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Classification of Lipolytic Enzymes and their Biotechnological Applications in the Pulping Industry

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Abstract

In the pulp and paper industry, during the manufacturing process the agglomeration of pitch particles (composed of triglycerides, fatty acids and esters) leads to the formation of black pitch deposits in the pulp and on machinery which impacts on the process and pulp quality. Traditional methods of pitch prevention and treatment are no longer feasible due to environmental impact and cost. Consequently, there is a need for more efficient and environmentally friendly approaches. The application of lipolytic enzymes, such as lipases and esterases could be the sustainable solution to this problem. Therefore, an understanding of their structure, mechanism and sources are essential. The microbial sources for the different groups of lipolytic enzymes, differences between lipases and esterases and their potential applications in the pulping industry are reviewed here.

Draft

Key words: Lipases, esterases, lipolytic, microbial, biopulping

1. Introduction

Enzymes are a vital component of numerous industrial processes, such as pulp and paper, food, beverage, detergent, clothing, fuel and pharmaceutical manufacturing (Gurung et al. 2013). Currently, hydrolases are the most popular enzymes in biotechnology (Gurung et al. 2013), with carbohydrases, proteases and lipases forming more than 70% of all enzyme sales in the current market, which is expected to reach 10.7 billion USD by 2024 (Global Market Insights, August 2016; Li et al. 2012). Hydrolases are a class of enzymes that demonstrate broad substrate specificity. They are capable of hydrolyzing peptides, halides, amides, as well as esters and triglycerides (Fojan et al. 2000). One of the most valuable classes of hydrolases in biotechnological applications are lipolytic enzymes, which comprise of lipases (EC 3.1.1.1, triacylglycerol hydrolases) and 'true' esterases (EC 3.1.1.3, carboxyl ester hydrolases) (Casas-Godoy et al. 2012; Fan et al. 2012; Hudlicky and Reed 2009; Bornscheuer 2002). Lipases and esterases are known to catalyze both the hydrolysis and synthesis of ester compounds. They are characteristically resilient enzymes that can withstand harsh industrial processes with wide pH ranges, high temperatures and the presence of organic solvents (Gupta et al. 2004). They also exhibit characteristic chemo-, stereo- and regio- selectivity that are of particular interest in applications, such as the synthesis of optically pure compounds (Haki and Rakshit 2003). Their catalytic flexibility, robustness and high specificity attract a lot of attention as industrial biocatalysts. This report is a review of the different types of lipolytic enzymes, their mechanisms of action, and their potential application in various industrial processes, with an emphasis on the pulp and paper industry.

2. Lipolytic Families

The three-dimensional structure of lipases and esterases displays the characteristic α/β -hydrolase fold (Ollis et al. 1992) and a definite order of α -helices and β -sheets. Bacterial lipolytic enzymes are classified into eight families (Families I – VIII) based on variances in

their amino-acid sequences and biological properties (Arpigny and Jaeger 1999). Of the eight different lipolytic families, Family I ('true' lipases- interfacial activation and presence of a lid is characteristic of this family) is the largest and is further divided into six subfamilies. Carboxyl esterases along with various other lipases are grouped into the other seven families, e.g. Family II (also called GDSL [sequence motif] family), Family III, Family IV (also called HSL [hormone sensitive lipase] family), Family V, Family VI, Family VII, and Family VIII (Arpigny and Jaeger 1999).

2.1 Lipolytic Family I

Lipolytic family I is the most represented family and is divided into seven subfamilies, with an estimated total of 27 members. Lipases of this family share a Gly-Xaa-Ser-Xaa-Gly consensus sequence (Kanaya et al. 1998; Osterlund et al. 1996). Family I include the 'true' lipases, e.g. Gram negative *Pseudomonas* lipases and Gram positive bacteria, such as *Bacillus*, *Staphylococcus*, *Propionibacterium* and *Streptomyces* (Arpigny and Jeager 1999). The expression of active lipases belonging to subfamilies I.1 and I.2 is dependent on a chaperone protein called lipase-specific foldase (Lif). Two aspartic residues are implicated in the Ca-binding site found at homologous positions in all sequences and the two cysteine residues forming a disulphide bridge are conserved in most sequences. These four residues are believed to be important in stabilizing the active site of these enzymes (Kim et al. 1997). An example of such a lipase (LipC12) (Ogierman et al. 1997) belongs to family I.1 of bacterial lipases, which has a chaperone-independent folding, does not possess disulfide bridges and is calcium ion dependent. LipC12 is stable from pH 6 to 11 and has activity from pH 4.5 to 10, with higher activities at alkaline pH values. Stability is observed up to 3.7 M NaCl environments and temperatures ranging from 20 to 50°C, with maximum activity at

30°C over a 1-hour incubation period. The pure enzyme has specific activities of 1767 U/mg and 1722 U/mg against pig fat and olive oil, respectively (Glogauer et al. 2011).

