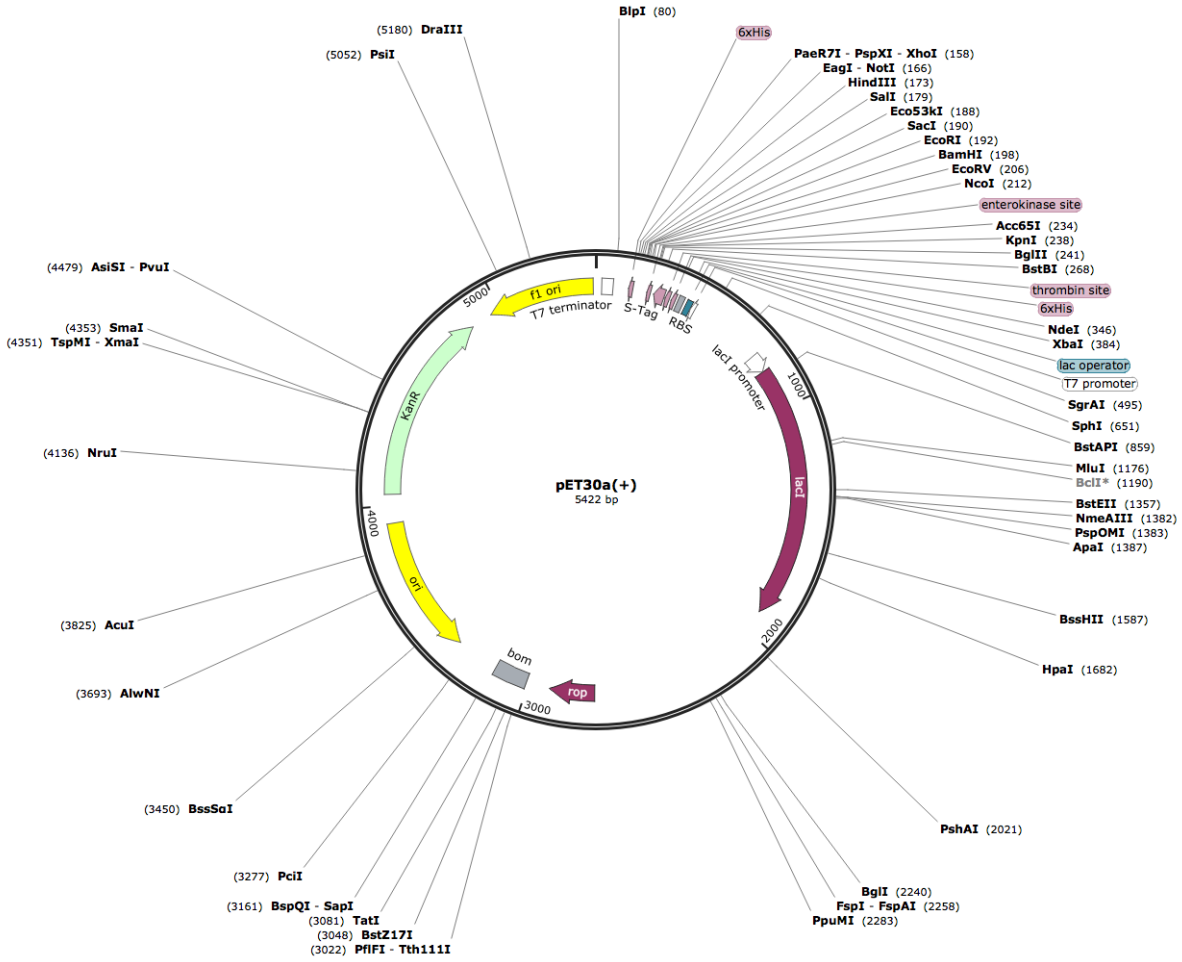

Figure 5.1 The map of pET 30a (+) with all the features displayed.

Due to the importance of high level production of recombinant protein in enzymatic studies, the genes were cloned in pET30a (+) expression vector (**Figure 5.1**). The pET is the most powerful system that has so far been developed for the cloning and expression of the recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals where expression is induced by providing a source of T7 RNA polymerase in the host cells. The T7 RNA polymerase is so active that when fully induced, almost all of the cell's resources are converted to the target gene expression. This vector encodes the N-terminal and C-terminal HisTag sequences that allow for easy purification, quantification and detection of target proteins.

