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BIOPROSPECTING FOR NOVEL LIPASES FROM INDIGENOUS OLIVE WASTEWATER BIOFILMS

by

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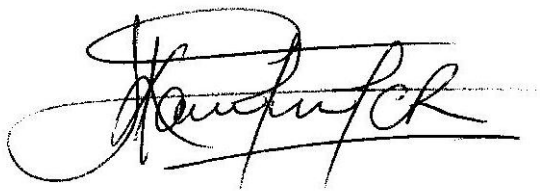
DECLARATION

I, James Kagaba, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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SUMMARY

Lipase-catalysed chemical transformations are today routinely considered by synthetic organic chemists as economical and competitive “green chemistry” alternatives. Although lipases can effortlessly be produced on a large-scale by fermentation, their industrial application was, until recently, limited to the detergent, oleo-chemistry and dairy industry. However, during the last few decades, the biotechnological application of lipases has expanded significantly, becoming indispensable in the manufacture of pharmaceuticals, pesticides, single cell protein production, biosensor preparations and waste management. Similarly, lipases have become a vital ingredient in the contemporary food processing industry with applications ranging from fruit juice production to baked foods, vegetable fermentations and dairy enrichment. Furthermore, lipases are routinely used as flavour development agents in cheese, butter and margarine products. Lipases are also applied in the leather industry for processing hides and skins and for treatment of activated sludge and other aerobic waste product treatments where its action enhances oxygen transfer. While lipases currently account for less than 21 % of the enzyme market, a growing interest in lipases is reflected by the publication of an average of 1000 research papers per year and the growing number of available lipases since the 1980s. There is a sustained interest to bioprospect for novel lipase enzymes from available unexplored biodiversity.

This study aimed to screen for lipase-producing microorganisms resident in olive wastewater biofilms. Lipase activity of positive isolates was subsequently also quantitatively determined to select for the highest producers of true lipases. A *Geotrichum candidum* isolate from olive mill wastewater biofilms was selected for subsequent studies based on its superior lipase production phenotype. Using a yeast mediated ligation approach the *G. candidum GCL1* lipase gene was cloned and heterologously expressed in *Saccharomyces cerevisiae* as an enzyme production host. The recombinant lipase was purified and analysed in terms of substrate specificity, pH optima, temperature optima and stability as well as organic solvent tolerance. The *G. candidum* gcl1 lipase presented enhanced thermo- and organic solvent-stability that are highly sought after traits for industrial application.

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CHAPTER ONE:
GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction - The continued quest for lipases

Lipases (EC 3.1.1.3), also known as triacylglycerol acylhydrolases, belong to the serine hydrolase family, responsible for the hydrolysis and synthesis of natural (found in mono-, di- and triglycerides) and non-natural ester bonds (Bornscheuer, 2002). Lipases are ubiquitously found throughout all domains of life, but microbial lipases have found particular commercial importance as versatile industrial biocatalysts. Some of the reasons why microbial lipases are preferred industrial biocatalysts include the fact that lipolytic microorganisms secrete lipases typically at high extracellular levels and these enzymes can be relatively easily produced at large scale. Furthermore, microbial lipases show various characteristics amenable to industrial use (Choudhury and Bhunia, 2015). Microbial lipases catalyse reactions under mild or extreme conditions, e.g. pH and temperature, are stable in aqueous and non-aqueous media, show broad substrate specificity and high regio- and/or stereoselectivity during catalysis. For this reason, lipases have found application in various multifaceted biotechnological processes. For example, the hydrolytic action of lipases is relevant to the detergent industry aiming at developing cost-efficient and energy-saving washing processes (Tang *et al.*, 2017). Transesterification reactions of lipases play an essential role in the food, nutraceutical and biofuel industries (Zhao *et al.*, 2015; Rivero *et al.*, 2017), while the regio- and/or stereoselectivity of lipases make them highly applicable in the synthesis of pharmaceuticals and other pure compounds (Sun *et al.*, 2017).

Driven by the catalytic versatility of the lipases in industrial applications and the quest of manufacturing industries to develop alternative greener, safer and sustainable processes studies on the identification, isolation, production and characterization of novel lipases, as evidenced by the vast body of annual published research, persist (De Godoy Daiha *et al.*, 2015; Sarmah *et al.*, 2017). Over the last few decades, bioprospecting for microbe-associated lipases, from culturable and non-culturable microorganisms, from extreme or less explored environments, have provided promising novel lipase candidates for tailored industrial applications (Lopez-Lopez, Cerdan and Siso, 2014; Sarmah *et al.*, 2017; Tang *et al.*, 2017).

1.2 Olive wastewater (OWW) as a potential source of lipases

The extraction of olive oil and manufacture of table olives are economically important in many Mediterranean countries, as well as in South Africa (Fernandez, Adams and Fernandez-Diez, 1997). Olive oil and table olive processing industries generate enormous volumes of dark coloured effluent, generally termed olive wastewater

(OWW), and solid residues, which are significant environmental pollutants due to the high organic load and recalcitrant polyphenol content (Khoufi, Aloui and Sayadi, 2006).

OWW, rich in oil residues, has been shown to host a diverse population of indigenous microbial species in the form of biofilms (Ntougias, Bourtzis and Tsiamis, 2013). It is acknowledged that biofilm microorganisms have adapted to and survive in their environments by producing enzymes that provide them with a nutritional resource or protect them from toxic environments (Boltz *et al.*, 2017). The milieu of OWW could be considered a relatively high-stress environment to survive in, i.e. fluctuations in pH, salinity, toxic phenolic content for the resident microbial population. It is hypothesised, as the basis for undertaking this study that the microbial inhabitants of OWW biofilms would produce enzymes with unique adaptations to function optimally under these conditions. For this reason, the microorganisms within the olive wastewater biofilms could be considered a valuable resource for bioprospecting lipase enzymes with potential industrial applications.

1.3 Aims of the Study

The study aimed to screen the microbial population present in indigenous olive wastewater biofilms for novel lipases.

The specific objectives were as follows:

1. Screening of the culturable microbial fraction of OWW biofilms for lipase activity using traditional plate screening methods
2. Characterisation and quantification of lipase activity by different OWW biofilm isolates
3. Cloning and sequencing of a lipase gene(s)
4. Heterologous expression and characterisation of the lipase gene(s) in the yeast *Saccharomyces cerevisiae*.

CHAPTER TWO:
LITERATURE REVIEW: MICROBIAL LIPASES AND ITS INDUSTRIAL APPLICATIONS

2.1 Lipases - General definition and enzymatic properties

Lipases are water-soluble enzymes classified as triacylglycerol acylhydrolases, pertaining to their ability to catalyse reactions of hydrolysis (Fig. 2.1 A), with the release of carboxylic acids and alcohols, of ester bonds in acylglycerols as their natural substrates (Lopes *et al.*, 2011). Under low water conditions, however, ester synthesis reactions known as esterification or transesterification reactions may also occur (Fig 2.1 B). Lipolytic enzymes and lipases are generic terms describing two distinct groups of enzymes associated with the lipid bioconversion: carboxylesterases (CEs; EC 3.1.1.1) and true lipases (TLs; EC 3.1.1.3) (Fojan *et al.*, 2000). Several criteria (Table 2.1) have been compiled to differentiate TLs from CEs, however, substrate specificity remains the only criterion entirely valid currently for this distinction due to the existence of several exceptions. CEs exhibit a preference towards short-chain acyl molecules (< ten carbons), while TLs have a substrate preference for long-chain acyl molecules (≥ ten carbons) (Jaeger, Dijkstra and Reetz, 1999; Bornscheuer, 2002). Furthermore, TLs have an increased ability to act on aggregated non-dissolved lipids, as opposed to CEs which act on water-dissolved molecules (Neves Petersen, Fojan and Petersen, 2001). Nevertheless, it should be noted that most TLs are still capable of hydrolysing carboxylesterases substrates, and *visa versa* (Jaeger, Dijkstra and Reetz, 1999; Fojan *et al.*, 2000).

A. Hydrolysis:

$$RCOOR' + H_2O \rightarrow RCOOH + R'OH \quad (1)$$

B. Synthesis:
Reactions under this category can be further classified:

(a) Esterification

$$RCOOH + R'OH \rightarrow RCOOR' + H_2O \quad (2)$$

(b) Interesterification

$$RCOOR' + R''COOR_* \rightarrow R''COOR_* + R''COOR' \quad (3)$$

(c) Alcoholysis

$$RCOOR' + R''OH \rightarrow RCOOR'' + R'OH \quad (4)$$

(d) Acidolysis

$$RCOOR' + R''COOH \rightarrow R''COOR' + RCOOH \quad (5)$$

Figure 2.1. The catalytic actions of lipases.

Lipases are classified as serine hydrolases with a catalytic triad composed of serine, histidine and aspartate or glutamate, which is also found in serine proteases. The mechanisms involved in lipase catalysis are illustrated in Fig. 2.2. In the first step, serine is activated by deprotonation, for which histidine and aspartate are required (Fig. 2.2 a). Consequently, the nucleophilicity of the hydroxyl residue of serine is enhanced and attacks the carbonyl carbon of the ester bond forming an acyl-enzyme intermediate (Fig. 2.2 b). The presence of an oxyanion hole contributes to the stabilisation of charge distribution and reduction of the ground state energy of the tetrahedral intermediate. The deacylation step in Fig. 2.2 c is shown which is controlled by the electronegativity of the molecules populating the interface. In this process, a nucleophile (e.g. H_2O or monoglyceride) attacks the acyl-enzyme intermediate leading to the product (a carboxylic acid) release and regeneration of the catalytic site (Reis *et al.*, 2009; Adlercreutz, 2013).

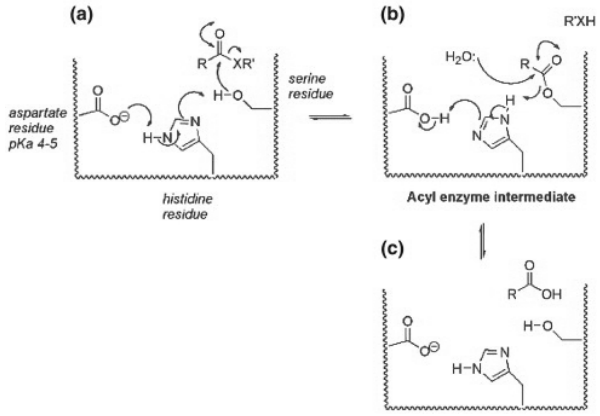

Figure 2.2. Mechanism of lipase catalysis (adapted from Reis *et al.*, 2009).

Lipases together with esterases, phospholipases, proteases, amidases, epoxide hydrolases, nitrilases and glycosidases fall in the structural superfamily α/β hydrolases (group EC 3), composed of a conserved pentapeptide Gly-X-Ser-X-Gly motif, a core of predominantly eight parallel β strands forming a super-helically twisted central β sheet surrounded by varying number of α helices (Gupta *et al.*, 2015). The active serine (in the conserved pentapeptide) is usually located in a central β sheet termed the catalytic elbow.

In the past interfacial activation was used to assign an enzyme to a TL (Table 2.1). Briefly, it describes a sharp increase of lipase activity when the substrate forms micellar aggregates or emulsion droplets (i.e. in the presence of an interface) when the substrate reaches a specific higher concentration (Reis *et al.*, 2009). This differs to the Michaelis-Menten reaction kinetics displayed by CEs, where activity increases as substrate concentration increases (Brzozowski *et al.*, 1991; Bornscheuer, 2002). This

phenomenon is usually correlated with the presence of a “lid” in the three-dimensional enzyme structure. The lid corresponds to a surface loop of the protein covering the active site of the enzyme which moves away (lid-open conformation) when in contact with a lipid-water interface, to allow substrates access to its active site(s). Thus, the enzymatic reactions catalysed by true lipases are sensitive to the lipid-water interface where the substrates usually form an equilibrium between monomeric, micellar and emulsified states (Jaeger, Dijkstra and Reetz, 1999). On the contrary, carboxylesterases activity is highest on more water-soluble substrates, and it thus depends on substrate concentration (Fojan *et al.*, 2000; Bornscheuer, 2002). However, these criteria are not entirely suitable to define TLs as some do not show interfacial activation and/or do not have a lid loop (Jaeger, Dijkstra and Reetz, 1999).