Bacillus lipases are generally classified under two subfamilies, I.4 and I.5 (Arpigny and Jaeger 1999). Several *Bacillus* lipases are known to have a common alanine residue that replaces the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. However, *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus licheniformis* lipases from the I.4 subfamily are the smallest true lipases (approx. 19-20 kDa) and they share very little similarity at the amino acid level (approx. 15%) with the other *Bacillus* and *Staphylococcus* lipases (Arpigny and Jaeger 1999). Temperatures above 45°C result in decreased activities for subfamily I.4 lipases, however, they display high activity at basic pH (between 9.5 and 12) (Guncheva and Zhiryakova 2011). Some exceptions include the lipases of *Bacillus thermocatenulatus* which have a molecular mass of about 43 kDa and display maximal activity at approx. 65°C and pH 9.0 (Schmidt-Dannert et al. 1994; Carrasco-Lopez et al. 2009). Another *Bacillus* sp. known as *Bacillus stearothermophilus*, also produces a lipase with a molecular mass of 67 kDa and optimal activity at pH 11 and 55°C (Bacha et al. 2015). Massadeh and Sabra (2011) reported maximal lipase activity of 90.57 U/ml from *B. stearothermophilus* at 45°C and pH 8. Other examples of lipases in this family are produced by staphylococcal isolates. They produce larger lipases than *Bacillus* sp. (approx. 75 kDa) that are secreted as precursors and cleaved in the extracellular medium by a specific protease, generating a protein of approximately 400 residues. The pro-peptide (207±267 residues) seemingly operates as an intramolecular chaperone and assists in translocation of the lipase across the cell membrane (Sorensen and Mortensen 2005; Götz et al. 1998).

2.2 Lipolytic Family II

Lipolytic family II has five members that have been characterized and reported. Enzymes categorized as Family II do not demonstrate the usual pentapeptide (Gly-Xaa-Ser-Xaa-Gly) but exhibit a Gly-Asp-Ser-(Leu) [GDS(L)] motif comprising the active-site serine residue. This residue lies much closer to the N-terminus in these proteins than in other lipolytic enzymes (Upton and Buckley 1995). Unlike conventional lipases, GDSL enzymes do not possess a nucleophile elbow, which is a region containing a beta-beta-alpha structural motif and holds the nucleophilic and the oxyanion hole amino acid residues that form the catalytic site in various enzymes. The nucleophile (Ser, Asp or Cys) is found in a sharp turn, the so-called nucleophile elbow. They are known to have flexible active sites that are able to modify its structure in the presence of specific substrates, thus increasing its substrate specific range (Akoh et al. 2004). Included in this family are esterases of *Streptomyces scabies*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Photobacterium luminescens*, *Vibrio mimicus*, *Escherichia coli*, *Aeromonas hydrophila* (Loveless and Saier 1997; Henderson et al. 1998) and lipases of *A. hydrophila*, *Vibrio parahaemolyticus*, *Xenorhabdus luminescens* and *Streptomyces rimosus* (Akoh et al. 2004). An example of an extracellular lipase from this family was isolated from *P. aeruginosa* with a molecular weight of 30 kDa. The lipase was stable up to 45°C and maintained activity in the alkaline pH range (Sharon et al. 1998). The enzyme was found to be highly stable in the presence of methanol and ethanol, and the cationic surfactants, such as Triton X-100 and Tween-80, substantially increased activity. The presence of a calcium-binding site in this *Pseudomonas* lipase is predicted as its activity was stabilized significantly by Ca^{2+} and inactivation of the enzyme by EDTA was overcome by subsequent CaCl_2 treatment (Sharon et al. 1998).

