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CLONING AND CHARACTERISATION OF THREE NOVEL LIPASES FROM *Thermomyces lanuginosus*

Submitted in fulfilment for the Master of Applied Sciences (Biotechnology) degree in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Kugen Permaul** and **Dr Nokuthula Peace Mchunu**

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Student's signature

Table of Contents

Acknowledgements i

Abstract ii

List of Figures iv

List of Tables ix

1. Introduction 1

2. Literature review 3

2.1 Lipases 3

2.1.1 Sources of microbial lipases 6

2.1.2 Screening methods for - lipase producing micro-organisms 9

2.1.3 Microbial production process of lipases 12

2.1.4 Applications of lipases 17

2.1.5 Thermostable lipases 25

2.2 Protein engineering 26

2.2.1 Bioinformatics on lipases 28

2.3 Recombinant lipases 32

2.3.1 *Pichia pastoris* as a suitable host for expression of recombinant lipases 33

2.3.2 *Escherichia coli* as a host for expression of recombinant lipases 37

2.4 Scope of study 42

3. Materials and methods 43

3.1 Bioinformatical analysis 43

3.2 Growth and maintenance of *T. lanuginosus* 43

3.3 Total RNA isolation 44

3.4 RNA quantification 45

3.5 Agarose gel electrophoresis (RNA) 45

3.6 cDNA synthesis 46

3.7 Plasmids, bacterial and yeast strains 46

3.8 Plasmid isolation 55

3.9 Agarose gel electrophoresis 56

3.10 DNA purification 56

3.11 Polymerase chain reaction 57

3.12 Cloning of lipase putative genes 58

3.13 Preparation of *E. coli* competent cells 58

3.14 Transformation and screening of positve *E. coli recombinants* 59

3.15 Preparation of *P. pastoris* competent cells 60

3.16 Transformation and screening in *P. pastoris* 60

3.17 Lipase expression 61

3.18 Purification with a His-Bind resin affinity chromatography 62

3.19 SDS-PAGE analysis 63

3.20 Assay for lipase activity 63

3.20.1 Temperature optima and stability 64

3.20.2 pH optimum assays 65

3.20.3 Lipase substrate specificity 66

3.20.4 Effect of metal ions on lipases 66

4. Results 67

4.1 Sequence analysis 67

4.1.1 Protein BLAST analysis 67

4.1.2 Amino acid sequence comparison 68

4.1.3 Conserved domain analysis 70

4.1.4 Protein modelling 70

4.2 Cloning of the *Thermomyces lanuginosus* SSBP lipases in different *P pastoris* vectors 75

4.2.1 Amplification of lipases from cDNA 75

4.2.2 Construction of *Pichia* recombinants 77

4.3 Lipase expression in heterologous hosts (*P. pastoris* and *E. coli*) 80

4.3.1 Expression in *P. pastoris* 80

4.3.2 Expression in *E. coli* BL21 (DE3) 82

4.4 Characterisation of lipases 86

4.4.1 Temperature optima 86

4.4.2 pH optima 87

4.4.3 Thermostability of lipases 88

4.4.4 Substrate affinity 92

4.4.5 Effect of metal ions on lipases 93

5. Discussion 94

6. Conclusion 101

7. Reference 102

Appendix A 130

Appendix B 131

Appendix C 132

Appendix D 133

Appendix E 136

Appendix F 137

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ABSTRACT

Although other *Thermomyces lanuginosus* lipases have already been reported in the literature, genome sequencing resulted in three different lipases being identified. Thus this study aimed to characterise these novel *T. lanuginosus* lipases. The three lipase gene sequences were analysed to investigate their novelty, similarities and to compare them to existing lipases. It was found that they were different from each other and had low identity to existing lipases.

Conserved domain analysis showed that all three genes belong to the abhydrolase superfamily, the family in which lipases and esterases belong. Furthermore, lipase C was also part of another family, PLNO2877 superfamily, another conserved protein domain family specifically for triacylglycerol lipases. Protein sequence alignment analysis also revealed that lipases A and B are more similar to each other compared to lipase C. SWISS protein models were also created using the best template matches for each protein sequence, the protein models further indicated the distinctness of lipase C and the similarity between lipases A and B were further demonstrated by superimposing their ribbon.

The cDNA of *Thermomyces lanuginosus* SSBP was used to amplify the lipase A and lipase B genes using primers designed for pPICZαA and pPIC9K cloning and expression vectors. Lipase B gene was also cloned into pPBG1. When the PCR products were analysed for amplification with gel electrophoresis. Lipase B amplification produced a single distinct band of approximately 1100 bp which was the expected PCR product for all three *Pichia* cloning vectors. Amplification for lipase A proved to be unsuccessful as three bands were produced instead of a single distinct band. Plasmid pET100/D-TOPO containing the artificially synthesised three putative lipases were synthesised for expression in *E.coli* BL21 (DE3). This method yielded higher expression levels for all three lipases when compared to *Pichia*.

After purification, the recombinant lipases from *E. coli* produced lipase yields of 176.2 ± 1.2 for lipase A; 184.1 ± 0.46 for lipase B; and 181 ± 0.13 for lipase C. This was much higher than the activity obtained from *P. pastoris* expression. Enzyme characterisation was performed using *E. coli* only. The temperature optimum of all three lipases was identical at 60˚C. All three lipases had preference for alkaline conditions, with an optimum of pH 8, and activity was stable between pH 7.0-10.0. All three lipases preferred longer chain substrates, with *p*-nitrophenyl palmitate (C16) being the most favourable, with an exception of lipase C which preferred *p*-nitrophenyl stearate (C18) with activity 7% higher than that on *p*-nitrophenyl palmitate. These lipases therefore have temperature and pH properties that will be useful for thnumerous industrial applications of lipases which will be investigated in future studies.

LIST OF FIGURES

Fig 2.1: Trigylceride hydrolysis reaction by lipases to produce fatty acids and glycerol (Naya and Imai, 2013). 3

Fig 2.2: Different reactions catalysed by lipases. (Ribeiro *et al*., 2011). 5

Fig 2.3: (a) Lipolytic activity shown by a zone of hydrolysis on tributyrin agar; (b) Lipolytic activity shown by orange fluorescence upon ultraviolet radiation on Rhodamine B plate; and (C) Lipolytic activity shown by dye indicators on agar (Salwoom *et al*., 2019). 11

Fig 2.4: Various industrial applications of lipases (Angajala *et al*., 2016). 18

Fig 2.5: Lipase catalysed transesterification illustration (Chen and Kwong, 2015). 23

Fig 2.6: Phylogenetic tree representing evolutionary analysis of microbial lipases from bacteria, fungi, algae and archaea (Verma *et al*., 2021). 29

Fig 2.7: Protein structure showing controlled lid-opening in Thermomyces lanuginosus lipase (Skjold-Jørgensen *et al*., 2017). 31

Fig 2.8: Sequential approach for recombinant lipase production (Kormanová, *et al.,* 2020)..33

Fig 3.1: Map of pPICZαA used for expression of lipase in *P. pastoris* showing location of the replication region. Restriction sites on the plasmid are shown and their exact positions indicated in brackets and other regions (Novagen). The lipase gene was inserted into pPICZαA using the *Xba*I and *Kpn*I restrictions sites……....48

Fig 3.2: Map of pPIC9K used for expression of lipase in *P. pastoris* showing location of the replication region. Restriction sites on the plasmid are shown and their exact positions indicated in brackets and other regions (Novagen). The lipase gene was inserted into pPIC9K using the *Xba*I and *Sna*BI restrictions sites. 49

Fig 3.3: Map of the *Pichia* vector, pBGP1, showing the location of the ampicillin resistance (Amp^R) gene, zeocin resistance (Zeo^R) gene, *GAP* promoter (pGAP), secretion signal (α-factor) and multiple cloning sites (MSC) and *E. coli* replicon (colE1) and *Pichia* autonomous replication sequence (PARS1) (Lee *et al*., 2006). The lipase gene was inserted into pBGP1 using the *Xho*I and *Eco*RI restrictions sites……...…50

Fig 3.4: Map of *E.coli* vetor pET100/D-TOPO® showing the ampicillin resistance gene, the multiple cloning site (MCS) and the T7 primer site. It also confers a polyhistidine (6×His) tag which enables a simple purification method.....52

Fig 3.5: Map of plasmid LipA-pET100 containing the synthesised lipase gene A (1119 bp) from *Thermomyces lanuginosus* SSBP.....53

Fig 3.6: Map of plasmid LipB-pET100 containing the synthesised lipase gene B (1371 bp) from *Thermomyces lanuginosus* SSBP.....53

Fig 3.7: Map of plasmid LipC-pET100 containing the synthesised lipase gene C (1446 bp) from *Thermomyces lanuginosus* SSBP.....54

Fig 4.1: Protein BLAST analysis of lipase genes LipA (a), LipB (b) and LipC (c) showing putative lipases similarities and identities from the NCBI database……..68

Fig 4.2: MUSCLE TOOL Amino Acid Alignment for all three lipase genes (LipC, LipA and LipB). *=conserved residues, : = strongly similar groups, .= weakly similar groups. Red = hydrophobic, blue = negatively charged, magenta = positively charged, green = polar residues…….69

Fig 4.3: Domain analysis for lipases LipA (A), LipB (B) and LipC (C) showing specific and non-specific superfamily hits. All three genes belong to the Abhydrolase superfamily. 70

Fig 4.4: SWISS MODEL protein folding structures for LipA based on the highest sequence analysis in Table 4.1. Liquorice (stick) model is on the left and cartoon (ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines.....71

Fig 4.5: SWISS MODEL protein folding structures for lipase LipB based on the highest sequence analysis in Table 4.2. Liquorice (stick) model is on the left and cartoon (ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines. 73

Fig 4.6: Cartoon (ribbon) model showing superimposed models of LipA and LipB. The superimposed image shows a lot of similarities between the two where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines. 73

Fig 4.7: SWISS MODEL protein folding structures for lipase LipC based on the highest sequence analysis in Table 4.3. Liquorice (stick) model is on the left and cartoon (ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines. 74

Fig 4.8: Amplification of LipA and LipB from cDNA using pPICZα primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: LipA PCR product from cDNA. Lane 3: LipB PCR product from cDNA…….…….75

Fig 4.9: Amplification of LipA with pPICZαA and pPIC9K primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2-3: LipA PCR product for pPICZαA after PCR optimisation attempts. Lane 4-5: LipA PCR product for pPIC9K..... 76

Fig 4.10: Amplification of LipB with all three *P. pastoris* vector primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: Negative amplification. Lane 3-4: LipB PCR product using pPICZαA primers. Lane 5: PCR product using pPIC9K primers. Lane 6: PCR product using pPBGP1 primers……..76

Fig 4.11: Restriction analysis confirming ligation of pPICZαA to LipB by restriction of the transformed plasmid with endonucleases *Xho*I and *Xba*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: LipB control. Lane 3: Unrestricted pPICZαA control. Lane 4: Single digest of pPICZαA. Lane 5-6: Empty lanes. Lane 7-8: Restricted LipB-pPICZαA plasmid showing the correct 3.3 kb and 1.1kb fragments respectively. 77

Fig 4.12: Restriction analysis confirming ligation of pPIC9K to LipB by restriction of the transformed plasmid with endonucleases *Sna*BI and *Xba*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: Restricted LipB-pPIC9K plasmid showing the correct 9.2 kb and 1.1 kb fragments respectively. LipB-pPBG1. 78

Fig 4.13: Restriction analysis confirming ligation of pPBG1 to LipB insert by restriction of the transformed plasmids with endonucleases *Eco*RI and *Xho*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2-3: negative clones Lane 4-8: Restricted LipB-pPBG1 plasmid showing the correct 4.6 kb and 1.1 kb fragments, respectively. 79

Fig 4.14: BMMY-rhodamine B-olive oil plate used to qualitatively confirm the expression of lipases in positive transformants. The image was captured after 48 hours of methanol induction.

An orange fluorescence emission on ultraviolet exposure indicate expression of the three clones……80

Fig 4.15: Biomass production over a period of 120 hours for LipB-pPICZαA, LipB-pPIC9k and a *P pastoris* GS115 negative control.....81

Fig 4.16: Recombinant lipase production by LipB-pPICZαA and LipB-pPIC9K expressed in *P. pastoris* GS115 in BMMY and induced with methanol every 24 hours over 120 hours at 30 C with shaking at 250 rpm. A negative control is also shown.....82

Fig 4.17: Qualitative plate assay showing lipase expression by *E. coli* clones epressing LipA, LipB and LipC. A negative control plate is also shown. 83

Fig 4.18: A 12% SDS-PAGE showing crude recovery of lipases A, B and C. Lane 1-3: Protein from clones A, B and C respectively, all exhibiting thick bands ranging from ~30 to ~35kDA, typical of the target lipase size. Lane 4: Thermo Fisher Scientific PageRuler. 84

Fig 4.19: A 12% SDS-PAGE showing purified lipases A, B and C. Lane 1: Lipase from clone LipA. Lane 2: Lipase from clone LipB. Lane 3: Lipase from clone LipC. Lane 4: Thermo Fisher Scientific PageRuler.....85

Fig 4.20: Effect of temperature on enzyme activity of lipases A, B and C at pH 8 over a 40 – 90˚C temperature range. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol in 15 min by UV-Vis spectrometry (410 nm). 87

Fig 4.21: Effect of pH on enzyme activity by lipases A, B and C at 60˚C from pH 3 to pH 10. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol in 15 min by UV-Vis spectrometry (410 nm).....88

Fig 4. 22: Temperature stability of Lipase A at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm.....89

Fig 4. 23 Temperature stability of Lipase B at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm. 90

Fig 4.24: Temperature stability of Lipase C at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm. The activity at 100% was 181.0 ± 0.13 U/ml……..91

Fig 4.25: Determination of the specificity of lipases A, B and C towards *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl palmitate (C16), and *p*-nitrophenyl stearate (C18). All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol by UV-Vis spectrometry (410 nm)……...92

LIST OF TABLES

Table 2.1: Summary of bacterial lipase producers found in recent literature 7

Table 2.2: Summary of fungal lipase producers found in recent literature (Mehta *et al*., 2017; Chandra *et al*., 2020) 9

Table 2.3: Microbial strains cited in the recent literature as potential lipase producer and their industrial applications 19

Table 2.4: Protein models and crystal structures available in literature for some funal lipases (Verma *et al.*, 2021). 30

Table 2.5: Summary of recent recombinant lipases cloned and expressed in *Pichia pastoris*...35

Table 2.6: Summary of recent recombinant lipases cloned and expresses in *Escherichia coli* BL21 (DE3)……. 39

Table 3.1: Plasmids vectors used in for *P. pastoris* expression in this study……..47

Table 3.2: Plasmids vectors used in for *P. pastoris* expression in this study synthesised by ThermoFisher (https://apps.thermofisher.com/apps/geneart-qa) 51

Table 4.1: SWISS MODEL protein sequence template match for LipA 71

Table 4.2: Swiss Model Protein sequence template match for LipB 72

Table 4.3: SWISS-MODEL protein sequence template match for LipC 74

Table 4.4: Lipase activity for LipA, LipB and LipC..... 86

Table 4.5: Relative lipase activity of lipases A B and C demonstrating the effect of metal ions……..... 93

1. INTRODUCTION

There is vast evidence of the inherent commercial and environmental drawbacks of chemo-synthetic or chemo-conversion processes employed by numerous industries (De Gonzalo and de María, 2017; Sheldon and Brady, 2018). The disadvantages of these methods emanate from their heavy reliance on harsh and energy-intensive processes (Hughes and Lewis 2018). Furthermore, these conventional chemo-synthetic methods often lack specificity and selectivity, thus leading to poor yields and production of excessive levels of waste (Arroyo *et al.,* 2017; Su *et al.,* 2018). Therefore, the ever increasing pressure and number of concerns over the hostile environmental impacts, waste and safety of chemo-synthetic methods has put a strain on industries to continue to seek environmentally benign and commercially viable processes (Sheldon, 2016).

On the contrary, enzymatic reactions have been shown to minimise waste due to high specificity and stereo- and regio-selectivity. These enzymatic conversion reactions can be carried out safely and with high energy efficiency because they require moderate temperatures, atmospheric pressure and pH (Li *et al.,* 2018; Su *et al.,* 2018). Furthermore, owing to the abundance of enzymes in all living organisms, they have evolved to satisfy the metabolic demands of an extensive range of cell types (Bell *et al.,* 2021). To avoid metabolic chaos in cells teeming with numerous chemical reactions, the enzymes have had to be highly specific to the reactions they catalyse and target substrates (Adams *et al.,* 2019). Thus they can be adapted to various industrial process.

Microbial enzymes continue to attract lots of interest in industrial applications as they have great potential of replacing harsh chemical industrial processes that are partially responsible for global warming. Microbial lipases are one of these important enzymes and hence they have been studied extensively over the past decades. Some lipases have even been commercialised for different industrial applications such as the production of detergents, modification of oils and fats, pre-treatment of wool and production of biodiesel among others (Fernandez-Lafuente, 2010), and one such lipase was isolated from a thermophilic fungus, *Thermomyces lanuginosus* (Hasan *et al.,* 2006).

The genome *Thermomyces lanuginosus* SSBP has been sequenced to acquire the necessary information required to utilise and improve its industrial application potential (Mchunu *et al*., 2013). Although other *T. lanuginosus* lipases have already been reported in the literature, genome sequencing of *Thermomyces lanuginosus* SSBP identified. Thus this study aims to characterise these novel *T. lanuginosus* lipases. Their nucleotide and protein sequences were analysed to establish their similarity to each other and to confirm their novelty. The lipase genes were cloned for expression in *Pichia pastoris* and *Escherichia coli* using different plasmid vectors. The recombinant lipases were then characterised to investigate their physicochemical properties.

2. LITERATURE REVIEW

2.1 Lipases

Lipases are one of the most ubiquitous enzymes in nature being produced from animal, plant and microbial sources. Lipases (triacylglycerol hydrolases, E.C 3.1.1.3) are a group of hydrolases that catalyse the hydrolysis of triglycerides to produce glycerol and fatty acids (Fig 2.1). They are part of the same hydrolase family as esterases, and are characterised by long-chain triacyl glycerols which distinguishes them from esterases and the rest of the hydrolases (Bharathi and Rajalakshmi, 2019).

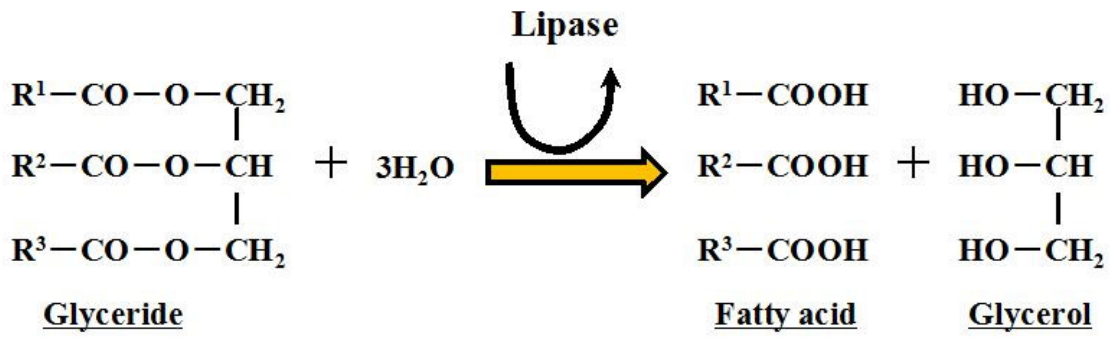

Fig 2.1: Trigylceride hydrolysis reaction by lipases to produce fatty acids and glycerol (Naya and Imai, 2013).

Literature has illustrated and proven the stability of lipases in extreme temperatures and pH. This important trait makes them very efficient in catalysing reactions, both in aqueous and non-aqueous conditions (Tan *et al*., 2018). However, in non-aqueous conditions, lipases can cause the reverse of the hydrolysis reaction, namely, esterification. They are also further responsible for transesterification reactions where an exchange of groups occurs, a summary is illustrated

in Fig 2.2 (Bhardwaj and Gupta 2017). Idustrial lipase applications are developed based on the following lipase - catalysed reactions:

i) Hydrolysis

ii) Esterification

iii) Transesterification: These reactions are further subcategorised as follows:

   a. Interesterification

   b. Alcoholysis

   c. Acidolysis

Because of their versatility, lipases can accept a wide range of compounds as substrates, making them very desirable for industrial applications (Bhardwaj and Gupta, 2017). They have become increasingly popular in the food, chemical, fuel and pharmaceutical industries due to their ability to perform very specific chemical transformation or biotransformation (Singh and Mukhopadhyay, 2012). Recent studies focused on cloning and sequencing, characterization and the mechanism of action of lipases. Current predictions show that lipases will occupy a significant stake along with proteases and carbohydrates in industrial applications (Hernández-García *et al.*, 2017; Angajala *et al*., 2016). Lipase ability to catalyse these different reactions with ease, efficiency and stability makes them commercially desirable.

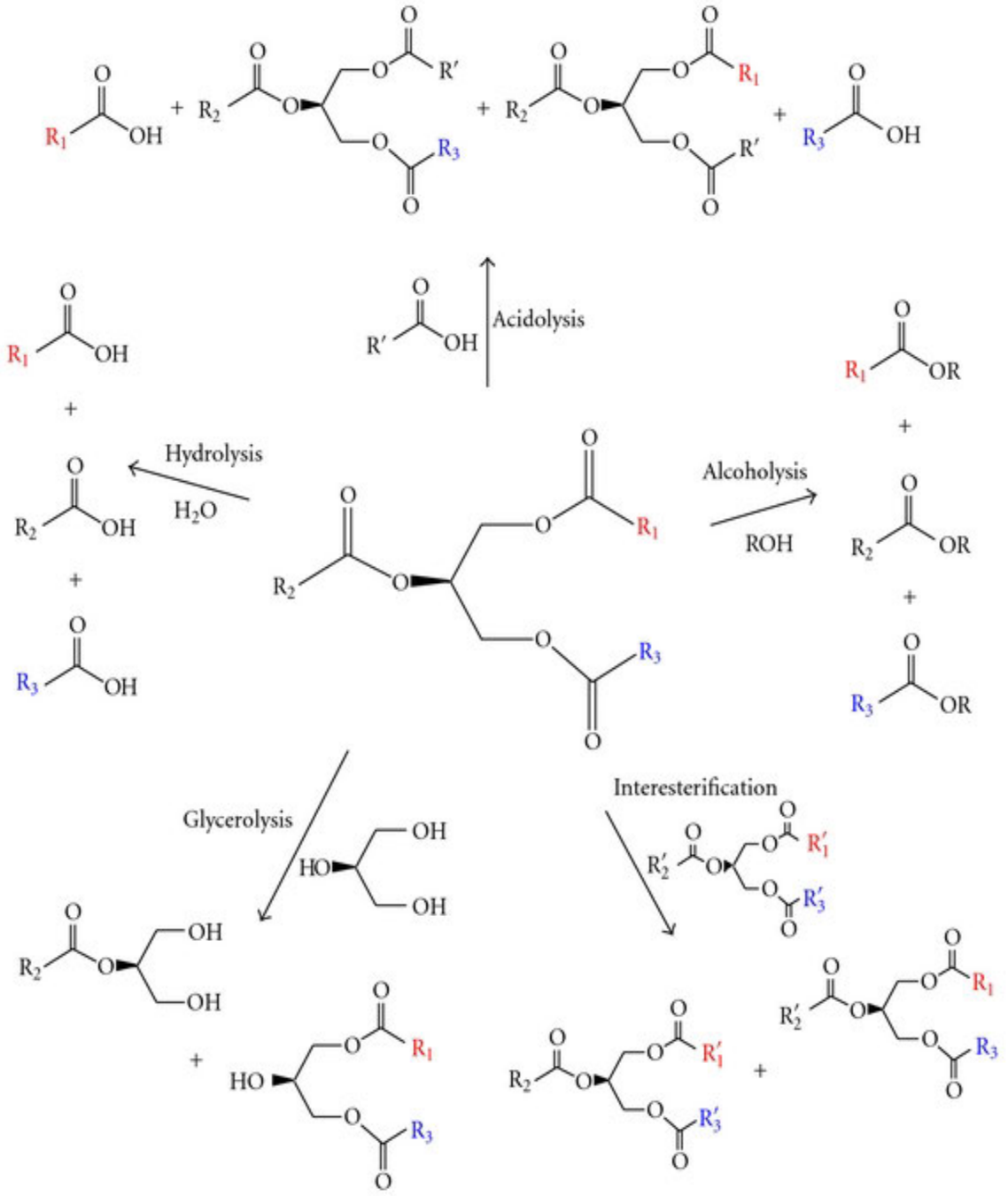

Fig 2.2: Different reactions catalysed by lipases. (Ribeiro *et al*., 2011).

2.1.1 Sources of microbial lipases

Lipases are versatile and ubiquitous enzymes available from animal, plant and microbial sources. In the field of biotechnology, microbial sources have been identified as a major sources of enzymes with industrial potential including lipases. The main microbial sources which have been identified for in literature are bacteria and fungi (Table 2). Lipases from microbial sources are considered more suitable to withstand extreme environmental conditions; stability in organic solvents; and generally, do not require assistance of co-factors (Javed *et al.,* 2018).

2.1.1.1 Bacterial lipases

The first bacterial lipases were detected from *Bacillus priodigiousus* and *Bacillus fluorescens* in 1901, and currently, *Serratia marcescens* and *Pseudomonas fluorescens* have been identified as the best producers of bacterial lipases (Chandra *et al*., 2020; Kuebutornye *et al.,* 2020). Some bacterial lipases are thermostable making them ideal for industrial applications at high temperatures, although, some have been reported to be non-specific in terms of substrate specificity (Tembhurkar *et al*., 2012).

Table 2.1: Summary of bacterial lipase producers found in recent literature

Bacterial strain	Reference
Aeribacillus 096201	Lokre and Kadam (2014)
Aneurinibacillus thermoaerophilus	Masomian *et al.* (2016)
Bacillus pumilus	Kandasamy *et al*. (2021)
Bacillus stratosphericus	Gricajeva *et al*. (2016)
Bacillus subtilis	Suci *et al*. (2018)
Burkholderia ubonensis	Yang *et al.* (2016)
Enterococcus faecium	Ramakrishnan *et al.* (2016)
Geobacillus stearothermophilus	Dror *et al.* (2015); Ekinci *et al.* (2016)
Geobacillus thermoleovorans	Abol Fotouh *et al*., (2016)
Janibacter	Castilla *et al.* (2017)
Lactococcus	Konkit and Kim (2016)
Pelosinus fermentans	Biundo *et al.* (2016)
Pseudomonas fluorescens	Yuan *et al*., (2021
Staphylococcus warneri	Yele and Desai, 2015
Serratia marcescens	Chen *et al*., 2021
Thalassospira permensis	Kai and Peisheng, 2016
Xanthomonas oryzae	Mo *et al.,* 2016
Yersinia enterocolitica	Ji *et al.,* 2015

2.1.1.2 Fungal lipases

Fungal lipases have been of great research interest since the early 1950's due to their valuable temperature and pH stability; activity in organic solvents; substrate specificity, and ease of downstream processing (Iftikhar *et al*., 2010; Chandra *et al*., 2020). Fungal lipases have been found to be superior over bacterial lipases in some industrial applications. This is because, latest technology and machinery favour batch fermentation and economical extraction methods, both of which favour fungi over bacteria (Basheer *et al*., 2011; Costa *et al*., 2017).

Recently, *Colletotrichum gloeosporioides* isolated from the savanna soil in Brazil has been found to be the most productive fungal strain, producing an impressive 27 700 U/l of microbial lipase (de Almeida *et al*., 2013; Chandra *et al*., 2020). A good number of fungal organisms have also been identified to produce high amounts of lipases which have been processed in commercial scales. Amongst others, *Aspergillus niger, Candida rugosa, Thermomyces lanuginosus* and *Rhizopus oryzae* are the principal producers of commercial lipase (Chandra, 2019a; 2019b). Table 2.2 shows a summary of some lipase – producing fungi found in recent lirerature.

