GEOBACILLUS AND OTHER BACTERIAL LIPASES: A REVIEW ON EXPRESSION, PURIFICATION, AND CHARACTERIZATION

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ABSTRACT: *Microorganisms have been known to produce highly thermostable enzymes which can be isolated for use in industrial processes. The lipase is a highly stable serine hydrolase in the organic solvent. The lipase is multi-faceted with strong applications in chemical synthesis and biomedical science, as well as in biotechnology. A review of the expression, purification, and characterization of Geobacillus bacteria and other bacteria lipases was conducted across 25 studies in order to provide a collective source of information in regard to lipases and biomedical, biochemical, and biotechnology applications. The expression, purification, and characterization of Geobacillus bacteria and other bacteria lipases using a diversity of strains is presented. The studies reflect the growth of research which enzymatic transformations in the organic solvent is broad, as the number of industrial and biomedical applications are discovered.*

Keywords: lipase, Geobacillus, bacteria, enzyme, purification, genome sequencing, thermophilic

INTRODUCTION

Microorganisms which produce highly thermostable enzymes are isolated for use in the industrial process [1]. Microbial enzymes are more convenient to be used for application purposes as compared to enzymes derived from plants or animals due to the diverse range of catalytic activities available, high yields obtained, low cost, abundant supply and the ease of genetic manipulation. Moreover, microbial enzymes have structures which enable them to be stable and production method is straight forward as compared to plant and animal enzymes.

The lipase triacylglycerol acyl hydrolase (EC 3.1.1.3) is an enzyme produced in the lipidic carbon, which acts on carboxylic ester bonds, and that may be used to catalyze *in vivo* a diversity of reactions in controlled environments [2, 3, 4, 5, 6, 7, 1, 8, 9, 10]. The lipase expresses esterase activity, to include cholesterol amidase, phospholipase, cutinase and lysophospholipase [7]. The expression, purification, and characterization of *Geobacillus* bacteria and other bacteria lipases using a diversity of strains is reviewed..

Bacteria genera which produce lipase include *Pseudomonas*, *Burkholderia*, Staphylococcus, and *Bacillus* [3, 5]. The most commonly used genera for biotechnical applications are the *Pseudomonas* and *Burkholderia* [3]. The bacterial lipases are intracellular, extracellular, or membrane-bound (integral or peripheral). The lipase is a part of the α/β hydrolases structural superfamily and is secreted by the bacteria through ++type I (T1SS) and type 2 (T2SS) secretory systems [11, 9]. Lipase research develops a novel, improved lipase through directed evolution and metagenomics approach [2]. The bacteria lipase may be isolated from ocean sediment and the soil [12]. The selectivity enables the manufacture of agrochemicals, biosensors, and novel pharmaceuticals [6, 11].

Lipase Bacterial Strains

Masomian, et. al. [8] and Golaski, *et. al.* [13] described the lipases as "the most significant biocatalysts used in biotechnological applications" (p.2). Sangeetha, et. al. [11] described lipases as hydrolytic enzymes which are valued due to functionality on micro-aqueous and non-aqueous environments. Moreover, the lipase does not require a cofactor in order to serve as a catalyst for hydrolytic reactions and to sustain activity in harsh organic solvents. Ekinci, et. al. [7] supported that the attractiveness of the bacterial lipase lies in the high catalytic efficiency, pH dependency, high specificity, temperature, and biodegradability. Gupta, *et. al*. [2] also supported that the lipase was able to outlast stable in an organic solvent.

Nagarajan [9] described the lipase as a"fat-splitting enzyme" which "facilitate a diversity of applications" (p. 1163). The show, *et. al*. [5] supported that lipase (EC 3.1.1.3) is a "carboxylesterase that catalyzes carboxyl ester bond hydrolytic cleavage in long-chain acylglycerols" (p. 19). Lan, et. al. [14] described lipases as multi-faceted with strong applications in chemical synthesis and biomedical science, as well as for biotechnology. Interfacial activation occurs when the lipases come in contact with an insoluble substrate that is above the critical micellar concentration and the active site lid mobilizes and exposes the hydrophilic pocket toward enzyme catalysis [9]. Interfacial activation is a unique catalytic capacity of the lipase, along with the capacity to produce fatty acids and valuable glycerides from oil, and an additive in detergent.

Applications of Lipases

Lipases are among the most "important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. The mass production of lipases has only been possible due to genetically bioengineered microorganisms, such as bacteria. These microorganisms have short generation times and the conditions required for optimum growth and hence, optimum productivity, are easily achieved. Microbial lipases "are considered to be the third-largest group based on total sales volume". By the turn of the millennium, the industrial production of lipases was worth billions. Lipases are very much suited for mass industrial production as they work under mild conditions, are highly stable in organic solvents, exhibit broad substr**ate** specificity and commonly show high region- and/ or stereoselectivity in catalysis [15].

