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Cloning, Expression and Biochemical Characterization of a Thermostable Lipase from Locally Isolate *Geobacillus stearothermophilus* BSR3

A Dissertation

Submitted to the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of philosophy in Biotechnology

By

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Summary:

In order to isolate *Bacillus stearothermophillus* , fifty soil samples were collected from the southern region of Iraq representing five Iraqi provinces including Basrah , Thiqar , Misan , AL_Mothanna , and Karbalaa respectively . From these soil samples, fifty four local isolates were obtained and subjected to identification according to their morphological, cultural, and biochemical tests.

Results showed that twenty nine of these isolates were identified as *Bacillus stearothermophillus*. The ability of these local isolates in lipase production was examined on LB-trybutyrin agar medium. Results showed that two of these isolates were lipase producers according to the diameters of hydrolysis zones. *B.stearothermophillus* BSR3 was selected for the molecular study because it was efficient in the production of thermostable lipase.

In attempt to clone thermostable lipase gene of BSR3 isolate in most familiar gram negative host (*E. coli*), lipase gene was amplified using specific primers designed to include genetic elements upstream and downstream lipase gene of the chromosomal DNA of standard strain of *B. stearothermophillus* (U78785) according to the information data [illegible] portion of nitrate reductase of *Geobacillus* sp.WCH70.

As a consequence of this result new sets of primers were designed according to the complete sequence of lipase gene of *Geobacillus* sp.WCH70 to amplify lipase gene of BSR3 to consist of specific control sequences upstream and downstream of lipase gene. The amplified gene fragment was cloned in pPCRScriptSK (+) cloning vector and sequenced according to chain termination method. Results showed that the amplified lipase gene was identical (99% identity) to lipase gene of *Geobacillus* sp.WCH70.

According to results of biochemical tests and DNA sequencing of lipase gene, locally isolated *B. stearothermophillus* BSR3 was regarded as *Geobacillus stearothermophillus* BSR3.

To study the expression system of BSR3 lipase gene in *E. coli*, different strategies were designed for gene amplification to consist of open reading frame with and without control elements needed for gene expression in new host and as follows :

1. Open reading frame without both leader sequence $(ll)^-$ and stop codon $(stp.cd)^-$, with *Nco*I/*Xho*I cohesive ends.
2. Open reading frame without leader sequence $(ll)^-$, but with stop codon $(stp.cd)^+$, with *Nco*I/*Xho*I cohesive ends.
3. Open reading frame with both leader sequence $(ll)^+$, and with stop codon $(stp.cd)^+$, with *Nco*I/*Xho*I cohesive ends.
4. Open reading frame with leader sequence $(ll)^+$, but without stop codon $(stp.cd)^-$, with *Nco*I/*Xho*I cohesive ends.

These fragments were amplified and cloned in pPCRScript cloning vector and sequenced to determine the exact coding sequence of lipase gene. The presence of [illegible] allows gene expression in host in comparison with coding region of *Geobacillus* sp. WCH70 lipase [illegible]. Results showed that the de[illegible] sequences for these four amplified fragments with coding region of *Geobacillus* sp. WCH70 was 99.98% and the molecular size of each of these fragments are 721, 721, 796, and 796 bp respectively after sequencing and analyzing on agarose gel.

To study the optimal gene expression of the four fragments of lipase gene these four fragments were double digested with *Nco*I/*Xho*I and *Nde*I/*Xho*I restriction enzymes respectively to generate these four fragments and subcloned in pET20b(+) expression vector , and transformed in *E.coli* BL21(DE3). Results showed that the clone harboring recombinant pET20b(+) containing lipase gene fragment with leader sequence$(ll)^+$ and stop codon $(stp.cd)^+$of native lipase gene gave maximum degree of gene expression and lipase production among other

clones of genetically engineered *E. coli* containing other forms of lipase gene; this clone was selected to study optimum conditions for lipase production .

Optimum conditions for lipase production by genetically engineered *E.coli* BL21(DE3) was studied under different nutritional and cultural conditions. Results showed that maximum lipase production was achieved after induction genetically engineererd *E. coli* BL21(DE3) with 1 mM of IPTG for 8 hours at 18C^{o} and pH8. Expression of lipase gene under these conditions was studied by analyzing protein profile of cell fractions on SDS-PAGE. Results showed that, the approximate molecular mass of lipase enzyme was about 25 Kb with sharp protein band on polyacrylamide gel in comparison with same band of cell fractions without induction with IPTG.

abbreviation	Mean
Blast	Basic local alignment search tools
CCRC	Culture Collection and Research Center
Da	Dalton
dNTP	Deoxyribonucleotide trisphosphate
DTT	Dithiothreitol
DI	Deionized water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Eth	Ethidium bromide
EDTA	Ethylenediaminetetracetic acid
GSC	Genetic Stock Center
IDT	Integrated DNA technologies
LB	Luria-bertani broth
mRNA	Messenger ribonucleic acid
MR-VP	Methyl red- Voges Proskauer
NCBI	National Center of Biotechnology Information
O.D	Optical density
Omp	Outer membrane proteins
PCR	Polymerase chain reaction
PUFAs	Poly Unsaturated Fatty Acids
RDL	*Rhizopus delemar* lipase
RBS	Ribosomal binding site
rpm	Round per minuet
S	Svedberg
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sp.	Species
TEMED N,N,N,N-	Tetramethylene diamine
TCAG	Centre for Applied Genomics institute
TAE	Tris- acetate electrophoresis buffer
TBE	Tris Base EDTA buffer
UV	Ultraviolet

Chapter One

1: INTRODUCTION

Over the past decade, there is an interest in thermophilic endospore forming bacteria of the genus *Bacillus*, because of their possible contamination of heated food products and of their biotechnological importance as sources of thermostable enzymes and other products of industrial interest. Genus *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive aerobic or facultative bacteria that are characterized by their ability to produce robust endospores in response to adverse environmental conditions. There is great diversity in physiology among members of the genus, whose collective features include degradation of many substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, antibiotic production, nitrification, denitrification, nitrogen fixation. *Bacillus* species are important organisms for both fundamental research and industrial applications (Smith *et al*., 1952; Slepecky, 1992; Senesi *et al*., 2001). Bacilli currently account for 60% of the commercially available proteins synthesized on an economical scale (Harwood, 1989). The majority of these proteins are naturally secreted into the growth medium, such as alkaline proteases amylases and lipases (Schallmey *et al*., 2004). Lipases are glycerol ester hydrolyses (E.C.3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface. During hydrolysis lipases pick acyl group from glycerides forming lipase-acyl complex, which then transfers its acyl group to OH group of water. However, in non-aqueous conditions, these naturally hydrolytic enzymes can transfer acyl groups of carboxylic acids to nucleophiles other than water (Martinelle and Hult, 1995). Thus lipases can acylate alcohols, sugar esters, thioesters and amides (Schallmey *et al*., 2004). To employ a lipase on synthetic job, it must be immobilized because soluble lipases lose their activity in non-aqueous reaction media. These synthetic properties allow wide spread applications in various field of biochemical and organic conversions (Poonam *et al*., 2002; Bruno, 2004). It is well known that lipases are the most widely

used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases (Gitlesen *et al.*, 1997). In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceuticals, food and leather industries. Promising fields for the application of racemic mixtures to produce optically active compounds (Gombert *et al.*, 1999). These functions of lipases owe to their broad specificity for a wide spectrum of substrates, stability in organic solvents and enantioselectivity (Snellman and Colwell, 2004; Gulati *et al.*, 2005). In view of variety in applications, there has been a renewed interest in the development of sources of lipases. Numerous species of bacteria, yeasts and moulds produce lipases with different enzymologyical properties and specificities but moulds are known to be more potent lipase producer. These microorganisms produce lipases both by solid substrate and submerged fermentations. *Bacillus* species have a huge ability to secrete enormous quantities of proteins directly into the growth medium, a factor that greatly facilitates downstream processing, production of heterologous proteins using bacterial expression [illegible] 1999; He *et al.*, 2004). The expression vector and host are important factors for achieving maximal expression of cloned genes however; molecular cloning of a foreign gene does not ensure that the gene will be expressed successfully. The most difficult problems in bacterial expression are proteolytic degradation and production of proteins that accumulate in misfolded forms, most often as inclusion bodies. However, *E. coli* expression systems are being overlooked as tools to produce recombinant proteins from both prokaryotic and eukaryotic origin. *E. coli* is not ideal due to its inability to secreted proteins in to the surrounding medium since they are localized in the periplasmic space resulting in the formation of inclusion bodies (Schallmey *et al.*, 2004). The formation of protein aggregates within the cell imposes metabolic burdens on the host and also complicates downstream processing which result in poor yield of proteins.

In contrast, Bacilli are well-known high-level producers of a variety of extracellular proteins. Their capacity to produce and secrete large quantities (20-25 g/l) of extracellular proteins has placed them amongst the most important industrial protein producers. These organisms continue to be dominant bacterial workhorses in microbial fermentations and have been extensively applied in the production of useful biochemicals, antibiotics, insecticides, and industrial enzymes. Certain species from this genus have been produced by commercial thermostable enzymes e.g. thermostable lipases (Vasantha *et al*., 1984; Chung *et al.*, 1992; Wong and Wu, 1999; Li *et al*., 2004). According to those mentioned above, this study is aimed:

1. Isolation of *Geobacillus stearothermophilus* from different environmental sources.
2. Identification of the local isolates according to their cultural, morphological, and biochemical characteristics.
3. Screening the ability of the isolates [illegible] lipase.
4. Molecular cloning of thermostable lipase gene from the selected isolate of *Geobacillus stearothermophilus* using suitable vector and hosts.
5. Studying some optimum condition for thermostable lipase produced by the new host.

Chapter Two

2: Literature Review

2.1: Genus *Bacillus*

Scientific classification
Kingdom : Bacteria
Phylum : Firmicutes
Class : Bacilli
Order : Bacillales
Family : Bacillaceae
Genus : *Bacillus*

Genus *Bacillus* is a large and diverse collection of aerobic and facultative anaerobic, rod-shaped, Gram-positive (to Gram-variable), endospore-forming bacteria. The genus includes thermophilic and psychrophilic, acidophilic and alkalophilic, freshwater and halophilic bacteria that utilize a wide range of carbon sources for heterotrophic growth or grow autotrophically. 16S rRNA gene sequence analysis has revealed high phylogenetic heterogeneity in this genus. Within phylogenetic groups that include the genera *Alicyclobacillus*, *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus*, *Virgibacillus*, *Salibacillus* and *Gracilibacillus*. Currently, the mesophilic aerobic spore-forming [illegible] the temperature [illegible] the genera *Bacillus*, *Alicyclobacillus*, *Brevibacillus*, *Aneurinibacillus*, *Sulfobacillus*, *Thermoactinomyces* and *Thermobacillus*. The majority of thermophilic species described belong to the genus *Bacillus* genetic groups 1 and 5, as judged from their 16S rRNA sequences. The thermophilic species *Bacillus smithii* and *Bacillus coagulans* fall into group 1, along with the type species of the genus, *Bacillus subtilis*, and other mesophilic species. *Bacillus tusciae* is related to the genus *Alicyclobacillus* and *Bacillus thermocloaceae* and *Bacillus thermosphaericus* represent distinct lineages. Group5 is a phenotypically and phylogenetically coherent group of thermophilic bacilli displaying very high similarity among their 16S rRNA sequences. This group comprises established species of thermophilic bacilli (*Bacillus stearothermophilus*, *Bacillus* thermocatenulatus, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus*

thermoglucosidasius and *Bacillus thermodenitricans*), species that have not been validly published (*Bacillus caldolyticus*, *Bacillus caldotenax*, *Bacillus caldovelox* and *Bacillus thermoantarcticus*) and the asporogenous species *Saccharococcus thermophilus*, representing a separate line of descent. (Ash et *al*., 1991; Rainey *et al*., 1994; Nicolaus *et al*.,1996; Sunna *et al*., 1997; Manachini *et al*., 2000).

Phylogenetic analysis has revealed that the genus *Bacillus* and its thermophilic members require extensive taxonomic revision characterization of these bacteria based on phenotypic features indicated that they belong to the genus *Bacillus*. Comparative 16S rDNA analysis revealed that isolates clustered with group 5 of the genus *Bacillus* and might represent two novel species. A new species, *Geobacillus subterraneus* and *Geobacillus uzenensis*, of the new genus *Geobacillus*, which comprises group 5 of the genus *Bacillus* and contains the transferred species *Geobacillus stearothermophilus*, *Geobacillus thermoleovorans*, *Geobacillus thermocatenulatus*, *Geobacillus kaustophilus*, *Geobacillus thermoglucosidasius* and *Geobacillus thermodenitrificans* (Stackebrandt *et al*., 1987; Razak *et al*., 1994; Rainey *et al*., 1994).

In the pioneering studies, several species have been validly described including *B. acidocaldarius*, *B. pallidus*, *B. thermocloacae* (Kosugi *et al*., 1988), *B. thermoglucosidasius* and *B. thermoleovorans* (Zarilla and Perry, 1987). Moreover, some other species such as *B. caldolyticus* , *B. caldotenax* and *B. caldovelox*, *B. thermocatenulatus* and *B. thermoflavus* (Heinen, 1972; Nazina *et al*., 1992, 1993, 1995, 2000, 2001; Rahman *et al*., 1994) were excluded from the Approved Lists of Bacterial Names and have lost their standing in bacterial nomenclature. This proliferation of species suggests that the thermophilic bacilli may be more diverse than originally considered and yet they have not been subject to comprehensive, taxonomic studies to the extent of their mesophilic counterparts (Heinen *et al,* 1972; Razak et *al,* 1994). This is surprising given the biotechnological importance of these bacteria as

sources of thermostable enzymes and other products of industrial interest. There are different species in Genus *Geobacillus* (*Bacillus*) (Daniel and Zeigler, 2001) as shown in figure (2-1).

Figure (2-1): Origin of *Geobacillus* species (Daniel and Zeigler, 2001).

2.2: Extracellular products of thermophilic microorganisms and applications

The application of thermostable enzymes in biochemical processes has shown many obvious advantages that include: enhancement of the reaction rate constant, increasing the diffusion and mass transfer rate as the medium viscosity decreases at high temperatures, increasing the solubility of the hydrophobic substrates and lowering the risk of contamination by pathogenic microorganisms. Furthermore, these thermophilic microorganisms, which may be used as safe cloning hosts, usually for producing enzymes of increased shelf-life via enhancement their thermal stability and resistance to chemical denaturation. (Kluskens *et al*., 2002) As a consequence, programs to isolate natural thermophilic microorganisms or genetic modification techniques to produce α-amylase and other thermostable enzymes are increasing around the world.

Amylase, for instance, has been produced by different bacterial species such as: *Geobacillus* (formerly *Bacillus*) *thermoleovorans*, *Bacillus licheniformis*, *Bacillus* sp., *Bacillus subtilis*, *Bacillus amyloliquefaciens*. The present contribution in this context, and deals with the isolation and characterization of amylase-producing bacteria from hot springs. (Nazina, 2001; Kudryashova *et al*., 1998)

The drawbacks of the industrial application of lipases such as their high cost of production and low stability can be overcome by exploring new sources of immobilization and activation of the biocatalyst among the numerous lipases described in the literature, only the enzymes belonging to few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions, and hence, they may be considered industrially relevant enzymes (Kim *et al*., 1997, 2002). Therefore, attempt to isolate microorganisms that produce thermostable lipase always gains attention since this

enzyme can be used in numerous, biotechnological processes of thermostable lipases from *Bacillus sp*. that are active in alkaline (pH 9.0–10.0).

Proteases are unusually stable under high temperature due to the fact that organisms producing them are adapted to high temperature environments. They are suitable candidates for applications in industrial processes that are performed under high temperature condition, such as leather processing, detergents, peptide synthesis, food industry and biotechnological applications. (Haki and Rakshit, 2003) They are also ideal model molecular for investigations aimed at elucidating the mechanisms of chemicophysical stability of proteins. Up to now, many thermophilic proteases have been isolated and characterized from thermophilic organisms (optimum growth temperature above 55 ◦C) (Kudryashova *et al*., 1998) as well as hyperthermophilic archaea. Some of them have been investigated owing to their physiological significance as well as their promise in biotechnological applications. A thermophilic bacterium strain YMTC 1049, which was identified as *Geobacillus sp*. (Hawumba *et al*., 2002). Strain YMTC 1049 enters the onset of stationary phase after growth for 24 h under [illegible] study of thermophilic extracellular protease, with reasonable growth yield in 24 h, these characteristics offer great promise for applications in industries such as thermostable extracellular protease named protease RH-1 purified from the thermophilic strain YMTC 1049. In order to explore the application of protease RH-1 in leather industry, detergents, sewage treatment and other industries, results showed that protease RH-1 was very stable and had high activity at high temperature. Resistance to denaturing agents and detergents also offers promise for protease RH-1 to be a good candidate for biotechnological and industrial applications. (Morikawa *et al*., 1994; Hawumba *et al*., 2002)

2.3: Enzymes thermostability and thermoactivity

Proteins from hyperthermophilic micro-organisms (living at>85°C) are thermostable in that they are usually able to withstand temperatures that would rapidly denature most proteins from mesophiles (living at 20-40°C) such as *Escherichia coli* or humans. (Cowan *et al*., 1987; Yokoyama and Matsui, 2005). In most cases, the stability is intrinsic to the protein, although in some hyperthermophilic Archaea this stability is augmented *in vivo* by the presence of high concentrations of intracellular metabolites. Thus, enzymes from hyperthermophiles are of considerable biotechnological interest as their enhanced stability could greatly reduce enzyme replacement costs or permit processes to be carried out at high temperatures. With the discovery of the Archaea, and in particular those members that can grow at temperatures in excess of 100°C, hyperthermophilic enzymology is a field of intense current interest and activity (Pignede *et al*., 2000). Enzymes from hyperthermophiles are [illegible] thermoactive. Almost all show temperature optima at or slightly above the growth temperature of the source organism (Unsworth *et al*., 2007). However, the specific activity of a thermoactive enzyme at its optimum temperature is often comparable with that of the homologous enzyme from a mesophile at its optimum. That is, it is generally observed that the temperature coefficients (the factor by which the rate of a reaction is increased on raising the temperature by 10°C) are usually around 2 for enzymic reactions, and from this one would predict a more than a 60-fold higher rate for the thermoactive enzyme at 100°C than that of the mesophile at37°C (Chavez *et al*., 1999). A variety of structural and compositional factors have been implicated in the origins of protein thermostability, but there is as yet no overall consensus explanation. However, it is clear that molecular flexibility is essential both for binding of substrates and for catalysis, and all enzymes must balance this need for flexibility with the requirements of stability (Zhu *et al*., 2003). Thermophilic proteins are thought to be less flexible than mesophilic

proteins at mesophilic temperatures. However, it is also proposed that both thermophilic and mesophilic proteins attain a similar degree of flexibility at their respective optimum temperatures (Charton *et al.*, 1991) Nevertheless, it could be that hyperthermophilic enzymes cannot achieve the degree of flexibility of their mesophilic counterparts and catalytic efficiency is thus reduced. However, in seeking a structural explanation for the thermostability and thermoactivity of enzymes from hyperthermophiles, an important and universal phenomenon has been overlooked, namely the temperature dependence of chemical equilibria (Scmid and Verger, 1998). All chemical equilibrium are dependent on temperature to some degree, and those of the ionizable amino acid side chains that are frequently found to be involved in enzymic catalysis are no exception. Although the pKa values of the carboxylic acid side chains of aspartate and glutamate alter little with temperature, the side chains of the basic residues histidine, lysine and arginine experience a downfield shift in pKa by as much as 2.5 units on going from 25 to 100°C (Jaenn and Berg, 1989). It is known that enzymes can fine-tune the pKa values of the ionizable residues by interac[illegible] native protein on folding, and this may have consequences for the enzyme's stability Therefore, it may be that hyperthermophilic enzymes cannot achieve the payment of this cost whilst retaining the necessary stability at extreme temperatures. Such temperature-induced downfield shifts in pKa values would have a detrimental effect on catalytic efficiency in the case of a histidine residue, for example, that was involved in proton abstraction and donation in the catalytic mechanism, and which therefore requires a pKa value close to the intracellular pH (Dennis, 1994). Hyperthermophilic organisms might solve the pKa problem in another way, that is, by adjusting their internal pH to a lower value to counterbalance the shifts in the pKa values of their proteins' essential amino acids (Fierke *et al.*, 1987). However, although few direct measurements have been made

on hyperthermophiles, it is thought that their internal pH values are generally between pH 6 and 7. The potential shifts in pKa values of amino acids with temperature will pose a difficult problem to the enzyme technologist who wishes to engineer a mesophilic enzyme to produce a thermophilic version able to operate at high temperatures, and such considerations might open up new areas of investigation in the field of hyperthermophilic enzymatic catalysis. However, in posing the question, 'Why then are thermophilic enzymes apparently such poor catalysts? Perhaps we are making an incorrect assumption, namely that they might be regarded as poor enzymes. Growth at 100°C is by no means feat, and the fact that some micro-organisms can achieve this is indicative of their possessing some very good and remarkable enzymes. Indeed, one might presume that these cells have enzymatic activities commensurate with the environments in which they grow (Walker *et al*., 1979; Tilbeurgh *et al*., 1993; Schmidt-Dannert *et al*., 1996). Given that life is thought to have arisen and initially evolved at high temperatures (>85°C), although that mesophily is an evolutionary development from thermophily (Stetter, 1996), one should consider [illegible] their high catalytic rates at [illegible]. Furthermore, given that the majority of the biosphere lives at 10°C or less, that consideration might be better addressed towards psychrophilic enzymes (Schrag *et al*., 1997).

2.4: Thermophiles in industry

The use of renewable resources and of thermophilic organisms for industrial processes is not new (Norton *et al*., 2007). During the early 1930s and during World War Π, as well as in the 1950s, biomass and thermophilic organisms were used in patented processes (McClements *et al*., 2009). The knowledge about the used microbes was not always very detailed and, in many cases, mixed anaerobic and aerobic cultures were used. Thermophilic microbes and their biochemistry have brought back and broadened the possibilities of the use of thermophiles and

extreme thermophiles in industrial processes (Fave *et al*., 2004). General aspects of the use of thermophilic organisms for technical applications and includes the following areas:

1. Thermophiles as sources of thermo stable proteins and enzymes.
2. Thermophiles as biocatalysts for conversion of biomass into fuel - related compounds and/or feedstock chemicals.
3. Thermophiles in leaching processes and waste management (Yu et *al*., 2010).

2.5: Lipases

Lipases are lipolytic enzymes found in many microorganisms, plants, and higher animals. The characteristic feature of all lipases is that although they are water-soluble, they catalyze heterogeneous ester hydrolysis at the lipid-water

Fats and oils are interface important macronutrients, they provide energy, deliver essential fatty acids and lipid soluble nutrients, and [illegible] component to many foods. The regulation of lipid bioavailability is receiving increasing attention because of the need [illegible] nutritional value of our food (Lundin and Gol[illegible]

Erlanson and Borgstrom, (1970) classify the lipids on the basis of their tendency to interact with water. The group of non polar lipids do not interact with water and will be present in aqueous environment either as a lens of oil or as a crystal (e.g. paraffin). The group of polar lipids can be subdivided into three classes:

- Class (I): is also known as insoluble non swelling amphiphiles and are exemplified by triglycerides, diglycerides and proton a Ted long-chain fatty acids.
- Class (II): polar lipids are insoluble swelling amphiphiles, such as phospholipids and monoglycerides.

In addition to forming a stable monolayer on the top of an aqueous

solution, these lipids can penetrate into the bulk aqueous phase in the form of liquid crystals.

- Class III polar lipids are the so called micelle forming or soluble amphiphiles (e.g. sodium or potassium salts of long-chain fatty acids and lysophosphatidylcholine) that form unstable monolayer as shown in figure (2-2) (Reis *et al*., 2008, 2010).