Table 2.2 Summary of fungal lipase producers found in recent literature (Mehta *et al*., 2017; Chandra *et al*., 2020)

Fungi	pH stability	Thermostability
Aspergillus niger	2.2 - 6.8	50
Candida	5.0 - 7.5	45
Geotrichum candidum	4.5 - 10.0	50
Penicillium cylopium	6.5 - 9.0	40
Penicillium roqueforte	-	50
Rhizopus delemar	3.0 - 8.0	65

2.1.2 Screening methods for - lipase producing micro-organisms

Different screening methods are used to identify lipase producing microorganisms. These methods involve culturing microbial strains on solid agar medium or in liquid media containing different substrates. Lipase screening can be broadly classified as either direct or indirect screening methods.

2.1.2.1 Qualitative methods

Solid agar media with added substrates or selective dyes can be used for screening of lipolytic microorganisms. This is a an essential screening method to evaluate microorganisms for their lipase enzyme production potential (Mateos-Díaz *et al.,* 2012). These are futher classified into two categories based on the substrates and indicator dyes. This assay is mostly qualitative as they do correspond to lipase levels and sometimes give false negative or positive results.

However, their advantage is that they rapidly indicate whether an organisms has a potential to produce lipase.

2.1.2.1.1 Gel diffusion assays using lipid substrates and indicator dyes

Gel diffusion methods are used to screen for lypolytic microbes based on their lypolysis on lipids incorporated into solid media. This is characterized by the development of a clear zone of lipolysis around the colonies (Lee *et al.,* 2015). Based on the substrates utilized, a variety of agar plate assays have been developed and they incorporate substrates such olive oil, tributyrin and tweens (Kumar *et al.,* 2012; Lanka and Trinkle 2017; Bharathi and Rajalakshmi, 2019). Among those lipolytic tests, the tributyrin agar plate assay is the most popular lipase screening plate assay due to its ease in preparation and quick results (Bharathi and Rajalakshmi, 2019).

They involve use of indicator dyes with various substrates to screen the lipase producing microbial strains. The dyes like phenol red, Rhodamine B, Victoria blue, night blue can be used as indicator. Rhodamine B added to olive oil agar plates is a very common, sensitive, and effective assay to identify active lipases. The basic principle and mechanism in this method involves interaction of hydrolysedi substrates with Rhodamine B (fluorescent dye) resulting in the formation of orange, fluorescent halos around microbial colonies which can be visible upon UV irradiation (Kumar *et al.,* 2012; Demera, Barahona and Barriga, 2019). An example of this assay is shown in Fig 2.3 for the assay of a lipase - producing *Pseudomonas* sp. LSK25 isolated from Antarctica (Salwoom *et al*., 2019).

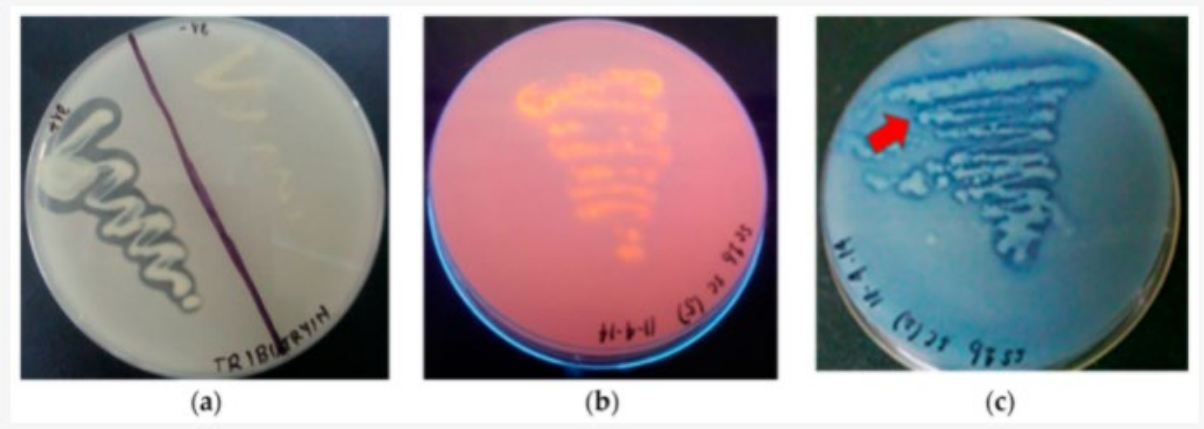

Fig 2.3: (a) Lipolytic activity shown by a zone of hydrolysis on tributyrin agar; (b) Lipolytic activity shown by orange fluorescence upon ultraviolet radiation on Rhodamine B plate; and (C) Lipolytic activity shown by dye indicators on agar (Salwoom *et al*., 2019).

2.1.2.2 Quantitative methods - liquid medium

Lipase producing ability of micro-organisms can be determined using various quantitative methods such as titrimetric method, spectrophotometric method, chromatographic methods, and molecular screening methods (Bharathi and Rajalakshmi, 2019).

Titrimetric methods were routinely subjected for estimation of lipase activity more than half a century. Recently, spectrophotometric method using *p*-NPP (*p*-nitrophenyl palmitate) as a substrate for quantitative screening potential lipase procedure has gained much attention due to their simple procedures (Velu *et al.,* 2012; Balseiro-Romero *et. al.,* 2017).

2.1.2.2.1 Titrimetric method

2.1.2.2.1.1 *p*-NPP substrate assay

The *p*-nitrophenyl palmitate (*p*-NPP) assay is commonly used to determine the esterelytic activity of these enzymes (Yao *et al.,* 2008). The colorimetric estimate of *p*-nitrophenol (*p*-

NP) released after enzymatic hydrolysis of *p*-NPP at 410 nm forms the basis of this assay detection protocol. The amount of enzyme that liberates 1 µmol of *p*-nitrophenol per minute is known as one unit of enzyme activity. Researchers all over the world use this assay protocol to estimate the esterelytic activity of both lipases and esterases (Sivaramakrishnan and Incharoensakdi 2016; Wei *et al.,* 2021). A variety of (*p*-NP) esters having C2 to C18 (*p*-NP acetate to *p*-NP stearate) in their fatty acid side chain can be hydrolyzed by bacterial lipases (Javed *et al.,* 2018). Hence, this approach can also be used to estimate the esterase activity of certain proteases. Moreover, other *p*-NP esters such as *p*-NP-caprylate, *p*-NP-acetate, and *p*-NP-valerate are used in addition to top *p*-NP palmitate. The speed and simplicity of this assay procedure over other commonly used protocols like titrimetric are its main advantages.

However, a turbid solution, due to immiscibility of *p*-NPP in water or the fatty acids, is often obtained, interfering with spectrophotometric measurements. Following the completion of the reaction, calcium chloride ($CaCl_2$) is usually added to the reaction mixture to alleviate turbidity for reproducible absorbance readings. Fatty acids precipitate as calcium salts, which can then be filtered or centrifuged to get a transparent solution. When dealing with many samples, however, the fatty acid precipitation stage makes the assay time consuming, laborious, and inconvenient. Another challenge is that $CaCl_2$ does not stop the enzymatic reaction, as a result of the intervening precipitation, centrifugation, or filtration phases, there is a lag between the end of the reaction and the measurement of the product's optical density, thus leading to overestimated enzyme titer values (Gupta *et al*, 2002).

2.1.3 Microbial production process of lipases

Microbial lipases are produced intracellularly and extracellularly, however most lipases, eukaryotic and prokaryotic, are produced extracellularly via submerged fermentation

processes. Consequently, their production is greatly influenced by the medium composition in which they are produced. This includes the carbon, nitrogen and trace element sources. They are also greatly influenced by physicochemical factors such as temperature; pH; and dissolved oxygen. These factors can either support or inhibit lipase enzyme production (Javed *et al.,* 2018).

2.1.3.1 Carbon source

Microbial lipase is the resultant protein product after the activation of lipase-producing genes. Carbon sources, especially olive oil, play an important role in lipase enzyme action in all types of microbial lipases. However, the induction of lipases with oils (olive, palm, and other vegetables) has influenced their recovery. When mustard seed oil was used as a carbon source, *Aspergillus terreus* produced a high yield of lipase. In fungal strains, combining olive oil cake and sugar - cane bagasse increases lipase enzyme activity. Olive oil has been shown to increase lipase activity in bacteria strains as compared to other oils. The use of Tween 80, on the other hand, has helped in the recovery of lipase provided by *Acinetobacter* sp. Vegetable oils, in addition to being inexpensive and readily available, are commonly used to improve the production of higher lipases (Bharathi and Rajalakshmi, 2019).

2.1.3.2 Nitrogen source

A nitrogen supply is needed for microbial growth and lipase development. Ammonium salts, yeast extract, sodium nitrate, urea, peptone and tryptone have mostly been used to produce higher lipases from a variety of microorganisms. The addition of urea to the culture medium for lipase synthesis increased the lipolytic function of *Rhizopus* sp. harvesting. *Aspergillus* sp. has also used peptone in conjunction with other nitrogen extracts to produce lipase (Bharathi and Rajalakshmi, 2019)

2.1.3.3 Temperature and pH

Most enzymes perform best at optimal conditions of their host cells, so naturally lipases produced by mesophollic microbes would perform best at mesophilic temperatures and thermophilic microbes would produce thermostable lipases (Zheng *et al*., 2011). The formation of microbial lipases is also influenced by temperature. In the shake flask process, the optimal temperature is critical for enzyme secretion. Bharathi and Rajalakshmi (2019) reported that at 37˚C, the biomass concentration of lipase is found to be higher. An increase in temperature up to 38˚C can also enhance lipase production. Lower temperatures reduce the synthesis of lipase enzymes, whereas higher temperatures affect enzyme activity (Bharathi and Rajalakshmi, 2019).

Lipases, in general, act in a wide pH range. Typically, bacterial lipolytic enzymes tend to act in alkaline pH conditions, while fungal lipases prefer acidic conditions (Mehta *et al*., 2017). *Rhodotorula glutinis* HL25 produced a high yield of lipase when the pH of the processing medium was held at a near-neutral level. In contrast, acidic pH usually enhances the production of fungal lipases, however there has been huge variations in pH optima as research on lipases continues (Ramakrishnan *et al.,* 2016).

2.1.3.4 Fermentation techniques

Submerged fermentation and solid-state fermentation systems are the most commonly used techniques to produce lipase enzyme from microorganisms (Treichel *et al.,* 2009). The major factor or limitation for the expression of lipase enzyme is the carbon source since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid or any

other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts, and glycerol (Teng and Xu, 2008).

2.1.3.4.1 Submerged fermentation system.

The growth of microorganisms in a mostly liquid environment is referred to a submerged fermentation (Suci *et al*., 2018). This form of fermentation involves growing of the microorganism as a suspension in a liquid medium containing various and essential nutrients that are either dissolved or suspended as particulate solids. Submerged fermentation is common in the industrial synthesis of enzymes, antibiotics, and other products (Lima *et al.,* 2019). The breakdown of the nutrients by the microorganism being cultivated results in the formation of biomass, enzymes and secretion of bioactive compounds (Sharma *et al.,* 2017).

Fed-batch fermentation and continuous fermentation are the two most effective ways for submerged fermentation. Sterilized growth nutrients are applied to the culture in fed-batch fermentation. It is most common in the biology specialized industry because it happens as biomass grows or multiplies in the fermenter. It aids in increasing cell density in the bioreactor and is usually extremely condensed to avoid dilution. Through supplying nutrients to the culture, the rate of growth is sustained, and the chance of overflow metabolism is reduced. For continuous fermentation, an open system is used. Then, at the same rate as the converted nutrient solution is being recovered, sterilized liquid nutrients are steadily and constantly added to the bioreactor. Consequently, the fermentation broth is regenerated at a constant rate. Parameters such as temperature, pH, dissolved oxygen and carbon dioxide levels must be controlled to ensure optimal fermentation (Ogidi *et al.,* 2020).

Submerged fermentation has previously been shown to be extremely successful in the large-scale development of industrially essential enzymes such as lipases, pectinases, and cellulases. (Snellman and Colwell, 2004). Submerged fermentation has a number of benefits over traditional solid-state fermentation, particularly incorporating the natural environment for many microorganisms, improved oxygen circulation, lower energy and cost requirements, less operational issues, less impact on downstream production, higher efficiency, compactness of the fermentation vessel, and lower initial and recurring costs (Castilho *et al.,* 2000).

2.1.3.5 Purification of lipases

Most industrial processes require enzymes, including lipases, in the purified and highly concentrated form, hence it is important to purify lipases after the fermentation process. The main constraints in traditional purification strategies include low yields and lengthy processing time. Industries look for purification strategies that are inexpensive, rapid, high yielding and amenable to large-scale operations. (Palekar *et al*., 2000)

Most of the microbial lipases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted thus far have used a precipitation step, with 60% of these using ammonium sulphate and 35% using ethanol, acetone or an acid followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography. Precipitation is usually used as a fairly crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation (Saxena *et al*., 2003).

Ion exchange chromatography is the most common chromatographic method used in 67% of the purification schemes analysed and in 29% of these procedures. The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange (58%) and the carboxymethyl (CM) in cation exchange (20%). Strong ion exchangers based on triethylaminoethyl groups and Q-Sepharose are becoming more popular in lipase purification. Gel filtration is the second most frequently employed purification method, used in 60% of the purification schemes and more than once in 22% of them. Affinity chromatography has been used as a purification step in 27% of the schemes (Bharathi and Rajalakshmi, 2019 ; Saxena *et al*., 2003).

The usual procedures of lipase purification are sometimes troublesome, time consuming and result in low final yields. Some novel purification technologies have recently been applied to the purification of lipases. These include membrane processes, immunopurification, hydrophobic interaction chromatography employing epoxy-activated spacer arm as a ligand and polyethylene glycol–Sepharose gel, poly(vinyl alcohol) polymers as column chromatography stationary phases and aqueous two-phase systems. (Bharathi and Rajalakshmi, 2019).

2.1.4 Applications of lipases

Microbial lipases are considered very important for several applications (Fig 2.4) and it has accelerated the search for new lipases and variations from natural sources (Chandra *et al*., 2020). Due the enantioselective catalytic behaviour and region-selective properties of lipases, they are one of the most versatile catalysts in lipid biotechnology. Lipases can be used in several industrial processes, such as the production of emulsifiers and pharmatueticals, development of flavours in dairy products, hydrolysis of fat, biodegradation and synthesis of

thermoplastics, transesterification of oils and fats to produce biodiesel, as well as the synthesis of chiral organic compounds, detergents and the bioremediation of industrial and domestic waste Table 2.3 summarises some potential lipase – producing strains and their industrial applications (Almeida *et al*., 2019).

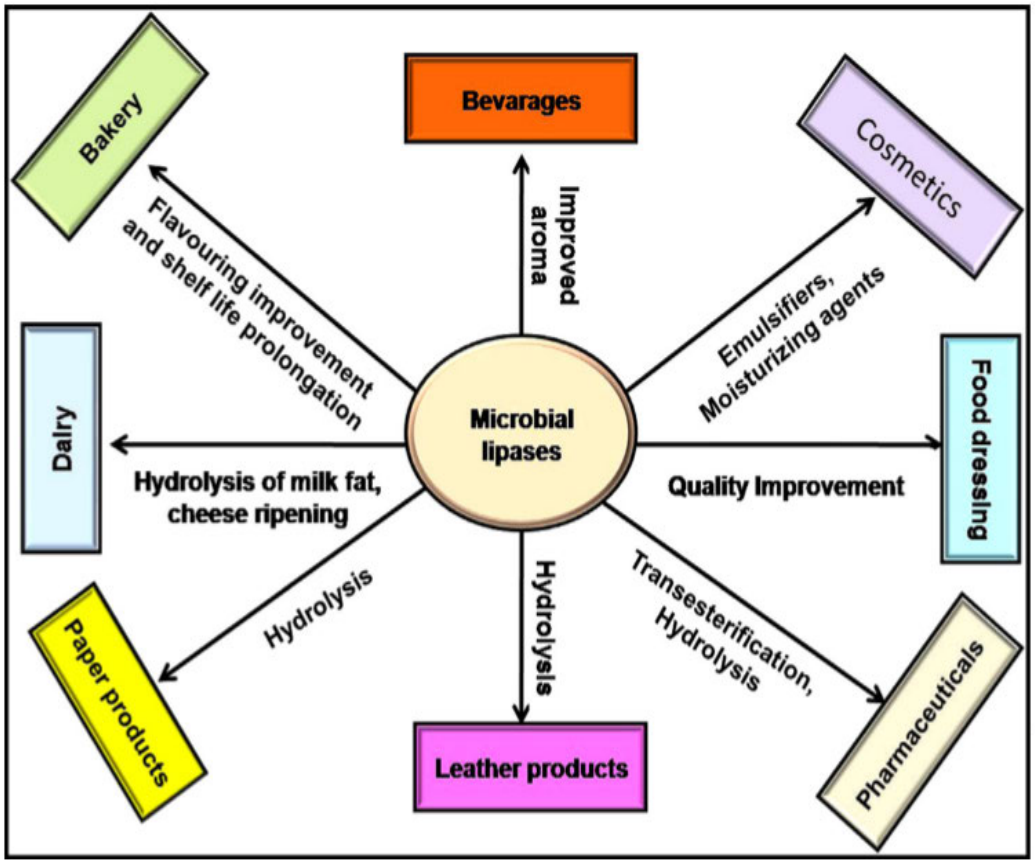

Fig 2.4: Various industrial applications of lipases (Angajala *et al*., 2016).

Table 2.3: Microbial strains cited in the recent literature as potential lipase producers and their industrial applications (Almeida *et al*., 2019; Chandra *et al*., 2020).

Microbial Source	Applications	Reference
Aspergillus flavus	Accelerated stain elimination; pharmaceutical, polymers and biodiesel production	Skagerlind *et al*. (2007)
Aspergillus niger	Accelerated cheese production and dough conditioning	Knob *et al*. (2020)
***Aspergillus nigei* GZUF36**	Potential in the synthesis of functional oils	Contesini *et al*. (2010)
Aspergillus oryzae	Synthesis of saturated fatty acid, crude oil hydrocarbons degradation and cheese production	Celligoi *et al.,* 2017
Candida antarctica	Enrichment of fats and oils, pitch control in pulp and paper industry, elimination of bad cholesterol for treatment of Alzheimer's disease	Tecalão *et al.* (2012); Andualema and Gessesse, (2012)
Candida lipolytica	Production of fatty acids and cheese ripening	Sarmah *et al*. (2018)

Candida parapsilosis	Food additive	Lan *et al*. (2011)
Candida rugosa	Aerobic waste treatment andhuman milk fat substitute	Glogauer *et al*. (2011)
Candida tropicalis	Degradation of hydrocarbons in crude oil	Farag and Soliman (2011)
***Fusarium solani* NFCCL 4084**	Biodiesel production	Fickers *et al*. (2005)
Geotrichum candidum	Preparation of chiral intermediates for pharmaceutical compounds	Andualema and Gessesse (2012)
Meyerozyma guilliermondii	Animal feed industry (cheese whey)	Knob *et al*. (2020)
Penicillium abeanum	Enrichment of tuna oil	Li and Zong (2010)
Penicillium camembertii	Saturated triacyl glycerides synthesis and glycerolglycolipids production	Khumalo *et al*. (2002)
Penicillium chrysogenum	Treatment of waste in the food industry	Chinedu *et al*. (2010)
Penicillium roquefortii	Production of characteristic flavours of blue cheese in the cheese industry	Li and Zong (2010)

Rhizomucor miehei	Biocatalyst in cosmetic and personal care products, synthesis of flavour esters and surfactant for baking and dairy industry	Khumalo *et al*. (2002); Woodley (2008)
Rhizopus arrhizus	Production of flavour esters	Akacha and Gargouri (2015)
Rhizopus nodosus	Fur and fat removal in leather production	Khambaty (2020)
Thermomyces lanuginosus	Detergent production, biodiesel production, modification of fats and oils for healthy food production	Skjold-Jørgensen *et al*. (2017)

2.1.4.1 Food industry

Today, the modification of fat and oils is one of the key areas that requires innovative and eco-friendly technologies in the food processing industry. Lipases play a vital role in oil transformation in the food industry. Vegetable oils are tailor made by altering physiochemical properties and with nutritionally significant structured triacylglycerols have immense potential in the future market (Hasan *et al*., 2006).

Lipases are used in the production of baked goods, dairy products and fruit juices as well as to produce modified acylglycerols through interesterification of oils and fats (Ghorai *et al*., 2011). Lipases are mostly used in the production of food aromas that imitate complex natural aromas (Trbojević Ivić *et al*., 2016). Lipases from *Penicillium roquefortii* have been used in

the enhancement of blue cheese, because they can synthesize methyl ketones that give the distinctive flavour of blue cheeses (Cao *et al*., 2014). *Burkholderia cepacia* lipase has been applied in the food and bakery industry for the synthesis of furfuryl acetate which is used as flavouring agent (Mathpati *et al*., 2016). Trans-fatty acid free margarine can be produced from triacylglycerides using lipases and as well as the hydrolysis of food waste for biomethane production (Sellami *et al*., 2012; Meng *et al*., 2015).

2.1.4.2 Biodiesel production

Lipases are the cog of biodiesel production and other structured lipids through the modification of fats and oils. The reaction (Fig 2.5) occurs in a highly aqueous environment that favours the waste oils that are used, which usually contain a high-level of water molecules (Yu *et al*., 2016). The disadvantage of this reaction is the presence of glycerol, therefore, to overcome this problem, researchers have been focusing on the production of biodiesel like fuel that maintains glycerol as monoglyceride. Lipases that are specific for sn-1,3-position are applied to produce a biofuel that contains a mixture of glycerol derivatives and fatty acid methyl esters or fatty acid ethyl esters. The latter can therefore inhibit the need to separate glycerol from biodiesel thereby enhancing the process yield (Calero *et al*., 2014; Escobar *et al*., 2014). An example of this the patented product called Ecodiesel-100, which is produced from using a lipase from pig pancreas that is partial 1,3-regiospecific alcoholysis (Caballero *et al*., 2009).

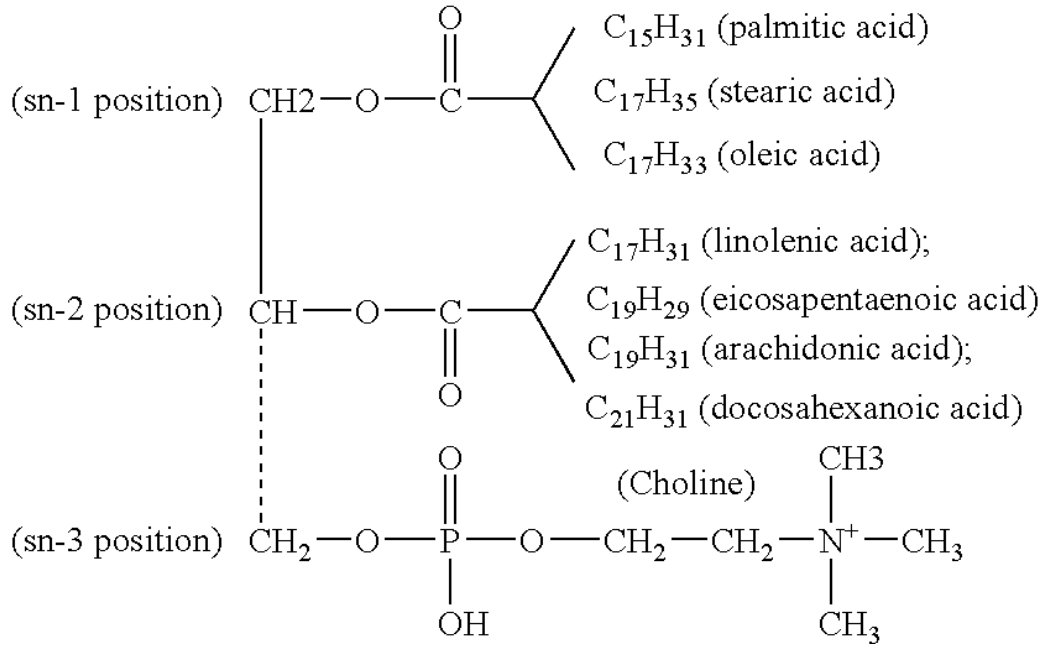

Fig 2.5: Lipase catalysed transesterification illustration (Chen and Kwong, 2015).

2.1.4.3 Bioremediation

The search for more sustainable and eco-friendly waste treatment and environmental preservation techniques has increased. One technique that can be applied is the use of extracellular enzymes such as lipases, proteases and esterases to reduce the waste footprint. These enzymes are able to hydrolyse ester bonds which are normally hard to attack assisting many other microorganisms to be able to utilise the waste as nutrient source. These enzymes can be able to improve the bioremediation of oily effluents producedby food industries, health resorts or hospitals (Basheer *et al*., 2011). Lipases are broadly used mediator enzymes in organic synthesis because of their vast range of substrate selectivity, they do not require a cofactor and are stable in organic solvents. A thermoalkalophilic bacterial lipase was used in the development of biosensors that can detect water pollution (Herranz *et al*., 2018). Okino-Delgado *et al*. (2017) reported the use of orange lipase for the bioremediation of cooking oils.

2.1.4.4 Pharmaceutical industry

The application of lipases in the pharmaceutical industry has increased due to their therapeutic benefits. Numerous pharma products have been certified or are in clinical trials for the treatment of cardiovascular, inflammation and pain, anxiety and obesity (Nomura and Casida, 2016). The use of lipases in the synthesis of optically active compounds (pure alcohols, carboxylic acids and amines) proves that lipases can be applied in practical synthetic methods (Zhu and Wang, 2011). Polixatel (anticancer drug) can be produced through the synthesis of chiral intermediate compounds by lipases (Fukaya *et al*., 2016)

The enrichment of the mono and diacylglycerides of poly-unsaturated fatty acids by microbial lipases can be applied in the production of a variety of pharmaceutical products (Patel *et al*., 2018). Liposomes can be applied to reduce drug wastage and overcome other anatomical barriers by delivering the drug to the target areas (Lee *et al*., 2015). Lipases can be used to synthesise pure ibuprofen via hydrolysis and esterification (Xie *et al*., 1998). *Bacillus* lipase that is selective to fatty acids chain length of ester has been used in the synthesis of enantiopure compounds (Guncheva and Zhiryakova, 2011).

Lipases are used to enrich polyunsaturated fatty acids (PUFAs) and their by-products monoacylglycerides and diacylglycerides are widely used to produce different pharmaceuticals (Sarmah *et al*., 2018). Because of the metabolic benefits of polyunsaturated fatty acids as many of these polyunsaturated fatty acids are essential fatty acids, they are increasingly used as pharmaceuticals over years. For example, many polyunsaturated fatty acids are essential for the synthesis of lipid membranes and prostaglandins in human bodies. Most of drugs produced are chiral and only few drugs have the desired biological activities in certain stereoisomers while the others may contain harmful side effects to human bodies (Xu *et al*., 2019). So, one of the most important properties of lipase is that they are able to differentiate between

enantiomers, which makes them a promising and useful biocatalyst in the pharmaceutical industry.

2.1.5 Thermostable lipases

Thermostable lipases have growing importance for different applications. Mesophilic producers have been used in most of the studies so far. Thermostability of enzymes presents advantages allowing higher operation temperatures which provide higher reactivity (higher reaction and diffusional rates), higher process yield, lower viscosity and the reduced risk of microbial contamination. Thermostable enzymes can be obtained from mesophilic and thermophilic organisms. The obvious source of thermostable enzymes is thermophilic organisms because they will confer their proteins a high thermal stability (Verma *et al*., 2021). In recent years, there has been a great demand for thermostable enzymes in industrial fields. They have been applied to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavours. Therefore, thermostable lipases from various sources have been purified and characterized (Kim, *et al*., 1998; Kormanová *et al*., 2020). Since they can be produced at low cost and exhibit improved stability, thermostable lipases from microorganisms are very advantageous. The optimum temperature for a thermostable lipase from *Thermomyces lanuginosus* was reported at 45˚C (Omar *et al*., 1987). In another study, a lipase from *T. lanuginosus* Y-38 and found that the enzyme was found to have an optimal temperature of 60˚C, at pH 8.0. It also had stability in the pH range of 4 to 11. It showed increasing activity at up to 65˚C but was inactivated on heating at 80˚C for 20 min (Maheshwari *et al*., 2000).