Table 2.1: Differences between TLs and CEs

Characteristic	True Lipases (TLs)	Carboxylesterases (CEs)	Reference
Preferred substrate	Long-chain acylglycerols and esters	Short-chain acylglycerols and simple esters	(Jaeger, Dijkstra and Reetz, 1999)
Standard substrate	Triolein; p-nitrophenyl palmitate	Tributyrin; p-nitrophenyl butyrate	(Fojan *et al.*, 2000; Bornscheuer, 2002)
Substrate chain length preferred	More than ten carbons	Less than ten carbons, usually less than six	(Ribeiro *et al.*, 2011)
Preferred hydrophobicity of substrate	High	High to low	(Bornscheuer, 2002)
Binding site for acyl chains	Long	Short	(Pleiss, Fischer and Schmid, 1998)
Interfacial activation and mobile lid	Yes (exceptions do occur)	No	(Brzozowski *et al.*, 1991)
Solvent-exposed amino acids	More hydrophobic, non-polar amino acids	Less hydrophobic, non-polar amino acids	(Fojan *et al.*, 2000)
Active site amino acids	More small, non-polar amino acids	Less small, non-polar amino acids	(Fojan *et al.*, 2000)
Optimum pH	8 to 9, with a few exceptions preferring 5 to 6	5.5 to 7	(Hasan, Shah and Hameed, 2006; Ramani *et al.*, 2010)
Stability and activity in organic solvents	High	High to low	(Bornscheuer, 2002)
Range of substrates	Broad	Broad to tight	(Fojan *et al.*, 2000)
Regio- and stereoselectivity	High	High to zero	(Fojan *et al.*, 2000)
Reaction kinetics	Certain minimum substrate level required for activity	Michaelis-Menten kinetics	(Bornscheuer, 2002)

Lipases from a large number of bacterial, fungal, plant and animal sources have been purified to homogeneity (Javed *et al.*, 2017). Enzymes hydrolysing triacylglycerols have been studied for well over 300 years, and the ability of the lipases to catalyse the hydrolysis and also the synthesis of esters were recognised nearly 70 years ago. In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them to soluble products (Hasan, Shah and Hameed, 2006). Lipases have traditionally been obtained from mammalian pancreas or digestive tracts and were used as digestive aid for human intake and flavour modifications in milk-fat, but it later became apparent that the use of these partially pure lipases could include unfavourable tastes, hormones and viruses (Macrae and Hammond, 1985; Hasan, Shah and Hameed, 2006). Lipases have also been studied and isolated from fish (which uniquely show preference towards poly-unsaturated fatty acids), snails (functioning as a digestive aid), scorpions (preferring alkaline conditions) and crabs (digestive aid with thermophilic qualities) (Zouari *et al.*, 2007; Cherif and Gargouri, 2009; Kurtovic *et al.*, 2009; Amara *et al.*, 2010). Lipases are indeed common among plants, but knowledge regarding these enzymes is limited, primarily due to the challenges linked with their extraction. These challenges are associated with the observation that plant lipases are mainly membrane-related and predominantly present in germinating oilseeds and cereals. Various reports and reviews have demonstrated the applications of specific plant lipases, but these all conform to the view that plant lipases provide numerous obstacles concerning their extraction, yields and scalability (Barros, Fleuri and MacEdo, 2010; Nagarajan, 2012; Rivera, Mateos-Díaz and Sandoval, 2012).

Microbial lipases first gained attention in 1901 when the microbiologist Eijkman observed their presence in *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Serratia marcescens* (Eijkman, 1901). The first crystal structure of a fungal lipase (that of *Rhizomucor miehei*) and a bacterial lipase (that of *Pseudomonas glumae*) was completed towards the end of the 20th century, respectively (Brady *et al.*, 1990; Noble *et al.*, 1993). Microbial lipases have gained particular industrial attention due to their broad substrate specificity, robustness under extremes of temperature, pH and organic solvents, and chemo-, regio and enantioselectivity (Mehta, Bodh and Gupta, 2017). Short generation times, ease of genetic manipulations, scale-up, and purification is a further significant benefit of working with lipases from microorganisms (Nagarajan, 2012). Microbial lipases can be membrane-bound (Najjar *et al.*, 2011), intracellular (Jermsuntiea *et al.*, 2011) or, most commonly, extracellular (Sharma, Chisti and Banerjee, 2001). They are inducible enzymes, with only a few being expressed constitutively (Nagarajan, 2012). Monomeric lipases can

have molecular weights of between 19 and 60 kDa, with oligomeric forms more than 300 kDa (Salameh and Wiegel, 2007). Crude and purified lipases are known to frequently form high molecular weight aggregates (Sharma, Chisti and Banerjee, 2001). They usually do not require cofactors to function, although Lu *et al.* (2013) reported that calcium ions have been able to stimulate the activity of specific lipases by forming long-chain fatty acid salts, which can decrease fatty acid interference at the interface. Although there are several commercially exploited producers of lipase, the main microbial sources are the fungal genera *Mucor*, *Geotrichum*, *Candida*, *Aspergillus*, *Rhizopus* and *Penicillium*, and the bacterial genera *Bacillus*, *Pseudomonas* and *Burkholderia* (Nagarajan, 2012).

2.2 Physiological role and diversity of microbial lipases

Microbial lipases originate mostly from mesophilic and thermophilic microbes and can function in a broad range of temperatures, exhibiting activity and stability in temperatures between 27°C and 70°C. The optimal temperatures for lipase activity are usually between 30°C and 40°C (Ribeiro *et al.*, 2011), although cold-active and thermotolerant lipases have been reported (Maiangwa *et al.*, 2015; Javed *et al.*, 2017). Microbial lipases are functional over a broad pH range (pH 4 – 12), generally exhibiting optimum activity in neutral conditions (Hasan, Shah and Hameed, 2006), but acidic and alkaline lipases have recently been the focus of increased research (Anobom *et al.*, 2014). Their preferences regarding pH and temperature are usually linked to the environment the host organism inhabits.

2.2.1 The Physiological Role of Microbial Lipases

The physiological function of lipases is not yet clear for many of them, although it has been postulated that they offer microorganisms a considerable physiological advantage (Ruiz, 2001), as fungi and bacteria may secrete lipases to facilitate nutrient absorption from the external medium or in examples of pathogenic microbes, to promote invasion of a new host. Thus they may be considered crucial microbial virulence factors (Stehr *et al.*, 2003). Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an essential role in the turnover of these water-insoluble compounds.

In general, lipases as lipolytic enzymes break down and mobilise lipids mainly triacylglycerols within the cells of the individual organism as well as in the transfer of lipids from one organism to another (Beisson *et al.*, 2000). Lipases also play other significant physiological roles within their host cells, which includes adhesion to host cells and host tissues, synergistic interactions with other enzymes, initiation of the inflammatory processes by affecting immune cells and self-defence mediated by lysing competing microflora (Gácser *et al.*, 2007).

2.2.2 Diversity of Microbial Lipases

Although a large number of lipases has been obtained, purified and applied from animal and plant tissues, it does not compare to the rich and diverse source that microorganisms have provided (Sharma, Chisti and Banerjee, 2001). Microbial lipases isolated from different sources have a wide range of properties depending on their sources concerning thermostability, pH optimum, etc., providing enzymes which possess a broad range of applicability in the industrial sector (Saxena *et al.*, 2003). In recent years, lipases from extremophilic organisms including thermophiles, acidophiles, alkaliphiles, halophiles, psychrophiles and basophiles of various origins have been exploited for their biocatalytic properties, as microbial lipases isolated from harsh environments often show higher inherent enzyme stability and catalytic efficiency (Moura *et al.*, 2017).

Filamentous fungal and yeast lipases have become the most industrially used enzymes in the world. This is due to their high substrate specificity, high catalytic efficiency, temperature and pH tolerance, activity in organic solvents, and general stability under various chemical and physical industrial conditions, combined with the fact that fungal enzymes are secreted for easy and cost-effective recovery (Mehta, Bodh and Gupta, 2017). Filamentous fungi are amenable to cultivation by solid substrate fermentation (SSF) using cheap substrates and therefore have the advantage of considerably reducing the cost of lipase production. Even when cultured in submerged fermentation (SmF) mode, fungal lipases are easily amenable to extraction thereby significantly reducing the recovery cost (Table 2.2). Fungal lipases do not require other accessory proteins (molecular chaperones) as opposed to their bacterial counterparts for proper enzyme activation (Hobson *et al.*, 1993).

The first commercial lipase enzyme appeared in 1994 (Novo Nordisk). The enzyme originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. Since then, fungal lipases of genera *Geotrichum*, *Rhizopus*, *Fusarium*, *Penicillium*, *Aspergillus*, *Thermomyces Candida rugose*, *Candida antarctica*, *Candida cylindrecea* and *Yarrowia lipolytica* have been extensively exploited for biotechnological applications, constituting more than 50 % of available commercial lipases (Maldonado, 2017; Mehta, Bodh and Gupta, 2017) (Table 2.2).

Table 2.2. Various fungal strains for lipase production through fermentation adapted from Mehta, Bodh and Gupta (2017).

Microorganism	Production Time (h)	Lipase activity (U/ml)	Type of fermentation	Raw material	Reference
Penicillium aurantiogriseum	48	25	SmF	Soya bean oil	(Lima *et al.*, 2003)
Rhizopus rhizopodiformis	24	43	SSF	Olive oil cake-Bagasse	(Cordova *et al.*, 1998)
Rhizopus pusillus	25	10.8	SSF	Olive oil cake-Bagasse	(Cordova *et al.*, 1998)
Penicillium restrictum	24	30	SSF	Babassu oil cake	(Gombert *et al.*, 1999)
Penicillium simplicissimum	36	30	SSF	Babassu oil cake	(Gutarra *et al.*, 2007)
Rhizopus oligosporus TUV-31	48	76.6	SSF	Egg yolk	(Iftikhar *et al.*, 2010)
Rhizopus oligosporus ISUUV-16	48	81.2	SSF	Almond meal	(Awan *et al.*, 2003)
Aspergillus carneu	96	12.7	SSF	Sunflower oil	(Kaushik *et al.*, 2006)
Candida cylindracea	179.5	23.7	SmF	Oleic acid	(Kim and Hou, 2006)
Candida rugosa	50	3.8	SmF	Olive oil	(Rajendran, Palanisamy and Thangavelu, 2008)
Penicillium verrucosum	48	40	SSF	Soybean bran	(Kempka *et al.*, 2008)
Geotrichum sp.	24	20	SmF	Olive oil	(Burkert, Maugeri and Rodrigues, 2004)
Rhizopus homothallicus	12	826	SSF	Olive oil	(Rodriguez *et al.*, 2006)
Penicillium chrysogenum	168	46	SSF	Wheat bran	(Kumar *et al.*, 2011)
Fusarium solani FS1	120	0.45	SmF	Sesame oil	(Maia *et al.*, 2001)
Penicillium simplicissimum	48	21	SSF	Soy cake	(Di Luccio *et al.*, 2004)
Aspergillus awamori	96	495	SmF	Rice bran oil	(Basheer *et al.*, 2011)
Candida cylindracea NRRLY-17506	175	20.4	SmF	Olive mill wastewater	(Brozzoli *et al.*, 2009)

SSF = Solid state fermentation, SmF = Submerged fermentation

The filamentous ascomycetous mesophilic yeast-like fungus, *Geotrichum candidum*, is well-known for its production of lipases with a high specificity for unsaturated long-chain fatty acids [long-chain *cis* (Δ9)] unsaturated fatty acid groups and esters (Maldonado, 2017). The genus *Geotrichum,* inhabitants of soil, plant tissues, digestive tracts of humans, insects and other mammals, produce lipases of different isoforms and mostly extra-cellular. However, purification and characterisation of these potential lipase isoforms have been reported somewhat tricky on account of overlap in their physical and biochemical properties. *G. candidum* lipases have been purified to homogeneity and characterised, as well as cloned and expressed into *S. cerevisiae* and *Picha pastoris* (Shimada *et al.*, 1990; Bertolini *et al.*, 1995; Catoni, Brocca and Schmid, 1997; Maldonado, 2017).

2.3 Industrial Applications of Lipases

Lipases occupy a prominent place among biocatalysts, currently considered the third largest group and estimated to represent 21% of the global industrial enzyme market (De Godoy Daiha *et al.*, 2015). The enzymatic properties and substrate specificity

versatility of lipases has led to their frequent use as biocatalysts in the industrial scale production of fine chemicals, involving organic solvents and other industrial applications such as detergent formulations, food, oleochemical, pulp and paper industries, nutrition, cosmetics and pharmaceutical processing (Kato *et al.*, 2007; Ericsson *et al.*, 2008; Mehta, Bodh and Gupta, 2017). However, although considerable advances have been achieved, commercial lipases still suffer from low stability under operational process conditions, i.e. high temperatures and salinity. Additionally, the cost of lipase enzymes is often still prohibitive, compared to cheaper chemical methods. Hence, the search for novel lipases with high stability and increased catalytic turnover under process conditions is ongoing (De Godoy Daiha *et al.*, 2015; Madhavan *et al.*, 2017).

Despite their broad applicability, microbial lipases are employed in industry via two methods: *in situ*, which entails cultivating the microbial lipase-producer in a medium with suitable substrates (mostly used in the food industry), or *ex situ*, which generally entails immobilising pure enzymes (mostly used in the production of fine chemicals) (Pandey *et al.*, 1999). The commercial application scope of lipases is extremely diverse. This review will highlight specific applications in the agro-biotechnology, biodiesel, chemical and pharmaceutical, food, pulp and paper and detergent industries.