The following table provides a brief overview of the arious applications of lipases on the industrial scale [15]**.**

Table 1: Thermophilic lipase producing bacteria which include the *Geobacillus* genus

Industry	*Effect*	*Application*
Detergent	Hydrolysis of fat	Removing oil stains from clothes
Dairy	Hydrolysis of milk fat, the ripening of cheese, conversion of butterfat	Developing flavoring agents
Bakery	Improvement of flavors	Increasing shelf-life and storage
Beverages	Improvement of aroma	Beverages
Food dressings	Improvement of quality	Mayonnaise, whippings
Health foods	Transesterification	Health foods
Meats	Development and intensification of flavors	Fat removal from meats
Fats and oils	Transesterification	Cocoa butter substitutes, margarine production, fatty acids, and glycerol production
Chemicals	Enantio-selection; synthesis	Chemicals
Pharmaceutical	Transesterification	Specialty lipids
Cosmetic	Synthesis	Moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with better quality
Cleaning	Hydrolysis	Removal of fats

Thermophiles are microorganisms capable of surviving in temperatures above 50^0C which is above the range of temperatures required by mesophiles [16]. In order to thrive in such extreme temperatures, the microorganisms have evolved to adopt several different survival strategies and mechanisms. These include oligomerization and a large hydrophobic center, which are essential for protein stability and folding, a high number of disulfide linkages, which are necessary for the stability of the tertiary structure of proteins. It also preserves the quaternary structure, an increased in salt-bridging, which increases the stability of the enzymes; and an increase in surface charges. Thermophiles are also known to be lipase producers and one such bacterial genus which falls under this category would be *Geobacillus*.

Sequencing of enzyme producers is often carried out with the *Geobacillus* species as aerobic organisms that can be isolated during the process of cultivation [11]. *Geobacillus,* or "rod-shaped soil-derived bacteria", exhibits optimum growth temperatures range between 55°C and 65°C [17]. The *Geobacillus* genus has been divided into bioremediation and biotechnology; and reclassified according to the 16S rRNA sequence, DNA-DNA, phenotypic characterizations, and as lipids or fatty acids [17, 18]. *Geobacillus* has been successfully isolated from a diversity of harsh industrial environments, to include extreme temperatures, aquatic environments, and in soil.

The bacterial lipase is primarily alkaline and produced by submerged and solid-state fermentation. The lipases enzyme hydrolyzes the triglyceride to free fatty acid and to glycerol [11]. *Geobacillus stearothermophillus* has been the focus of many studies in regard to the xylan-degrading enzyme. Surfactants increase the interface between the lipid and water; therefore, the lipolysis is enhanced. The *Geobacillus* is comprised of endospore-forming obligate thermophiles, and the *Geobacillus bacteria* is widespread due to the pores [12, 19]. Aerobic, rod-shaped, or anaerobic in appearance, the *Geobacillu*s spp is gram-positive, thermophilic, and may sustain growth in extreme temperatures between 35°C and 80°C, which attracts the biotechnologist as a source of the thermostable enzyme [19]. The catalytic performance of the bacterial lipases is improved by bioimprinting [11]. The genomes of 6 strains were isolated by the research group, which revealed enzymes characterized by carbohydrate degradation (C5-6 Technologies [18, 19]. The key enzymes were cloned from *Geobacillus* strains in *Escherichia coli* and purified using column chromatography.

Isolation of bacterial strains

Isolated bacteria is characterized by antibiotic resistance, Gram staining, metal resistance, or morphology methodologies [20]. Antibiotic resistance levels in *Enterococci* and *Escherichia coli* pose risks to the natural environment; and in many cases where the bacteria origin is unknown, the significance of the antimicrobial-resistant strains of *Escherichia coli* remains unquantified [21]. The morphological staining method consists of observations of *cocci*, *bacilli*, and *vibrio* using a Light Microscope. Lan et al., [14] aimed to extract lipase 10 from the *C. Albicans* in order to identify the biochemical properties. Among the non-universal serine codons (CTGs) that were transformed into universal TCT serine codons through overlapping extension Polymerase Chain Reactions. CaLIP10 was expressed in the *Pichia pastoris* bacteria and optimized using response surface method. The CaLIP10 experienced purification and characterization by ion, pH, temperature, and solvent effect observations of changes in lipase activity and coconut oil hydrolyze. Saeed et al., (2010) isolated *Escherichia coli* bacterial strain from desert soil, and proceeded with the purification approach of morphological and biochemical test with 165 rRNA *Geobacillus stearothermophillus*. The lipase was isolated with Polymerase Chain Reaction (PCR) using 5' CATATGATGAAAKGCTGYCGGGT-3' forward primer, and 5'- GGATCCTTAAGGCCGCAARCTCGCCA-3' reverse lipase gene primers.