A lipase from a probable *Geotrichum* strain was also found to be thermostable and showed very high activity after 60 minutes incubation at 60˚C (Ginalska, *et al*., 2004). Alkalophilic

thermophiles are mainly preferred in detergent and leather industries. Lipases which are stable at pH ranges from pH 10 to 11, at temperatures from 30 to 60˚C and in the presence of surfactants, are preferred as ingredients of laundry detergents (Maheshwari *et al*., 2000). In various fields of industry, such as chiral molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents, alkaline enzymes should find additional uses (Horikoshi 1999).

Reactions catalysed by lipases are often carried out in mixtures of the reactants to avoid the use of the solvents and for this, reaction mixture has to be heated from 50˚C to 80˚C for the fat to be liquid. Therefore, lipases must be thermostable for optimal performance (Zamost *et al*., 1991). This has led to a search for more thermostable lipases for industrial applications. Unfortunately, most wild type sources of lipases cannot fulfil this demand, leading to an increase of research in protein engineering.

2.2 Protein engineering

Although biological catalysts are highly attractive for chemical synthesis, many of them are not employed in most industrial processes due to various limitations (Agouridas *et al*., 2019). These limitations include demanding physiological parameters such as high temperature, low or high pH, high salt concentration, low stability, poor activity in non-aqueous media and many additional requirements that may increase production costs (Oh *et al*., 2018). Thermostability is generally one of the prerequisites for any enzyme to be applicable in industrial processes because thermostable enzymes commonly have greater resistance to chemical denaturants, high alkalinity or extreme acidity, and increased substrate solubility (Kim *et al*., 2015). The effects of salt concentration and inhibitor concentration on the enzyme have a profound impact on its suitability and performance as an industrial biocatalyst (Santhanam *et al*., 2011; Mate and Alcalde, 2015).

Protein engineering offers a compelling pathway towards designing enhanced industrial enzymes through directed evolution (Rodgers *et al*., 2010; Santhanam *et al*., 2011). Direct evolution has been the strategy of choice for tailoring the catalytic, biophysical and molecular properties of target proteins. This technique mimics natural evolution but in a shorter period, and involves an iterative two-step protocol that initially generates molecular diversity by random mutagenesis and in-vitro recombination, followed by the screening of library members with improved properties (Lutz, 2010). These iterative cycles of mutagenesis and screening have been reported to successfully enhance thermostability, tolerance to organic solvents, enantio-selectivity, and substrate specificity (Jäckel and Hilvert, 2010). However, this creates a need for functional expression in a host suitable for the production of large quantities of the improved enzyme. There's still a serious bottleneck in the expression and production of acceptable levels of enzymes for industrial applications (Arnold, 2018; Morrison and Podracky, 2020).

Many scientists have looked at using different expression systems to improve the production rate of recombinant enzymes. Numerous hosts that have been used are not natural hosts of the target recombinant enzyme (Arnold, 2018). Therefore, may not improve productivity because the enzyme is either produced at low amounts, stored where it is not easily accessible (and may require expensive downstream processing) or the host does not have a good transport system to export the enzyme (Wang *et al*., 2021). To overcome this problem, expressing in a host that secretes most of its proteins or a natural host is generally preferred because these hosts allow for easier purification and downstream processing. Thus preventing feedback inhibition that may be facilitated by the build-up of the enzyme in the cell (Arnold, 2018; Morrison and Podracky, 2020). Further, when using a natural host there is no need for added promoter genes or secretion signals because the host is finely tuned to produce the recombinant protein (Wang *et al*., 2021).

2.2.1 Bioinformatics on lipases

Bioinformatics and functional metagenomics are fast becoming very useful tools with great potential. These tools are very successful in identification of novel enzymes and explore their natural benefits even further. Recently, some lipolytic enzymes have been screened from the metagenomics database using "Reconstruction of Ancestral Sequence", is an advanced bioinformatics approach to categorise, analyse and manipulate microbial lipases of interest (Verma *et al*., 2019). By 2020, more than 75000 true lipase sequences had been reported in the public domain database UniProt (www.uniprot.org), out of which only 182 were reviewed. This translates to thousands of possibilities for better performing lipases for commercialisation thus enhancing healthy competition towards greener technologies. A phylogenetic tree, showing some explored microbial lipases from algae, archaea, bacteria and fungi which have been analysed and categorised into families and superfamilies, (Fig 2.6) gives an insight in their potential evolutionary patterns (Verma *et al*., 2021).

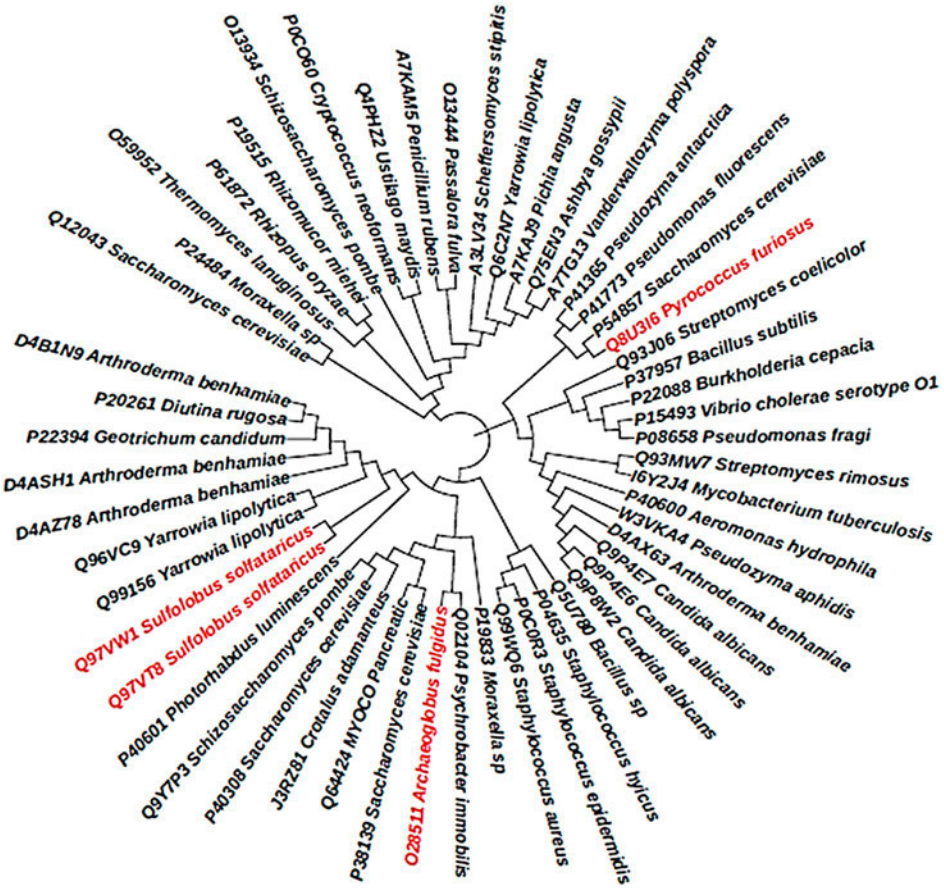

Fig 2.6: Phylogenetic tree representing evolutionary analysis of microbial lipases from bacteria, fungi, algae and archaea (Verma *et al*., 2021).

Over the past years, scientists have also been able create some lipase crystal structures to enhance in-depth understanding of the biochemical functionality of the enzyme, these are available over a number of international protein databases (Verma *et al*., 2021). The availability of these protein models have found greater user over the past few years and play an instrumental role in bioinformatical studies related to the enzymes of interest. A summary of some of the fungal lipase molecular structures available in the protein database, dating back to 1992 when the protein model of a lipase from *Rhizomucor miehei* was discovered by Derewenda *et al.* (1992), is represented in Table 2.4.

Table 2.4: Protein models and crystal structures available in the literature for some fungal lipases (Verma *et al.*, 2021)

Fungal Sources / Protein Database Identity	**Publication Title**	**References**
***Moesziomyces antarctica* / 3ICV**	Structural consequences of a circular permutation on lipase B from *Candida antartica*	Qian *et al*., 2009
***Diutana rugosa* / 1GZ7**	Crystal structure of the closed state of lipase 2 from *Candida rugosa*	Mancheño *et al*., 2003
***Fusarium graminearum* / 3NGM**	Crystal structure of lipase from *Gibberella zeae*	Lou *et al*., 2010
***Geotrichum candidum* / 1THG**	A refined structure of the lipase from *Geotrichum candidum*	Schrag and Cygler, 1993
***Penicillium camembertii* / 5CH8**	Crystal structure of MDLA N225Q mutant from *Penicillium cyclopium*	Tang *et al.*, 2016
***Rhizomucor miehei* / 3TGL**	Structure and molecular model refinement of *Rhizomucor miehei* triacylglyceride lipase: a case study of the use of	Derewenda *et al.*, 1992

	simulated annealing in partial model refinement	
***Thermomyces lanuginosus* / 5AP9**	Controlled lid-opening in *Thermomyces lanuginosus* lipase - a switch for activity and binding	Skjold-Jørgensen *et al*., 2017

A protein structure for a *Thermomyces lanuginosus* by Skjold-Jørgensen *et al*. (2017) is depicted Fig 2.7.

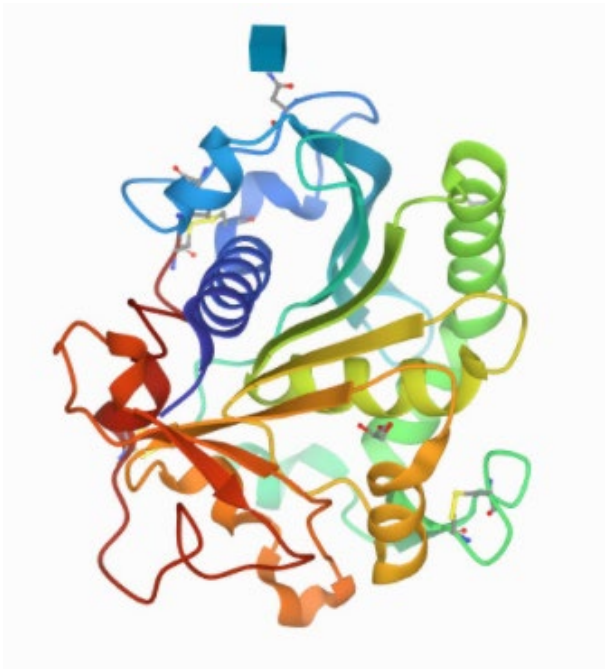

Fig 2.7: Protein structure showing controlled lid-opening in Thermomyces lanuginosus lipase (Skjold-Jørgensen *et al*., 2017).

2.3 Recombinant lipases

Microbial lipases are one of the most commonly researched enzymes in the field of protein engineering. This is due to their use of inexpensive culture media, practical handling, ease of scale-up cultivations and the existence of sufficient knowledge for genetic manipulation for expression hosts such as *Pichia pastoris, Saccharomyces cerevisae* and *Escherichia coli* (Samoylova *et al*., 2019). Most lipases that have been researched are of bacterial origin such as *Achromobacter, Alcaligenes, Bacillus, Geobacillus, Pseudomonas and Streptomyces* among others. Lipases from *Bacillus* species seem to be the most widely used due to their thermostability and high tolerance to organic solvents (Kormanová, *et al*., 2020)

Lipases produced by yeasts and filamentous fungi are more attractive for industrial applications due their unique thermal stability and substrate specificity (Ghasemian and Moradpour, 2019). Lipases from *Aspergillus, Candida, Geotrichum, Penicillium, Rhizopus, Rhizomucor, Thermomyces, Trichosporon* and *Yarrowia* have been identified as having the highest potential for industrial applications and commercialisation (Borrelli and Trono, 2015). Some of these fungal lipases have already been optimised for industrial production (Fig 2.8) and have been commercialised. These are as follows (Atalah *et al*., 2019):

- Lipase B (CALB) from *Candida antarctica* (Novozym®435 and NS 88011);
- Lipozyme TL IM from *Thermomyces lanuginosus* (Novozymes, Copenhagen, Denmark);
- Novozym 40.086 from *Rhizomucor miehei*; and
- Lipase FE-01 from *A. oryzae* (ASA Spezialenzyme GmbH, Wolfenbüttel, Germany).

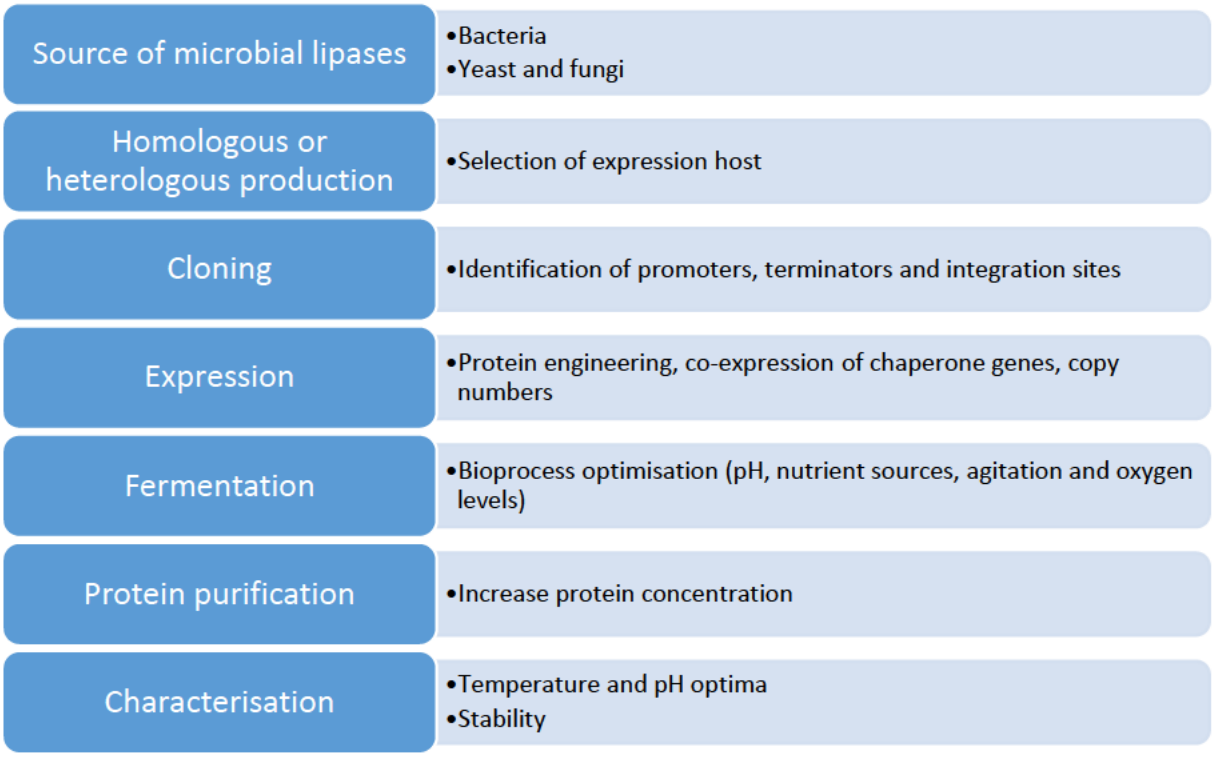

Fig 2.8: Sequential approach for recombinant lipase production (Kormanová, *et al.*, 2020).

2.3.1 *Pichia pastoris* as a suitable host for expression of recombinant lipases

A methylotrophic yeast capable of growing in methanol as its sole carbon source *P. pastoris* is one of the midst studied eukaryotic expression systems accounting for 35% of all heterologously produced lipases according to Borello and Trono in 2015. This is due to the ease of manipulation; ability to perform complex post-translational modifications; and high expression levels associated with *P. pastoris* (Kormanová *et al*., 2020).

Over the years *P. pastoris* has been used extensively to express recombinant proteins which can be scaled up to fermentation to meet larger demands. It can be produced in higher cell densities compared to other expression hosts, thus resulting in increased protein productivity (Yang and Zhang, 2018). It has adapted to using raw carbon and nitrogen sources thus requiring low nutritional requirements. These properties conquer *P. pastoris* very cost effective for

commercial production of lipases since its nutrient sources are easily available as industrial by-products (Kormanová *et al*., 2020).

This yeast was not always considered to be genetically amenable as *S. cerevisiae*, which is another suitable eukaryotic host for heterologous expression, but genetic advances over the years has paved the way for *P. pastoris* (Spohner *et al.*, 2015). Over the last decade, research has focused towards genetics and use in the lipase application industry (Borrelli and Trono, 2015; Demain and Vaishnav, 2009).

The yeast *P. pastoris* has two major advantages over *S. cerevisiae*. Firstly, it posesses of the methanol-inducible alcohol oxidase 1 gene (*AOX1*). This is a tightly regulated gene and is repressed in the absence of methanol, and when used to drive heterologous protein production, avoids toxic effects of heterologous protein expression until expression of the product is induced by methanol. Secondly, *Pichia* can be grown to higher densities than *S. cerevisiae* (Hartner and Glieder, 2006). Table 2.5 summarises the recent lipases cloned and expressed in *P. pastoris* from different microbial sources.

Table 2.5: Summary of recent recombinant lipases cloned and expressed in *Pichia pastoris*

Enzyme	Enzyme source	Expression Host	Expression Vector	Molecular Mass (kDa)	Optimal Temperature and pH	References
Lipase MAS1	Marine *Streptomyces* sp. strain W007	*P. pastoris* X-33	pPICZαA	30	25˚C, 8.0	Lan *et al*. (2016)
r27RCL	*Rhizopus chinensis*	*P. pastoris* GS115	pPIC9K	37	37˚C, 8.0	Sha *et al.* (2013)
CALB	*Candida Antarctica*	*P. pastoris* GS115	pPICZαB	33	40˚C, 7.0	Samuel *et al*. (2013)
CALB	*Candida Antarctica*	*P. pastoris* X-33 and M12	pBluescript II SK	33	40˚C, 7.0	Robert *et al*. (2019)
ROL	*Rhizopus oryzae*	*P. pastoris* GS115	pPICZα-ROL	35	35˚C, 8.0	Jiao *et al*. (2018)

ROL	*Rhizopus oryzae*	*P. pastoris* X-33	pPICZFLDα ROL	35	30˚C, 7.25	Resina *et al*. (2005)
FSL	*Fusarium solani*	*P. pastoris* X-33	pPICZαA-FSL and pGAPZα-FSL	30	35˚C, 7.0	Wongwatanapaiboon *et al.* (2016)
TDL	*Thermomyces dupontii*	*P. pastoris* X33	pPICZαA	30	60˚C, 9.5	Wang *et al*. (2019)
LN	*Thermomyces lanuginosus*	*P. pastoris* GS115	pPIC9K	33	60˚C, 9.0	Fang *et al*. (2014)

2.3.2 *Escherichia coli* as a host for expression of recombinant lipases

One of the most popular and most widely used expression systems is the Gram-negative bacterium *Escherichia coli*, which from the point of view of total general costs and effectivity usually stands as a first choice. High level of gene expression, fast growth, high cell density and possibilities for scale-up applications are advantages of this host platform. *E coli* is considered as the easiest, quickest, and cheapest host with a fully known genome. However, high-level production of recombinant proteins still suffers from multiple bottlenecks. A significant portion of the genes of interest is not efficiently expressed in this system due to low expression levels, the fact that expressed proteins are accumulated within aggregates, product is degraded or lacks its biological activity. Thus, numerous modifications have been carried out on this expression host to optimize it as a good candidate for protein expression and; as a result, several engineered strains of *E. coli* have been designed (Hayat *et al*., 2018).

There is a wide spectrum of designed *E. coli* strains with various genotypes that can potentially solve above-mentioned problems. However, in some cases, the only effective approach is the implementation of new alternative hosts with unique features. One of the most important factors of the *E. coli* platform is its rapid growth. Wild-type *E. coli* has a doubling time of about ~ 30 min in nutrient rich medium. The approach of adaptive laboratory evolution has been used for development of new fast-growing *E. coli* strains (LaCroix *et al*., 2015; Kormanová, *et al*., 2020).

Successful protein expression in *E. coli* requires a good knowledge of physicochemical properties of recombinant proteins, good selection among common strains of *E. coli* and vectors, as well as factors related to growth media including time, temperature and inducers

(Hayat *et al*., 2018). Table 2.6 summarises recent lipases that have been cloned and expressed in *E. coli* BL21 (DE3).

Table 2. 6: Summary of recent recombinant lipases cloned and expresses in *Escherichia coli* BL21 (DE3)

Enzyme	Enzyme Source	Expression Host	Expression Vector	Molecular Mass	Optimal pH and Temperature	References
Lipase KV1	*Acinetobacter haemolyticus*	*E. coli* BL21 (DE3)	pET-30a (+)	39 kDa	40˚C, 8.0	Batumalaie *et al*. (2018)
Lipase BaG7Lip	*Bacillus amyloliquefaciens G7*	*E. coli* BL21-Star (DE3)	p15TV-L	26 kDa	50˚C, 8.0	Khan *et al*. (2020)
Lipase LipBC (LipA) + foldase LipBC (LipB)	*Burkholderia contaminans* LTEB11	*E. coli* BL21 (DE3)	pET28a(+) and pT7-7 for LipA and LipB, respectively	36 kDa (LipA), 37 kDa (LipB)	25-45˚C, 6.5-10.0	Alnoch *et al*. (2020)

Lipase LipBT and foldase LifBT	*Burkholderia territorii* GP3	*E. coli* DH5α, *E. coli* DH10β, *E. coli* BL21 (DE3) pLysS, *E. coli* Origami B, *E. coli* Shuffle B, and *E. coli* Shuffle K	pGEM-T Easy (*E. coli* DH5α), pET15b (*E.coli* DH10β)	30 kDa	80˚C, 11.0	Putra *et al*. (2019)
Lipase Ca-Est	*Clostridium acetobutylicum* (ATCC 824)	*E. coli* BL21 (DE3)	pMCSG7	29 kDa	60˚C, 7.0	Nagaroor and Gummadi, 2020
Lipase Lip3	*Drosophila melanogaster*	*E. coli* BL21 (DE3)	pETMCSIII	43 kDa	-	Alfaro-Chávez *et al*. (2019)
Lipase HT1-5M	*Geobacillus zalihae*	*E. coli* BL21 (DE3) pLysS	pLysS	44 kDa	70˚C, 9.0	Ishak *et al.* (2019)

Lipase GnMgl	*Glaciecola nitratireducens* FR1064	*E. coli* BL21 (DE3)	pET22b	39 kDa	30˚C, 9.0	Li *et al.* (2020)
Lipase LipPN1	*Proteus* sp. NH 2-2	*E. coli* BL21 (DE3)	pET-28a(+)	31 kDa	40˚C, 9.0	Shao *et al*. (2019)
G55y, T52Y and AMS8 recombinant lipases	*Pseudomonas fluorescens* AMS8	*E. coli* BL21 (DE3)	pET32b	-	-	Yaacob *et al*. (2019)
Lipase LSK25	*Pseudomonas* sp. LSK25	*E. coli* BL21 (DE3)	pET32b(+)	65 kDa	30˚C, 6.0	Salwoom *et al*. (2019)

2.4 Scope of study

The Enzyme Technology Research Group in the Department of Biotechnology at the Durban University of Technology has been doing extensive research on the different enzymes produced by *Thermomyces lanuginosus* SSBP strain since it was isolated over a decade ago (Singh *et.al*., 2000). Previous studies have revealed that this microbe utilises various enzymes that have a significant impact in improving a lot of industrial applications. Some of these enzymes include amylases, glucoamylases, xylanases, lipases, phytases, and chitinases. Whilst the group has substantially researched most of these enzymes, minimal research has been focussed on the *T. lanuginosus* lipase.

The genome of *T. lanuginosus* SSBP has been sequenced to acquire the necessary information required to utilise and improve its industrial application potential (Mchunu *et al*., 2013). Although other *T. lanuginosus* lipases have already been reported in the literature, three novel lipases were identified after analysis of the genome. Thus this study aims to clone and express three the lipase genes from *Thermomyces lanuginosus* SSBP in *Pichia pastoris* GS115 and *Escherichia coli* BL21, and characterise the recombinant lipases.

The objectives of the study were to: a) conduct a bioinformatics study of the lipase; b) synthesise the lipase genes from the cDNA of *Thermomyces lanuginosus* SSBP; c) clone and express the lipase genes into *Pichia pastoris* GS115 and *Escherichia coli* BL21 (DE3); d) purify the lipases; e) characterise the lipases in order to establish optimal production; f) investigate the lipases' thermostability profile; and g) investigate the lipases' substrate affinities.

3. MATERIALS AND METHODS

3.1 Bioinformatical analysis

T. lanuginosus SSBP predicted nucleotide and amino acid sequences of three lipase genes were selected from previous data set produced in our research group (Mchunu *et al*., 2013). DNA and protein sequences were edited using CLC Main Workbench V21.0.5 (Qiagen). The sequences were analysed using SignalP (Binert *et al*., 2017), to identify the presence of signal peptides and MUSCLE (Edgar, 2004a; Egdar, 2004b) for sequence alignment and comparison. Conserved domain analysis of the putative proteins was performed using the NCBI (National Center for Biotechnology Information) Conserved Domain Search (https://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi) and InterProScan (http:// www.ebi.ac.uk/Tools/Inter ProScan) (Waterhouse *et al*., 2018). The three-dimensional (3D) structures of the three putative lipases were predicted using the SWISS MODEL web tool using their top selected models as templates (Guex *et al*., 2009; Studer *et al*., 2020). No evaluation or validation of the models were performed as most of the templates had low sequence identity.

3.2 Growth and maintenance of *T. lanuginosus*

T. lanuginosus SSPB (isolated in South Africa) was grown on potato dextrose agar at 50˚C for 5 days before inoculation into liquid medium containing 0.1% KH_2PO_4, 2% glucose, 3% yeast extract at pH 6.5 (Singh *et. al.*, 2000). *T. lanuginosus* was maintained in potato dextrose agar slants and sub-cultured after two months. A one cm^2 plug of the culture was excised from a plate inoculated into liquid medium supplemented with 1% olive oil and grown for 2 days at 50˚C with shaking at 150 rpm. Twenty millilitres of culture was harvested by centrifugation at

2000 ×*g* for 10 min. Pellets were snap frozen in liquid nitrogen then stored at -80˚C for total RNA isolation.

3.3 Total RNA isolation

Total RNA from *T. lanuginosus* was isolated using a combination of Trizol reagent (Sigma) and Plant Total RNA Isolation kit from Qiagen. This was done by adding 5 ml of Trizol reagent to a frozen pellet of *T. lanuginosus,* followed by vortexing until the pellet thawed and was resuspended in Trizol reagent. Thereafter 200 µl phenol-chloroform-isoamyl alcohol (25:24:1) of was added to the sample for every 1 ml of trizol. The tube was then vortexed for 15 s and incubated at room temperature for 5 min. This was followed by centrifugation at 4˚C for 10 min at 10 000 ×*g*. The aqueous top layer of the mixture was transferred to a new microcentrifuge tube, followed by addition of an equal volume of ice - cold ethanol. The resulting mixture was transferred to a spin column in 700 µl aliquots and centrifuged at 5000 ×g for 15 s. The columns were washed with 500 µl of ethanol-containing RPE wash buffer. To remove contaminating DNA, on-column digestion was performed by adding 100 µl DNase supplied by Qiagen on to the column and incubating for 30 min at room temperature. The columns were washed again with 500 µl of RPE wash buffer followed by centrifugation at 5000 ×*g* for 2 min. The columns were dried by centrifuging them at 10 000 ×*g* for 1 min. The total RNA was eluted with 30 µl of RNase - free water. Thereafter, the samples were kept on ice or stored at -70˚C for further analysis. Total RNA quantification and integrity verification was done using a NanoDrop 2000 analyzer (Thermo Scientific), and by 1.2 % agarose gel electrophoresis, before proceeding with cDNA synthesis.

3.4 RNA quantification

Quantification of nucleic acids was done using the NanoDrop 2000 analyzer. The quantification was based on the assumption that an absorbance of 1 at optical density (OD) of 260 nm corresponds to 40 ng RNA/µl (Sambrook and Russell, 2006). Purity of the RNA samples was estimated using the absorbance ratio at 260 nm and 280 nm and RNA a ratio of more than 2 was used for further analysis.