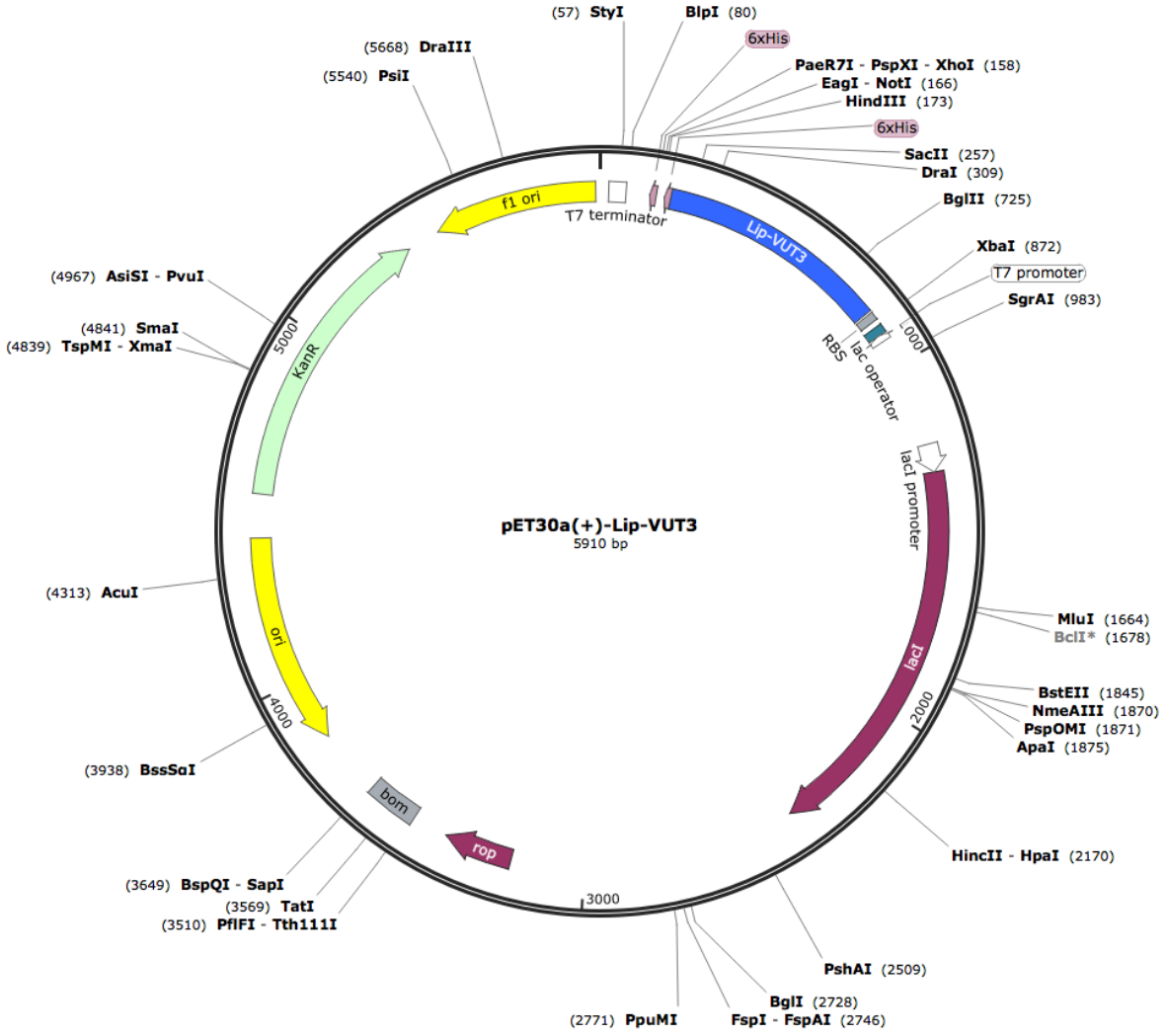

Figure 5.2 Map of *In-silico* pET 30a(+)-Lip-VUT3 construct using SnapGene software. The cloning strategy is restriction-digestion with *HindIII* & *NdeI* and fill up of the overhang with dNTPs.

The cloning strategy relied on linearizing the pET 30a(+) by restriction digestion at the *HindIII* and *NdeI* restriction sites. The genes of interest were then inserted and ligated to fill in the gap (**Figure 5.2** and **5.3**). The plasmid constructs pET30a(+)-Lip-VUT3 and pET30a(+)-Lip-VUT5 were transferred into *E. coli* BL21 DE3 containing T7 RNA polymerase, for expression studies. Two media were used for plating the transformed *E.coli*. The SOC agar plates had a high number of transformed *E.coli* as compared to LB agar plates (Appendix 2). The result simply confirms that SOC medium promotes high transformation efficiency. Some clones were selected to test the success of the cloning step. The transformed *E.coli* had the recombinant plasmids digested with restriction enzymes *XbaI* and *XhoI* to confirm the success of ligation of

the genes of interest to the expression vector. The restriction digestion was analysed on 0.8% agarose gel (**Figure 5.4**).

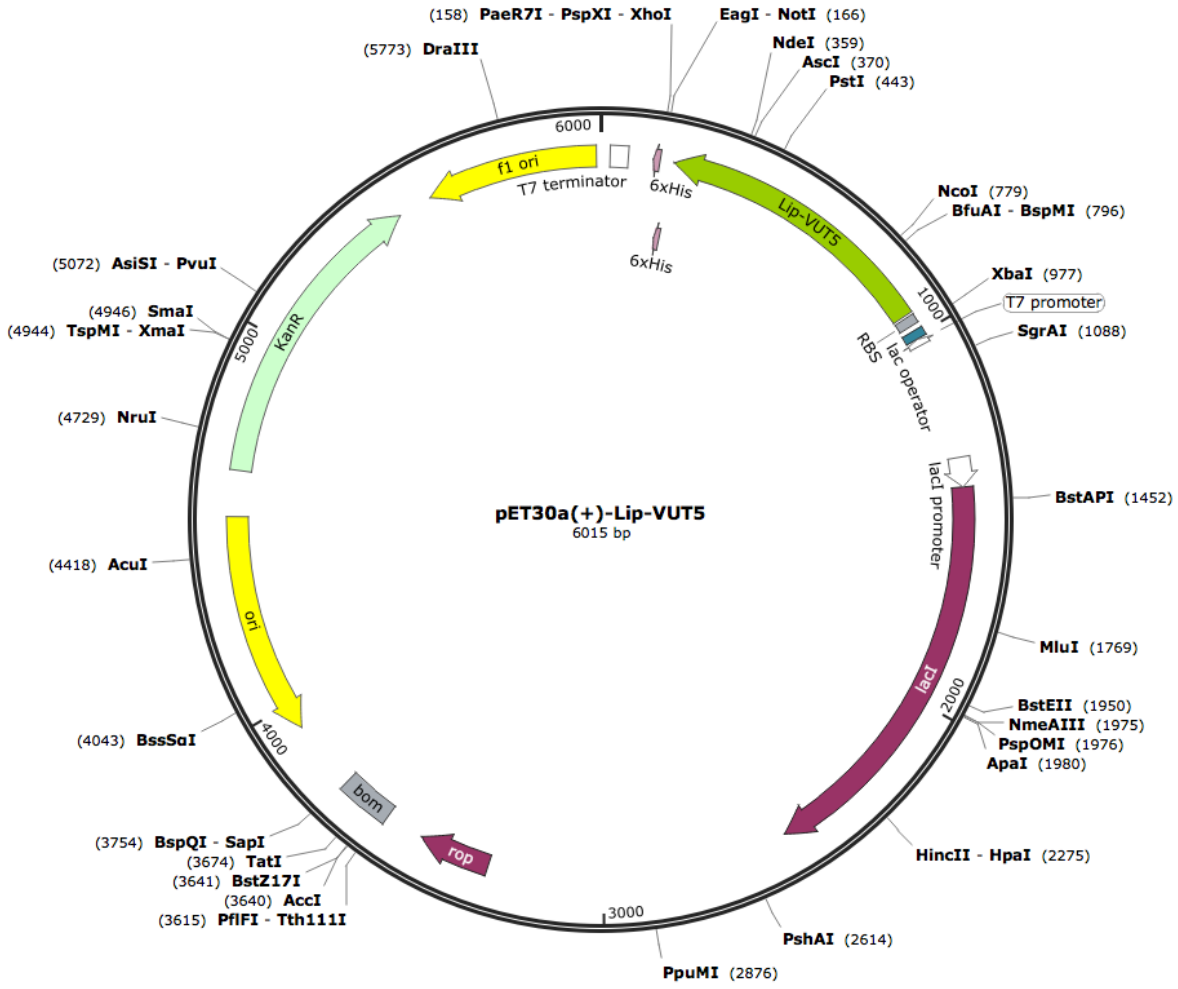

Figure 5.3 Map of *In-silico* pET 30a(+)-Lip-VUT5 construct using SnapGene software. The cloning strategy is restriction-digestion with *HinIII* & *NdeI* and fill up of the overhang with dNTPs.

