T.C.

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MASTER'S PROGRAM IN BIOLOGY

2022-YL-064

THE EFFECT OF CULTURE CONDITIONS ON LIPASE PRODUCTION BY *Geobacillus thermodenitrificans* HBB268

PRINCESS SINDISWA SOKHULU

MASTER'S THESIS

SUPERVISOR

Prof. Dr. Kubilay METIN

AYDIN–2022

ACKNOWLEDGEMENTS

I would like to extend a word of gratitude to my advisor, Prof. Dr. Kubilay METİN, for the continuous support during my Master's program and overall research, I appreciate your patience, motivation and the vast scientic knowledge you have provided. For providing guidance, support and recommendations on this thesis, I would also like to express my respect and endless thanks to the Head of Biology Department Prof. Dr. H. Halil BIYIK for his contributions and valuable information towards my study.

My sincere thanks to PhD student Sezgin KARAMAN and Research Assintant Zehra Burcu BAKIR for sharing thier knowledge and support at every stage of my study particularly thier assistance with statistical evaluations, insightful comments and recommendations throughout this thesis program.

I thank my lab mates and friends, Research Assistant Gizem ANTIKA, Aslı ÇANAKÇI, Goncagül ÖZGER, PhD studunt Mustafa TOURAY, and Ruth Maseko PHIRI for thier moral support. I cannot forget to thank Şebnem KARANFIL and family for thier love and endless support, Nalan Çakır and Ahmet Asal thank you for making life easy during the period of my study.

I would like to express my gratitude to the thesis committee and jury members for their valuable time and consideration.

Last but not least I would like to thank My mother Mano Henriatta SOKHULU together with my sisters Balungile and Khwezi SOKHULU for thier support and prayers. Though we were separated by two continents during my study, I still felt your love.

Princess Sindiswa SOKHULU

TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

A	: Absorbance
ANOVA	: Analysis of Variance
°C	: Centigrade degree
EDTA	: Ethylenediaminetetraacetic Acid
g	: Gram
g	: Gravity
HBB	: Halil Bıyık Bacteria
IUB-MB-EC	: International Union of Biochemistry and Molecular Biology
kDa	: kilodalton
Km	: Michaelis-Menten Constant
LB	: Luria-Bertani
L	: Litre
mL	: Millilitre
Mm	: Millimolar
M	: Molar
NCBI	: National Center for Biotechnology Information
µL	: Microlitre
OD	: Optical Density
pNPL	: Para-Nitrophenol Luarate
U	: Enzyme Unit
V	: Volume
Vmax	: Maximum Enzyme Activity
%	: Percentage

LIST OF FIGURES

LIST OF TABLES

ÖZET

Geobacıllus thermodenıtrıficans HBB268'İN LİPAZ ÜRETİMİ ÜZERİNE KÜLTÜR KOŞULLARININ ETKİSİ

Sokhulu P. S. Aydın Adnan Menderes Üniversitesi, Fen Bilimleri Enstitüsü, Biyoloji Programı, Yüksek Lisans Tezi, Aydın, 2022.

Amaç: *Geobacillus thermodenitrificans* HBB268 tarafından üretilen lipazın, üretim koşullarının optimizasyonu yapılarak en yüksek verimde enzim üretiminin sağlanması amaçlanmıştır.

Materyal ve Yöntem: Substrat olarak p-NPL (p-nitrofenil laurat) kullanılarak spektrofotometrik olarak lipaz aktivite tayini yapılmıştır.

Bulgular: Bu çalışmada Adnan Menderes Üniversitesi Biyoloji Bölümü kültür stoklarında kayıtlı HBB268 izolatı Aydın ili sıcak su kaynaklarından izole edilmiştir. Önceden lipolitik aktiviteye sahip olduğu bilinen *Geobacillus thermodenitrificans* HBB268 izolatı; termofilik, fakültatif, gram-pozitif, çubuk şeklinde bir bakteridir. HBB268 suşunun kültür koşulları optimize edildiğinde en iyi enzim üretimi; karbon kaynağı olarak %0,5 maltoz, nitrojen kaynağı olarak %0,5 meat extract içeren besi ortamında pH 7,00 ve 55 °C koşulları olarak belirlenmiştir. HBB268 izolatı optimum koşullarda büyütüldüğünde, logaritmik fazın başlangıcında enzim üretimi başlamış ve durgunluk fazında (18-24 saat) maksimuma ulaşmıştır. *Geobacillus thermodenitrificans* HBB268'in lipaz enzimi pH 8,0 ve 55 °C'de optimum aktivite gösterdiği belirlenmiştir.

Sonuç: Bu tezden elde edilen sonuçlar *Geobacillus thermodenitrificans* HBB268 tarafından büyük ölçekli ve uygun fiyatlı lipaz üretimini içeren daha ileri bir araştırma için temel teşkil edecektir.

Anahtar Kelimeler: Enzim Karakterizasyonu, *Geobacillus*, Lipaz, Termofilik Enzim, Volüm Aktivite.

ABSTRACT

THE EFFECT OF CULTURE CONDITIONS ON LIPASE PRODUCTION BY *Geobacillus thermodenitrificans* HBB268

Sokhulu P. S. Aydın Adnan Menderes University, Institution of Natural and Applied Sciences, Biology Program, Master's Thesis, Aydın, 2022.

Objective: The aim of the study is to optimize culture conditions for enhanced maximum lipase production by *Geobacillus thermodenitrificans HBB268*.

Material and Methods: Lipase activity was assayed spectrophotometrically assay using pNPL (p-nitrophenyl laurate) as substrate.

Results: In this study, HBB268 isolate, registered in Adnan Menderes University Biology Department Culture Stocks, was isolated from the hot water sources of Aydın province. HBB268 isolate, previously known to have lipolytic activity; is a thermophilic, gram-positive, rod-shaped bacterium. When the culture conditions of the HBB268 strain are optimized, the best lipase enzyme production is obtained from; 0,5% maltose as carbon source and 0,5% meat extract as nitrogen source. When HBB268 isolate was grown under optimum conditions, enzyme production started at the beginning of the logarithmic phase and reached its maximum in the stagnation phase (18-24th hour). It was determined that the lipase of *Geobacillus thermodenitrificans* HBB268 showed optimum activity at pH 8,00 and 55 °C.

Conclusion: The obtained results of this study, will serve as a baseline for a further inquiry that includes the large-scale and affordable production of lipase by *Geobacillus thermodenitrificans* HBB268.

Key Words: Enzyme Characterization, *Geobacillus*, Lipase, Thermophilic Enzyme, Volume Activity.

1. INTRODUCTION

Life is maintained by biochemical reactions taking place within an organism. Enzymes are vital biological catalysts produced by living cells to regulate reactions and pathways of cellular metabolism (Gurung et al., 2013). Under biologically relevant conditions, uncatalyzed reactions like metabolism, catabolic processes, decomposition of compounds, protein synthesis, cell renewing, growth, etc., may occur at a slower rate, inaccurately, or even form undesired by-products. Enzymes circumvent such problems by providing a specific environment in which a given reaction can occur accurately in fractions of seconds provided the catalysis is carried by an appropriate enzyme. (Nelson and Cox, 2017).

Preservation of food, cheese making, and fermentation of beverages were among the early enzymatic processes carried out by man long before the mechanism of enzymes was understood (Sutay Kocabas and Grumet, 2019). Today, enzymes are employed in a large number of industrial processes greatly replacing traditional chemical catalysts. Most industrial enzymes are from microbes, possessing multifaceted catalytic activities i.e., substrate selectivity, stability, suitability for genetic modifications, and most importantly their use require less energy consumption due to their simplified production routes and they prove to reduce environmental toxicity (Chapman et al., 2018). Fıne and bulk chemical industries, pharmaceutical, food, detergent, medicine, textiles, and other industries have benefited from these advantages for years while natural gas conversion and biofuel industries have only recently broadened enzyme exploitation (Choi et al., 2015).

The majority of industrial enzymes are hydrolyses, as they are used to break down proteins, lipids, and carbohydrates, however, enzymes belonging to other classes are also used. Two major enzymes of hydrolases, lipases (EC 3.1.1.3, triacylglycerol hydrolases) and esterases (EC 3.1.1.1, carboxyl ester hydrolases) are important biocatalysts with high industrial potential as they hydrolyze the carboxyl ester bonds in acyl-glycerides (Castilla et al., 2017). Lipases especially can catalyze the hydrolysis and the reverse reaction of synthesis reactions and have a wider range of applications for use in many processes (Gandhi, 1997).

Proteases account for the largest share of the global industrial enzyme market (Sarrouh, 2012). Carbohydrases and proteases account for 70% of revenue generated by industrial enzymes, whereas lipases and phytases account for 8% and 7%, respectively (Arbige et al., 2019). The global enzyme market is currently estimated at $5.5 billion, and it is expected to reach $7.0 billion by 2023 (Kocabas and Grumet, 2019). The enzyme market is dominated by top producers like Novozymes, DSM, BASF, and DuPont, with a 75% contribution of all industrial enzymes (Arbige et al., 2019).

Thermostable enzymes are of industrial and biotechnological interest, better suited for harsh industrial processes (Leuschner and Antranikan, 1995). Biotechnological processes require elevated temperatures to reduce contamination, easy solubility of organic compounds and accelerated reaction rates (Becker, 1997; Krahe et al., 1996). Lipases are great candidates for the vast majority of these harsh industrial processes especially lipases produced by thermophilic bacteria as their structure is extremely stable at high temperatures in organic solvents, and they have a high level of activity. Additionally, these lipases exhibit strong resistance to chemical denaturation and may be highly active in alkaline pHs, for these reasons lipases have found immense use, especially in the detergent industry (Bakir and Metin, 2017; Uğraş, 2017; Van Den Burg, 2003).

Thermomonas hydrothermalis, Geobacillus thermoleovorans, and *Bacillus licheniformis* have all been shown to be promising sources of thermophilic lipases up to this point (Lee, et al., 1999; Kambourova et al., 2003; Mohammad et al., 2017). Studies on the discovery of new microorganisms that may produce these enzymes, however, are continually expanding due to the relevance of thermophilic enzymes in both biotechnology and different industries. In order to keep up with rising demands, enzymes must be produced in large quantities. Though there are significant underlying middle-cost issues. Agricultural wastes or by-products are employed as inexpensive enzyme synthesis substrates for microorganisms to circumvent such issues. Waste materials including lemon peel, coconut cake, soya residues, coffee husk, and melon waste, have been used as affordable substrates for the synthesis of microbial lipases by studies such as (Alkan et al., 2007; Parihar, 2012). There also have been reports that waste-frying oil can be also utilized as cheap substrates in the production of microbial lipases (Ferreria, et al., 2017). Also, nutritional factors (carbon and nitrogen sources, minerals) and physical factors (pH, temperature, surfactants, inoculum concentration, and incubation time) can influence lipase production (Hasan-Beikdashti, et al., 2012; Nunes, et al., 2014). It can be concluded that

choosing an inexpensive substrate and optimizing the culture conditions of production mediums is the primary element to take into account when producing lipase.

1.1. History of Enzymes

Though the role of microbes was understood when brewing alcohol, less could be explained as to what was happening at a molecular level. In the late 1700s, biological catalysis was brought into spectrum by studies such as the digestion of meat by gastric juices, an experiment carried out by L. Spallanzan (Baron, 1979). Extensive studies continued in the 1800s, chemist Anselme Payen discovered the first enzyme, diastase (Payen and Persoz, 1833). In 1862 Louis Pasteur, Ferdinand Cohn and Robert Koch reported that the fermentation of sugar to alcohol was catalyzed by ferments and that these ferments were inextricably linked to the structure of live yeast cells (Drews, 2000). In contrast to Pasteur's conclusion, Eduard Buchner proved that fermentation was promoted by molecules that continued to function independently when he fermented glucose to produce ethanol from cell-free yeast extracts in the mid-1850s. The molecules discovered by Buchner were eventually given the term enzymes (from the Greek enzymes, "leavened") by Frederick W. Kühne (Aehle, 2007). James Sumner crystallized and isolated urease from jack bean and found that urease crystal consisted entirely of protein, and he postulated that all enzymes were proteins in 1926. The idea remained controversial until J. Northrop and M. Kunitz Isolated and crystallized digestive enzymes and found them also to be proteins (Nelson and Cox, 2017). As a result of these initial studies, it is now known that all enzymes are proteins in nature with just a few classes of ribosomal RNA molecules as exceptions that were discovered to also possess the ability to catalyze chemical reactions (Blanco, 2017). Enzymes have been purified, immobilized, produced in large quantities, and practically applied industrially (Duza and Mastan, 2013).

1.1.1. Nomenclature and Classification of Enzymes

Enzymes are often named by adding the suffix-ase to the name of the substrate that they modify. For example, lipase, esterase, and protease are enzymes that catalyze reactions involving lipids, esters, and proteins (Nelson and Cox, 2017). Gastric pepsin, saliva ptyalin, pancreatic trypsin, and chymotrypsin are enzymes that have arbitrary names to avoid confusion created by the use of names according to various criteria, the International Union of Biochemistry and Molecular Biology (IUBMB) proposed a classification system, to assign each enzyme a descriptive name and a number that allows its unequivocal identification (Schomburg, 2002). The Enzyme Commission number (EC number) classifies enzymes numerically based on the chemical reaction they catalyze (McDonald et al., 2015). In 1961, the (EC) number system classified enzymes into six categories: oxidoreductases (EC1), transferases (EC2), hydrolases (EC3), lyases (EC4), isomerases (EC5), and ligases (EC6). For many years, these six groups remained unaltered until August 2018, when a new class, translocases (EC7), was added (Tao et al., 2020).

1.1.2. Enzyme Structure and Mechanism

Some enzymes are simple proteins composed only of amino acids. Others however form associations with various nonprotein molecules for significant catalytic functions. An apoenzyme is an inactive enzyme, its activation occurs upon binding to a cofactor. Common cofactors may be either inorganic metal ions (Zn, Fe) or coenzymes which are organic compounds such as vitamins that bond, either loosely/noncovalently to the enzyme or covalently, forming complexes that are hard to separate. A coenzyme bound this way is called a prosthetic group. The cofactor and apoenzyme complex is called a holoenzyme.

1.1.3. Active Site

The basic mechanism of an enzyme action depends on its ability to bind the substrate at the domain of the enzyme molecule known as an active site where catalysis takes place. The enzyme active sites are a three-dimensional chiral groove or crevice buried within the

tertiary structure of the protein. This feature protects the bound substrate from solvent molecules that could interfere with catalysis. The active site's location varies according to the protein family. On the surface and within the active site are different amino acid residues. Enzymes specificity is due to the identity and spacial arrangement of these residues which dictate substrate recognition, binding, and stability, other residues are primarily involved in the formation and cleavage of chemical bonds to substrates and products (Blanco, 2017; Ghanem and Raushel, 2012).

1.2. Enzyme Activity

The amount of substrate converted by the enzyme in moles per unit of time is referred to as enzyme activity. It quantifies the amount of active enzyme in a mixture at a given time. The conditions used to evaluate enzyme activity in vitro frequently differ from those found in vivo. The goal of measuring enzyme activity is typically to determine the amount of enzyme present under defined conditions so that activity can be compared between samples and laboratories. The conditions chosen are usually those with the best pH, substrate concentrations, and temperature control. Nonetheless, with a thorough examination of the factors influencing enzyme activity, it should be feasible to extrapolate to the activity expected to occur in vivo. Substrate concentrations (s), pH, ionic strength and nature of salts present, and temperature are all factors that influence enzyme activity (Scopes, 2002).

1.2.1. Temperature

The rate of reaction is directly affected by temperature such that an increase in temperature results in an increase in the rate of the reaction. This is due to the increase in the kinetic energies of the molecules. The increase in this kinetic energy exceeds the energy that causes the weak hydrogen and hydrophobic bonds to break in the enzyme's structure. At high temperatures, the structure of proteins deteriorates and their efficient catalytic activities are destroyed. Hence, when performing an enzyme assay, it is critical to ensure that the temperature remains constant and that you know exactly what to compare with other results that may have been reported at different temperatures. (Ghanem and Raushel, 2012).