3.5 Agarose gel electrophoresis (RNA)

The quality of RNA samples was also analysed using agarose gel electrophoresis.using 1.2% was used. The gels were prepared by mixing appropriate amounts of agarose and 1× TAE buffer, which was prepared from a 50× TAE stock (242 g Tris, 57.1 ml acetic acid, 100 ml of 0.5 M EDTA, pH 8) and microwaved for 45 s. The gel was allowed to cool to less than 50˚C, then SYBR Green I (Invitrogen) was added to the gel. This was poured into a casting tray with well combs and allowed to solidify at room temperature. The RNA samples containing loading buffer were heated for 5 min at 60˚C to break RNA self-priming then loaded into the gel. An aliquote of a 1000 bp DNA Molecular Weight Marker (Fermentas) was loaded next to the samples and the gel was run at 120 V for approximately 1 h (Bio-Rad Gel Electrophoresis). Gels were then viewed under UV and images captured and stored using a Gel Doc XR System (Bio-Rad).

3.6 cDNA synthesis

For cDNA library preparation, mRNA was isolated from total RNA using the Oligotex mRNA Mini Kit protocol (Qiagen). cDNA was synthesized from mRNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) with 100 µM random hexamer primers (Fermentas). After the second strand synthesis, the cDNA was purified using Qiagen Quick PCR MinElute column (Qiagen) and eluted in 50 ul elution buffer. The cDNA was stored at -20˚C for further use.

3.7 Plasmids, bacterial and yeast strains

The plasmids that were used for *P. pastoris* expression in this study are listed in Table 3.1 and include pPICZαA (Fig 3.1), pPIC9K (Fig 3.2) and pBGP1 (Fig 3.3). *E. coli* strains DH5α (Thermofisher) and BL21 (DE3) (Thermofisher), were maintained on LB media (10 g/l bacteriological peptone, 5 g/l yeast extract, 5 g/l NaCl and 15 g/l agar). *Pichia pastoris* GS115 (Invitrogen) was maintained on YPD media (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose, 20 g/l agar). Selection and maintenance of *E. coli* clones was done on LB media containing 100 µg/ml of ampicillin. *Pichia* clones were selected in YPD media with 100 µg/ml of zeocin for pPICZαA and pBGP1 and 0.25 – 1.0 mg/ml geneticin (G418) for pPIC9K.

Table 3.1: Plasmids vectors used in for *P. pastoris* expression in this study

Plasmid	Characteristics	Source
pPICZα	Ampicillin^{R}, Zeocin^{R}, AOX1 promoter and terminator	Novagen
pPIC9K	Ampicillin^{R}, Kanamycin^{R} that confers resistance to geneticin reagent in *Pichia,* AOX1 promoter and terminator	Novagen
pBGP1	Ampicillin^{R}, Zeocin^{R}, *GAP* promoter and termination cassettes	Lee *et al*., 2006

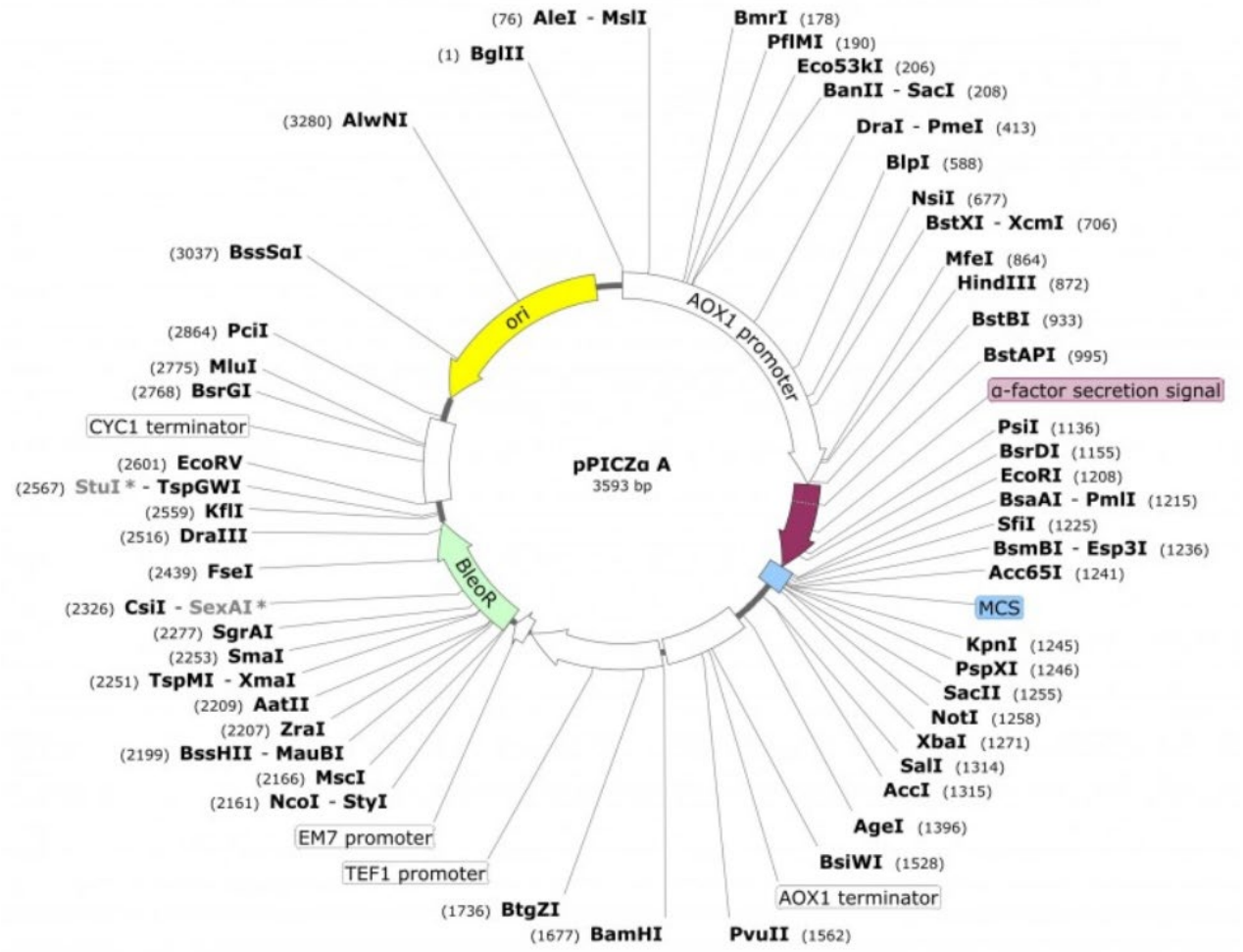

Fig 3.1: Map of pPICZαA used for expression of lipase in *P. pastoris* showing location of the replication region. Restriction sites on the plasmid are shown and their exact positions indicated in brackets and other regions (Novagen). The lipase gene was inserted into pPICZαA using the *Xba*I and *Kpn*I restrictions sites.

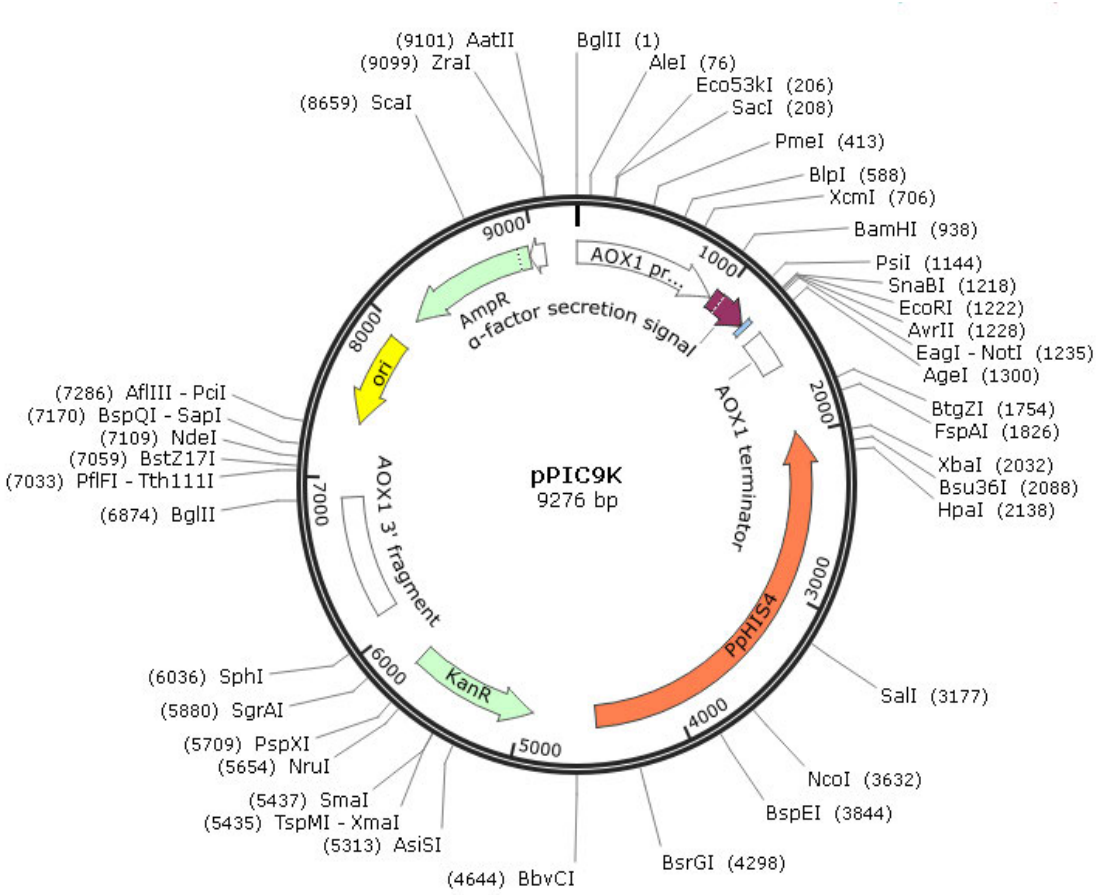

Fig 3.2: Map of pPIC9K used for expression of lipase in *P. pastoris* showing location of the replication region. Restriction sites on the plasmid are shown and their exact positions indicated in brackets and other regions (Novagen). The lipase gene was inserted into pPIC9K using the *Xba*I and *Sna*BI restrictions sites.

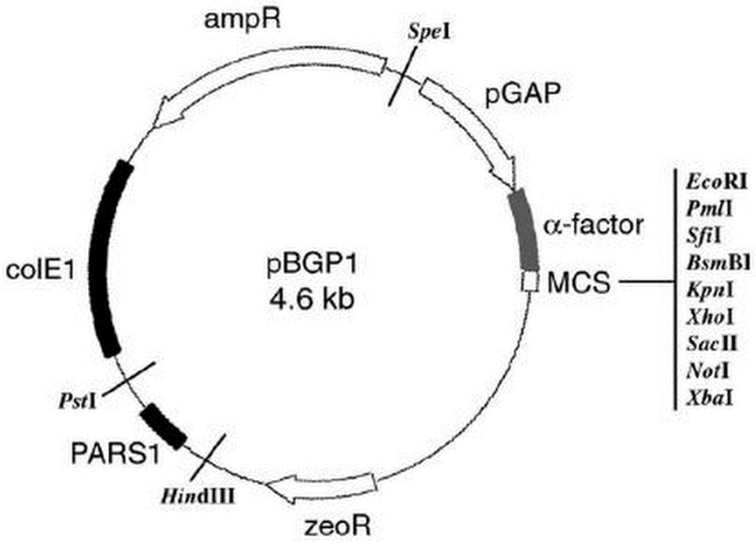

Fig 3.3: Map of the *Pichia* vector, pBGP1, showing the location of the ampicillin resistance (Amp^R) gene, zeocin resistance (Zeo^R) gene, *GAP* promoter (pGAP), secretion signal (α-factor) and multiple cloning sites (MSC) and *E. coli* replicon (colE1) and *Pichia* autonomous replication sequence (PARS1) (Lee *et al*., 2006). The lipase gene was inserted into pBGP1 using the *Xho*I and *Eco*RI restrictions sites.

Plasmids pET100/D-TOPO containing the three putative lipase were synthesised by Thermofischer Scientific (Table 3.2 and Fig 3.4) for expression in *E. coli* BL21 (DE3). These plasmids were designed using three lipase sequences identified in the *T. lanuginosus* SSBP genome by Mchunu *et al*., (2013). Synthesis was carried out by the GeneArt division of Thermofisher, and the plasmids were designated: 18AEWU4C_SiphiA_pET100/D-TOPO (Fig 3.5), 18AEWU3C_SiphiB_pET100/D-TOPO (Fig 3.6), 18AEWU3C_SiphiCB_pET100/D-TOPO (Fig 3.7) (https://apps.thermofisher.com/apps/geneart-qa). These plasmids were renamed as LipA-pET100, LipB-pET100 and LipC-pET100 respectively, and will be referred to as such in this study. These plasmids did not require any further cloning steps and were transformed into *E. coli* for the expression and induction steps.

Table 3.2: Plasmids vectors used in for *P. pastoris* expression in this study synthesised by ThermoFisher (https://apps.thermofisher.com/apps/geneart-qa)

Plasmid	Characteristics
18AEWU4C_SiphiA_pET100/D-TOPO (LipA-pET100)	Ampicillin^{R}, T7 promoter, transcription and termination site, polyhistidine (6×His) tag, insert size 1119 bp, plasmid size 6883 bp.
18AEWU3C_SiphiB_pET100/D-TOPO (LipB-pET100)	Ampicillin^{R}, T7 promoter, transcription and termination site, polyhistidine 6×His tag insert, size 1371 bp, plasmid size 7135 bp.
18AEWU2C_SiphiCB_pET100/D-TOPO (LipC-pET100)	Ampicillin^{R}, T7 promoter transcription and termination site, polyhistidine (6×His) tag, insert size 1446 bp, plasmid size 7210 bp.

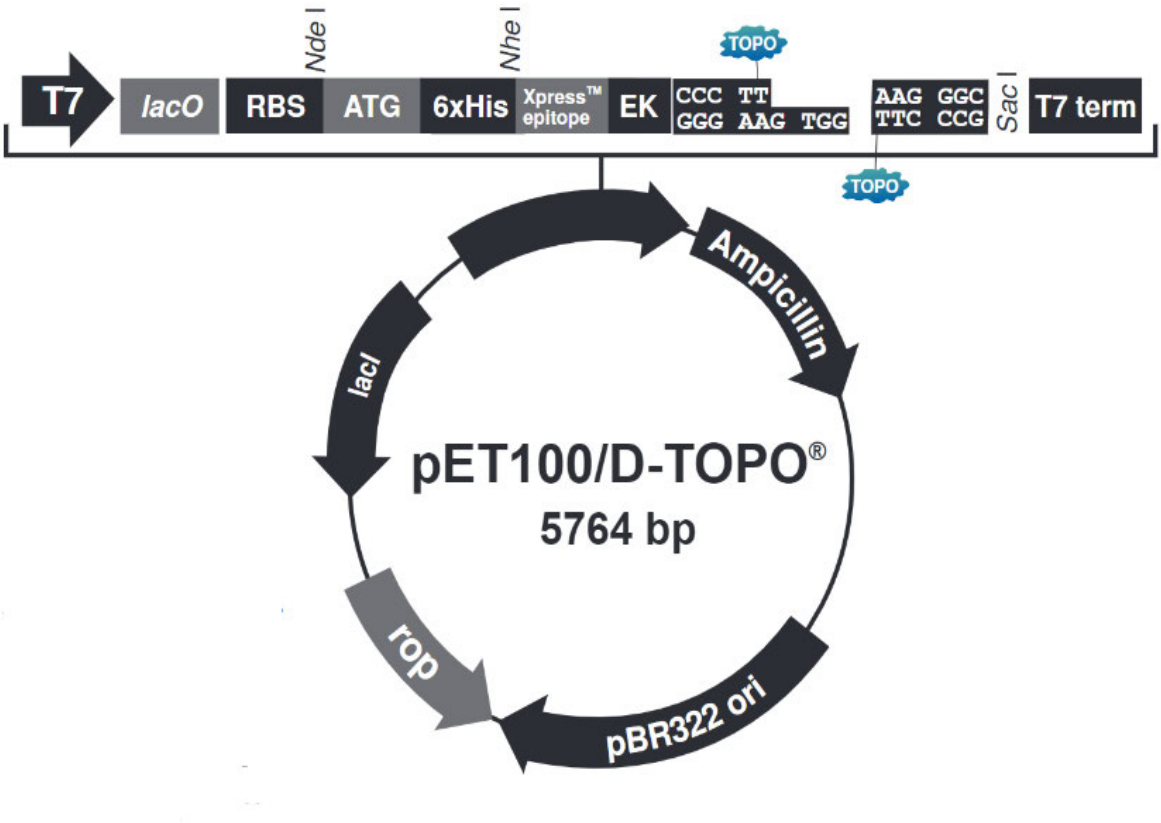

Fig 3.4: Map of *E.coli* vetor pET100/D-TOPO® showing the ampicillin resistance gene, the multiple cloning site (MCS) and the T7 primer site. It also confers a polyhistidine (6×His) tag which enables a simple purification method.

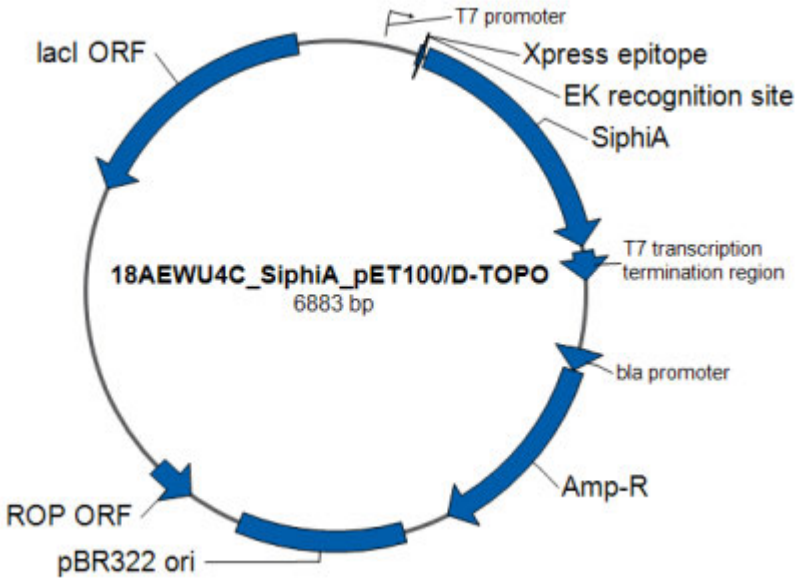

Fig 3.5: Map of plasmid LipA-pET100 containing the synthesised lipase gene A (1119 bp) from *Thermomyces lanuginosus* SSBP.

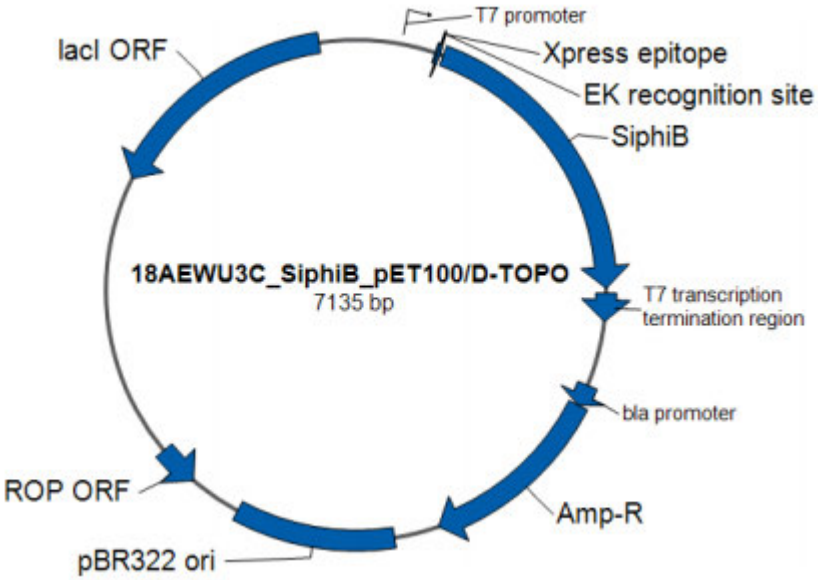

Fig 3.6: Map of plasmid LipB-pET100 containing the synthesised lipase gene B (1371 bp) from *Thermomyces lanuginosus* SSBP.

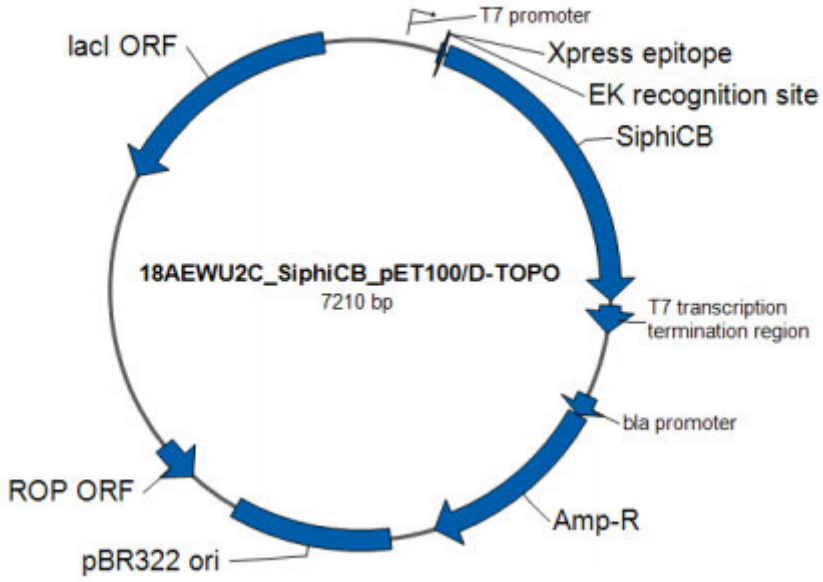

Fig 3.7: Map of plasmid LipC-pET100 containing the synthesised lipase gene C (1446 bp) from *Thermomyces lanuginosus* SSBP.

3.8 Plasmid isolation

Plasmid isolation from *E. coli* was carried out using the GeneJET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instruction manual. *E. coli* cultures containing plasmids were grown overnight in 5 ml LB broth supplemented with 100 µg/ml of ampicillin at 37 C. The cultures were then centrifuged and the pellets resuspended in 250 µl ice cold lysis solution, followed by the addition of neutralizing buffer with gentle mixing. The mixture was then incubated at room temperature for 3 min, followed by centrifugation in a microcentrifuge for 5 min at 10 000 ×*g*. The supernatant was transferred into spin columns. The spin columns were centrifuged for 1 min followed by two washing steps with ethanol. The plasmid DNA was eluted using 50 µl of sterile double distilled water. Five microlitres of the plasmid suspension was used to verify the presence of the plasmid DNA using agarose gel electrophoresis. The plasmid DNA was measured using the NanoDrop 2000 for quantification and quality. The rest of the suspended solution was stored at -20˚C.

Plasmid isolations from yeast were done using a modified procedure of the GeneJET Plasmid Miniprep Kit (Fermentas). The yeast cells were resuspended and after addition of acidified glass beads (400 µm - Sigma), the cells were vortexed for 3 min to lyse the cells. Thereafter, the isolation kit procedure was followed as mentioned above. The DNA solution obtained from this isolation was added to *E. coli* competent cells for transformation to amplify DNA and plasmid DNA was recovered from *E. coli* by plasmid isolation.

3.9 Agarose gel electrophoresis

Visual analysis and confirmation of plasmid DNA isolation, restriction digestion analysis, PCR analysis and DNA purification was performed using agarose gel electrophoresis. The separation of DNA molecules in this technique is based on the size of the negatively charged DNA molecules. Agarose gels were prepared by microwaving 0.8% agarose in 1×TAE buffer for 40 seconds at high power. The molten agarose was mixed thoroughly and poured into a casting tray fitted with well combs and allowed to set. The gel was then placed in the electrophoresis tank and covered with 1×TAE electroporation buffer, prior to applying the samples. Samples were mixed with 6×gel loading buffer (0.0375 g bromophenol blue, 4.0 g sucrose, 1.5 ml 10% SDS and 3 ml 0.5 M EDTA, pH 8), in a ratio of 5:1. The DNA samples, along with DNA molecular weight markers (Thermo Scientific) were loaded into the wells of the gel and electrophoresis was conducted at a field strength of 50 - 100 V for 60 - 120 min. The gels were stained in ethidium bromide (50 µg/ml) for 15-20 min and destained in distilled water for a further 15-20 min. The gels were then viewed using a UV transilluminator and the desired bands were identified by comparison with the marker bands. Gel images were captured and analysed using the Gel Imager XR gel documentation System (BioRad).

3.10 DNA purification

PCR products were purified using the DNA Clean and Concentrator Kit (Zymo Research), according to the manufacturer's instructions. Two volumes of DNA binding buffer was added to each volume of double-stranded DNA. The mixture was loaded into a Zymo-spin column and placed in a collection tube. This was followed by centrifugation for 30 s at 10 000 ×g and the flow through was discarded. 200 µl of wash buffer was added to the column and centrifuged for 30 s. The wash step was repeated, after which the column was placed in a sterile Eppendorf

tube and subsequently 20 µl of sterile deionized water was added to the column followed by centrifugation to elute the purified DNA then measured using the NanoDrop 2000 (Thermo Scientific) for quantification and quality. The DNA was stored at -20˚C, until required. DNA fragments were purified from agarose gels by excising the bands and purifying using the Zymoclean Gel DNA Recover Kit (Zymo Research), similar to the procedure described above.

3.11 Polymerase chain reaction

The lipase genes were amplified in order to have adequate concentrations for further DNA manipulation. The amplification was performed using the PCR cycling conditions ; denaturation at 95˚C for 30 s, annealing at the respective temperatures for 60 s, extension at 72˚C for 90 s and final extension at 72˚C for 10 min. The PCR primers used for cloning LipA and LipB into pPICZαA, pPIC9K and pPGB1 were synthesised by Inqaba Biotec (Appendix A). The PCR reaction mixtures contained 1 µl of DNA (10 - 50 ng/µl); 2.5 mM $MgCl_2$; 1 × PCR buffer (NEB); 0.5 µM primers, 0.1 mM dNTPs and 1 U of *Taq* polymerase (Fermentas).

PCR reactions were carried out for 30 cycles using a PCR Genius thermal cycler (Techne). After amplification, which was confirmed using agarose gel electrophoresis, DNA purification was performed and followed by DNA quantification agarose gel electrophoresis for verification of DNA presence after purification. The PCR products were stored at -20˚C in a biofreezer and used for subsequent cloning experiments.

3.12 Cloning of lipase putative genes

Plasmid cloning vector pPICZα, and Lipase B PCR products were digested with Fast Digest endonucleases, *Xho*I and *Xba*I; and pPIC9K and Lipase B were digested with *SnaB*I and *Xba*I; and pBGPI and Lipase B were digested with *Eco*RI and *Xho*I (Fermentas), to create compatible sticky ends for ligation with using 1 unit of ligase per 1 µg of DNA (Sambrook *et al.*,1989). The resultant reaction mixtures were incubated at 37˚C for five minutes and the reactions were terminated by incubation at 65˚C for 15 min. Restriction reactions were purified using either the DNA Clean and Concentrate Kit (Zymo Research) or the Gel Extraction Purification Kit (Zymo Research), according to the manufacturer's instructions, and small aliquots were examined on agarose gels to confirm successful purification. The DNA was subsequently quantified using the using the NanoDrop 2000.

The vector and PCR products were ligated at a molar ratio of 1:3 vector to insert. The ligation mixtures contained vector, insert, 1× ligation buffer and T4 DNA ligase (Fermentas) and the reaction was incubated at 22 C overnight using a thermal heating block (BIOER).

3.13 Preparation of *E. coli* competent cells

Prior to transformation, electrocompetent bacterial cells were prepared by growing a single colony of *E. coli* DH5α and *E. coli* BL21 (DE3) (Novagen) in 5 ml LB broth overnight at 37˚C in a shaking incubator. One ml of this culture was used to inoculate 29 ml of fresh LB broth and further shaken at 37˚C, until an optical density of 0.500 at 590 nm was reached. Cells were centrifuged at 4000× *g* for 10 minutes and the supernatant was discarded. Throughout the subsequent procedures, the cells were kept chilled by placing on ice. Cells were re-suspended in 10 ml of ice-cold 10% glycerol. The cell suspension was once again centrifuged at the same speed for a further 10 min, the supernatant was discarded, and the cells were re-suspended in 5

ml ice-cold 10% glycerol. This step was repeated once more using 2 ml ice-cold 10% glycerol. The final step involved re-suspension of the competent cells in 200 µl of ice-cold 10% glycerol and dispensing 50 µl aliquots into sterile 1.5 ml tubes. These cells were stored at -80˚C, and used within 3 months, as prolonged storage decreases the transformation efficiency (Ausubel, 2002).