The appearance of bands on the gel shows that the genes were sucessfully ligated (**Figure 5.4**). The two lipase genes Lip-VUT3 and Lip-VUT5 were expressed after using a concentration of 1mM of IPTG as inducer.

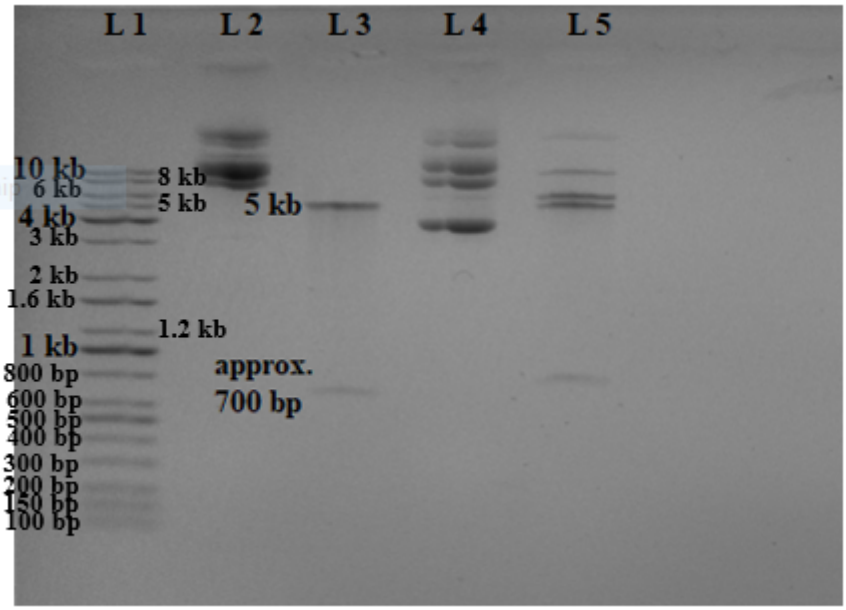

Figure 5.4 Agarose 0.8% gel analysis of restriction digestion of recombinant plasmids extracted from transformed *E.coli* to confirm the presence of the gene of interest. L1: 1kb DNA Ladder; L2: Undigested plasmid from transformed *E.coli*-pET 30a(+)-Lip-VUT3; L3: *XbaI* and *XhoI* digested plasmid from transformed *E.coli*-pET 30a(+)-Lip-VUT3; L4: Undigested plasmid from transformed *E.coli*-pET 30a(+)-Lip-VUT5; L5: *XbaI* and *XhoI* digested plasmid from transformed *E.coli*-pET 30 a (+)-Lip-VUT5.

5.3.2 Overexpressed recombinant lipase analysis by SDS-PAGE

The Lip-VUT3 and Lip-VUT5 recombinant proteins were successfully expressed after induction with 1 mM IPTG. Expected Lip-VUT3 and Lip-VUT5 protein size of 24.8 kDa and 28.4 kDa was detected after induction of the culture with IPTG and most of it was found to be soluble because no sonication was required to extract the proteins using B-Per regent.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the induced and un-induced bacterial lysates and the purified Lip-VUT3 and Lip-VUT5 proteins indicated the expected molecular mass of 24.8 and 28.4 kDa (**Figure 5.5**). The results demonstrated that the Lip-VUT3 and Lip-VUT5 proteins were expressed in BL21 (DE3) cells

correctly. The Lip-VUT3 and Lip-VUT5 were overexpressed for a period of 16 hours at temperature at 25 °C. These parameters were revealed to be the optimum ones for overexpression of these recombinant proteins because the proteins were soluble and were not stored in inclusion bodies. The proteins were purified by affinity to nickel ions using AKTA system. However, the eluted proteins were too dilute and had to be concentrated. But since there was a shortage of protein concentrator columns in the laboratory, it was decided to perform the characterisation with the purified enzymes obtained from GenScript. The purified recombinant Lip-VUT3 and Lip-VUT5 were 99% pure as confirmed on SDS-PAGE analysis (**Figure 5.5**). The quantity of the purified Lip-VUT3 and Lip-VUT5 protein was estimated about 2.76 mg and 570 µg in total. The SDS-PAGE also demonstrates that the 6× His-tag that was added to the C-terminal facilitated the purification process and was validated by the presence of a single band corresponding to the proteins of respective size.