Lipases are lipolytic enzymes that play a key role in fat metabolism. They are catalysts for the hydrolysis of triacylglycerides, which contribute for major portion of calories (Bihovsky and Boudepudi, 1990). Due to the polar nature of oils and fats, the oil–water interface is where the control of lipolytic conversion and finally digestion takes place. It might come as a surprise that despite past efforts the interfacial behavior of lipases is not completely understood. The catalytic of lipases

carbonyl carbon of the esterified substrate forming an acyl-enzyme intermediate (Kim *et al*., 1998). Another important component of the catalytic mechanisms is the oxyanion-hole which is composed of properly arranged H-bond donors (mostly main-chain NH groups). The oxyanion hole helps to stabilize a reaction intermediate during catalysis when the carbonyl oxygen carries a partial negative charge. The active serine residue is embedded in the short consensus sequence GXSXG (with X being any amino acid), a motif also found in esterases and proteases (Kim *et al*., 2000). The active site of lipases in the "closed" form is shielded from the surface by protective surface loops called the "lid". Upon activation, the lid undergoes a conformational rearrangement exposing the active

site serine and creating the active, open form of the enzyme. In the past, the interfacial influence on lipase activity has been described through the "quality of the interface" which determines the behavior of the lipases and the outcome of the enzymatic reaction. However, determining the quality of the interface is ambiguous and has proven difficult to define. Additionally, a variety of interfacial phenomena were reported to have detrimental influence on both enzymes and reactions (Rua *et al*., 1998; Li *et al*., 2005; Leow *et al*., 2007). For example, enzymes irreversibly denature at interfaces under high interfacial tension conditions (low surface pressure) or are excluded from the interface under low interfacial tension conditions, respectively. On the other side, a large number of studies of lipases in micro emulsions show that lipases can be part of the interface and stay fully active at even very low interfacial tension. To better understand their biological roles, it is important to determine how the expression of their activities depends on the surrounding environment. In the case of lipases, this is f[illegible] defining experimental conditions such as concentrations of enzymes and substrates, [illegible] and temperature [illegible] substrates distributed non-uniformly within t[illegible]

Most thermostable lipases are from thermophilic *Bacillus* species. The characteristic pentapeptide sequence Gly-X-Ser-X-Gly around the catalytic serine residue is changed into an Ala-X-Ser-X-Gly sequence and the sequences around catalytic Asp and His are quite different from the other families and these lipases are the smallest proteins (MW 20,000) among all lipases (Kim *et al*., 2000). In addition, they have alkaline pH optima and have preference toward short chain triglycerides as substrates. Understanding the structural basis of thermostability and thermoactivity, and their interdependence, is central to the successful future exploitation of extremophilic enzymes in biotechnology (Choi *et al*., 2004).

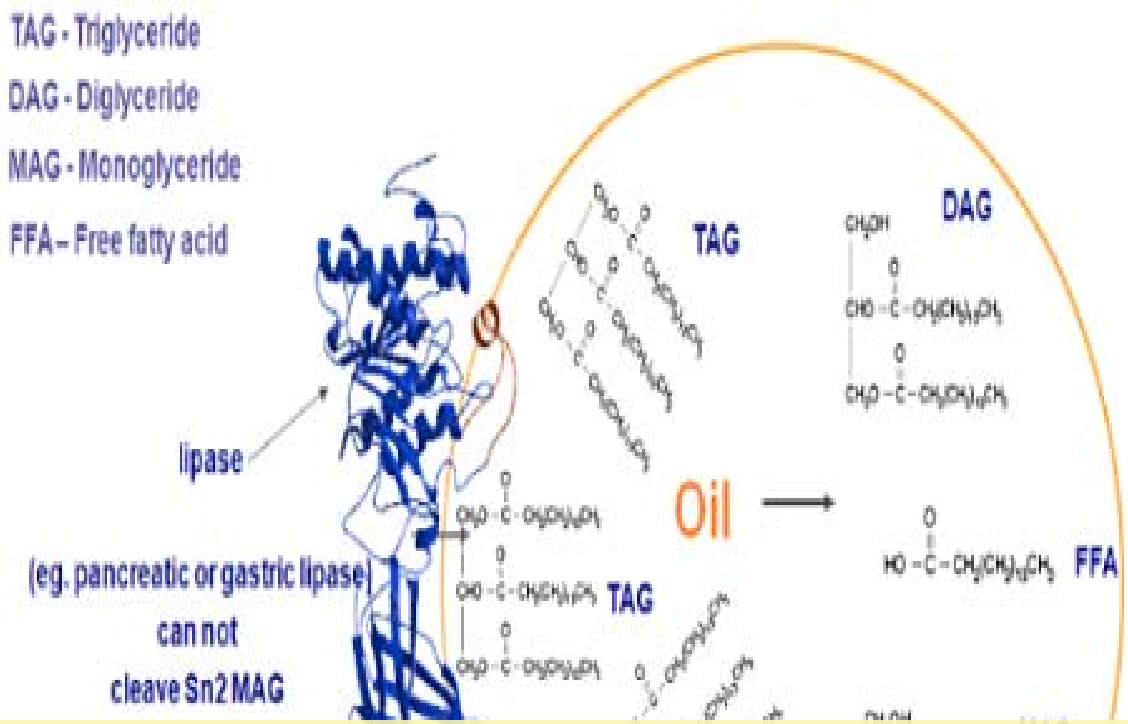


Figure: (2-2) Scheme of enzymatic reaction of pancreatic lipase with triglycerides, leading to triglycerides (TAG), which are mainly oil-soluble, monoglycerides (MAG) and free fatty acids (FFA), which are mainly water-soluble. (Reis *et al*., 2008, 2010)

However, the structural basis of thermostability is still not fully characterized. Ionizable residues play essential roles in proteins, modulating protein stability, folding and function. *Bacillus* extracellular lipase (*lip*A) is encoded by the *lip*A gene and is one of the smallest lipases with a molecular weight of 19.4 kDa. Lip A lipase does not show interfacial activation in the presence of oil ± water interfaces and is a typical thermophilic enzyme, showing optimum activity at 308 K and pH

8.0. *lip*A lipase activity varies between pH 7.0 and 10.0, decreasing strongly above pH 10.5 or below pH 6.5. However, *lip*A lipase exhibits remarkable thermostability at or below pH 5.0. The enzyme preheated at 328 K for 20 min at pH 4.0±5.0 retained almost 100% activity when assayed at pH 8.0, whereas when preheated at pH 10 it completely loses activity in 2 min. The ability of the protein to withstand high temperature decreases when the pH of preheating was beyond 4.0 to 10.0 (Shoeb *et al*., 2008).

2.6: Lipases in biotechnology

For several decades, lipases are already used in industry although the number of applications and therefore their importance to the enzyme manufacturing industry was rather small. It has been estimated earlier that from the worldwide enzyme market of 600 million US $, only some 20 million is accounted for by lipases. However, the production technology of lipases has made great progress in the last 5 years, mainly as a result of recombinant DNA technology by which it is possible to overproduce lipases in suitable microbial systems in an economically attractive way. As a consequence, different lipases from as many different microorganisms are now available and especially the detergent industry did benefit from this development and is by now the largest application area of industrial lipases. Remarkably, all commercial applications of lipases thus far concern enzymes of fungal or yeast origin. This does not imply inferior characteristics of bacterial lipases, but can be explained by the fact that the food applications, which until recently exceeded the number of non-food applications, prefer the use of lipases from fungal origin because of their proven GRAS-status (Generally Regarded As Safe) (Stead, 1986; Arbige and Pitcher, 1998; Li *et al*., 2004). However, there are various applications, bacterial lipases are as good as, or sometimes to be preferred to, their eukaryotic counterparts, in the following, the actual application of lipases is stated below:

In detergents, only the hydrolyzing capability of lipase is relevant, whereas in the processing of fats and oils as well as in organic synthesis both hydrolysis and synthesis reactions are of importance. This product contains the extracellular lipase from *P. alcaligenes*, at which the enzyme is active at pH 7-11 and at temperatures up to 60°C hydrolyzing triglycerides with chain lengths varying from C2 to C18 (Lenting *et al*., 1993). However, the highest activity is observed with longer chains (> C12). Many literatures describe several other bacterial lipases which were screened for detergent applications such as those from *Pseudomonas* and *Bacillus sp*. processing of fats and oils, both hydrolysis and synthesis take place. The number of applications in which lipase is used as in Table (2-1), however, is still limited when compared with the cheaper chemical processes.

2.7: Lipase applications

2.7.1: Flavour and food industry:

The variety of flavours and fragrances used in foods and perfumery are esters, some of which are [illegible] esters. Many [illegible] synthesized [illegible] lipase biocatalysis [illegible] and mild reactions. Although many flavour esters are composed of acids and alcohols very different from fatty acids and glycerol (branched, cyclic, aromatic, very short chain) lipases may still accept these. Synthesis of terpene alcohol esters by various lipases has been reported, as well as the continuous synthesis of ethyl butyrate (pineapple/banana flavour) by immobilized *Candida rugosa* lipase. By alcoholysis of butter fat and ethanol, e.g. with *Candida rugosa* lipase. This type of reaction provides a mixture of fatty acid ethyl esters, which may be combined with natural components in foods, e.g. margarine and cookies (Gupta *et al*., 2004). The oil industry is widely interested in the application of lipase to the neutralization of oil acidity.

The acidity of tropical oils is due to the presence of free fatty acids. The content of these acids can be depressed by partial esterifications by lipases. The

Soya-bean oil changes its flavour during storage and during the frying process giving rise to a typical fish smell. The Soya-bean oil flavour instability is due to the linoleic acid. At low temperature *Rhyzomucor miehei* lipase has shown specificity towards polyunsaturated fatty acid, by decreasing the linoleic acid to 3% content by biocatalised transesterefication at 10 ˚C (Nthangeni *et al*., 2005). It is in the prostaglandin path-synthesis and it is rare in edible oils. In order to obtain a fraction with a higher content of γ-linoleic acid, from evening primrose oil as ester, turnip lipase was employed (Wentzel *et al*., 2001). Using similar techniques it is possible to get hydrochloric products enriching oil with short and medium chain-length fatty acids. Specific enzyme catalysts may be used for interesterification, in this field, another example of lipase application in the cookies industry is cocoa butter production. This lipid contains a high amount of stearic acid and is composed of 1-palmitoyl-2-oleyl-3-stearoyl glycerol (Geresh *et al*., 1990).

Table (2-1): Biotechnological application of bacterial lipases.

Type of reaction	Origin of lipase	Product (application)	References
Hydrolysis of fats and oils	*Pseudomonas*		Kosugi et *al*., 1988
Glycerolysis of fats and oils	*Pseudomonas*	Monoacylglycerols (surfactants)	Yang *et al*., 1994
Estraification to glyceride	*Chromobacterium riscosum*		Yu *et al*., 2010
	Pseudomonas flaoresceus		Yu *et al*., 2010
(Trans) esterification to Immobilizedglycerol	*Chromobacterium Riscosum*		Berger and Schneider, 1992, 1993
Acylation of sugar Alcohols	*Chromobacterium Riscosum*	Sugar monoacylesters (surfactants)	Chopineau *et al*., 1988
Acidolysis / Alcoholysis Of fish oils	*Pseudomonas*		Zuyi and W[illegible]3; Ada[illegible] *et al*., [illegible]
Resolution of racemic	*Arthrobacter*	Building blocks for	Margolin, 1993; Milsuda *et al*., 1988
		Insecticides	
		Drugs	
Diesters with diols	*Pseudomonas*	(alkyds polyester intermediates)	1991
	Pseudomonas	Macrocyclic Lactones	Zhi-wei *et al*., 1988
Transesterification of Monosaccharides	*Pseudomonas* (*cepacia*)	Acrylate esters (polyacrylate Intermediates)	Margolin et *al*., 1987; Ikeda *et al*., 1993
Intramolecular Esterification	*Pseudomonas*	Macrocrlic lactones	Makita et *al*., 1987; Zhi-wei *et al*., 1988

An alternative source of it at cheaper cost is palm oil. In fact palm oil is composed of 1, 3- dipalmitoyl-2-oleyl glycerol. The 1, 3 regioselective transesterification, catalyzed lipase from *Humicola lanuginose* lipase show the same reagoselectivity, it is able to biotransform olive oil into 1, 3-distearyl-2-oleyl glycerol. In the same way the immobilized lipase from *Humicola lanuginose*, a thermostable enzyme, catalyses the transesterification of olive oil into 1, 3-distearyl-2-oleyl glycerol at 50°C in 30 h with 65% yield in stearic acid (Stead ,1986; Babayan, 1987). Flavour development of cheeses is to a great extent due to the ability of lipases to modify milk fat by partial hydrolysis. Specific free fatty acid profiles are generated by naturally occurring microbial lipases or enzymes added for flavour enhancement. In the first case, moulds involved in the ripening of certain varieties of cheeses, e.g. *Penicillium sp.*, produce lipases. *Penicillium roqueforti* lipases have short-chain fatty acid specificity and the liberation of a suitable (Sarma *et al.*, 1991)

Balance of mainly short-chain, volatile fatty acids seem to be the general effect behind characteristic flavor. Pre-gastric esterases are used in the manufacture of Italian cheeses to produce the characteristic piquant flavour.

Flavour development is usually the result of a very small degree of lipid hydrolysis (few percent) whereas a higher degree may lead also to a change in the physical properties of the fat. Such partial hydrolysis of oils and fats can improve the palatability and digestibility of the lipid component in feeds and pet foods. Hard fats may be softened due to mono- and di-glycerides and more energy supplied to the animals if the lipid is "pre-digested" (Stead, 1986).

2.7.2: Detergent industry

Fatty acids and oil soiling are difficult to remove from fabrics at low washing temperatures. Therefore, intensive research efforts have been directed towards

development not only of new detergent compounds and surfactants but also toward enzymes which could facilitate removal of this type of soiling under wash conditions (Venegas, 1993). Hydrolysis of triglycerides and other fatty esters does not increase the water solubility of the lipids as readily as in the case of protein and starch hydrolysis. A detergent lipases require a high alkaline stability (common powder detergents pH is 9-10), are thermostable enzymes, stable enzymes, stable in the presence of protease and compatible with common detergents (Sarma *et al*., 1991). The first detergent lipase on the market was lipolase, introduced early in 1988 which was also the first industrial enzyme produced by recombinant technology (Venegas, 1993).

2.7.3: Pulp and paper

Enzyme treatments, including the hydrolytic application of lipases, have recently been taken up by the paper and pulp industry. Although lipids constitute a very small part of wood, they may have important effects. In spruce, about 2 % resin is found. The resin is a mixture of [illegible] components. The resin content is reduced to [illegible] processes, however a more complete resin removal is possible by the addition of lipase in the process, with improved water absorption characteristics of cellulose fibers as the result (Shah *et al*., 2007; Nawani and Kaur, 2007). The resin content may also influence other properties, e.g. paper strength or color and result in paper making processes (the formation of suspension of wood fibers). A lipase preparation, Resinase A is produced by Novo and used in pulping processes. Printed paper can be treated by lipase in order to facilitate removal of color from the printing ink, containing vegetable oil base. This application can be useful for the improvement of recycle paper (Mohana *et al*., 2008).

2.7.4: Chiral switches

There is an increasing trend towards the use of optically pure steroisomers with pharmaceutical activity, which are more target-specific and show fewer side-

effects than racemic mixtures of isomers. This leads to an increasing demand for efficient processes for the industrial-scale synthesis of optically active compounds and to an increased research and production of chiral drugs, for instance, lipases or esterases employment the biocatalytic strategy to the resolution of racemic mixture can be applied to compounds containing alcohols, esters and acid moieties. If chiral or prochiral substrates are being used, it is usually just one enantiomer which undergoes reaction, thus leading to the chiral resolution of racemates. A number of wild-type and recombinant lipases for the ability to catalyze the transesterification reactions between a secondary alcohol and vinylacetate (Makita *et al*., 1987; Milsuda *et al*. 1988; Zhi-wei *et al*., 1988; Margolin, 1993).

2.8: Lipase and lipid chemistry

The natural substrates of lipases are triacylglycerol and, as a consequence, most applications of lipases deal with the modification of lipids. The reaction chemistry of lipase hydrolysis allows using these enzymes for hydrolysis as well as for the synthesis or interesterification of triacylglycerols. Besides lipase regioselectivity, chain-length and fatty acid specificity, the production of structured lipids with a defined distribution of fatty acids along the glycerol backbone (e.g. coca-butter equivalent) and the synthesis of emulsifiers like mono- or diglycerides (MAG or DAG) were extensively investigated. As shown by Gupta *et al*. (2004), lipases from *Rhizopus sp*. Have a strict 1, 3-regioselectivity in nonpolar organic solvents where acyl migration is slow.

Recently, structured lipids containing essential fatty acids, especially polyunsaturated fatty acids (PUFA) like docosahexaenoic or eicosapentaenoic acid have been targets of lipase research. Fish oils contain up to 30% PUFA, mainly in the form of triacylglycerols, and are sensitive to elevated temperatures. Resulting

in side reactions such as oxidation, *cis-trans* isomerization or double- bond migrations (Zuyi *et al.*, 1993; Adaehi *et al.*, 1993).

2.8.1: Hydrolysis

The conventional chemical fat-splitting processes require rather harsh conditions with respect to temperature (240-260°C) and pressure (60 bar). This inevitably produces undesirable side effects, like product discoloration and degradation of some fatty acids. However, due to the cheapness and efficiency of the chemical process, enzyme applications may be economically competitive only in some special cases. Thus far, only in one case application of lipase for common fat or oil hydrolysis on small industrial scale has been reported (Franken *et al.*, 1991). Oil and Fat Companies. has used a fungal lipase for the manufacturing of soap. However, fungal lipases are not necessarily the best choice, since *Pseudomonas* lipase(s) were shown to be superior to various fungal lipases for large scale hydrolyses of beef tallow and castor oil (Sugi *et al.*, 1988; Martinelle et al., 1993; Adachi, 1993).

2.8.2: Glycerolysis

Glycerolysis are of fats and oils are used for the commercial production of monoglycerides, which are applied as emulsifiers in wide range cosmetic and pharmaceutical products (McNeill and Berger, 1993). It is not likely that this process will be replaced by an enzymatic process in the near future (McNeill and Yamane, 1991). Mixtures of mono- and diacylglycerols are formed; high yields of monoglyceride are obtained with *Pseudornonas* lipases.

2.8.3: Esterification

Glycerides can also be obtained by direct esterification of free fatty acids to glycerol, as in figure (2-4) (Karl-Erich *et al.*, 1994). However, esterification

catalyzed by various microbial lipases always resulted in mixtures of glycerides, with yield and composition of the mixture depending on the source of lipase.

A process resulting in regioisomerically pure glycerides has been developed comprising as an essential step in the adsorption of glycerol onto a solid support. Lipase-catalyzed glyceride synthesis with the immobilized glycerol and various acyl donors (e.g. free fatty acids, fatty acid alkylesters, natural fats and oils) yielded multigram quantities of regio-isomerically pure di- and monoacylglycerols. lipases producing the desired glycerides with high yield. Monoglycerides were separated from the other reactants in a separate vessel and the undesired products were fed back to the reactor (Hassan et *al*., 2010).

2.8.4: Alcoholysis / acidolysis

Using acylacceptors other than glycerol additional mono-acylcompounds can be synthesized. For example, alcoholysis of sugar alcohols with various plant and animal oils has been shown to yield sugar monoesters of fatty acids. Among various lipases tested, [illegible] is the [illegible] hampering commercial application of enzymes also in this field. On the contrary, refinement of oils containing highly unsaturated fatty acids (PUFAs, poly-unsaturated fatty acids) may be a process with prospects for enzyme application on a commercial scale, because PUFAs are easily subject to decomposition in the chemical process, yielding undesirable oxidation products and polymers. Since fish oils possess poly-unsaturated fatty acids predominantly at the 2-position and this bond is relatively resistant to lipase attack, 1, 3-specific lipases can be particularly useful in the concentration of polyunsaturated fatty acids in monoglycerides (Zuyi *et al*., 1993), this has been shown in the case of enzymatic alcoholysis of cod liver oil and acidolysis of sardine oil, using various microbial lipases (Karl-Erich *et al*., 1994).

2.8.5: Interesterification

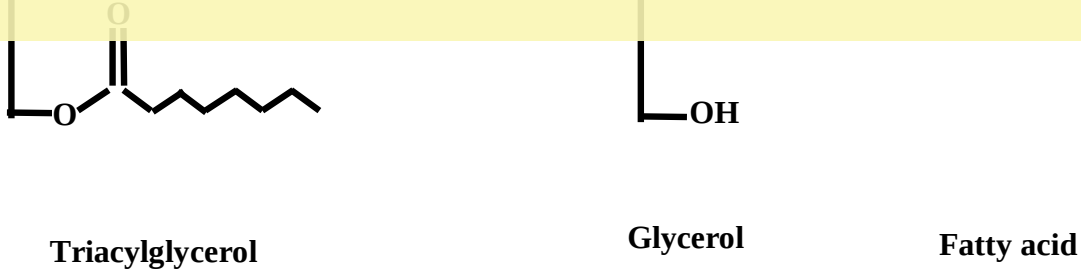
A few lipase-catalyzed synthesis reactions in low-water environment have found (limited) application on commercial scale. An example is the transformation of low-value oils, like the palm-oil mid fraction, into high-value cocoa butter triglycerides by interesterification.This process is carried out using the immobilized lipase from *Rhizomucor miehei*. Another class of so-called structured triglycerides which may be a potential product of a lipase-catalyzed interesterification reaction are glycerides containing medium-chain fatty acids on the 1- and 3-positions and an essential fatty acid on the 2-position. They can form an alternative for the medium chain triglycerides which are currently used to meet the nutritional needs of patients with real absorption problems, because shortage of essential fatty acids in these patients can easily occur due to sole consumption of medium-chain lipids (Babayan, 1987). *Rhizopus delemar* (RDL) was used to produce a cocoa-butter like fat by interesterification between olive oil and stearic acid in hexane. The highest activity was found for RDL adsorbed on silica followed by entrapment with photo cross-linkable resin polymer resin [illegible] stearic acid. figure (2-5) showed structured lipids [illegible] of linseed oil with caprylic acid (Karl-Erich., *et al.*, 1994).

2.9: Mechanism of lipase action

Hydrolysis of fats and oils (triacylglycerols) is an equilibrium reaction and therefore it is possible to change the direction of the reaction to ester synthesis by modifying the reaction conditions, as it shown in figure (2-3).

The equilibrium between forward and reverse reactions in this case is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyze ester synthesis reactions. Different types of synthesis reactions can be distinguished: common ester synthesis from glycerol and fatty acids and the biotechnologically more important transesterification reactions in which the acyl donor is an ester as it shown in figure (2-4).

Transesterifications involving fats and oils can further be specified depending on the type of acyl acceptor. Glycerolysis and alcoholysis refer to the transfer of an acyl group from a triglyceride to either an alcohol or glycerol. In interesterifications, the acyl group is exchanged between a (tri) glyceride and either a fatty acid (also called acidolysis) or a fatty acid ester (more specifically another (tri) glyceride). In teresterifications require a small amount of water, in addition to the amount needed for the enzyme to maintain an active hydrated state. As the presence of (too much) water will decrease the amount of ester synthesis products, the water content should be carefully adjusted to achieve accumulation of desired reaction products.