1.2.2. pH Effect on Enzymes

For most enzymes, optimum activity is usually recorded between pH 6.00 and 8.00. Below or above these ranges, the reaction rate decreases more or less rapidly. However, there are exceptions such as gastric pepsin which exhibits maximal activity at acidic pH 1.50 roughly, acid phosphatase in male prostate, presenting optimum activity at pH 5.00 while alkaline phosphatase from other organs exhibits optimal activity at a pH of 9.50. The state of ionization of functional groups on the enzyme molecule and the substrate is affected by changes in the pH of the medium. The proper distribution of electrical charges in both molecules is required for the formation of the enzyme-substrate complex. The optimum pH is that at which the essential group dissociation is most conducive to the interaction of the enzyme with the substrate to form the enzyme-substrate complex. Extreme pH changes, either above or below the ideal range, may result in the denaturation and consequent deactivation of the enzyme. (Joshi, et al., 2000; Talley and Alexov, 2010).

1.2.3. Substrate Concentration

Most enzyme molecules are free at very low substrate concentrations. As the substrate concentration rises, more enzyme molecules collide, resulting in the formation of the ES complex. If the substrate concentration continues to increase, a point is reached in which virtually all the enzyme molecules are occupied by the substrate that's if the concentration of enzyme remains constant. The enzyme has become saturated with the substrate at this point. If the substrate concentration continues to rise and far exceeds the amount of enzyme, a constant state is reached in which the rate of reaction does not show any further increase. Any additional increase in substrate no longer results in increased enzyme activity, and the reaction behaves in zero order.. (Akutsu et al., 2009).

1.2.4. Enzyme Concentration

As the concentration of enzymes rises, more enzymes will be interacting with substrate molecules, speeding up the reaction. However, this will also only work up to a

certain concentration, at which point the enzyme concentration ceases to be the limiting factor.

1.2.5. Enzyme Inhibitors

Additionally, some chemical agents can inhibit enzyme activity in various ways. Some of them work by binding to critical sites or functional groups in the enzyme. Inhibition can be reversible whereby an inhibitor inactivates an enzyme by bonding covalently to a particular group at the active site or reversible, through noncovalent, reversible interactions, an inhibitor inactivates an enzyme. (Blat, 2010; Srinivasan, 2022).

1.3. Lipase

Claude Bernard pioneered the discovery of lipase in 1846 while studying human pancreatic cells. Lipase was observed in pancreatic secretion to emulsify or hydrolyze insoluble fatty substances into more soluble products. The animal pancreas used to be the main source of lipase and it was widely used as a digestive supplement, either as a crude mixture with other hydrolase enzymes or in purified grade. Unfortunately, the pancreas became insufficient for the demand and there were hardships encountered in collecting available materials (Hasan et al., 2006). When microbial sources for lipase acquisition were discovered, the industrial potential of lipases expanded, which increased demand from suppliers. The early bacterial production of lipase was observed in *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens* in 1901 (Jaeger and Reetz, 1998). *Serratia marcescens, Pseudomonas aeruginosa,* and *P. fluorescens* bacteria have been identified for large-scale lipase production (Homaei et al., 2013). Over the years, genes responsible for lipase synthesis have been genetically modified through recombinant DNA and lipase makes the first commercial recombinant lipase industrialized from the fungus *Thermomycesl anugiwnosus* and expressed in *Aspergillus oryzae* in 1994 (Blamey et al., 2017).

1.3.1. Structure and Mechanism of Lipases

Lipases belong to an α/β-hydrolase fold family first defined by (Ollis et al., 1992). A superfamily with a wide range of enzymes whose activities rely mainly on a catalytic triad usually made of Ser, His, and Asp amino acid residues (Arpigny and Jaeger, 1999).

1.3.2. Mechanism of Lipase Action

In water, lipases have only a marginal but measurable activity towards dissolved substrates but as soon as an aggregated substrate starts to form an emulsion, a dramatic increase in lipase activity is observed which is called interfacial activation. This Phenomenon distinguishes lipases from esterases which display normal Michaelis-Menten activity (Anthonsen et al., 1995). The 1990 X-ray crystallographic three-dimensional structures of several lipases, including *Rhizomucor miehei* lipase and human pancreatic lipase, revealed the presence of an α helical fragment (lid) that shields the active center of lipase (Figure 1.1) (Schmid and Verger, 1998). From these 3-D strucrtures it was deduced that in the presence of a hydrophobic surface, the flap/lid of the lipase enzyme is displaced, converting it from its closed state to an open state that facilitates interaction between its hydrophobic internal face. The hydrophobic residues that usually surround the lipase active center with the substrate are lipases that have a lid but do not exhibit interfacial activation (Lorente et al., 2020). lipase from *Pseudomonas glumae* (Kim et al., 1997), lipase from *Pseudomonas aeruginosa (*Jaeger et al., 1993), and lipase from *Staphylococcus hyicus* showed interfacial activation with only some substrates (López-Fernández, 2020; Van Oort et al., 1989).

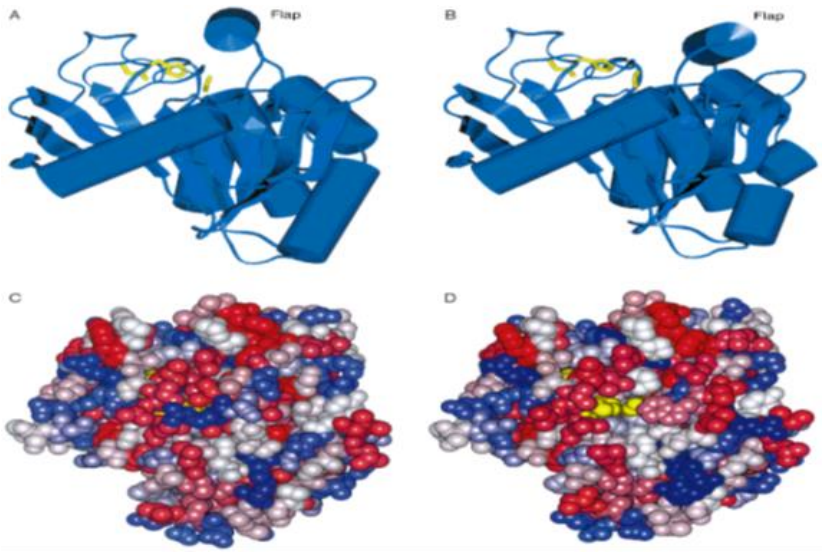

Figure 1.1. *Mucor miehei* lipase structure in closed (A, C) and open forms (B, D). Adapted from Schmid and Verger, (1998).

1.3.3. Specificity and Classification of Lipase

For industrial applications, the specificity of lipase is of signicant importance. Lipases are the third largest group of enzymes after proteases and amylases source. Lipases catalyze the hydrolysis of fats at the water-lipid interface in aqueous media by cleaving ester bonds while consuming water molecules and producing the corresponding fatty acids and glycerol or alcohol. Furthermore, due to the principle of microreversibility, they perform reverse reaction in non-aqueous media called synthesis reactions. These reactions are further classified as esterification, which occurs when an alcohol and a carboxylic acid undergo a double displacement reaction, resulting in esters and water; and transesterification, which occurs when an ester's acyl group switches with an alkyl group of an alcohol, acid, or another ester, resulting in alcoholysis, acidolysis, and interesterification reactions, respectively.

Lipases are classified based on sources they obtained from and based on specificity. Specificity of lipase is further subdevided into three subclasses, substrate specific lipase, wh selectively acting on a specific substrate in a mixture of crude raw material, enantioselective lipases which preferentially hydrolyse one of the isomers of a racemate over the other and finally regioselective (positional) lipases which favour their activity depending on positional

specificity and can be divided, in three classes 1-3 specific, non specific lipases and , fatty acid specific. It has been observed that most lipases belong to sn-1,3 specific lipase (Ribeiro et al., 2011), these hydrolyse or esterify fatty acid explicitly at the either/both sn-1 and sn-3 position. The steric hindrance effect prevents sn-2 fatty acids from binding to the active site of lipase (Anthonsenl et al., 1995; Fernández et al., 2020).

1.3.4. Sources of Lipase

Lipases are abundant enzymes naturally produced by animals, plants as well as several microorganisms. In animals, lipases are obtained from stomach and pancreatic juices. While animals use lipase for lipid metabolism including fat digestion, adsorption, reconstitution, etc., industrially it has versatile uses including the production of lysolecithin by phospholipase, a natural emulsifier largely used in food, cosmetic, and pharmaceutical sectors. However, animals are no long the main source for commercial lipases due to the difficulties encountered in extracting lipases (Hasan et al., 2006; Sarmah et al., 2018). In plants, lipases can be found in grains, fruits, leaves, and energy reserve tissues in oilseeds such as canola, and castor beans. These are active during germination and they catalyze the hydrolysis of stored triacylglycerols into fatty acids which are then converted to sugars to aid the development of plant embryos. Plant lipase production is low in costs and presents good stability in interesterification reactions and is wildly used in agriculture (Meshramet al., 2019; Sarmah et al., 2018). Lastly, lipases of microbial origin are obtained from filamentous fungi, yeast, and bacteria (Filho, 2019). Microorganisms are known to produce a variety of lipases, with distinct substrate specificity, optimum pH, and temperature range (Palekar et al., 2000). In comparison to their plant and animal derivatives, they can be exploited for their simplified production routes and fast growth which saves time plus energy, easy genetic manipulation through recombinant DNA technology for desired catalytic qualities, and most importantly they are environmentally benign (Dey et al., 2014, Hasan et al., 2006, Lee et al., 2015; Priji et al., 2015; Ullah et al., 2015). Nutritionally these microorganisms utilize cheap lipids either from animals or vegetables as carbon and energy sources for their growth (Hasan et al., 2006). Their diverse enzymatic properties make them prominent sources for industrial applications (Gurung et al., 2013). Though many lipase-producing microorganisms have been reported in the scattered literature, the genera

Candida, Rhizopus, and Pseudomonas are considered the authentic industrial sources of lipases (Table 1.1) (Larios et al., 2004; Singh and Mukhopadhyay, 2012; White and White, 1997). The yeast Candida rugosa is the most employed microorganism for lipase production (Larios et al., 2004; Singh and Mukhopadhyay, 2012).

Table 1.1. Some microbial sources of lipase and their industrial applications adapted from Chandra et al., (2020).

Microbial Sources	Applications	References
Fusarium solani NFCCL 4084	Halophilic lipase for biodiesel production	Fickers et al. (2005)
Yarrowia lipolytica	Degrades very efficiently hydrophobic and unusual substrates	Sarmah et al. (2018)
Aspergillus oryzae	Saturated fatty acids synthesized, faster cheese ripening, flavour customized cheese	Celligoi et al. (2017)
Rhizomucor miehei	Cocoa-butter equivalents	Houde et al. (2004)
Candida antarctica	Oils and fats enriched, removal of size lubricants, denim finishing	Yamaguchi et al. (1991)
Candida rugosa	Human Milk fat substitute	Glogauer et al. (2011)
Candida lipolytica	Cheese ripening, Fatty acid production	Sarmah et al (2018)
Trichoderma lanuginosus	Produceda lipase containing detergent 'LipoPrime	Adrio and Demain, (2005)
Rhizomucor meihei	As a biocatalyst in personal care products such as skin and sun-tan creams, bath oils etc	Undurraga et al. (2001)
Pseudomonas mendocina	Dishwashing/laundry Removal of fat strain	Hasan et al. (2007)
Acinetobacter radioresistens; Bacillus sp. FH5	Used in detergent industry	Kiamarsi et al. (2019)
Staphylococcus pasteuri	Ysing in oil degradation	Aleub et al, (2006)

1.3.5. Lipase Catalyzed Reactions

Primarily lipases are responsible for the hydrolysis of triacylglycerides; however, this unique group of enzymes accepts a variety of other low and high-molecular-weight esters, thiol esters, amides, polyol esters, and so on as substrates (Gandhi, 1997). Lipases act at the organic-aqueous interface in natural conditions, such as the presence of excess water, to catalyze the hydrolysis of carboxylate ester bonds and release free fatty acids and organic alcohols. Because the equilibrium between the forward and reverse reactions is controlled by the water activity of the reaction mixture, the reverse reaction, esterification, can occur under limiting water conditions. Different transesterification reactions can also occur at low water activity. The exchange of groups between an ester and alcohol (alcoholysis), an ester and an acid (acidolysis), an ester and an amine (aminolysis), or two esters is referred to as transesterification (interesterification) (Figure 1.2) (Rahman et al., 2006).

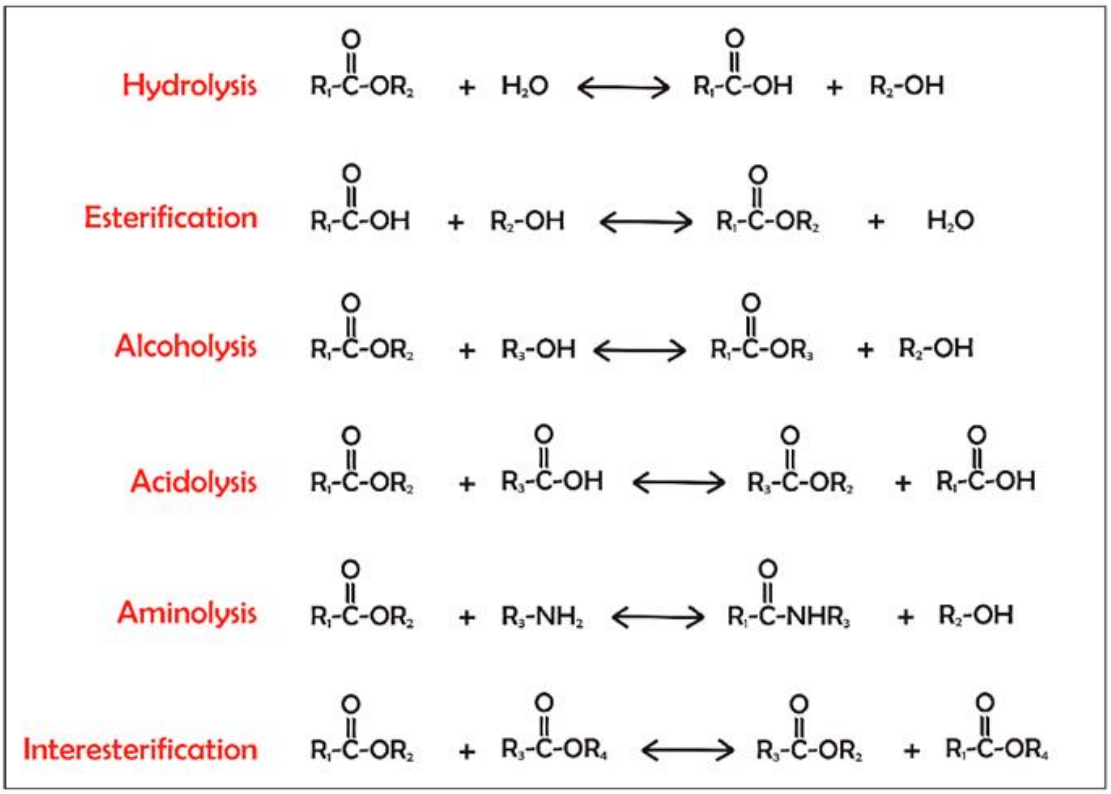

Figure 1.2. Lipase catalyzed reactions**,** adapted from Rahman et al., (2006).

1.3.6. Industrial Applications of Lipases

Food and beverages (dairy, bakery, fruit juices, beer, wine), detergents, biofuel production, animal feed, pharmaceuticals, and other applications such as textiles, leather, and paper processing are among the most common uses for industrial enzymes. Overall, food and feed applications account for 55-60% of the global enzymes market, and the market is expected to grow at a 6-8% annual rate (Figure 1.3 and Table 1.2) (Guerrand, 2017). Lipase use varies according to the requirements and properties of the reactions for each application (Kamini et al., 2000). The uniqueness of lipases in chemo- regio- and enantio-selectivity, enable their use in the also in production of novel drugs, agrochemicals, and fine chemicals. They are a great preference for biocatalysts (Saxena et al., 2003).