2.3.1 Lipases in agro-biotechnological processes

Lipases are also used in a wide range of agro-biotechnological processes (Marchetti, Miguel and Errazu, 2007), such as enzyme-assisted silage fermentation, bioprocessing of crops and crop residues, biodiesel production, fibre processing and production of feed supplements to improve feed efficiency (Royon *et al.*, 2007; Han *et al.*, 2017). In fact, it is anticipated that agricultural waste biomass may soon turn into a valuable and viable resource for the production of chemicals and fuels (Beilen and Li, 2002).

2.3.2 Lipases in biodiesel production

The production of biodiesel from a renewable natural source (oil from plants, such as canola) has helped alleviate the pressure on our fossil-fuel energy sources. Biodiesel is considered a cheap, biodegradable and non-toxic source of energy. The trans-esterification reaction catalysed by lipases in organic solvents can result in the conversion of vegetable oils to short-chain alcohol esters, and provide an alternative to petroleum-based energy for the public transport sector (Hama, Noda and Kondo, 2018). Commercially, biodiesel production involves the use of chemical catalysts in aqueous reactions. Strong alkalis such as sodium and potassium hydroxides are added to methanol or ethanol as well as any other refined, crude or frying oils from palm, corn, soybean, peanut, sunflower olive, palm and palm kernel. Alternatively strong acids, especially sulphuric acid, are used as catalysts for biodiesel production (Marchetti,

Miguel and Errazu, 2007). These conventional methods require high temperatures and further purification to remove glycerol, water and other by-products. Lipases catalyse the hydrolysis of glycerol, alcoholysis, and acidolysis, as well as transesterification and esterification with high specificity, and are thus considered desirable for biodiesel production. The majority of yeast and fungal lipases involved in biodiesel production are *A. niger*, *C. antartica*, *C. rugosa*, *R. miehei*, *R. oryzae*, and *T. lanuginose* (Nie *et al.*, 2006).

2.3.3 Lipases in chemical and pharmaceutical industries

The use of enzymes in chemical and pharmaceutical sectors takes an important position. There is continued demand for more selective and efficient catalysts and processes for the manufacture of fine chemicals (Gotor-Fernández, Busto and Gotor, 2006). Interest in industrial lipases continues to grow, and this trend is hardly surprising considering the increased integration of biological catalysts into the production of a variety of commodity chemicals. Processes range from the production of simple chemicals such as acrylamide, fructose, malic acid and aspartic acids, to the synthesis of highly complex pharmaceutical intermediates and drug substances (Hasan, Shah and Hameed, 2006). Chemical methods have traditionally been used to synthesise key intermediates in the production of pharmaceuticals and agro-chemicals. This is a difficult and expensive process, with most compounds only functional when it is in a specific enantiomeric form. Reactions catalysed by lipases provide a suitable alternative to resolve racemic mixtures, with the chemo-, enantio- and regio-selectivity characteristics of lipases providing enantiomerically pure building blocks for the synthesis of antibiotics, pesticides, anti-inflammatory compounds (Ghanem and Aboul-Enein, 2004). Similarly, the asymmetric hydrolysis, esterification, transesterification, alcoholysis, acidolysis, aminolysis and ammoniolysis reactions of lipases in nonpolar organic solvents are used for the synthesis of compounds such as polyols, chiral alcohols, chiral carboxylic acids, amines and amides (Henket and Bornscheuer, 2003; Hasan, Shah and Hameed, 2006; Marchetti, Miguel and Errazu, 2007; Seddigi *et al.*, 2017). In the aroma and fragrance industry, aromatic compounds which are used in perfumes are often volatile. Cold-active lipases have found application in the synthesis of these compounds at low temperatures (Zimmer *et al.*, 2006).

2.3.4 Lipases in food industry

Fats and oils are important constituents of foods, and their modification is one of the prime areas of focus in food processing industry. Lipases can be applied *in situ* (together with other enzymes) to improve their shelf-life or flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked goods and beer (Choudhury and Bhunia, 2015). Lipases such as those of *G. candidum*, *Mucor*

meihei, *A. niger* and *A. oryzae* are extensively used in the dairy industry for the hydrolysis of milk fat. Lipases are used to break down milk fats and give characteristic flavours to cheeses. The flavour comes from the free fatty acids produced when milk fats are hydrolysed. They are used for flavour enhancement in cheese, acceleration of cheese ripening, in the manufacture of cheese like products and lipolysis of butterfat and cream (Liu, Holland and Crow, 2004; Konkit and Kim, 2016; Kadhum and Shamma, 2017).

Lipases catalyse the synthesis of short-chain fatty acids and alcohols, which contribute as flavour and fragrance compounds and prolong the shelf life, particularly in the baking industry where chemical emulsifiers have been substituted with lipases (Cherry and Fidantsef, 2003; Singh *et al.*, 2016). Monoesters such as propylene glycerol (1,2-propanediol) are a good substitute or supplement emulsifiers in the preparation of cakes, cake mixes, margarine, coffee whitener and bread. The application of emulsifiers improves dough stability and conditioning. These emulsifiers are usually produced through transesterification of triglycerides with propylene glycerol by using inorganic catalysts at temperatures >220°C. However, these higher temperatures result in dark by-products with off-flavours. Hence, microbial lipases have been an desirable alternative (Moayedallaie, Mirzaei and Paterson, 2010).

Lipase-catalysed hydrolysis of fats and oils is seen as a promising process for the production of fatty acids and glycerol since the reaction can be carried out under mild conditions. The conventional process for fat and oil hydrolysis (Colgate Emery process) requires a pressure of about 4.82 MPa and temperature of about 220-250°C or higher, which gives a high conversion (97–98%) but is highly energy intensive (790 MJ/kg of the fat split). To reduce the energy consumption and minimise thermal degradation of the products, researchers have employed enzyme-catalysed hydrolysis or whole-cell biocatalyst as an alternative, which transforms lipids at room temperature (Pugazhenthi and Kumar, 2004).

The position, chain length and degree of unsaturation significantly influence not only the physical properties but also the nutritional and sensory value of a given triglyceride. Fats and oils are essential food constituents, and lipase-based technology involving hydrolysis and synthesis reactions are commercially used to upgrade less desirable fats. Enzymes can modify lipids within glyceride along a fatty acid chain or can be replaced by new ones, hence producing higher valued fat. The synthesis reactions of lipases, such as esterification and trans-esterification, are utilised to convert cheap oil to more high-value products such as cocoa butter (Hasan, Shah and Hameed, 2006; Guerrand, 2017).

2.3.5 Lipases in the pulp and paper industries

The use of enzymes in the pulp and paper industry include cellulases, hemicellulases, resinases, xylanase, and lipases. Hydrophobic components of wood which are mainly triglycerides, waxes and lipophilic extractives such as sterol esters, resin acids, free fatty acids and sterols cause severe problems, known as pitch, in pulp and paper processing. Lipolytic enzymes are used as pitch control, removing the lipid fraction of wood that interferes with the downstream processing of paper pulp. Lipases are also used in combination with/without other cellulolytic enzymes in the deinking process in wastepaper recycling by degrading oil-carrier-based inks (Chang and Gong, 2016; Nik Raikhan Nik Him, Caroline Apau and Nurul Shafika Azmi, 2016).

2.3.6 Lipases in the detergent industries

Lipases, such as those from *T. lanuginose*, demonstrate high activity under alkaline elevated temperature conditions and are used as additives in laundry and household detergents to enable the hydrolysis of a variety of triglycerides in fat stains, while withstanding the harsh washing conditions (pH 10-11) and temperatures (30-60°C) (Singh *et al.*, 2016). Lipases used in the detergent industry must be able to withstand chemical denaturation or proteolytic degradation caused by damaging surfactants such as linear alkyl benzene sulfonate present in detergents (Sharma, Chisti and Banerjee, 2001).

2.4 Modification and Applications of Lipases

Enzymes are often modified to alter their reaction mechanism to include new reaction catalysis, improved and expanded substrate specificity or stability at high temperature and in strong polar organic solvents (Hult and Berglund, 2003). The high cost of commercial lipases drives research efforts to increase enzyme catalytic performance, long-term operational stability and regeneration/reuse of the enzyme. Consequently, the application of different techniques of enzyme chemical modification, immobilisation and genetic engineering has introduced considerable improvements in the efficiency of lipases in biocatalysis and overall improvements of particular processes to mitigate the enzyme costs (Boutureira and Bernardes, 2015).

2.4.1 Chemical modification and immobilisation

Free lipase enzymes, soluble in aqueous solution, will be denatured and inactivated under conditions such as improper pH and temperature, with a concomitant loss of activity even if it is preserved in buffers. For these reasons, free lipase suffers from the difficulty of reusability, which impedes the easier and more expansive commercial utilities of lipase. To solve the problems of reuse and stability, the chemical modification combined with immobilisation techniques have been explored with great success (Shuai *et al.*, 2017).

Modification of protein surface characteristics by chemical binding with modifiers can improve or modulate biocatalyst performance (Díaz-Rodríguez and Davis, 2011). Lipases have been modified by chemical means, introducing metals or modified cofactors that interact with specific amino acids which helps to elucidate protein structure such as active sites and to alter and improve their native properties, and endow them with useful new functions (Beilen and Li, 2002). The modification of the carboxylate groups in Asp and Glu residues in *Candida antarctica* B and *T. lanuginose* lipases by chemical amidation using ethylenediamine positively influenced enzyme activity (Díaz-Rodríguez and Davis, 2011). However, the success of these non-specific modification strategies is not always predictable and could have the opposite effect in other lipase enzymes (Palomo *et al.*, 2007).

Immobilization is the most common modification method applied to lipases to increase the stability, selectivity, activity of lipases as catalysts and the yield of synthetic reactions in organic media (Marchetti, Miguel and Errazu, 2007; Fernandez-Lafuente, 2010). The immobilisation process typically involves attaching the enzyme by physical adsorption, covalent bonding, onto solid inorganic or organic carriers, cross-linking or entrapment (Adlercreutz, 2013; Yücel *et al.*, 2014). Adsorption involves the physical interactions generated by the carrier and enzyme that include van der Waals forces, ionic interactions and hydrogen bonding. The binding is weak, but more importantly, does not change the native structure of the enzyme, i.e. prevents the active sites of the enzyme from being disturbed and allows the enzyme to retain its activity (Jesionowski, Zdarta and Krajewska, 2014). Entrapment utilises sol-gel methodology to entrap the enzyme solution in silica-based materials, or the enzyme is entrapped in a polymer network. For covalent bonding, carriers are modified with aldehyde groups, succinimidyl ester groups, or amino groups (with glutaraldehyde used as a coupling agent) to react and bond to the amino groups in lipase, as well as quinone groups that can react with amino or thiol groups of lipase (Shuai *et al.*, 2017). Covalent bond immobilisation of lipases benefits the enzyme by increasing the stability of the immobilised lipase as well as increased recyclability. Furthermore, most studies where covalent bonding has been applied to lipases have observed an added enhanced function of lipase, i.e. most of the studies observed broader pH range and higher thermal stability and long-lasting lipase activity (Shuai *et al.*, 2017).

Various lipases have been immobilised using different chemistries, and carrier supports to date (Panke and Wubbolts, 2002; Deng *et al.*, 2005; Peng, 2014; Yücel *et al.*, 2014). Organic carriers seem to be preferred for the immobilisation of lipases. They include a wide and highly diverse gamut of polymers, such as cross-linked PVA and epoxy activated PVA, poly(N-methylol acrylamide), small and large poly(hydroxybutyrate)

beads and polyacrylonitrile electrospun fibres, organic matrices of natural origin, including chitosan beads, MANAE-agarose and cellulose ultrathin film, commercial polymer products, e.g. polypropylene membranes Accurel EP100 and Accurel MP1000, adsorbent Purasorb. Lipases were also immobilised on other materials such as butyl and octadecyl sepabeads, cotton flannel cloth, olive pomace powder and commercial ion exchange resin Lewatit (Jesionowski, Zdarta and Krajewska, 2014). Recently, the use of nanomaterials as carriers for lipase immobilisation has received growing interest. Nano-particles and nanofibres are used to achieve high enzyme loading, surface to volume ratio and catalytic efficiency for large-scale applications (Shuai *et al.*, 2017). Lipase enzyme immobilisation has made significant contributions to the use of this enzyme in offering easier product recovery, recycling of expensive enzymes, flexibility of reactor design, better operation control, enhanced thermal storage and conformational stability.