Sharma, et. al. [4] isolated lipase production bacteria isolated from soil and lipase enzyme was purified with Sephadex G-100 gel column chromatography and ammonium sulfate

precipitation. In the purified state, the lipase activity became positively stimulated by the introduction of Triton X-100, SDS with Mg^{2+}, and Ca^{2+} and was able to outlast stable in an organic solvent. Narasimhulu, et. al. [20] found that the strain selection affects the resistance of the isolated strain, which is commonly used in wastewater treatment applications.

Fotouh, et. al. [6] isolated more than 23 bacterial strains of Egypt *Geobacillus thermoleovorans* DA2 with 16S rRNA identification. The optimal environment for the production was recorded as 60°C, pH 10 for 48 hours in incubation. The researchers reported that the TA cloning lipase may substitute for many chemical leather processes to minimize the resulting environmental footprints and ultimately increase the quality of the materials. Sakaff, et. al. [19] highlighted the genome *Geobacillus thermoleovorans* CCB_US3_UF5 similarities to the *Geobacillus kaustophilus* HTA 426 for applications using orthologous or synteny genes.

Bacterial lipase expression

The lipase may exhibit regioselectivity, chemoselectivity, or enantioselectivity [2]. Krzesiak, et. al. [23] investigated the proposition that the *Pseudomonas alcaligenes* lipase expression could be activated with the addition of soybean oil, or other medium components.The *lipR* and *lipQ* genes were identified. The lipase promoter fragment was retarded by lLpR-overexpressing strain cell extracts in band shift assay. Krzesiak, et. al. [23] concluded that the LipQR 2 component system controlled the *Pseudomonas alcaligenes*.

Liu, et. al. [3] investigated a heterologous expression and characterization of *Pseudomonas fluorescens* Pf0-1 lipase. After 10 cycles in a non-solvent, the immobilized lipases remained at 70% and 82%. Optimum temperature was reported as 70°C and the optimal pH was 8.0 after 12 hours of incubation. Golaki, et. al. [13] investigated the *Cohnella* sp. A01 lipase in the context of the flexibility of use in industrial application. Lipase 3646 from *Cohnella* sp. A01 was expressed in the *Escherichia coli,* and purified using 2-step anion exchange chromatography. The researchers reported the molecular weight of the purified lipase to be about 29.5 kDA on SDS-PAGE, with Michaelis-Menten values of 61.94 U/mg V_{max} and 1077 μM K_m. Moreover, the sample exhibited high stability in the alkaline pH. The researchers concluded that lipase 3646 could be useful in detergents and organic solvents.

Bacterial lipase purification

Lipase purification is commonly achieved by chromatographic methods, organic solvent liquid-liquid extractions, and separations of the membrane [24]. Show, et. al. [5] argued that microbial lipases require "purification methods which are effort-intensive due to culture broth complexity and the requirements of bioactive state retainment" (p. 19). Therefore, the chromatographic separation and precipitation methods are common purification strategies.

Ekinci, et. al. [7] partially purified a lipase through ion exchange and gel filtration chromatography from *Geobacillus stearothermophilus* AH22 and characterized in a diversity of surfactants, cations, substrates, and inhibitors. Initial rates with partially purified lipase measured 30 μg/mL, constant temperature 25°C, pH 8.0, and increasing substrate concentration *p*-NP esters between 0.01 and 1.0 mM. The kinetic parameters determined consisted of the K_m Michaelis-Menten constant and V_{max}. maximum reaction rate. The purification produced a 2.1-fold lipase purification with 20.8% final yield with maximum hydrolytic activity at 50°C and pH 7.0. Javed, et. al. [25] published a similar review of processes and results from bacterial lipase purification and characterizations and described several improved properties of bacterial lipases in surface hydrophobicity, enzyme activity, substrate tolerance, proteolytic resistance, and stability.

Bacterial lipase enzyme characterization

Gram stains characterize the bacteria as either Gram-negative or Gram-positive by the subjection of the bacterial smear to an iodine solution, crystal violet, decolorizing alcohol, and Safranin [20]. Gram-positive typically exhibit a deep purple or violet color in the laboratory condition, and the Gram-negative bacteria become counterstained by Safranin and exhibit a red color. Interfacial activation is a characteristic of the lipase; therefore, the lipase reaction typically is incompatible to the Michaelis-Menten model. Lipases which are closely characterized to HZ lipase gene include *Bacillus* and *Geobacillus* from the subfamily 1.5, identity < 57%.