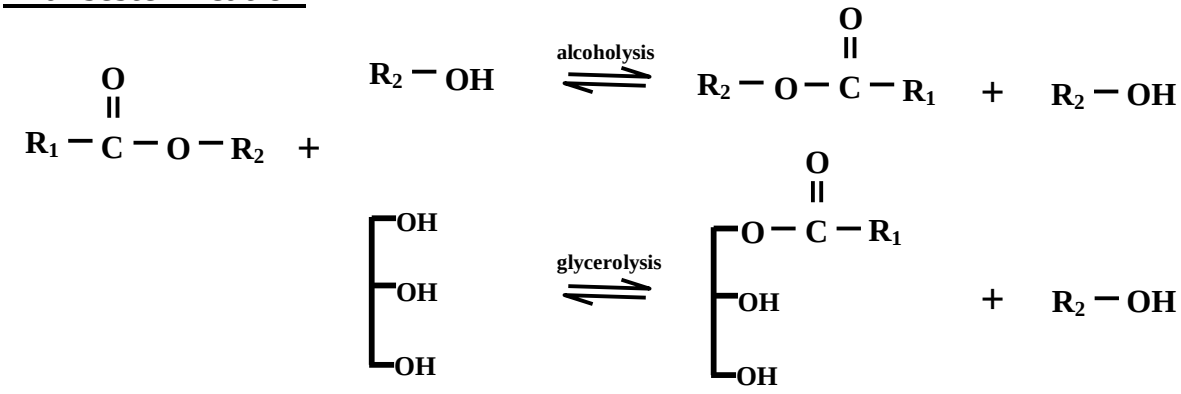
Triacylglycerol **Glycerol** **Fatty acid**

Figure (2-3): Enzymatic reaction of a lipase catalyzing or synthesis of a triacylglycerol substrate. (Karl-Erich *et al.*, 1994)

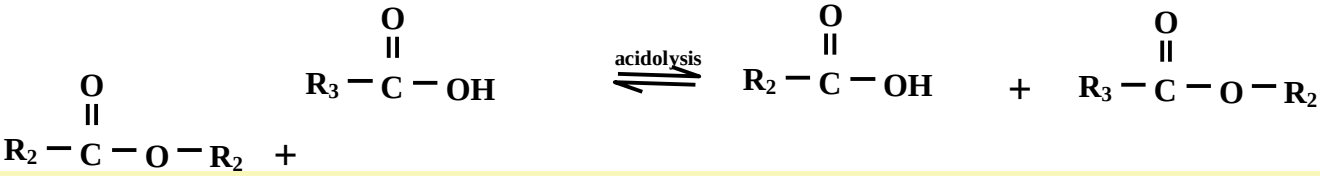
2.10: Over expression of lipase

cDNA-derived clones of *E. coli* expressing sometimes showed low activity on tributyrin plates. Under of the *lac*-promotor on pUC8-2.14 cloning vector, a low-level expression of a clone lipase from *Rhizopus oryzae* (ROL) in *E. coli* was achieved (Wingender *et al*., 1987). Visualization of the expression of a clone lipase on SDS-gels by immunoblotting resulted in bands with sizes 45 and 39 kDa, corresponding to the preproenzyme, respectively. Similar results were found for recombinant ROL (Ransac *et al*., 1999), indicating that *E. coli* is unable to fully process the preproenzyme to its mature product. The genes encoding the ROL and RDL were therefore cloned into high-level expression vectors, in order to allow for the production of amounts of recombinant lipase upon translocation into the periplasmic space is toxic for the *E. coli* cells. Expression of mature lipase was possible only if a tightly controlled promoter was used, or cytoplasmically, which resulted in expression of mature inactive lipase as inclusion bodies [illegible] *et al*., 1995). However, expression of the active prolipase into the periplasm was not toxic for *E. coli*. Using a pET vector under a [illegible] as inactive inclusion bodies (Tabor and Rich [illegible] Both mature and precursor lipase can fuse to an *omp*A-leader sequence, which directs expression of the lipase into the periplasm. Using this system, expression levels of 20-30% and 10-20% can be obtained for the mature (Studier *et al*., 1986; Fierke *et al*., 1987).

Transesterification



Interesterification



2.11: Gene cloning and characterization of thermostable lipase from *B. stearothermophilus*

Lipase is an enzyme that hydrolyzes the ester bonds in triglyceride to liberate fatty acids and glycerol in aqueous systems. This enzyme is also active in organic solvents, greatly expanding its industrial application range.

Bacillus stearothermophilus L1 lipase is a well characterized thermoalkalophilic lipase. (Kim *et al*. 1998) expressed L1 lipase in a recombinant *Escherichia coli* and investigated its properties. Recombinant L1 lipase produced by *E. coli* showed its thermoalkalophilic enzyme properties were most active at 60–65°C and around pH 9–10. (Kim et *al*., 2002)

The gene coding for an extracellular lipase of *Bacillus stearothermophilus* L1 was cloned in *E. coli*. (Kim et *al*., 2002) Sequence mentioned in figure (2-5) analysis showed an open reading frame of 1254 bp, which encodes a polypeptide of 417 amino acid residues. The polypeptide of a signal sequence (29 amino acids) and a mature protein of 388 amino acids. An alanine replaces the first glycine in the conserved pentapeptide (Gly--X-Ser-Gly) around the active site serine. The expressed lipase can be purified by hydrophobic interaction and ion exchange chromatography and this lipase had highest activity to hydrolyzed beef tallow and palm oil more than olive oil at 50 C. Strain (L1) of *B. stearothermophilus* produce extracellularly both esterase (50 kDa) and lipase (43kDa) which were found through electrophoresis of culture and [illegible] zymogram before it, because of its low le[illegible]

The mature *B. stearothermophilus* L1 lipase was secreted into the culture medium as an active form from *S. cerevisiae* and the secreted enzyme showed thermoalkalophilic enzymatic properties similar to that produced by *E. coli* (Rhee *et al*., 2005).

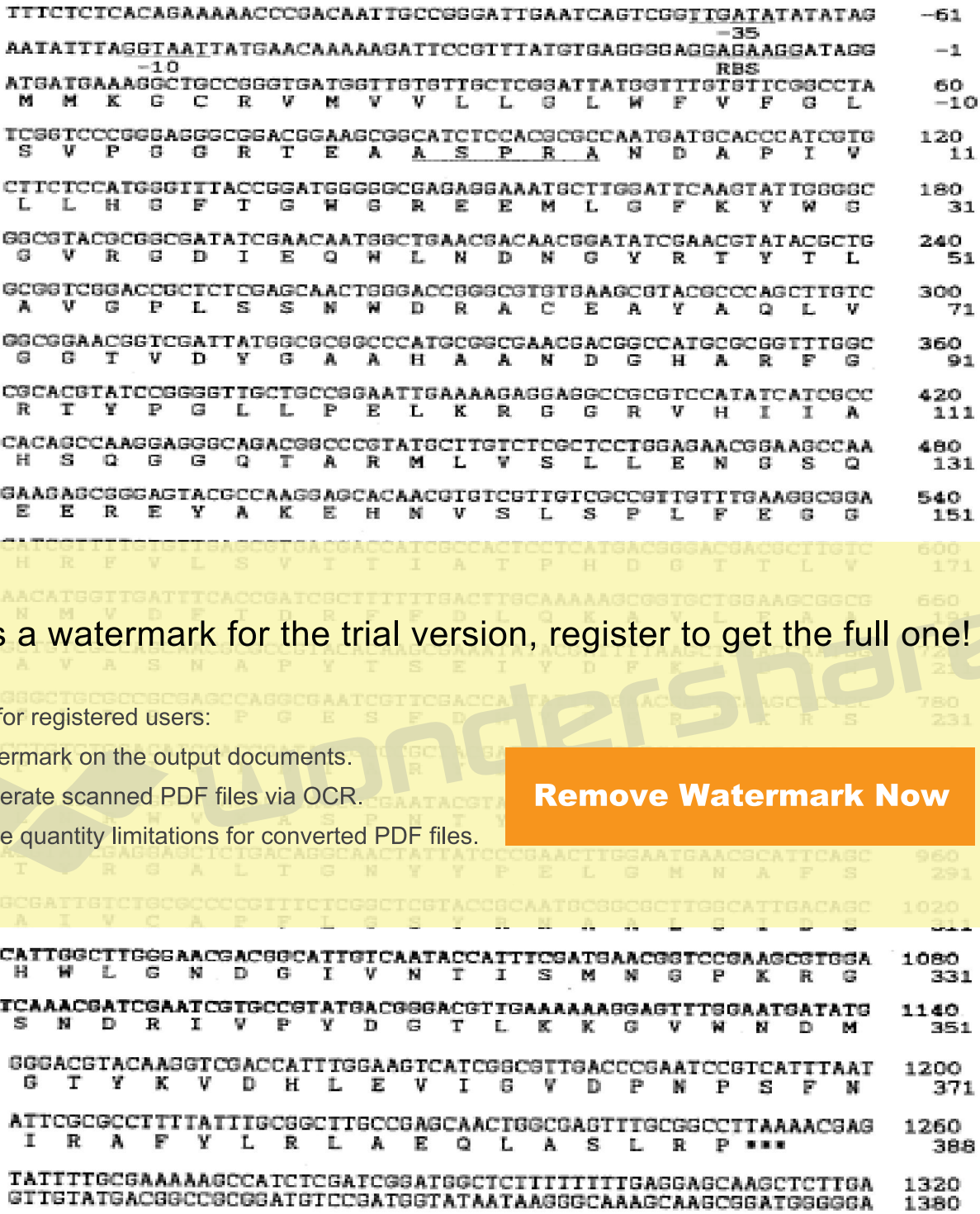


Figure (2-5): Nucleotide sequence of the *B. stearothermophilus* L1lipase gene and its deduced amino acid sequence. The numbering of nucleotides starts at the 5' end of the lipase gene and that of amino acids at the N-terminus of mature enzyme. The putative -35, -10, ribosomal binding site (RBS), and stop codon(*) are shown. The N-terminal amino acid sequence from the purified L1 lipase is underlined. (Kim et *al*., 2002)

2.12: The pET Expression System

Expression systems are designed to produce many copies of a desired protein within a host cell. In order to accomplish this, an expression vector is introduced into a host cell. This vector contains all of the genetic coding necessary to produce the protein, including a promoter appropriate to the host cell, a sequence which terminates transcription, and a sequence which codes for ribosome binding site (Oehler *et al.*, 1990). One expression system was developed in 1986 by Studier and Moffatt, who created RNA polymerase expression system which was highly selective for bacteriophage T7 RNA polymerase. The initial system involved two different methods of maintaining T7 RNA polymerase into the cell - in one method, a lambda bacteriophage was used to insert the gene which codes for T7 RNA polymerase, and in the other, the gene for T7 RNA polymerase was inserted into the host chromosome (Dubendorff and Studier, 1991). This expression system

2.13: Producing Proteins with the pET Expression System

2.13.1: Designing the pET Plasmid:

A pET vector is a bacterial plasmid designed to enable the quick production of a large quantity of any desired protein when activated. This plasmid mentioned in figure(2-6) contains several important elements - a *lac*I gene which codes for the *lac* repressor protein, a T7 promoter which is specific to only T7 RNA polymerase (not bacterial RNA polymerase) and also does not occur anywhere in the prokaryotic genome, a *lac* operator which can block transcription, a polylinker, an f1 origin of replication (so that a single-stranded plasmid can be produced when co-infected with M13 helper phage), an ampicillin resistance gene, and a *Col*E1

origin of replication (Rosenberg *et al*., 1987). To start the process, your favorite gene (lipase) is cloned into a pET plasmid at the polylinker site. Both the T7 promoter and the *lac* operator are located 5' to lipase. When the T7 RNA polymerase is present and the *lac* operator is not repressed, the transcription of lipase proceeds rapidly. Because T7 is a viral promoter, it transcribes rapidly and profusely for as long as the T7 RNA polymerase is present, as shown in Figure (2-6): the map of pET Vector. The expression of your favorite protein (lipase) increases rapidly as the amount of mRNA transcribed from lipase increases. Within a few hours, lipase is one of the most prevalent components of the cell (Studier, 1991).

Figure (2-6): The pET Vector. contains a drug resistant marker for ampicillin resistance (green), the *lac*I gene (blue), the T7 transcription promoter (red), the lac operator region (pale green) 3' to the T7 promoter, and a polylinker region (black). Also, there are two origins of replication - one is the f1 origin which enables the production of a single stranded vector under appropriate conditions, and the other is the conventional origin of replication (Tabor *et al*., 1985).

2.13.2: Controlling the pET Expression System

One of the most important parts of the pET Expression System involves the fact that lipase is not transcribed unless the T7 RNA polymerase is present. Prokaryotic cells do not produce this type of RNA, and therefore the T7 RNA polymerase must be added. Usually, the host cell for this expression system is a bacterium which has been genetically engineered to incorporate the gene for T7 RNA polymerase, the *lac* promoter and the *lac* operator in its genome. When lactose or a molecule similar to lactose is present inside the cell, transcription of the T7 RNA polymerase is activated typically, the host cell used is *E. coli* strain BL21(DE3) (Dubendorff and Studier, 1991). The diagram below Figure (2-7) shows the genome of the host cell.

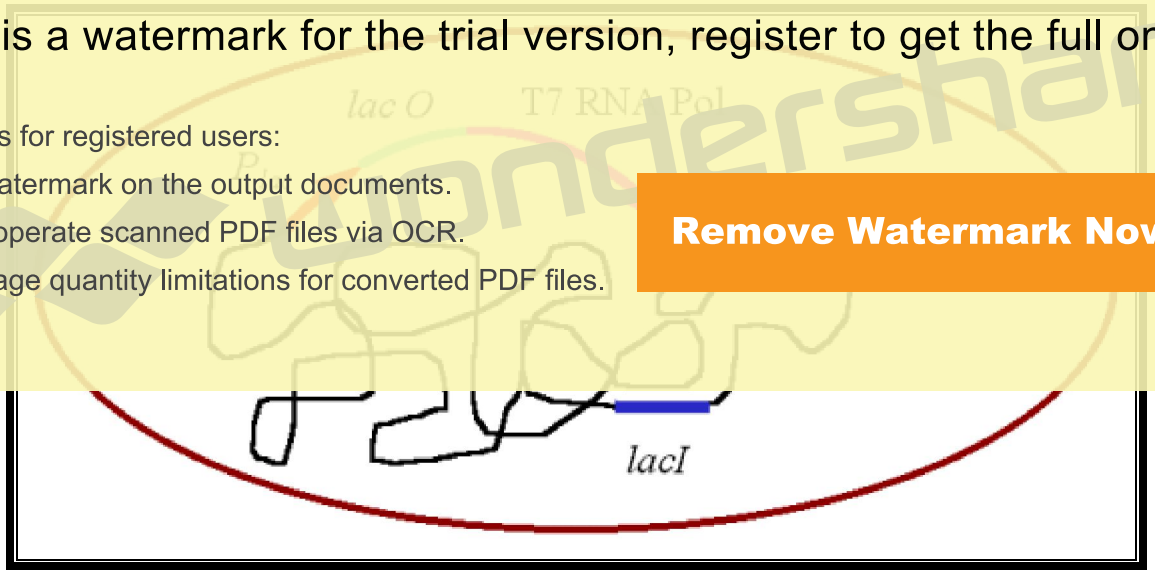


Figure (2-7): *E. coli* BL21 (DE3) the host chromosome. The *lac* promoter is in red, the *lac* operator is green, the gene which encodes the T7 RNA polymerase is pink, and the *lac* inducer is blue. The host cell genome is represented by the black line, and the brown line represents the cell membrane (Dubendorff, and Studier, 1991)

.

T7 RNA polymerase can be introduced to the cell through methods other than incorporating a gene in the host chromosome. It can be introduced through infection of the original host cell with lambda CE6 (Studier and Moffatt, 1986, 1987). Control of the pET expression system is accomplished through the *lac* promoter and operator. Before lipase can be transcribed, T7 polymerase must be present. The gene on the host cell chromosome usually has an inducible promoter which is activated by IPTG. This molecule, IPTG, displaces the repressor from the *lac* operator. Since there are *lac* operators on both the gene encoding T7 polymerase and lipase, IPTG activates both genes (Dubendorff and Studier, 1991). Therefore, when IPTG is added to the cell, the T7 polymerase is expressed, and quickly begins to transcribe lipase which is then translated as lipase. IPTG works to displace a *lac* repressor since IPTG is an analogue of lactose. The *lac* genes express enzymes which are involved in the breaking down of lactose, and therefore, the presence of lactose (or its analogue) would trigger the induction of transcription of *lac* genes. (Tabor and Richardson, 1985).

The typical prokaryotic expression vector represented in Figure(2-6) consists of an inducible promoter, ribosome binding site, Shine Dalgarno sequence (SD), target gene, transcription terminator, a replication origin that determines the plasmid copy number, and an antibiotic resistance marker for plasmid selection and propagation (Baneyx, 1999). A strong tightly regulated promoter that can be induced in a simple and cost effective manner is usually desired to achieve high level of protein synthesis (Hippel, 1982). When a promoter is used for high level protein expression in *E. coli*, it should be able to accumulate protein within the cell up to about 30% of the total cellular proteins. For large scale production, the cells should be able to grow to a high cell density. Tightly regulated promoters are used for expressing proteins that are toxic to the host cell. The overexpressed genes may result in misfolding of the proteins, decrease the cell growth rate, cause cell lysis,

and result in the loss of the recombinant protein production. Still the most widely used promoters for large-scale protein production use thermal induction (*pL*) or chemical inducers (*trp*).When cold response promoters are used, protein folding will be only slightly affected at about 15 to 20 °C, whereas the rates of transcription and translation will be substantially decreased. This in turn, will provide sufficient time for protein refolding, yielding active proteins, and avoiding the formation of inclusion bodies (Oppenheim et *al*., 1996). The pH promoter has been reported to be very strong for recombinant proteins produced at levels of up to 40 to 50% of the total cellular protein and is another attractive option for high level gene expression (Chou *et al*., 1995). However this might be just a typical as protein synthesis depends on several other factors other than promoter strength. Another *lac* derived promoter system is the pET vector system. Here the T7 RNA polymerase is under the control of the prophage (λDE3) encoding the IPTG-inducible *lac* UV5 promoter (Tabor and Richardson, 1985). A single copy of RNA polymerase within the cell is sufficient for gene expression. In many cases the overproduced protein cannot reach a biologic[illegible] form insoluble inclusion bodies. In other case[illegible] the cell (Studier *et al*., 1986).

2.15: Expressing Genes in Different *E. coli* Compartments

2.15.1: Cytoplasmic Expression

For most applications, it is desirable to achieve maximal production of the target protein in the cytoplasm where the nascent polypeptides are first synthesized, problems related to the formation of insoluble inclusion bodies is a significant barrier in the expression of genes in the cytoplasm. However the inclusion bodies offer certain advantages (Cheng *et al*., 1981), as they facilitate the isolation of protein in high purity and concentration for refolding *in vitro* (Rudolph and Lilie, 1996); the target protein is also protected from the proteases in the host. Proteins that are toxic to the cell when in the active form can also be produced first as

inclusion bodies and then refolded separately (Hellebust *et al.*, 1989). However, the protein folding *in vitro* is an arduous task and therefore not an attractive alternative. It is also not certain if the refolded protein would be able to regain its biological activity. The inclusion body formation of human T-cell receptor has been modeled on the parameters: charge average, total number of residues, turn forming residues, cysteine and proline fraction (Adari *et al.*, 1995). Several strategies have been developed to minimize the formation of inclusion bodies in the cytoplasm. The most common approach is to reduce cultivation temperature during fermentation (Schein *et al.*, 1989) thereby reducing the rate of transcription and translation. This also reduces the hydrophobic interactions that are responsible for protein misfolding. Several alternatives to this end have been investigated; 1) using a cold inducible promoter (Oppenheim et *al.*, 1996) 2) selection of suitable host strain (Kenealy *et al.*, 1987) 3) supplementation of non-metabolizable sugars for improving the protein solubility (Bowden *et al.*, 1988) 4) [illegible] (Sugimoto *et al.*, 1991) etc.

Protein Targeting to Periplasm

In contrast to the cytoplasm, the periplasm offers several advantages for protein targeting. The oxidizing environment of the periplasm that contains Dsb-enzymes catalyzing the formation of disulfide bonds facilitates the proper folding of the proteins, and the protein degradation in the periplasm is also less extensive. Only 5 % of the total cellular proteins are present in the periplasm (Nossal *et al.*, 1966; Swamy *et al.*, 1982) therefore the recombinant protein can be efficiently concentrated and the purification is considerably less difficult.

All the periplasmic proteins in *E. coli* require the secretion of their corresponding precursors across the cytoplasmic membrane mediated by a signal sequence. A wide variety of signal peptides, such as *Sp*A (Moks *et al.*, 1987), *Pel*B (Better *et al.*, 1993), *Pho*A (Tong *et al.*, 2000), and *Omp*A (Xu *et al.*, 2002), have been adopted successfully as a genetic strategy to secrete recombinant proteins for

expression in the periplasm or outer membrane of *E. coli*. The transport of proteins to the bacterial periplasm is an incompletely understood process and the presence of a signal peptide does not always ensure efficient protein translocation through the inner membrane. For example, despite the correct cleavage of the signal peptide the T-cell receptor protein could not be detected in the periplasm (Novotny *et al.*, 1991). Coexpression of the periplasmic transport components like signal peptidaseI (Van Dijl., 1991), *sec*E, and *prl*A4 genes (Perez-Perez *et al.*, 1995), and use of *prl*F mutant strains (Snyder and Silhavy, 1992) are certain strategies for improving the translocation of proteins.

2.15.3: Extracellular Secretion

Secretion of recombinant proteins to the extracellular medium of *E. coli* has several advantages over intracellular expression. They include simpler downstream processing and purification, least proteolysis, improved protein folding and disulfide bond formation in the oxidizing environment outside the cell, enhanced solubility and accessibility of the secreted protein to antibodies, correct cleavage of the signal peptide. Unfortunately, very few proteins are secreted into the culture medium due to the barrier from the outer membrane. The known secretion pathways in gram negative bacteria are Type I, II, III, IV, and V with varying mechanisms. The Type I, II, and III mechanisms are most commonly used in *E. coli* for secretion of toxins, degradative enzymes, and pathogenicity factors to the exterior of the cell. The Type II secretion system is the commonly known Sec-dependant translocation of the proteins from the cytoplasm across the inner membrane to the cell envelope. This class includes most of the periplasmic and outer membrane proteins that are synthesized as a propeptide in the cytoplasm with a cleavable signal peptide. Genetic strategies have been developed in the past to secrete the intracellular proteins extracellularly including the use of leaky mutant strains (Ray *et al.*, 2002) and cell wall-less L-form *E. coli* (Gumpert and Hoischen 1998), outer membrane permeabilization by chemical or biochemical agents including addition of

magnesium, calcium, TritonX, EDTA, and lysozyme (enzymatic) (Shokri *et al.*, 2003; Choi and Lee, 2004). Coexpression of protein release factors such as bacteriocin release protein (BRP) (Van der *et al.*, 1995), colicin E1 lysis protein (Kil), *tol*AIII and out genes (Wan and Baneyx, 1998) have been successfully used to secrete recombinant proteins such as penicillin amidase, *β*-glucanase, *β*-lactamase, phytase etc.