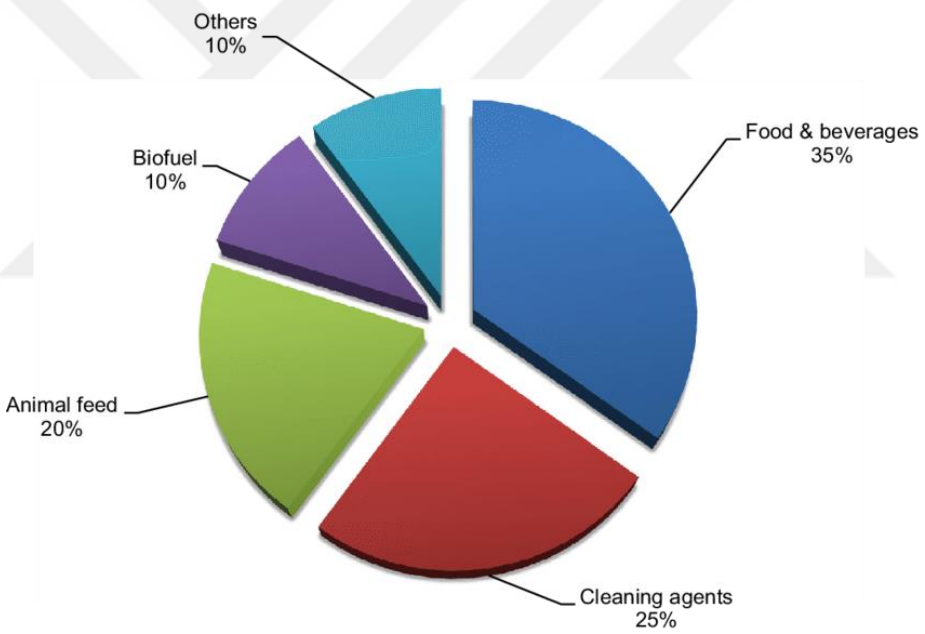

Figure 1.3. Industrial applications of lipases

Table 1.2. Industrial applications of lipases

INDUSTRY	APPLICATIONS
Foods processing	Lipases are incoropeated in food processing such as tenderising and ripnening of foods, cocoa butter processing, in dairy products for flavor development (Jooyandeh, 2009).
Fats and oleo-chemistry	In resent studies immobilised lipase is used as an alternative to physico-chemical processes to catalyze the hydrolysis, esterification, and inter-esterification of oils and fats (Guerrand, 2017).
Oily-wastewater treatments	Lipases are added to waste water reservoirs to dissolve the thin layers of fat that have accumulated on the surface in order to maintain the best conditions for biomass development. (Sarac and Ugur, 2015).
Pharmaceutical sector	Due to their high level of specificity, lipases can be used to produce novel drugs with active and pure compounds. Enantioselective enzymes, for instance, are employed as a chiral chromatography substitute technology (Francotte, 2001).
Detergents	Lipases find use in laundry and dishwashing formulations in commercial detergents where they have been optimized to operate under various pH and temperature conditions They have the ability to remove stubborn oil or grease stains in cloth materials. They are prefered industrially over phosphate-based detergents because they reduce environmental pollution (Sahoo et al., 2020).
Pulp and paper	Lipase are used in wood treatments to reduce pulp triglycerides and waxes which hinders the processes involved in the production of paper and pulp (Gutiérrez et al., 2009).
Biodiesel	Thermostable lipases are used to produce biodiesel from various feedstocks such as waste oils, vegestable oils, animal fats and many more. Moderate reaction conditions, the use of less alcohol during the reaction, and the need for less water and energy during product isolation are all advantages of lipase-catalyzed biodiesel synthesis (Cavalcante et al., 2021).

1.4. General Characteristics of *Geobacillus* Genus

The *Geobacillus* bacteria usually survive in environments with temperatures in the range of 45-75°C (optimum at 55– 65°C) with good stability at acidic to slightly alkaline pH (5.00–9.00). They are thermophilic, gram-positive, spore-forming bacilli, and are capable of aerobic or facultatively anaerobic respiration (Manachini et al., 2000). Their genome size is reported to be always less than 4 Mb (Aliyu et al., 2016). *Geobacillus* bacteria used to be classiefied as species of *Bacillus,* a strain named *Bacillus stearothermophilus*. However, in 2001 the beginning of the twenty-first century, (Nazina et al., 2001) carried out a multi-level taxonomic study on the fifth group of *Bacillus* species that led to revisions and

reclassifications of this species. The species *B. kaustophilus*, *B. stearothermophilus*, *B. thermocatenulatus*, *B. thermodenitrificans*, *B. thermoglucosidasius*, and *B. thermoleovorans* were reported to constitute a group of bacteria with similarities in phylogenetic, morphological and physiological traits (Najar and Hakur, 2020). As a result, a new genus. At present, the genus *Geobacillus*, which includes 20 species and 21 strains, has been sequenced and made available publicly (Table 1.3). With the development of bioinformatics and sequencing technology, the results of *Geobacillu*s classification are constantly updated. The species *Geobacillus pallidus* was reclassified into a novel genus, *Aeribacillus* in 2010 (Galbis et al., 2010). *Geobacillus tepidamans* was reclassified as *Anoxybacillus tepidamans* in 2016 (Aliyu et al., 2016). Recent research suggested that the genus *Geobacillus* consists of two distinct genera, *Geobacillus* and the novel genus *Parageobacillus* found in 2012. The latter genus contains P. *caldoxylosilyticus, P. thermoglucosidasius, P. antartcicus, P. toebii*, and *Parageobacillus* species all of which were known as genus *Geobacillus* (Aliyu et al., 2016). Furthermore, the study by Bezuidt et al., (2016) indicates that horizontal gene transfer is a major factor in the evolution of *Geobacillus* from *Bacillu*s, with genetic contributions from other phylogenetically distant taxa. When comparing genomes, *Geobacillus* and *Anoxybacillus* share a large number of genes (Bezuidt et al., 2016).

1.4.1. Nutritional Requirements

The nutritional requirements of *Geobacillus* are straightforward. In contrast to many other microorganisms, the genus. *Geobacillus* does not require growth stimulants, vitamins, NaCl, or KCl. Organic acids, peptone, tryptone, and yeast extract are among the substrates frequently used by culturable strains. Furthermore, this genus has a broad ability to use hydrocarbons as carbon and energy sources (Eunhye et al., 2021; Nazina et al., 2001). *Geobacillus* sp. typically has higher ideal development temperatures than the majority of industrial microorganisms, which has the advantage of lower bacterial contamination risk and lower sterilization and cooling costs. The basic nutritional needs and higher culture temperature render *Geobacillus* sp. into appealing thermo-tolerant chassis cells. In general, high efficiency is achieved with the optimization of the culture medium (Eunhye et al., 2021).

Table 1.3. The basic characteristics of *Geobacillus* species, adapted from Lin et al., (2015)

	Cell width (μm)	Cell length (μm)	NaCl range (%, w/v)	pH range	Temperature range (°C)	DNA G+C content (mol%)	Typical strain
Geobacillus caldoxylosilyticus	0.5–1	4–6	ND	ND	42–70	44–50.2	DSM 12041^T
Geobacillus debilis	0.5–1	1–14.2	ND	ND	50–70	49.9	DSM 16016^T
Geobacillus galactosidasius	ND	ND	0.1–0.2	6–8	50–75	53.5	DSM 18751^T
Geobacillus gargensis	1.0–1.5	6.0–12	0–1	5.5–8.5	45–70	52.9	DSM 15378^T
Geobacillus jurassicus	About 2	ND	0–1	6.4–7.8	40–75	53.8–54.5	DSM 15726^T
Geobacillus icigianus	1–1.6	6–14	0–5.5	5–9	45–65	52	DSM 28325^T
Geobacillus kaustophilus	<0.9	<3	0–5	6–8	37–68	51–55	NCIMB 8547^T
Geobacillus lituanicus	1.1–1.4	4.4–5.8	ND	ND	55–70	52.5	DSM 15325^T
Geobacillus pallidus	0.8–0.9	2–5	ND	6.5	30–70	39–41	DSM 3670^T
Geobacillus stearothermophilus	0.6–1	2–3.5	0–5	6.0–8.0	37–65	46–52	NCDO 1768^T
Geobacillus subterraneus	0.8–1.5	4.6–8.0	0–5	6.0–7.6	45–70	49.7–52.3	DSM 13552^T
Geobacillus tepidamans	0.9–1.2	3.9–4.7	ND	6–9	39–67	42–43.2	DSM 16325^T
Geobacillus thermantarcticus	ND	ND	<5	5–9	30–70	ND	DSM 9572^T
Geobacillus thermocatenulatus	0.9	6–8	0–4	ND	35–78	55.2	DSM 730^T
Geobacillus thermodenitrificans	0.5–1.0	1.5–2.5	0–3	6–8	45–70	48.2–52.3	DSM 465^T
Geobacillus thermoglucosidasius	0.5–1.2	3–7	0–1.5	6.5–8.5	42–69	45–46	ATCC 43742^T
Geobacillus thermoleovorans	1.5	3.5	ND	6.2–7.5	45–70	52–58	ATCC 43513^T
Geobacillus toebii	0.5–0.9	2.0–3.5	0–5	6–9	45–70	43.9 (by HPLC)	SK-1^T
Geobacillus uzenensis	0.9–1.7	4.7–8.5	0–4	6.2–7.8	45–65	50.4–51.5	DSM 13551^T
Geobacillus vulcani	0.6–0.8	2.0–3.5	0–3	5.5–9.0	37–72	53.0	DSM 13174^T

1.5. Analytical Methods for Lipase Activity Determination

The accelerated industrial demand for lipases requires constant identification of new potential microbial strains with high lipase production as well as unique industrial serving properties. Different analytical methods that are practical, reliable, rapid, substrate-specific, selective, and sensitive have been developed for the evaluation of lipase activities. These analytical methods generally focus on analyzing the formation of free fatty acids, the products of the lipase-catalyzed triglycerides. Among these quantitative volumetric analysis, calorimetric analysis, fluorescence, various chromatographic procedures, and other methods are used (Stoytcheva et al., 2012).

1.5.1. Titrimetric Method

The technique is based on the titrimetric analysis of the free fatty acids liberated from triacylglycerols during hydrolysis catalyzed by lipase (Stoytcheva et al., 2012).

1.5.2. Colorimetric Method

The colorimetric method like the titrimetric is a quantitative method based on the principle that free fatty acids form a complex with metal in an organic solvent. The metal in the organic phase is measured spectrophotometrically.

1.5.3. Synthetic Substrates

Hydrolysis of the substrate by lipase releases p-nitrophenol, which can be measured spectrophotometrically in the visible range (about 410 nm) (Becker et al., 1997). The fluorometric methods display fatty acids esterified with a chromogenic substrate such as coumarin umbelliferone, pyrenic compounds, fluoresceine, etc spectrophotometrically.

1.5.4. Turbidimetry and Nephelometry

Lipase activity is measured by observing the decrease in absorbance of a triacylglycerol emulsion over time due to de-emulsification with free fatty acids released.

1.5.5. Other Methods

Such chromatographic methods, measurement of interfacial tension, test kits, Immuno applications, and conductivity methods can be used to determine lipase activity.

1.6. Aim of This Study

The ever increasing global population and the developing economies worldwide, strike a need to manufacture goods used in daily life, such as foods, clothes, paper, textiles, fuels, chemicals, detergents and medications. The processes invloved use a lot of energy and raw materials, producing a lot of waste and this in turn has a negative impact on our environment and overall quality of life. It is obvious that an urgent solution is needed to lower the impact associated with each unit of produced goods in oder to sustain human needs without compromising the environment.

Industrial biotechnology is one such alternative technology that could be used instead of current technologies in the transition to cleaner production processes as it uses bio-based materials. Among biotechnologies, enzymatic processing is regarded as one of the most promising and long-term alternatives to conventional processing among biotechnologies.

Lipases are one of the enzymes employed in many biotechnological processes especially in the hydrolization of lipophilic materials. Lipase mediated processes are said to reduce energy consumption and have replaced many harmful chemicals contributing to global warming (Jegannathan et al., 2013).

For this reason, the presented study is aimed to determine the optimum culture conditions for *Geobacillus thermodenitrificans* HBB268 (previously confirmed for lipase activity) to acquire higher yields of lipase enzyme. For optimization, various physical and nutritional factors were studied to understand their effect on lipase production.

2. LITERATURE REVIEW

It has been observed that microbial lipase production is strongly influenced by media composition like nutritional and physical-chemical factors. As mentioned by Chandra et al., (2020), high enzyme productivity is frequently attained through scaling up production and optimizing the culture environment but one of the major hindrances industrially to the production of lipases is the process costs involved. To bridge this gap however lot of work has been dedicated to utilizing cheap sources even wastes as raw materials to produce lipase. Carbon supply is said to be not only a primary nutritional component but also an inducer of lipase production in the process of fermentation hence various carbon sources such as glucose, sucrose, maltose, and galactose are widely incorporated in certain concentrations in lipase production mediums (Chandra et. al., 2020).

A medium's constituents must meet the basic needs for cell biomass and metabolite production by providing an adequate supply of energy for biosynthesis and cell maintenance. Nitrogen can be either inorganic (as in ammonium salts) or organic (as in amino acids, proteins, or urea) (Costa et al., 2002).

E. Dalmau (2000) used batch cultures to investigate how various carbon sources affect the growth and overall lipase production in *Candida rugosa*. The study revealed that Carbohydrates and acids that are not of a lipid nature had no induction effect on lipase enzyme production. They reported that the use of lipids or fatty acids yielded the highest enzyme in quantity. Tween 80 took the role of being a stimulant for lipase biosynthesis and extracellular secretion of lipase. Combining fatty acids and carbohydrates as substrate showed an increase in lipase synthesis, and sometimes their consumption occurred sequentially. Glucose presented a repressing effect on lipase production. Moreover, glucose was found to be an effective stimulant for the secretion of lipase by cells with a high level of cell-bound lipase activity due to their previous growth in oleic acid (E. Dalmau, 2000).

Abdel-Fattah and Gaballa (2008) isolated a thermophilic strain producing thermostable lipase. The isolate was identified based on 16S rRNA sequencing, the results obtained from the phylogenetic analysis show high similarities to *Geobacillus thermoleovorans*. Lipase from this isolate was cloned and over-expression in *Escherichia coli*, subclone pET vector was used to sub-clone the lipase gene. The Lipase enzyme

activity was reported to be roughly 4.5-times higher when compared to the wild-type strain in *Geobacillus thermoleovorans* Toshki lipase enzyme was reported to be thermostable at a temperature 60 °C and pH 8 (Abdel-Fattah and Gaballa, 2008).

Bakir and Metin, (2015) used 201 thermophilic bacteria isolated from natural hot springs in Aydın/Turkey and registered in the culture stocks of Adnan Menderes University Biology Department in their study. It was stated that 43 of these bacteria showed lipolytic activity and 22 showed lipase activity. In this study, 22 isolates were determined to be lipase positive and their quantitative lipase activities were determined by growing in LB broth medium. HBB 134 strain was selected as the best lipase-producing isolate with a lipase activity of 19,925 U/mL. It was determined that the isolate showed maximum similarity (99%) with *Anoxybacillus flavithermus* according to 16S rRNA sequences. The best enzyme production obtained from HBB 134 was determined at pH 6.50 and 45 °C in an environment containing 0.5% olive oil as a carbon source and 0.5% peptone as a nitrogen source. They revealed that lipase production starts at the beginning of the logarithmic growth phase and reaches its maximum level in the middle of the logarithmic phase (12 hours) when the HBB 134 isolate is grown under optimum culture conditions (Bakir and Metin, 2015)

Geobacillus thermodenitrificans strain AV-5 isolated from the Mushroom Spring of Yellowstone National Park (USA) produced lipase with a maximum activity of 330 U/mL from a medium supplemented with 2% waste cooking oil at pH 8.0 and 50 °C (Christopher, 2015).

Soleymani, (2017) investigated *Bacillus* sp. ZR-5 for lipase activity. After 24 hours of incubation, the maximum lipase activity was attained using low-cost carbon and nitrogen supplies, such as 1.5 percent glucose syrup (160 U/mg), 1 percent fish powder (1238 U/mg), and 1407 U/mg of olive oil. The results obtained indicated a significant increase in lipase activity with the usage of low cost (Soleymani, 2017).

Sreelatha et al., (2017) used submerged fermentation to investigate lipase production using (GSLMBKU-10, GSLMBKU-13, and GSLMBKU-14) strains of thermophilic *Thermomyces lanuginosus*. The basal medium with olive oil and triacetin (0.1%) concentration was reported to stimulate lipase production. GSLMBKU-10 and GSLMBKU-13 yielded maximum lipase production in yeast extract starch medium supplemented with 0.1% triacetin (Sreelatha et al., 2017).