2.3 Lipolytic Family III

Family III of lipolytic enzymes is comprised of 3 characterized members so far. Enzymes possess the conserved consensus sequence Gly-Xaa-Ser-Xaa-Gly. Members of this family are very closely related (Lee, 2016). Enzymes exhibit the canonical fold of α/β -hydrolase and contain a typical catalytic triad. High activities at low temperature (less than 15°C) were believed to originate from conserved sequence motifs of these enzymes (Feller et al. 1991; Langin et al. 1993). However, distinct sequence similarity between esterases from psychrophilic (*Moraxella* sp. *Psychrobacter immobilis*), mesophilic (*Alcaligenes eutrophus*, *Escherichia coli*) and thermophilic (*Alicyclobacillus acidocaldarius*, *Archeoglobus fulgidus*) microorganisms suggest that temperature variation is not responsible for such considerable sequence conservation (Arpigny and Jaeger 1999). A comparative study of these enzymes would be beneficial in resolving the unique properties of this family of hydrolases. An example of an enzyme from this family is the extracellular lipase from *Serratia marcescens*, ECU1010, with a molecular mass of 65 kDa, a pI of 4.2, and pH and temperature optima of 8.0 and 45°C, respectively (Zhao et al. 2008).

2.4 Lipolytic Family IV

Family IV, otherwise known as the hormone-sensitive lipase (HSL) lipolytic family (family IV), due to its high sequence similarity to the mammalian HSL (Hausmann and Jaeger 2010), has six characterized members in its family. The hydrolysis reaction of triacylglycerols in adipose tissue is catalyzed by HSL, in addition to being a rate-limiting enzyme in the exclusion of fatty acids from stored lipids (Østerlund et al. 1996). This family has two highly conserved consensus motifs, the common GX SXG and HGG, which plays a role in the oxyanion hole formation (Mohamed et al. 2013). Enzymes are known to exhibit an α/β -hydrolase fold (Ngo et al. 2013). Enzymes from this family have been identified in *Pseudomonas* sp., *Alcaligenes eutrophus*, *Moraxella* sp. *Alicyclobacillus acidocaldarius*,

Escherichia coli and *Archaeoglobus fulgidus* (Manco et al. 2000; Choo et al. 1998; Kanaya et al. 1998; Manco et al. 1998; Feller et al. 1991). An esterase of *Pyrobaculum calidifontis* is considered as a member of the HSL family. Activities reported for this enzyme are similar if not higher than previous reports with 1050 U/mg at 30°C and 6410 U/mg at 90°C (Hotta et al. 2002; Rashid et al. 2001; Manco et al. 2000).

2.5 Lipolytic Family V

Enzymes in this family are made up of six characterized members to date. Some of them originate from mesophilic (*Acetobacter pasteurianus*, *Pseudomonas oleovorans*, *Haemophilus influenza*), psychrophilic (*Sulfolobus acidocaldarius*) and thermophilic microorganisms (Arpigny and Jaeger 1999). Typically, this family possesses the conserved motif GX SXG, as well as the other common motif PTL (Nacke et al. 2011). A lipolytic enzyme (EstV) from *Helicobacter pylori* has been isolated, cloned, purified and classified as a Family V hydrolase. This enzyme was predominantly active with short-chain substrates (*p*-nitrophenol acetate, *p*-NP butyrate, *p*-NP valerate) and did not display interfacial activation, but was stable and had a maximum activity at 50°C and pH 10 (Ruiz et al. 2007).

2.6 Lipolytic Family VI

There are five well characterized members that are classified as family VI lipolytic enzymes based on their size. This family comprises of the smallest esterases, with a molecular mass of 23±26 kDa. The subunit has the α/β -hydrolase fold and a classical Ser-Asp-His catalytic triad. A carboxylesterase from *Pseudomonas fluorescens* is an example of such an esterase (Kim et al. 1997). This enzyme hydrolyses small ester-containing compounds with a broad specificity and demonstrates no activity towards long-chain triglycerides (Hong et al. 1991). In another example, an extracellular alkaline lipase produced by *P. fluorescens* AK102 was

stable between pH 4 and 10 with an optimum pH between 8 and 10 and an optimum temperature of 55°C. This enzyme could have a potential application in the pulping industry for the removal of pitch (Kojima et al. 1994). There is a lack of information on other enzymes in this family. Interestingly, esterases from this family show 40% homology at the amino acid level to eukaryotic lysophospholipases (Bornscheuer, 2002), which are responsible for the liberation of fatty acids from lysophospholipids (Schmiel and Miller 1999).