2.15.4: Bacterial Surface Display

Bacterial surface display is becoming an increasingly important research area for creating libraries of biomolecules that can be screened for desired properties, antibody production, bioadsorbents for removal of harmful chemicals and heavy metals, whole cell biocatalysts, vaccines, and biosensors (Kornacker *et al.*, 1990; Lee *et al.*, 1999, 2003). For a heterologous protein to be displayed on the outer membrane, it has been fused to a carrier protein that can be anchored in the outer membrane of *E. coli* and a signal peptide that can translocate the fusion protein across the cytoplasmic membrane. Passenger [illegible] N-terminal, C-terminal, or as a sandwich (Chatterjee, 2006). The first example was the display of short gene fragments inserted into the genes for the *Escherichia coli* outer membrane proteins *Lam*B, *Omp*A and *Pho*E, and the gene fusion products were found to be accessible on the outer surface of the recombinant bacteria (Francisco *et al.*, 1993; Chang and Lo, 2000). Since then, not only outer membrane proteins, but also *E. coli* lipoproteins (*Lpp*) (Francisco *et al.*, 1993) and (*Tra*T) (Chang and Lo, 2000), lipoprotein pullulanase from *Klebsiella pneumonia* (Kornacker *et al.*, 1990), *E. coli* adhesion protein AIDA-I autotransporter (Jose *et al.*, 2002), *Eae*A intimin from pathogenic *E. coli* (Wentzel *et al.*, 2001), fimbria proteins *Fim*A and *Fim*H, and flagellar protein *Fli*C. (Westerlund-Wikström *et al.*, 1997), have been employed to achieve heterologous surface display on *E. coli*. Functionally inactive esterase from *Pseudomonas aeruginosa* has been used to display lipases on the cell surface

membrane of *E. coli* (Becker and Craig, 1994). The ice nucleation protein from *Pseudomonas syringae* has been reported to accommodate large size heterologous protein for *E. coli* surface display (Jung *et al*., 1998; Kwak *et al*., 1999; Levy *et al*., 2001). An interesting alternative is described about a novel system that allows the display of recombinant proteins Staphylokinase protein on the cytoplasmic membrane using the L-form cells of *E. coli* (Fahnert *et al*., 2002). Cell-surface display has not been useful for high level gene expression to date. It has also been associated with deteriorated cell physiology resulting from outer membrane perturbation due to insertion of heterologous proteins. Figure (2-8) shows the cell surface display systems.

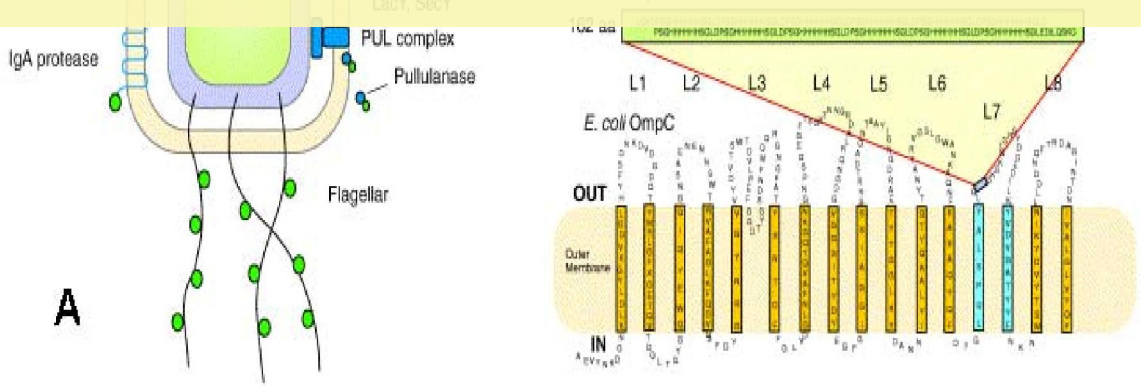


Figure (2-8): Cell surface display systems in gram negative bacteria (A) surface display systems (B) cell surface display using ice nucleation protein (INP) (C) surface display using *E. coli Omp*C (Esposito and Chatterjee, 2006).

Chapter Three

Methods

3.: Material and Methods

3.1: Materials

3.1.1: Equipment and apparatus

The following equipment and apparatus were used in this study:

Equipment and apparatus	Company /origion
Nanodrop-Spectrophotometer	Bio Rad/USA
SDS-PAGE unit	Bio Rad
Power supply	LKB /Sweden
UV Transillaminator	Vilber Lourmat /France
GeneAmp Thermocycler	Bio-Rad
Micropulser	Bio-Rad
pH-state	Bio-Rad
Autoclave	Gallenkamp /England
Compound microscope	Olympus /Japan
[illegible]	[illegible]
Distillator	Gallenkamp/Germany
Hot plate with magnetic stirrer	Gallenkamp
[illegible]	[illegible]
[illegible]	[illegible]
pH- meter	Metter- GmpH Toledo/U.K.
Sensitive balance	Sartorius /Germany
Shaker incubator	GFL/ Germany
Vortex	Stuart scientific/U.K.
Water bath	Memmert/ Germany
Automatic pipettes	Wittege/ Germany
Eppendrof bench centrifuge	Netherler and Hinz/ Germany
Laminar air flow	Memmert
Millipore filter paper unit	Miliporecorp /USA
Micro centrifuge	Eppendorf/ Germany
French Press	Bio-Rad

3.1.2: Chemicals

The following chemicals were used in this study:

Chemicals	Company/Origin
K_2HPO_4, KH_2PO_4, Na_2HPO_4, $MgCl_2$, Sucrose, $CaCl_2$, NaOH, KCl, Sodium Dodecyl sulphate(SDS), Tris base, Glucose, MgSO4	BDH/UK
Agar, Hydrogen peroxide, N,N,N,N-tetramethylene-diamine dihydrochloride, Acrylamide, bisacrylamide, ammonium sulfate, NaCl, Crystal violet, Methanol, Acetic Acid, Gelatin, Methanol, Ampicillin, Kanamycin, Chloramphenicol, Ethidium bromide, Agarose, Peptone, Yeast extract	Difco/UK
H_2SO_4, HCl	Hopkin and Williams /UK
Glycerol, Isopropanol, phenol, hydrogen peroxide, α-naphthol, tetramethyl*p*-phenylenediamine dihydrochloride, Potassium hydroxide	Riedel-Dehaeny/UK
Lysozyme , Bovine serium albumin , X-Gal, IPTG , ethidium bromide	Biolab/USA
Pfu polymerase, DNA ladder, agarose,	Stratagene/ USA

3.1.3: Restriction enzymes

The following restriction enzymes were used in this study

Restriction enzyme	Restriction site	Source / origin
*Nco*I	5′-C↓CATGG-3′	Sigma/USA
*Nde*I	5′-CA↓TATG-3′	Sigma
*Xho*I	5′-C↓TCGAG-3′	Sigma
*Hind*III	5′-A↓AGCTT-3′	Sigma
*Kpn*I	5′-GGTAC↓C-3′	Sigma

3.1.4: Antibiotics

The following antibiotics were used in this table.

Antibiotic	Solvent	Stock Conc. (mg/ml)	work Conc. (µg/ml)
Ampicillin	DI water	50	50
Chloramphenicol	Ethanol	34	34
Kanamycin	DI water	50	50

3.1.5: Kits used in this study:

The following kits were used in this study:

Kit	Company/origin
Genomic DNA Isolation Kit.	Qiagen/U.S.A
Plasmid DNA Isolation Kit (Qiaprep Spin Miniprep Kit)	Qiagen
Gel-Extraction of DNA Kit (Qiaquick Gel Extraction Kit)	Qiagen
PCR Purification Kit (Qiaquick Gel Extraction Kit)	Qiagen
PCR Cloning Kit (pPCR-Script Cm Cloning Kit)	Stratagene/U.S.A

These Kits were provided with different buffers and lysis solutions which include the following:

Buffers	Lysis solutions
Bacterial lysis buffer solution (P1)	RNaseA (50 mg/ml)
Bacterial lysis buffer solution (P2)	
Bacterial lysis buffer solution (PB)	
Elusion buffer(EB) solution	Proteinase
Wash Buffer (AW1)	
Wash Buffer (AW2)	Lysozyme
Binding buffer solution (QG)	
Neutralization Buffer(N3)	

3.1.6 : Bacterial strains and plasmids

The following bacterial strains and plasmids were used in this study:

Strains and Plasmids	Relevant Genotype	Reference
E. coli		
BL21(DE3)	F⁻ *ompT dcm lon hsdS*$_B$ (r_B^-, m_B^-) *gal λ*(DE3[*lacI ind1 sam7 nin5 lacUV5-T7* gene 1])	CGSC
DH5α	*supE44Δ (lac)U169 hsdR17recA1 endA1 gyrA96 thi-1relA deoR(ψ80 lacZDM15)*	CCRC
Plasmid		
pET20b(+)	*PT7::pelB*, Ap^R	NEB
pPCRscript SK(+)	PCR cloning vector, Cm^R	Stratagene

Oligonucleotide Primer	Sequence	Source/Origin
pPCRscript		
Forward Reverseward	5'-GCAGGAAAAAGCTGAAGCGG-3' 5'-TTTCGACAATGCAGCCGATATGG-3'	IDT/CD
pET20b(+)		
Forward Forward	5'-TTGCCATGGGCATTCAAGATCAA-3' 5'-GATCATATGAGACGCGGTATTG-3'	IDT
Reverseward Reverseward	5'- CTCGAGTTGTTTGTCCTCCTCC-3' 5'-CTCGAGTCATTGTTTGTCCTCC-3'	IDT

All primers were designed in this study and provided by integrated DNA technologies (IDT).

3.1.8: Culture media

3.1.8.1: Ready to made media

Media	Company
Nutrient agar	Biolife /Italy
Nutrient broth	Biolife

This media were prepared according to the instructions of the manufacturer.

3.1.8.2: Prepared media

A. Nutrient Gelatin medium (Stolp and Gadkari, 1984)

This medium was prepared according to the instructions of the manufacturer by dissolving 120 g of gelatin in 950 ml of distilled water, pH was adjusted to 7.0, then volume was completed to 1000 ml with distilled water and sterilized by autoclaving.

C. Luria-Bertani broth (LB) medium (Nazina *et al.*, 2000)

This medium was prepared so that it consists of the following components.

Component	Weight(g)
trypton	10
yeast extract	5
NaCl	5

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.0, then volume was completed to 1000 ml with distilled water and sterilized by autoclaving.

D. Luria-Bertani (LB) agar medium (Nazina *et al.*, 2001)

This medium was prepared by adding 1.5% agar-agar to Luria broth medium prepared in item (3.1.8.2.C).

E. Luria -Tributyrin agar medium (Xu *et al.*, 2008)

This medium was prepared so that it consists of the following components.

Component	Weight(g)
trypton	10
yeast extract	5
NaCl	5
Tributyrin	10
Agar	16

to 7.0, the volume was completed to 1000 ml with distilled water and sterilized by autoclaving.

F. Medium (Maniatis *et al.*, 1982)

This medium was prepared so that it consists of the following components.

Component	Final concentration
Bacto tryptone	2%
Bacto yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

pH was adjusted to 7.0 and sterilized by autoclaving except glucose which was sterilized by filtration.

G. Methyl Red and Voges-Proskauer (MR-VP) medium (Atlas *et al.* 1995)

This medium was prepared so that it consists of the following components.

Component	Weight(g)
K_2HPO_4	5
peptone	5

All components were dissolved in 900 ml distilled water, pH was adjusted to 7.6, the volume was completed to 950 ml with distilled water and sterilized by autoclaving. , then 50 ml of 10% glucose solution was added (sterilized by filtration).

3.1.9: Reagents, Dyes and solutions

2.1.9.3: Methyl red indicator (Collee *et al.*, 1996)

This indicator was prepared by mixing the following components:

Component	Amount
Methyl red	0.05 (g)
Ethanol (95% v/v)	150 (ml)

The volume was completed to 1000 ml with distilled water.

3.1.9.4: Lugal's solution (Fad, 1976)

It was prepared by dissolving 10 g of potassium iodide in 25 ml of distilled water; 5 g of iodine was then added with stirring until completely dissolved,

volume was then completed to 100 ml with distilled water in volumetric flask and kept in dark bottle at 4ºC.

3.1.9.5: Bromophenol blue (0.5%)

It was prepared according to Blackshear (1984) by dissolving 0.5g of bromophenol blue in 100 ml distilled water.

3.1.9.6 : Ethidium bromide stock solution (10 mg/ ml)

It was prepared according to Maniatis et *al*. (1982) by dissolving 1g of ethidium bromide in 100ml of distilled water, mixed gently then stirred by magnetic stirrer for several hours to complete dissolving. Container was wrapped in aluminum foil and stored at 4ºC.

3.1.9.7: Loading Buffer 6X (Maniatis *et al*., 1982)

It was prepared to be consist of 0.25% bromophenol blue and 40% (w/v) sucrose, and stored at 4 ºC.

3.1.9.8: Enzymatic lysis buffer solution (1M) (DNeasy Blood &Tissue Handbook), (Sambrook *et al*., 2001)

It was prepared so that it consists of EDTA 2mM; Tris-HCl 20mM, and 1.2% Triton-X100; pH was adjusted to 8, and stored at 4 ºC.

3.1.9.9: Tris- acetate solution (50X) (Maniatis *et al*., 1982)

This solution was prepared so that it consists of 2M Tris-base, 0.5M EDTA, and 57 ml glacial acetic acid in 1000 ml distilled water, pH was adjusted to 8.0 and stored at 4 ºC until use.

3.1.9.10: Tris-EDTA (TE) buffer solution (Maniatis *et al*., 1982)

This buffer solution was prepared so that it consists of 10 Mm Tris-HCl,

and 1mM EDTA, pH was adjusted to 8.0 and stored at 4ºC.

3.1.9.11: Sodium phosphate buffer (Maniatis *et al*., 1982)

It was prepared to be consisting of sodium phosphate buffer 0.05M, pH was adjusted to 7.5.

3.1.9.12: EDTA Solution of (0.5 M) (Maniatis *et al*., 1982)

It was prepared by dissolve 14.61g EDTA in 80 ml distilled water, pH was adjusted to 8.0, then volume was completed to 100 ml with distilled water, sterilized by autoclaving and stored at 4ºC.

3.1.9.13: TAE buffer solution (50X) (Maniatis *et al*., 1982)

20ml of 0.5 M EDTA and 11.42 ml of glacial acetic acid was added volume was completed to 200 ml of distilled water and stored at 4°C.

3.1.9.14: Tributyrin Emulsion (Xu *et al*., 2008)

It was freshly prepared so that it consists of 2% (v/v) tributyrin, pH was adjusted to 8.0.

3.1.10: Buffers and solutions for protein electrophoresis (Oberoi *et al*., 2001)

3.1.10.1: Sodium dodecyl sulphate (SDS) solution (10%)

It was prepared by dissolving 10 g of SDS in 90 ml of distilled water, then volume was completed to 100 ml with distilled water.

3.1.10.2: Tris-HCl (1.5 M)

It was prepared by dissolving 27.23 g Tris-base**,** in 90 ml of distilled water, pH was adjusted to 8.8 then volume was completed to 150 ml with distilled water and stored at 4ºC.

3.1.10.3: Tris-HCl (0.5 M)

It was prepared by dissolving 6g Tris base, in 90 ml of distilled water, pH was adjusted to 6.8 then volume was completed to 100 ml with distilled water and stored at 4ºC.

3.1.10.4: Acrylamide- Bisacrylamide solution (40%)

It was prepared by dissolving 38g of acrylamide and 1.34 g of bisacrylamide in 100 ml of distilled water. Solution was filtered through wattman filter paper no.1, and kept in dark bottle at 4ºC until use.

3.1.10.5: Ammonium persulfate solution (10%)

This solution was freshly prepared by dissolving 1 g ammonium sulfate in 10 ml of distilled water.

3.1.10.7: Resolving Gel Buffer solution

This solution was prepared so that it consists of the following components:

Component	Volume
Distilled water	6.03ml
Tris-HCl(1.5 M)	4.5ml
SDS (10%)	180µl
Acrylamide- Bisacrylamide soln.	7.2ml
APS 10 %	90 µl
TEMED	9 µl
Total	18ml

3.1.10.8 Stacking Gel Buffer solution

This solution was prepared so that it consists of the following components:

Component	Volume
Distilled water	3.03ml
Tris-HCl(0.5 M)	1.25ml
SDS (10%)	50µl
Acrylamide- Bisacrylamide soln.	0.665ml
APS 10 %	25 µl
TEMED	5 µl
Total	5ml

3.1.10.9 Coomassie Blue (1mg/ml)

This solution was prepared so that it consists of the following components:

Component	Volume
Coomassie Blue	1g
Methanol	400 ml
Acetic acid	100 ml

acetic acid was added and the volume was completed to 1L with distilled water.

3.1.10.10 Destaining Solution

This solution was prepared so that it consists of the following components:

Component	Volume (ml)
Methanol	800 ml
Acetic acid	200 ml

3.1.10.11 Sample Buffer Solution (2X)

This solution was prepared so that it consists of the following components:

Component	Volume (ml)
Distilled water	3.55
Tris-HCl(0.5 M), pH 6.8	1.25
Glycerol	2.5
SDS (10%)	2.0
Bromophenol blue (0.5%)	0.2
Total volume	9.5

β-mercaptoethanol (5%) was added to the solution immediately before use. Protein sample was diluted at ratio (1:1) with this buffer solution and heated at 95 ºC for 4 minutes, then cooled and stored at -20ºC.

3.1.10.12 Tris/ Tricin / SDS Running Buffer solution (10X)

It was prepared by dissolving 60.55g of Tris base, and 89.6 g Tricine in 400 ml distilled water, 10 g of SDS was completed to 500 ml with distilled water, and stored at 4°C until use.

3.1.11 Buffers and Solutions for Bacterial Transformation

3.1.11.1 Glycerol solution (10%) Xu *et al.* (2008)

This solution was prepared by adding 12.6 g glycerol to 90 ml of distilled water, mixed thoroughly then volume was completed to 100 ml with distilled water, and sterilized by autoclaving.

3.2 Methods

3.2.1 Collection of soil samples

For isolation of *Bacillus stearothermophilus*, soil samples were collected from different locations of southern Iraqi provinces (Basrah, Karbalaa, Thiqar, Misan, and Al-Mouthana). 1 g of each soil sample was taken and incubated in an oven at 65ºC for 24 hours, then suspended in 5 ml of sterilized distilled water and mixed vigorously. After settling, 0.1 ml aliquots of aqueous phase were taken, spread on nutrient agar plates, and incubated at 65ºC for 24 hours. After incubation, the growing colonies were selected and identified according to their cultural, morphological characteristics, and biochemical tests.

3.2.2 Identification of isolates

3.2.2.1 Cultural and morphological characteristics

To identify *B. stearothermophilus*, cultural and morphological characteristics (size, shape, edge, gram staining, color, spore forming) were examined according to Atlas *et al.* (1995).

3.2.2.2 Biochemical tests

3.2.2.2.1 Gram's stain (Harely and Prescott, 1996)

Single colony of each isolate was transferred and smeared on a clean slide and stained with crystal violet, then slides were treated with iodine, decolorized with absolute alcohol, and counterstained with safranine, and examined under light microscope.

3.2.2.2.2 Catalase test (Atlas *et al.*, 1995)

This test was performed by adding a few drops of hydrogen peroxide (3%) on single colony of each isolate on clean slide. Production of gaseous bubbles indicates a positive result.

3.2.2.2.3 Oxidase Test (Atlas *et al*., 1995)

This test was done by using moistened filter paper with few drops of freshly prepared solution of tetramethyl-ρ-phenylenediamine dihydrochloride. Aseptically, a clump of cells was picked up from the slant growth with a sterile wooden stick applicator and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result.

3.2.2.2.4 Methyl Red Test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolate and incubated at 55°C for 48 hours, then five drops of methyl red reagent was added and mixed gently. Presence of bright red color indicates a positive result.

3.2.2.2.6 Gelatin hydrolysis test (Stolp and Gadkari, 1984)

This test was performed to demonstrate the ability of bacterial isolates to hydrolyze gelatin. Test tubes of gelatin medium were inoculated with a fresh culture of each isolate by stabbing, then tubes were incubated at 37ºC for five days. Liquefaction of gelatin medium indicates positive results.

3.2.2.2.7 Growth at pH 6.8 (Atlas *et al*., 1995)

Single colony of each isolate was picked up and streaked on nutrient agar plates at pH adjusted previously to 6.8, then plates were incubated at 55 ºC for 24 h. Presence of growing colonies indicates positive results.

3.2.2.2.8 Growth at thermophilic conditions (Atlas *et al.*, 1995)

This test was performed to demonstrate the ability of bacterial isolates to grow in different thermal temperatures. Single colony of each bacterial isolate was taken and streaked on nutrient agar plates and incubated at 40, 50, 55, 60, 65 ºC. Presence of growing colonies indicates a positive result.

3.2.2.2.9 Starch hydrolysis test (Fad, 1976)

This test was performed by inoculating starch agar medium with each of bacterial isolates, and incubated at 55 ºC for 24 h then plates were flooded with lugal's iodine solution prepared in item (3.1.9.4). Development of clear zones around colonies indicates a positive result.

3.2.3 Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al.* (1982) as follows:

Bacterial isolates were maintained for a period of a few weeks on LB agar slants; they were tightly wrapped with parafilm, and stored at 4˚C.

3.2.3.2 Medium-Term Storage

Bacterial isolates were maintained in stab culture for a period of few months. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of LB agar medium and stored at 4˚C.

3.2.3.3 Long-Term Storage

Bacteria can be stored for many years in nutrient broth containing 15% glycerol at low temperature without significant loss of viability. This was done

by adding (1.5 ml) of sterilized glycerol to an exponential growth of bacterial isolates in a screw-capped bottle with final volume (10 ml) and stored at -20˚C.

3.2.4 Sterilization Methods

3.2.4.1 Moist Heat Sterilization (Autoclaving)

Media and solutions were sterilized by the autoclaving at 121ºC (15Ib/in) for 15 min.

3.2.4.2 Dry Heat Sterilization

Glassware were sterilized at 180 ºC in oven for 3 hours.

detected according to (Xu *et al*., 2008) using tributyrin as suitable substrate and as follows:

3.2.5.1 Detection of Lipase production on solid medium

Lauria-tributyrin agar medium was used to assay the ability of locally isolated *B. stearothermophilus* in lipase production. This was achieved by transferring each of the isolates on plates of this medium, and were incubated at 55˚C for 24 hours then, Diameter of hydrolysis zones were measured and used as an indicator for the selection of efficient isolate in lipase production.

3.2.5.2 Lipase assay

Lipase activity was measured by titrating free fatty acids released by hydrolysis of tributyrin using pH state method. An appropriate volume of the soluble fraction of cell lysate was added to 5 ml of 2% tributyrin emulsion prepared in item (3.1.9.14) at 50 °C and the reaction solution was maintained at pH 8.0 by controlled addition of 0.02 N NaOH. The release rate of fatty acid was measured with a pH titrator for 10 min. Activity unit was defined as the amount of enzyme required to liberate 1 µmole of fatty acids per min.

$$\text{Enzyme activity} = \frac{\text{Volume of base consumption} \times \text{base normality}}{\text{Volume of enzyme solution} \times \text{reaction time}} \times 1000$$

3.2.6 Isolation of Genomic DNA

Genomic DNA of *Bacillus stearothermophilus* was isolated according to (DNeasy Blood & Tissue Handbook) protocol using Qiagen Kit and as follows:

1- Aliquot of 1 ml was taken from overnight culture of the selected isolate of *Bacillus stearothermophilus* according to [illegible]

2- Cells were resuspended in 180 µl of enzymatic lysis buffer solution prepared in item (3.1.9.8), and incubated at 30 ºC for 30 min. to generate protoplasts.

3- protoplasts were resuspended in 180 µl of buffer AL in ependorff tubes, then 20 µl of proteinase K solusion was added, and incubated at 55ºC in a shaker water bath until complete lysis.

4- Microtubes were incubated at 56ºC for 1 – 2 hours, then 20 µl of RNase solution (50 mg / ml) was added and incubated at a room temperature for 30 min.

5- Mixture from Step 4 was pipette into DNeasy Mini-spin column placed in a 2 ml collection tube, and centrifuged at 8000 rpm for 1 min., then flow-through was discarded.

6- DNeasy column was placed in a new 2ml collection tube, then 500µl of Buffer AW1 was added and centrifuged at 6000 rpm for 1 min. then the flow-through was discarded.

7- DNeasy column was placed in a new 2ml collection tube, then 500 µl of Buffer AW2 was added, and centrifuged at 14000 rpm for 3 min to dry the DNeasy membrane. The flow-through was discarded.

8- DNeasy column was placed in a clean 1.5 ml microcentrifuge tube, then 200µl of Buffer AE was added directly onto the membrane and incubated at room temperature for 1 min., then centrifuged at 6000rpm for 1 min to elute genomic DNA.