Masomian, et. al. [8] isolated an organic-solvent tolerant, thermostable lipase from *Aneurinibacillus thermoaerophilus* strain HZ. The lipase was purified and characterized using anion exchange chromatography and gel filtration on Q Sepharose and Sephadex-G75, respectively. The researchers reported a purification fold of 15.6 and pH of 7.0. The activity of 43.5 U/mg with 19,7% recovery was obtained with an optimum temperature of 65°C. In a subsequent study, Masomian, et. al. [8] isolated a novel gene with thermophilic *Aneurinibacillus thermoaerophilus* strain HZ in an Escherichia coli vector. The optimal activity was recorded at 65°C, retained activity $\geq$ 97% following a 1-hour incubation.

Pandey, et. al. [26] isolated *Geobacillus stearothermophilus* bacteria from sediment samples collected from an Indian Himalayan hot spring. The researchers reported growth between the temperatures of 55°C and 95°C in the laboratory environment. The cell biomass production occurred most fluently at the high temperatures. After the subcultures, coilage from Gram-negative cells were observed in clusters and chains with swollen sporangia endospore at the terminals. The maximum activity was recorded 65°C with 25.8 U/L after a total of 14 hours in incubation. Brumm, et. al. [27] isolated *Geobacillus* strains and reported 6 sequences of *Geobacillus xylanolytic* and the performance in the degradation of carbohydrates. The range for the *Geobacillus* species was between 43.9% and 52.5%. The capacity of the bacterial strains to use α-1, sucrose, inositol, 4-glucosides, arabinan, and lactose exhibited significant differences in the sequencing.

Summary of Strategies

The strategies used for the characterizing or purifying of bacteria lipases are summarized according to the studies selected for this review. Table 2 illustrates the strain investigated in the studies, the method used to investigate the strains, and the authors:

Table 2: Strategies to characterize or purify lipases

Strategies for Bacteria Lipase		
Strain	**Method to characterize and/or purify**	**Study**
Burkholderia cepacia ST8	Submerged fermentation	Lee et al., 2017
Geobacillus sp.	165 rRNA sequence; bioimprinting	Sangeetha et al., 2011; C5-6 Technologies, 2018; Zeigler, 2014; Sakaff et al., 2012
Geobacillus stearothermophilus	Morphology; bilochemical test; 16S rRNA; autoclave	Sifour et al 2010; Ekinci et al., 2017; Pandey et al., 2014; Burgess et al., 2017
Cohnella sp.	2-step anion exchange chromatography	Golaki et al 2015
Candida Antartica; Escherichia coli BL21	Surface methodology	Badillo-Zeferino et al., 2017
C. albicans	Light Microscope	Lan et al., 2011
Bacillus methylotrophicus PS	Sephadex G-100 gel column chromatography; ammonium sulfate precipitation; submerged fermentation	Sharma et al ., 2017
Geobacillus thermoleovorans; *Geobacillus thermoleovorans* CCR11	165 rRNA; surface methodology	Fotouh et al., 2016; Sakaff et al., 2012; Badillo-Zeferino et al., 2017
Geobacillus kaustophilus	Modified phenochloroform; Roche 454; Solexa paired-end sequence	Sakaff et al., 2012
Micobial Lipase; Rhodotorula glutinis PTCC 5256; Aspergillus niger	Aqueous 2-phase system; Aqueous micellar 2-phase system	Show et al .,2015
Pseudomonas alcaligenes	lLpR-overexpressing strain cell extracts	
Pseudomonas fluorescens		Liu et al., 2017
Aneurinibacillus thermoaerophilus	anion exchange chromatography; gel filtration	Masomian et al .,2016; Masomian et al .,2013
Serratia marcesscens	ultra-filtration; carboxymethyl-cellulose chromatography; Fourier transformation-infrared spectroscopy	Mohanasrinivasan et al., 2016

CONCLUSIONS

This study provides a review of 27 studies related to lipase and bacteria. The main focus was on Geobacillus in the context of biotechnology. However, the scope of Geobacillus is vast therefore the taxonomy has been ambiguous and DNA hybrid studies have a remarkable value among the researchers. This study concludes that for the purification of lipase, the classical strategies have been criticized and considered to be non-specific, complex, with the poor quality and laborious. Moreover, this study reveals that the polyphasic methodology for explaining bacteria is not possible for the Geobacillus stearothermophilus when it comes with the variation in the characteristics of phenotypical.

In addition to that this review concludes that the enzymatic transformations of the organic solvent is vast in literature since its industrial and biomedical applications have been found. The value of lipases in industrial use has been increased by the tolerance of proteases and organic solvents. Whereas the detailed understanding of the natural environment and bacterial pathogens which specifically transfer the ecological systems are required for the ecological resistance enhancement. Likewise, there is a need for the evidence-based researches for the contextual application, catalytic characterization and purification procedures for bacterial lipases.

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