2.7 Lipolytic Family VII

Family VII esterases are a smaller family with only three well known members. They have an estimated size of ± 55 kDa, and share substantial amino acid sequence homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases (Arpigny and Jaeger 1999). This family has the classical triad G-X-S-X-G pentapeptide. The esterase from *Arthrobacter oxydans* is classified under this family and is specifically active against phenylcarbamate herbicides by hydrolysing the central carbamate bond (Pohlenz et al. 1992). The esterase is plasmid-encoded and thus has the potential to be more easily transferred to other strains or species. Interestingly, the esterase of *B. subtilis* is capable of hydrolyzing *p*-nitrobenzyl esters, and may be applied in the final removal of *p*-nitrobenzyl ester utilized as a protecting group in the synthesis of β -lactam antibiotics (Zock et al. 1994). Another esterase isolated from a *Bacillus* strain was found to be thermostable and had maximum activity at 60°C and maintained 100% activity at 75°C for 30 min (Andualema and Gessesse 2012). These characteristics would be appropriate for application in the reduction of pitch during the pulping process (high process temperatures) where glyceride lipids may be targeted.

2.8 Lipolytic Family VIII

Family VIII consists of three characterized members. These enzymes are approximately 380 amino acids in length with similarity to many class C β -lactamases. A 150 amino acid fragment (from positions 50 to 200) showed 45% similarity at the amino acid level to an *Enterobacter cloacae ampC* gene product (Galleni et al. 1988). *AmpC* codes for resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and beta-lactamase inhibitor-beta-lactam combinations (Jacoby 2009). This trait indicates that the active site (Ser-Xaa-Xaa-Lys) conserved in the N-terminal, belongs to class C β -lactamases (Lobkovsky et al. 1993; Nishizawa et al. 1995). Contrary to this, the esterase/lipase consensus sequence (Gly-Xaa-Ser-Xaa-Gly) of the *P. fluorescens* esterase was proposed to be involved in the active site of β -lactamases (Kim et al. 1994). Esterases from *Streptococcus chrysomallus* also possess this motif; but, it is not conserved in the *Arthrobacter globiformis* esterase. Also, the motif is situated in close proximity to the C-terminus of the *P. fluorescens* and *S. chrysomallus* enzymes with an absence of the histidine attachment (amino acid used in the synthesis of proteins). This demonstrates the unconventional nature of these enzymes as the Ser-Asp-His residue sequence is conserved throughout the entire superfamily of lipases and esterases. Site-directed mutagenesis studies have demonstrated that the Gly-Xaa-Ser-Xaa-Gly motif does not play a significant role in enzyme functioning of an esterase (EstB) from *Burkholderia gladioli* (Petersen et al. 2001).

A number of genera, such as *Pseudomonas* and *Streptomyces*, are known to produce hydrolases which are classified into different families (Figure 1). Arpigny and Jaeger (1999) first classified bacterial lipolytic enzymes into the eight families according to their amino acid sequences and biological properties. This became the reference point for classification of novel lipolytic enzymes to a family. However, unique families are being discovered through

the use of metagenomics (Fu et al. 2011; Kim et al. 2009; Lee et al. 2006). Table I summarizes the different classes of lipolytic enzymes currently described.

Figure 1: Phylogenetic tree based on a novel amino acid sequence of EstCS2 and closely related proteins. Protein sequences for previously identified families of bacterial lipolytic enzymes retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). The units at the bottom of the tree indicate the number of substitution events (adapted from Kang et al. 2011).

3. Lipases

Lipases (e.g. triacylglycerol hydrolase, EC 3.1.1.3) hydrolyze long-chain acyl groups (7C10) to fatty acids and acylglycerols (Litthauer et al. 2002; Ollis et al. 1992; Verger 1997; Ellaiah et al. 2004; Bornscheuer 2002). Lipases can be differentiated from esterases by the occurrence of interfacial activation, which is only observed in lipases, whilst esterases obey classical Michaelis-Menten kinetics. Structural analysis revealed that this interfacial activation is due to a hydrophobic domain (lid) covering the active site of lipases - only in the presence of a minimum substrate concentration, i.e. only in the presence of triglycerides or hydrophobic organic solvents, does the lid move apart, making the active site available (Sharma 2001; Jaeger et al. 1999). Therefore, lipases have altered properties from esterases, which have an acyl binding pocket (Pleiss et al. 1998).

Microbial lipases specifically have unlimited potential in commercial applications, such as additives in fine chemicals, wastewater treatment, food processing, cosmetics, detergents, pharmaceuticals, degreasing formulations, paper manufacture and accelerated degradation of fatty wastes and polyurethane (Kamini et al. 2000; Liu et al. 2012; Shu et al. 2012; Brabcova et al. 2013; Lailaja and Chandrasekaran 2013; Nerurkar et al. 2013; Whangsuk et al. 2013; Zhang et al. 2013; Adulkar and Rathod 2014; Gerits et al. 2014; Li et al. 2014; Saranya et al. 2014; Fulton et al. 2015; Rodrigues et al. 2016; Speranza et al. 2016). Even though a large number of lipases have been described in the literature, a limited number of enzymes belonging to a few species have proved their stability and biosynthetic activity amenable to use in organic solvents, and thus their consideration as industrially applicable enzymes (Cardenas et al. 2001; Jaeger et al. 1999). Their biotechnological potential is dependent on