3.2.7 Quantitation of DNA concentration

Purity and concentration of DNA solutions was measured according to (Maniatis *et al.*, 1982) using computerized nanodrop-spectrophotometer by adding 2µl of DNA solution into the photocell of the apparatus, then results of DNA purity and concentration (ng/µl) were displayed.

Nanodrop spectrophotometer measures DNA purity and concentration according to the following equations:

$$\text{DNA purity} = \frac{\text{Abs. at 260 nm}}{\text{Abs. at 280 nm}}$$

DNA concentration (ng/ µl) = Abs (260 nm) **x** 50 µg/ml

Note: Absorbency (260nm) of 1 equal to 50 µg/ ml of pure DNA.

3.2.8 Amplification of *B. stearothermophilus* lipase gene (Maniatis *et al.*, 1982)

To amplify lipase gene of the locally isolated *B. stearothermophilus*, database of DNA sequences in NCBI was gave the basic information to design

specific primers for lipase gene of *B. stearothermophilus*. These primers were used to amplify lipase gene, then PCR products of the gene were DNA sequenced. Results of sequencing and DNA alignment with the same sequences mentioned by NCBI/Blast bioinformatics data base (accession number cp001638.1) showed that the sequence amplified was identical to nitrate reductase of *Geobacillus sp.* WCH70 (degree of sequence similarity was 97%), but don't share any sequence similarity of genomic DNA of *B.stearothermophilus*. According to these results lipase gene of *Geobacillus sp*. WCH70 was used to design specific primers for amplification of lipase gene of the local isolate.

The reaction mixture (50 µl) for lipase gene amplification by PCR contains the following components:

Component	Final Conc.	Volume (µl)
10X PCR Buffer	1X	5µl
10 mM dNTP Mix	1X	5µl
Forward prime 20 µM	0.4µM	1µl
Reverseward primer 20 µM	0.4 µM	1µl
pfu polymerase		0.5 µl
DNA template (50 ng)	1 µg	1.5 µl

The volume was completed to 50 µl with molecular biology grade distilled water. PCR reaction was achieved in gene Amp thermocycler using *pfu* polymerase to amplify lipase gene with blunt end terminals. Amplification procedure was achieved according to the following program:

Step	Temp. (°C)	Time (min.)	No. of cycles
Initial denaturation	95	3	1
Denaturation	95	30 Sec.	35
Annealing	×	30 Sec.	35
Extension	72	×	35
Final extension	72	5	1

X: depends on optimum temperature for each primer.

3.2.9 Gel Electrophoresis for PCR products

PCR products were analyzed on agarose gel (0.8%) using horizontal [illegible] gel was immersed in 1X TAE buffer, then they were loaded into the wells. Electrophoresis was [illegible] Agarose gel was stained with [illegible] solution for 10 minutes, then DNA bands were visualized by using U.V transilluminator at 365 nm. (Maniatis *et al*., 1982)

3.2.10 Elution and Purification of DNA fragments from agarose gel (Vogelstein *et al*., 1979)

1- DNA fragments were excised from agarose gel with a clean, sharp scalpel, then gel slices were minimized by removing extra-blank agarose gel.

2- Gel slices were weighted in colorless ependorff tubes, then 3 volumes of Buffer QG were added to 1 volume of gel containing DNA fragments.

3- Ependorff tubes were incubated at 50°C for 10 min with vortexing for 2 minutes intervals (or until the gel slice has completely dissolved).

4- After complete dissolving of gel slices, 0.3 volume of isopropanol was

added and mixed thoroughly, then the total volume was transferred to QIAquick spin column in a provided 2collection tube to bind DNA to membrane, by centrifugation for 1 min at 12.000 rpm.

5- The flow-through was discarded and the QIAquick column was placed back in the same collection tube.

6- Amount of 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min. at 12.000 rpm, then 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min. at 12.000 rpm.

7- The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min. at 12000 rpm, then it was placed into a clean 1.5 microcentrifuge tube to elute DNA.

8- 50 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane and centrifuged for 1 min. at 12.000 rpm.

features of this vector was mentioned in figure (3-1) and table (3-1) respectively.

The amplified gene was cloned in pPCRScriptCamSK(+) according to Stratagene protocol for pPCRScriptCamSK(+). Cloning was achieved in a 0.5ml microcentrifuge containing the following:

1 µl of the pPCR-Script Cm SK(+) cloning vector (10 ng/µl), 1 µl of PCR-Script 10X reaction buffer, 0.5 µl of 10 mM ATP, 2–4 µl of the blunt-ended PCR product/ or 4 µl of the control PCR insert, 1 µl of *Srf* I restriction enzyme (5 U/µl), and 1 µl of T4 DNA ligase (4U/µl). The volume was completed with molecular biology grade distilled water to 10 µl.

The ligation reaction was mixed gently then microfuges were incubated for 1hour at room temperature.

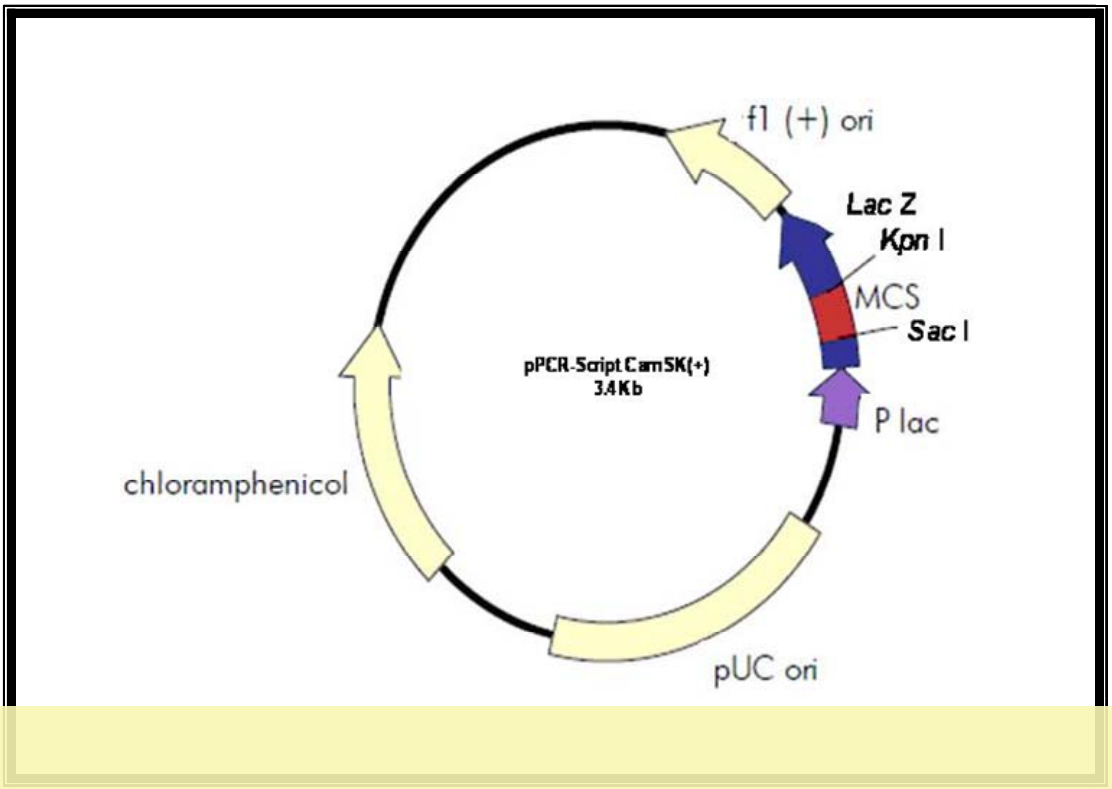


Figure (3-1):Genetic map of pPCRScriptCamSK(+) cloning vector, the nucleotide sequence is available both from the GenBank® database (Accession #U46017) and from www.stratag

Table (3-1): Main features of pPCR-ScriptCamSK(+) cloning vector.

Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication	135–441
multiple cloning site	653–760
lac promoter	817–938
pUC origin of replication	1158–1825
chloramphenicol resistance gene	2125–2676

3.2.12 Transformation of *E. coli* with recombinant pPCRScript SK(+)

E. coli DH5α was transformed with recombinant pPCRScriptCamSK(+) as follows:

3.2.12.1 Preparation of competent *E. coli* DH5α (Chung and Miller, 1993)

1- From overnight fresh culture (seed culture) *E. coli* DH5α, 1ml was taken and used to inoculate 100 ml of LB-broth in conical flask, then culture was incubated at 37ºC with shaking (200 rpm) to OD(600nm) equal to 0.5-0.7.

3- After incubation, flask was placed immediately in ice bath for 20 min., then cells were transferred into precooled centrifugal bottle (60 ml) and spinet at 6000 rpm for 15 minutes at 4ºC.

4- Supernatent was discarded gently, then pellet was resuspended in 50 ml of ice-cooled 10% glycerol prepared in item (3.1.11.1). Cells were then recentrifuged at 6000rpm for 15 min at 4ºC, then supernatant was discarded gently. This step was repeated twice.

6- Pellet was resuspended in ~4 ml of ice- cooled 10% glycerol, and transferred to 2ml sterile microfuge tubes and centrifuged at 6000 rpm for 15 minutes at 4 °C, then supernatant was discarded gently.

7- Pellet was resuspended again in 400µl of ice- cooled 10% glycerol, then 40ul aliquots of cell suspension was tra[illegible] co[illegible] at least for 6 months.

3.2.12.2 Electroporation (Chung and Miller, 1993)

Competent cells obtained from previous steps were subjected to electroporation according to the following procedures:

1- Competent cells stored at -80ºC were thawed in ice bath, Then 1-2 µl of recombinant pPCRScript(Cam) was added, mixed well, and incubated in ice bath for 1 min.

2- Contents of ependroff tubes were transferred into precooled electroporation cuvettes, and subjected to electroporation in micropulser for 5 milliseconds.

3- Cuvettes were taken immediately, then 1 ml of SOC medium prepared in

item (3.1.8.2 F) was added to each cuvette, mixed gently and transferred into eppendroff tubes and incubated at 37ºC for 1 hour with shaking (225 rpm). (The period between applying the pulse and transferring the cells to outgrowth medium is crucial for recovering *E. coli* transformants). Delaying this transfer by even 1 minute causes a 3fold drop in transformation. This decline continues to a 20- fold drop by 10 minutes.

3.2.13 Plasmid DNA extraction from wild type and transformed *E. coli* DH5α (QIAprep® Miniprep Handbook)

pET20b (+) and recombinant pPCRScript were isolated from *E. coli* DH5α according to the following procedure:

1- Single colony of *E. coli* DH5α transformed with both pET20b(+) and recombinant pPCRScript and pET20b(+) were picked up and used to inoculate 5 ml LB-broth containing the appropriate antibiotic, and incubated at 37°C for 16 hours.

2- After overnight, cells were harvested by centrifugation and the supernatant discarded. The pellet was resuspended in 250 µl buffer P1 (containing RNase) in ependorff tubes.

3- Amount of 250 µl of lysis buffer P2 was added and mixed thoroughly by inverting for 4–6 times.

4- 350µl of buffer N3 was added and mixed thoroughly by inverting for4–6 times.

5- Ependorff tubes were then centrifuged for 10 min at 13000 rpm until a compact white pellet was formed.

6- Supernatants from step 5 were applied into the QIAprep spin column by decanting or pipetting, and centrifuged for 30-60 sec. at 13000rpm.

7- Pellet in QIAprep spin column was washed by adding 0.5 ml of buffer PB and centrifuged for 30–60sec., then flow-through was discarded.

8- QIAprep spin column was washed by adding 0.75 ml buffer PE

(containing ethanol) and centrifuged for 30–60 sec. at 13000rpm.

9- Flow-through was discarded, then tubes were recentrifuged for an additional 1 min to remove residual washing buffer.

10- QIAprep column was placed in a clean 1.5 ml microcentrifuge tube to elute DNA by adding 50 µl of Buffer EB or molecular biology grade distilled water to the center of each QIAprep spin column, let to stand for 1 min, and centrifuged for 1 min. at 13000rpm.

3.2.14 Selection of transformants

Transformants of *E. coli* DH5α harboring pPCRScript(Cam) were selected on LB agar medium containing chloramphenicol (34µg/ml), 80µM of 5-bromo-4-3-indolyl-β-D-galactopyranoside (X-gal), and 20mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG). This medium was plated in Petri dishes, then plates were left to stand for 30 min. at room temperature, then 100 µl of transformed *E. coli* was spread on surface of the medium, and incubated at 37ºC for 24 hours. After incubation, white colonies were picked by touching chloromphenicol (34µg/ml) and incubated at the same conditions and regarded as stock culture.

Touch of each white colony was taken and used to inoculate LB broth medium containing chloramphenicol (34µg/ml) and incubated at 37ºC for 16 hours, then DNA was extracted according to procedure mentioned in item (3.2.13).

3.2.15 Restriction analysis of recombinant pPCR-Script (+) from transformed *E. coli* (Maniatis *et al.*, 1982)

Recombinant pPCRScript extracted from transformant *E. coli* DH5α was subjected to restriction digestion using *Hind*III to confirm the exact insert of lipase gene. Reaction mixture for restriction digestion contains the following

solutions:

1- For analytical purposes, the reaction mixture must be contained in microfuge tube as indicated below:

Component	Stock Conc.	Require Conc.	Volume (µl)
D.I. Water	X µl	-	-
Reaction buffer	10X	1X	1 µl
DNA	50-200ng/µl	10~30ng/µl	2 µl
Restriction enzyme	1U/µl	~1 U	~1 µl
Total volume	-	-	10 µl

For most of the commercial restriction enzymes, 1 Unit of enzyme is defined as the amount required to digest 1µg of DNA (e.g. λ DNA) in one hour at 37 °C.

2- All components were mixed in microfuge tube and incubated at 37°C (or other recommended temperature for specific restriction enzyme) for 1 hour.

3- Reaction was then inactivated by heating at 65°C for 15 min.

4- Gel Electrophoresis for Restriction digestion products of recombinant pPCR-Script(+) from transformed *E. coli* was analyzed on agarose gel (0.8%) as in item (3.2.9) to detect the exact size of recombinant plasmid.

3.2.17 Sequencing of lipase gene

Lipase gene of *B. stearothermophilus* cloned in pPCR-Script(+) was sequenced to determine the complete sequence of the gene.

Recombinant plasmid with lipase gene was first extracted and purified from transformed *E. coli* DH5α, then 250 ng/ µL of DNA was sent for sequencing to The Centre for Applied Genomics (TCAG) institute.

3.2.18 Subcloning of lipase gene (Maniatis *et al*. 1982)

After sequencing of lipase gene of *B. stearothermophilus*, this gene was subcloned in pET20b (+) in order to study the expression of the gene in *E. coli*. Genetic map and main features of pET20b (+) was described in figure (3-2) and table (3-2) respectively. This vector was designed to contain T7 promoter for overexpression of the desired gene in *E. coli* BL21(DE3) as suitable host. It also contained His-tag sequence for affinity purification of fusion protein (lipase enzyme).

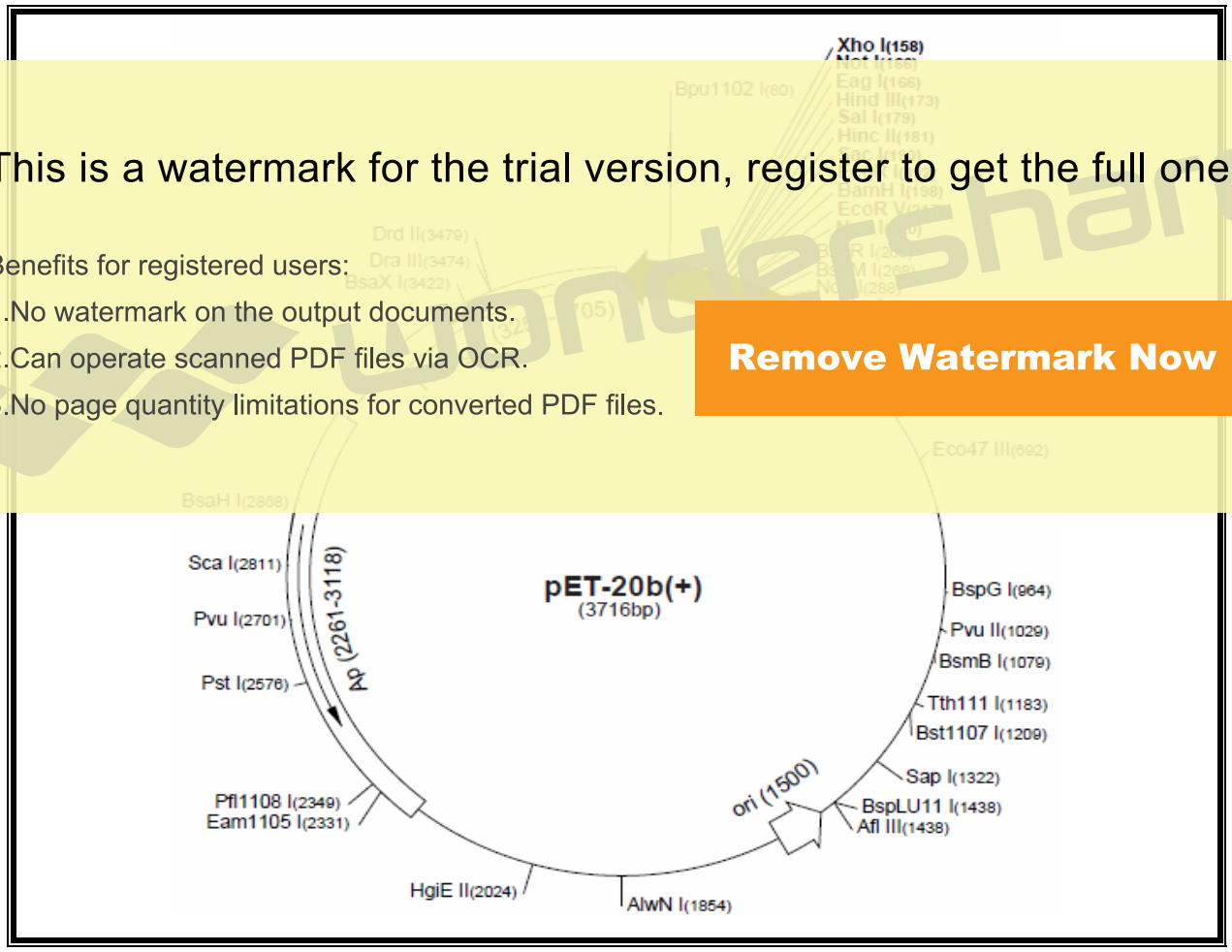


Figure (3-2): Genetic map of pET20b (+) (*www.novagen.com*).

Table (3-2): Main features of pET-20b (+)

Feature	Nucleotide Position
T7 promoter	353-369
T7 transcription start	352
pelB coding sequence	224-289
Multiple cloning sites	
His•Tag coding sequence	140-157
T7 terminator	26-72
pBR322 *ori*V	1500
f1 origin	3250-3705

Subcloning of lipase gene in pET20b(+) was achieved as the following steps:

Reverseward-1: **5'- CTCGAGTTGTTTGTCCTCCTCC-3'**

Reverseward-2: **5'-CTCGAGTCATTGTTTGTCCTCC-3'**

These primers were designed to include:

1- Native signal peptide of the mature lipase gene with and without His-tag sequence, (Forward-1 against Reverseward-1 and Reverseward-2 primers respectively).

2- Leader peptideless (ll) of mature lipase gene with and without His-tag sequence (Forward-2 against Reverseward-1 and Reverseward-2 primers respectively).

3.2.18.2 Restriction digestion of pET20b (+)

pET20b (+) was prepared for subcloning of lipase gene according to (Maniatis et *al*., 1982). Two ependorff tubes were used to achieve the reaction, the first containing pET20b digested with *Nco*I/*Xho*I respectively, while the other containing pET20b then digested with *Ndo*I/*Xho*I respectively, in order to obtain two patterns of double digested linearized pET20b with sticky ends of *Nde*I/*Xho*I and *Nco*I/*Xho*I terminals. Restriction digestion in both microfuge tubes was prepared as in item (3.2.15).

3.2.18.3 Gel electrophoresis and elution of restricted pET20b

Gel electrophoresis for restriction digestion products of pET20b with both pairs of restriction enzymes (*Nde*I/*Xho*I and *Nco*I/*Xho*I) was analyzed on agarose gel (0.8%) as mentioned in item (3.2.9) to separate the DNA bands generated after digestion, and then to elute these DNA bands from agarose gel separately according to procedure mentioned in item (3.2.10).

Amplified lipase gene fragments obtained after PCR amplification step were ligated with both of the two patterns of pET20b (+) generated after digestion with both pairs of restriction enzymes (*Nde*I/*Xho*I and *Nco*I/*Xho*I), then gel electrophoresis, and elution steps described above. Both two patterns of inserts and cloning vector have compatible cohesive ends (*Nde*I/*Xho*I and *Nco*I/*Xho*I terminis). Ligation reaction was carried out by adding three amounts of insert (lipase gene) and one of vector into ependorff tubes and set to contain the following in ligation buffer**:**

Component	Amount
Insert	0.1-1µg
Vector	0.1-1µg
T4 DNA ligase	0.1 Weiss Units*

***One Weiss Unit is defined as the amount of enzyme that catalyzes the exchange of 1 nmole of P^{32} from pyrophosphate into ATP in 20 minutes at37˚C.**

Volumes of ligation mixture in ependorff tubes were then completed to 10µl with molecular biology grade distilled water. The reaction mixture was mixed gently and incubated at 16ºC for 2 hours.

3.2.18.5 Purification of recombinant pET20b (+)

Recombinant pET20b(+) was purified using Qiagen Gel Purification Kit. The plasmid was transformed into competent cells of *E. coli* DH5α and *E. coli* BL21(DE3).

1- An amount of 5 volumes of buffer PB was added to 1 volume of ligation mixture and mixed via from the

2- Ependroff tube contents were then transferred into QIAquick spin column provided with 2 ml collection tube and centrifuge for 30–60 sec. at 12.000 rpm.

3- Flow-through was discarded, and the precipitate DNA was washed by adding 0.75 ml of buffer PE and centrifuge for 30–60 sec. at 12.000 rpm.

4- Supernatant was discarded, and the precipitate DNA was recentrifuged for additional 1 min.

5- QIAquick column was transferred into a clean 1.5 ml microcentrifuge tubes to elute DNA by adding 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5) to the center of the QIAquick membrane and centrifuged for 1 min., 30 µl of elution buffer was added to the center of the QIAquick

membrane, and let the column to stand for 1 min., then it was centrifuged for 1 min. at 12.000 rpm.

6- Supernatent (DNA solution) was used for transformation of *E. coli* DH5α.

3.2.19 Transformation of *E. coli* DH5α with recombinant pET20b (+)

Recombinant pET20b (+) was transformed in *E. coli* DH5α according to the procedure mentioned in item (3.2.12). Transformants were selected according to the expression of selectable marker (ampicilin resistance) on LB-agar medium.

3.2.20 Extraction of Recombinant pET20b (+) from transformed *E. coli* DH5α

Recombinant pET20b (+) was extracted from transformed *E. coli* DH5α according to the procedure mentioned in item (3.2.13). This step was achieved to confirm the colonies of pET20b (+) contain

3.2.21 Restriction digestion of recombinant pET20b (+)

Recombinant pET20b (+) extracted from transformants of *E. coli* DH5α was double digested with *Nco*I/*Xho*I and *Nde*I/ *Xho*I in ependorf tubes according to the procedure mentioned in item (3.2.18.2). This step was done to detect the true insert of lipase gene in pET20b(+), then digestion products were analyzed as in item (3.2.9).