2.5 Bioprospecting for Novel Lipases

The classical approach to isolate new lipase biocatalysts is to cultivate microorganisms from different environments and subsequent screening for the desired phenotype, i.e. lipolytic activity (Nagarajan, 2012). Several screening methods exist for the isolation of lipolytic microorganisms, including gel diffusion assays using various lipid substrates (such as tributyrin, tricaprylin, triolein, olive oil, Tween 20) incorporated into the growth media or gel diffusion assays using indicator dyes (including phenol red, Rhodamine B). Lipase activities can be qualitatively assessed through these methods through readily detectable degradation haloes or fluorescent zones (Hasan, Shah and Hameed, 2009; Lanka and Latha, 2015). However, about 99.9% of microorganisms in environmental niches cannot be cultivated by standard laboratory techniques (Amann *et al.*, 1990).

It is estimated that only about 2% of microbes on Earth have been utilised as enzyme producers by industry, and it is clear that access to a wider range of microbial enzymes from novel niches would be potentially beneficial for industries. To address this problem, high-throughput sequence-based and functional metagenomics has been greatly successful in mining for lipases from metagenomics sequences. These methods bypass the requirement for the isolation or cultivation of microorganisms by directly isolating genomic DNA from environmental samples, archiving it in appropriate heterologous hosts, and finally screening these libraries for a gene of interest (Distaso *et al.*, 2016; Ferrer *et al.*, 2016; Madhavan *et al.*, 2017).

2.5.1 Desirable Properties of New Novel Lipases

Over the last few decades, the enzyme market and a number of competitive enzyme-based industrial processes have grown exponentially due to the benefits of novel

enzyme activities and application fields as well as lower-cost production (Wahler and Reymond, 2001; Singh *et al.*, 2016). As mentioned, microbial lipases represent the most versatile and widely used enzymes in biotechnological applications and organic chemistry, however, only comparatively few lipases have been well-characterised for industrial applications (Guerrand, 2017). In order to be commercially competitive and successful, industries are increasingly seeking novel microbial lipases with increased robustness, catalytical efficiency, increased substrate specificity, enantioselectivity and stability (temperature, pH, organic solvent, etc.) (Akoh, Lee and Shaw, 2004). Some of these aspects will be discussed in more detail below.

2.5.1.1 Activity and stability in organic solvents

Lipases perform synthesis (esterification and transesterification) reactions in the presence of organic solvents, i.e. in the absence of water where alternative nucleophiles, such as alcohols, amines and thiols, leads to transesterification, aminolysis and thiotransesterification, respectively (Kilbanov, 2001). Enzymatic reactions in organic solvents provide numerous industrially attractive advantages, including higher product yields, higher substrate conversion rates, increased solubility of non-polar substrates, increased product recovery, exclusion of side-reactions and minimisation of substrate and product inhibition (Kumar *et al.*, 2016). Industries therefore prefer bioconversions and biocatalysis in organic solvents over enzymatic hydrolysis in water, as can be seen with the production of pharmaceuticals. Besides organic solvents, industries use ionic liquids as environmentally friendly alternative solvents for synthesis reactions catalysed by lipases. However, the catalytic activity displayed by enzymes in neat organic solvents is usually far lower (up to four or five orders of magnitude) than in water and all enzymes are insoluble in almost all organic solvents (Adlercreutz, 2013; Stepankova *et al.*, 2013). The loss of lipase activity can be due to the disruption of tertiary structure due to changes in medium hydrophobicity, restricted conformational flexibility, deformation of the active site geometry resulting in limited enzyme-substrate formation and interfacial denaturation of the enzyme due to interfacial tension (Salihu and Alam, 2015). Extremophilic microorganisms, for example thermophiles and hyperthermophiles, and those isolated from phenol contaminated environments have been shown to contain lipases that are more active/resistant in organic solvents, i.e. a positive correlation with the stability of their enzymes in organic solvents (Kumar *et al.*, 2016; Moura *et al.*, 2017).

2.5.1.2 Activity at high temperatures and thermostability

Industrial processes employing lipases, as well as lipases in detergents, are predominantly carried out at elevated temperatures exceeding 45°C (Li and Zhang, 2005; Moura *et al.*, 2017). Higher temperatures result in the increase of reaction rates,

diffusion rates and substrate solubility, as well as the reduction of viscosity and mesophilic contamination (Elias *et al.*, 2014). Industrial bioconversion reactions therefore prefer lipases that are stable and active at high temperatures.

2.5.1.3 Enantioselectivity of lipases

The synthesis of optically pure compounds requires lipases to be highly enantioselective. Lipases are essential towards the production of functional, optically pure enantiomers of high pharmaceutical valuable. Lipases are interesting biocatalysts which generally exhibit satisfactory enantioselectivity at low temperatures (even down to −80°C), while some lipases show a decrease in enantioselectivity at higher temperatures (Yang *et al.*, 2017).

2.6 Olive Wastewater (OWW) as Potential Source of Novel Lipases

The production of table olives and olive oil leads to the production of large volumes of wastewater. The composition of table olive wastewater (TOWW) is mainly influenced by the alkaline steps during debittering stages, the washing or rinsing water fraction and finally the acidic water fraction from the spent brine solution (Beltran-Heredia *et al.*, 2000). On the other hand, olive mill wastewater (OMWW) is derived from water used for washing and oil extraction. Even though there are substantial differences between the olive wastewater produced from different styles of processing and oil extraction, all have certain underlying similarities. In general olive wastewater has a green to blackish-brown colour, is malodorous, contains potassium and phosphate salts, and is rich in organic substrates such as polysaccharides, fats, sugars, organic acids and phenols (Mulinacci *et al.*, 2001).

High organic content characterises the wastewater produced during edible olive production processes (TOWW) (up to 40 g/L COD), rich in polyphenols (up to 6 g/L), a high salt (NaCl) content up to 2% with a broad pH range from 3.6 to 13.2 (Tsioulpas *et al.*, 2002). OMWW more toxic than TOWW, is rich in residual olive oil from the extraction process, has excessive high organic loads (45-220 g/L COD) and a phenol and polyphenol content ranging from 0.5 to 24 g/L (Tsioulpas *et al.*, 2002; Paraskeva and Diamadopoulos, 2006). Furthermore, OMWW is an acidic (pH 5 – 5.5) wastewater.

Given the composition of TOWW and OMWW, it is hypothesised that microorganisms, exposed to these extreme conditions in terms of pH, phenol content and salinity found in the toxic olive wastewater environment, could be a valuable resource of “extremophilic” microorganisms able to produce lipases with robust characteristics regarding, catalytical efficiency and stability (pH, organic solvents, etc.).

CHAPTER THREE:
MATERIALS AND METHODS

3.1 Screening for lipases from indigenous olive wastewater (OWW) biofilms

3.1.1 Culturing and harvesting of OWW biofilms

Fresh OWW (5 - 10 L) was obtained from both Buffet Olives (producers of table olives) and Vesuvio Estate (producers of olive oil) in the Paarl region (Western Cape, South Africa) during the active harvesting and processing seasons of 2006. The OWW, both from Buffet Olives and Vesuvio Estate, was supplemented with 0.2% (w/v) D(+)-glucose, 5% (w/v) di-ammonium hydrogen phosphate (Merk Chemicals (PTY) LTD, Germany), 2% (v/v) of commercially available olive oil and 2% (w/v) pureed black olives. Supplementation of OWW was done not only to ensure biofilm development and growth but, more importantly, to stimulate or enrich for lipase-producing microorganisms in the biofilm matrix. OWW biofilms were cultured in the supplemented OWW using modified Pederson devices, containing 12 microscope slides (as attachment surfaces for the growing biofilm) at 25°C for four weeks with mild stirring. The modified Pederson devices were suspended in a 1-litre glass beaker filled with fresh supplemented OWW as required.

After four weeks, biofilms were harvested by sonication. Individual microscope slides were soaked in 25 ml sterile water in a 250 ml sterile Erlenmeyer flask and sonicated for 3 min using a Soniclean sonicator (Ultrasonic Engineering (Pty) Ltd, South Africa). The sonication step was repeated three times with fresh 25 ml sterile water to ensure maximum removal of all the attached biofilm from the slides. The resulting pooled biofilm cell suspension was centrifuged at 8000 rpm, for 15 min at 20°C. The biofilm pellet was subsequently subjected to the following two procedures: (1) serial dilutions of biofilm suspension were plated on lipase screening media; (2) biofilm suspensions were also stored at -80°C in 40 % (v/v) glycerol solution for future use.

3.1.2 Phenotypic lipase screening

In order to screen for lipase activity from the culturable fraction of OWW biofilms the following nutrient media screening approach was prepared: A 100 ml lipoidal emulsion (100 ml per 900 ml of base medium) was prepared by blending 30% (v/v) olive oil (commercial grade) and 0.25% (v/v) Tween 80 for 30 sec in a Waring blender, followed by autoclaving for 15 min at 121˚C. Filter sterilised (using 0.22 µm Cameo acetate filters, Lasec SA (Pty) Ltd) Rhodamine B solution (20 mg/20 ml sterile water [0.02% w/v of total media prepared]) was added to the cooled lipoidal emulsion. After thorough vortex mixing, the Rhodamin B-containing lipoidal emulsion (100 ml) was added to 900

ml autoclaved fungal and bacterial base media, mixed by vortexing and poured into Petri dishes. The fungal base medium contained 3.9% (w/v) potato dextrose agar (PDA; Biolab Diagnostics, Wadeville, South Africa) supplemented with 20 mg/l filter-sterilised streptomycin (Sigma-Aldrich, USA), while the bacterial base medium, contained 4.5% (w/v) Luria Bertani agar (LB; Biolab Diagnostics), supplemented with filter-sterilised nystatin (50 µg/ml) (Sigma-Aldrich, USA). After autoclaving, the base media were cooled to 50°C followed by mixing with the lipoidal emulsion described above.

The biofilm suspensions prepared in section 3.1.1 was diluted from 10^{-2} to 10^{-6} in PBS, and the respective dilutions were plated out using technical grade sterile 3 mm glass beads (Merck, South Africa) on lipase screening plates (described in section 3.1.2). PDA and LB agar plates were incubated at 30°C and 37°C for 3 and five days, respectively. Lipase positive isolates were sub-cultured to pure culture and maintained on PDA and LB agar, respectively. The lipase activity was monitored by fluorescence with UV light at 350 nm. Fluorescent colonies and clearing zones around the colony indicated positive lipase activity. *Candida cylindracea* CBS 6330 was used as a positive control for screening on plates (Kim and Hou, 2006).

3.1.3 Liquid lipase activity assays

All positive lipase isolates from OWW biofilms were subjected to quantitative lipase activity assays to determine the volumetric lipase activity, substrate specificity, pH and temperature optima of the extracellular lipase activity. All lipase assays were executed in triplicate to ensure statistical validation.

The different isolates of bacteria and fungi were cultured in 5 ml LB or YPD broth for 48 hours at 37°C or 30°C, respectively, for optimum growth with shaking at 230 rpm. The media (LB and YPD broth) were supplemented with 3% (v/v) olive oil to induce lipase enzyme production. After 48 hours, the cells were harvested by centrifugation (13000 rpm, 5 min) and supernatants containing the lipase were used for lipase activity assays. Lipase activity was detected by measuring the hydrolysis of *p*-nitrophenyl (p-NP) esters (C4-C18) to *p*-nitrophenyl and other fatty acid derivatives. The microwave-assisted assays, conducted according to Jain, Jain and Gupta, (2005), started with the addition of the reaction mixture (220 µl) in flat-bottom microtitre plates, consisting of 100 µl of 0.1 M sodium phosphate buffer (containing 0.27 M NaCl and 0.9% v/v Triton X-100, pH 7.0), 100 µl of the enzyme (supernatant) and 20 µl of a 0.005 M p-NP ester dissolved in DMSO. As a negative control, a similar reaction mixture was prepared, containing 100 µl milliQ water, 100 µl 0.1 M sodium phosphate buffer and 20 µl of the corresponding p-NP esters. On the other hand, the blank reaction mixture consisted of 100 µl LB or YPD sterile broth and 100 µl 0.1 M sodium phosphate buffer together with

20 μl milliQ water. The reaction mixtures were incubated at 37°C for 30 min, after which the solution was microwaved in microwave oven for 30 sec at a frequency of 2.45 GHz. The absorbance was measured immediately at 415 nm using a microplate reader (Bio-Rad Laboratories, Japan). One enzyme unit was defined as the amount of enzyme that liberates 1μmole of p-NP per min at pH 7 and 37°C.