3.14 Transformation and screening of positve *E. coli recombinants*

After ligation, one microlitre of the reaction mixtures was used to transform 50 µl electrocompetent *E. coli* DH5α and *E. coli* BL21. The electrocompetent cells and DNA were incubated on ice and then transferred to chilled 0.2 cm GenePulser electroporation cuvettes (BioRad). The cells were then electroporated using the GenePulser Xcell (BioRad) using the following conditions: 2.5 kV, 200 Ω and 25 µF, for approximately 5 milliseconds. One ml of LB broth was immediately added to the cuvette, the contents transferred to 1.5 ml tubes and incubated at 37˚C for 1 h. During this incubation period, the transformed cells were allowed to recover from the shock treatment and express the antibiotic resistance genes. One hundred microlitres of the transformation mixtures wwere plated on LB-ampicillin agar plates (1% peptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, supplemented with 100 µg/ml ampicillin). Colonies that grew on the plates were deemed resistant to the antibiotic and could contain the recombinant plasmid. These were grown on ampicillin - supplemented LB broth and the plasmids (LipB-pPICZαA, LipB-pPIC9K, LipB-pBGP1) isolated and linearised with appropriate restriction endonucleases (excluding LipB-pBGP1, which did not require linearisation) for subsequent transformation into *P. pastoris*.

Synthesised plasmids LipA-pET100, LipB-pET100, LipC-pET100 were also plated out on isopropyl β-D-1-thiogalactopyranoside (0.25 mM) induced LB-ampicillin containing 1% (v/v) emulsified olive oil and 0.001% rhodamine B, to confirm expression in *E. coli* BL21. Colonies that showed an orange fluorescence under UV light were selected for further analysis.

3.15 Preparation of *P. pastoris* competent cells

P. pastoris GS115 was made competent by treating the cells with dithiothreitol (DTT) and subsequent stabilization in sorbitol (Scorer *et al.,* 1994). The *P. pastoris* cells were grown overnight on YPD plates at 30 C. A single colony was inoculated into 200 ml of YPD broth and the cells were further incubated overnight at 30 C to an OD_{600} of approximately 1.4. The cells were harvested by centrifugation at 2750 ×*g* for 10 min. Cells were resuspended in 40 ml of YPD broth, 1 ml of 1 M DTT and 8 ml of 1 M HEPES buffer (pH 8) and incubated at 30˚C for 15 min. The cells were then sequentially washed three times by re-suspending and centrifugation at 2750 ×*g* for 10 min, with 200 ml of sterile water, 100 ml and 8 ml of 1 M sorbitol, respectively. The cells were finally resuspended in 400 µl of 1 M sorbitol and stored in aliquots of 50 µl at -80˚C.

3.16 Transformation and screening in *P. pastoris*

Integrative plasmids LipB-pPICZαA and LipB-pPIC9K were linearised by restriction endonuclease *Sac*I and *Sal*I, respectively. LipB-pBGP1, an episomal plasmid does not require linearisation (Lee *et al*., 2005). Linear plasmids were then transformed into *P. pastoris* GS115 by electroporation. Electroporation of *P. pastoris* was performed using a Gene Pulser Xcell (BioRad) using the following pulsing conditions: 2.5 kV, 200 Ω and 25 µF. Fifty microlitres of yeast competent cells were mixed with up to 5 µl of the plasmid DNA for transformation in a

sterile 1.5 ml tube. The electrocompetent cells were transferred into 0.2 cm electroporation cuvettes (BioRad) that had been kept on ice, and the pulse was delivered. Ice cold sorbitol solution (1 M) was added immediately after electroporation and the cells were incubated on ice for 15 min.

One millilitre of YPD (2% peptone, 1% yeast extract, 1% dextrose) was added followed by further incubation for 1 h at 30 C without shaking. Aliquots (100 µl) were plated onto YPD selective plates supplemented with 100 µg/ml of zeocin for LipB-pPICZαA and LipB-pPBGP1 transformed *P. pastoris* and 0.25 – 1.0 mg/ml geneticin (G418) for LipB-pPIC9K transformed *P. pastoris* and incubated at 30˚C for 3-4 days. Positive transformants were further plated onto BMMY-rhodamine B-olive oil plates (1.34% YNB, 0.5% (v/v) methanol, 0.02% biotin, 1% olive oil, 0.001% rhodamine and 1.5% agar) (Fang *et al*., 2014). *P. pastoris* transformed with pPIC9K was used as a negative control.

3.17 Lipase expression

Positive yeast clones were first inoculated in 3 ml BMGY and after incubation at 30˚C for 24 h, were resuspended into 500 ml BMGY and incubated at 30˚C, until the OD_{600} reached a value of about 2.0. The cells were then centrifuged, resuspended in 250 ml of BMMY and grown in shaking flasks for 120 h at 30˚C. To maintain induction, 1% (v/v) methanol was added to the culture every 24 h. The cells were harvested by centrifugation and the supernatants were collected.

The selected *E. coli* clones showing lipase activity were used to inoculate 20 ml LB with ampicillin. After overnight growth, these cultures were used to inoculate 200 ml liquid media for lipase production. Expression in *E. coli* lasted for approximately 8 h, including a 6.5 h period of induction with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30˚C then 4˚C

overnight. The enzyme was extracted using Bugbuster Protein Extraction Reagent (Novagen) according to the suppliers' manual. The *E. coli* cells were harvested by centrifugation at 10 000 ×*g* for 10 min at 4˚C. The Bugbuster reagent was added at a ratio of 1: 30, and the cells were incubated for 20 min with slow shaking at room temperature. The cells were then centrifuged at 13 000 × *g* for 30 min at 4˚C. The supernatants were retained as they contained the enzyme fraction and stored at 4˚C for purification.

3.18 Purification with a His-Bind resin affinity chromatography

The His-bind resin slurry (BioRad) was thoroughly mixed and poured into a fretted column to a final resin bed volume of 6 ml. The resin was allowed to pack the top of the column bed with gravity flow. The column was washed with 3× column volumes (CVs) of sterile Milli-Q water, followed by 5× CVs of a 1× charge buffer (50 mM $NiSO_4$). The column was then washed with 3× CVs of a 1× binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.0). After the binding buffer had drained out, 30 ml of crude protein was added into the column at a flow rate of ~10× CVs/h. This was followed by the addition of 10× CVs of 1× Binding buffer. The column was then washed with 6× CVs of a 1× wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.0). The bound protein was eluted with 6× CVs of a 1× elution buffer (0.5 M NaCl, 1 M Imidazole, 20 mM Tris-HCl, pH 7.0) at a flow rate of 1 ml/min. The collected fractions of the eluent (1 ml/fractions) were subjected to a Lowry protein assay (Lowry *et al*. 1951) and a lipase assay. The fractions showing high specific activity were combined and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.19 SDS-PAGE analysis

SDS-PAGE was carried out as per the Laemmli 1970 method to estimate the molecular weight of the enzyme and visualise and evaluate protein purification efficiency. Before SDS-PAGE analysis, the samples (40 µg in 15 µl distilled water) were prepared by mixing them with 5 µl of sample buffer (25 mM Tris-HCl buffer, pH 8.0, 2% SDS, 2% dithiothreitol, 20% glycerol and 0.02% bromophenol blue). The samples were then heated at 100˚C for 5 minutes and cooled at room temperature. Samples were loaded on the gel (5% stacking gel and 10% resolving gel) and electrophoresed at 20 mA per gel at room temperature, until the dye front reached the end of the gel. Coomassie brilliant blue R250 (0.25% dye, 10% acetic acid, 45% ethanol in distilled water) was used to stain the gels. The gels were destained (7.5% acetic acid, 5.% ethanol in distilled water) and photographed with a Gel Doc XR system (BioRad).

3.20 Assay for lipase activity

After 24 hours incubation, the samples were centrifuged at 8000 ×g for 20 minutes and the cell-free culture supernatant were used as the source of extracellular enzyme. Lipase activity was determined spectrophotometrically at 410 nm using *p*-nitrophenyl palmitate (*p*NPP) as a substrate (Gupta *et al*., 2002). The substrate was prepared by combining one part of solution A [Thirty milligrams of *p*-nitrophenyl palmitate (Sigma – Aldrich N2752) dissolved in 10 ml Isopropanol (Sigma – Aldrich 34863)] to nine parts of solution B [Sodium deoxycholate 0.2 g, 0.1 g of Gum Arabic and 0,4 ml Triton X-100 all dissolved in 90 ml of 0.05 mM Tris-HCl buffer pH 8].

The reaction mixture consisted of 900 µl substrate (mixture of 1ml from solution A and 9ml from solution B) and 100 µl of enzyme. The sample mixture was then incubated at 50˚C for 15 minutes. For the enzyme blank, 900 µl of substrate plus 100 µl of enzyme production media

and absorbance was read at 410 nm. Lipase activity was calculated using the following formula based on the release of *p*-nitrophenol and using *p*-nitrophenyl palmitate as a substrate (Gupta *et al*., 2002):

$$\textbf{Activity (U/mL)} = \frac{\left[\frac{(\mathbf{A_S} - \mathbf{A_c}) - \mathbf{c}}{\mathbf{m}} \times \mathbf{Df}\right]}{(\mathbf{V} \times \mathbf{T})}$$

A_s: Absorbance of test sample at 410 nm

A_c: Absorbance of the control at 410 nm

Df: Dilution factor

V: Total volume of reaction

T: Total reaction time.

c: Y – intercept of the *p*-nitrophenol standard curve

m: Gradient of the *p*-nitrophenol standard curve

The *p*-nitrophenol standard curve was constructed using *p*-nitrophenol concentrations ranging from 5 to 75 µM and the absorbance was measured at 410 nm (Appendix B).

All lipase assay reactions were carried out in triplicate, and the reported values were means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol in 15 min by UV-Vis spectrometry (410 nm).

3.20.1 Temperature optima and stability

The purified enzyme was used to investigate the effect of temperature and pH on the three *E. coli* BL21 - expressed *T. lanuginosus* lipases (A, B and C). Temperature optimum was investigated using the *p*-nitrophenol palmitate spectrophotometry assay at a temperature range of 40˚C to 90˚C at pH 8.0 for 15 minutes, due to the thermophilic nature of the enzyme. The substrate solution (900 µl) was pre-incubated at each relevant temperature for 15 minutes

followed by the addition of the enzyme (100 µl). The reaction buffer (Tris-HCl, pH 8) was used as a blank for each temperature. The reaction was allowed to occur over a period of 15 minutes and the release of *p*-nitrophenol was measured spectrophotometrically at 410 nm. One unit (U) of enzyme activity was defined as the amount enzyme that released 1 µmol of *p*-nitrophenol per minute (Gupta *et al*., 2002)

After the temperature optima for the three cloned lipase enzymes was determined, their temperature stability was investigated at varying temperatures (thermostability). Thermostability was investigated by incubating the purified enzymes at temperatures ranging from 50˚C to 80˚C over a period of 120 minutes. An enzyme assay was performed at 15 minutes intervals for each enzyme over a period of two hours, under the same conditions as mentioned in the previous paragraph.

3.20.2 pH optimum assays

The optimum pH for each lipase was determined at the pH range from pH 3 to pH 10 at 60˚C. This was performed using different buffer solutions having pH values ranging from 3 to 10 (0.05 M citrate-phosphate, pH 3-6; 0.05 M Tris-HCl, pH 7-8; 0.05 M sodium carbonate, pH 9-10). The *p*-nitrophenyl palmitate substrate solution prepared in different pH buffers (900 µl) was pre-incubated at 60˚C for 15 minutes, followed by the addition of the enzyme (100 µl). The appropriate buffer was used as a blank for each pH and the reactions were analysed after a 15-minute period.

3.20.3 Lipase substrate specificity

A total of five *p*-nitrophenol substrates were used to determine the specificity or the preference of the *T. lanuginosus* lipases. A concentration of 10 mM of *p*-nitrophenyl acetate (C2) , *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl palmitate (C16) and *p*-nitrophenyl stearate (C18), all purchased from Sigma-Aldrich, were dissolved in isopropanol. All substrates were prepared as explained under section 3.20 and pre-incubated at 60˚C for 15 minutes followed by the addition of the test lipase enzyme (100 µl). The reaction was allowed to occur over a period of 15 minutes and the release of *p*-nitrophenol was measured spectrophotometrically at 410 nm.

3.20.4 Effect of metal ions on lipases

The effect of metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Na^{+}) on enzyme activity were analysed by pre-incubation of the lipase enzyme in 10mM of metal chloride solution at 60˚C for 15. This was followed by the lipase activity assay described in section 3.20.

4. RESULTS

4.1 Sequence analysis

4.1.1 Protein BLAST analysis

Sequences that were annotated as putative lipase genes were obtained from the genome sequencing of *T. lanuginosus* SSBP by Mchunu *et al*., 2013. Before cloning and chemical characterisation, sequences were analysed to compare them to existing lipases using nucleotide and amino acid sequence databases as described in section 3.1 of the methodology.

Putative lipases were analysed using the nucleotide BLAST (BLASTN) function of the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) which showed nonspecific matching of sequences to their existing database (Appendix D) as most sequence identity and coverage was low. This proved that these proteins were novel and have not been described or deposited in any database except for the genome sequencing work as explained above. However, protein BLAST (BLASTP) of the putative lipase aligned with known lipases in the NCBI database although with low identity and similarity as can be observed in Fig 4.1 with between 60 to 77% identity. The highest hits, however, were to filamentous fungi closely related *Thermomyces* (Samson *et al*., 2011).

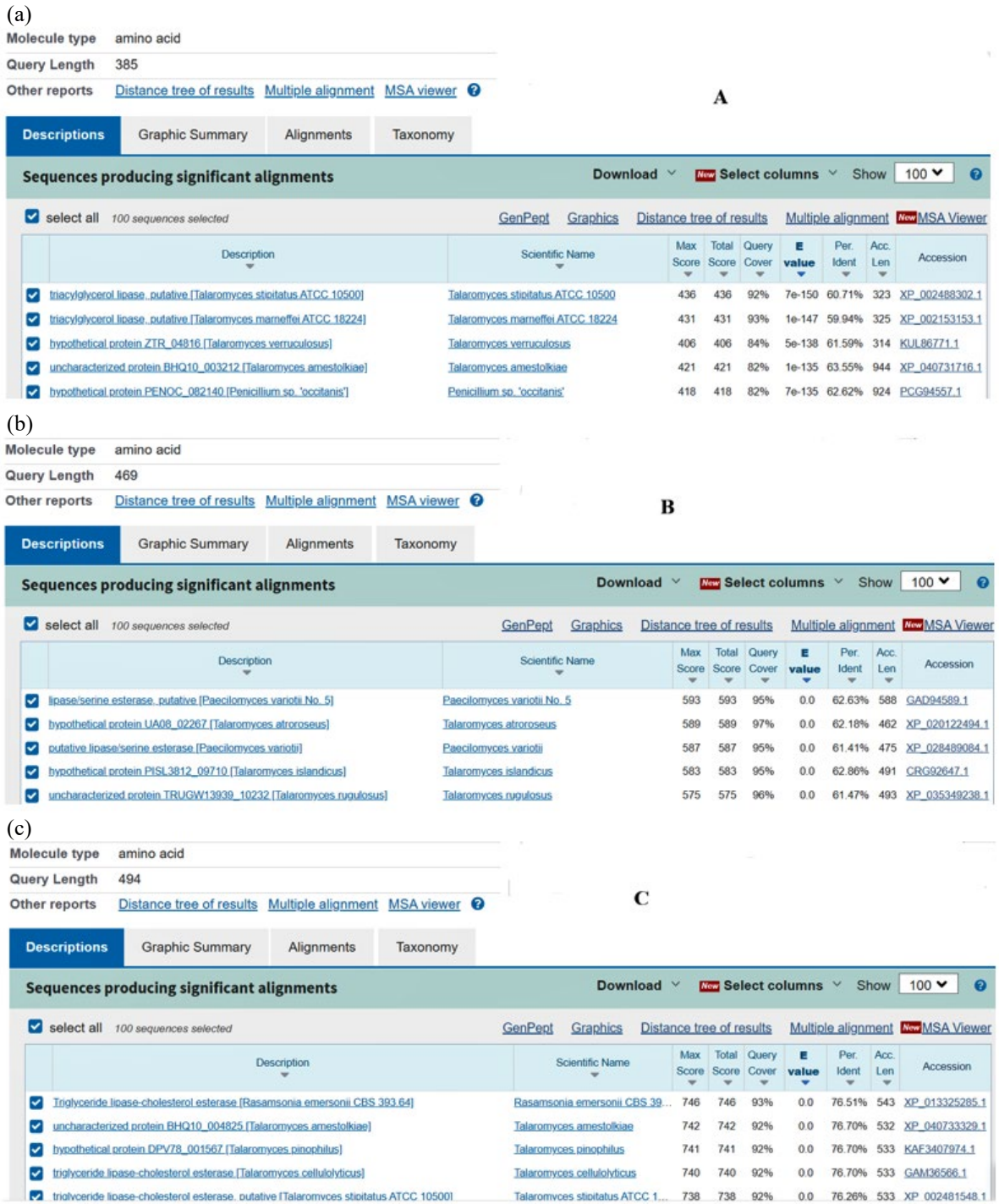

Fig 4.1: Protein BLAST analysis of lipase genes LipA (a), LipB (b) and LipC (c) showing putative lipases similarities and identities from the NCBI database.

4.1.2 Amino acid sequence comparison

Comparison of the three lipases using the MUSCLE multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/muscle/) revealed that all three lipase genes from *T.*

lanuginosus SSBP were different from each other, thus pointing to them arising from different sources, not duplication events (Fig 4.2). The three lipase genes were further compared to a commercial *T. lanuginosus* lipase (Lipolase), and the alignment revealed that none of the three lipases are similar to Lipolase (Appendix F). This confirms that lipases A, B and C from *T. lanuginosus* SSBP are novel.

Fig 4.2: MUSCLE TOOL Amino Acid Alignment for all three lipase genes (LipC, LipA and LipB). *=conserved residues, : = strongly similar groups, .= weakly similar groups. Red = hydrophobic, blue = negatively charged, magenta = positively charged, green = polar residues.

4.1.3 Conserved domain analysis

Conserved domain analysis (Fig 4.3) showed that all three genes contain the lipase family conserve domain hydrolase family namely the abhydrolase superfamily. Lipases A and B had similar family profiles with LipA having more specific hits and LipC with non-specific hits. Lipase C showed specific hits and non specific query matches of abhydrolase superfamily, but it also matched with a different superfamily namely, the PLNO2872 superfamily, which would require further analysis in future studies.

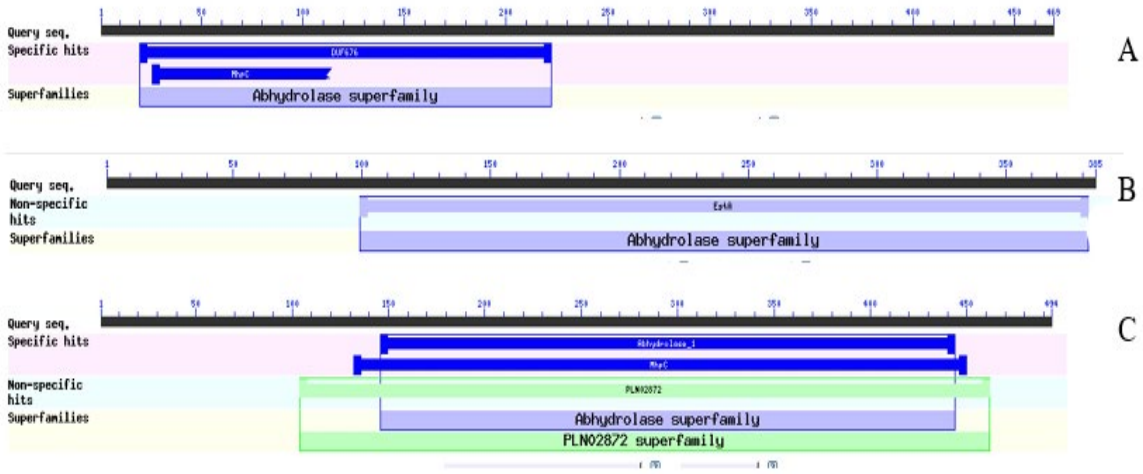

Fig 4.3: Domain analysis for lipases LipA (A), LipB (B) and LipC (C) showing specific and non-specific superfamily hits. All three genes belong to the Abhydrolase superfamily.

4.1.4 Protein modelling

Protein sequences were further analysed using the SWISS Model database to visualise possible protein folding based on available templates.The models that were created were not validated as this analysis was performed to merely obtain data of possible protein folds. Thus, no binding pockets or substrate binding sites were identified except using what is known in literature. Created protein models for each of the three *T. lanuginosus* SSBP putative lipases confirmed

that the matched lipase sequences from different organisms and are probably monomers (https://swissmodel.expasy.org/interactive).

The templates that had some identity to LipA were mostly bacterial enzymes. Table 4.1 shows the top five templates that showed the best hits for which are all putative lipases with sequence identities below 30%. The selected protein template (Lipase C12) had a match of 25.70% sequence identity. The protein model for LipA was created using the top matching template and can be seen in Fig 4.4.

Table 4.1: SWISS MODEL protein sequence template match for LipA

A total of 1249 templates were found to match the target sequence. This list was filtered by a heuristic down to 50. The top templates are:

Template	Sequence Identity	Biounit Oligo State	Description
6cl4.1	25.70	monomer	Lipase C12 LipC12 - Lipase from metagenomics
6ksl.1	26.74	homo-dimer	Lipase 2 Staphylococcus aureus lipase - S116A inactive mutant
3w9u.1	29.44	monomer	Putative lipase Crystal structure of Lipk107
4gw3.1	29.44	monomer	Putative lipase Crystal Structure of the Lipase from Proteus mirabilis
4hs9.1	30.24	monomer	Lipase Methanol tolerant mutant of the Proteus mirabilis lipase

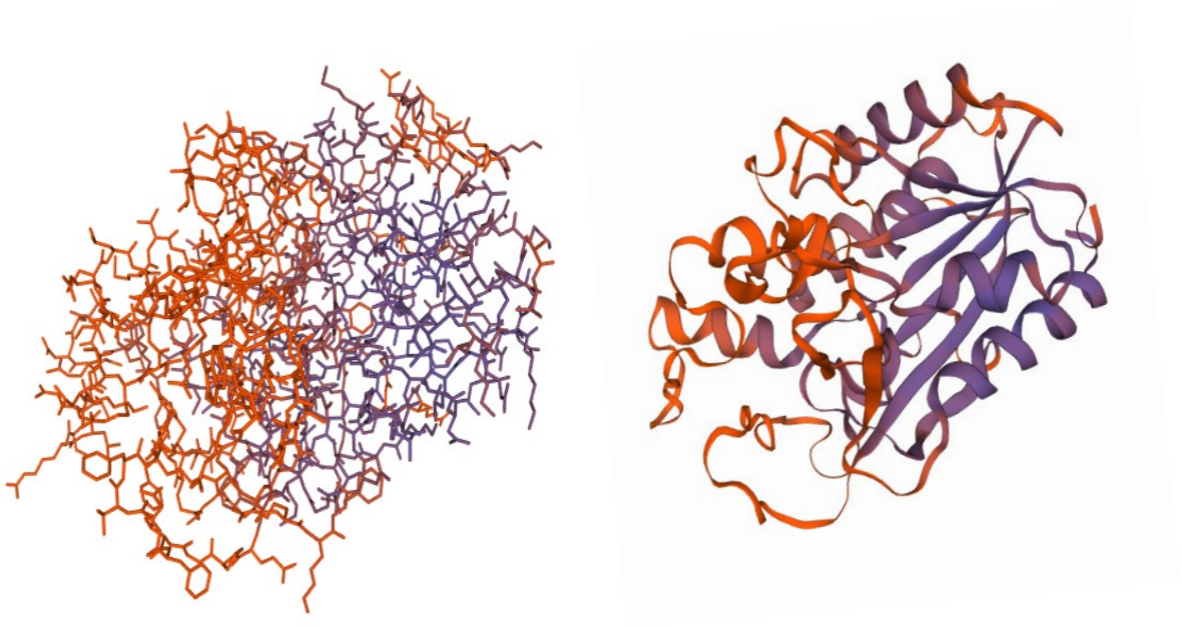

Fig 4.4: SWISS MODEL protein folding structures for LipA based on the highest sequence analysis in Table 4.1. Liquorice (stick) model is on the left and cartoon

(ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines.

Lipase B had a total of 1739 templates that probably matched lipase its sequence, these were further filtered down to a total of 50. Table 4.2 shows the top five templates that showed the best similarities for which are all either lipases or estearases from hydrolase family thus corroborating the data acquired from the MUSCLE tool database in fig 4.3. All templates had low sequence identity with the top matching template (*Burkholderia cepacia* lipase) only having a match of 27.78% sequence identity.

Table 4.2: Swiss Model Protein sequence template match for LipB

A total of 1739 templates were found to match the target sequence. This list was filtered by a heuristic down to 50. The top templates are:

Template	Sequence Identity	Biounit Oligo State	Description
2nw6.1	27.78	monomer	Lipase Burkholderia cepacia lipase complexed with S-inhibitor
1tah.1	25.93	monomer	LIPASE THE CRYSTAL STRUCTURE OF TRIACYLGLYCEROL LIPASE FROM PSEUDOMONAS GLUMAE REVEALS A PARTIALLY REDUNDANT CATALYTIC ASPARTATE
4gw3.1	24.53	monomer	Putative lipase Crystal Structure of the Lipase from Proteus mirabilis
7cof.1	27.52	monomer	Alpha/beta hydrolase Cholesterol esterase from Burkholderia stabilis (orthorhombic crystal form)
1qge.1	25.93	hetero-dimer	PROTEIN (TRIACYLGLYCEROL HYDROLASE) NEW CRYSTAL FORM OF PSEUDOMONAS GLUMAE (FORMERLY CHROMOBACTERIUM VISCOSUM ATCC 6918) LIPASE

Protein models for LipB were created using the top matching template, lipase from *Burkholderia cepacia* complexed with S-inhibitor, and can be seen in Fig 4.5.

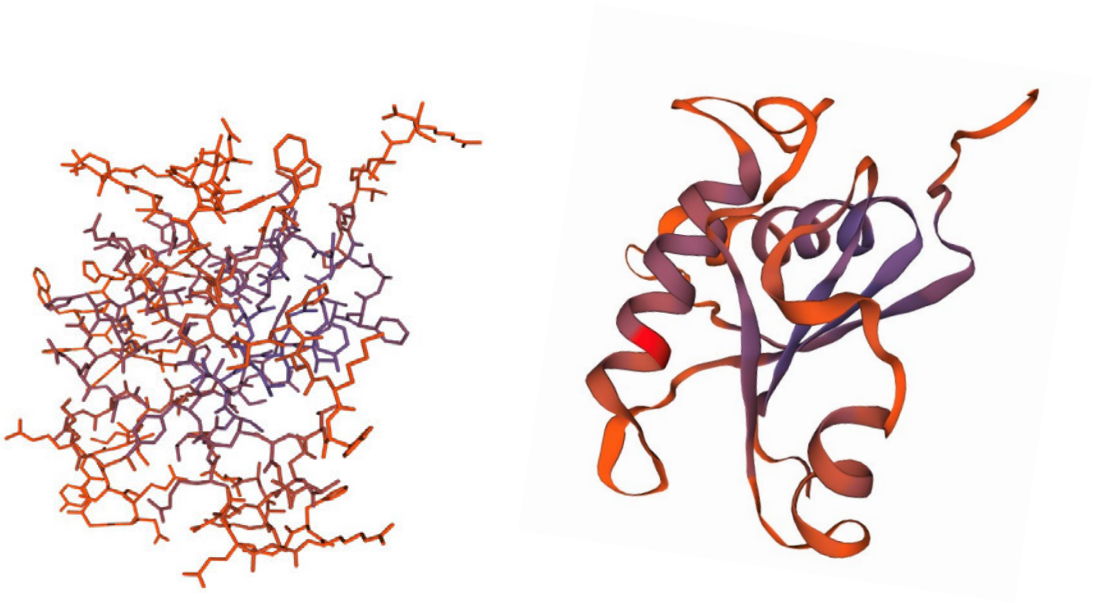

Fig 4.5: SWISS MODEL protein folding structures for lipase LipB based on the highest sequence analysis in Table 4.2. Liquorice (stick) model is on the left and cartoon (ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines.