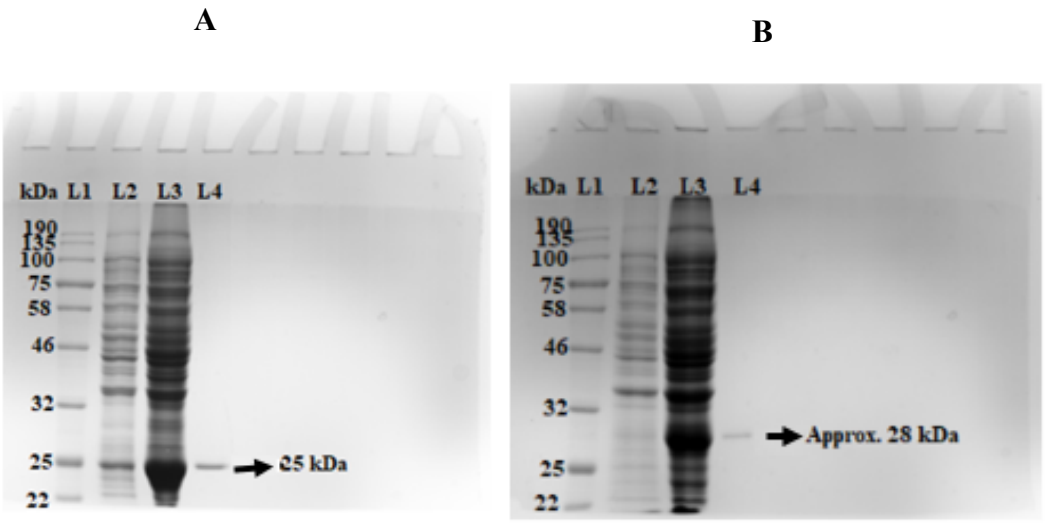

Figure 5.5 SDS-PAGE analysis of Induced and purified recombinant Lip-VUT3 and Lip-VUT5 lipases with protein bands corresponding to 25 kDa for Lip-VUT3 and 28 kDa for Lip-VUT5. (A) Lane 1: protein molecular weight marker; Lane 2: Uninduced Lip-VUT3; Lane 3: Induced Lip-VUT3 with 1 mM IPTG; Lane 4: purified Lip-VUT3. (B) Lane 1: protein molecular weight marker; Lane 2: Uninduced Lip-VUT5; Lane 3: Induced Lip-VUT5 with 1 mM IPTG; Lane 4: purified Lip-VUT5.

5.3.3 Characterisation of recombinant proteins

5.3.3.1 pH and Temperature profiles of Recombinant Lipases

The effect of pH and temperature on Lip-VUT3 and Lip-VUT5 activities was determined at various pH and temperatures, respectively (**Figure 5.6** and **5.7**) below. The pH and temperature profiles were studied against purified and un-purified recombinant lipases just to display the effectiveness of the purification process. The activities of the two purified and un-purified lipases (**Appendix C2 and C3**) were presented as relative activity with the highest activity taken as 100%. The effect of pH on lipase activity was assayed at 1 pH unit increments and of temperature was tested at 10 °C increments. Activity was assayed for a 20 minutes period.

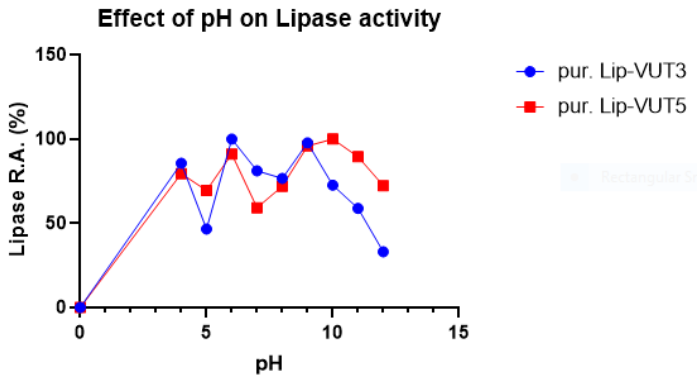

Figure 5.6: The effect of pH on purified Lip-VUT3 and Lip-VUT5 determined by measuring enzyme activity using olive oil as a substrate. The highest activity obtained was defined as 100%.

The highest activity of the purified Lip-VUT3 was at pH 6.0 while the un-purified had the highest activity at pH 5.0 (**Appendix C2**) and the purified and un-purified lipases showed more than 60% of maximum activity at the pH range of 4.0-11.0. Purified Lip-VUT5 exhibited the highest activity at pH 10.0 while the un-purified (**Appendix C2**) had the highest activity at pH 9.0. The purified and un-purified Lip-VUT3 lipase showed more than 60% of maximum activity at the pH range of 4.0-10.0. Therefore, Lip-VUT3 exhibited maximal activity in slightly acidic to neutral pH (5.0-9.0), retaining 100% activity at pH 6.0 while the activity

dropped at pH10. The purified and un-purified Lip-VUT5 lipases showed more than 60% of maximum activity at the pH range of 6.0-12.0 just like the lipase isolated from *Serratia* sp.W3 strain. The SmL was found to be stable at a large scale of pH from pH 5-12 (Eddehech et al., 2019). Therefore, Lip-VUT5 exhibited maximal activity in slightly alkaline pH (9.0-11.0), retaining 100% activity at pH 9.0 while the activity remained constant showing that Lip-VUT5 lipase activity is not really affected by the pH. Based on the data obtained above, it could be observed that there was not much difference in activity between the purified and un-purified recombinant lipases in terms of pH. It could be concluded that the other host proteins did not act as inhibitors of the recombinant lipase

The highest activity for Lip-VUT3 was at 40 ºC for the un-purified (**Appendix C3**) while being at 60 °C for the purified. The highest activity for Lip-VUT5 was at 60 °C for the un-purified (**Appendix C3**) one while being at 70 ºC for the purified one. There was a remarkable regression in activity after 40 ºC for un-purified Lip-VUT3 (**Appendix C**). The activity dropped from 100% to less than 50% whereas for the purified Lip-VUT3 (**Figure 5.7**), the activity remained stable throughout the rest of the temperatures. The results can can lead to the hypothesis that purification improves the activity of the enzyme. Lip-VUT5, however had the same activity pattern for the purified and un-purified. The purification also led to an improvement in the temperature profile of the lipase. The highest activity for the un-purified Lip-VUT5 was at 60 ºC while the highest activity of the purified Lip-VUT5 was at 70 ºC. Therefore, Lip-VUT3 and Lip-VUT5 are said to be thermophilic lipases because their maximal activities take place at high temperatures.

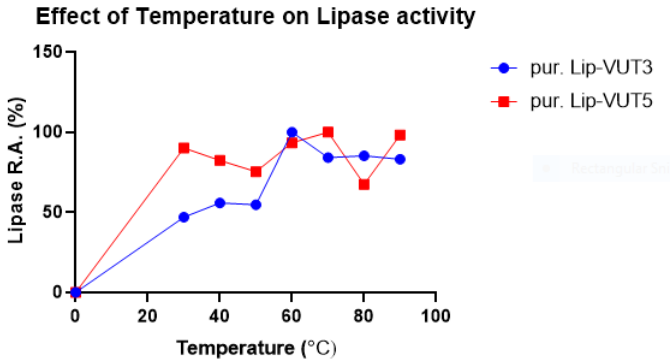

Figure 5.7: The effect of Temperature on purified Lip-VUT3 (A) and Lip-VUT5, determined by measuring enzyme activity using olive oil as a substrate. The highest activity obtained was defined as 100%.