3. MATERIAL AND METHOD

3.1. Chemicals

All used chemicals have been obtained in c.p. (chemically pure) quality from the following suppliers.

Table 3.1. List of chemicals used in this study.

Chemical	**Supplier**
Acetone	Merck
Agar	Fluka
Agarose	Sigma
Almond oil	Fluka
Ammonium Nitrate	Merck
Ammonium Sulphate	Merck
Beef extract	Merck
Corn oil	Fluka
Cooton oil	Fluka
Ethanol	Merck
Galactose	Fluka
Lactose	Fluka
Luria-Bertani	Sigma
Maltose	Sigma
Methanol	Merck
Oleic Acid	Riedel- de Haen
Olive oil	Sigma
Peptone	Fluka
p-Nitrophenol Laurate	Fluka
Skim milk	Fluka
Soya oil	Soyala
Starch	Fluka
Sucrose	Merk
Sunflower oil	Yurdum
Triptone	Fluka
Urea	Riedel-de Haen
Yeast Extract	Merck

Table 3.2. Buffer solutions.

Buffer Stock	pH Range
Trisodium Citrate	3,00 – 6,00
Sodium Phosphate	5,00 -6.5
Tris-HCl	7 - 8.5
Glysine Sodium Hydroxide	9 - 10
Di-sodium Hydrogen phosphate	11
Potassium chloride/ Sodium hydroxid	11.8 - 12

3.2. Methods

3.2.1. Sample Stocking and Maintenance

The microorganism used in this study, *Geobacillus thermodenitrificans* HBB268 was obtained from previously isolated stock cultures registered in the Biochemistry laboratory of Adnan Menderes University, Faculty of Arts and Sciences, Department of Biology. Prior to the study, the sample was grown in Luria-Bertani (LB) agar plate medium at 55 ºC for 24 hours, the contents of the solid media were 1.5% agar, 1% NaCl, 0.5% tryptone, and 0.5% yeast, all sterilized at 121°C for 15 minutes. The culture was then transferred to sterile cryotubes containing 20% skim milk solution under sterile conditions and stored at -80 ºC in the ADU Department of Biology Biochemistry laboratory for use in future experiments.

3.2.2. Inocula Development

To activate the dormant HHB268 strain, the nutrient broth was prepared in 50 mL. The medium was transferred to 50 mL Erlenmeyer flasks adjusting the final volume to be 20 mL. The medium was then sterilized in an autoclave at 121°C for 15 minutes. The medium was left to cool slightly at room temperature before inoculation. The mediums were inoculated with a single swap of respective HHB268 bacterial solid culture taken from (LB) agar plate, this seed culture was then incubated (Newbrunswick Scientific Innova 43) for 24 hours in an orbital shaker at 55°C with a shaking speed of 150 rpm. The optical density (OD_{600}) of the 24-hour activated seed culture was measured using a spectrophotometer

(Shimadzu UV-1700, Japan) and was adjusted to 0.1 at 600 nm (approximately $8x10^7$ cell/mL) with sterile physiological saline. This seed culture was freshly prepared before every experiment and was used for inoculum.

From the adjusted seed culture solution 1% was inoculated into 250 mL Erlen flask containing 50 mL enzyme production medium. The cultures were incubated for 24 hours in an orbital shaker at 55°C with a shaking speed of 150 rpm. At the end of incubation, lipase activity and cell density (OD_{600}) in cultures were determined spectrophotometrically

3.2.3. Quantitative Determination of Lipase Activity

A spectrophotometric assay was used for the determination of lipase activity using p-nitrophenyl laurate (pNPL) as substrate (Lesuiss et al., 1993). The reaction mixture consisted of 0.1 mL of enzyme solution, before optimization (0.8 mL of 50 mM Glycine-NaOH buffer pH 9.00), under optimum conditions (0,8 mL of 50 mM Tris-HCl, pH 8.00) and 0.1 mL of 10 mM substrate (pNPL) solution dissolved in ethanol. As described in the scattered lipase literature, the temperature was set to 55 °C, and the hydrolytic reaction was conducted for 30 min. Following the incubation, 0.25 mL of 0.1 M Na_2CO_3 was added to quench the reaction. The mixture was centrifuged at 10 000 ×g, for 15 min (Heraeus-Biofugepico, Germany), and the absorbance of the cell-free culture filtrate at 410 nm was assayed for lipase activity against enzyme-free substrate solution as blank on a spectrometer (Table 3.3 and 3.4) (Shimadzu UV-1700, Japan) (Sigurgisladottir et al.,1993).

Table 3.3. Components used in lipase activity assay

Blank	**Enzyme**
0,8 mL Glycine-NaOH (50 mM, pH 9.00)	0,8 mL Glycine-NaOH (50 mM, pH 9.00)
0,1 mL LB broth	0,1 mL enzyme solution
0,1 mL pNPL (10 mM, dissolded in ethanol)	0,1 mL pNPL (10 mM, dissolved in ethanol)
incubation at 55º C for 30 minutes	
0.25 mL of 0.1 M Na_2CO_3 (quench the reaction)	
centrifuge at 10.000 x g, +4º C for 15 minutes	
measurement in spectrophotometer at 410 nm	

Table 3.4. Reaction components in standard quantitative lipase determination under optimum conditions

Blank	**Enzyme**
0,8 mL Tris-HCl Buffer (50 mM, 8.00 pH)	0,8 mL Tris-HCl Buffer (50 mM, pH 8.00)
0,1 mL LB broth	0,1 mL enzyme solution
0,1 mL pNPL (10 mM, dissolded in ethanol)	0,1 mL pNPL (10 mM, dissolved in ethanol)
incubation at 55º C for 30 minutes	
0.25 mL of 0.1 M Na_2CO_3 (quench the reaction)	
centrifuge at 10.000 x g, +4ºC for 15 minutes	
measurement in spectrophotometer at 410 nm	

One unit of lipase activity was expressed as the amount of enzyme that releases 1µmol of p-nitrophenol from pNP-laurate in 1 minute under standard experimental conditions.

Below is the formula used to calculate the Volume Activity after the enzyme activity is measured spectrophotometrically;

$$VA = \left(\frac{V}{l.\varepsilon.v.t}.A\right).SF$$

SA : Specific Activity (U/mg)

VA : Volume Activity (U/mL)

V : Reaction Volume (mL)

l : Light Path (1cm)

ε : Molar Absorbance Coefficient (mM-1 cm-1)

v : Sample Volume (mL)

t : Incubation Time (30 min)

A : Absorbance

DF : Dilution Factor

3.3. Effect of Culture Conditions on *Geobacillus thermodenitrificans* HBB268 Lipase Production

3.3.1. Effect of Initial pH on Lipase Production

In order to determine the effect of pH on bacterial growth and lipase production, the pH of the production medium was adjusted at 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 8.0 8.5, 9.0 and 10 values, and the optimum pH value for lipase production was determined. The production process was carried out as specified in 3.2.2. At the end of incubation, lipase activity assay and cell density (OD_{600}) in cultures were determined spectrophotometrically. In the studies, each experiment was repeated 3 times and the average values were given in the graphs and tables.

3.3.2. Effect of Incubation Temperature on Lipase Production

The enzyme production from HBB268 was subjected to different temparatures at 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85 ºC and the optimum temperature value for lipase production was determined. The production process was carried out as specified in 3.2.2. At the end of incubation, lipase activity (410 nm) and cell density (OD_{600}) in cultures were determined spectrophotometrically. In the studies, each experiment was repeated 3 times and the average values were given in the graphs and tables.

3.3.3. Effect of Carbon Sources on Lipase Production

Various carbon sources (0.5%): lipids such as Analytical grade (A) olive oil, commercial (C) cotton oil, maize oil, groundnut oil, olive oil, sunflower oil and sugars including galactose, glucose, lactose, maltose, starch and sucrose, were used to determine their effect on lipase production.

LB broth medium containing 0.5g arabic gum was used as control for mediums containing oil. The contents of the media used is specified in Table 3.5. The carbon sources

were added to the media at concentration of 0.5%, media containing oils were emulsified separately for 3 minutes at the highest speed of the mixer (WARING,57 Commercial Blender 8011ES, USA). The production media were sterilized in an autoclave at 121 °C for 15 minutes

The production process was carried out as specified in 3.2.2. At the end of incubation, lipase activity (410 nm) and cell density (OD_{600}) in cultures were determined spectrophotometrically. In the studies, each experiment was repeated 3 times and the average values were given in the graphs and tables.

Table 3.5. Control media used in trials of carbon sources

Chemical substance	Sugars	Oils
Trypton	0.1%	0.1%
Yeast extract	0.5%	05%
NaCl	1%	0.1%
Arabic gum	-	0.5%
Carbon source	0.5%	0.5%
pH 7.00, 55 °C		

3.3.4. Effect of Nitrogene Sources on Lipase Production

To investigate the effect of various nitrogen sources on enzyme production, yeast extract and tryptone were removed from the production medium. İnstead (0,5% w/v) of various organic nitrogen sources (casein hydrolaze, peptone, beef extract, tryptone, urea, yeast extract) and inorganic nitrogen sources (ammonium sulfate, ammonium nitrate) were added to the production medium (Table 3.6).

The production process was carried out as specified in 3.2.2. At the end of incubation, lipase activity and cell density (OD_{600}) in cultures were determined spectrophotometrically. In the studies, each experiment was repeated 3 times and the average values were given in the graphs and tables.

Table 3.6. Media used in nitrogen source trials.

Chemical substance	Quantity
Nitrogen Source	% 0,5
NaCl	% 1
pH 7.00, 55 °C	
Sterilized at 121°C for 15 minutes	

3.3.5. Determination of Incubation Time for Bacterial Growth and Lipase Activity of *Geobacillus thermodenitrificans*

In order to determine the effect of time course and the efficiency of the new medium on enzyme production, *Geobacillus thermodenitrificans* HBB268 was incubated under the determined optimum culture conditions (pH 8 and 55 °C including the optimum physical and nutritional factors) in an orbital shaker with a shaking speed of 150 rpm for up to 36 h. Samples were aseptically withdrawn at 3-hour intervals during incubation and analyzed spectrophotometrically for cell density (OD_{600}) and lipase activity. The production process was carried out as stated in 3.2.2. then volume activity was calculated and interpreted in a graphs and tables.

3.4. Characterization of *Geobacillus thermodenitrificans* HBB268 Lipase

3.4.1. The Effect of pH on Enzyme Activity

To determine the effect of pH on enzyme activity, buffer solutions were prepared at pH values of 3.00-11.00 in a hot water bath at 55°C (Polyscience 911, USA) with 0.5 unit intervals. McIlvaine (citric acid-Na2HP04) buffer for pH 3.00-6.00, Tris-HCl buffer for pH 6.00-8.50 and Glycine-NaOH buffer for pH 9.00-10.00 were prepared. Enzyme activity was measured spectrophotometrically at 410 nm. Calculations were made using the molar absorbance coefficients of pNPL at different pHs.

3.4.2. Effect of Temperature on Enzyme Activity

In order to determine the effect of temperature on enzyme activity, Tris-HCl buffers prepared at pH 8.00 were used by adjusting temperatures between 35-80 °C. Enzyme activity was determined spectrophotometrically under standard experimental conditions by which the only variable was temperature as a parameter.

3.5. Statistical Analysis

The results of lipase activity are expressed as mean±standard deviation (SD). Every experiment was repeated at least three independent times. All data were analyzed using one-way ANOVA with Tukey post test to determine statistical significance. The level of significance was set at $p<0.05$.

4. RESULTS

4.1. The Impact of Media Conditions on Lipase Production by *Geobacillus thermodenitrificans* HBB268

4.1.1. Detection of Initial pH on Bacterial Growth and Lipase Production of HBB268 Strain

HBB268 was incubated at different pH points (3.00- 7.50) to determine the optimum pH for bacterial growth (OD_{600}) and lipase production. After 24 hours of incubation, maximum enzyme activity (137,300 U/mL) was obtained at pH 7.14, followed by pH 6 and 6.5 with 53,500 and 80,600 U/mL respectively ($F=23.77$; $df=4, 44$; $p<0.001$) (Table 4.1 and Figure 4.1). The enzyme activity could not be detected at pH 3.00 to 5.00 due to lack of bacterial growth.

Table 4.1. Effect of initial pH of growth medium on HBB268 lipase production

pH	OD_{600} (Mean ± SD)	x	Volume Activity (U/mL) (Mean ± SD)	x
5.50	0,53 ± 0,06	d	9,23 ± 5,27	c
6.00	1,15 ± 0,14	c	53,50 ± 33,90	b
6.50	1,26 ± 0,35	c	80,60 ± 40,20	b
7.00	2,80 ± 0,06	a	137,30 ± 35,00	a
7.50	2,41 ± 0,18	b	88,28 ± 13,38	b
	Pooled StDev = 0,19		Pooled StDev = 28,98	

Mean.: Each data is the average of three replicates.
SD: Standard deviation.
x : The statistical differences between the means of each group was showed with different letters in the x column ($p < 0,05$).

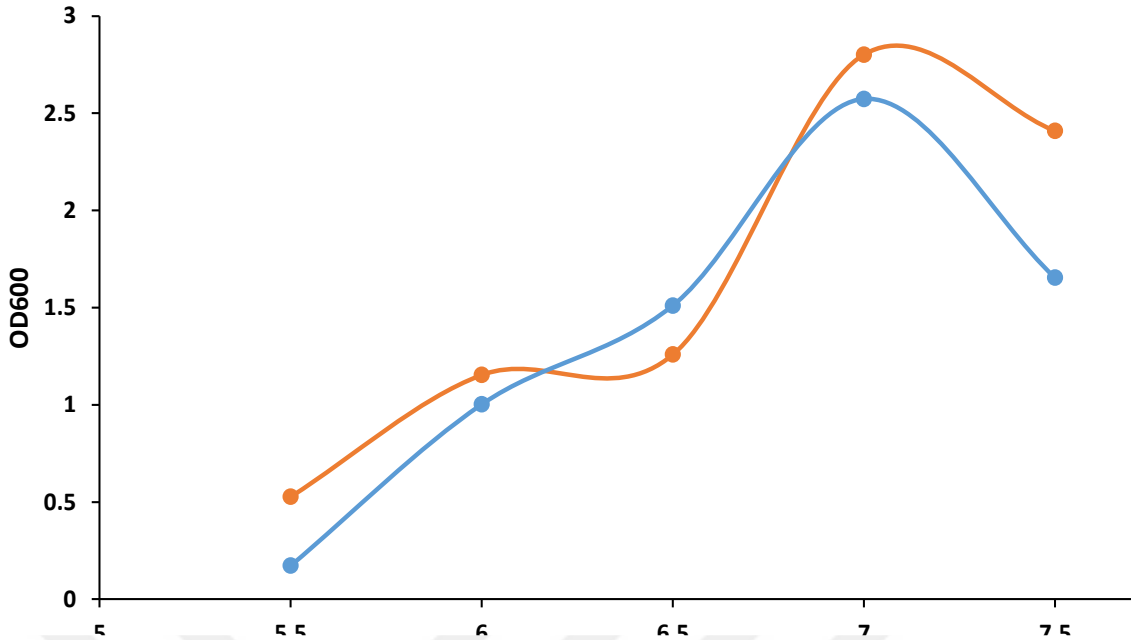

Figure 4.1. Effect of initial pH on the production of lipase in HBB268 strain.

4.1.2. Detection of Optimum Temperature for Bacterial Growth and Lipase Production of HBB268 Strain

HBB268 strain was incubated at 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 ºC for 24 hours to determine the effects of temperature on bacterial concentration and lipase production. According to the spectrophotometric measurements, the optimum temperature was detected at 55 ºC with 4,289 U/mL absorbance and 200,240U/mL of lipase production reported. The enzyme activity could not be detected at 35, 40, 70, and 80 ºC due to no bacterial growth (Table 4.2 and Figure 4.2).