Of the three lipases, lipase A and lipase B shared the greatest similarity in structure and this is depicted in Fig 4.6.

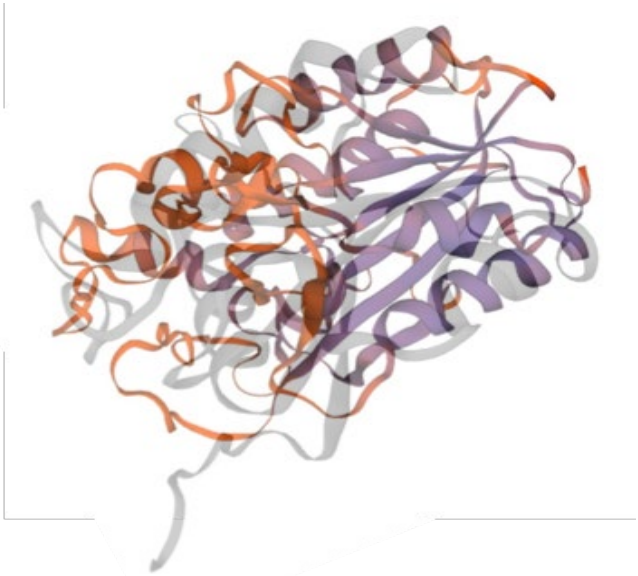

Fig 4.6: Cartoon (ribbon) model showing superimposed models of LipA and LipB. The superimposed image shows a lot of similarities between the two where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines.

The SWISS Model database found a total of 1648 templates that probably matched lipase LipC that were further filtered down to a total of 50. Table 4.3 shows the top five templates that showed the best similarities for which are all either lipases or estearases from hydrolase family thus corroborating the data acquired from the MUSCLE tool database. All templates had low sequence identity with the top matching template (Lysosomal acid lipase / cholesteryl ester hydrolase) only having a match of 31.84% sequence identity. Protein models for LipC were created using the top matching template, Lysosomal acid lipase / cholesteryl ester hydrolase and can be seen in fig 4.7.

Table 4.3: SWISS-MODEL protein sequence template match for LipC

A total of 1648 templates were found to match the target sequence. This list was filtered by a heuristic down to 50. The top templates are:

Template	Sequence Identity	Biounit Oligo State	Description
6v7n.1	31.84	monomer	Lysosomal acid lipase/cholesteryl ester hydrolase Crystal Structure of a human Lysosome Resident Glycoprotein, Lysosomal Acid Lipase, and its Implications in Cholesteryl Ester Storage Disease (CESD)
1k8q.1	32.96	monomer	Triacylglycerol lipase, gastric CRYSTAL STRUCTURE OF DOG GASTRIC LIPASE IN COMPLEX WITH A PHOSPHONATE INHIBITOR
1hlg.1	31.83	homo-dimer	LIPASE, GASTRIC CRYSTAL STRUCTURE OF HUMAN GASTRIC LIPASE
6v7n.1	35.98	monomer	Lysosomal acid lipase/cholesteryl ester hydrolase Crystal Structure of a human Lysosome Resident Glycoprotein, Lysosomal Acid Lipase, and its Implications in Cholesteryl Ester Storage Disease (CESD)
1k8q.1	35.92	monomer	Triacylglycerol lipase, gastric CRYSTAL STRUCTURE OF DOG GASTRIC LIPASE IN COMPLEX WITH A PHOSPHONATE INHIBITOR

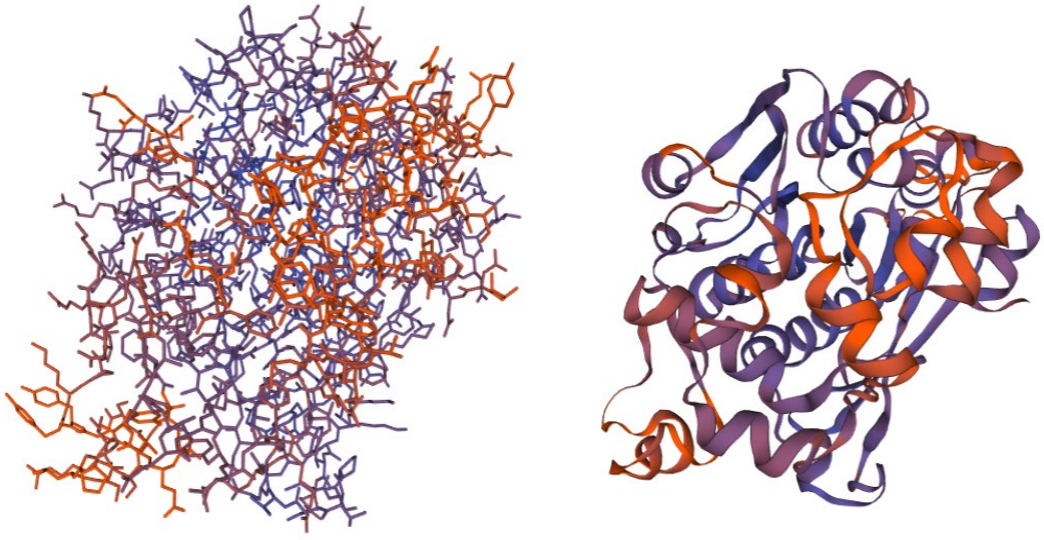

Fig 4.7: SWISS MODEL protein folding structures for lipase LipC based on the highest sequence analysis in Table 4.3. Liquorice (stick) model is on the left and cartoon (ribbon) model on the right where α-helices are shown as coiled ribbons, β-strands as flat ribbons, and non-repetitive coils or loops as thin lines.

4.2 Cloning of the *Thermomyces lanuginosus* SSBP lipases in different *P pastoris* vectors

4.2.1 Amplification of lipases from cDNA

The cDNA of *T. lanuginosus* SSBP, produced using olive oil - supplemented media was synthesised from *T. lanuginosus* RNA (Appendix C). It was used to amplify the LipA and LipB genes using gene - specific primers designed to match pPICZαA and pPIC9K cloning and expression vectors flanking sequences. Lipase gene LipB was also cloned into pPBG1. A small aliquot of the PCR products was analysed for amplification with gel electrophoresis. LipB amplification produced a single distinct band of approximately 1100 bp which was the expected PCR product as can be seen in (Fig 4.8) and (Fig 4.10). Amplification for LipA showed three bands were produced instead of a single distinct band, this was observed for both primers for pPICZαA (Fig 4.8) and pPIC9K (Fig 4.9).

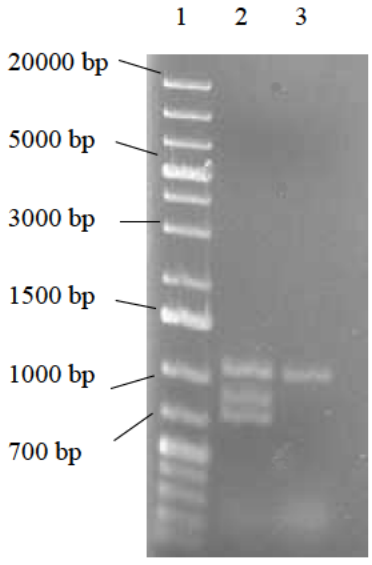

Fig 4.8: Amplification of LipA and LipB from cDNA using pPICZα primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: LipA PCR product from cDNA. Lane 3: LipB PCR product from cDNA.

After numerous amplification attempts, the LipA gene still produced three bands. The DNA bands corresponding with the correct PCR product size were cut out, gel purified and amplified again for subsequent cloning experments. All further attempts to amplify the LipA gene were unsuccessful and further studies were carried only carried out using LipB for cloning in *P. pastoris* and all three genes were synthesised and cloned into *E. coli* vectors.

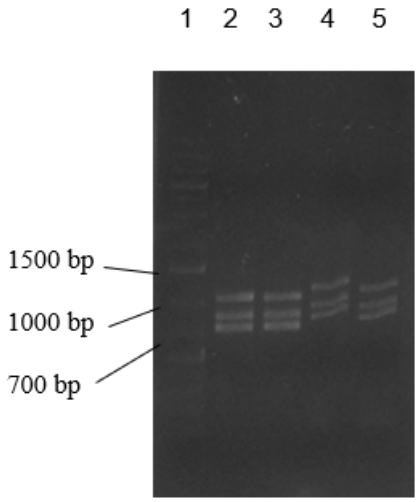

Fig 4.9: Amplification of LipA with pPICZαA and pPIC9K primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2-3: LipA PCR product for pPICZαA after PCR optimisation attempts. Lane 4-5: LipA PCR product for pPIC9K.

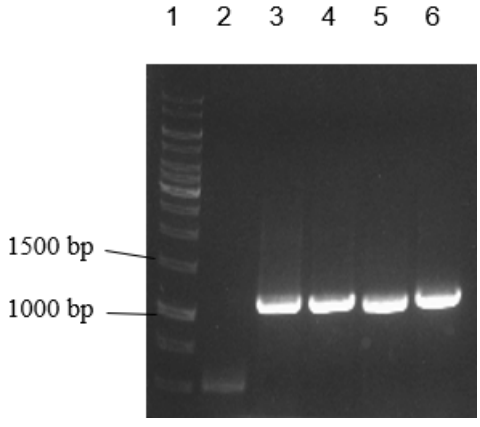

Fig 4.10: Amplification of LipB with all three *P. pastoris* vector primers. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: Negative amplification. Lane 3-4:

LipB PCR product using pPICZαA primers. Lane 5: PCR product using pPIC9K primers. Lane 6: PCR product using pPBGP1 primers.

4.2.2 Construction of *Pichia* recombinants

4.2.2.1 Lip-pPICZαA

PCR products obtained using pPICZαA primers (Fig 4.8 and 4.10) were ligated to the pPICZαA vector after restriction with endonucleases. Plasmid pPICZαA and lipase B PCR product were restricted with *Xho*I and *Xba*I to create sticky ends. The ligation products were transformed in *E. coli* DH5α and the plasmids from the positive transformants were isolated. Restriction analysis of the LipB-pPICZαA plasmid restricted with *Xho*I and *Xba*I endonucleases confirmed the presence of the gene of interest, LipB, in the positive transformants. Fig 4.11 (lane 7 and 8) below clearly demonstrates the presence of pPICZαA with an approximate size of 3.3 kb and LipB insert with an approximate size of 1.1 kb. Positively confirmed LipB-pPICZαA plasmids were linearised by *Sac*I and purified for transformation in *P. pastoris.*

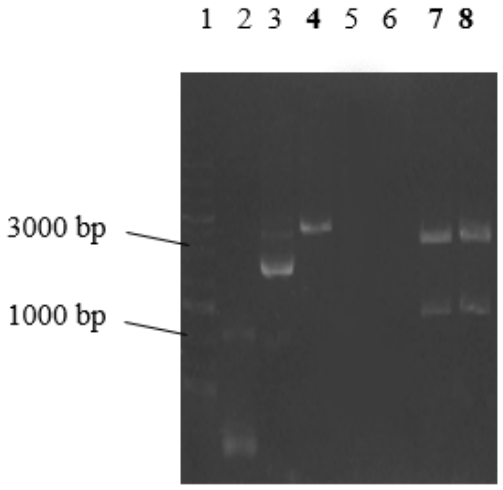

Fig 4.11: Restriction analysis confirming ligation of pPICZαA to LipB by restriction of the transformed plasmid with endonucleases *Xho*I and *Xba*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: LipB control. Lane 3: Unrestricted

pPICZαA control. Lane 4: Single digest of pPICZαA. Lane 5-6: Empty lanes. Lane 7-8: Restricted LipB-pPICZαA plasmid showing the correct 3.3 kb and 1.1kb fragments respectively.

4.2.2.2 LipB-pPIC9K

Plasmid pPIC9K and the LipB PCR product (lanes 3 – 4 in Fig 4.10) were restricted with *Sna*BI and *Xba*I to create sticky ends and then ligated to each other. The ligation products were transformed in *E. coli* DH5α and plasmids from the positive transformants were isolated. Restriction analysis of the LipB-pPIC9K plasmid cleaved with *Sna*BI and *Xba*I endonucleases confirmed the presence LipB. Lane 2 in Fig 4.12 below clearly demonstrates the presence of the pPIC9K fragment with a size of 9.3 kb and LipB insert with an approximate size of 1.1 kb. Positively confirmed LipB-pPIC9K plasmids were linearised with *Sal*I and purified for transformation into *P. pastoris.*

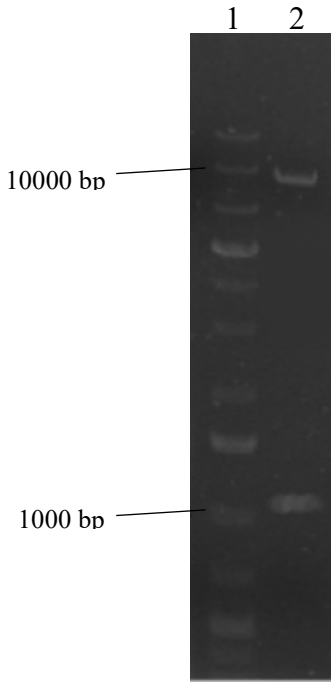

Fig 4.12: Restriction analysis confirming ligation of pPIC9K to LipB by restriction of the transformed plasmid with endonucleases *Sna*BI and *Xba*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2: Restricted LipB-pPIC9K plasmid showing the correct 9.2 kb and 1.1 kb fragments respectively. LipB-pPBG1.

4.2.2.3 LipB-pPBG1

Plasmid pPBG1 and the LipB PCR product (lane 6 in Fig 4.10) were restricted with *Eco*RI and *Xho*I to create sticky ends and then ligated to each other. The ligation products were transformed in *E. coli* DH5α and plasmids from the positive transformants were isolated. Restriction analysis of the LipB-pPBG1 plasmid cleaved with *Eco*RI and *Xho*I endonucleases confirmed the presence of LipB, in the positive transformants. Lanes 4 – 8 in Fig 4.13 below clearly demonstrates the presence of pPBG1 with an approximate size of 4.6 kb and LipB insert with a size of 1.1 kb. Positive LipB-pPBG1 plasmids were transformationed into *P. pastoris.*

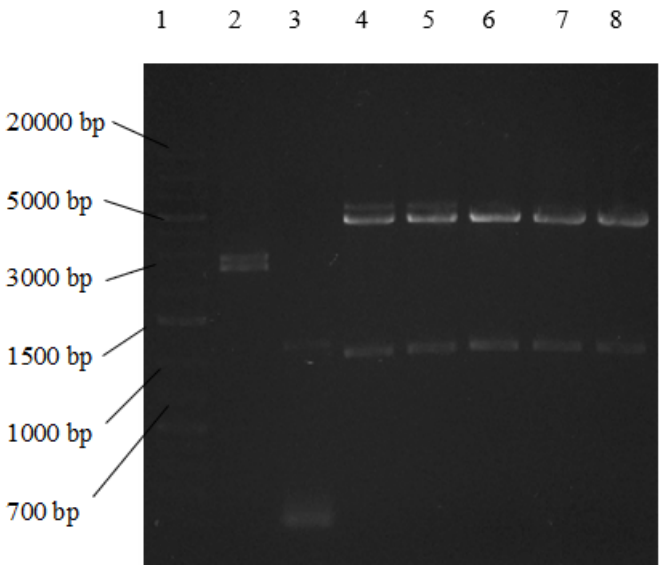

Fig 4.13: Restriction analysis confirming ligation of pPBG1 to LipB insert by restriction of the transformed plasmids with endonucleases *Eco*RI and *Xho*I. Lane 1: Thermo Scientific 1 kb Plus DNA ladder. Lane 2-3: negative clones Lane 4-8: Restricted LipB-pPBG1 plasmid showing the correct 4.6 kb and 1.1 kb fragments, respectively.

4.3 Lipase expression in heterologous hosts (*P. pastoris* and *E. coli*)

4.3.1 Expression in *P. pastoris*

4.3.1.1 Rhodamine B plate lipase screening

Positive *Pichia* transformants were selected on YPD selective plates supplemented with 100 µg/ml of zeocin for pPICZαA and pPBGP1 transformants, and 1.0 mg/ml geneticin (G418) for pPIC9K transformants. Positive transformants were further plated onto BMMY-rhodamine B-olive oil plates, induced with a 100 µl of 100% methanol every 24 hours for three days. Colonies that showed orange fluorescence under ultraviolet (UV) light indicated positive lipase expression (Fang *et al*., 2014). Positive transformants containing LipB-pPICZαA, LipB-pPIC9K and LipB-pBGP1 all showed expression of the lipase enzyme (Fig 4.14).

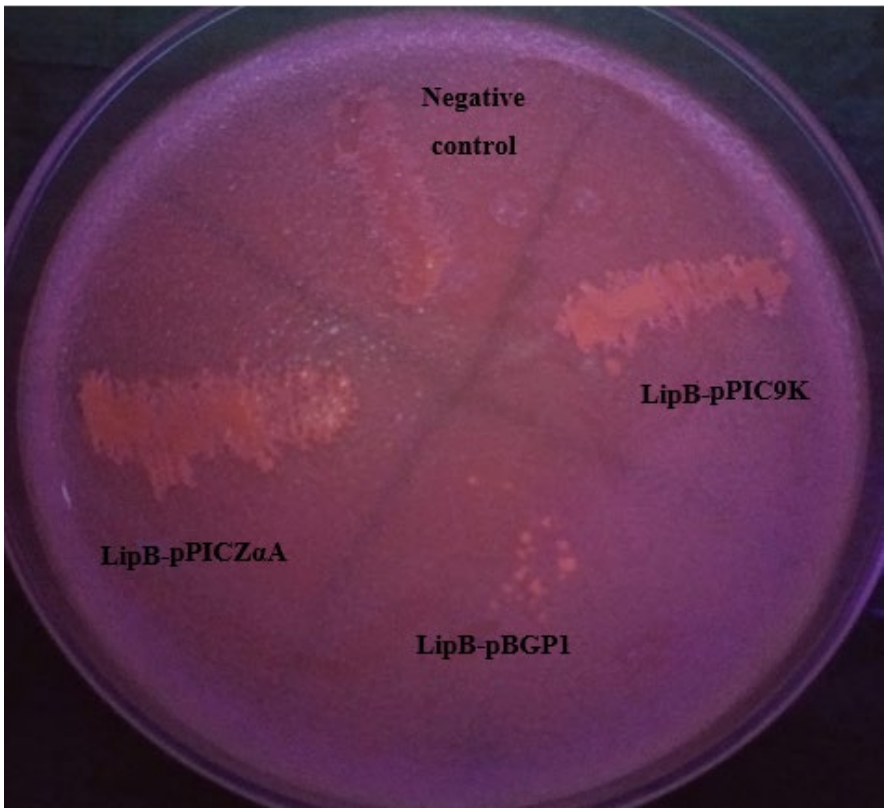

Fig 4.14: BMMY-rhodamine B-olive oil plate used to qualitatively confirm the expression of lipases in positive transformants. The image was captured after 48 hours of methanol induction. An orange fluorescence emission on ultraviolet exposure indicate expression of the three clones.

4.3.1.2 Recombinant lipase production in *P. pastoris*

Presumptive recombinant *P. pastoris* colonies containing LipB-pPICZαA and LipB-pPIC9K were cultivated in shake flasks for 120 hours in BMMY media. Lipase production was induced by the addition of 1% v/v 100% methanol in flasks at 24-hour intervals. There was no induction required for *P. pastoris* with LipB-pBGP1 cultures because of the constitutive glyceraldehyde phosphate promoter in this vector, instead, the selective pressure was maintained with zeocin. However, lipase enzyme expression in shake flasks was undetectable for LipB-pBGP1.The highest biomass was obtained at 48 hours for the two LipB clones (Fig 4.15).

The highest lipase B expression was obtained after 48 hours of incubation with lipase activity of 12.69 U/ml for LipB-PICZαA and 4.33 U/ml for LipB-pPIC9K. Expression with pPIC9K was significantly lower and only less than a unit higher than the negative control (Fig 4.16). The low level of lipase expression in the control could be due to background expression of endogenous lipases.

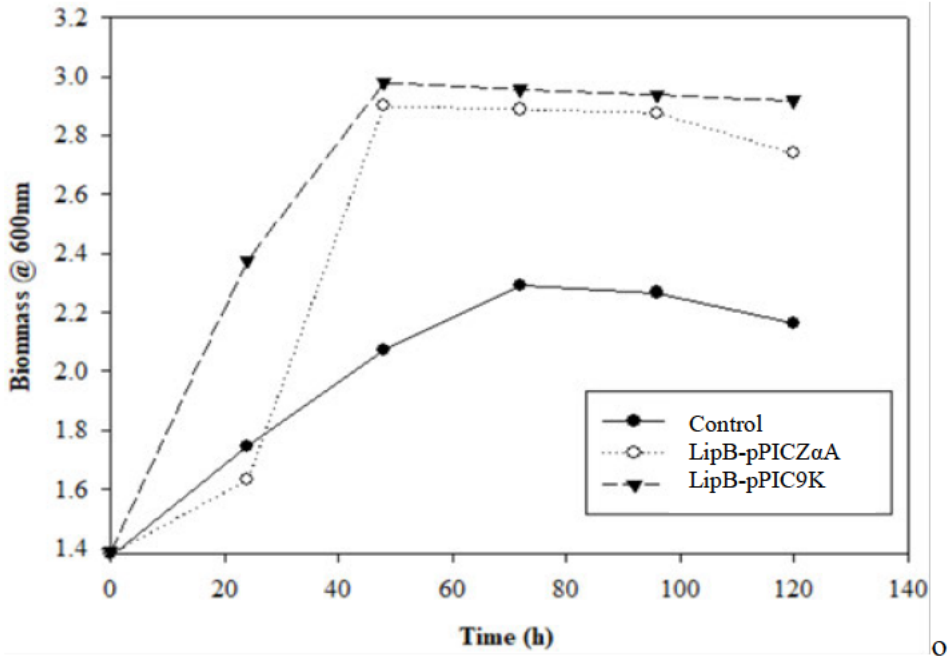

Fig 4.15: Biomass production over a period of 120 hours for LipB-pPICZαA, LipB-pPIC9k and a *P pastoris* GS115 negative control.

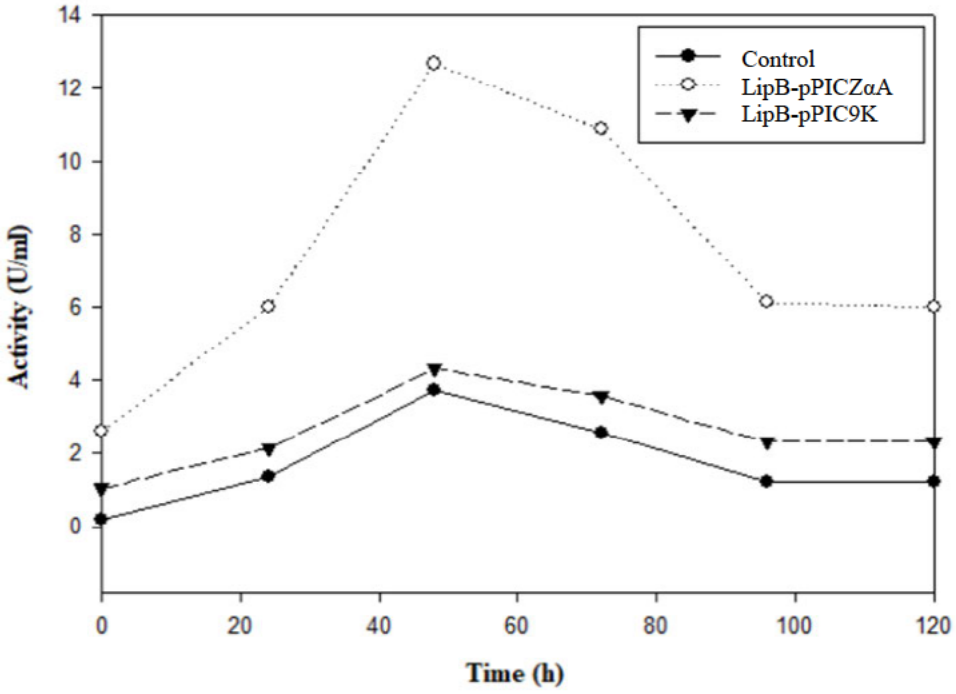

Fig 4.16: Recombinant lipase production by LipB-pPICZαA and LipB-pPIC9K expressed in *P. pastoris* GS115 in BMMY and induced with methanol every 24 hours over 120 hours at 30 C with shaking at 250 rpm. A negative control is also shown.

4.3.2 Expression in *E. coli* BL21 (DE3)

4.3.2.1 Rhodamine B plate lipase screening

Clones containing synthesised genes LipA, LipB, LipC in pET100 were plated on IPTG (0.25 mM) induced LB-amp containing 1% (v/v) emulsified olive oil and rhodamine B, to confirm expression in *E. coli* Colonies that showed orange fluorescence under UV light confirmed lipase expression (Fang *et al*., 2014). Due to the precision of synthesised plasmids, all transformed colonies showed a positive lipase expression (Fig 4.17). The negative control fluorescence under UV light indicating background lipase activity.

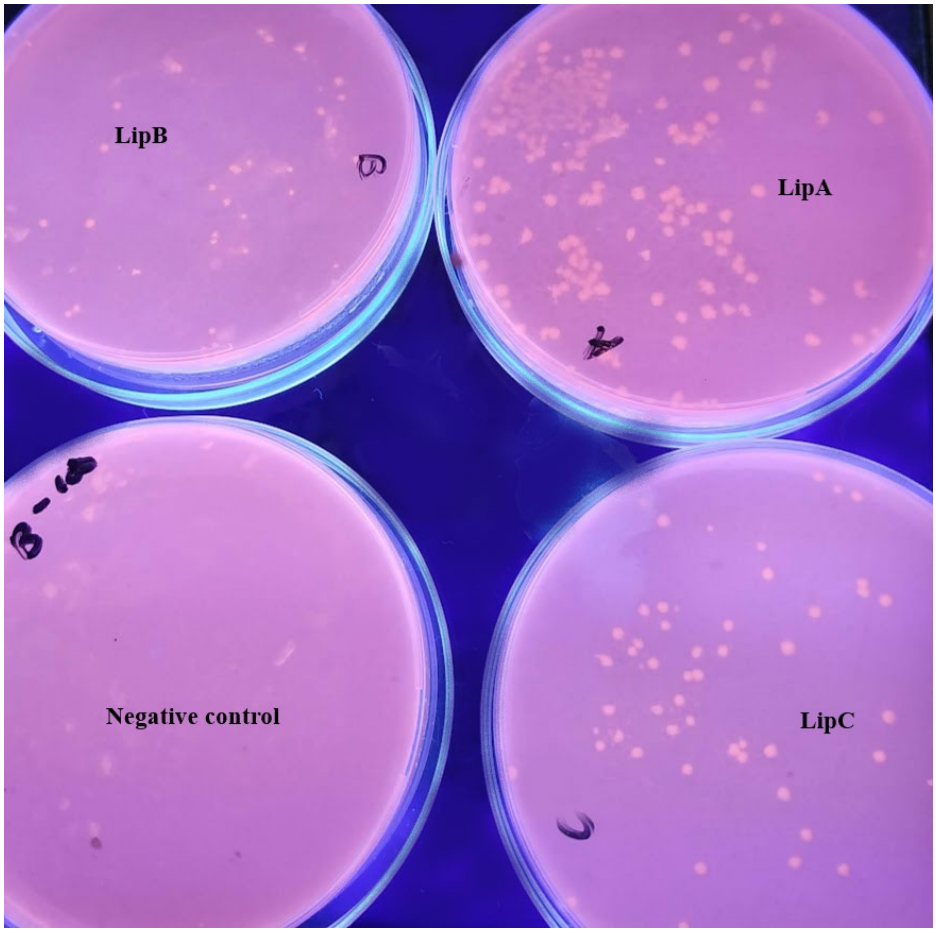

Fig 4.17: Qualitative plate assay showing lipase expression by *E. coli* clones epressing LipA, LipB and LipC. A negative control plate is also shown.