3.2.22 Transformation of *E. coli* BL21(DE3)

Recombinant pET20b (+) was transformed in *E. coli* (DE3) according to the procedure mentioned in item (3.2.12). Transformants were selected on the basis of the expression of selectable marker (ampicilin resistance) on LB-agar

medium. This step was achieved to study the expression of *B.stearothermophilus* gene in the new host (*E. coli*).

3.2.23 Induction of lipase production in genetically engineered *E.coli* BL21(DE3) (Xu *et al.* 2008)

Single colony of transformed *E. coli*BL21 (DE3) was used to inoculate LB-broth medium containing ampicilin (50µg/ml) and incubated at 37ºC for 15 hours.

1- Aliquot of 1 ml of fresh culture of transformed *E. coli* BL21(DE3) was taken and used to inoculate 25ml of LB-broth in 100 ml conical flasks and incubated at 37ºC till O.D was 0.4-0.6.

2- Different concentrations of IPTG were added to a final concentration of (0.05, 0.2, 0.5, 1 mM) respectively and incubated at 28ºC for 4 hours.

3- Aliquots of bacterial culture were taken and diluted to $O.D_{600}$ = 1.0 using LB-broth, and stored in ice bath for a few hours, then centrifuged in cooled centrifuge (4ºC) at 5000 rpm for 10 minutes.

3.2.24 Lipase assay in cell fractions

Lipase activity of cell fractions was assayed on solid medium according to the procedure mentioned in item (3.2.5.1), while enzyme activity (u/ml) was determined according to the procedure mentioned in item (3.2.5.2).

This step was done after induction of lipase production by *E. coli* BL21(DE3) using IPTG.

3.2.25 Determination of lipase molecular weight Towbin *et al.* (1979)

Lipase produced by genetically engineered *E. coli* BL21 (DE3) was applied in polyacrylamied gel to determine the molecular weight under nondenaturing conditions. Protein ladder from biolabs was used to estimate the

molecular weight of separated proteins,   and as follows:

1- Resolving gel was prepared by adding 10 ml of acrylamide/ bisacrylamide solution prepared in item (3.1.10.7), then separating gel was transferred gently to the gel cassette using Pasteur pipette. The top of gel was covered by distilled water and allowed the gel to polymerize about 1h. at room temperature. Then the layer of distilled water poured off.

2- Stacking gel prepared as in item (3.1.10.8), then 60µl of 10% ammonium persulphate and 10 µl of TEMED prepared in item (3.1.10.5) and (3.1.10.6) were added and mixed gently. Using Pasteur pipette, staking gel was transferred gently over the separating gel at a level which allows the comb to be inserted with 5mm between the bottom and well, the gel was allowed to polymerize about 2h. at room temperature.

3- SDS-PAGE samples were prepared by adding an equal volume of sample buffer solution (2X) prepared in item (3.1.10.11) to the sample and mixed well, the samples were incubated in thermal block at 90°C for 5 min. and cooled to 25 °C, then 20µl of the prepar[illegible]

after loading the PAGE cassette in [illegible] in item (3.1.10.12).

The usual running time is about 45 min for 200v supply. After the run was done, the gel glass plate sandwich was gently separated and then stained by coomassie blue prepared in item (3.1.10.9) then destained using destaining solution prepared in item (3.1.10.10).

Chapter Four

Results

4. Results and Discussion

4.1 Isolation of *Bacillus stearothermophilus*

In order to isolate *Bacillus stearothermophilus*, fifty soil samples were collected from hot regions southern of Iraq. Results mentioned in table (4-1) showed that ten soil samples were taken from each Iraq province including Basrah, Karbala, Thiqar, Misan, and Al-Mothanna. A total of 54 isolates were obtained from these soil samples, these isolates were suspected to be *Bacillus stearothermophilus* maybe because of their ability to grow after incubation at 65ºC for 24 hours to kill any other contaminant bacterial growth, then to allow the sporulation of *Bacillus stearothermophilus* spores after incubation at suitable conditions. All of the isolates were subjected to identification according to their morphological and cultural characteristics and biochemical tests.

Isolation is the most important step specially when isolates were mesophilic *Bacillus stearothermophilus* because it has a large number of thermostable enzymes such as amylase, [illegible] industrial applications, moreover, it is strain the most attractive for isolation and study (Paulin *et al*., 1987).

Thermophiles are adapted to survive at high temperature in hot springs. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes. The enzymes from thermophiles, hence, are of great interest for industrial applications (Akanbi *et al*., 2010).

Table (4-1): Soil samples used for isolation of *Bacillus stearothermophilus*

Source of sample	No. of sample	No. of isolates
Basrah	10	12
Karbalaa	10	11
Thiqar	10	10
Misan	10	11
Mothanna	10	10
Total	50	54

4.2 Identification of bacterial isolates

4.2.1 Morphological characteristics

Different morphological characteristics of bacterial isolates were studied to identify these isolates. Results showed that most of these isolates appeared as rod in shape, singles or chains, spore forming, spores are round, terminal; colonies are [illegible] and can be degraded.

4.2.2 Biochemical identification:

Local isolates suspected to be members of *Bacillus stearothermophilus* were subjected to a number of biochemical tests.

Results, indicated in table (4-2), showed that 29 out of 54 of these isolates are positive for catalase, methyl red, and have the ability to hydrolyse gelatin, casein and starch, while they gave negative results for oxidase, indol, voges-proskauer. On the other hand, results mentioned in table (4-2) showed that these isolates have the ability to grow in presence of 2 and 5% NaCl, while they are unable to grow under higher salt concentration (7%) because this concentration affects microbial cell growth by making a blasmogenic to cell protoplast so that leads to stop cells anabolism then cause cells to die as mentioned by Rao *et al.* (1979).

Table (4-2): Biochemical tests for the local isolates of *Bacillus stearothermophilus.*

Biochemical test	Result
Gram stain	+
Catalase	+
Oxidase	-
Indol	-
Voges-Proskauer	-
Methyl red	+
Hydrolysis of	
Casein	+
Gelatin	+
Starch	+
[illegible]	[illegible]
6.8	+
[illegible]	+
5.7	+
[illegible]	[illegible]
2%	+
5%	+
7%	-
Growth at	
40 ºC	+
55 ºC	+
60 ºC	+
65 ºC	+

(+): Positive test.

(-): Negative test.

Results indicated in table (4-2), also showed that most of these isolates have the ability to grow. optimally at different thermophilic temperatures ranging between 40-65ºC, on the other hand these isolates were also able to grow under moderate acidic pH and nutral pH values (pH 6.8 and pH 7.0 respectively), while they are unable to grow at less pH value (pH 5.7). These results agreed with Rao *et al*., 1979; Carpenter, 1977; Eltayib *et al*., 2010).

After identification of the local isolates according to their morphological and physiological characteristics and biochemical testes, these 29 isolates are regarded as *Bacillus stearothermophilus,* and screened for their ability in lipase production.

4.3 Ability of locally isolated *B. stearothermophilus* for lipase

Gram-positive *Bacillus stearothermophilus* (Bayoumi *et al*., 2007)

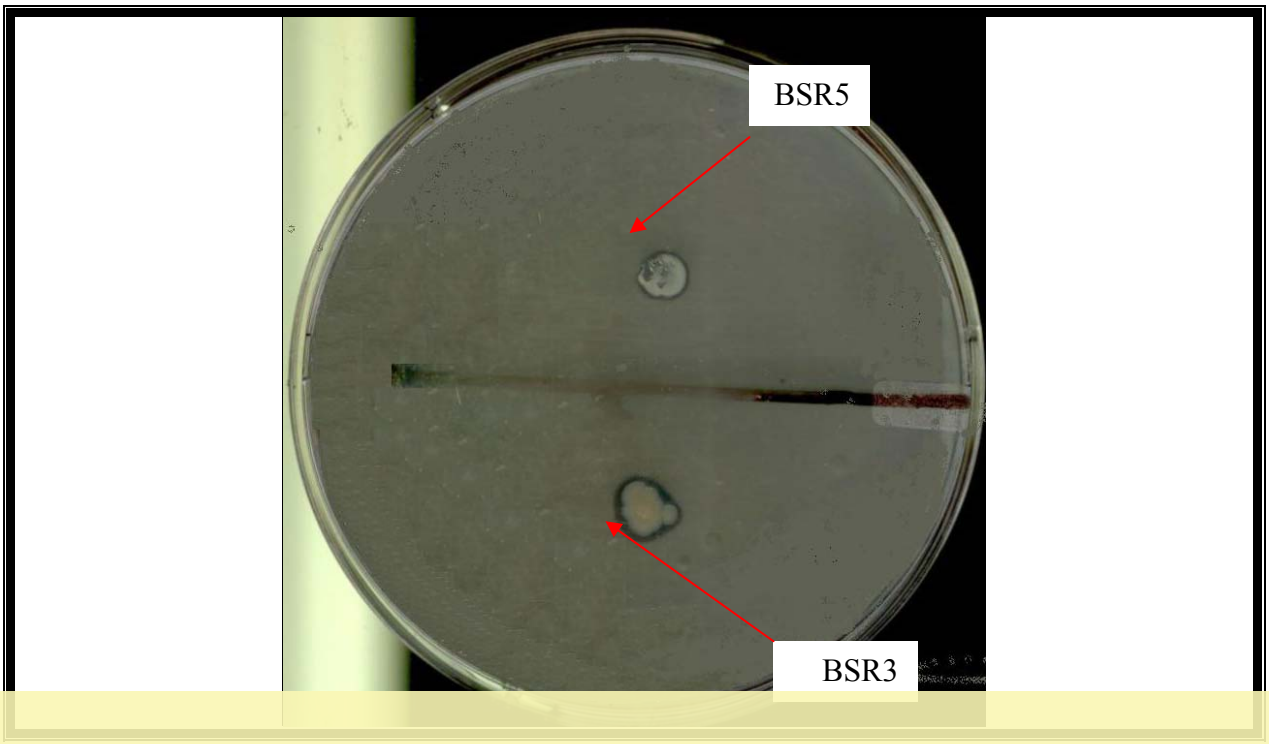
Results showed that two out of 29 isolates are lipase producers because of the presence of halo zones of lipolysis around colonies of each isolate with variable degrees. Lipase had the specificity for action on insoluble emulsified substrate by the adsorption of the enzyme to the surfaces of these hydrophobic substrates allowing lipolysis to occur as it was mentioned by Yu, *et al*. (2010).

According to select the most efficient isolate in lipase production, these local isolates were screened by determining the diameter of visible halos developed around each of the isolates on LB-tributyrin agar medium. Results indicated in table (4-3) showed the differences between these isolates in lipase production and lipolytic activity on LB-tributyrin agar medium expressed by the diameter of zone of hydrolysis as it was shown in figure (4-1). From these results it was found that the local isolate symbol BSR3 was the efficient in lipase production, because of the formation of highest diameter of hydrolysis zone around its colony (6mm), other isolate symbol BSR5 formed lower diameter of hydrolysis around its colony (4mm) on LB-tributyrin agar medium, while the other 27 isolates haven't the ability to produce lipase, so there are no zones of hydrolysis formed around there colonies. According to the results of zones of hydrolysis formed around BSR3 and BSR5 isolates, the isolate BSR3 (*Bacillus stearothermophilus* BSR3) was selected for cloning of lipase gene.

Genomic DNA of the *B. stearothermophilus* BSR3 was extracted using Qiagen Kit. Results showed that DNA extracted from this isolate was obtained in pure form because the ratio of the absorbance at 260nm divided by the absorbance at 280nm (260 /280 ratio) was 1.8 which refers to pure DNA. It was clear that purity of DNA ranged between 1.8 to 2.0 due to the 260 /280 ratio that will vary somewhat with the relative amounts of G/C and A /T in the DNA sample (Nazina, 2001). On the other hand results showed that the concentration of this pure DNA was 50 µg /µl. from this DNA concentration, aliqoutes of DNA were taken and used for amplification of lipase gene.

Table (4-3): Diameter of lypolysis by local isolates of *B. stearothermophilus* on LB-tributyrin agar medium after incubation at 65ºC for 16 hours.

Isolate no.	Diameter of clear zone(mm)
BSR1	0
BSR2	0
BSR3	**6**
BSR4	0
BSR5	4
BSR6	0
BSR7	0
BSR8	0
BSR9	0
BSR10	0
BSR11	0
BSR12	0
BSR13	0
BSR14	0
BSR15	0
BSR16	0
BSR17	0
BSR18	0
BSR19	0
BSR20	0
BSR21	0
BSR22	0
BSR23	0
BSR24	0
BSR25	0
BSR26	0
BSR27	0
BSR28	0
BSR29	0



Forward primers:

(1) 5´- TGATGGAACGCTGCCATGCCAGAG-´3

(2) 5´- ATGATGAAAGGCTGCCGGGTGATG -´3

Reverse primers:

(1) 5´- GTGCCATGTTGTAAGGTACGCCGTTG-´3

(2) 5´- TTAAGGCCGCAAACTCGCCAGTTG -´3

These primers were designed to include genetic elements upstream and downstream of lipase gene of *B. stearothermophilus* standard strain accession no. U78785 mentioned in NCBI DNA database, then four reactions were achieved by PCR and four products were obtained with different lengths and control elements as mentioned in table (4-4). The predicted lengths for these fragments are 1500, 1200, 1400, and 1400bp after amplification of lipase gene using the two sets of primers (forward (1) against reversward (1) and (2), and forward (2) against reversward (1) and (2) respectively).

Reaction products of PCR were analyzed on agarose gel to identify the amplified fragments in presence of 10 Kb DNA landmark ladder. Results of gel electrophoresis mentioned in figure (4-2) showed the presence of products for these four amplified fragments at exact lengths and positions predicted [illegible] the 1400bp fragment in lane (4) and (5)).

Other amplified fragments than the expected presents in different lanes on [illegible] to the am[illegible] *[illegible]thermophilus* genomic DNA, in which the selected primers may share the complementary base pairing then annealing and amplification of other undesirable regions.

The expected and desired fragment (1200bp) may represent lipase gene fragment, extracted from agarose gel, purified, and cloned in pPCRScriptCamSK(+) cloning vector, then the recombinant vector was used to transform *E. coli* DH5α by electroporating competent cells of this strain in SOC medium for two hours at 37ºC . Transformants were selected on LB-agar medium containing the selectable marker (X-gal and chloramphenicol).

Table (4-4): Primer design for amplification of *B. stearothermophilus* BSR3 lipase gene.

Reac. No.	Oligonucleotide sequence	Genetic elements contained	Fragment size (pb)
1	Forward(1) : 5´-TGATGGAACGCTGCCATGCCAGAG-´3 Reversward(1): 5´-GTGCCATGTTGTAAGGTACGCCGTTG-´3	P - ORF - T	1500
2	Forward(2): 5´- ATGATGAAAGGCTGCCGGGTGATG -´3 Reversward(2): 5´- TTAAGGCCGCAAACTCGCCAGTTG -´3	ORF	1200
3	Forward(1): 5´-TGATGGAACGCTGCCATGCCAGAG-´3 Reversward(3): 5´-GTGCCATGTTGTAAGGTACGCCGTTG-´3	ORF - T	1400

P: promoter; ORF: open reading frame; T: terminater

Single of the white colonies of transformant *E. coli* was taken and propagated in LB- broth medium with chloramphenical, extracted the recombinant pPCRScript SK(+) cloning vector containing the desired lipase fragment to insure the exact insert of lipase gene then sequencing the complete nucleotide sequence of the gene. Results indicated in figure (4 - 3) showed the complete nucleotide sequence of the expected amplified lipase gene; from this figure, it was found that there are 40 codons coding for different 40 amino acids, starting with ATG start codon for Methionine. Although GUG and UUG have been reported to function as initiation codons in *Bacillus* (Wang and Doi, 1986;

Wong *et al*., 1999) since most prokaryotic and eukaryotic genes initiate with the AUG start codon (Smith *et al*., 1952; Schmidt *et al*., 1996).

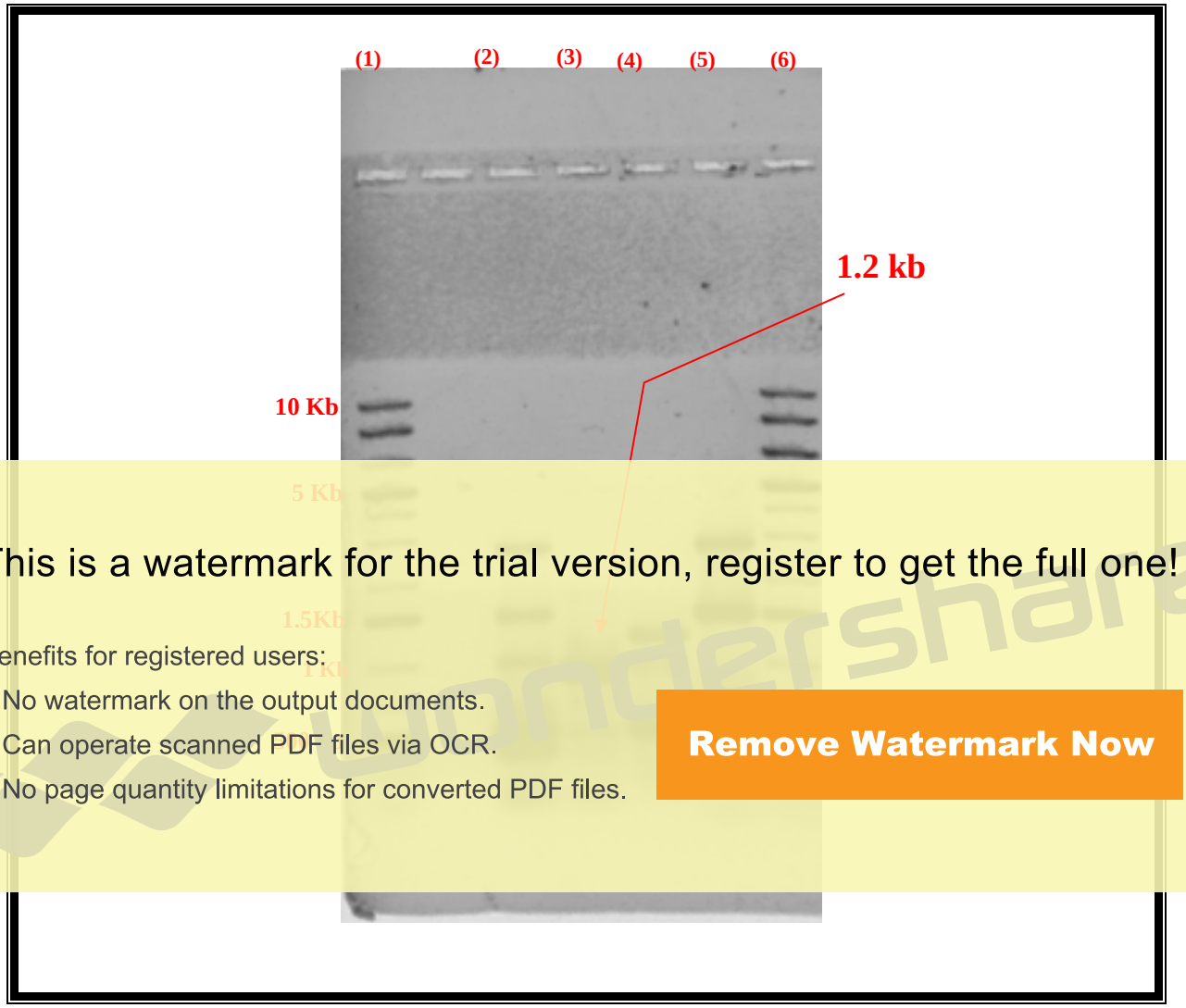


Figure (4-2): Aagarose Gel electrophoresis for amplified products of *B. stearothermophilus* BSR3 lipase gene on (0.8%) agarose gel for 1 hour, 60 voltage.

Lane (1) and (6): 10Kb DNA landmark ladder.

Lane (2): Amplified products using forward (1) and reversward (1) primers.

Lane (3): Amplifid products using forward (1) and reversward (2) primers.

Lane (4): Amplified products using forward (2) and reversward (1) primers.

Lane (5): Amplified products using forward (2) and reversward (1) primers.

1 <u>atg</u> atgaaag gctgccggga ggacgata t c ggaatacag c c c tgttccgt 50
51 tcatgc g aaa atcg atg ttt accatta a a t cgagcttt c c ttcgggcgca 100
101 t c ct cat tcc aaacgat ttc gctcgtc t t t aactcatt a t ttt gct cgct 150
151 taaa ttc g ca tgatggg tcc ctaacaga t g tttta aaa a a tat tca tgt c 200
201 ct tt t gc cga gctgccg atc aaattggc g c gcc aaacaaa cat cac tctc 250
251 ggaaag ttg a tcgggtcgga cggattt t c g atcgc aaacg tta aat tcc c 300
301 tt gt t tt a t t tcattgac c a catattg a a t cacttctt c g ttt gtc t tt g 350
351 cgttt g c ct t ttccacta a c gtgatgct g t ttgcgtt a a a ttg cggataa 400
401 gatggga gc c accctaaacg aaccgctaa a tagtta t a g t cgccgccatg 450
451 ctgataa c g c ggctgatcaa acaacgggga a atatg tt c g tcc atc t ttt 500
501 gatc ttc g t a tttc cac tgc tctgtta c a a aa ta gaa aaa cgacgttccg 550
551 ttt t gca ga c gcggcggtcc tccccagtcg c gcg cca tcg caa tcgtctg 600
601 ccag cct tcc agcggccgca [illegible] 650
651 c tcc acc att tact cct tg c gaacccgtta [illegible] 700
701 [illegible] 750
751 [illegible] tcgaatcga t [illegible] 800
801 g cg[illegible] ttg cgccgcc gt g tttcgatca a tgc c a g t a a t cgc ttcctgc 850
851 cat gcc ggcg t ata cg g ttt cggatcatt a ta a t c ga c a g ga<u>taa</u>tcacc 900
901 cgg cag cct t tcatcat 917

Figure (4-3): The complete nucleotide sequence of the expected amplified lipase gene of *B. stearothermophilus* BSR3 from NCBI. First underline sequences represents atg start codon, while the other represents taa stop codon.

4.6 Alignment and analysis of lipase gene fragment

The complete nucleotide sequence of lipase gene mentioned in figure (4-3) was aligned with DNA sequence of different microorganisms found in NCBI / Blast (WWW.NCBI.net) to determine the maximum similarity and identity of this fragment with similar sequences of other microorganisms. Results showed that sequence of the amplified fragment expected to be lipase gene was identical to portion of nitrate reductase of *Geobacillus sp*. WCH70 as it was shown in table (4-5), figure (4-4) and in Table (4-6).

According to this results, renamed the locally isolate of *B. stearothermophilus* BSR3 to *Geobacillus stearothermophilus* BSR3

Results obtained from table (4-5) showed that *G. stearothermophilus* BSR3 lipase gene have 67% degree of similarity to portion of nitrate reductase gene of [illegible] other hand results mentioned in figure (4-4), table (4-6) showed that the similarity was 67% for the query (lipase fragment of *G. stearothermophilus* BSR3) to portion of nitrate reductase of *Geobacillus sp.* WCH70 while the degree of similarity was decreased when compared with other sequences for different microorganisms such as *Geobacillus stearothermophilus* GSA17K (66%); *Bacillus licheniformis*ATCC 14580, (41%); *Bacillus licheniformis*DSM13 (41%); *B. cereus* subsp. Cytotoxis NVH 391.98(52%); *B. cereus* E33L(36%); *Staphylococcus aureus* subsp. *aureus* genome (32%); and *staphylococcus aureus* subsp, *aureus* COL (32%).

Table (4-5): Alignment of nucleotide sequence of *G. stearothermophilus* BSR3 lipase gene with portion of *Geobacillus* sp. WCH70 nitrate reductase gene.