their capacity to catalyze not only the hydrolysis of triglycerides, but also their synthesis from glycerol and fatty acids (Jaeger et al. 1999). Lipases are known to hydrolyze up to 90% of triglycerides in pitch to glycerol/monoglycerides and fatty acids, which are considerably less sticky and more hydrophilic (simple to wash) than the triglycerides (Jaeger and Reetz 1998).

3.1 Classification

Classification of lipolytic enzymes as 'true' lipases requires the fulfilment of two criteria: (i) they should be activated by the presence of an interface, i.e. their activities should dramatically increase as soon as the triglyceride substrate forms an emulsion. This occurrence is termed "interfacial activation"; (ii) they should also contain a "lid", which is a surface loop of the protein covering the active site of the enzyme and moves away on contact with the interface (Brzozowski et al. 1991; Derewenda et al. 1992; van Tilbeurgh et al. 1993). The 'true' lipase family (Family I) cover the 6 subfamilies which predominantly catalyze the hydrolytic reactions of substrates with long acyl chains (Messaoudi et al. 2011).

3.2 Mechanism of action of Lipases

Lipases take effect on ester bonds present in acylglycerols to release free fatty acids and glycerol in a liquid medium (Villeneuve et al. 2000). In limited liquid environments, these enzymes are capable of reversing this reaction (esterification), *via* acidolysis, interesterification, and alcoholysis (Villeneuve et al. 2000). Elucidation of their structures revealed that the interfacial activation observed is due to a hydrophobic domain (lid) covering the active site of lipases and that high levels of activity was observed only in the presence of a minimum substrate concentration (Araújo et al. 2008).

3.3 Microorganisms producing lipases

Lipases have originated from plants, animals and microorganisms, however, bacterial lipases are the most versatile, stable and reactive in organic medium (Andualema and Gessesse 2012). Numerous microorganisms are known to produce lipases when incubated with lipid substrates (Haba et al. 2000). The majority of bacterial lipases originate from Gram-negative bacteria, the most valuable being *Pseudomonas* which includes at least seven lipase-producing species, which are *Pseudomonas aeruginosa* (Tielen et al. 2013; Prasad 2014), *Pseudomonas cepacia* (Badgujar et al. 2016; Cao et al. 2016; Sasso et al. 2016), *Pseudomonas alcaligenes* (Chen et al. 2014; Patel et al. 2014), *Pseudomonas glumae* (Knapp et al. 2016), *Pseudomonas fluorescens* (Xun et al. 2012; Guldhe et al. 2015; Lima et al. 2015), *Pseudomonas fragi* (Santarossa et al. 2005; Dey et al. 2014) and *Pseudomonas putida* (Fatima et al. 2014).

3.4 Heterologous production of lipases

Lipases from *Pseudomonas* species require the functional assistance of about 30 different cellular proteins prior to recovery from the culture supernatant in an enzymatically active state, demonstrating that folding and secretion are highly specific processes that generally do not function properly in heterologous hosts (Rosenau et al. 2004; Rosenau and Jaeger 2000). Extracellular enzymes, such as lipases, must be translocated through the bacterial membrane to a suitable location to fulfil their function. Gram-positive bacteria secrete enzymes which cross a single cytoplasmic membrane. These proteins generally contain a signal sequence, directing translocation *via* the secretion machinery (Fekkes and Driessen 1999). The TAT pathway is a second translocation pathway found in lipase secreting Gram-negative and Gram-positive bacteria (Shruthi et al. 2010; Heravi et al. 2009). Proteins utilizing this pathway contain a unique twin arginine translocation motif in their signal sequence (Tjalsma et al. 2000). In general, active expression of lipases from *Pseudomonas* and *Burkholderia*

requires the presence of a chaperone protein known as the lipase-specific foldase (Lif), for precise folding of the lipase (Quyen et al. 2012; Wu et al. 2012). A text book example would be that of the cold-active lipase gene isolated from *Psychrobacter* sp. which was expressed in *E. coli* BL21 yielding a specific activity of 66.51 U/mg. When the recombinant plasmid was co-expressed with a “chaperone team” the lipase displayed a specific activity of 108.77 U/mg (Cui et al. 2011). Other expression hosts, such as *Bacillus* species, have also been explored. A lipase isolated from *P. vulgaris* was expressed in *B. subtilis* WB800, which displayed a high lipase activity of 356.8 U/ml after a 72-hour induction with sucrose (Lu et al. 2010). Lipolytic genes have also been isolated from metagenomes and expressed in different host strains (Liaw et al. 2010). A lipase from a metagenome has been cloned in *Streptomyces lividans* with maximal activity of 4287 U/mg towards *p*-nitrophenyl butyrate at 60°C and pH 8.5 (Cote and Shareck 2010).