3.2 Molecular identification of fungal isolate VES 43.1

For preliminary identification of VES 43.1, the fungal isolate was cultivated in liquid SC medium containing 2% glucose at 30°C on a rotary shaker at 100 rpm for five days. Biomass was then harvested by filtration, and genomic DNA was isolated with the ZR Fungal/Bacterial DNA MiniPrepTM kit (Zymo Research), according to the manufacturer's instructions. VES 43.1 was putatively identified by amplifying the internal transcribed spacer regions (ITS1 and ITS2) of the nuclear ribosomal RNA gene using the primers ITS1 and ITS4 and the isolated genomic DNA template (White *et al.*, 1990). The 25 μl PCR reaction mix contained 100 ng genomic DNA, 0.2 μM of each primer, 10 μM deoxynucleotides and 1 x buffer (Whitehead Scientific, South Africa). The PCR reaction was carried out in a GeneAmp PCR System 2400 (Perkin Elmer) and the PCR conditions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min; followed by final extension at 72°C for 7 min. The reaction mixture was separated on a 0.8% (w/v) agarose gel at 80V followed by ethidium bromide staining and visualised using UV light. The ±600 bp amplicon was excised and gel purified using the ZymocleanTM Gel DNA Recovery Kit (Zymo Research, USA), according to the manufacturer's instructions. This amplicon was then cloned using the InsTAcloneTM PCR Cloning Kit (Fermentas), according to the manufacturer's instructions. Sequence determination was carried out at the Central Analytic Facility, Stellenbosch University. The sequences were analysed using DNAMAN software and subjected to similarity search by BLAST (Basic Local Alignment Tool).

3.3 Cloning, expression and characterisation of the *Geotrichum candidum* VES 43.1 lipase I (*GCL1*) gene product

Standard recombinant DNA methods were carried out according to the methods described in Sambrook and Russell (2001). All PCR reactions, restriction enzymes and deglycosylation enzymes were setup using standard protocols as instructed by the manufacturers. The conditions for all of the PCR reactions were kept standard according to the manufacturer (Kapa Biosystems, Boston, US). All sequence-related analysis was done in CLC Genomics Workstation (v 7.9.1).

3.3.1 PCR amplification primer design

To amplify the full open reading frame of the *GCL1* gene (based on the *G. candidum GCL1*; Genbank Accession: ACX69980), the following degenerate primer set containing universal inosine-bases was designed and synthesised by Integrated DNA Technologies. The primers design ensured that the first 57 bp of the ORF was excluded (to ensure removal of the native 19 aa signal peptide) and that a start codon (ATG) (in bold) is provided in-frame. These oligonucleotides contained the *Sal*I restriction site (underlined).

5′-GeoLipI: 5′-GTCGAC**ATG**AACCGIAGAGAITAACGTCAGICTCAAAG-3′

3′-GeoLipI: 5′-GTCGACGTITCCAAAAICITIITITTIGCIGCIGC-3′

3.3.2 *Geotrichum candidum* VES 43.1 genome DNA isolation and PCR amplification.

PDA-cultured (50 ml) *G. candidum* mycelia were collected by centrifugation (5000 g, 3 min), frozen at −70°C and ground in liquid nitrogen with a mortar and pestle. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrepTM kit (Zymo Research). To do so, the powder (about 100 mg) was dissolved in 400 µl of lysis buffer. The manufacturer's protocol was then followed. Total DNA was eluted from silicon columns in two lots of 100 µl of preheated (65°C) elution buffer. A 10x diluted gDNA was used as a template for KAPA HiFi DNA polymerase (Kapa Biosystems, Boston, US) using 5' and 3'GeoLipI primers. Following the denaturation at 94°C for 4 min, the samples were subjected to 30 cycles of amplification in a MultiGene II thermocycler (Labnet International Inc.), using the following cycle conditions: denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec and extension step at 72°C for 3 min followed by final extension at 72°C for 10 min

3.3.3 Cloning and sequencing of the *G. candidum* VES 43.1 GCL1 gene

The 1629 bp *GCL1* gene amplicon was gel purified using Roche High Pure PCR product purification kit (Roche, Germany) and cloned into pGEM T-easy vector system (Promega Corporation, USA) and transformed into *Escherichia coli* DH5α (New England Biolabs, Midrand, South Africa) using blue/ white selection plates, white colonies were individually picked and inoculated into 5 ml LB-Amp broth. Plasmid DNA (pDNA) from the transformants was extracted using Roche High Pure plasmid isolation kit. The pDNA was sent for sequencing at Central Analytical Facility (Stellenbosch University, South Africa) using the standard SP6 and T7 universal primers.

3.3.4. Homologous recombination-mediated cloning in *Saccharomyces cerevisiae*

3.3.4.1 Preparation of the *G. candidum* VES 43.1 *GCL1* PCR product and plasmid yXYNSEC

Plasmid DNA of the pGEM T-easy-*G.candidum GCL1* clone (section 3.2.3) was used as a template for PCR amplification. The yeast integration (YI) oligonucleotides:

5′-GeoLipYI:

(5′-
GAGGTCGAATCCGTGGCTGTGGAGAAGCGC**CATCATCATCATCATCAT**CAGGCCCCCA
CGGCCGTTCTTAATGG-3′) and

3′-GeoLipYI:

(5′-
GAGAAAAGAAAAAAATTGATCTATCGCAGATCCTTAACCGGAGAGAGTAACGTCAGGCTC
-3′)

were used together with KAPA HiFi DNA polymerase for PCR reaction. These primers were designed on the same principle as that in section 3.2.1. However, the GeoLipYI primers contain the flanking regions (underlined) homologous to the upstream and downstream regions of the yXYNSEC plasmid multiple cloning sites as well as an in-frame N-terminal HIS-tag (in bold). The PCR program consisted of pre-denaturation (94°C, 4 min), followed by 25 cycles of; denaturation, step (94°C, 30 s); annealing step (62°C, 40 s) and elongation step (72°C, 4 min) and the reaction was finally elongated at 72°C, 10 min. The yXYNSEC yeast expression shuttle vector contains URA selective marker, the *S. cerevisiae* constitutive *PGK1*p promoter and *PGK1*t terminator and the secretion signal of the *Trichoderma reesei xyn2* gene (Van Rooyen *et al.*, 2005). yXYNSEC plasmid DNA was linearised with *Xho*I restriction enzyme digestion. Digested yXYNSEC DNA was first purified using Roche High Pure PCR product purification kit (Roche applied sciences, Germany).

3.3.4.2 Transformation of the competent *S. cerevisiae* with yXYNSEC vector and PCR product

S. cerevisiae W303 (Wallis *et al.*, 1989) (genotype: *MATa ade2-1 trp1-1 can1-100 ura3-1 his3-11,15 leu2-3,112*) was transformed with the linearised yXYNSEC shuttle vector and GeoLipYI lipase PCR product and plated on SC^{-ura} drop media. Briefly, *S. cerevisiae* W303 was grown overnight with shaking at 230 rpm into 5 ml YPD broth at 30°C. Cells were harvested by centrifugation (10 sec at 1300 rpm), and the pellet was washed with 1ml Milli-Q water. The pellet was then, resuspended in 100 µl 1 X TEL buffer (0.1 M LiOAc, 0.5 M EDTA, 1 M Tris-HCl) and incubated for 20 min at 30°C.

Then 10 µl of 10 mg/ml salmon sperm DNA, together with linearised yXYNSEC and GeoLipYI lipase PCR product were added and incubated again for 20 min at 30°C. Finally, 560 µl 50% PEG-4000 and 140 µl of 5 X TEL buffer were added, resuspended using a Gilson and the reaction mixture was once again incubated for 20 min at 30°C. The reaction mixture was then subjected to heat shock for 15 min at 42°C and centrifuged for 10 sec at 13000 rpm. The supernatant was discarded, the pellet washed off with 1 ml milliQ sterile H_2O and centrifuged for 10 sec at 13000 rpm. The pellet was finally resuspended in 300 µl milliQ H_2O, and 100 µl of the re-suspension was plated out on to SC^{-ura} drop medium. The plates were incubated for four days at 30°C.

3.3.4.3 Verification of the correct cloning of *G. candidum* VES 43.1 *GCL1*

Plasmid DNA from yeast transformants were harvested by using the Smash-and-Grab method (Hoffman, 2001). The extracted plasmid DNA was amplified in *E. coli* DH5α, extracted with the Roche High Pure plasmid isolation kit, followed by PCR amplification confirmation with the 5′- and 3′-GeoLipYI primers to verify the presence of the correct cloning of the 1635 bp *GCL1* gene. Purified plasmid DNA was also digested with *Eco*RV and *Bam*H1 restriction enzymes (Fermentas Life Sciences) to confirm correct insert size. Finally, final confirmation with sequencing at Central Analytical Facility (Stellenbosch University, South Africa) with primers pMBRE4-1′F (5′-GTTT AGTA GAA CCT C GTG AAA CTTA-3′) and pMBRE4-3′R (5′-ACTTA AAA TACG CTGAA CCC GAA CAT-3′) (Lilly *et al.*, 2009) was done.

3.4 Characterisation of the recombinantly produced *G. candidum* VES 43.1 gcl1

3.4.1 *G. candidum* VES 43.1 gcl1 purification

S. cerevisiae W303 transformed yXYNSEC-*GCL1* (Sc-yXYNSEC-*GCL1*) was cultured in 25 ml SC^{-ura} drop medium for 72 h to allow for enzyme expression. Cells were harvested (6 000 rpm, 4°C for 10 min) and the supernatant filtered through a 0.45 µm cellulose acetate filter (Sartorius, Goettingen, Germany). The flow-through was concentrated using Pierce™ Protein Concentrators PES, 10 KDa MWCO (ThermoFisher, USA) according to the manufacturer's instructions. The concentrated protein was loaded onto a 5 ml Ni-IMAC column at 1 ml/min using an AKTA FPLC. The bound protein was washed with 5 column volumes of wash buffer (105 mM imidazole) before elution (250 mM imidazole). Ni-IMAC column eluate was exchanged into a low salt buffer (20 mM Tris pH 8.0, 150 mM KCl). The eluted proteins were subject to SDS-PAGE and stained with Coomassie. The purified protein was dialysed overnight against pH 6, 10 mM sodium phosphate buffer with 50 mM NaCl and stored at 4°C until further analysis.

3.4.2 SDS PAGE analysis

Protein samples were mixed with 2x loading buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol and bromophenol blue) and boiled at 100°C for 5 min. The SDS-PAGE analysis was performed with 10% polyacrylamide resolving and 5% stacking gels in a Tris-glycine buffer system (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS) (Gallagher, 1996). Gels were run at 120V for 1h and stained with either silver-stained or Coomassie stain (Sasse and Gallagher, 2009). Both crude and deglycosylated gcl1 protein was analysed by SDS PAGE with the Mark12 Unstained Standard (Invitrogen). PNGase F (Sigma-Aldrich) treatment was carried out according to the manufacturer's instructions. Protein samples were quantified using the Pierce BCA protein assay kit (ThermoFisher, USA).

3.4.3 Substrate specificity to various *p*-nitrophenyl esters

The substrates, *p*-nitrophenyl fatty acid esters, of varying chain length (saturated:C4, C8, C12, C16, C18 and unsaturated: C18:1 *cis*-9) were used at the final concentration of 0.3 mg/mL and the lipase activity was measured according to the method described in section 3.1.3 using the purified *G. candidum* VES 43.1 gcl1 protein.

3.4.4 Effect of pH and temperature on lipase activity and thermal-stability of lipase

The effect of pH and temperature on the purified *G. candidum* VES 43.1 gcl1 protein was conducted using the C18 *p*-nitrophenyl ester as substrate. Optimum pH of the lipase was determined by measuring the enzyme activity over a pH value ranging from 3.6, 4, 4.6, 5, 5.6, 6, 6.5, 7, 8 and 10 in different 0.1 M sodium phosphate buffers at 45°C. The effect of temperature on the lipase was studied by carrying out the enzymatic reactions (using 0.1 M sodium phosphate buffer at pH 8) at different temperature in the range of 0°C, 25°C, 37°C, 55°C, 65°C and 80°C for 1 hour. The thermal stability of the lipase was assayed at various temperatures ranging from 30, 55 and 80°C for a different time interval from 0 to 4 h. At each time interval, 1 mL sample was withdrawn and then assayed for residual activity. The enzyme activity was expressed as percentage relative activity, where the enzyme activity at the optimum temperature was taken as 100%.

3.4.5 Stability of lipase in different organic solvents

The effects of different organic solvents on the activity and the stability of the purified lipase were investigated following method defined by Ogino *et al.* (2000). The stability of lipase in organic solvents (methanol, ethanol, butanol, isopropanol, toluene, acetone, isooctane, cyclohexane, n-hexane, chloroform) was investigated by appropriately mixing 200 µL of purified enzyme and 67 µL of solvent in 96-well microtiter plates to obtain a final solvent concentration of 25% (v/v). The solution was incubated in shaker

(150 rpm) at 45°C for 0–4 h at pH 8 and lipase activity was assayed in the aqueous phase.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Screening for lipase-producing microorganisms from olive wastewater biofilms

4.1.1 Phenotypic lipase screening

OWW biofilm isolates produced a significant amount of extracellular lipase on both Rhodamine B and Tween 80 lipase activity screening plates. The use of solid media supplemented with emulsified triglycerides is a standard methodology for screening for lipolytic enzyme activity in microorganisms (Hasan, Shah and Hameed, 2009). A total of 233 pure microbial isolates (based on unique colony morphology) was obtained from OWW biofilms using screening plates containing emulsified fluorescent Rhodamine B, Tween 80 and olive oil as well as on Tween 80 supplemented with calcium chloride. Of the 57 fungal isolates from OWW, 27 isolates (47%) showed lipolytic activity on the Rhodamine B screening plates (Table 4.1, Appendix A1). A total of 4 out of the 35 Buffet Olives isolates (BUF11, 41, 42 and 57) and 3 out of the 22 Vesuvio fungal isolates (VES 1, 43, 74) showed better lipase activity comparable to the positive control *C. cylindracea* CBS 6330. Screening for fungal lipase activity was more efficient on Rhodamine B media compared to Tween 80, with only isolate VES 43.1 showing significant lipase activity on Tween 80 (Table 4.1, Appendix A1).