Reaction No.	Aligned Region	Identity (%)	Coverage (%)
2	ORF	67	92

ORF: Open Reading Frame

Figure (4-4): Nucleotide sequence alignment of locally isolated *G. stearothermophilus* BSR3 lipase gene with *Geobacillus sp* WCH70 nitrate reductase gene. NCBI / Blast (WWW.NCBI.net)

(1): *Geobacillus sp* WCH70
(2): *Geobacillus stearothermophilus* genomic DNA.
(3): *Bacillus licheniformis*ATCC 14580.
(4): *Bacillus licheniformis*DSM13.
(5): *B. cereus* subsp. Cytotoxis NVH 391.98.
(6): *B. cereus* E33L.
(7): *Staphylococcus aureus* subsp. *aureus* .
(8): *Staphylococcus aureus* subsp, *aureus* .

Table (4-6): Percentage of similarity and coverage for the nucleotide sequence alignment of *G. stearothermophilus* BSR3 lipase gene with other DNA sequences of different microorganisms.

Accessory No.	Description	Similarity %	Coverage %
Cp001638.1	*Geobacillus* sp.WCH70	67	92
AB126615.1	*Geobacillus stearothermophilus*	66	92
CP000002.3	*Bacillus licheniformis*ATCC 14580	41	72
AE017333.1	*Bacillus licheniformis* DSM13	41	72
CP000764.1	*B. cereus* subsp. Cytotoxis NVH 391-98	52	70
CP000001.1	*B. cereus* E33L.	36	66
AP009351.1	*Staphylococcus aureus* subsp. *aureus*	32	75
CP000046.1	*Staphylococcus aureus* subsp. *aureus*	32	75

indicated in appendix (D) showed the expected lipase gene of *G. stearothermophilus* BSR3 and portion of nitrate reductase of *Geobacillus* sp. WCH70. Results of alignment for DNA sequences of open reding frame of lipase gene was similar (67%) to region of nitrate reductase covering between 1345 bp and 2261 bp of nitrate reductase gene. Similarity between the two sequences expressed by stars (*), while the non similar sequences appeared as gaps between these stars.

According to results of similarity, lipase gene of *G.stearothermophilus* BSR3 was amplified using two newly designed primers according to the DNA sequence of *Geobacillus*. Sp.WCH70 lipase gene (1400bp) from NCBI (accessory no. CP001638)

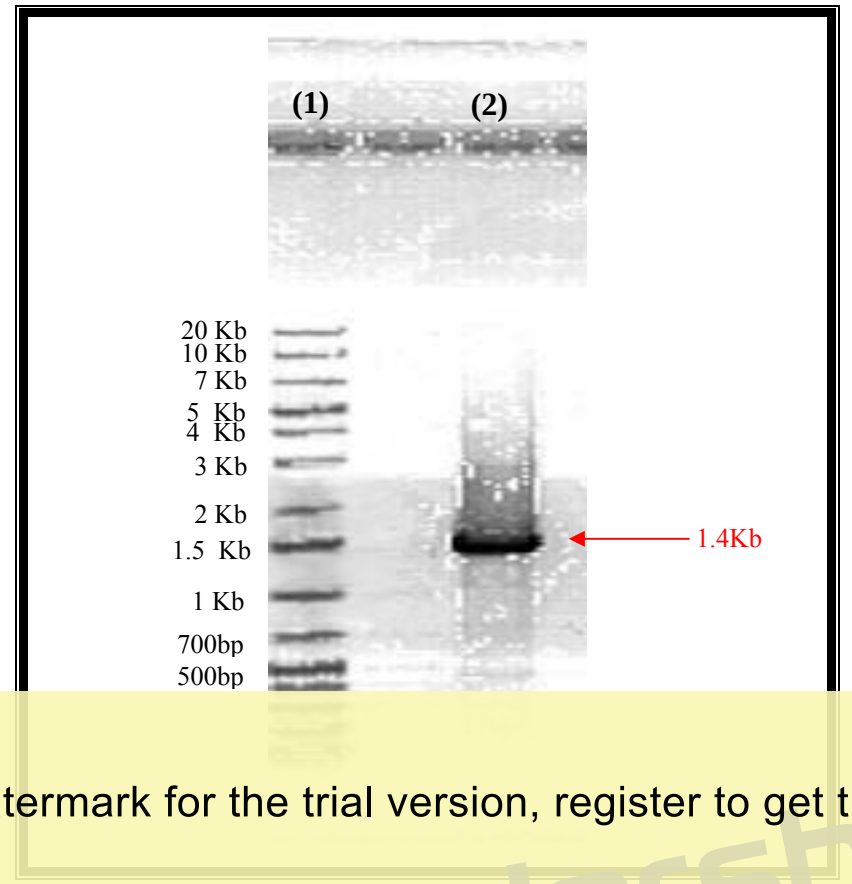
4.7 Amplifiction of *Geobacillus stearothermophilus* BRS3 lipase gene

In order to amplify lipase gene of *Geobacillus stearothermophilus* BRS3, two primers were designed according to the DNA sequence of lipase gene of *Geobacillus* WCH70 with upstream and downstream genetic element sequences(-35 and -10 promoter sequences, shine dalgarno, leader sequence and hairpin loop terminator sequence). These two specific primers have the following sequences.

Forward primer: 5´-GCAGGAAAAAGCTGAAGCGGC-3´

Reversward primer: 5´-TTTCGACAATGCAGCCGATAGG-3´

To explore the exact amplification of lipase gene fragment with the exact molecular size. Results mentioned in figure (4-5) showed that only one band appeared on agarose gel after amplification of genomic DNA of *Geobacillus stearothermophilus* BSR3 using these two specific primers. From this figure it was found that the molecular weight of this fragment (lipase gene) was 1400 bp when compared with the position of DNA landmarker fragment of 1400 bp appeared together on the same gel.



Lane (2): lipase gene fragment

The amplified fragment was eluted from agarose gel, purified, and cloned in pPCRScriptCam SK(+) cloning vector, then the recombinant vector was used to transform *E. coli* DH5ɒ by electroporation to obtain higher concentration of recombinant vector after transformation and cell division. Transformants were selected on LB-agar medium containing the selectable marker (X-gal and chloramphenicol), then a single of the white colonies of transformant *E. coli* was taken and propagated in LB- broth medium with chloramphenical, recombinant vector containing the desired lipase gene fragment was extracted.

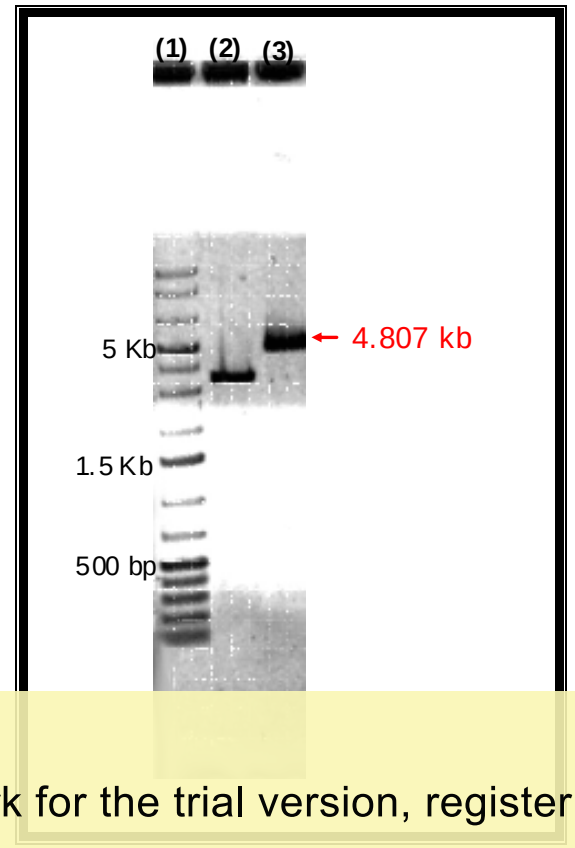
Recombinant vector [pPCRScriptCam SK(+) with the insert] was digested with *Kpn*I to get linearized vector for easy electrophoresis on agarose gel with one form of plasmid DNA(linear) and to compare the exact molecular weight of lipase gene insert after amplification in presence with the linear landmark (10Kb).(Sambrook and Russell, 2001).

Results, indicated in figure (4-6), showed DNA profile of recombinant vector after electrophoresis for one hour on agarose gel, from this figure it was found that were two plasmid DNA bands which appeared on agarose gel, the first represented the linearized pPCRScriptCam SK(+) cloning vector (3407bp) when compared with the landmark ladder occurring at the same position; this molecular weight calculated from agarose gel is the exact molecular weight of pPCRScriptCam SK(+), while the second band on agarose gel represented the molecular weight of this band was 4807bp in comparison with the molecular weight of landmark ladder fragment occurring at the same position on the same gel and this band represented [illegible] pPCRScriptCam SK(+)(3407bp) together with lipase gene insert (1400bp).

4.8 Nucleotide sequence of *G. stearothermophilus* BSR3 lipase gene

Lipase gene of BSR3 inserted in recombinant pPCRScript(+)was sequenced to determine the complete nucleotide sequence of the gene. Results, indicated in figure (4-7), showed that lipase gene was 1400 bp in length, rich in GC (45.7 %). Codes for 264 amino acid are mentioned in figure (4-8).

Results of DNA sequence of lipase gene mentioned in figure (4-7) also showed promoter sequence (with both -35 and -10 regions) which represent region of DNA in front of a gene that binds RNA polymerase and so promotes



Lane (1): landmark ladder (10Kb)

Lane (2): pPCRScriptCam SK(+) cloning vector digested with *Kpn*I

Lane (3): Recombinant pPCRScriptCam SK(+) cloning vector digested with *Kpn*I

gene expression, and shain-dalgarno sequence close to the front of transcription mRNA that is recognized by the ribosome, and stop codons that signals the start and the end of protein transcription respectively, and leader sequence (84 bp in length) coding for leader peptide containing 27 amino acids. This leader peptide which a short peptide coded by the leader region of a lipase gene controlled by the attenuation mechanism was rich in hydrophobic amino acids (16 hydrophobic amino acids) such as isoleucine, valine and leucine respectively as shown in figure (4-8).

RBS usually contains a sequence GGAGG and has an average free energy of about -17 nucleotide for binding between the 3´ end of the 16S rRNA and the RBS region of the mRNA (Band and Henner, 1984; Sifour *et al*. 2010).

A promoter is a specific region just upstream from a gene that acts as a binding site for transcription factors and RNA polymerase during transcription initiation. This region is characterized by sequences at positions -10 and -35 base pairs upstream from the transcription start site in prokaryotes organisms. The consensus sequence 5´-TTGACA- N_{17}-TATAAT-3´ that is recognized by σ^A in *Bacillus* species is identical to the consensus recognized by σ^{70} in *Escherichia coli* (Kazuo and Ogasawara, 2002). There are at least three intrinsic parameters that affect promoter engagement during transcription: the hexamer DNA between the two boxes (spacer region). The strength of the promoter refers to the frequency of the promoter to initiate transcription. A strong promoter is substitutions resulting in infrequent initiations. Strong promoters are usually applied to improve the level of gene expression (Wang and Doi, 1984; Suriana *et al*., 2009).

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1	cgcaagctgt	atgaagaag t	cagcgcttt c	ttgcaagaaa	gaaatatttc	accgacatca	60
61	gaagcgtatg	acatcatccg	tctcgat tt a	caagtat gtt	cgatgggaat	tgtagat t a t	120
121	ttgcaaaaac	ggtaaggcac	tagaaatt t g	tttgaaca ta	atccagtaag	aacggcttg g	180
181	tgaaatcacc	aagctt tt t c	attgtggcg g	ctgtt tt at a	acggcgtgct	t tttacta t c (35-)	240
241	aaatgcggag	agtctaaag g	tgtagcctg g	atgaaaatat (10-)	tgtatgat a a	gtaacaaaga	300
301	taagaaagg**a** (RBS)	**tg**attgca t g	agacgcggta	ttgtaagc a c	cat cacgatt	gtatccgt g c	360
361	tggcgggaat	attatgg c t t	ggcggttt tg	ccatgggca t	tcaagat caa	ttt t t tt cc g	420
421	ccgcgatgcc	gccagcaggc	acgacgaaat	atacaaaaga	gaaaaaagcg	gacaaccgtg	480
481	aaatt tat a t	tgtcgctc t t	ggcgactcgc	tgacaagggg	aacgggagac	gaaagcggaa	540
541	aagggtat a t	tggctata t g	gtcgattc t c	tccgtaa aaa	aacaacgaaa	ccgattcg t g	600
601	taacaaac t t	ggctattaa a	ggacagcgat	ctgacgg act	cct taa gcaa	t taggacaa g	660
661	ctgagat a a a	gaggcagcta	aaaatggct g	acatcat cgt	gat gacgatc	g gcgggaatg	720
721	attt gt t t c a	aggcggagaa	gcgctaaagc	ttgcgccaaa	gcaaatcgag	caggcaaaaa	780
781	acgcgta t t t	acacaact t a	gaccgtat t t	ttcaaac gat	tcg cagcgtc	aataaaga t g	840
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
901	catccgcg a t	cgtaaggcag	tggaa tt t t g	cttcggc ag[illegible]	aacgg[illegible]	cgcta[illegible]cc a[illegible]	960
961	atatcat t g c	t g tgccgata	tt cg a tt tgt	[illegible]t tca	[illegible] gaa [illegible]gat	ta ttt gt a c a	1020
1021	[illegible]	[illegible]	[illegible]	ataaacgca[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	gacggag[illegible]	gacaaacaat	gacgacaa[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	cgatttacg c	aaaacaattc	gccgcaaaga	aattatca a a	gga att tcat	ttg aa ct g aa	1200
1201	ggaaggtgaa	g tatttggg t	ttttagga c c	gaatggcgc g	ggaaaaacga	cca cca tccg	1260
1261	catgcttg t c	gggcttatca	agccaacatc	gggacata t t	t c t atttgcg	gct atg ac c t	1320
1321	agagcgtcag	tttaccaaag	cgattcgcc a	tatcggc t g c	a t tgtcgaaa	atc ca g a att	1380
1381	atatccg ta t	ttaagtggat	ggg				1403

Figure: (4-7) Nucleotide sequence of lipase gene fragment of *G. stearothermophilus* BSR3.

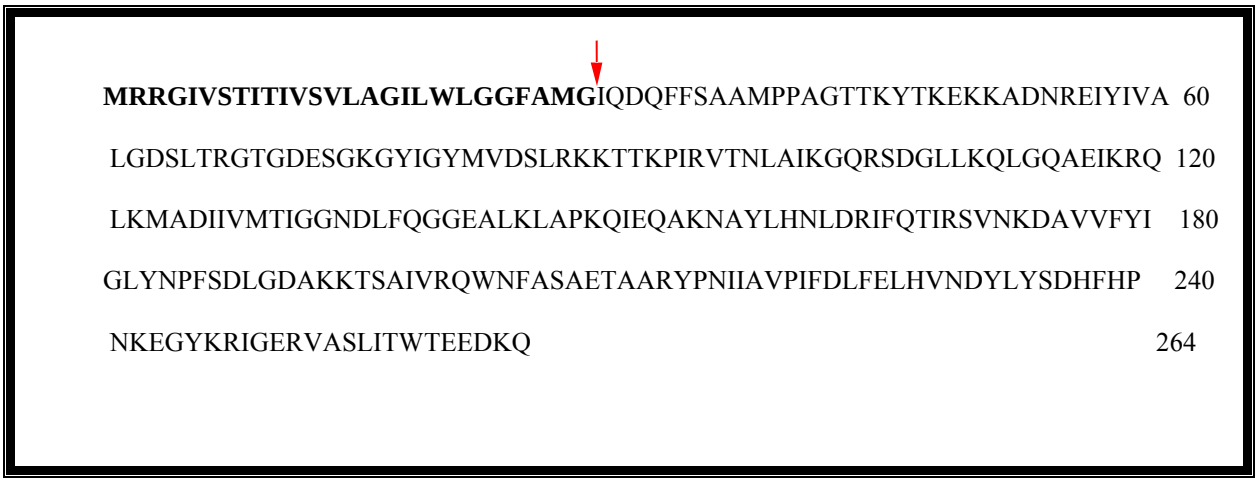


Figure (4-8): Deduced amino acid sequence of lipase enzyme from locally isolated *Geobacillus stearothermophilus* BSR3.
• Bold letters represents leader peptide for whole enzyme.
• Red arrow represents cleavage site for removing leader peptide between amino Acid residues 27 and 28.

Like other *Bacillus* lipase, an Ala replaces the first Gly residue in the conserved amino acid sequences Gly-Xaa-Ser-Xaa-Gly, which is conserved among microbial and mammalin lipases (Leow *et al*., 2004; Li and Zhang 2005; Leow *et al*., 2007).

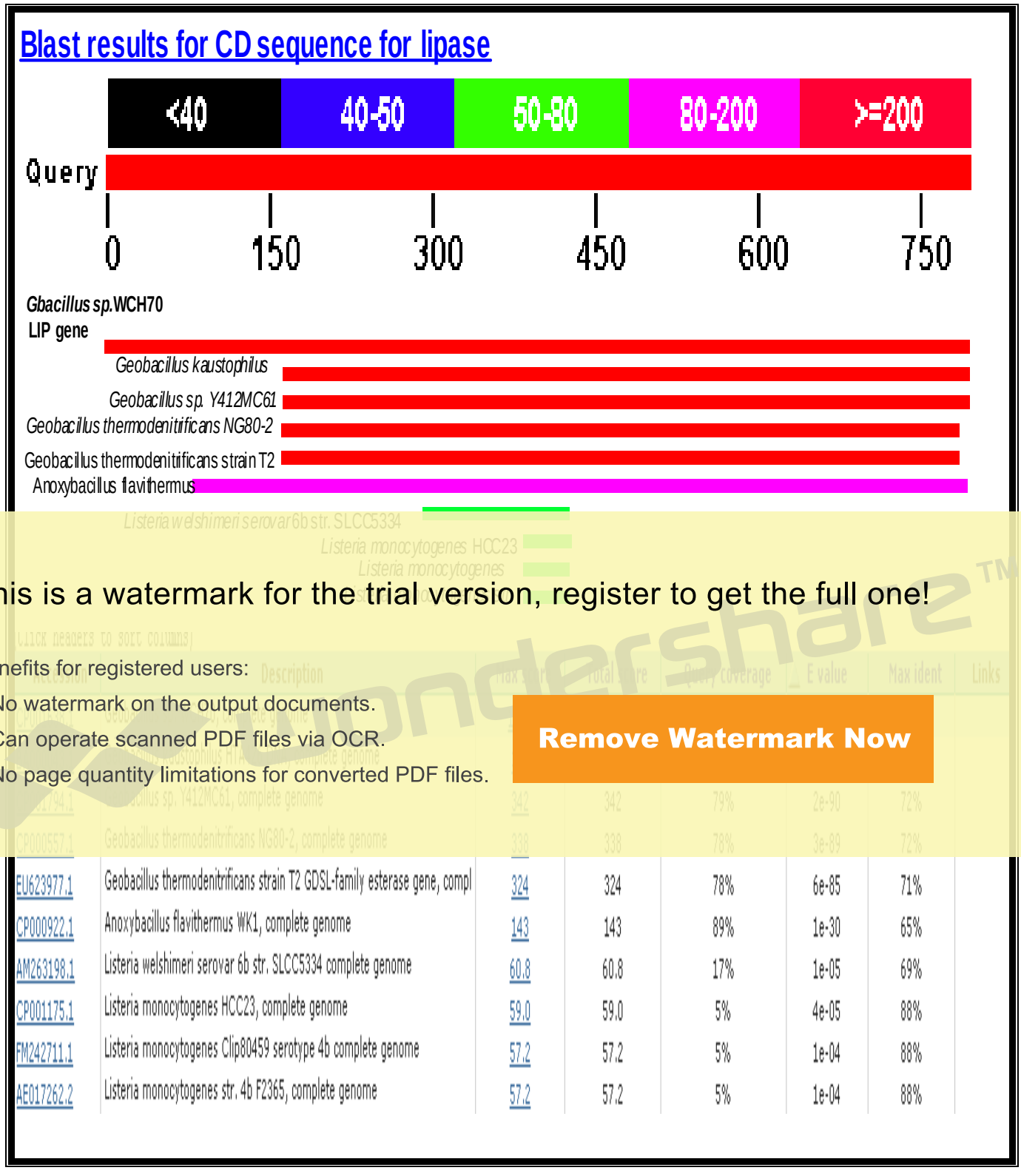
From these results, it was found that the nucleotide sequence and deduced amino acid sequence of *G. stearothermophilus* BRS3 was absolutely identitical (100%) to the nucleotide sequence and deduced amino acid sequence of *Geobacillus sp.* WCH70 lipase gene as shown in figure (4-8).

Results mentioned in this figure showed that *G. stearothermophillus* BSR3 lipase fragment have 99% degree of similarity to lipase gene of *Geobacillus sp* WCH70, covering 100% DNA sequence of the gene.

On the other hand results mentioned in figure (4-9) showed that the sequence similarity decreased when it was compared with other related gene sequences of other microorganisms.

From these results it was also found that the degree of similarity between the nucleotide sequence of lipase gene BSR3 and *Geobacillus* WCH70 was 99%, but not 100% due to the presence of two silent mutations found in lipase gene of *G. stearothermophilus* BSR3 (query) at position 170 (thimine) and 606 (adenine) in comparison with the same position 170 (cytosine) and 606 (thymine) respectively.

and their deduced amino acids, the locally isolated BSR was regarded as *Geobacillus stearothermophilus* BSR3 that have the ability to produce extracellular lipase enzyme.



EU623977.1	Geobacillus thermodenitrificans strain T2 GDSL-family esterase gene, compl	324	324	78%	6e-85	71%
CP000922.1	Anoxybacillus flavithermus WK1, complete genome	143	143	89%	1e-30	65%
AM263198.1	Listeria welshimeri serovar 6b str. SLCC5334 complete genome	60.8	60.8	17%	1e-05	69%
CP001175.1	Listeria monocytogenes HCC23, complete genome	59.0	59.0	5%	4e-05	88%
FM242711.1	Listeria monocytogenes Clip80459 serotype 4b complete genome	57.2	57.2	5%	1e-04	88%
AE017262.2	Listeria monocytogenes str. 4b F2365, complete genome	57.2	57.2	5%	1e-04	88%

Figure (4-9): Nucleotide sequence alignment of *G. stearothermophilus* BSR3 lipase gene with related sequences of different microorganisms. NCBI / Blast (WWW.NCBI.net).

In contrast to Gram-negative bacteria, proteins that are secreted to the extracellular environment by Gram-positive bacteria only need to travel through a single membrane to enter the surrounding medium (Van-Wely *et al*., 2001; Nawani *et al*., 2006) While targeting, a protein for export appears to be straightforward through the use of signal peptides, suitable factors need to be understood to achieve maximum efficient translocation of a particular protein (Simonen and Palva, 1993). To differentiate cytosolic proteins from extracellular ones, proteins destined for secretion are synthesized as precursors with a cleavable amino terminal signal peptide (Bron *et al*., 1999). These signal peptides ensure proper targeting of the polypeptide to the translocation machinery at the cytosolic membrane. The general secretory pathway mediates the translocation of proteins in an unfolded conformation (van Roosmalen *et al*., 2004).

In other studies it was found that lipase gene from thermophilic *Geobacillus* sp. TI containing an open reading frame of 1023 bp coding for lipase enzyme with molecular mass of 37 kDa consisting of mature [illegible] with a leader peptide of 28 amino (Nielsen *et al*., 1997).

4.9 Amplification of *G. stearothermophillus* BSR3 lipase gene with control elements

Lipase gene of *G. stearothermophilus* BSR3 sequenced previously was reamplified to contain different genetic elements in order to study the over expression system of lipase gene in suitable host of *E. coli* at a transcriptional level under the control of strong promoter of the expression vector pET20b(+).