4. Esterases

Esterases retain significant applications in various biotechnological processes due to their stability in organic solvents, extensive substrate specificity, stereoselectivity, regioselectivity, and lack of requirement for cofactors (Jaeger and Eggert 2002; Akoh et al. 2004; Faber 2000; Patel 2000). Esterases (EC 3.1.1.1) hydrolyze the ester bonds of water soluble fatty acid esters with short-chain acyl groups (6C8) (Ollis et al. 1992; Verger 1997). Several methods have been developed to screen and isolate novel esterases (Kumar et al. 2012; Elend et al. 2006; Kim et al. 2006; Choi et al. 2003; Suzuki et al. 2003), including metagenomic techniques (Henne et al. 2000). A range of esterase characteristics have been described, primarily in molecular biology, targeted synthesis, purification, quantitation, production, and distribution

4.1 Mechanism of action of esterases

Esterases boast a diversity of substrate specificities; however, they typically possess a catalytic triad composed of Ser, His, and Asp/Glu in the polypeptide chain (Ollis et al. 1992; Verger 1997; Bornscheuer 2002). The active site Ser residue is integrated at the centre of the conserved pentapeptide sequence motif, Gly-Xaa-Ser-Xaa-Gly (Ollis et al. 1992). The motif is usually positioned in the sharp turn between a β -strand and α -helix, known as the nucleophilic elbow (Verger 1997). Ester bond hydrolysis is mediated by the nucleophilic attack of the active Ser on the carbonyl of the substrate in a charge-relay system with the two other amino acid residues (His and Asp/Glu) (Ollis et al. 1992). Ester hydrolysis or formation is fundamentally the same for lipases and esterases and involves four steps. Initially, the substrate is attached to the active serine, generating a tetrahedral intermediate which is stabilized by the catalytic His and Asp residues. Next, the alcohol is liberated and an acyl-enzyme complex is produced. Attack of a nucleophile forms a tetrahedral intermediate again, which following resolution generates the product (an acid or an ester) and free enzyme (Bornscheuer and Kazlauskas 1999). In some cases, esterases may appear to function in vitro as esterases, however they may end up functioning in vivo as transferases. An ideal example is that of the *O*-Acetyl peptidoglycan esterase 2 (Ape2) protein in *Neisseria gonorrhoeae*, which was thought to be an *O*-acetylPG esterase but instead functioned as a PG *O*-acetyltransferase (Moynihan and Clarke, 2010). This may prove to be a major obstacle in the implementation of enzymes for biotechnological applications.

4.2 Microorganisms producing esterases

Esterases are produced by an array of organisms such as; *Streptomyces* sp. (Nishimura and Inouye 2000), *Pseudomonas* sp. (Prim et al. 2006; Tserovska et al. 2006), *Bacillus* sp. (Ding et al. 2014; Metin et al. 2006), *Lactobacillus* sp. (Choi and Lee 2001),

Thermoanaerobacterium sp. (Shao and Wiegel 1995), *Micrococcus* sp. (Fernandez et al. 2004), *Ophiostoma* sp. (Calero-Rueda et al. 2002), *Penicillium* sp. (Horne et al. 2002), *Aspergillus* sp. (Giuliani et al. 2001), *Humicola* sp. (Htzakis et al. 2003), *Sporotrichum* sp. (Topakas et al. 2003), *Saccharomyces* sp. (Lomolino et al. 2003), *Candida* sp. (Ghosh et al. 1991), plant (Pringle and Dickstein 2004) and animals (Finer et al. 2004) and may be applied in valuable biological processes. The efficient hydrolysis of both triglycerides and sterol esters using sterol esterase from *Ophiostoma piceae* has been successfully applied for pitch control in the pulp and paper industry (Calero-Rueda et al. 2002; Gutiérrez-Fernández et al. 2014; Coloma et al. 2015). Steryl and cholesteryl esterases from *Pseudomonas* sp. (Uwajima and Terada 1976; Svendsen et al. 1995; Sugihara et al. 2002), *Chromobacterium viscosum* (Kontkanen et al. 2004) and *Candida rugosa* (Rúa et al. 1993) are also reported to contribute a vital role in reducing pitch problems during paper manufacturing.