Table 4.2. Detection of optimum temperature on bacterial growth (OD_{600}) and lipase production (U/mL) in HBB268 strain

Temperature (°C)	**OD_{600} (Mean ± SD)**	**x**	**Volume Activity (U/mL) (Mean ± SD)**	**x**
45	2,02 ± 0,25	c	146,52 ± 15,74	c
50	2,52 ± 0,19	b	168,61 ± 8,88	b
55	4,29 ± 0,22	a	200,24 ± 18,77	a
60	2,79 ± 0,22	b	64,72 ± 4,54	e
65	1,78 ± 0,51	c	93,55 ± 4,54	d
70	0,18 ± 0,10	d	7,60 ± 5,88	f
	Pooled StDev = 0,28		Pooled StDev = 11,21	

Mean.: Each data is the average of three replicates.
SD: Standard deviation.
x : The statistical differences between the means of each group was showed with different letters in the x column ($p < 0,05$).

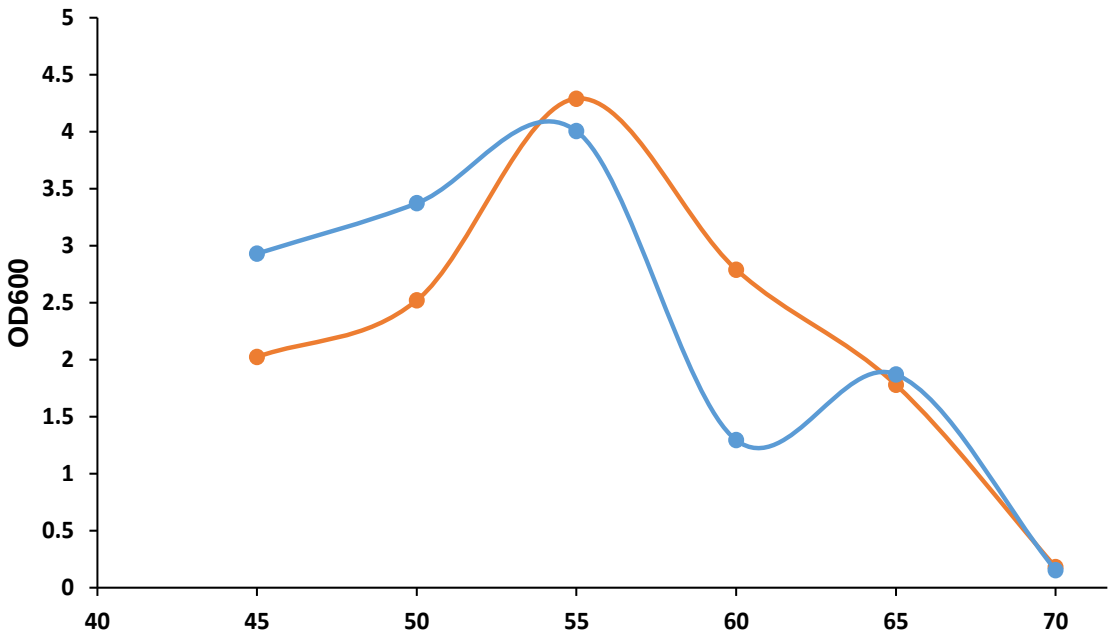

Figure 4.2. Effect of temperature on the production of lipase and bacterial growth in HBB268 strain**.**

4.1.3. Evaluation of Different Sugar Contents as Carbon Sources

Various carbon sources (0.5% w/v) including galactose, glucose, lactose, maltose, starch and sucrose were used to determine their effect on lipase synthesis. A statistical difference was observed after HBB268 was incubated in LB media supplemented with different sugars. The highest enzyme activity was recorded from treatments with maltose with 378,000 U/ml though the bacterial concentration was very low. This was followed by galactose, starch, surcose and lactose with enzyme activity ranging between 184,120 and 194,600 U/ml. The least activity occurred in media with glucose and control (Table 4.3, Figure 4.3 and 4.4).

Table 4.3. Effects of different sugar contents as a carbon source on lipase production and bacterial density.

Media	**OD600 (Mean ± SD)**	**X**	**Volume Activity (U/mL) (Mean±SD)**	**X**
Galactose	2,91 ± 0,33	b	184,93 ± 11,72	b
Glucose	0,48 ± 0,09	d	56,35 ± 26,71	c
Lactose	3,06 ± 0,73	b	185,20 ± 56,20	b
LB (Control)	3,27 ± 0,07	b	87,49 ± 16,35	c
Maltose	1,91 ± 0,46	c	378,00 ± 61,30	a
Starch	4,63 ± 0,37	a	184,12 ± 15,11	b
Sucrose	3,37 ± 0,25	b	194,60 ± 49,90	b
	Pooled StDev = 0,39		Pooled SD= 39,19	

Average.: Each data is the average of three replicates.
StDev: Standard deviation.
x: The difference between the means with different letters in the same column and the media with the highest activity is statistically significant ($p < 0.05$)

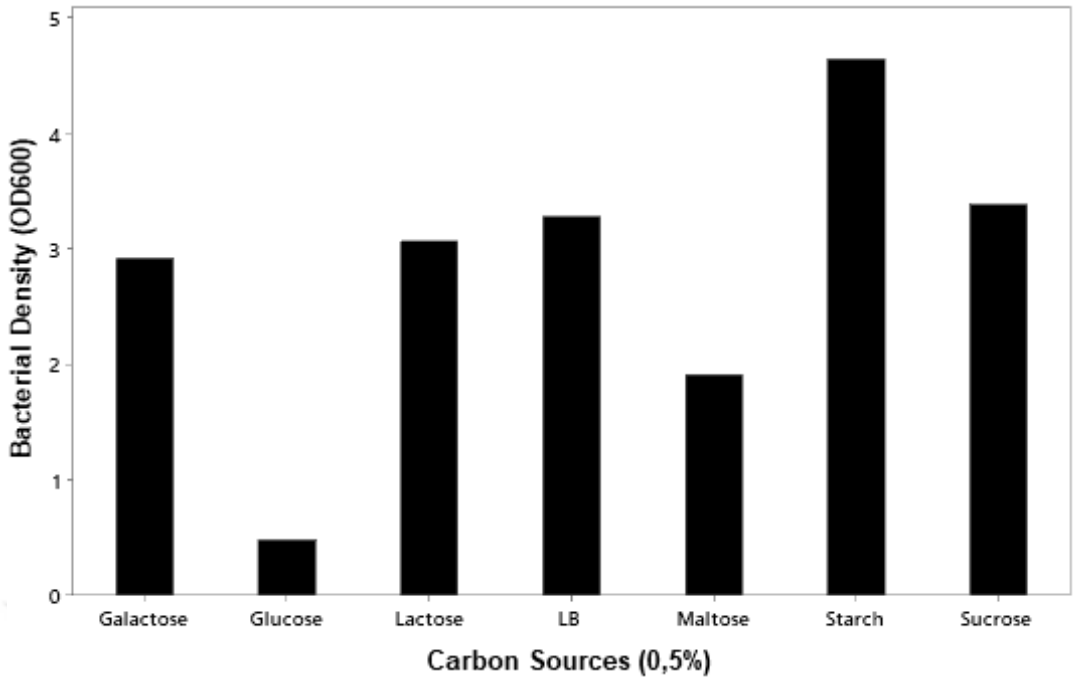

Figure 4.3. Effects of different carbon sources on bacterial growth of HBB268

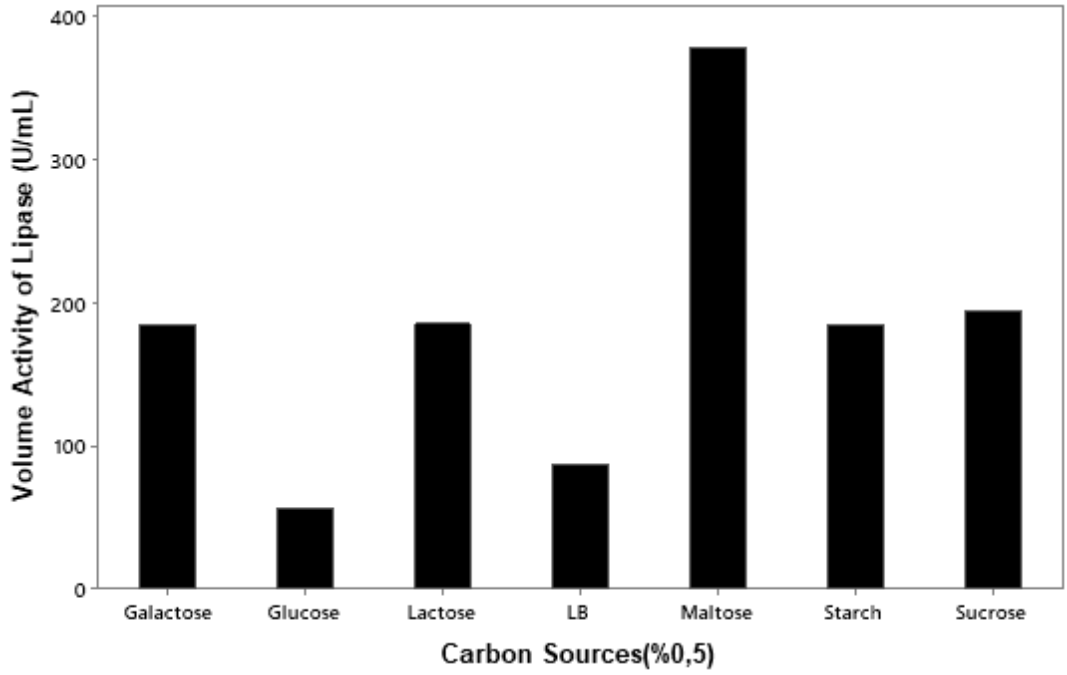

Figure 4.4. Effects of different carbon sources of sugar on lipase production level of HBB268.

4.1.4. Evaluation of Different Lipid Contents as Carbon Sources

Different lipids had a statistical significant effect on the lipase activity and bacterial density of *Geobacillus thermodenıtrıficans* HBB268. Bacteria was incubated in LB media supplemented olive oil, cotton oil, groundnut oil, sunflower oil and maize oil. The highest enzyme activity was recorded from treatments with olive oil and sunflower oil with 201,250 and 213,420 U/mL, respectively. Moderate activity was observed in treatments with ground nut, maize, and olive oil (enzyme activity ranged between 167,160 and 173,570 U/ml). The least activity occurred in media with cotton oil (Table 4.4, Figure 4.5 and 4.6).

Table 4.4. Effects of different lipid sources on the growth of HBB268 and the production of lipase (The quality of lipid sources was aligned as commercial (C) and analytical grade (A).)

0,5 % of Lipids Added in Media	**OD_{600} (Mean ± SD)**	**X**	**Volume Activity (U/mL) (Mean ±SD)**	**X**
Cotton Oil	86,42 ± 5,65	b	131,27 ± 8,01	c
Ground Nut Oil	83,38 ± 2,31	bc	167,16 ± 10,37	b
LB (Control)	47,87 ± 1,53	d	137,51 ± 5,43	c
Maize Oil	101,83 ± 2,73	a	169,29 ± 13,31	b
Olive Oil (A)	78,80 ± 2,80	c	173,57 ± 9,87	b
Olive Oil (C)	84,36 ± 3,82	b	201,25 ± 5,57	a
Sunflower Oil	100,61 ± 4,36	a	213,42 ± 6,69	a
	Pooled SD = 3,56		Pooled SD = 8,87	

Mean: Each data is the average of three replicates.
SD: Standard deviation.
x: The difference between the means with different letters in the same column and the media with the highest activity is statistically significant ($p < 0,05$)

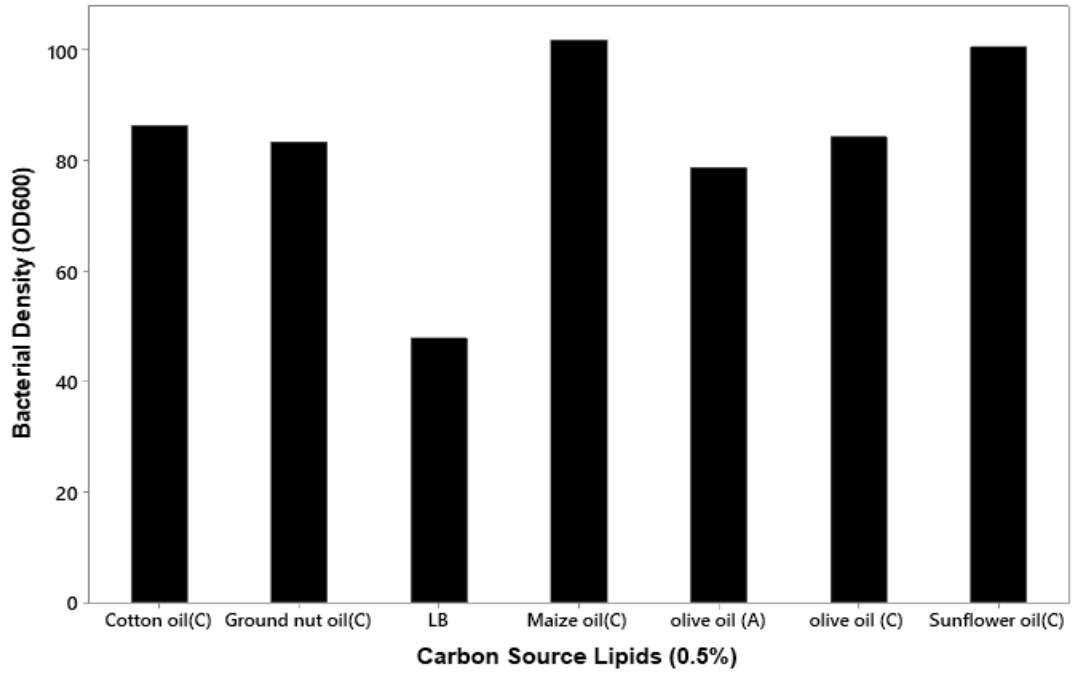

Figure 4.5. Effects of different lipid sources on the bacterial growth of HBB268 strain (The quality of lipid sources was aligned as commercial (C), analytical grade (A).)

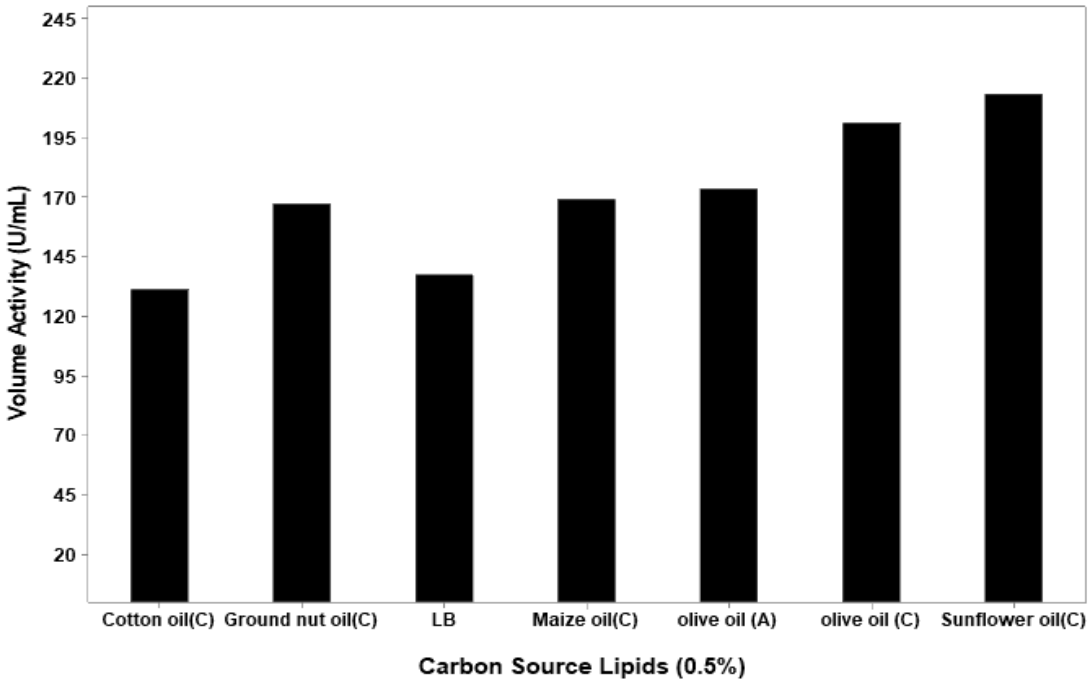

Figure 4.6. Effects of different sources of lipids on lipase production level of HBB268 (The quality of lipid sources was aligned as commercial (C), analytical grade (A).)