Expression in *E. coli* was preferred over *P. pastoris* expression since expression appeared to be higher, a longer time was required for expression, and *P. pastoris* endogenous lipase expression was higher. Consequently, further enzyme characterisation protocols were performed using the *E. coli* expressed enzyme. Furthermore,all three lipase genes were expressed in *E. coli* whereas only two genes were cloned in *P. pastoris.*

4.3.2.2 Recombinant lipase production in *E. coli*

Expression in *E. coli* for was carried out for 8 h, including a 6.5 h induction with 0.25 mM IPTG at 30˚C then 4˚C overnight (Mchunu *et al*., 2009). The enzyme was extracted using Bugbuster Protein Extraction Reagent (Novagen) according to the supplier's manual. All three lipase were successfully extracted as can be observed on the SDS - PAGE gel (Fig 4.18). Thick bands were observed in the approx. 35 kDa region. It can be discerned though, that LipA is smaller than LipB, which in turn is smaller than LipC. The numerous background bands are the cytoplasmic proteins from the *E. coli* BL21 strain.

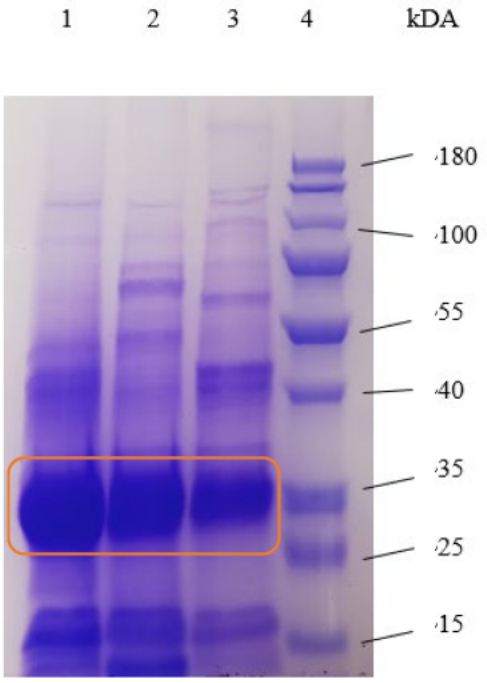

Fig 4.18: A 12% SDS-PAGE showing crude recovery of lipases A, B and C. Lane 1-3: Protein from clones A, B and C respectively, all exhibiting thick bands ranging from ~30 to ~35kDA, typical of the target lipase size. Lane 4: Thermo Fisher Scientific PageRuler.

Protein purification was performed using His-Bind resin affinity chromatography because of the polyhistidine (6×His) tag added during the design and synthesis of the expression constructs. Purification resulted in single discrete bands on SDS

The SDS-PAGE (Fig 4.19) shows purified bands of lipase protein bands LipA, LipB and LipC characterised by their distinct protein size of ~30 - ~35 kDA. Lipase A migrated as a ~30 kDA band, the smallest protein of the three, while Lipase C migrated as a ~ 35 kDA, the largest of the three lipases. Lipase B migrated as a ~33 kDa band, similar to other researched *T lanuginosus* lipases (Fang *et al*., 2014).

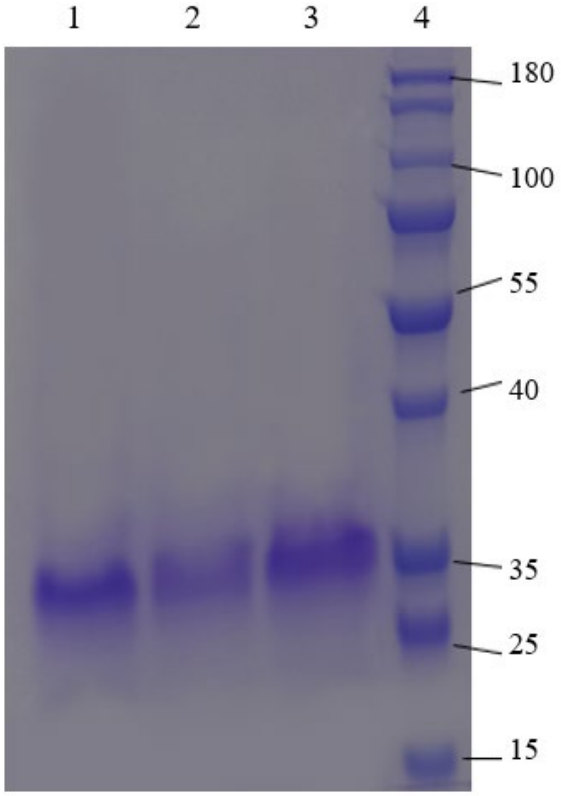

Fig 4.19: A 12% SDS-PAGE showing purified lipases A, B and C. Lane 1: Lipase from clone LipA. Lane 2: Lipase from clone LipB. Lane 3: Lipase from clone LipC. Lane 4: Thermo Fisher Scientific PageRuler.

Lipase B from bacterial expression system produced the highest lipase activity of the three yielding 184 U/ml. Overall expression of the lipases is summarised in Table 4.4 and shows better and significantly high expression in *E. coli* compared to *P. pastoris*.

Table 4.4: Lipase activity for LipA, LipB and LipC

Clones	Enzyme (U/ml) in *P. pastoris*	Enzyme (U/ml) in *E. coli*	Localisation
LipA	*ND	176.2 ± 1.2	Intracellular
LipB	12.7± 0.51	184.1 ± 0.46	Extracellular (*P. pastoris*) and Intracellular (*E. coli*)
LipC	*ND	181 ± 0.13	Intracellular

*ND- Not cloned/undetectable expression

4.4 Characterisation of lipases

4.4.1 Temperature optima

Enzyme characterisation was performed using only *E. coli* recombinants as *P. pastoris* exhibited much lower levels of lipase expression. The temperature optima of lipases was tested in the range from 40-90˚C.

The temperature optimum of all three lipases was similar, with the highest activity obtained at 60˚C for all three lipase variants. It was also observed that between 40-80˚C, these lipases

showed very good thermotolerance, with all three showing residual activity of over 80% at 50˚C. Over and above having an optimal temperature of 60˚C, their thermotolerance levels were impressive with LipA (Lipase A) having the highest residual activity of 80% followed by LipB (Lipase B) with 72% activity while lipase LipC (Lipase C) still had 62% residual activity at 70˚C. At 80˚C, Lipase A still had residual activity of 49% dropping slightly to 37% at 90˚C; Lipase B with residual activity of 47% also slightly dropping to 38%, while Lipase C had residual activity of 48% significantly dropping to 21% at 90˚C (Fig. 4.20).

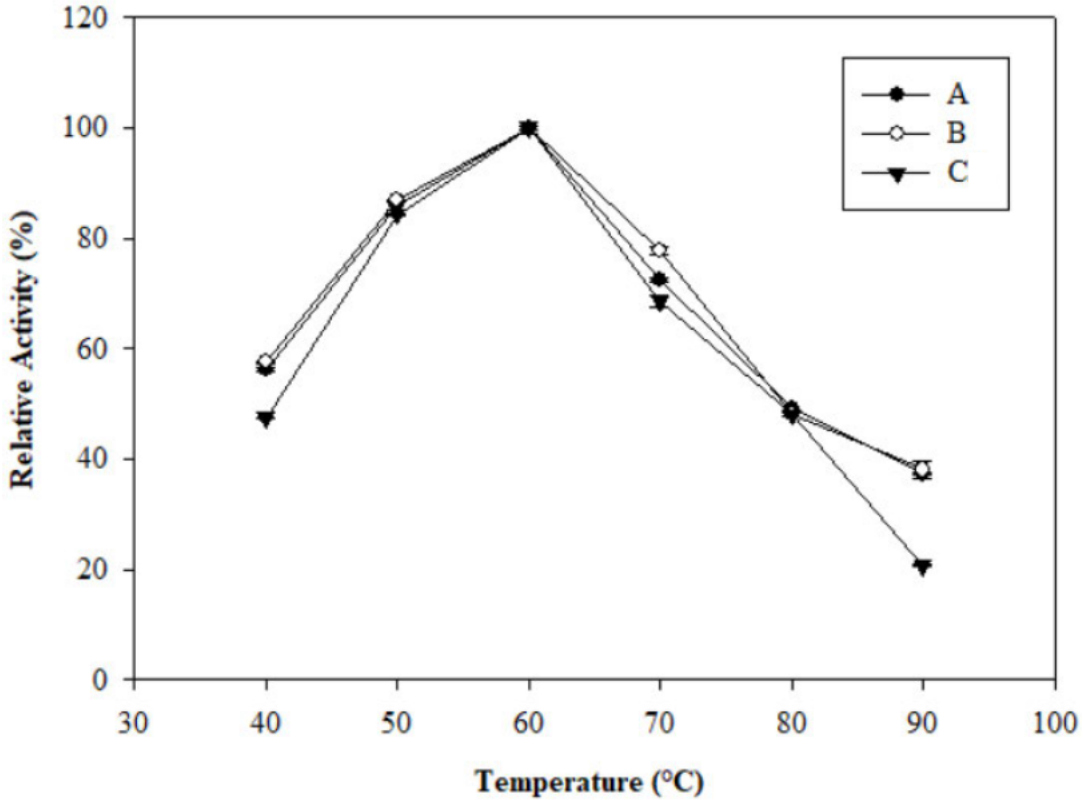

Fig 4.20: Effect of temperature on enzyme activity of lipases A, B and C at pH 8 over a 40 – 90˚C temperature range. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol in 15 min by UV-Vis spectrometry (410 nm).

4.4.2 pH optima

The pH optimum for each lipase was tested between pH 3 to pH 10. All three lipases had preference for alkaline conditions, with an optimum of pH 8, and activity was stable between

pH 7.0-10.0. Interestingly, these lipases retained more than 80% of the overall activity at pH 9, lipase A had 86% activity, lipase B with 89% and lipase C had 83% residual activity. At pH 10, lipase A still retained 54% residual activity followed by lipase B with 53% with lipase C dropping slightly below 50%. It is also important to note that acidic pH completely inhibited lipase activity for all three lipases, from pH 3 - 6 there was no or very low activities observed (Fig. 4.21).

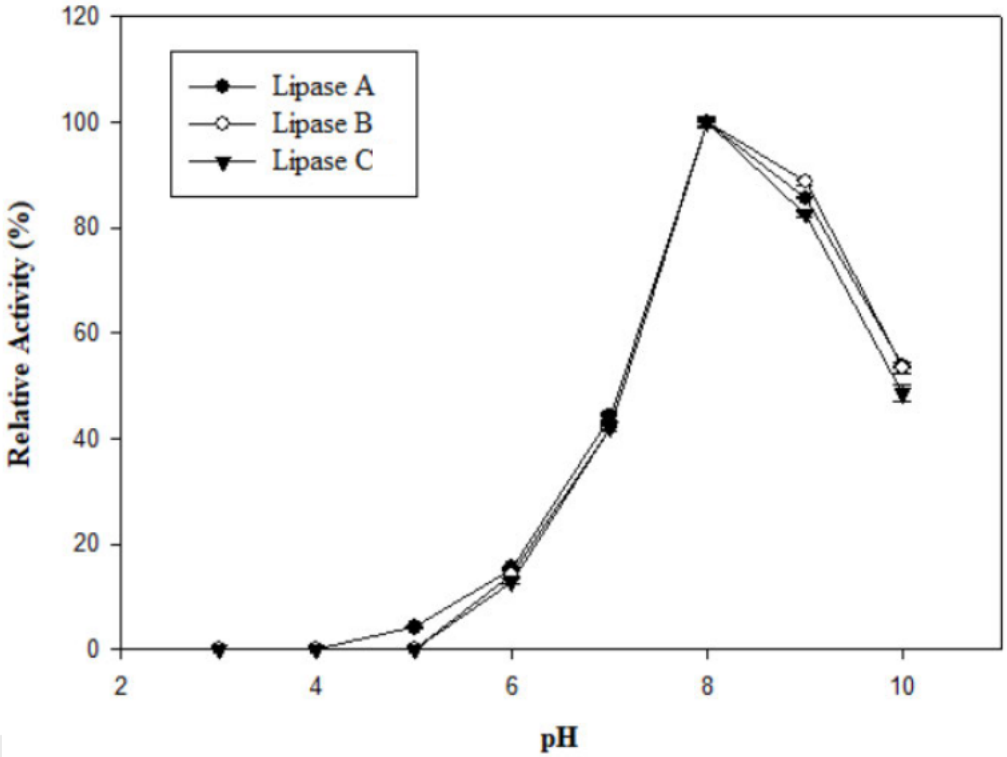

Fig 4.21: Effect of pH on enzyme activity by lipases A, B and C at 60˚C from pH 3 to pH 10. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol in 15 min by UV-Vis spectrometry (410 nm).

4.4.3 Thermostability of lipases

The thermostability of the three recombinant lipases was investigated at different temperatures in order to determine its potential for industrial applications that require processing at higher

temperatures. Each recombinant lipase was tested for stability at temperatures 50, 60, 70 and 80˚C over 120 minutes.

4.4.3.1 Lipase A

Lipase A maintained very good thermostability across all four tested temperatures. At 50˚C, it retained 93% activity after 60 min and 46% activity after 120 min, however, it is important to note that 86% activity was still retained after 90 min. At 60˚C, relative activity of 71% was still retained after 60 min, while 60% activity still remained after 120 min. At 70˚C, 88% of activity was retained after 60 min, with an impressive 70% thermostability activity still remaining after 120 min. At 80˚C, 72% activity was retained after 60 min while 57% activity still remained after 120 min (Fig. 4.22).

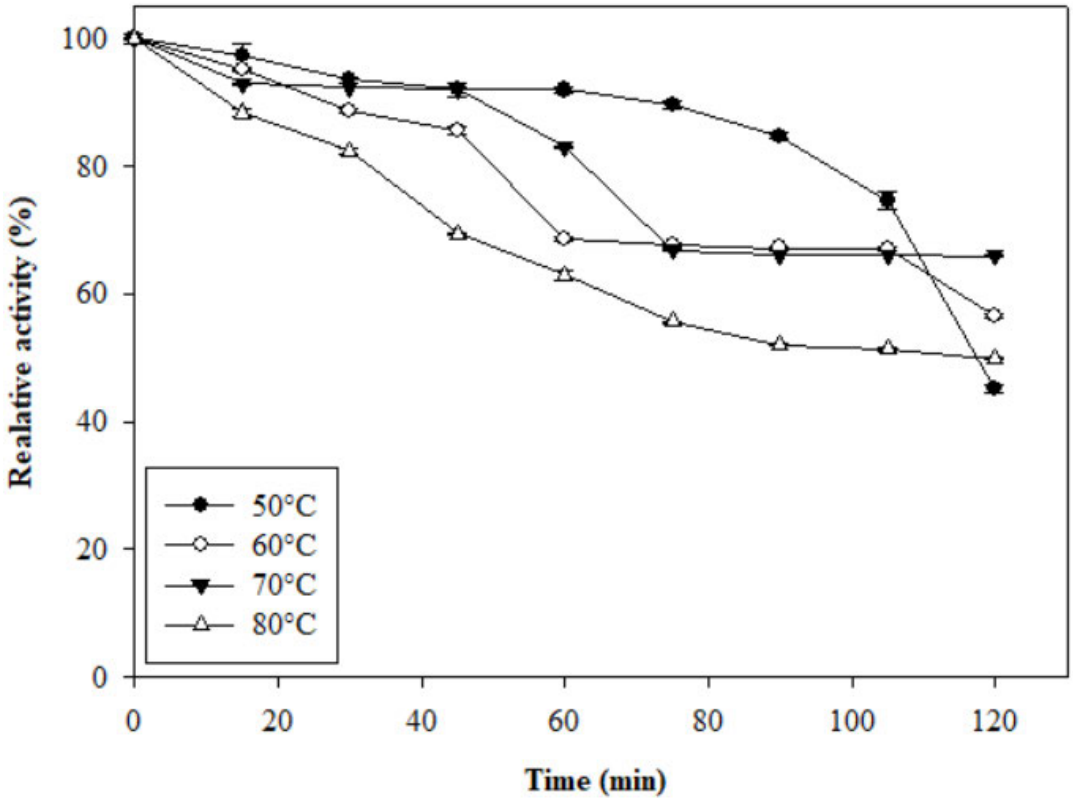

Fig 4. 22: Temperature stability of Lipase A at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm.

4.4.3.2 Lipase B

Lipase B maintained very good thermostability across all four temperatures. At 50˚C it retained 91% activity after 60 min and 52% activity after 120 min, however, it is important to note that 81% activity was still retained after 90 min. At 60˚C, activity of 88% was still retained after 60 min, while 56% activity still remained after 120 min. At 70˚C, 78% of activity was retained after 60 min with an impressive 68% thermostability activity still remaining after 120 min. At 80˚C, 86% activity was retained after 60 min while 59% activity still remained after 120 min (Fig. 4.23).

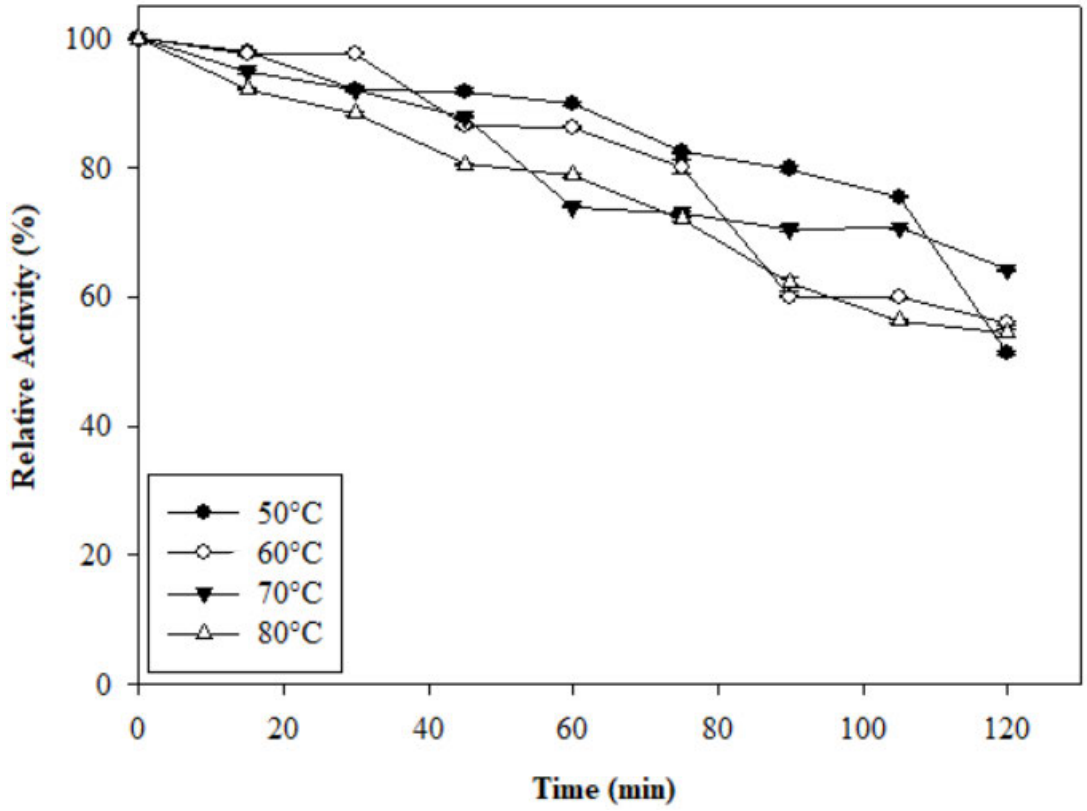

Fig 4. 23 Temperature stability of Lipase B at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm.

4.4.3.3 Lipase C

Lipase C maintained very good thermostability across all four temperatures. At 50˚C it retained 94% activity after 60 min and 61% activity after 120 min, it is important to note that it had much better thermostability after 120 min at this temperature compared to the other lipases in this study. At 60˚C, activity of 91% was still retained after 60 min, while 50% activity still remained after 120 min, which was unexpectedly lower. At 70˚C, 80% of activity was retained after 60 min, with an impressive 74% thermostability activity still remaining after 120 min. At 80˚C, 68% activity was retained after 60 min while 40% activity still remained after 120 min (Fig 4.24).

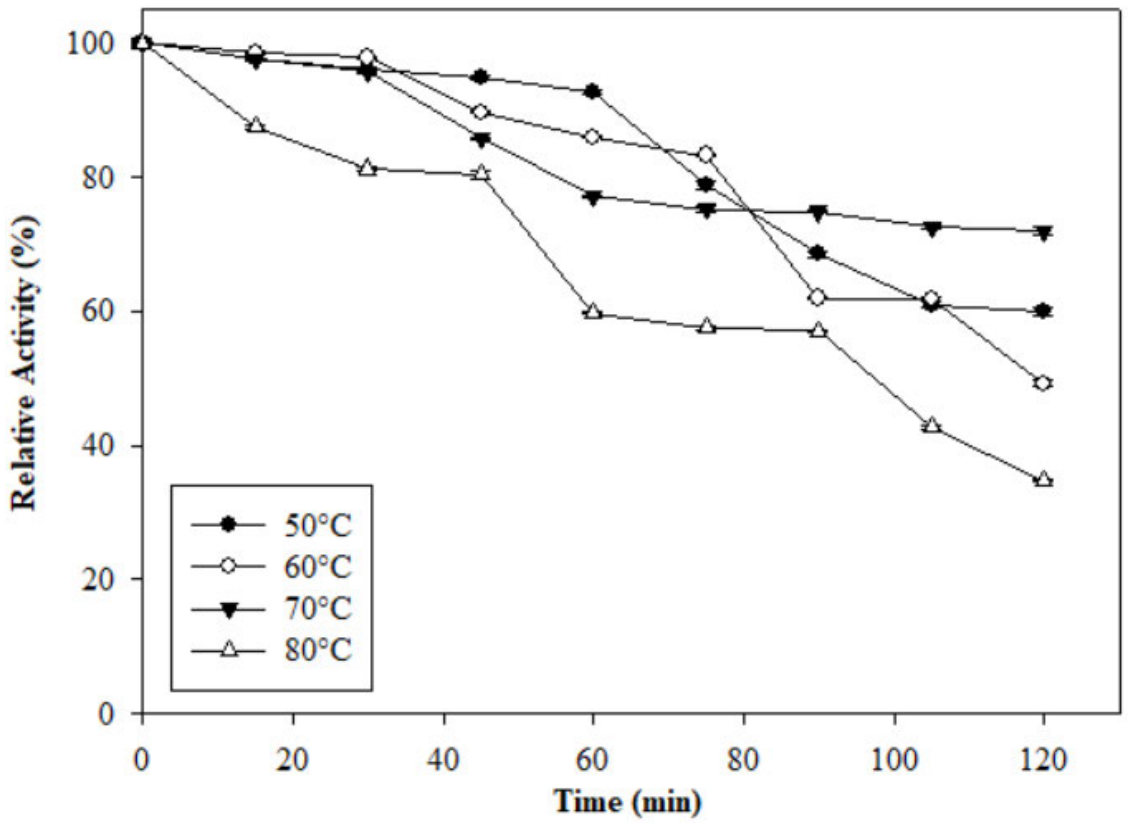

Fig 4.24: Temperature stability of Lipase C at temperatures 50, 60, 70 and 80˚C over a 120 min period. All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol at 410 nm. The activity at 100% was 181.0 ± 0.13 U/ml.

4.4.4 Substrate affinity

Substrate affinity analysis was performed to confirm if the enzymes were true lipases or esterases as the sequence analysis had hits that included esterases and lipases. In general, estearases have low activities on lipids with longer carbon chains while true lipases have high preference for them (Salameh and Wiegel., 2007).

A total of five *p*-nitrophenol substrates representing various lengths of fatty acids [*p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), p-nitrophenyl palmitate (C16) and *p*-nitrophenyl stearate (C18)] were used to determine the specificity or the preference of the three lipases. All three lipases preferred longer chain substrates, with *p*-nitrophenyl palmitate (C16) being the most favourable, with an exception of Lipase C which preferred *p*-nitrophenyl stearate (C18) with activity 7% higher than that on *p*-nitrophenyl palmitate (Fig. 4.25). This confirmed that they were all true lipases.

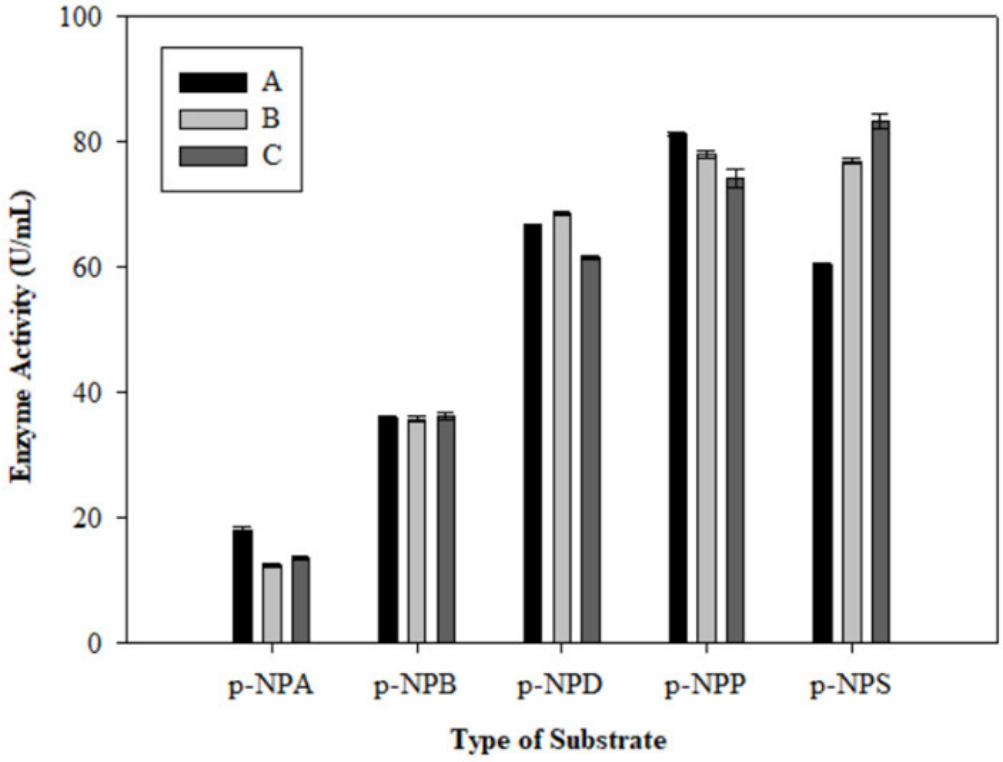

Fig 4.25: Determination of the specificity of lipases A, B and C towards *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), *p*-

nitrophenyl palmitate (C16), and *p*-nitrophenyl stearate (C18). All values are means of three replicates ± standard deviation obtained by monitoring the release of *p*-nitrophenol by UV-Vis spectrometry (410 nm).

4.4.5 Effect of metal ions on lipases

Among the metal ions tested, Ca^{2+}, Mg^{2+} and Mn^{2+} activated all three lipases, while Na^{+} had no significant effect on all the lipases. Co^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} had an inhibitory effect on the lipases with Fe^{3+} having the highest inhibitory effect on all three lipases.

Table 4.5: Relative lipase activity of lipases A B and C demonstrating the effect of metal ions

METAL IONS	A (%)	B (%)	C (%)
Ca^{2+}	144	146	150
Co^{2+}	42	45	41
Cu^{2+}	85	88	89
Fe^{2+}	50	53	51
Fe^{3+}	35	35	39
Mg^{2+}	123	125	125
Mn^{2+}	120	121	120
Na^{+}	98	97	99
Control	100	100	100

5. DISCUSSION

T. lanuginosus is well-known for producing a lipase that is widely used in industry. This enzyme (Lipolase) was first sold by Novozymes in 1998 as the first enzyme to be produced via genetic engineering (Novozymes, 2001). Engineered variants of this enzyme are now sold under the brand name Lipozyme TL, which also has wide application (Silva *et al*., 2015).