4.3 Heterologous production of esterases

Numerous reports have been published on the cloning and expression of microbial esterases (Khalameyzer et al. 1999; Ro et al. 2004; Brod et al. 2010; Huang et al. 2010; Terahara et al. 2010). When considering heterologous expression one needs to investigate the type of expression system to be applied as this could have a significant effect on the level enzymes produced. For instance, *Ophiostoma piceae* produces a sterol esterase with high activity in the hydrolysis of triglycerides and sterol esters, however, once expressed in *Pichia pastoris*, greater activity was observed due to higher solubility (Cedillo et al. 2012). This is due to the alteration in the N-terminal sequence of the protein expressed in *P. pastoris*, which included 4-8 additional amino acids which ultimately modified its aggregation performance (Vaquero et al. 2015).

In 2004, Choi and colleagues discovered a novel esterase gene (*estI*). The amino acid sequence indicated that it may be classified as a novel member of the GHSMG family of lipolytic enzymes. *E. coli* BL21 (DE3)/pLysS containing the *estI* gene expressed a novel 67.5-kDa protein corresponding to EstI in an N-terminal fusion with the S-tag peptide. The optimum pH and temperature of the purified enzyme were 7.0 and 37°C, respectively. The most specific substrate was *p*-nitrophenyl-caprylate (C8) with K_m and k_{cat} values of approximately 14 μM and 1,245 s^{-1} , respectively (Choi et al. 2004).

Moukoulis et al. (2008) successfully cloned a Type C feruloyl esterase (FAE) in *Saccharomyces cerevisiae* under the transcriptional control of the alcohol oxidase (AOX1) promoter and integrated it into *Pichia pastoris* X-33 to validate FAE activity. A recombinant protein with a molecular weight of 62 kDa and a pI of 6.8 was produced (Moukoulis et al. 2008). When incubated together with the xylanase from *Trichoderma longibrachiatum* in de-starched wheat bran, ferulic acid (FA) was effectively liberated. The esterase displayed broad pH stability making it a significant applicant for alkaline applications such as pulp treatment in the paper industry (Moukoulis et al. 2008).

The *axe* gene which encodes an acetylxylan esterase from *Thermobifida fusca* NTU22, has been cloned, sequenced and expressed in *E. coli*. The optimal pH and temperature of the purified esterase was 7.5 and 60°C, respectively. A significant increase in xylooligosaccharide production was observed when oat-spelt xylan was treated with a combination of the recombinant xylanase and acetylxylan esterase compared to independent treatment with xylanase or acetylxylan esterase (Huang et al. 2010). A sterol esterase from *O. piceae* was expressed in two hosts; *S. cerevisiae* and *P. pastoris*. The highest activity of 42 U/ml was produced by *P. pastoris* at 28°C, however low activity was observed in *S.*

cerevisiae. The heterologous expression of a functional fungal esterase in yeast is quite an accomplishment and opens up an opportunity to develop more robust enzymes (Vaquero et al. 2015).

5. Applications

Enzymes are currently being applied in multiple industries. The flexibility of enzyme properties enables application in a number of degradation and synthesizing processes such as the detergent, food, animal feed, beverage, textile, pulp and paper, organic synthesis and leather industries (Table II). An international initiative towards industrial biotechnology has opened up a niche for the development of novel enzymes. Enzymes are designed based on the limiting parameters of processes, and their stability and effectiveness in catalyzing specific reactions.

Lipases in particular have enormous potential in the lipid bio-industry due to their availability and stability in organic media (Sharma and Kanwar 2014). The application of lipases in the pulp and paper making industry is vital in the control of pitch formation. Pitch is described as the agglomeration of lipophilic extractives from wood material which causes black spots in the pulp and gumming up of machinery. Esterases on the other hand are used to break down the polyvinyl acetate in glues present in recycled paper processing (Jegannathan and Nielsen 2013). The use of such enzymes in the pulp and paper industry reduces the consumption of energy and amount of chemicals used in processing, thus limiting their negative contribution to the environment (Patrick 2004; Skals et al. 2008). In the recycling of newspaper, the paper needs to be deinked prior to production of newsprint and white paper (Bajpai 2013). When the ink is composed of vegetable oil, lipases are often used to break down the lipophilic components of the ink (Hasan et al. 2006; Yang et al. 1994). Yang and his colleagues (1994)

patented a specific combination of parameters and ratio of cellulases, xylanases and lipases for the removal of ink from various paper samples whilst preserving the quality of the salvaged pulp.