4.1.5. Determination of the Optimum Nitrogen Sources Included in Growth Medium of HBB268 for Lipase Production

To investigate the effect of various nitrogen sources on enzyme production, yeast extract and tryptone were first removed from standard LB medium and then various organic nitrogen sources (gelatin, casein hydrolaze, casein, peptone, meat extract, tryptone, urea, yeast) and inorganic nitrogen sources (ammonium sulfate, ammonium nitrate) were added (0,5% w/v) to the production medium. These nitrogen sources had a significant effect on the lipase activity and bacterial density. The highest enzyme activity was recorded from treatments with meat extract with 21,453 U/mL. The least activity occurred in media with yeast extract (Table 4.5, Figure 4.7 and 4.8). Casein hydrolyse, gelatine, urea and all inorganic sources bacteria density was not observed therefore enzymye activity was not measured.

Table 4.5. Effects of various nitrogen sources on lipase production of HBB268 strain.

Media	**OD_{600} (Mean ± SD)**	**X**	**Volume Activity (U/mL) (Mean ± SD)**	**X**
Casein	1,313 ± 0,02	a	2,51 ± 0,10	e
LB (Control)	2,79 ± 0,09	ab	13,42 ± 0,31	c
Meat extract	2,86 ± 0,03	b	21,45 ± 0,47	a
Peptone	2,71 ± 0,05	c	15,52 ± 0,16	b
Tryptone	2,49 ± 0,02	d	15,52 ± 0,16	d
Yeast extract	0,49 ± 0,04	e	1,55 ± 0,14	f
	Pooled SD = 0,05		Pooled SD = 0,27	

Mean: Each data is the average of three replicates.
SD: Standard deviation.
x: The difference between the means with different letters in the same column and the media with the highest activity is statistically significant ($p < 0,05$).

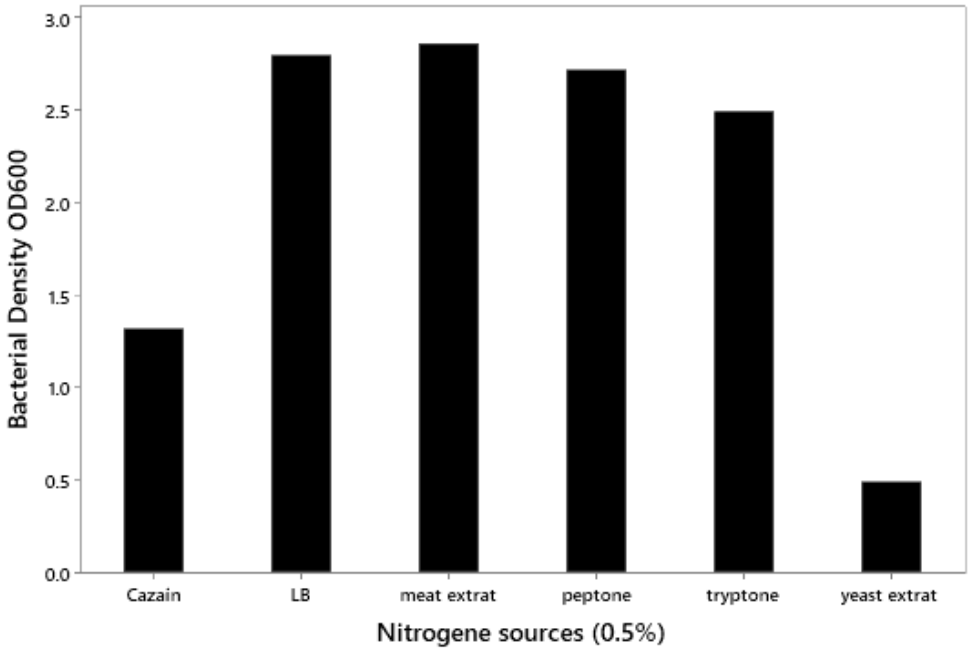

Figure 4.7. Effects of various nitrogen sources on lipase production of HBB268 strain

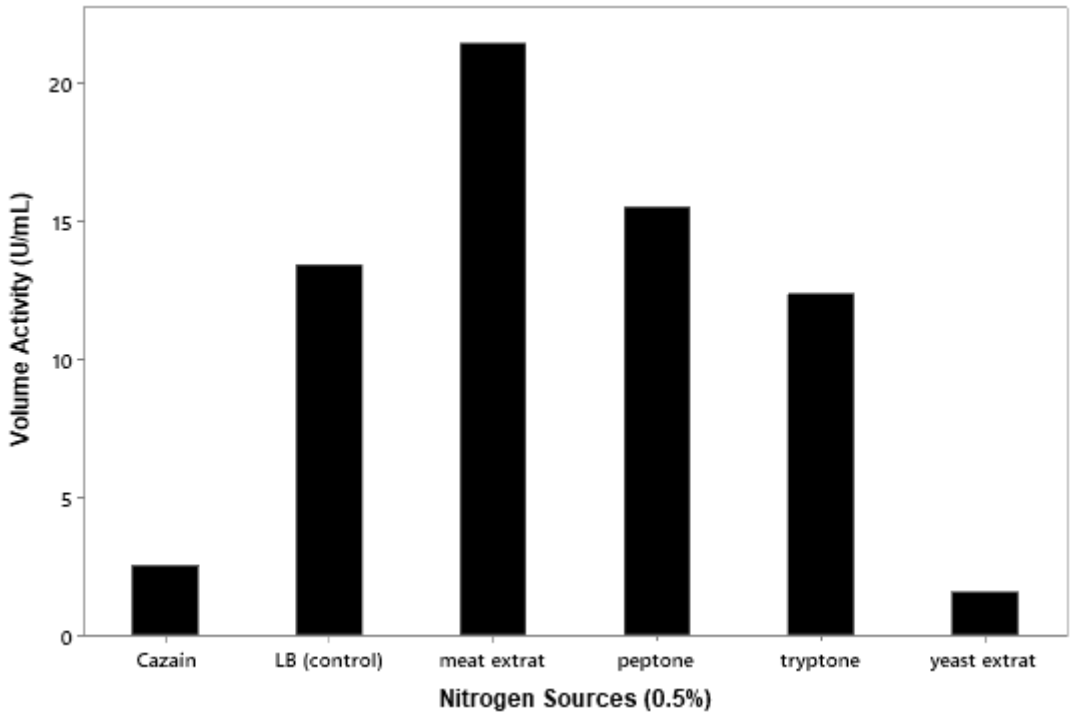

Figure 4.8. Bacterial density of HBB268

4.1.6. Effect of Incubation Time on Lipase Production

Geobacillus Thermodenitrificans HBB268 was subjected to a 36-hour incubation to determine the time-dependent development and enzyme production in the optimised medium. Cell density (OD_{600}) and lipase activity measurements were made by taking samples at 3-hour intervals during the incubation period.

According to the results the maximum volume activity (73.334 U/ml) was observed in the cell free supernatant of the culture taken after 18 h of incubation. The lipolytic activity increased up to 18 h and and a linear decrease was observed with further incubation up to 36 h (Table 4.6 and Figure 4.9).

Table 4.6. Effect Of Incubation Time On Lipase Production

Tıme (h)	**OD600 (Av. ± StDev)**	**x**	**Volume Aktivite (U/mL) (Av. ± StDev)**	**x**
3	1,30 ± 0,06	d	0,57 ± 0,08	f
6	1,13 ± 0,08	d	10,63 ± 0,70	e
9	2,16 ± 0.18	c	19,82 ± 1,95	d
12	2,61 ± 0,88	b c	28,16 ± 1,45	c
15	3,04 ± 0,08	a b	44,88 ± 5,49	b
18	3,09 ± 0,06	a b	73,33 ± 2,80	a
21	3,11 ± 0,11	a	69,10 ± 4,09	a
24	3,04 ± 0,21	a b	69,62 ± 1,23	a
36	2,93 ± 0,15	a b	44,31 ± 4,31	b

Average: Each data is the average of three replicates.
StDev: Standard deviation.
x : The difference between the means with different letters in the same column and the media with the highest activity is statistically significant ($p < 0.05$)

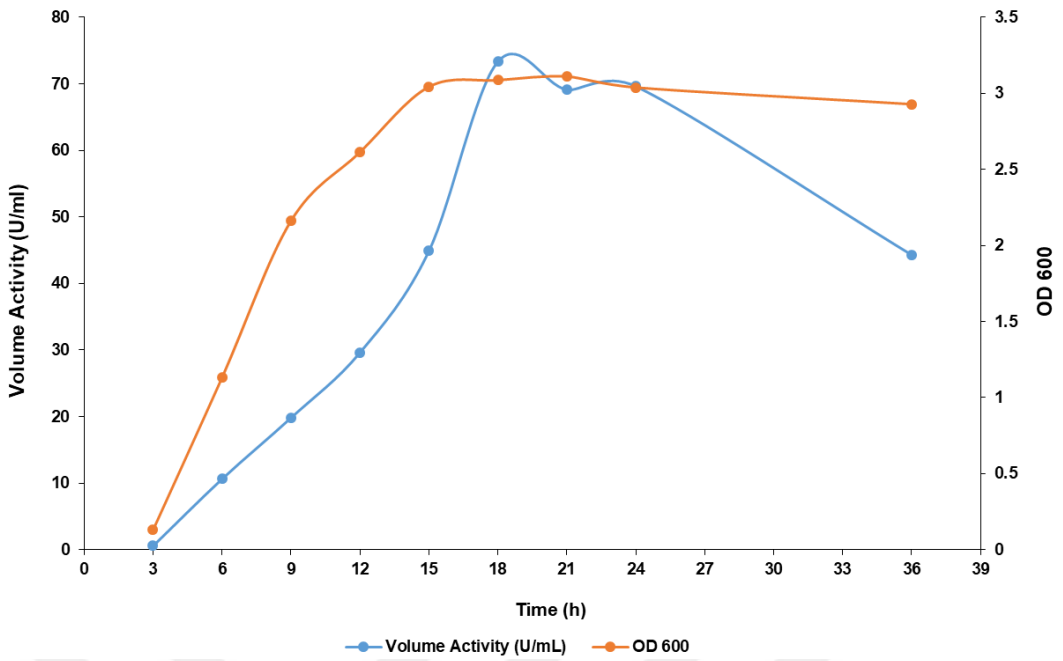

Figure 4.9. Effect of incubation time on bacterial growth and lipase avtivity of HBB268

4.2. Characterization of *Geobacillus thermodenitrificans* HBB268 Lipase

4.2.1. The effect of pH on enzyme activity

In order to examine the effect of pH on lipase enzyme activity, values between pH 3-11 were measured. The enzyme activity of lipase was pH dependent with the optimum pH for incubation ranging between 7.5 and 8.5. This was statistically higher than the other pH ranges ($F= 169.98$; $df=12, 38$; $p<0.001$). pH 8.00 showed the Higheste activity of 347,200 U/mL while moderate activity was observed at pH between 6,00-7.00 and at pH 10.00. Enzme activity at pH ≤ 5 was undesirable (Table 4.7 and Figure 4.10).

Table 4.7. HBB268 lipase enzyme optimum pH value

pH	Volume Activity (U/mL) (Mean ± SD)	X
3.00	2,69 ± 3,29	e
4.00	2,82 ± 4,96	e
5.00	23,61 ± 4,24	d e
5.50	81,86 ± 2,32	c
6.00	213,03 ± 3,57	b
6.50	237,49 ± 14,44	b
7.00	242,62 ± 14,47	b
7.50	307,16 ± 17,29	a
8.00	347,20 ± 46,70	a
8.50	320,37 ± 6,90	a
9.00	230,82 ± 13,83	b
10.00	206,60 ± 18,30	b
11.00	60,30 ± 11,55	c d
	Pooled StDev = 16,82	

Average.: Each data is the average of three replicates.
StDev: Standard deviation.
x : The difference between the means with different letters in the same column and the media with the highest activity is statistically significant ($p < 0.05$)

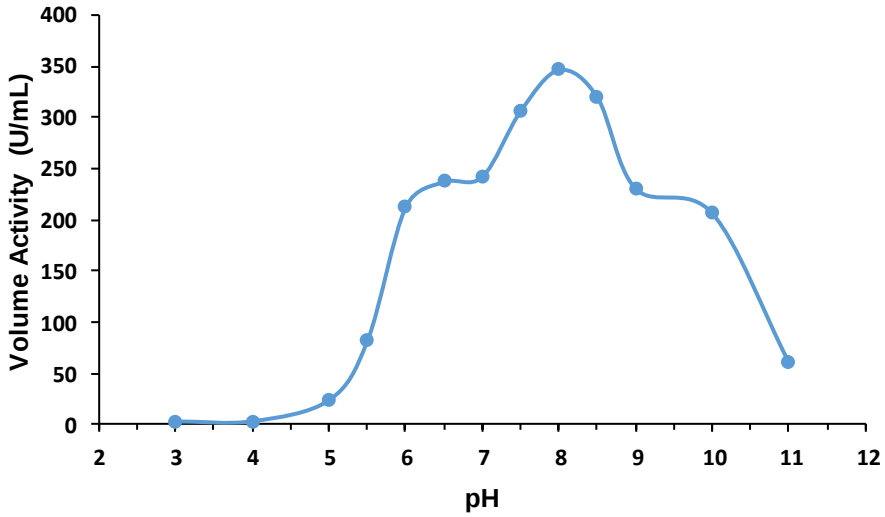

Figure 4.10. Effect of pH on lipase activity of HBB268

4.2.2. Effect of Incubation Temperature on Enzyme Activity

In order to determine the effect of temperature on lipase activity, measurements were made between 35-80 °C. There was a statistical difference in the effects of temperature on lipase activity (F=59.24; df=10, 32; p<0.001). Enzyme activity was found to have increased steadly until 55 °C with maximum lipase activity of (261.95 U/mL) and then it gradually decreased. At the lowest temperature 35 °C the lipase activty recorded was 68,950 U/mL (Table 4.8 and Figure 1.1).

Table 4.8. Effect of temparature on lipase activity of HBB268

Temperature	Volume Activity (U/mL) (Mean± SD)	X
35	68,95 ± 4,29	cd
40	99,20 ± 52,60	cd
45	118,29 ± 9,13	bc
50	164,30 ± 5,16	b
55	261,95 ± 3,45	a
60	112,78 ± 13,24	c
65	93,17 ± 9,91	cd
70	52,03 ± 8,22	de
75	15,64 ± 2,56	e
80	7,05 ± 2,12	e
85	2,43 ± 0,97	e
	Pooled StDev = 17,21	

Mean: Each data is the average of three replicates.
SD: Standard deviation.
x: The difference between the means with different letters in the same column and the media with the highest activity is statistically significant (p < 0.05)

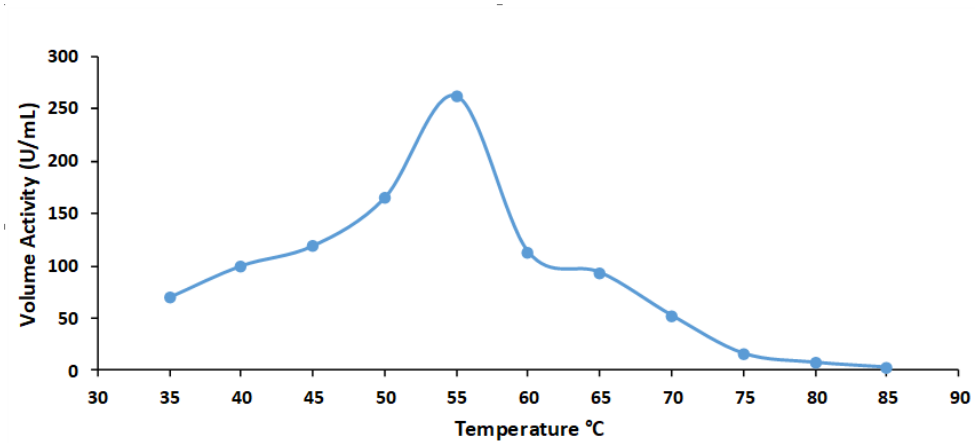

Figure 4.11. Effect of temperature on lipase activity of HBB268

5. DISCUSSION

Lipases (triacylglycerol hydrolase) occupy an important place in the biotecnological industry due to their multifaceted properties like substrate specificity, regioselectivity, and chiral selectivity (Hasan et al., 2006). They catalyze the hydrolysis of triacylglycerol to diacylglycerol, fatty acids and glycerol. These hydrolases find immense applications in many industries especially food processing, animal feed, fat and oils, detergent, pharmaceutical, biosensors and environment maintance and other. Lipase can be extracted from several sources: animal, plants, and microbes. Most of the enzymes currently developed for industrial applications are obtained from bacteria, yeast or fungi. The industrial demand for new sources of lipases with different catalytic characteristics stimulates the isolation and selection of new strains.