The majority of *Bacillus* lipases have their maximal activity at alkaline pH and temperature 45-50 ºC (Guncheva and Zhiryakova, 2011) . However in this study, Lip-VUT3 and Lip-VUT5 had been reported to have maximum activity at pH 6 and 10, and temperature 60 and 70 ºC, respectively. Temperature, pH and solvent tolerance parameters of an enzyme are features looked at for selection of enzymes for biotechnological applications. Because High or low pH and temperatures can negatively affect the ions or the conformation in the structures of enzymes resulting in activity degeneration (Faiz et al., 2007) .

5.3.3.2 Methanol tolerance

Effect of methanol (**Figure 5.8**) at various concentrations towards lipase activity was determined using purified Lip-VUT3 and Lip-VUT5 at their respective optimum pH and temperature (pH 6.0. and 10.0, respectively and 60 ºC and 70 ºC, respectively).

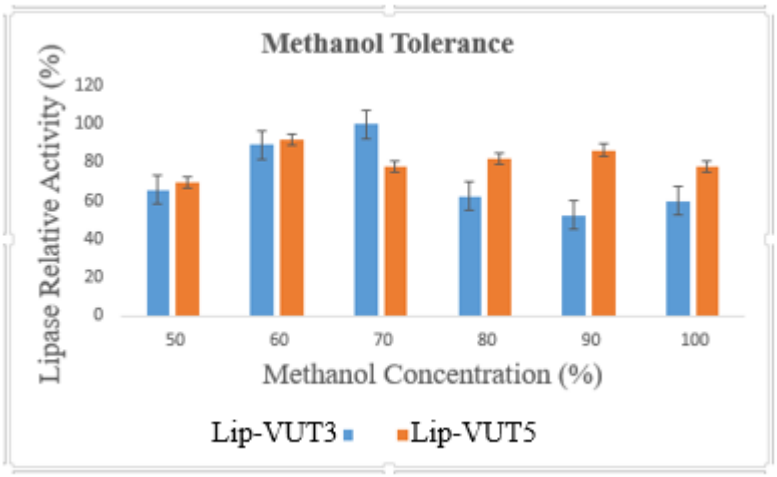

Figure 5.8 Methanol tolerance of Lip-VUT3 and Lip-VUT5 recombinant lipases. Activity of lipase in various concentration of methanol ranging from 50 to 100%.

The assay showed that Lip-VUT3 and Lip-VUT5 lipases were capable of maintaining 50% of their activity in various methanol concentrations (50-100%) after incubation for 20 minutes at 60 °C. The enzyme showed more than 50% of relative activity even at 100% methanol. Lip-VUT3 had an increase of 40% relative activity when exposed to different methanol

concentration (50%, 60% and 70%) and after 70%, the activity started to decline. On the other hand, Lip-VUT5 showcased that it could retain its activity throughout as compared to Lip-VUT5. Hence, the two recombinant lipases Lip-VUT3 and Lip-VUT5 showed more than 50% tolerance towards methanol. Contrary to the results obtained, most of microbial lipases were identified as non-tolerant towards hydrophilic solvents because the hydrophilic solvents seriously reduced their stability (Zhao et al., 2008; Doukyu and Ogino, 2010). But, the present study showed otherwise because the presence of methanol did not hinder the lipase to perform their hydrolysis function. Some other previous studies (Gaur et al., 2008; Zhao et al., 2008) had similar outcomes with the current one. Lipases isolated from *Psedomonas taiwanensis* could tolerate hydrophilic solvents because the stability was not altered. Additionally, the lipase isolated from Pseudomonas aeruginosa retained 97% of its activity after 24 h of incubation in methanol (Gaur et al., 2008; Zhao et al., 2008). Lipases displaying tolerance towards such solvents could be potential candidates for biodiesel production.

5.3.4 Kinetic studies of recombinant Lip-VUT3 and Lip-VUT5 lipases

The K_m value of Lip-VUT3 was of 0.287 mM while the K_m of Lip-VUT5 was of 0.556 mM (**Figure 5.9**). These results show that Lip-VUT3 lipase has almost double the affinity towards olive oil as a substrate then Lip-VUT5 lipase.

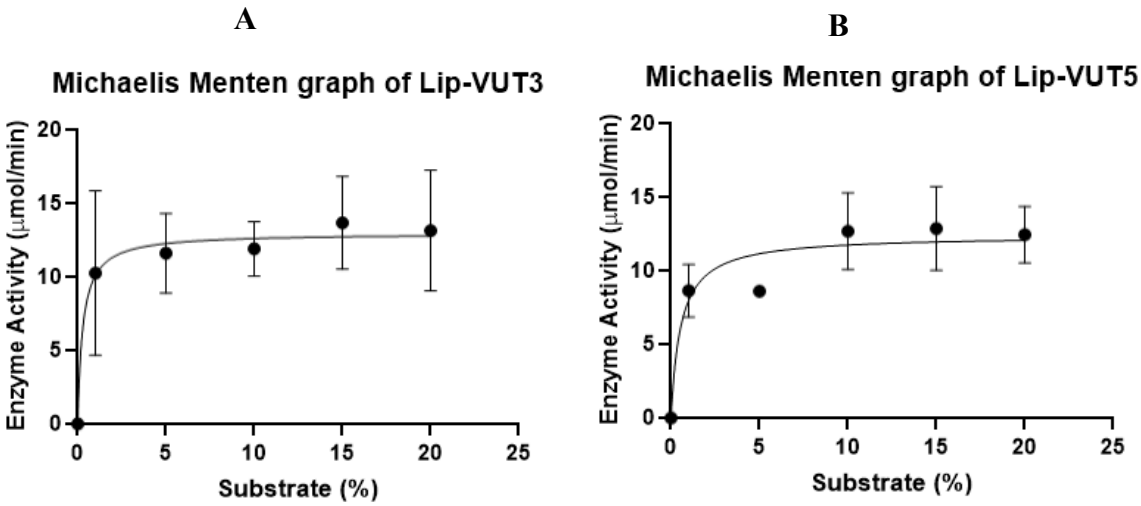

Figure 5.9: The kinetic parameters of recombinant lipases Lip-VUT3 and Lip-VUT5. (A) Michaelis-Menten Data graph curve of Lip-VUT3, (B) Michaelis-Menten Data graph curve of Lip-VUT5.