Recent advances in metagenomics and proteomics has supported the discovery of novel enzymes and genetic engineering of microbes (Adrio and Demain 2014). Future applications of enzymes from microbial, plant and animal resources will ensure a more feasible approach to bioprocessing, as well as reducing the amount of waste generated and overall impact on the environment.

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Table I: Current classification of bacterial lipolytic enzymes. Description of bacterial lipolytic families I-VIII in the Arpigny and Jaeger classification, and new families and subfamily discovered by functional metagenomics (*) (adapted from Arpigny and Jaeger 1999; Fu et al. 2011; Kim et al. 2009; Lee et al. 2006).

Family	Description
I	Group of true lipases subdivided into six subfamilies: <i>Pseudomonas</i> lipases and relatives (subfamilies I.1, I.2 and I.3), <i>Bacillus</i> and <i>Staphylococcus</i> lipases and relatives (I.4 and I.5) and other lipases (I.6).
II	Modified pentapeptide motif around the active serine: Gly- Asp-Ser-(Leu) [GDS(L)]. Secreted and membrane-bound esterases
III	Extracellular lipases and esterases.

EstA*	Related to family III but different conserved motifs (pentapeptide GHSMG). Discovered from surface seawater.
IV	Many members of this family show sequence similarity to mammalian hormone-sensitive lipase (HSL). Typical motif HGG. Lipolytic enzymes from psychrophilic, mesophilic and thermophilic origins.
EstB*:	New subfamily in family IV with second active site glutamate (conserved sequence EXLLD) instead of the aspartate (DPLXD) of the representative members of family IV. It was discovered in surface sea water.
V	Conserved motif HGGG upstream of the pentapeptide motif GDSAG. Sequence similarity with non-lipolytic enzymes: epoxide hydrolases, dehalogenases and haloperoxidases. Esterases from psychrophilic, mesophilic and thermophilic origins.
EstF*	Related to family V but with a modified pentapeptide, GTSXG, and different flanking regions around the HG motif and their own unique conserved sequence motifs. Isolated from deep sea sediments.
VI	The smallest esterases known (23–26 kDa). Sequence similarity to eukaryotic lysophospholipases.
VII	Large bacterial esterases (55 kDa). Sequence homology with eukaryotic acetylcholine esterases and intestine\ liver carboxylesterases.
VIII	Similarity to several class C β -lactamases.
LipG*	Presence of an Arg-Gly sequence in oxyanion hole instead of His-Gly, a signature sequence distinctive of filamentous fungal lipases. Isolated from tidal flat sediments.
LipEH166*	Comprise newly discovered lipase LipEH166 of psychrophilic origin, and three putative open reading frames. Isolated from intertidal flat sediments.
EstY*	Derived from pathogenic bacteria. First possible lipolytic virulence factors that do not belong to the GDSL family. Isolated from surface river water.

Table II: Enzymes used in various industrial processes and their application (adapted from Kirk et al. 2002; Margesin et al. 2002; Chang et al. 2004; Cavicchioli and Siddiqui 2004; Ramteke et al. 2005; Vijayalakshmi et al. 2011; Sarrouh et al. 2012; Gurung et al. 2013, Imran et al. 2012; Nigam 2013)

Industry	Enzyme	Application
Detergent	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour clarification
	Mannanase	Reappearing stains
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyliglucosidase	Saccharification

	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction
	Protease	Protease (yeast nutrition – fuel)
	Lipase	Synthesis of lipase-catalyzed biodiesel
Food	Protease	Milk clotting, flavour
	Lipase	Improvement of food texture
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning
	Phospholipase	Dough stability and conditioning
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility
	Xylanase	Digestibility
	B-Glucanase	Digestibility
Beverage	Pectinase	Depectinization, mashing
	Amylase	Juice treatment, low calorie beer
	B-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavour (beer)
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	Desizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch coating, deinking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	Deinking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	Degumming, lysolecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	Depickling
Personal care	Amyloglucosidase	Antimicrobial

Environmental application	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial
	Lipase	Degradation of lipid wastes
		Removal of solid and water pollution by hydrocarbons, oils and lipids

Draft

Figure 1: Phylogenetic tree based on a novel amino acid sequence of EstCS2 and closely related proteins. Protein sequences for previously identified families of bacterial lipolytic enzymes retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). The units at the bottom of the tree indicate the number of substitution events (adapted from Kang *et al.*, 2011).

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