On the other hand, of the 176 bacterial isolates screened for their lipase activity towards olive oil, Tween 80 and Rhodamine B, 100 bacterial isolates (57%) displayed lipolytic activity (Table 4.2, Appendix A2). A total of 5 out of the 106 Buffet Olives bacterial (4.7%) isolates and 9 out of the 70 Vesuvio fungal isolates (13%) showed a high lipase phenotype (Table 4.2, Appendix A2).

4.1.2 Volumetric lipase activity

All isolates which had strong lipolytic activity on plate screenings were cultured in YPD or LB broth supplemented with olive oil as the lipase inducer. Cells were harvested by centrifugation, and their supernatants (extracellular lipase) were used as lipase source for the *p*-NP stearate (C_{18}) hydrolysis assay. p-NP stearate (C_{18}) hydrolysis was chosen as an indicator of true lipase activity (representing a long-chain acyl molecule). The volumetric lipase activities (IU/ml) of the 27 chosen fungal isolates are shown in Fig. 4.1. Compared to the well-characterised *Candida cylindracea* CBS 6330 (Kim and Hou, 2006), ten of the OWW fungal isolates showed similar or higher volumetric lipase activity under the condition tested (BUF 11, BUF 3, BUF 32, BUF 38, BUF 41, BUF 42, VES 1, VES 12, VES 14, VES 43.1, VES 43 and VES 73). One fungal isolate VES 43.1 showed the highest volumetric lipase activity, with a 2.5 fold higher volumetric lipase

activity compared to the other OWW isolates and a five-fold higher volumetric lipase activity compared to *Candida cylindracea* CBS 6330 in the culture supernatant.

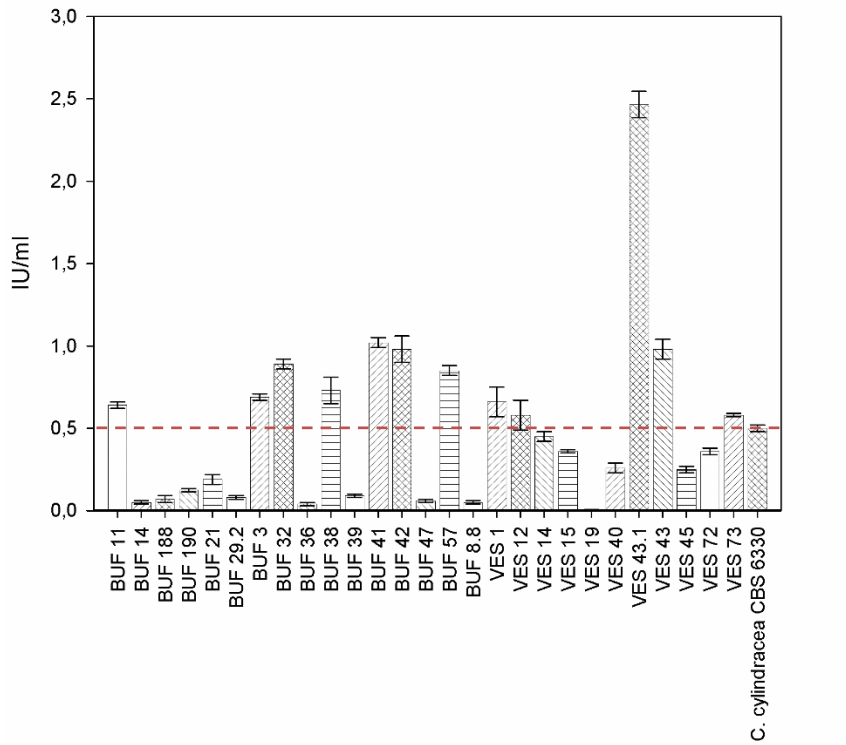

Figure 4.1. Volumetric lipase activity in (IU/ml) of the fungal isolates with the highest plate screen phenotype. BUF = Buffet Olives fungal isolates; VES = Vesuvio fungal isolates. *Candida cylindracea* CBS 6330 served as control lipase producer. Error bars denote standard error (SE), n=3.

Volumetric lipase activities of the 100 chosen OWW bacterial isolates (Fig. 4.2) were all significantly lower compared to the lipase activity measure for the OWW fungal isolates (Fig. 4.1). For both the fungal and bacterial OWW isolates there were no apparent trends between the solid media lipase screening plates and the volumetric lipase assays. High lipase activity on the lipase activity screening plates did not always correspond to high volumetric lipase activity of liquid cultures and *visa versa*. This observation is in line with the fact that although there is a linear relationship between the diameter of the haloes or fluorescent zones and the concentration of the produced lipase, false results can be obtained due to the acidification of the medium from the generation of acidic metabolism other than free fatty acids (FFAs), which are released by microbial lipases. This gel diffusion assays technique is therefore convenient as a rapid visual screening of lipolytic microorganisms (Hasan, Shah and Hameed, 2009).

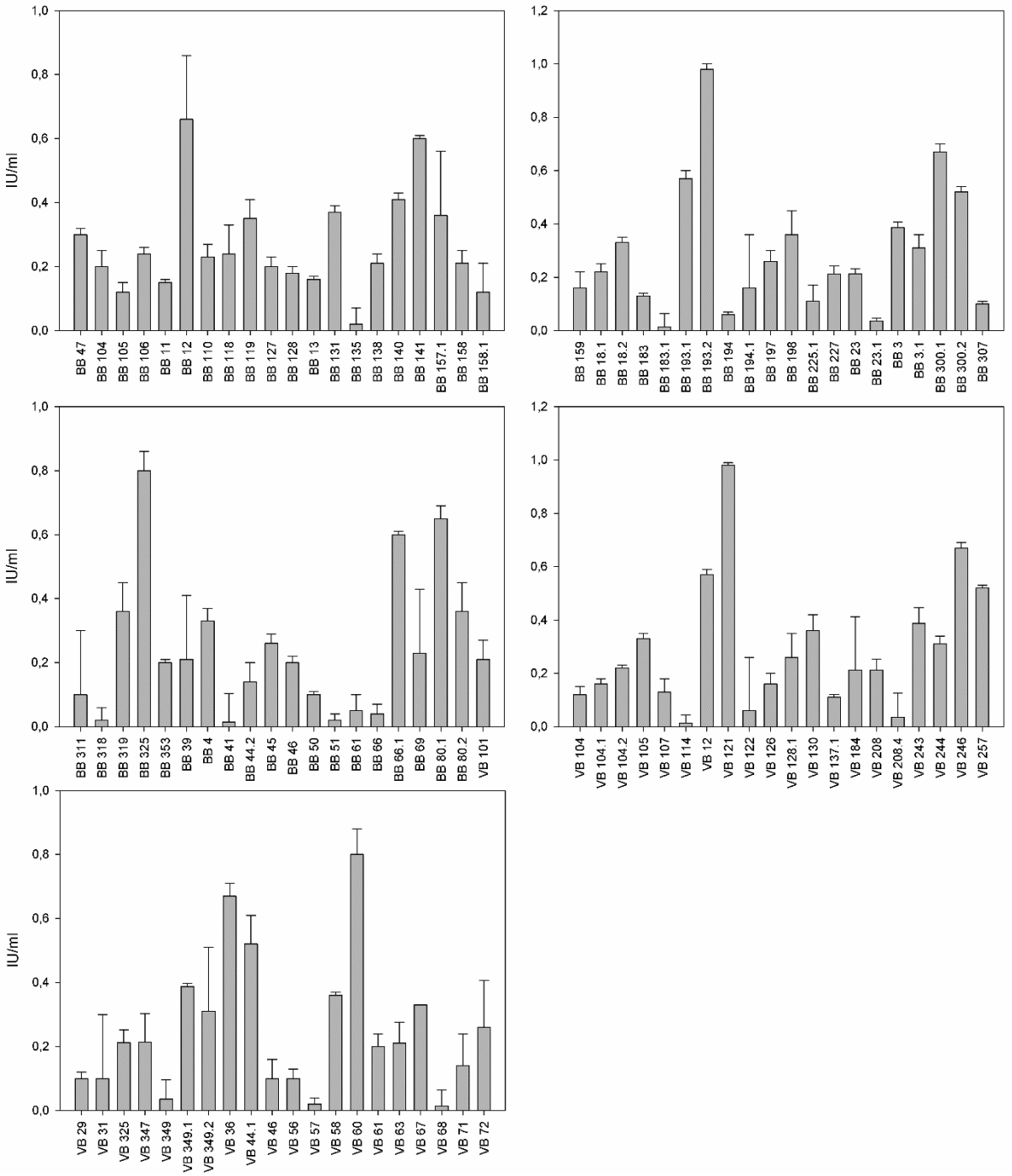

Figure 4.2. Volumetric lipase activity in (IU/ml) of the bacterial isolates with the highest plate screen phenotype. BUF = Buffet Olives fungal isolates; VES = Vesuvio fungal isolates. Error bars denote standard error (SE), n=3.

The result from both the qualitative solid media lipase screening plates and the quantitative volumetric lipase assays indicated that the fungal isolate VES 43.1 from OMWW was a superior lipase enzyme producer or produced an enzyme(s) with superior catalytic efficiency. The focus of this study shifted to characterise this enzyme further, to identify the fungal isolate, to clone the lipase gene and to heterologously produce the enzyme in *Saccharomyces cerevisiae* for in-depth characterisation.

The ±600 bp amplified ITS region was used for preliminary identification and showed 98% homology to the available internal transcribed spacer region sequence of *Geotrichum candidum*. The fungal isolate VES 43.1 was, therefore, preliminarily identified as *G. candidum*.

4.2 Cloning of the *G. candidum* VES43.1 *GCL1* gene

The *Geotrichum candidum* VES43.1 *GCL1* was successfully amplified from gDNA and cloned in yXYNSEC. Sequence analysis of the VES43.1 *GCL1* clone revealed that the open reading frame contained 1629 bp and encoded a protein of 543 aa with a theoretical molecular weight of 59.4 kDa. Compared to other gcl1 lipases from Genbank (Fig. 4.3) the *G. candidum* VES43.1 gcl1 protein is one amino acid shorter and contain nine unique amino acid changes not observed in closely-related gcl1 lipases (Fig. 4.3). The pentapeptide Gly-X-Ser-X-Gly active site signature motif was 100% conserved in the *G. candidum* VES43.1 gcl1 protein.

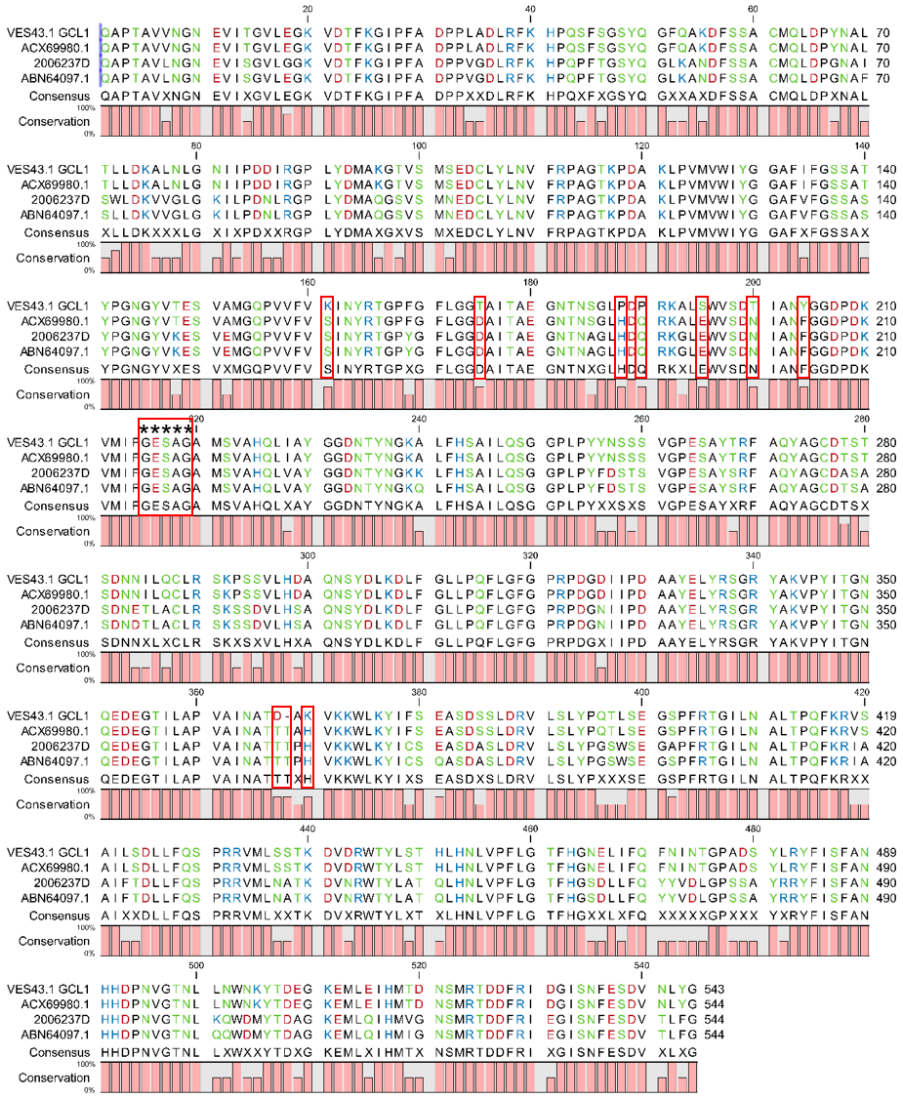

Figure 4.3. Amino acid sequence alignment of the *Geotrichum candidum* VES 43.1 gcl1 protein with other gcl1 enzymes in the Genbank database (Accession numbers: ACX69980.1, 20066237D and ABN64097.1). Red boxes indicated unique amino acid context in VES 43.1 gcl1 compared to the other gcl1 proteins. The asterisks indicate the conserved pentapeptide Gly-X-Ser-X-Gly motif of the active site.