The K_m represents the concentration of substrates in mM used by the enzyme when the reaction reaches half of maximal velocity (V_{max}). Since a small K_m indicates that the enzyme has high affinity for the substrates, Lip-VUT3 lipase is said to have higher affinity for olive oil as compared to Lip-VUT5 lipase. Just by using up 0.287 mM of olive oil, the Lip-VUT3 lipase reached half of V_{max}. These results support the data obtained from the *in-silico* analysis which predicted Lip-VUT3 to be a true lipase of the GDSL lipase family. However, the functional analysis ruled out the prediction or the possibility that Lip-VUT5 could be an esterase. It confirmed the identity of Lip-VUT5 being a lipase simply because Lip-VUT5 could hydrolyse olive oil which is an ester of long carbon chain.

5.4 Conclusion

This part of the study focused on the functional analysis of Lip-VUT3 and Lip-VUT5 obtained from the goat rumen metagenome. The study established the temperature and pH profiles of the isolated lipases. Lip-VUT3 and Lip-VUT5 were proven to withstand high concentration of methanol, making them potential candidates for the production of biodiesel (Fatty Acid Monoalkyl Ester: fame). A small experiment conducted in the transesterification of the chicken oil and cooking oil was conducted but it could not be reported because the data were simply not enough to publish the study. But, by observation, it could be noticed that some few volume of fatty acid monoalkyl ester were produced but coulod not be confirmed only by sight. Since enzymes vary in the way of binding to their substrate, kinetic studies are important to reveal such affinity. Olive oil of a concentration of 0.287 mM was enough to occupy half of the active sites of Lip-VUT3 while Lip-VUT5 required 0.556 mM to saturate the active site in order to initiate the hydrolysis of olive oil. Therefore, Lip-VUT3 only required a small amount of olive oil to perform its hydrolytic function while Lip-VUT5 required a higher concentration of olive oil for its hydrolytic process. It can be concluded that Lip-VUT3 had a higher affinity or olive oil than Lip-VUT5. The recombinant Lip-VUT3 and Lip-VUT5 had some interesting features like stability at high temperature, making them useful for industrial applications.

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GENERAL CONCLUSION AND FUTURE PROSPECTS

The main objective of the research study was to isolate mDNA from different goat rumen, construct a metagenomic library to recover novel genes that could possibly code for novel or isoforms of lipases. The discovery of enzymes like lipases is very important because lipases play a huge role in biotechnology. Therefore, hunting for novel or isoforms of lipases is very crucial for the development of a sustainable life.

In this study, total metagenomic DNA from the goat rumen was successfully extracted and ligated to PCC2FOS to create fosmid libraries that were subsequently screened for lipolytic activities. Sequence and functional-based metagenomics recovered one Lip-VUT3 lipase of 24.8 kDa belonging to the GDSL lipases of the bacterial family of lipases under the SGNH hydrolase superfamily while the other Lip-VUT5 being an hydrolase/lipase of 28.4 kDa belonging to the abhydrolase superfamily of the hydrolase family. Based on their temperature profiles, the following lipases can be used used in the fat and oil industry because it makes use of higher temperatures and for biodiesel production. A bonus to these discovered lipases comes with their alkalinity. Their alkalinity can make then useful for detergent formulations, synthesis of biopolymers and for the production of alkaline cosmetic products.

In conclusion, the outcome of this work proves that goat rumen is a potential source of isoforms of lipases and can be used for the bio-prospecting of thermostable biomolecules from any eco-niche. The *in-silico* analysis reported very low similarities with known or studied lipases and it could be concluded that the lipases isolated from this study were indeed novel. Functional metagenomics revealed itself a valuable technique for the discovery of isoforms of lipases of industrial potential. Further investigation done by applying the lipases on cooked oil and animal fats supported the data obtained.

Future work would include the immobilisation of the enzymes on nanoparticles to improve their stability and reusability. The crystallization of the enzymes could also provide additional information on the mechanism of action, folding pattern of the extracted enzymes.

APPENDIX

Appendix A: Materials used in the study

Appendix A1: List of Buffers and solutions used in the study

Buffers and solutions	Composition	pH
10X TBE	108 g Tris base; 55 g Boric acid; 7.45 g EDTA In 1000 ml of dH_2O	~8.3
10X TGS	30.0 g of Tris base; 144.0 g of glycine; 10.0 g of SDS In 1000 ml of dH_2O.	~8.3
6X DNA Loading dye	30% (v/v) glycerol 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF	-
Binding Buffer	20 mM sodium phosphate; 0.5 M NaCl; 20-40 mM imidazole In 1000 ml of dH_2O	7.4
Coomassie staining solution	10% (V/V) Acetic acid; 50% (V/V) Methanol; 0.1% (W/V) coomassie blue R250; 40% dH_2O	-

CTAB	100 mM Tris-HCl; 1.4 M NaCl; 20 mM EDTA; 2% CTAB In 1000 ml of dH_2O	8.0
Cupric-Acetate Pyridine	5g of copper acetate; 80 ml of dH_2O; Adjust pH to 5.0-5.6 with pyridine.	-
De-staining solution	10% (V/V) Acetic acid; 50% (V/V) Methanol; 40% dH_2O	-
1 M EDTA	186.1 g of disodium EDTA•$2H_2O$; In 400 mL of dH_2O Adjust the pH to 8.0 with NaOH	8.0
Elution Buffer	20 mM sodium phosphate; 0.5 M NaCl; 500 mM imidazole In 1000 ml of dH_2O	7.4
End-Repair 10X Buffer	330 mM Tris-acetate [pH 7.5]; 660 mM potassium acetate; 100 mM magnesium acetate; 5 mM DTT In 100 ul	7.5
Laemmli sample buffer	65.8 mM Tris-HCl; 26.3% (w/v) glycerol; 2.1% SDS; 0.01% bromophenol blue	6.8
1 M PBS	610 mL of 1M Na2HPO4; 390 mL of 1M NaH2PO4	7.0
Phage Dilution Buffer	10 mM Tris-HCl [pH 8.3]; 100 mM NaCl; 10 mM $MgCl_2$	8.3