The production of lipases is influenced by several nutritional and physico-chemical factors such as availability of carbon and nitrogen sources, incubation time, temperature, pH, presence of lipids as an inducers or substrates. Great demand in lipase enzymes has forced scientists to look for new sources of bacteria that can grow in cheaper media conditions. Optimization of medium compositions and use of suitable physico-chemical conditions are vital in biological processes to achieve a better yield at a low cost (Chandra et al., 2020).

The microorganism used in the experiments, *Geobacillus thermodenitrificans HBB268* previously isolated and screened for lypolyitic activity was obtained from stock cultures in the microbiology laboratory of Aydın Adnan Menderes University, Faculty of Arts and Sciences, Department of Biology. For the purpose of this study, optimization of culture conditions for high lipase production by *Geobacillus thermodenitrificans HBB268* was investigated using different fermentation parameters, these included carbon sources, nitrogen sources, pH, temperature and time-dependent bacterial developments. A quantitative lipase activity assay was determined spectrometrically with p-NPL (p-nitrophenyl laurate) as substrate.

After optimization of culture conditions (studying one factor at a time) the optimum initial pH range of the medium for lipase production was found to be between 6.50 and 7.50 with maximum HBB268 enzyme activity (137,300 U/mL) obtained at pH 7.14. The optimum temperature for growth and maximum lipase production was 55 °C with the highest lapase activity (200,240 U/mL). The most effective lipid carbon source in HBB268 lipase production was sunflower oil (0.5%) (213,420 U/mL) it was also a stimulant for bacterial growth. Sunflower oil is widely available and is a cheap carbon source. The highest enzyme activity was recorded from treatments with maltose with 378,000 U/ml. This was followed by galactose, starch, sucrose, and lactose with enzyme activity ranging between 184,120 and 194,600 U/ml. Meat extract (21,453U/mL) as nitrogen source increased the amount of enzyme the most. It was observed that the time-dependent development of HBB268 strain started at the beginning of the log phase 3rd h, reached and maintained a maximum value between 18-24 hours (73.334U/ml). The parameters gave higher yield than the optimised medium when studied separately which was quiet surprising, this will be further analysed in the continuation of this study in order to understand how different combinations and ratios affect HBB268 lipase production. The optimum hydrolytic reaction activity for HBB268 was found to be pH 8.00 at 55°C as optimum temperature.

Industrially, short incubation time is one of the most desirable factor for the production of lipase enzymes. We observed that the time-dependent development of HBB268 strain started at the beginning of the log phase 3rd hour, maximum lipase activity was achieved between 18-24 hours. The incubation time for other bacteria in literature differed greatly from our strain e.g. *Bacillus licheniformis* A7 needs 72 h for optimal lipase production (Tuysuz, (2019). The production of lipase by *Pseduomonas gessardiistarts* was seen after 24 hours of incubation and the highest enzyme activity was reported at 48 h (Veerapagu et al., 2013). Sreelatha et al., (2017) demonstrated that the highest lipase production for *Thermomyces lanuginosus GSLMBKU*-10 strain was obtained on the 9th day of incubation, while for GSLMBKU-13 strain maximum production was seen on the 12th day of incubation (Sreelatha et al., 2017).

In our study, *G. thermodenitrificans* HBB268 had the highest lipase activity when grown on media supplemented with sunflower oil at 0.5% concentration (213,420 U/mL), followed by olive oil (local) (201,250 U/mL), and olive oil analytical grade (173,570 U/mL). Similar to our study, Zhekova et al., (2015) found that *Aspergillus carbornarius* exhibited highest lipase activity from rapeseed (195.61 U/L), followed by soyabean oil

(155.00 U/L), and sunflower oil (149.00 U/L).

Optimum conditions for *Bacillus licheniformis* A7 was osberved to be on media supplemented with waste frying oil at 0.4 % concentration (Tuysuz, 2019).

The culture conditions of *Stenotrophomonas maltophilia* were optimized with statistical design method and the combination of peptone, yeast extract, olive oil, gave the highest lipolytic activity 4559 U/mL incomparison to 500 U/ml obtained from the basal medium (Hasan-Beikdashti, 2012)

Geobacillus thermodenitrificans AZ1 lipase production was studied by implementing Plackett–Burman statistical design and the best carbon source was D-sucrose and peptone as a nitrogen (Abdel-Fattah, 2012).

Among the various nitrogen sources (gelatin, casein hydrolaze, casein, peptone, meat extract, tryptone, urea, yeast, ammonium sulfate, ammonium nitrate), the highest enzyme activity was recorded from treatments with meat extract with 21,453 U/mL for our strain HBB268. These results are similar to Zhekova et al., (2015) who also found that *Aspergillus carbornarius* prefers meat extract (12236.03 U/mL) for lipase biosynthesis.

In industrial production, peptone, yeast, and tryptone are generally used as organic nitrogen sources (Almeida et al., 2013). In our study casein hydrolyse, gelatine, urea and all inorganic sources bacteria density was not observed this could be that these nitrogen sources probably does not contain the nutrients that satisfy the minimal requirement for HBB268 growth. *Bacillus licheniformis* prefered yeast extract as a nitrogen source (Larbidaouadi et al., 2014).

Studies on other *Geobacillus* species have shown that the optimum temperature for bacterial growth can vary. Jo et al. (2021) found the optimal activity of *Geobacillus thermocatenulatus* lipase production to be 50 °C and pH 9.5 (Jo et al., 2021). *Geobacillus thermoleovorans* reportedly had high lipase activity at 60 °C and pH 10. This species prefered galactose as carbon source and ammonium phosphate as nitrogen source at concentrations 0.5% (Abol Fotouh et al., 2016).

The optimum medium conditions for *Thermomyces lanuginosus* strains GSLMBKU-10, GSLMBKU-13 and GSLMBKU-14 of high bacterial density and lipase activity was at a pH range between 6.0-7.0 (Sreelatha et al., 2017). Optimum conditions for *Bacillus licheniformis* A7 were observed to be pH of 6,00 and at temperature of 55 °C (Tuysuz, 2019).

6. CONCLUSION AND RECOMMENDATIONS

Lipases are considered one of the most widely exploited enzymes in different industries especially lipases from thermophiles, due to their ability to remain stable in elavated temperature and their tolarence in harsh industrial conditions. They are also easily biodegradable and, when used in industrial manufacturing and released into the environment, they have low or no toxicity. Because of these characteristics, manufacturers can produce high quality goods while using less energy, water, chemicals, raw materials, and other resources than they would with traditional methods. This study has focused on the production, optimization, and characterization of the lipase enzyme from a thermophilic bacteria species *Geobacillus Thermodentrificans* HBB268. The optimum rearing conditions for HBB268 used in our study were found to be at pH 8.00, 55 °C temperature and 0.5% sunflower oil, maltose and meat extract. This study will be serve as a baseline for further studies on *Geobacillus thermodenitrificans* HBB268 lipase. It is a future plan to purify and characterize this lipase enzyme to be used in a vast number of industrial applcations especially in the turnover of waste oils for feedstocks and bio-friendly products.

REFERENCES

Abdel-Fattah, Y. R., Soliman, N. A., Yousef, S. M., and El-Helow, E. R. (2012). Application of experimental designs to optimize medium composition for production of thermostable lipase/esterase by *Geobacillus thermodenitrificans* AZ1. *Journal of Genetic Engineering and Biotechnology*, *10*(2), 193-200.

Aehle, W., Antrim, R. L., Buchert, J., Burrows, H., Roos, A. D., Grassin, C., ...and Wilms, J. (2007). *Discovery and development of enzymes*.

Akutsu, Y., Li, Y. Y., Harada, H., and Yu, H. Q. (2009). Effects of temperature and substrate concentration on biological hydrogen production from starch. *International Journal of Hydrogen Energy, 34(6),* 2558-2566. https://doi.org/10.1016/j.ijhydene.2009.01.048

Albayati, S. H., Masomian, M., Ishak, S. N. H., Mohamad Ali, M. S. B., Thean, A. L., Shariff, M., ... and Raja Abd Rahman, R. N. Z. (2020). Main structural targets for engineering lipase substrate specificity. *Catalysts, 10(7),* 747. http://dx.doi.org/10.3390/catal10070747

Alexander, D. R. (2003). Signal transduction and regulation. *BioEssays, 25(2).* https://doi.org/10.1002/bies.10213

Aliyu, H., Lebre, P., Blom, J., Cowan, D., and De Maayer, P. (2016). Phylogenomic re-assessment of the thermophilic genus *Geobacillus*. *Systematic and applied microbiology, 39(8),* 527-533. https://doi.org/10.1016/j.syapm.2016.09.004

Alkan, H., Baysal, Z., Uyar, F., and Dogru, M. (2007). Production of lipase by a newly isolated *Bacillus coagulans* under solid-state fermentation using melon wastes. *Applied biochemistry and biotechnology, 136(2),* 183-192. https://doi.org/10.1007/BF02686016

Anthonsen, H. W., Baptista, A., Drabløs, F., Martel, P., Petersen, S. B., Sebastião, M., and Vaz, L. (1995). Lipases and esterases: a review of their sequences, structure and evolution. *Biotechnology annual review, 1,* 315-371. https://doi.org/10.1016/S1387-2656(08)70056-5

Arbige, M. V., Shetty, J. K., and Chotani, G. K. (2019). Industrial enzymology: the next chapter. *Trends in biotechnology*, *37*(12), 1355-1366. https://doi.org/10.1016/j.tibtech.2019.09.010

Arpigny, J. L., and Jaeger, K. E. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochemical journal, 343(1),* 177-183. https://doi.org/10.1042/bj3430177

Baron, J. H. (1979). *The discovery of gastric acid. Gastroenterology, 76(5),* 1056-1064. https://doi.org/10.1016/S0016-5085(79)91338-6

Becker, P., Abu-Reesh, I., Markossian, S., Antranikian, G., and Märkl, H. (1997). Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus sp*. IHI-91 on olive oil. Applied *Microbiology and Biotechnology*, 48(2), 184-190. https://doi.org/10.1007/s002530051036

Bezuidt, O. K., Pierneef, R., Gomri, A. M., Adesioye, F., Makhalanyane, T. P., Kharroub, K., and Cowan, D. A. (2016). The *Geobacillus* pan-genome: implications for the evolution of the genus. *Frontiers in microbiology*, *7*, 723. https://doi.org/10.3389/fmicb.2016.0072

Blamey, J. M., Fischer, F., Meyer, H. P., Sarmiento, F., and Zinn, M. (2017). Enzymatic biocatalysis in chemical transformations: a promising and emerging field in green chemistry practice. *In Biotechnology of microbial enzymes (pp. 347-403).* Academic Press. https://doi.org/10.1016/B978-0-12-803725-6.00014-5

Blanco, A., and Blanco, G. (2017). *Medical biochemistry*. Academic Press.

Blat, Y. (2010). Non-competitive inhibition by active site binders. *Chemical Biology and Drug Design, 75(6),* 535-540. https://doi.org/10.1111/j.1747-0285.2010.00972.x

Bornscheuer, U. T. (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS microbiology reviews, 26(1),* 73-81. https://doi.org/10.1111/j.1574-6976.2002.tb00599.x

Brod, F. C. A. (2012). Expressão, purificação e caracterização bioquímica de lipase recombinante de *Staphylococcus xylosus* e esterase recombinante de *Lactobacillus plantarum.*

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., ... and Thim, L. (1991*).* A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature, 351(6326),* 491-494. https://doi.org/10.1038/351491a0

Burcu Bakir, Z., and Metin, K. (2017). Production and characterization of an alkaline lipase from thermophilic *Anoxybacillus sp*. HBB16. *Chemical and biochemical engineering quarterly, 31(3),* 303-312. https://doi.org/10.15255/CABEQ.2016.990

Castilla, A., Panizza, P., Rodríguez, D., Bonino, L., Díaz, P., Irazoqui, G., and Giordano, S. R. (2017). A novel thermophilic and halophilic esterase from Janibacter sp. R02, the first member of a new lipase family (Family XVII). *Enzyme and microbial technology*, *98*, 86-95. https://doi.org/10.1016/j.enzmictec.2016.12.010

Chapman, J., Ismail, A. E., and Dinu, C. Z. (2018). Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts*, *8*(6),238. https://doi.org/10.3390/catal8060238

Choi, J. M., Han, S. S., and Kim, H. S. (2015). Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnology advances*, *33*(7), 1443-1454. http://dx.doi.org/10.1016/j.biotechadv.2015.02.014

Choudhury, P., and Bhunia, B. (2015). Industrial application of lipase: a review. *Biopharm J*, *1*(2), 41-47.

Copeland, R. A. (2013). *Evaluation of enzyme inhibitors in drug discovery: a guide for medicinal chemists and pharmacologists.* John Wiley and Sons.

Cornish-Bowden, A. (2017). Enthalpy–entropy compensation and the isokinetic temperature in enzyme catalysis. *Journal of biosciences, 42(4),* 665-670. https://doi.org/10.1007/s12038-017-9719-0

Costa, E., Teixidó, N., Usall, J., Atarés, E., and Viñas, I. (2002). The effect of nitrogen and carbon sources on growth of the biocontrol agent *Pantoea agglomerans* strain CPA-2. *Letters in applied microbiology*, *35*(2), 117-120.

De Almeida, A. F., Taulk-Tornisielo, S. M., and Carmona, E. C. (2013). Influence of carbon and nitrogen sources on lipase production by a newly isolated *Candida viswanathii* strain. *Annals of Microbiology*, *63*(4), 1225-1234.

Drews, G. (2000). The roots of microbiology and the influence of Ferdinand Cohn on microbiology of the 19th century. *FEMS Microbiology Reviews*, 24(3), 225-249. https://doi.org/10.1111/j.1574-6976.2000.tb00540.x

Duza, M. B., and Mastan, S. A. (2013). Microbial enzymes and their applications–a review. *American Journal of Pharm Research*, *3*(8), 6208-6219.

Fernandez-Lorente, G., Rocha-Martín, J., and Guisan, J. M. (2020). Immobilization of lipases by adsorption on hydrophobic supports: Modulation of enzyme properties in biotransformations in anhydrous media. *In Immobilization of enzymes and cells (pp. 143-158)*. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-0215-7_9

Ferreira, A. N., Ribeiro, D. D. S., Santana, R. A., Santos Felix, A. C., Alvarez, L. D. G., Lima, E. D. O., ... and Nascimento Junior, B. B. D. (2017). Production of lipase from *Penicillium sp.* using waste oils and *Nopalea cochenillifera*. *Chemical Engineering Communications, 204(10),* 1167-1173. https://doi.org/10.1080/00986445.2017.1347567

Gandhi, N. N. (1997). Applications of lipase. *Journal of the American Oil Chemists' Society*, 74(6), 621-634. https://doi.org/10.1007/s11746-997-0194-x

Ghanem, E., and Raushel, F. M. (2007). Enzymes: *The active site*. eLS. https://doi.org/10.1002/9780470015902.a0000714

Guerrand, D. (2017). Lipases industrial applications: focus on food and agroindustries. *OCL Oilseeds and fats crops and lipids*, *24*(4), D403. 10.1051/ocl/2017031

Gupta, R., Kumari, A., Syal, P., and Singh, Y. (2015). Molecular and functional diversity of yeast and fungal lipases: Their role in biotechnology and cellular physiology. *Progress in lipid research, 57,* 40-54. https://doi.org/10.1016/j.plipres.2014.12.001

Gupta, S., Smith, T., Banaszak, A., and Boeckl, J. (2017). Graphene quantum dots electrochemistry and sensitive electrocatalytic glucose sensor development. *Nanomaterials, 7(10),* 301. https://doi.org/10.3390/nano7100301

Gurung, N., Ray, S., Bose, S., and Rai, V. (2013). A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed research international, 2013*. http://dx.doi.org/10.1155/2013/329121

Hasan, F., Shah, A. A., and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial technology, 39(2),* 235-251. https://doi.org/10.1016/j.enzmictec.2005.10.016

Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M. S., Ameri, A., Ghahremani, M. H., Khoshayand, M. R., and Faramarzi, M. A. (2012). Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. *Journal of the Taiwan Institute of Chemical Engineers, 43(5),* 670-677. https://doi.org/10.1016/j.jtice.2012.03.005

Homaei, A. A., Sariri, R., Vianello, F., and Stevanato, R. (2013). Enzyme immobilization: an update. *Journal of chemical biology, 6(4),* 185-205. https://doi.org/10.1007/s12154-013-0102-9

Hussein, A. H., Lisowska, B. K., and Leak, D. J. (2015). The genus *Geobacillus* and their biotechnological potential. *Advances in applied microbiology, 92*, 1-48.