4.3 Characterisation of the recombinantly-produced *G. candidum* VES 43.1 gcl1 lipase

After 72 hr culturing, crude supernatant and Ni-affinity chromatography-purified gcl1 protein from *S. cerevisiae* transformed with the yXYNSEC-*GCL1* plasmid were analysed by SDS PAGE gel analysis (Fig. 4.4 A). A single protein band was obtained after HIS-tag purification and deglycosylation (Fig. 4.4. A; lane 3) with an estimated molecular mass of ca. 59 kDa. In the crude supernatant (lane 2), the 59 kDa protein band is visible, but it appeared that most of the protein was hyperglycosylated (smear from 70-80 kDa). SDS-PAGE results confirmed that the *G. candidum* VES 43.1 *GCL1* gene was expressed and secreted in *S. cerevisiae* and the size of the HIS-tag purified recombinant gcl1 protein matched the predicted theoretical molecular mass.

To further characterise the activity of recombinant *G. candidum* VES 43.1 gcl1 lipase, HIS-tag purification was performed on non-deglycosylated (crude) Sc-yXYNSEC-*GCL1* supernatant (Fig. 4.4 B). The purified recombinant *G. candidum* gcl1 lipase showed the highest activity against longer-chain *p*-nitrophenyl esters, with the highest activity against the unsaturated C18 *p*-nitrophenyl substrate (C18:1 *cis* 9; oleic acid), which is consistent with previous reports that some *Geotrichum* lipases have a high substrate specificity for fatty acids with at least one *cis*-Δ9 double bond (Maldonado, 2017). Given the low activity on short-chain *p*-nitrophenyl esters, the *G. candidum* gcl1 can be classified as true lipase (Bornscheuer, 2002).

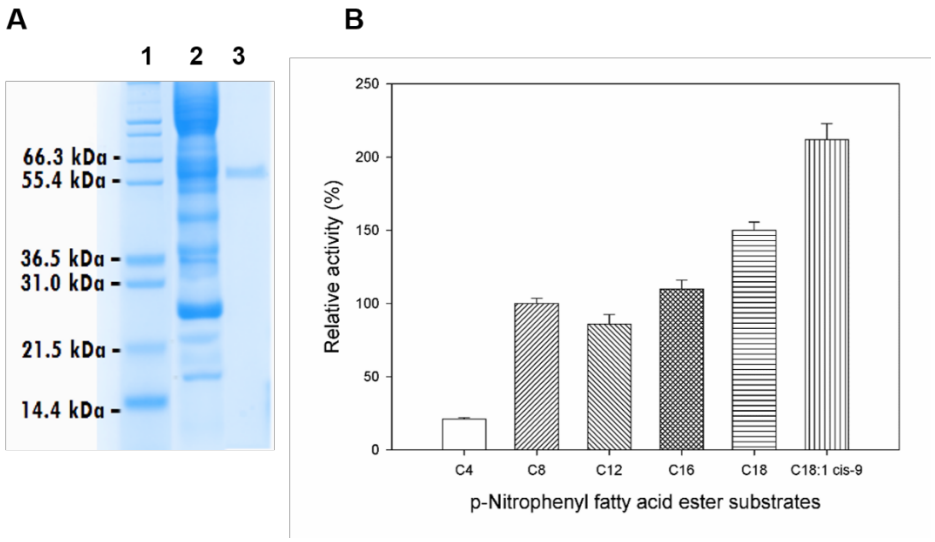

Figure 4.4. A Coomassie-stained SDS-PAGE gel showing the crude supernatant of Sc-yXYNSEC-*GCL1* (lane 2) and the HIS-tag purified recombinant *G. candidum* gcl1 protein (lane 3). Lane 1 is the Mark12 Unstained Standard. **B** Substrate specificity of purified recombinant *G. candidum* gcl1 lipase against various fatty acid esters. The activity of lipase toward different *p*-nitrophenyl esters was determined and calculated relative to the maximum activity measured toward p-nitrophenyl caprylate (C8) (taken as 100%). Error bars denote standard error (SE), n=3.

The purified recombinant *G. candidum* gcl1 lipase showed a pH optimum of 8, with 70% of activity remaining at pH 7, 65% activity at pH 6. Below pH 5 the gcl1 protein retained less than 40% of its activity (Fig. 4.5 A). The literature cites that most *Geotrichum* lipases have a pH optimum that varies between 6.0–7.0 (Omardien, Brul and Zaat, 2016). Yan *et al.* (2007) reported on the cloning and expression lipase gene from *Galactomyces geotrichum* Y05 (the teleomorph of *G. candidum*) also with a pH optimum of 8, but different from recombinant lipases of *G. candidum* CMICC 335426 and ATCC 34614 whose optimum pH was at a range of pH 7.0–7.5. The *G. candidum* VES 43.1 gcl1 lipase appears to not be unique in displaying a more alkaline pH optimum. Alkaline-active lipases are in high demand as most industrial processes operate at a relatively high temperature and alkaline pH conditions, such as the biodiesel and detergent industries (Aarthy *et al.*, 2014).

The optimum temperature for the *G. candidum* gcl1 lipase activity was observed to be 55°C as indicated in Fig. 4.5 C. *Geotrichum* lipases studied to date have been reported to have different temperature optima, ranging from 20–40°C (Maldonado, 2017). The temperature optimum of previously reported *Geotrichum* lipases was 50°C (*G. geotrichum* Y05) and 40°C (*G. geotrichum* CBS 772.71 and *G. candidum*) (Sugihara, Shimada and Tominaga, 1990; Phillips, Pretorius and van Rensburg, 1995; Holmquist, Tessier and Cygler, 1997; Yan *et al.*, 2007). Although, the *G. candidum* gcl1 lipase showed a 55°C temperature optimum, the enzyme appeared to be stable for 120 min at this temperature and lost ca. 60% of its activity. Compared to the *G. candidum* lipase characterised by Sugihara, Shimada and Tominaga (1990), which lost 95% of its activity at 55°C after 60 min, the *G. candidum* VES 43.1 gcl1 lipase appears marginally more thermostable at higher temperature. At 30°C the enzyme showed excellent stability. However, this temperature has little application in industries where lipases are required.

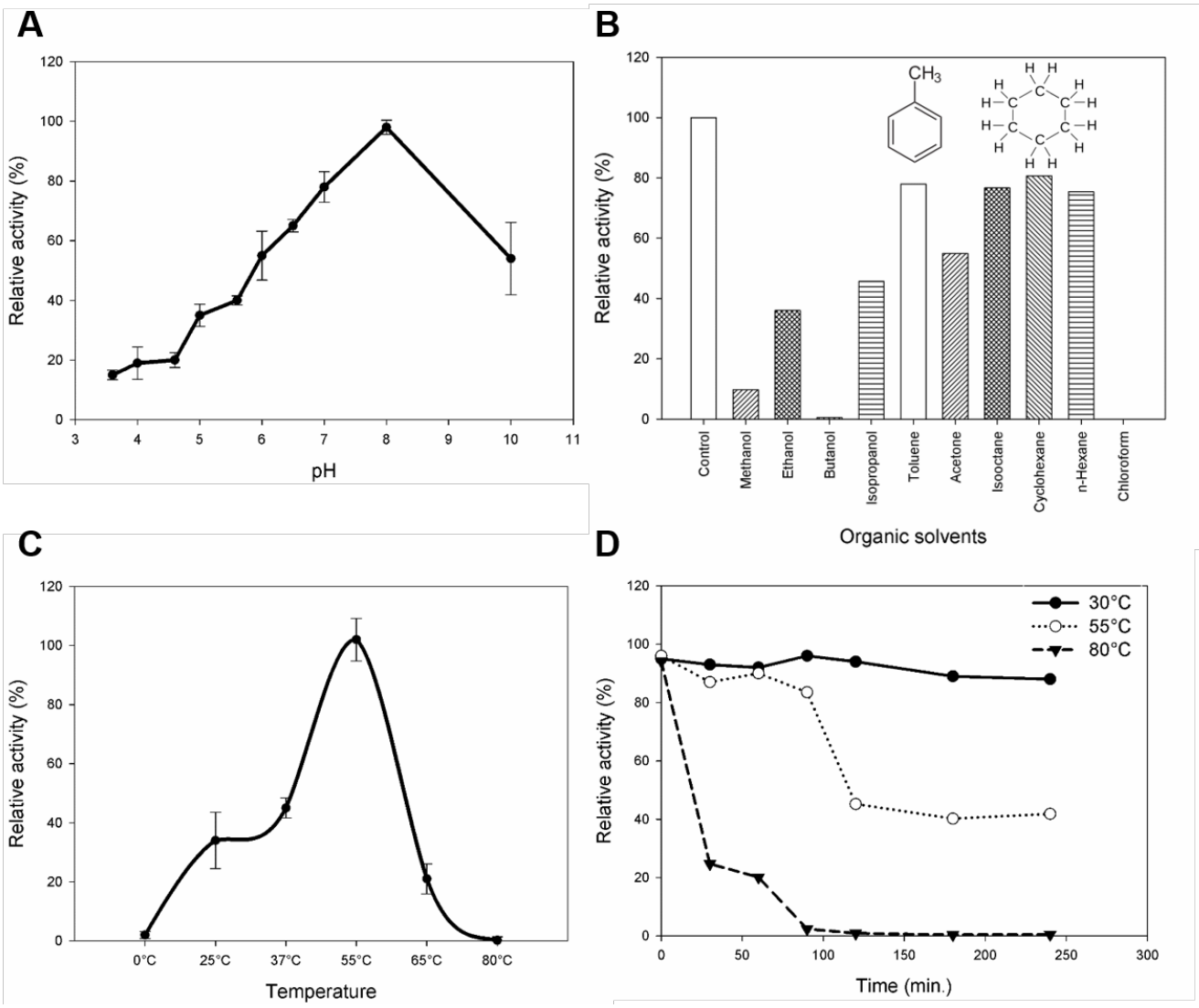

Figure 4.5. A Effect of pH on the activity of purified recombinant *G. candidum* gcl1 lipase. The enzyme was added to various buffer systems (pH 4.0–10.0) of 0.1 M at 45°C for 60 min. **B** Organic solvent stability of purified recombinant *G. candidum* gcl1 lipase at pH 8 and 45°C. **C** Temperature profile of purified recombinant *G. candidum* gcl1 lipase. The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range of 0–80°C at pH 8 using 0.1 M sodium phosphate. The reaction was carried out for 60 min. **D** Thermal stability of purified recombinant *G. candidum* gcl1 lipase was measured by incubating the lipase in 0.1 M sodium phosphate (pH 8) at 30, 55 and 80°C for 4 h. Residual lipase activity (%) was calculated relative to the initial activity. Error bars denote standard error (SE), n=3.