	In 1000 ml of dH_2O	
TE	10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0	8.0
1 M Tris-HCl	121.1 g of Tris base in 800 ml of H_2O; Adjust pH with 32% HCl	8.0

Appendix A2: List of antibiotics and inducers used in the study

Reagents	Preparation
Chloramphenicol (34 mg/ml): antibiotic	0.680g of chloramphenicol; 20 ml of 100% ethanol Filter-sterilised and aliquoted in 2 ml sterile microcentrifuge tubes. 12.5 µg/ml is the final concentration used.
Kanamycin (100 mg/ml): antibiotic	2g of kanamycin; 20 ml of distilled water Filter-sterilised and aliquoted in 2 ml sterile microcentrifuge tubes. 50 µg/ml is the final concentration used
Isopropyl-β-D-thiogalactopyranoside (IPTG) 1M: inducer	4.77g of IPTG 20 ml of distilled water Filter-sterilised and aliquoted in 2 ml sterile microcentrifuge tubes. 0.1 M is the final concentration used

Appendix A3: List of media used in the study

Media	Formulation per Liter	Preparation
LB agar	10 g Peptone; 5 g Yeast Extract; 10 g Sodium Chloride; 12 g Bacteriological Agar	All the components are mixed and autoclaved at 121 °C for 15 minutes. Medium is cooled at 50-60 °C to be poured in sterile petri dishes. (pH ~7.5)
LB broth	10 g Peptone; 5 g Yeast Extract; 10 g Sodium Chloride	All the components are mixed and autoclaved at 121 °C for 15 minutes. (pH ~7.5)
1% Tributyrin agar	10 g Peptone; 5 g Yeast Extract; 10 g Sodium Chloride; 12 g Bacteriological Agar	All the components are mixed and autoclaved at 121 °C for 15 minutes. Medium is cooled at 50-60 °C with vigorous shaking to allow the emulsification of the tributyrin and stabilization of the emulsion and pour plates. (pH ~7.5)
SOC agar	10 mM magnesium chloride, 10 Mm magnesium sulfate,	All components are mixed and autoclaved at 121 °C for

	2.5 mM potassium chloride, 10 mMsodium chloride, 2% tryptone, 0.5% yeast extract, 20 mM glucose (to be added after autoclaving)	15 minutes. The medium is cooled then 20 mM glucose is added after autoclaving.

Appendix A4: List of all microorganisms used in the study

Strains	Genotype/Features	Source
EPI300™-T1R Phage T1-resistant *Escherichia coli* Plating strain	*[F– mcrA Δ(mrr-hsdRMS-mcrBC) (StrR) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ– rpsL nupG trfA tonA dhfr]*	Epicentre
Escherichia coli BL21 (DE3)pLysS competent cells	*$F^–$ ompT gal dcm lon $hsdS_B(r_B^–m_B^–)$ λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) $[malB^+]_{K-12}(\lambda^S)$ pLysS[T7p20 ori_{p15A}](Cm^R)*	Lucigen

Appendix A5: List of all vectors used in the study

Vectors	Features	Selective marker	Source
pCC2FOS	Copy controlled vector, linearized and dephosphorylated at Eco72I restriction site. Requires EPI300™-T1R E. coli strain for high copy number induction, used for construction of fosmid library.	Chloramphenicol	Epicentre
pCC2FOS-Lip-VUT3	pCC2FOS derived constructs from the goat rumen metagenomic library harbouring Lip-VUT3 gene coding for potential lipase like activity.	chloramphenicol	This study
pCC2FOS-Lip-VUT5	pCC2FOS derived constructs from the goat rumen metagenomic library harbouring Lip-VUT5 gene coding for potential lipase like activity	chloramphenicol	This study
pET30a(+)	Expression vector of N and C-terminally His-tagged protein	Kanamycin	Merck
pET30a(+)-Lip-VUT3	pET30a(+) derived expression vector construct containing Lip-VUT3 synthesized gene.	Kanamycin	This study

pET30a(+)-Lip-VUT5	pET30a(+) derived expression vector construct containing Lip-VUT5 synthesized gene	Kanamycin	This study

Appendix A6: Preparation of 12% separating gels and 4% stacking gels for SDS-PAGE

Solution components	Volume (ml)	
	12% Resolving gel	4% stacking gel
Distilled water	3.3	3.4
30% Bis-acrylamide mix	4.0	0.83
1.5M Tris-HCl (pH 8.8)	2.5	0.63
10% SDS	0.1	0.05
10% Ammonium persulfate (APS)	0.1	0.05
TEMED	0.004	0.005

Appendix B: Supplementary results of the molecular part of the study

Appendix B1: Summary of statistical analysis of the *De novo* assembly and alignments of DNA sequences of pCC2FOS-Lip-VUT3 and pCC2FOS-LipVUT5 clones

- pCC2FOS-Lip-VUT3 clone

	Count	Average length	Total bases
Reads	214,180	236.36	50,624,387
Matched	211,497	236.36	49,988,933
Not matched	2,683	236.84	635,454
Contigs	663	367	243,479

- pCC2FOS-Lip-VUT5 clone

	Count	Average length	Total bases
Reads	307,000	255.34	78,390,505
Matched	304,880	255.28	77,829,265
Not matched	2,120	264.74	561,240
Contigs	465	394	183,611

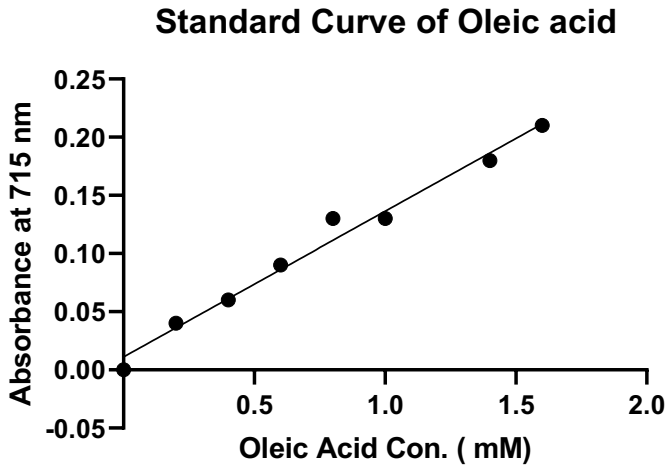
Appendix B2: Summary of BLAST search results of Lip-VUT3 and LipVUT5 ORFs