As previously mentioned, sequences of the three lipase genes were obtained from the genome sequencing of *T. lanuginosus* SSBP performed by Mchunu *et al*. (2013). The sequences were further analysed to investigate their novelty, similarities and to compare them to existing lipases. Analysis revealed that they were different and had low identity to existing lipases in the protein and nucleotide databases. The analysis further confirmed that they were putative lipases but none identified any of the lipase genes as from *T. lanuginosus*. This meant that all the lipase genes found in *T. lanuginisus* SSBP were novel and presented great potential for further research and application.

Conserved domain analysis (Fig 4.3) showed that all three genes belong to the abhydrolase superfamily, the family in which lipases and esterases belong. Furthermore, lipase C was also part of another family, PLNO2877 superfamily, another conserved protein domain family specifically for triacylglycerol lipases (Lu *et al*., 2020). Protein sequence alignment analysis (Fig 4.2) also revealed that lipases A and B are similar to each other compared to lipase C. SWISS protein models were also created usind the best template matches for each protein sequence (Figs 4.4, 4.5 and 4.7). The protein models further indicated the uniqueness of lipase C and the similarity between lipases A and B were further demonstrated by superimposing their ribbon models into each other (Fig 4.6). It thus became interesting to find out whether lipase C would posess different characteristics compared to its counterparts when cloned in *P. pastoris* GS115 and *E. coli* BL21.

Cloning studies for expression in *P. pastoris* was only successful with lipase B. Cloning was attempted for the lipases with three different vectors. The cDNA of *T. lanuginosus* SSBP was used to amplify the lipase B genes using primers designed for pPICZαA. pPIC9K and pPBG1 cloning and expression vectors. When the PCR products were analysed for amplification with gel electrophoresis, lipase B amplification produced a single distinct band of approximately 1100 bp which was the expected PCR product (Fig 4.8 and Fig 4.10). Amplification for lipase A proved to be unsuccessful as three bands were produced instead of a single distinct band, and purification and amplification of the correctly sized fragment was also unsuccessful.

Numerous factors could have contributed the formation of multiple bands during amplification of the LipA gene, including the annealing and extension temperature and time, DNA contamination, inappropriate use of PCR reagents and primer design. After eliminating and optimisation of PCR parameters, the resulting PCR product remained the same leading to the assumption that primer design was the cause of improper amplification. This could have been improved by increasing the length of the primers as the extra non - specific bands could have been from sequences similar to the target protein.

To allow cloning and expression of all three lipases in *E. coli* plasmids pET100/D-TOPO containing the three putative lipase was synthesised by Thermofischer Scientific (Table 3.2 and Fig 3.4) for expression in BL21 (DE3). These plasmids were designed using the three putative lipase sequences identified in the *T. lanuginosus* SSBP genome. As expected, this method created optimised *E. coli* expression using high copy number vectors.

Screening for transformation and expression of lipases in their respective hosts, was simultaneously performed using a chromogenic plate protocol coupled with induction using methanol for *P. pastoris* expression and IPTG for *E. coli* expression. Positive *P. pastoris* transformants were selected in BMMY-rhodamine B-olive oil plates. Colonies that showed

orange fluorescence under UV light indicated a positive lipase expression (Fang *et al*., 2014). Transformants cloned with LipB-pPICZαA, LipB-pPIC9k and LipB-pBGP1 were simply screened for positive expression of the lipase enzyme using this screen (Fig 4.14).

Positive *E. coli* transformants were selected on IPTG-induced LB-ampicillin plates containing emulsified olive oil and rhodamine B, to confirm expression in *E. coli.* All transformed colonies showed a positive lipase expression (Fig 4.17). This qualitative fluorescence lipase assay was based on the interaction of rhodamine B with fatty acids released during the enzymatic hydrolysis of triglycerides. This proved to be a more accurate assay compared to the commonly used tributyrin agar assay which lacks specificity for true lipases and often indicates the presence of esterases (Jette and Ziomek, 1994).

Recombinant lipase production by *P. pastoris* was studied by cultivating positively screened colonies transformed with LipB-pPICZαA and Lip-pPIC9K in shake flasks for 120 hours in BMMY media. Lipase production was induced with methanol in flasks at 24-hour intervals. There was no induction required for *P. pastoris* with LipB-pBGP1 cultivations because of the constitutive glyceraldehyde phosphate promoter in this vector. However, lipase enzyme expression in shake flasks was undetectable for LipB-pBGP1. This was surprising since low fluorescence was visible on screening plates. This suggests either a dilution effect in liquid cultures or that there was no gene expression and that fluorescence was due to host lipases.

The highest biomass for the expressed systems was obtained at 48 hours (Fig 4.15). The highest lipase expression for the other two vectors was obtained after 48 hours of incubation with lipase activity of 12.69 U/ml for LipB-pPICZαA and 4.33 U/ml for LipB-pPIC9K. Expression with pPIC9K was significantly lower and only less than a unit higher than the negative control (Fig 4.16). Since both vectors shared the AOXI promoter / terminator combination, this could be due to difference in the insertion sites in the *Pichia* genome.

Expression in *E. coli* was carried out for 8 h, including a 6.5 h period of induction with 0.25 mM IPTG at 30˚C then 4˚C overnight. The enzyme was extracted using Bugbuster Protein Extraction Reagent according to the supplier's manual. All three lipase were successfully extracted and were observed on the SDS – PAGE gel (Fig 4.18). Expression in *E. coli* was higher than in *P. pastoris.* Consequently, further enzyme characterisation protocols were performed using the *E. coli* expressed enzyme. Furthermore, the third lipase gene was also included in *E. coli* expression compared to *P. pastoris* expression where cloning of only two of the three genes was attempted.

The extracted enzyme solution was tested for protein presence by estimating the protein concentration (Lowry *et al*., 1951). The protein concentration was useful for loading equal concentration of protein on the SDS – PAGE gels and required for subsequent enzyme assays. Lipases A, B and C were produced at high levels. Production of the lipases occurred at much higher levels than the native *E. coli* proteins as evidenced by thick bands on SDS – PAGE gel. All three lipases showed thick protein bands ranging from approximately 30 kDa to 36 kDa, which is comparable to other *T. lanuginosus* lipases. Fang *et al*. (2014) characterised a recombinant *T. lanuginosus* lipase with a protein size of 33 kDa. LipA had the smallest size while LipC had the biggest protein size from the three lipases.

Protein sizes for lipases A, B and C were also calculated based on their sequences and the prediction showed a similer size range pattern. Since post- translational processing of eukaryotic proteins in prokaryotic hosts may not be optimal, predicted protein sizes were much higher than the ones obtained after expression in *E. coli*. Although the target proteins were identifiable based on their predicted sizes, it was clear that there were many protein impurities in the enzyme extracts. This is due to the fact that the target proteins were expressed intracellularly and in the process of extracting the lipase enzyme from *E. coli*, native proteins

from *E. coli* were also extracted (Studier, 2014). In order to prevent interferences with the enzyme assay, the extracted enzyme solutions were purified.

Protein purification was performed using His-Bind resin affinity chromatography since a polyhistidine (6×His) tag was added by the expression vector. This allowed for ease in the purification process which is often a time consuming process with proteins that do not have the poly-histidine tag. This would be an added advantage for specialised applications but a costly option as well. Pure enzymes are preferred for proper characterisation of cloned enzymes ensuring the absence of interfering contaminants which could lead to inaccurate results. Newer protein purification processes that utilise specificity and robustness are expected to guide the future of the purification market (Labrou, 2021). After purification, the recombinant lipases from *E. coli* produced lipase yields of 176.2 ± 1.2 for lipase A; 184.1 ± 0.46 for lipase B; and 181 ± 0.13 for lipase C. This was much higher than the activity yielded from *P. pastoris* expression, albeit without the purification step.

Enzyme characterisation was performed using only *E. coli* recombinants as *P. pastoris* exhibited relatively low levels of lipase expression. The temperature optima of lipases was determined using a temperature range from 40-90˚C. The temperature optimum profiles of all three lipases was similar, with the identical highest activity obtained at 60˚C for all three lipase variants. In literature, the optimum temperature for a thermostable lipase from *T. lanuginosus* was reported as 45˚C (Omar, *et al*., 1987). The commercial *T. lanuginosus* lipase Lipozyme Tl IM was also found to have a temperature optimum of 40˚C and it lost activity after this temperature (Koh et al., 2010). This is surprising since most hemicellulases from this fungus have temperature optima above 50˚C, with xylanase and mannosidase having remarkable optima of 70˚C and 80˚C, respectively (Singh *et al*., 2000).

It was also observed that between 40-80˚C, these lipases showed very good thermotolerance, with all three showing residual activity of over 80% at 50˚C. Over and above having an optimal temperature of 60˚C, their thermotolerance levels were impressive with lipase A having the highest residual activity of 80% followed by lipase B with 72%% activity while lipase C still had 62% residual activity at 70˚C. At 80˚C, lipase A still had residual activity of 49% dropping slightly to 37% at 90˚C; lipase B with residual activity of 47% also slightly dropping to 38%, while lipase C had residual activity of 48% dropping to 21% at 90˚C (Fig. 4.20).

The pH optimum was tested between pH 3 to pH 10. All three lipases had preference for alkaline conditions, with an optimum of pH 8, and activity was stable between pH 7.0-10.0. Interestingly, these lipases retained more than 80% of the overall activity at pH 9, lipase A had 86% activity, lipase B with 89% and lipase C had 83% residual activity. At pH 10, lipase A still retained 54% residual activity followed by lipase B with 53% with lipase C dropping slightly below 50%.

In another study, a purified lipase from *T. lanuginosus* strain Y-38 and found that the enzyme was optimally active at 60˚C, at pH 8.0 and was stable in the pH range of 4 to 11. It showed appreciable activity at up to 65˚C but was inactivated on heating at 80˚C for 20 min (Maheshwari, *et al*., 2000) . This lipase carries similar traits to the lipases in the current study, however, none of the lipases in this study were inactivated at 80˚C. Reactions catalysed by lipases are often carried out in mixtures of the reactants to avoid the use of the solvents and for this, reaction mixture has to be heated from 50˚C to 80˚C for the fat to be liquid. Therefore, lipases have to be thermostable for optimal performance (Zamost, *et al*., 2001). Hence it was important to investigate the thermostability of the lipases as they still showed relatively good activity at 80˚C.

Each recombinant lipase was tested for stability at temperatures from 50˚C to 80˚C over 120 minutes. All three lipases maintained very good thermostability across all four tested temperatures. Fang *et al*. (2014) also investigated the thermostability of a recombinant thermophillic lipase from *Thermomyces lanuginosus* $HSAUP_{03}80006$. This lipase also had similar traits to the lipases investigated in this study, with the an optimum temperature of 60˚C and a higher pH optimum of 9. However, the enzyme only retained 55% of it's activity at 70 ˚C and 45% at 80 ˚C in 60 min. This means that the thermostability of lipases A, B and C greatly outperformed the *T. lanuginosus* lipase by Fang *et al.* (2014), after 60 minutes as well as after 120 min.

Substrate affinity analysis was performed to confirm if the enzymes were true lipases or esterases as the sequence analysis had hits that included esterases and lipases. In general, esterases have low activities on lipids with longer carbon chains while true lipases have high preference for them (Salameh and Wiegel., 2007). A total of five *p*-nitrophenol substrates representing various lengths of fatty acids [*p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), p-nitrophenyl palmitate (C16) and *p*-nitrophenyl stearate (C18)] were used to determine the specificity or the preference of the three lipases. All three lipases preferred longer chain substrates, with *p*-nitrophenyl palmitate (C16) being the most favourable, with an exception of Lipase C which preferred *p*-nitrophenyl stearate (C18) with activity 7% higher than that on *p*-nitrophenyl palmitate (Fig. 4.25). This confirmed that they were true lipases.

The analysis on the effects of metal ions revealed that Ca2+ significantly activated the lipase enzymes while iron metals Fe2+ and Fe3+ significantly inhibited the enzymes when tested under optimal conditions.

6. CONCLUSION

The results from this study confirmed that the three putative lipase genes discovered when the *T. lanuginosus* SSBP genome was sequenced (Mchunu *et al*., 2014) are indeed different from each other. Conserved protein domain analysis confirmed that they all belong to the abhydrolase superfamily with lipase C further identified as part of a triacylglycerol lipases domain family – PLNO2872 superfamily (Lu *et al*., 2020). Most organisms do have multiple lipases, presumably to catalyse different substrates. Multiple enzymes can also arise from gene duplication events, but based on the differences in DNA sequences, it appears that these three lipases evolved independently, although lipases A and B share some structural similarity. It is also apparent that these three lipases are not related to the *T. lanuginosus* lipase (Lipolase) that has been commercialised.

Enzyme characterisation revealed that all three recombinant lipases were thermophilic with an optimum temperature of 60˚C and alkalophilic with an optimum pH of 8, both traits consistent with the fungal host, *T. lanuginosus* SSBP (Singh *et al*., 2000). Furthermore, they all showed very good thermostability at temperatures ranging from 50 – 90˚C at pH 8. The substrate affinity analysis further confirmed that all three genes are true lipases as they all yielded much higher activities when acting on longer chain triacylgylcerides, *p*-nitrophenol palmitate (C16)and *p*-nitrophenol stearate (C18) when compared to short chain triacylglycerides, *p*-nitrophenol acetate (C2) and *p*-nitrophenol butyrate (C4) (Salameh and Wiegel., 2007). The Ca^{2+} metal ion was shown to significantly activate the lipase enzymes, giving guidance on numerous possible industrial applications. The above properties suggest that these enzymes would be useful for the many industrial applications of lipases.

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APPENDIX A

Table A1: Sets of primers for cloning into pPICZαA, pPIC9K and pBGP1

Cloning vectors	Primers
pPICZαA	LipA forward primer: 5′ GGTACCAGCGGAGCTCCCTCGTGCT 3′ LipA reverse primer: 5′ TCTAGATTAATCACACTCTGAAATGGG 3′
	LipB forward primer: 5′CTCGAAGATGAGGAGCTCCCTTGTGCTGTTC 3′ LipB reverse primer: 5′ CTCAGACTACTAAAGACATGTCCCAATTAAC 3′
pPIC9K	LipA forward primer: 5′ TACGTAATGCGGAGGCTCCCTCGTG 3′ LipA reverse primer: 5′ TGCTCTAGACCCGTCGGTGGATTAATCACA 3′
	LipB forward primer: 5′ TACGTAATGAGGAGCTCCCTTGTGC 3′ LipB reverse primer: 5′ TGCTCTAGAACCCTAAAGACATGTCCCAATTAAG 3′
pPBGP1	LipB forward primer: 5′ GGAATTCTGTACGACGATGACG 3′ LipB reverse primer: 5′ GAGCTCGAGCTTTTATTCTG 3′

APPENDIX B

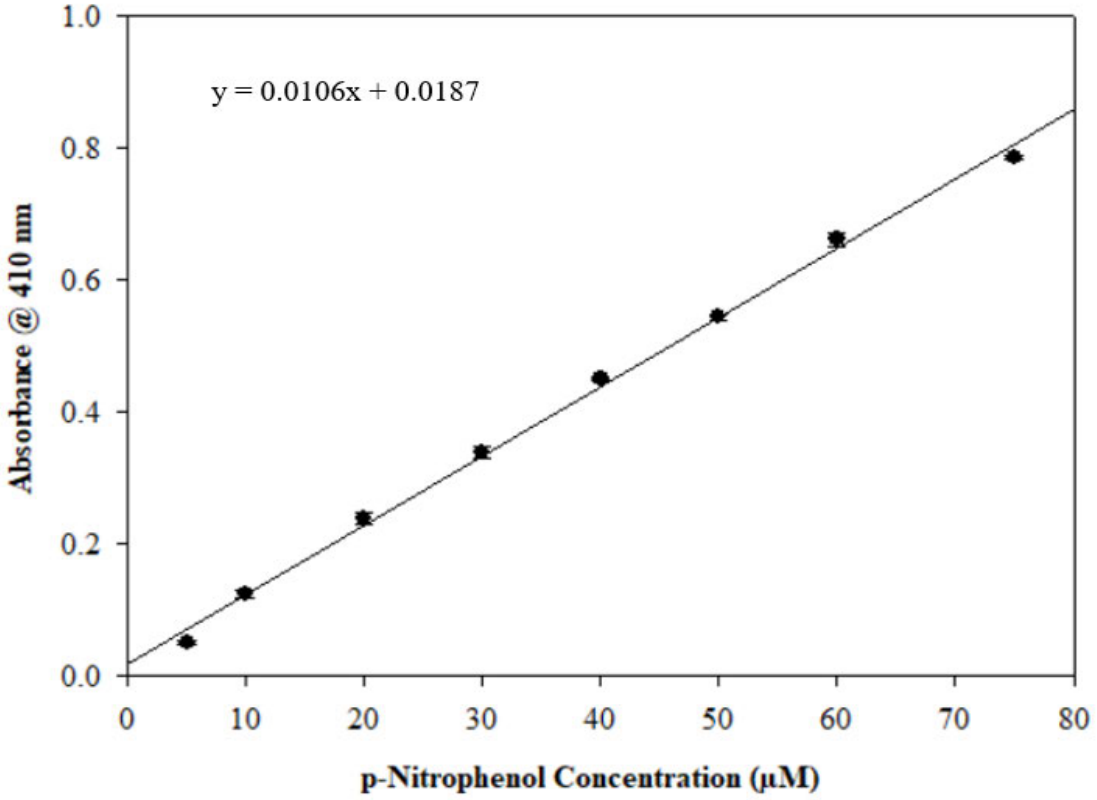

Fig B1: *p*-Nitrophenol standard curve used to calculate lipase enzyme activity.

APPENDIX C

T. lanuginosus RNA, bands show the 28s and 18s RNA

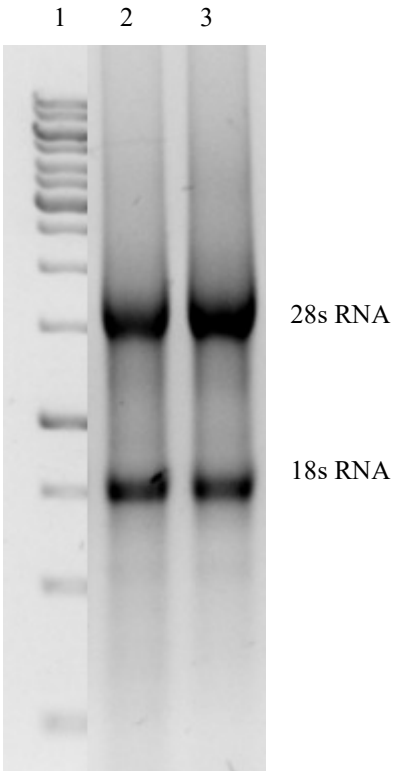

Fig C1: Total RNA isolation from *T. lanuginosus*. 28S and 18S rRNA is labelled.

APPENDIX D

Nucleotide sequences and BLAST results for lipases

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                    20                  40                  60                  80
lipA ATGAGGCCCCGTCCAGTTTTGCGCTCATCCTTGCGCTTCCTCTCAACATGCGCCGCCCGTCCAGTACTCCACTCCACATCGCTCAATTAT
                   100                 120                 140                 160                 180
lipA CGTGCCTCACGCGTGCCGCATGCTCGTAGCAGTGCCACGGCCACTACACCAGATCCACGCTTCGGCACTCTCATCAAAGACAAATTCGCA
                   200                 220                 240                 260
lipA CATATTCGGGATGACTATGGTATTTCTCGCGCTCTTGATACTGCCCCTTCTCATATAGATTTCACTGACTCCCGGGCCCCAGCGACGCCA
                   280                 300                 320                 340                 360
lipA AAGAACGCAATCGTGCTTGCCCATGGCCTACTGGGCTTCGACGAAATCCGACTTGCGGGACCATTTCTCCCCGCGATTCAATATTGGCGT
                   380                 400                 420                 440
lipA GGAATTCGCGACGCCCTGGCGCTGAAAGGTATCGAGGTGATTACGACGTCTGTGCCGCCGTACGGGACCATCGAAAAGCGTGCAGAGGCA
                   460                 480                 500                 520                 540
lipA TTGGCCATGAATATCGTCGCGCAGGCGCGGGGAAAGGATGTTAACATCATCGCGGGTACGACTGCCGAGGTAATTGGGCTCACGATGTCA
                   560                 580                 600                 620
lipA AAACTGATGGTTTTCAGAGGTCTCGATTCGCGCTTTATGATTAGCAGCCTTCGGCCGACGGAGTTTCGAGTGCGGTCCCTGACTACGGTT
                   640                 660                 680                 700                 720
lipA GCGACGCCTCATAGGGGTTCCGTCATTGCGGACTATTTCATGGAGCGCGTTAGTAACAATGTCTCACGCCTTCAGAAATTATCCCGCACT
                   740                 760                 780                 800
lipA CTGGATCGGATTGACTTAGACATGACTGCTTTTGGGCAATTGACCCGGTCGTATCTACAATCTGAATTCAATCAGTCCGTACCTGATCTG
                   820                 840                 860                 880                 900
lipA GATGAGGTCCGATACTTTTCGTACGGTGCCATGTTTCAGCCAGGGATCTTCAACGTATTCCGCCCCTTCTATCAGGTACTGGCCGAGGTG
                   920                 940                 960                 980
lipA GAGGGTCCGAACGACGGTCTCGTGAGCGTGGAGAGCAGCCGCTGGGGTGGTGACCGGGGCTACAAAGGCACCCTGGTCGGCGTCAGCCAT
                 1,000               1,020               1,040               1,060               1,080
lipA CTGGATCTGATCAATTGGACGAACCGATTGGAGTGGCTGACGGCGTTGATCACGGGAAACCGAAGGAAATTCAATGCCATTGCATTCTAC
                 1,100
lipA CTGGACATTGCCGATATGCTGGCCAAAGAGGGCCTATGA
```

Molecule type dna
Query Length 1199
Other reports Distance tree of results MSA viewer

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 100

select all 91 sequences selected — GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Synthetic construct HSP20 gene, complete cds	synthetic construct	65.8	65.8	2%	1e-05	100.00%	1115	MK829064.1
Plant transient expression vector pTK251, complete sequence	Plant transient e...	60.2	60.2	2%	5e-04	100.00%	12543	JX971625.1
Synthetic construct oleate hydratase gene, complete cds	synthetic construct	54.7	54.7	2%	0.025	100.00%	1929	MN563121.1
Cloning vector pEA206, complete sequence	Cloning vector p...	54.7	54.7	2%	0.025	100.00%	7861	MN161744.1
Cloning vector pBOB11_C-Term, complete sequence	Cloning vector p...	54.7	54.7	2%	0.025	100.00%	8509	MN991175.1
Synthetic construct SomaGCaMP6f1 gene, partial cds	synthetic construct	54.7	54.7	3%	0.025	90.70%	2658	MK695671.1

Fig D1: Nucleotide sequence and BLAST results for LipA.

Molecule type	dna
Query Length	1451
Other reports	Distance tree of results MSA viewer

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download | New Select columns | Show 100

select all *91 sequences selected* — GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Synthetic construct HSP20 gene, complete cds	synthetic construct	65.8	65.8	2%	1e-05	100.00%	1115	MK829064.1
Plant transient expression vector pTK251, complete sequence	Plant transient e…	60.2	60.2	2%	7e-04	100.00%	12543	JX971625.1
Synthetic construct SomaGCaMP6f1 gene, partial cds	synthetic construct	58.4	58.4	3%	0.002	89.58%	2658	MK695671.1
Synthetic construct SomaGCaMP6f2 gene, partial cds	synthetic construct	58.4	58.4	3%	0.002	89.58%	1701	MK695670.1
Synthetic construct calcium indicator jGCaMP7c gene, complete cds	synthetic construct	58.4	58.4	3%	0.002	89.58%	1353	MK749394.1
Synthetic construct calcium indicator jGCaMP7b gene, complete cds	synthetic construct	58.4	58.4	3%	0.002	89.58%	1353	MK749393.1

Fig D2: Nucleotide sequence and BLAST results for LipB.

Description	None
Molecule type	dna
Query Length	1526
Other reports	Distance tree of results MSA viewer

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download | New Select columns | Show 100

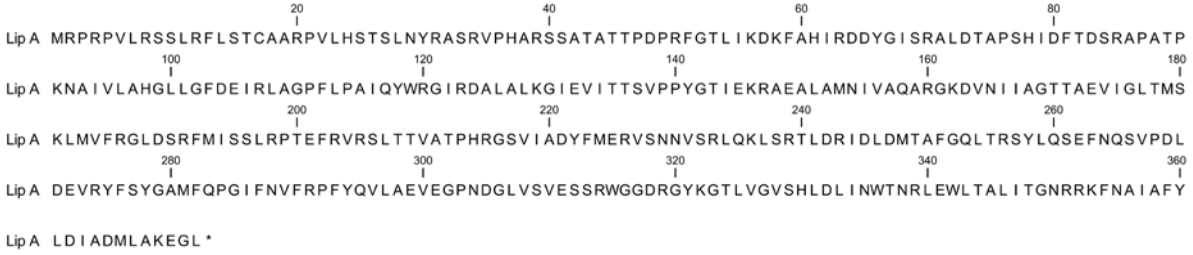
select all *100 sequences selected* GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus sydowii CBS 593.65 uncharacterized protein (ASPSYDRAFT_155695), partial mRNA	Aspergillus syd...	294	294	44%	2e-74	74.78%	1404	XM_040842485.1
Aspergillus puulaauensis cholesterol esterase (TGL1), partial mRNA	Aspergillus puul...	257	257	44%	2e-63	73.77%	1404	XM_041695905.1
Aspergillus puulaauensis MK2 DNA, chromosome 7, nearly complete sequence	Aspergillus puul...	257	257	44%	2e-63	73.77%	3323588	AP024449.1
Capronia coronata CBS 617.96 lysosomal acid lipase/cholesteryl ester hydrolase partial mRNA	Capronia coron...	254	254	44%	3e-62	73.71%	2283	XM_007728371.1
Colletotrichum aenigma putative lipase (CGCA056_v012222), partial mRNA	Colletotrichum ...	254	254	45%	3e-62	73.65%	1962	XM_037327466.1
Colletotrichum fructicola putative lipase (CGMCC3_g15677), partial mRNA	Colletotrichum f...	231	231	45%	1e-55	73.09%	1938	XM_032025717.1

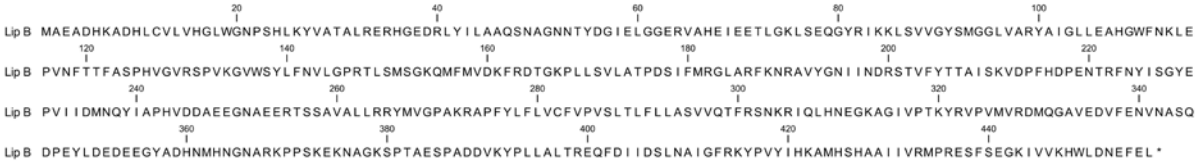
Fig D3: Nucleotide sequence and BLASTresults for LipC.

APPENDIX E

(a)



(b)



(c)

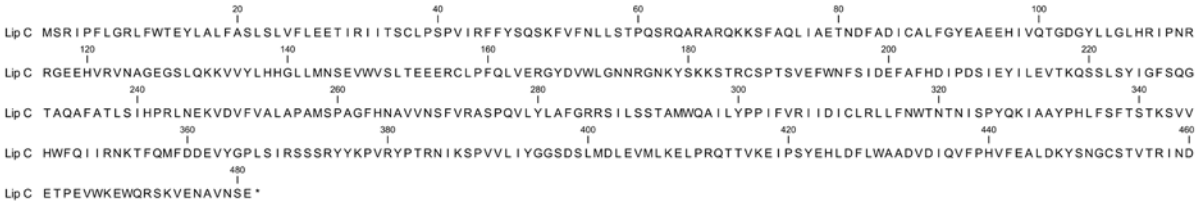


Fig E1:Amino acid sequences for LipA (a), LipB (b) and LipC (c).

APPENDIX F

Fig F1: Alignment of protein sequences of Lipases A, B and C to commercial Lipolase