An exciting phenotype displayed by the purified recombinant *G. candidum* gcl1 lipase is its high activity and stability in organic solvents, considered as a novel attribute for industrial application (Fig. 4.5 B). The recombinant gcl1 lipase retained high levels of activity (ca. 80%) in toluene, isooctane, cyclohexane and n-hexane, and lost most/all activity in butanol and chloroform. Stability of this lipase in organic solvents suggests it could be used as a biocatalyst in a non-aqueous medium such as used in the pharmaceutical industry, and the tolerance to toluene is unique for the *G. candidum*

VES 43.1 gcl1 lipase, i.e. has not been reported for any *Geotrichum* lipase studied before. Given the environment the *G. candidum* VES 43.1 was isolated initially from, i.e. olive mill wastewater, and the high phenol and polyphenol content of this environment, it can be reasoned that the novel toluene tolerance displayed by *G. candidum* VES 43.1 gcl1 lipase has been acquired through an adaptive evolutionary process. This deduction is also supported by the divergence in the amino acid sequence of the *G. candidum* VES 43.1 gcl1 lipase from other *Geotrichum* lipases (Fig. 4.3). Olive phenolic compounds and derivatives have been shown to inhibit various enzymes (Dekdouk *et al.*, 2015; Qin *et al.*, 2016).

CHAPTER 5:
GENERAL DISCUSSION AND CONCLUSIONS

Olive wastewater, generated from table olive and oil extraction processing plants, is a harsh environment rich in residual oil, phenolic compounds and extremes regarding pH and salinity. Although it is a toxic environment, microorganisms have adapted to grow and survive in these wastewater streams as biofilms and could serve as a potential resource of novel enzymes with unique characteristics that could benefit the industry. Microbial lipases have become one of the most important biocatalysts in several large industrial processes, able to function under mild or extreme conditions, e.g. pH and temperature, are stable in aqueous and non-aqueous media, show broad substrate specificity and high regio- and/or stereoselectivity during catalysis. Several industries actively use these unique characteristics of lipases for cost-effective production of high-value products. For example, transesterification reactions of lipases play a central role in the food, nutraceutical and biofuel industries (Zhao *et al.*, 2015; Rivero *et al.*, 2017), while the broad substrate specificity and regio- and/or stereoselectivity of lipases make them highly valuable in the synthesis of pharmaceuticals and other pure chemicals (Sun *et al.*, 2017).

This study aimed to exploit the microbial diversity in olive wastewater biofilms, to screen for lipase enzyme producers and clone, express and characterise a potential novel lipase gene using *Saccharomyces cerevisiae* as a production host. The strategy employed to achieve this aim followed a classical microbiology methodology to isolate and screen culturable bacteria and fungi for the ability to produce secreted extracellular lipases. Through this approach, we isolated 233 pure microbial isolates from olive wastewater biofilms of which 27 fungi and 100 bacteria produced detectable lipase activity. To quantitatively compare between the 127 lipase-producers, all isolates were subjected to volumetric lipase assays using the *p*-NP stearate (C_{18}), thus exploiting the long-chain ester substrate preference of true lipases to eliminate carboxylesterases producers.

The lipase bioprospecting approach led to the identification of a fungal strain (*Geotrichum candidum* VES 43.1) originating from an olive mill biofilm environment with a superior lipase production/secretion phenotype. Using standard recombinant DNA technology approaches, the gene encoding the lipase I (*GCL1*) was cloned and functionally expressed in *S. cerevisiae*. The heterologously expressed lipase protein was purified to homogeneity, which enabled the further characterisation of the lipase regarding substrate specificity, pH optima, temperature optima and stability, as well as the stability in organic solvents.

The *G. candidum* VES 43.1 lipase exhibited a preference for longer-chain *p*-nitrophenyl ester substrates, with the high activity against the unsaturated C18 *p*-nitrophenyl substrate. Substrate specificity analysis confirmed the true lipase nature of the gcl1 protein and corroborated previous findings regarding the high preference for unsaturated acyl molecules by lipases from the genus *Geotrichum* (Maldonado, 2017).

Regarding pH optima, the *G. candidum* VES 43.1 lipase did not display any significant novelty, but the enzyme's temperature optima, thermostability and tolerance for organic solvents was unique for *Geotrichum* lipases reported previously (Maldonado, 2017). *G. candidum* VES 43.1's gcl1 lipase had a temperature optima of 55°C, and the enzyme remained active for two hours before losing activity. This temperature phenotype is different from previously reported lipases from various *Geotrichum* strains, which typically functions at lower optimum temperatures and are more labile at elevated temperatures. Lastly, the *G. candidum* VES 43.1 lipase showed stable activity in organic solvents (at 75% v/v concentration). Although organic solvent tolerant *Geotrichum candidum* lipases have been reported before (Yan *et al.*, 2007), the lipase in this study was high toluene-stable, never reported previously.

Provided the environmental context from which the *G. candidum* VES 43.1 isolate originated, it can be reasoned that the toluene tolerance of the gcl1 lipase is possibly linked to the toxic concentrations of phenolic compounds in olive mill wastewater. To survive the fungus had to adapt its secreted lipase to tolerate phenolic compounds to facilitate efficient nutrient absorption from its environment. Further evidence for this hypothesis is provided by the significant number of unique amino acids found only in the *G. candidum* VES 43.1 lipase protein.

In the enzyme engineering field, screening limitations often necessitate the construction of semi-rational protein variant libraries instead of extensive variant libraries, which require expensive high-throughput infrastructure, such as robotics. The unique amino acid variations observed in this study in *G. candidum* VES 43.1 lipase protein could in future studies be used as lead targets for semi-rational protein engineering to investigate the determinants of temperature and toluene-tolerance in lipases.

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APPENDICES A: Lipase screening results of fungal isolates

Table 4.1. Lipase screening results of fungal isolates on Rhodamine B and Tween 80 plates.

No	Isolate	Rhodamine B	Tween 80	No	Isolate	Rhodamine B	Tween 80
1	BUF 10	-	+	30	BUF 56	-	nd
2	BUF 11	+++	+	31	BUF 57	+++	+
3	BUF 14	+	+	32	BUF 59	-	-
4	BUF 188	+	-	33	BUF 6	-	-
5	BUF 190	++		34	BUF 7	-	nd
6	BUF 21	++	-	35	BUF 8.8	+	+
7	BUF 22	-	-	36	VES 1	+++	-
8	BUF 28	-	nd	37	VES 12	++	+
9	BUF 29	-	-	38	VES 14	++	+
10	BUF 29	-		39	VES 15	++	+
11	BUF 29.1	-	-	40	VES 16	-	+
12	BUF 29.2	+	-	41	VES 18	-	+
13	BUF 3	++	-	42	VES 19	+	nd
14	BUF 32	++	+	43	VES 20	-	nd
15	BUF 33	-	-	44	VES 25	-	-
16	BUF 36	+	-	45	VES 26	-	-
17	BUF 37	-	-	46	VES 30	-	-
18	BUF 38	++		47	VES 35	-	
19	BUF 39	+	nd	48	VES 40	++	-
20	BUF 4	-	-	49	VES 43	++	nd
21	BUF 41	+++	+	50	**VES 43.1**	**+++**	**++**
22	BUF 42	+++		51	VES 45	++	nd
23	BUF 44	-	-	52	VES 46	-	nd
24	BUF 47	+	-	53	VES 58	-	nd
25	BUF 49	-	-	54	VES 72	++	+
26	BUF 50	-	-	55	VES 73	+++	nd
27	BUF 51	-	-	56	VES 8	-	-
28	BUF 53	-	nd	57	VES 9	-	nd
29	BUF 55	-	nd	58	*C. cylindracea* CBS 6330	+++	nd

Abbreviations: VES = Vusuvio; BUF = Buffet Olives; + (weak lipase activity); +++ (strong lipase activity); - (no lipase activity); nd (not determined).

APPENDICES B: Lipase screening results of bacterial isolates

Table 4.2 (b) Lipase screening results of bacterial isolates on Rhodamine B and Tween 80 plates.

No	Isolate	Rhodamine B Results	Tween 80 results	No	Isolate	Rhodamine B results	Tween 80 Results
1	BB 47	++	nd	89	BB 46	++	nd
2	BB 104	+	-	90	BB 49	-	+
3	BB 105	+	-	91	BB 50	+	-
4	BB 106	+	+	92	BB 51	+	-
5	BB 11	+	-	93	BB 55	-	-
6	BB 12	+++	-	94	BB 56	-	-
7	BB 110	+	-	95	BB 61	+	++
8	BB 118	+	+	96	BB 64	-	+
9	BB 119	++	+	97	BB 64.1	-	nd
10	BB 123	-	+	98	BB 66	+	+
11	BB 123.1	-	nd	99	BB 66.1	+	nd
12	BB 127	+	nd	100	BB 69	+	+
13	BB 128	+	+	101	BB 7	-	nd
14	BB 13	-	nd	102	BB 75	-	nd
15	BB 13	+	nd	103	BB 8	-	++
16	BB 131	++	-	104	BB 80.1	+	nd
17	BB 133	-	nd	105	BB 80.2	+	++
18	BB 135	+	-	106	BB13.1	-	nd
19	BB 136	-	nd	107	VB 101	+	++
20	BB 137	-	-	108	VB 102	-	+
21	BB 138	+	+	109	VB 104	+	nd
22	BB 139	-	+	110	VB 104.1	+	nd
23	BB 14	-	nd	111	VB 104.2	+	nd
24	BB 140	++	nd	112	VB 105	+++	nd
25	BB 141	+	nd	113	VB 106	-	+
26	BB 142	-	-	114	VB 106.1	-	nd
27	BB 143	-	-	115	VB 107	+	+
28	BB 157	-	nd	116	VB 108	-	nd

29	BB 157.1	+	+	117	VB 109	-	+
30	BB 158	+	+	118	VB 109.1	-	nd
31	BB 158.1	+	nd	119	VB 11	-	-
32	BB 159	+	-	120	VB 114	+	-
33	BB 16	-	nd	121	VB 12	+	+
34	BB 165	-	-	122	VB 121	+++	+
35	BB 166	-	nd	123	VB 122	+	-
36	BB 17	-	nd	124	VB 123	-	-
37	BB 172	-	-	125	VB 126	+	+
38	BB 173	-	-	126	VB 128	-	+
39	BB 18	-	nd	127	VB 128.1	+	nd
40	BB 18.1	++	+	128	VB 13	-	+
41	BB 18.2	++	nd	129	VB 130	+	++
42	BB 183	+	+	130	VB 137	-	+
43	BB 183.1	+	+	131	VB 137.1	+	++
44	BB 184.2	-	-	132	VB 156	-	+
45	BB 186	-	+	133	VB 184	+	-
46	BB 19	-	nd	134	VB 19	-	nd
47	BB 193.1	+	nd	135	VB 19	-	nd
48	BB 193.2	+++	-	136	VB 192.2	-	nd
49	BB 194	+	-	137	VB 208	+++	nd
50	BB 194.1	+	nd	138	VB 208.4	+++	nd
51	BB 197	+	-	139	VB 243	+++	+
52	BB 198	+	+	140	VB 244	+	+
53	BB 215	-	+	141	VB 246	+++	++
54	BB 218	-	+	142	VB 246.1	-	-
55	BB 223	-	+	143	VB 257	+	+
56	BB 225.1	+	nd	144	VB 259	-	-
57	BB 225.2	-	-	145	VB 29	+	+
58	BB 227	+	-	146	VB 31	+	nd
59	BB 23	+	nd	147	VB 325	+	nd
60	BB 23	++	nd	148	VB 347	+	nd
61	BB 3	++	+	149	VB 349	+	++
62	BB 3	+	nd	150	VB 349.1	+	nd

63	BB 300.1	+++	nd	151	VB 349.2	+	+
64	BB 300.2	++	+	152	VB 36	+++	nd
65	BB 304	-	-	153	VB 400	-	nd
66	BB 305	-	++	154	VB 44.1	+++	+
67	BB 307	+	-	155	VB 46	+++	++
68	BB 310	-	-	156	VB 5	-	nd
69	BB 311	+	-	157	VB 56	+	nd
70	BB 314	-	-	158	VB 57	+	++
71	BB 315	-	-	159	VB 58	+	nd
72	BB 318	+	-	160	VB 60	+	+
73	BB 319	+	nd	161	VB 61	++	nd
74	BB 325	+++	+	162	VB 63	+	+
75	BB 329	-	++	163	VB 67	+	nd
76	BB 33	-	++	164	VB 68	+	+
77	BB 331	-	+	165	VB 68.1	-	nd
78	BB 34	-	++	166	VB 69	-	nd
79	BB 340	-	nd	167	VB 70	-	+
80	BB 341	-	+	168	VB 71	++	-
81	BB 346	-	+	169	VB 72	-	+
82	BB 353	+	++	170	VB 72	+	+
83	BB 39	+	-	171	VB 73	-	+
84	BB 4	++	-	172	VB 74	-	+
85	BB 41	+	-	173	VB 97	-	-
86	BB 42	-	-	174	VB 99	-	+
87	BB 44.2	++	nd	175	VB 16	-	nd
88	BB 45	+++	+	176	VB 300	-	nd

Abbreviations: VB = Vusuvio; BB = Buffet Olives; + (weak lipase activity); +++ (strong lipase activity); - (no lipase activity) and nd (not determined).