Genes	Nucleotide Sequences	Amino acids sequences	Family	Best HIT
Lip-VUT3	ATGATGATGATGATGATGACCTTCCAGGCGGCCATAGCCC GCCGCGCGCAGTTTTTCGCTCATCGCTTCATAGCCGCGGCT GCTCGGATGCACGCGATCAATGCTCAGATATTCTTTTTCGC GGCCTTTAAACACCGCATAGGTATCAATCACTTTAATCTGC GGCGCGCTTTCCAGCTGTTTCAGATGGCGGTTAAAGCCGCT AATCCATTTATCCGCCAGTTCAATGCTCGGAAACGGGTTAT ACAGGTTCAGCAGGCGCACCAGATAGCGGGTATCTTTTTCG CCTTTAATTTCGCGAATTTTTTCCAGCATGCCGCTATAGTTT TTCTGGCAATGGCTGCTCGCTTCCAGAAACACATGTTCATC TTTTTCTTTTTCATAAATTTCCAGGCTCTGCAGCAGATCGTT GCCGCAGCCGGTAATGGTAATCACATCCGCTTTTTTCACCT GTTCCATAATAAACGGTTCGTTCAGCATCGCCAGAATTTCG CTGGTTTCCAGGCCGCTTTTCGCAAACACAATCAGGCTCAC TTCTTCGTTCAGATCTTCTTCCATTTTGCGTTTATAGCGCTG CACAAAGCCCGGTTCAAACAGGCCCGCGCCCACGCCCACG GTCAGGCTATCGCCCAGCGCGGTATACTGCAGGGTCAT	TFQAAIARRAQFFAHRF IAAAARMHAINAQIFFF AAFKHRIGINHFNLRRA FQLFQMAVKAANPFIRQ FNARKRVIQVQQAHQIA GIFFAFNFANFFQHAAIV FLAMAARFQKHMFIFFF FINFQALQQIVAAAGNG NHIRFFHLFHNKRFVQH RQNFAGFQAAFRKHNQ AHFFVQIFFHFAFIALHK ARFKQARAHAHGQAIA QRGILQGH	Esterase/Lipase family	Chain A, Crystal Structure Of Extracelular Lipase From *Streptomyces Rimosus* [**5MAL_A**]

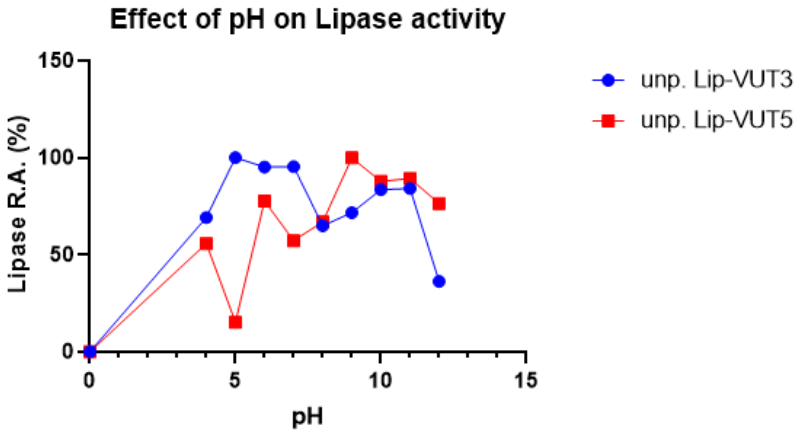
Lip-VUT5	GGGTCTGCCAATCCAGGGTTTCCAGAAACTGATACACATCT TTATGCAGGGTTTCGCGTTCTTTATCCAGGGTAATCGCATG GCCGCTTTCTTCATACCATTTCAGCTGTTTATCATCGGTTTC CACTTCGTTATAAATAATGTTCGCGCTATCGGTGTTAATCA TATGATCATGGCGCGCCTGCACCACAAAGGTCGGGCTATA AATCATATCCACATGTTCGCGCACATCCGCAATCAGATCCT GCAGCGCTTTCAGGGTGTTCATCGGGGTTTTTTTAAATTCTT CCATTTCCGCGTTAATCTGTTCCGCGGTTTTGCCTTCAAATT TTTTATAGTTGCGCGCATAATCCAGCACGCCTTCATACATG GTTTCTTCGCTTTTAATATACATCGGCGCGCACATCGGCAC AATGCCTTTAATCGGCACGGTATAGCCCAGTTTCAGGCTAA ACACGCCGCCCAGGCTCAGGCCGCACGCCGCAATCTGTTC ATAGCCTTCGCTTTTCAGATATTCATAGCCATCCATCACAT CTTTCCACCAATCTTCCGGGCCGGTGCTCAGCAGTTCTTCC GGCGGCACGCCATGGCCTTTATACTGCGGCGCATGGCAGG TATAGCCTTTTTCGTTCAGATAGCGGCCCAGCATGCGCACA TCCGCGGTGTTGCCGGTAAAGCCATGCAGCAGCAGCACCG CTTTTTTGCCGCCTTTAAAGGTAAACGGCTGCGGTTTCACA ATTTTCAT	MKIVKPQPFTFKGGKK AVLLLHGFTGNTADVR MLGRYLNEKGYTCHAP QYKGHGVPPEELLSTGP EDWWKDVMDGYEYLK SEGYEQIAACGLSLGGV FSLKLGYTVPIKGIVPM CAPMYIKSEETMYEGV LDYARNYKKFEGKTAE QINAEMEEFKKTPMNTL KALQDLIADVREHVDM IYSPTFVVQARHDHMIN TDSANIIYNEVETDDKQ LKWYEESGHAITLDKER ETLHKDVYQFLETLDW QT	Esterase/Lipase family	Chain A, thermostable Monoacylglycerol Lipase From *Geobacillus* sp. [**3XKS_A**]

Appendix C: Supplementary data for functional analysis of recombinant proteins

Appendix C1: Standard curve of Oleic acid used for the lipase assay based on the copper-soap method



Appendix C2: pH profiles of unpurified Lip-VUT3 and Lip-VUT5



Appendix C3: Temperature profiles of unpurified Lip-VUT3 and Lip-VUT5