Jaeger, K. E., and Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in biotechnology, 16(9),* 396-403. https://doi.org/10.1016/S0167-7799(98)01195-0

Jaeger, K. E., Dijkstra, B. W., and Reetz, M. T. (1999). Bacterial Biocatalysts: Molecular. Annu. *Rev. Microbiol, 53,* 315-51.

Jaeger, K. E., Ransac, S., Koch, H. B., Ferrato, F., and Dijkstra, B. W. (1993). Topological characterization and modeling of the 3D structure of lipase from *Pseudomonas aeruginosa*. *FEBS letters, 332(1-2),* 143-149. https://doi.org/10.1016/0014-5793(93)80501-K

Jegannathan, K. R., and Nielsen, P. H. (2013). Environmental assessment of enzyme use in industrial production–a literature review. *Journal of cleaner production, 42,* 228-240. https://doi.org/10.1016/j.jclepro.2012.11.005

Jo, E., Kim, J., Lee, A., Moon, K., and Cha, J. (2021). Identification and characterization of a novel thermostable GDSL-type lipase from *Geobacillus thermocatenulatus.* https://doi.org/10.4014/jmb.2012.12036

Jooyandeh, H., Amarjeet, K., and Minhas, K. S. (2009). Lipases in dairy industry: a review. *Journal of Food Science and Technology (Mysore), 46*(3), 181-189.

Joshi, M. D., Sidhu, G., Pot, I., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2000). Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *Journal of molecular biology, 299(1),* 255-279. https://doi.org/10.1006/jmbi.2000.3722

Kambourova, M., Kirilova, N., Mandeva, R., and Derekova, A. (2003). Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. *Journal of Molecular Catalysis B: Enzymatic, 22(5-6),* 307-313. https://doi.org/10.1016/S1381-1177(03)00045-6

Kamini, N. R., Fujii, T., Kurosu, T., and Iefuji, H. (2000). Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2. *Process biochemistry*, *36*(4), 317-324. https://doi.org/10.1016/S0167-7012(02)00161-6

Kapoor, M., and Gupta, M. N. (2012). Lipase promiscuity and its biochemical applications. *Process Biochemistry, 47(4),* 555-569. https://doi.org/10.1016/j.procbio.2012.01.011

Kim, K. K., Song, H. K., Shin, D. H., Hwang, K. Y., and Suh, S. W. (1997). The crystal structure of a triacylglycerol lipase from *Pseudomonas* cepacia reveals a highly open conformation in the absence of a bound inhibitor. *Structure, 5(2),* 173-185. https://doi.org/10.1016/S0969-2126(97)00177-9

Krahe, M., Antranikian, G., and Märkl, H. (1996). Fermentation of extremophilic microorganisms. *FEMS Microbiology Reviews, 18(2-3),* 271-285. https://doi.org/10.1111/j.1574-6976.1996.tb00243.x

Kuisiene, N., Raugalas, J., and Chitavichius, D. (2009). Phylogenetic, inter, and intraspecific sequence analysis of spo0A gene of the genus *Geobacillus*. *Current microbiology, 58(6),* 547-553. https://doi.org/10.1007/s00284-009-9366-2

Larbidaouadi, K., Benattouche, Z., and Abbouni, B. (2015). Screening selection identification production and optimization of bacterial lipase isolated from industrial rejection of gas station. *International Journal of Biotechnology Allied Fields*, *3*(9), 146-153.

Larios, A., García, H. S., Oliart, R. M., and Valerio-Alfaro, G. (2004). Synthesis of flavor and fragrance esters using *Candida antarctica* lipase. *Applied microbiology and biotechnology, 65(4),* 373-376. https://doi.org/10.1007/s00253-004-1602-x

Lee, D. W., Koh, Y. S., Kim, K. J., Kim, B. C., Choi, H. J., Kim, D. S., ... and Pyun, Y. R. (1999). Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiology Letters, 179(2),* 393-400. https://doi.org/10.1111/j.1574-6968.1999.tb08754.x

Lentini, V., Gugliandolo, C., and Maugeri, T. L. (2007). Identification of enzyme-producing thermophilic bacilli isolated from marine vents of Aeolian Islands (Italy). *Annals of microbiology, 57(3),* 355-361. https://doi.org/10.1007/BF03175073

León, I., Alonso, E. R., Mata, S., Cabezas, C., Rodríguez, M. A., Grabow, J. U., and Alonso, J. L. (2017). The role of amino acid side chains in stabilizing dipeptides: the laser ablation Fourier transform microwave spectrum of Ac-Val-NH 2. *Physical Chemistry Chemical Physics, 19(36),* 24985-24990.

Lesuisse, E., Schanck, K., and Colson, C. (1993). Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *European Journal of Biochemistry, 216*(1), 155-160.

Leuschner, C., and Antranikian, G. (1995). Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. *World Journal of Microbiology and Biotechnology, 11(1),* 95-114. https://doi.org/10.1007/BF00339139

Lin, J. H., Zhang, K. C., Tao, W. Y., Wang, D., and Li, S. (2019). *Geobacillus* strains that have potential value in microbial enhanced oil recovery. *Applied microbiology and biotechnology*, *103*(20), 8339-8350. https://doi.org/10.1007/s00253-019-10115-7

López-Fernández, J., Benaiges, M. D., and Valero, F. (2020). *Rhizopus oryzae* lipase, a promising industrial enzyme: Biochemical characteristics, production and biocatalytic applications. *Catalysts, 10(11),* 1277. https://doi.org/10.3390/catal10111277

Manachini, P. L., Mora, D., Nicastro, G., Parini, C., Stackebrandt, E., Pukall, R., and Fortina, M. G. (2000). *Bacillus thermodenitrificans* sp. nov., nom. rev. *International journal of systematic and evolutionary microbiology, 50(3),* 1331-1337. https://doi.org/10.1099/00207713-50-3-1331

McDonald, A. G., Boyce, S., and Tipton, K. F. (2015). Enzyme classification and nomenclature. *eLS, 1-11*. DOI: 10.1002/9780470015902

Meshram, A., Singhal, G., Bhagyawant, S. S., and Srivastava, N. (2019). Plant-derived enzymes: a treasure for food biotechnology. *In Enzymes in food biotechnology (pp. 483-502)*. Academic Press. https://doi.org/10.1016/B978-0-12-813280-7.00028-1

Mohammad, B. T., Al Daghistani, H. I., Jaouani, A., Abdel-Latif, S., and Kennes, C. (2017). Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. *International journal of microbiology, 2017*. https://doi.org/10.1155/2017/6943952

Najar, I. N., and Thakur, N. (2020). A systematic review of the genera *Geobacillus* and *Parageobacillus*: their evolution, current taxonomic status and major applications. *Microbiology, 166(9*), 800-816. https://doi.org/10.1099/mic.0.000945

Nardini, M., and Dijkstra, B. W. (1999). α/β Hydrolase fold enzymes: the family keeps growing. *Current opinion in structural biology, 9(6),* 732-737. https://doi.org/10.1016/S0959-440X(99)00037-8

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., ... and Ivanov, M. V. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermodenitrificans to Geobacillus* as the new combinations *G. stearothermophilus, G.* th. *International journal of systematic and evolutionary microbiology, 51(2),* 433-446. https://doi.org/10.1099/00207713-51-2-433

Nelson, D., and Cox, M. (2017). *Lehninger PRINCIPLES of BIOCHEMISTRY* (7 ed.). New York: W. H. Freeman and Company.

Nunes, P., Martins, A. B., Santa Brigida, A. I., Leao, M. M. D. R., and Amaral, P. (2014). Intracellular lipase production by Yarrowia lipolytica using different carbon sources. *Chemical Engineering Transactions, 38*, 421-426. https://doi.org/10.3303/CET1438071

Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., ... and Goldman, A. (1992). The α/β hydrolase fold. Protein Engineering, *Design and Selection, 5(3),* 197-211. https://doi.org/10.1093/protein/5.3.197

Palekar, A. A., Vasudevan, P. T., and Yan, S. (2000). Purification of lipase: a review. *Biocatalysis and Biotransformation, 18(3),* 177-200. https://doi.org/10.3109/10242420009015244

Parihar, D. K. (2012). Production of lipase utilizing linseed oilcake as fermentation substrate. *Int. J. Sci. Environ. Technol, 1(3),* 135-143.

Payen, A. P. J. F., and Persoz, J. F. (1833). Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels. *Ann. chim. phys*, *53,* 73-92.

Poli, A., Laezza, G., Gul-Guven, R., Orlando, P., and Nicolaus, B. (2011*). Geobacillus galactosidasius* sp. nov., a new thermophilic galactosidase-producing bacterium isolated from compost. *Systematic and applied microbiology*, *34(6),* 419-423. https://doi.org/10.1016/j.syapm.2011.03.009

Rahman, R. N. Z. R. A., Salleh, A. B., and Basri, M. (2006). Lipases: introduction. *New Lipases and Proteases*, *1*, 1-22. Nova Science Publishers, Inc

Ribeiro, B. D., Castro, A. M. D., Coelho, M. A. Z., and Freire, D. M. G. (2011). Production and use of lipases in bioenergy: a review from the feedstocks to biodiesel production. *Enzyme research, 2011*. https://doi:10.4061/2011/615803

Rios, N. S., Pinheiro, B. B., Pinheiro, M. P., Bezerra, R. M., dos Santos, J. C. S., and Gonçalves, L. R. B. (2018). Biotechnological potential of lipases from *Pseudomonas*: Sources, properties and applications. *Process biochemistry*, *75*, 99-120. https://doi.org/10.1016/j.procbio.2018.09.003

Sarmah, N., Revathi, D., Sheelu, G., Yamuna Rani, K., Sridhar, S., Mehtab, V., and Sumana, C. (2018). Recent advances on sources and industrial applications of lipases. *Biotechnology progress, 34(1),* 5-28. https://doi.org/10.1002/btpr.2581

Sarrouh, B., Santos, T. M., Miyoshi, A., Dias, R., and Azevedo, V. (2012). Up-to-date insight on industrial enzymes applications and global market. *J Bioprocess Biotech*, *4*, 002. http://dx.doi.org/10.4172/2155-9821.S4-002

Saxena, R. K., Sheoran, A., Giri, B., and Davidson, W. S. (2003). Purification strategies for microbial lipases. *Journal of microbiological methods*, *52*(1), 1-18. https://doi.org/10.1016/S0167-7012(02)00161-6

Schmid, R. D., and Verger, R. (1998). Lipases: interfacial enzymes with attractive applications. *Angewandte Chemie International Edition, 37(12),* 1608-1633. https://doi.org/10.1002/(SICI)1521-3773(19980703)37:12%3C1608::AID-ANIE1608%3E3.0.CO;2-V

Schomburg, I., Chang, A., and Schomburg, D. (2002). BRENDA, enzyme data and metabolic information. *Nucleic acids research*, *30*(1), 47-49. https://doi.org/10.1093/ nar/30.1.47

Scopes, R. K. (2001). *Enzyme activity and assays*. eLS.

Sigurgísladóttir, S., Konráòsdóttir, M., Jónsson, Á., Kristjánsson, J. K., and Matthiasson, E. (1993). Lipase activity of thermophilic bacteria from icelandic hot springs. *Biotechnology Letters, 15(4),* 361-366. https://doi.org/10.1007/BF00128277

Silva, A. G., and Guidini, C. Z. (2019). Lipases: sources, immobilization methods, and industrial applications. *Applied microbiology and biotechnology, 103(18),* 7399-7423. https://doi.org/10.1007/s00253-019-10027-6

Singh, A. K., and Mukhopadhyay, M. (2012). Overview of fungal lipase: a review. *Applied biochemistry and biotechnology, 166(2),* 486–520. https://doi.org/10.1007/s12010-011-9444-3

Sivamaran, P. (2021). Effectiveness of the proposed awareness teaching curriculum for behavioural support in caregivers with chronic obstructive pulmonary. *REX-ISSN 2321-1067,* 98.

Srinivasan, B. (2022). A guide to enzyme kinetics in early drug discovery. *The FEBS Journal.* https://doi.org/10.1111/febs.16404

Stoytcheva, M., Montero, G., Zlatev, R., A Leon, J., Gochev, V. (2012). Analytical methods for lipases activity determination: a review. *Current Analytical Chemistry*, 8(3), 400-407. https://doi.org/10.2174/157341112801264879

Sutay Kocabaş, D., and Grumet, R. (2019). Evolving regulatory policies regarding food enzymes produced by recombinant microorganisms. *GM crops and food, 10(4),* 191-207. https://doi.org/10.1080/21645698.2019.1649531

Talley, K., Alexov, E. (2010). On the pH-optimum of activity and stability of proteins. *Proteins: Structure, Function, and Bioinformatics, 78(12),* 2699-2706. https://doi.org/10.1002/prot.22786

Tao, Z., Dong, B., Teng, Z., Zhao, Y. (2020). The classification of enzymes by deep learning. *IEEE Access,* 8, 89802-89811.

Ugraş, S. (2017). Characterization of a thermophilic lipase from *Bacillus licheniformis* ht7 isolated from hayran thermal springs in giresun. *Romanian Biotechnological Letters*, 22(1), 12297-12306.

Underkofler, L. A., Barton, R. R., and Rennert, S. S. (1958). Production of microbial enzymes and their applications. *Applied microbiology,* 6(3), 212-221.

Van Den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current opinion in microbiology, 6(3),* 213-218. https://doi.org/10.1016/S1369-5274(03)00060-2

Van Oort, M. G., Deveer, A. M., Dijkman, R., Tjeenk, M. L., Verheij, H. M., De Haas, G. H., ... and Goetz, F. (1989). Purification and substrate specificity of S*taphylococcus hyicus* lipase. *Biochemistry*, *28*(24), 9278-9285.

Veerapagu, M., Narayanan, A. S., Ponmurugan, K., and Jeya, K. R. (2013). Screening selection identification production and optimization of bacterial lipase from oil spilled soil. *Asian J Pharm Clin Res*, *6*(3), 62-67.

Wang, J. L., Liu, P. (1996). Comparison of citric acid production by *Aspergillus niger* immobilized in gels and cryogels of polyacrylamide. *Journal of industrial microbiology,* 16(6), 351-353. https://doi.org/10.1007/BF01570114

White, J. S., White, D. C. (1997). *Source book of enzymes*. CRC Press.

Yada, R. Y. (Ed.). (2015). *Improving and tailoring enzymes for food quality and functionality*. Elsevier.

T.C.

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

SCIENTIFIC ETHICAL STATEMENT

I hereby declare that I composed all the information in my master's / doctoral thesis entitled THE EFFECT OF CULTURE CONDITIONS ON LIPASE PRODUCTION BY *GEOBACILLUS thermodenitrificans* HBB268 within the framework of ethical behavior and academic rules, and that due references were provided and for all kinds of statements and information that do not belong to me in this study in accordance with the guide for writing the thesis. I declare that I accept all kinds of legal consequences when the opposite of what I have stated is revealed. ……..

Princess Sindiswa SOKHULU

… / … / …

CURRICULUM VITAE

PERSONAL INFORMATİON

Last name First name **:** SOKHULU Princess Sindiswa

EDUCATION

Level	**Istitute**	**Date of graduation**
Bachelor's Degree Life-sciences (BIOLOGY)	Aydin Adnan Menderes University	21/09/2018