For this purpose, two sets of primers shown in table (4-7) were designed to obtain coding region (ORF) of lipase gene with and without control elements in

four different strategies described according to the knowledge as follows:

1. Amplifying open reading frame of lipase gene without both leader sequence (ll) and stop codon $(Stp.cd)^-$, with *Nco*I / *Xho*I cohesive ends. In this case the expression of lipase gene must occur in the presence of leader sequence and T7 terminator of pET20b expression vector upstream and downstream of lipase gene respectively.
2. Amplifying open reading frame without leader sequence $(ll)^-$, but with stop codon $(Stp.cd)^+$ control elements, with *Nco*I / *Xho*I cohesive ends. In this case the expression of lipase gene must occur in the presence of leader sequence of pET20b and terminates at lipase gene stop codon.
3. Amplifying open reading frame with both leader sequence $(ll)^+$and stop codon $(Stp.cd)^+$, with *Nde*I/ *Xho*I cohesive ends. In this case the expression of lipase gene must be occur in the presence of native signal peptide of lipase gene and with T7 terminator of pET20b(+) by deleting lipase gene stop codon.

In these different cases, the expression must occur under the control of T7 promoter of pET20b(+) expression vector. The genetic and restriction map of pET20b(+) expression vector is indicated in appendix (V).

Table (4-7): primers design for amplification of *G. stearothermophilus* BSR3 lipase gene with the desired characteristics.

Reac. No	Type of primer	Nucleotide sequence	Fragment discription	expected size (bp)
1	Fwd	5-TTGCCATGGGCATTC AAGATCAA-3	*Lip*(II)$^-$,Stp cd$^-$	721
	Rwd	5-CTCGAG TCATTG TTTGTCCTCC-3		
2	Fwd	5-TTG CCATGGGCATTCAAGATCAA-3	*Lip*(II)$^-$,Stp cd$^+$	721
	Rwd	5-TTGCCATGGGCATTCAAGATCAA-3		
3	Fwd	5-GATCATATGAGACGC GGTATTG-3	*Lip*(II)$^+$,Stp cd$^+$	796
	Rwd	5-CTCGAGTCATTGTTTGTCCTCC-3		
4	Fwd	5-GATCATATGAGACGCGGT ATT G-3	*Lip*(II)$^+$,Stp cd$^-$	796
	Rwd	5-CTCGAGTTGTTTGTCCTCCTC C-3		

Lip (II)$^-$:Lipase gene without leader sequence,
Lip (II)$^+$:Lipase gene with leader sequence

These amplified fragments were designed to study the expression system of lipase gene under the control of both indigenous control elements of lipase gene and the control elements of pET20b expression vector in different experiments.

Results indicated in figure (4-10) showed the DNA profile of the amplified lipase gene fragments mentioned above, this figure shows the four amplified DNA bands with the exact molecular weight on agarose gel which were identical to the expected molecular weight of the symmetrical mentioned in table (4-7) after designing the specific primers for obtaining the desired fragments of lipase gene.

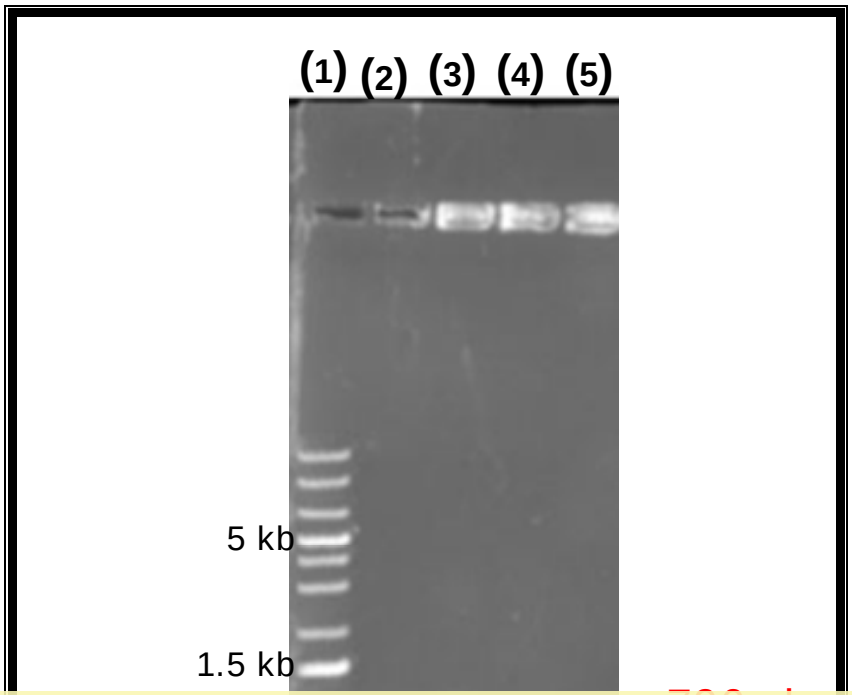


Figure (4-10): DNA profile of amplified lipase fragments with and without control sequence after electrophoresis on agarose gel (0.8%) for one hour.

Lane (1): landmark ladder 10 Kb.
Lane (2): lipase gene fragment without both leader sequence and stop codon.
Lane (3): lipase gene fragment without leader sequence, but with stop codon control element.
Lane (4): lipase gene fragment with both leader sequence and stop codon.
Lane (5): lipase gene fragment with leader sequence, but without stop codon control element.

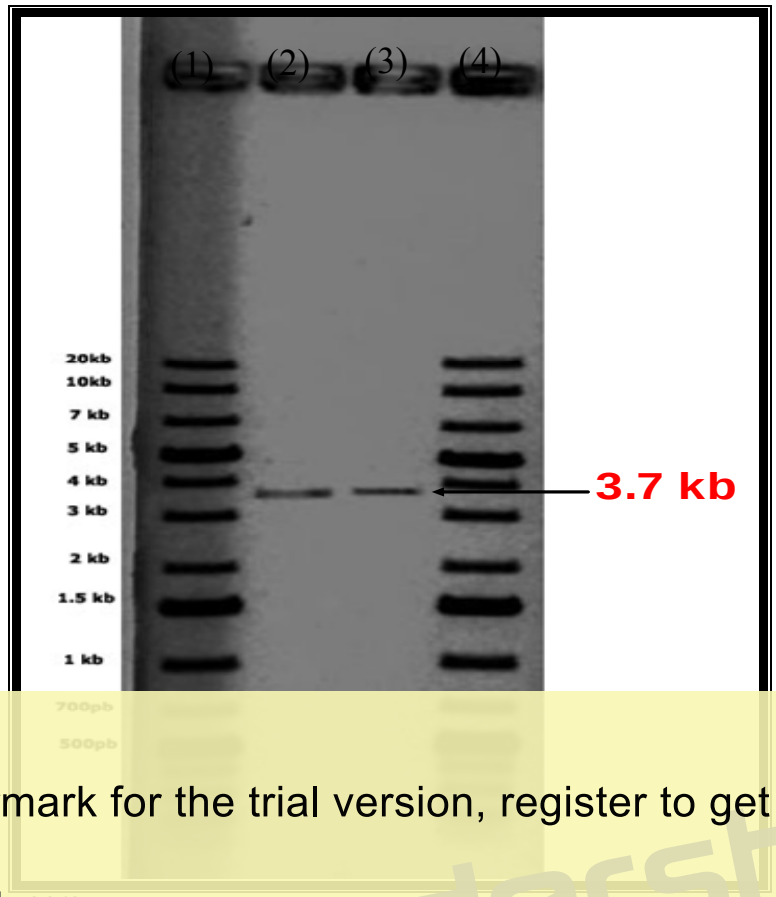
These four amplified fragments were eluted, purified from agarose gel, and cloned separately in pPCRScriptCam(+) cloning vector; these recombinant vectors were transformed in *E. coli* DH5α to obtain much more concentration of each vector after bacterial propagation and extraction of these vectors.

This step was done to achieve double digestion for recombinant vectors in order to obtain the desired lipase fragments with suitable cohesive termines (*Nco*I /*Xho*I and *Nde*I /*Xho*I respectively) designed previously.

It is much better to obtain DNA fragments with cohesive ends by digestion recombinant circular vectors rather than restriction the linearized fragments especially when the restriction sites was occurred at specific extra ends of any DNA fragment .

4.10: Restriction analysis of lipase gene fragments

Recombinant vectors containing the desired lipase fragments were double digested with two sets of restriction enzyme(*Nco*I/*Xho*I) and (*Nde*I/*Xho*I) to generate the amplified four fragments of lipase gene and were cloned separately in two patterns of pET20b(+) expression vector double digested with the same two sets of restriction enzymes(*Nco*I/*Xho*I) and (*Nde*I/*Xho*I) used to generate lipase fragments from recombinants pPCRScript Amp SK(+) to get compatible ends for both vectors and DNA fragments and [illegible] indicated in figure (4-11) showed the two patterns of pET20b(+) expression vector after digestion with both sets of restriction enzyme and electrophoresis on gel. From this figure, it was found that the molecular size of both patterns of double digested pET20b(+) was about 3700 bp out of the total molecular weight of this vector (3716 bp) according to the supplier company novagen (www.novagen.com), that means the digestion of this vector with both sets of restriction enzymes in multiple cloning site (MCS) generates two linearized DNA molecules, the major was about 3700bp which appeared on agarose gel and the very small fragment of about 16 bp which disappeared after electrophoresis on agarose gel (1%) due to its very small size that needs to be run and visualized on polyacrylamide gel (Tolstoshev and Blackesely, 1982).

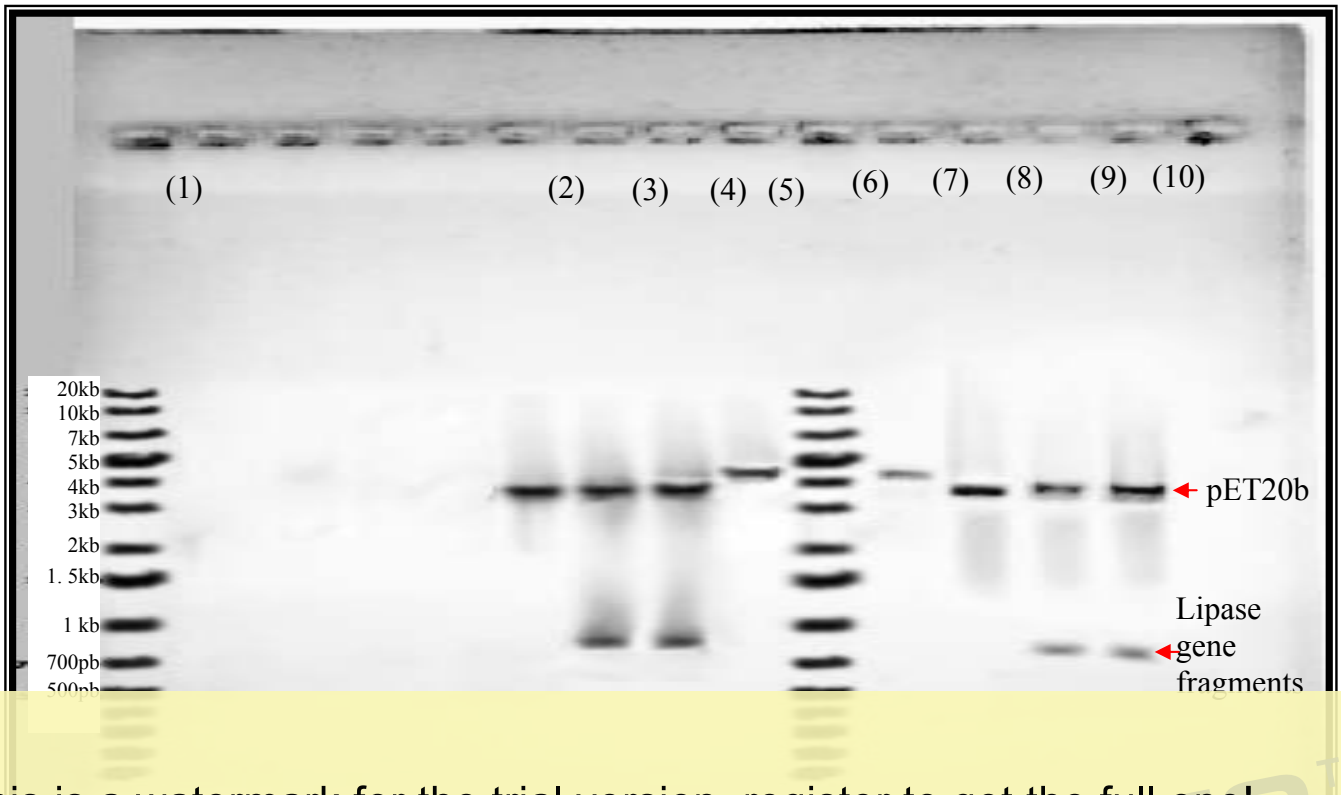


The two patterns of pET20b(+) expression vector restricted with both sets of restriction enzyme were eluted from agarose gel, purified, and ligated with similar cohesive ends of the four amplified fragments of lipase gene as it was mentioned in table (4-8).

Table (4-8): Types of lipase gene fragments and pET20b(+) cloning vector obtained after digestion with both sets of restriction enzymes (*Nco*I/*Xho*I) and (*Nde*I/*Xho*I).

Lipase gene fragment				Vector description	
Fragment No.	**Fragment Description**	**Fragment size (pb)**	**Type of terminies**	**Type of terminies**	**Size (bp)**
1	Lip(ll)$^-$, Stp cd$^-$	721	(*Nco*I/*Xho*I)	(*Nco*I/*Xho*I)	3700
2	Lip(ll)$^-$, Stp cd$^+$	721	(*Nco*I/*Xho*I)	(*Nco*I/*Xho*I)	3700
3	Lip(ll)$^+$,Stp cd$^+$	796	(*Nde*I/*Xho*I)	(*Nde*I/*Xho*I)	3700
4	Lip(ll)$^+$, Stp cd$^-$	796	(*Nde*I/*Xho*I)	(*Nde*I/*Xho*I)	3700

generated after double digestion of recombinant pET20b with suitable restriction enzyme. These four patterns of lipase gene were designed to study the best expression system in new host of *E. coli* BL21(DE3) and to determine the translocation of gene product (lipase enzyme) in the host cells.



Lane (2): pET20b double digested with (*Nde*I/*Xho*I).

Lane (3): Recombinant pET20b with lipase gene insert [lip (ll)$^{+}$, (Stp.cd)$^{+}$] double digested with *Nde*I and *Xho*I .

Lane (4): Recombinant pET20b with lipase gene insert (lip (ll)$^{+}$, (Stp.cd)$^{-}$) double digested with *Nde*I and *Xho*I .

Lane (5): Linearized recombinant pET20b with lipase gene insert (lip (ll)$^{+}$ digested with *Xho*I.

Lane (7): Linearized recombinant pET20b with lipase gene insert (lip (ll)$^{-}$,digested with *Xho*I.

Lane (8): pET20b double digested with *Nco*I and *Xho*I.

Lane (9): Recombinant pET20b with lipase gene insert [lip (ll)$^{-}$, (Stp cd)$^{-}$] double digested with *Nco*I and *Xho*I.

Lane (10): Recombinant pET20b with lipase gene insert [lip (ll)$^{-}$, (Stp cd)$^{+}$] double digested with *Nco*I and *Xho*I.

From this figure it was found that there are two fragments of about 796 bp and two of about 721 bp which represents the following:

1- Lipase gene (796 bp) with both leader sequence $(ll)^+$,and stop codon $(Stp.\ cd)^+$

2- Lipase gene (796 bp) with leader sequence $(ll)^+$,but without stop codon $(Stp.cd)^-$.

3- Lipase gene (721 bp) without both leader sequence $(ll)^-$, and stop codon $(Stop.cd)^-$.

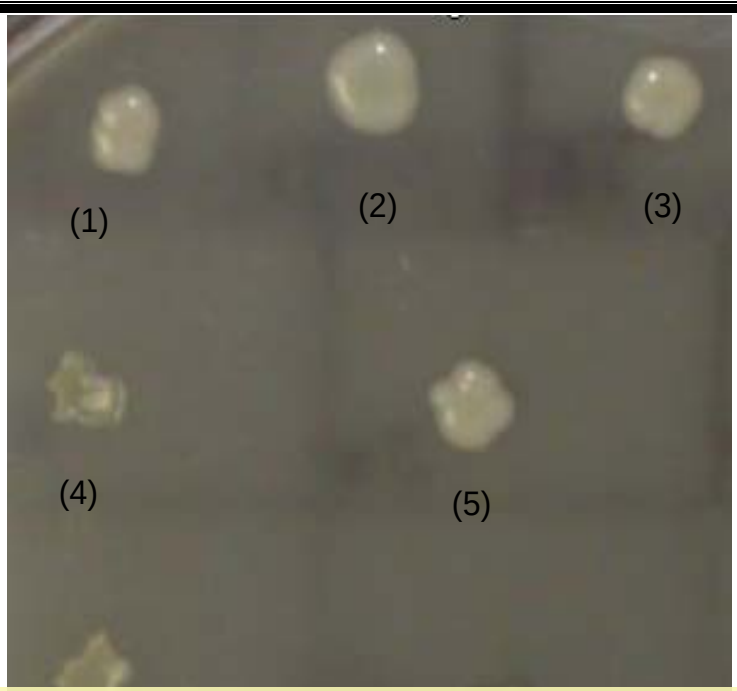
4- Lipase gene (721 bp) without leader sequence $(ll)^-$, but with stop codon $(Stop.cd)^+$.

These four fragments with genetic elements upstream and downstream coding regions for lipase gene were analyzed to confirm the exact sequence of each of these fragments. Results mentioned in appendix (IV), showed the complete nucleotide sequence for each of these four fragments of lipase gene.

4.4.2 Expression of lipase gene in *E. coli* BL21(DE3)

To study the expression system of *Geobacillus stearothermophilus* BSR3 lipase gene fragments cloned in pET20b(+) expression vector, recombinant vectors containing the desired fragments of lipase gene were used to transform *E. coli* BL21(DE3).This strain was the most common host for expression system of foreign genes and as a protein overproducer for industrial applications (Xu *et al*., 2008). Furthermore protein production can be induced with IPTG under the regulation of pET20b(+) T7 promoter.

Lipase production by transformed *E. coli* BL21 (DE3) was assayed on LB-tributyrin agar medium. Results indicated in figure (4-13) showed the expression of lipase gene according to the production of lipase enzyme by only one of the four types of transformed *E. coli* BL21(DE3) containing lipase gene with native leader sequence $(ll)^+$ and stop codon $(Stp.cd)^+$, this leader sequence codes for



Halo of hydrolysis

Figure (4-[illegible]): Screening of lipase enzyme produ[illegible] *E. coli* BL21 (DE3) on LB tributyrin agar medium after [illegible]

Clone (1): Wiled type of *E. coli* BL21 (DE3).

Clone (2) : *E. coli* DH5α harbouring pET20b (+).

Clone (3): *E. coli* BL21 (DE3) harbouring pET20b(+).

Clone (4): *E. coli* BL21 (DE3) harbouring recombinant pET20b (+) with lipase gene insert $(ll)^{+}$, $(Stp.cd)^{-}$.

Clone (5): *E. coli* BL21 (DE3) harbouring recombinant pET20b (+) with lipase gene insert $(ll)^{-}$, $(Stp.cd)^{+}$.

Clone (6): *E. coli* BL21(DE3) harbouring recombinant pET20b(+) with lipase gene insert $(ll)^{-}$, $(Stp.cd)^{-}$.

Clone (7): *E. coli* BL21(DE3) harbouring recombinant pET20b(+) with lipase gene insert $(ll)^{+}$, $(Stp.cd)^{+}$.

leader peptide which was rich in hydrophobic amino acids as shown in figure (4-8) which was essential for secretion of lipase enzyme into the periplasmic space of *E. coli* BL21(DE3) as explained by Pugsley (1993) who found that during transport of proteins out of the cytoplasm, signal sequence is cleaved by signal peptidase to yield a mature protein product. The cleavage site at the C- domain is usually less hydrophobic, containing a signal recognized by the signal peptidase as shown in figure (4-8).

On the other hand results mentioned in figure (4-13) showed that there is no expression of other forms of lipase gene fragments cloned in pET20b(+). Expression vector (ll^-, Stp. Cd^+ ; ll^+, Stp. Cd^-; ll^-, Stp. Cd^-). In this situation, the no expression of lipase gene in the last three forms mentioned above may be due to the non functional lipase gene, or may be due to the stable expression in the [illegible] explained by (Xu *et al*. 2008).

According to these results *E. coli* clone containing recombinant pET20b(+) [illegible] insert (ll^+ [illegible] lipase enzyme production under different cultural conditions.

4.12: Optimum conditions for lipase production by genetically engineered *E. coli* BL21 (DE3)

After success of lipase gene expression in *E.coli* BL21(DE3) containing recombinant pET20b(+) with lipase gene insert (ll^+, Stop cd^+), production of lipase enzyme by this clone was studied under different nutritional and environmental conditions.

4.12.1: Effect of IPTG on lipase production

High level expression of lipase by *E. coli* BL21(DE3) cells harboring the recombinant pET20b(+) expression vector was studied upon induction of cell suspension of bacterial cells with 1mM of isopropyl –β-D-thiogalactopyranoside

(IPTG) for 4 hours, then 50µl of cell suspension was added on LB-tributyrin agar plates, and incubated at 37ºC for 16 hours.

Results mentioned in figure (4-14) showed the halo of hydrolysis around colony of *E.coli* Bl21(DE3) clone because of the expression of lipase gene and production of lipase enzyme, due to the induction of T7 promoter of pET20b(+) that lipase gene lies under its control of expression. This result was agreed with Xu *et al*., (2005) who obtained similar results in their study on expression of penicillin acylase in *E. coli* under the control of T7 promoter of pET Kn29a.

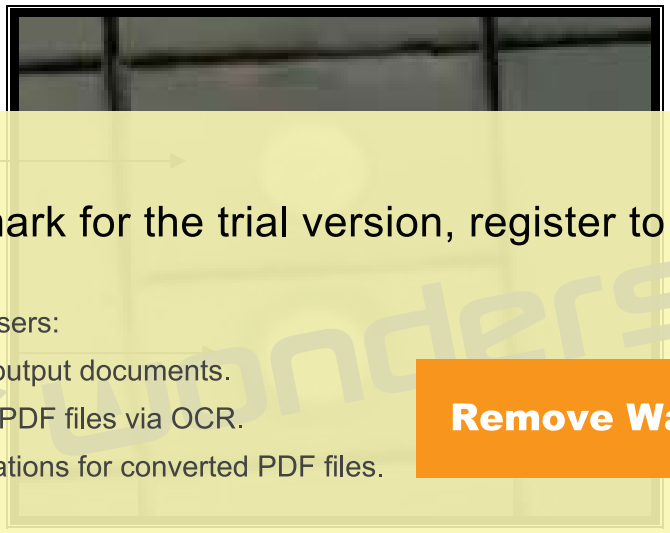


Figure (4-14): Production of lipase by *E. coli* BL21(DE3) induced by 1mM IPTG on LB-tributyrin agar medium and incubation at 37ºC for 16 hours.

4.12.2: Effect of different concentrations of IPTG on lipase production

Expression of lipase gene in transformed *E. coli* BL21 (DE3) was studied by induction of lipase production with different concentrations of IPTG (0.02, 0.05, 0.1, 0.2, 0.5, and 1Mm). Concentration of IPTG above 1 mM was not studied, because at these high concentrations, gene expression was decreased to low levels because not all mRNA transcripts are stable and translated to whole protein as described by Lopez *et al.*, (1994) and Iost and Dreyfus, (1995).

The expression of lipase gene was explored after induction with different concentrations of IPTG for 4 hours at 28ºC, then 20 OD unit of cell suspension was pelleted, and sonicated to obtain the soluble fraction, and was taken to detect lipase activity on LB-tributyrin agar medium.

Results mentioned in figure (4-15) showed different size of zone of hydrolysis due to the production of lipase under different levels of expression after incubation with different concentrations of IPTG.

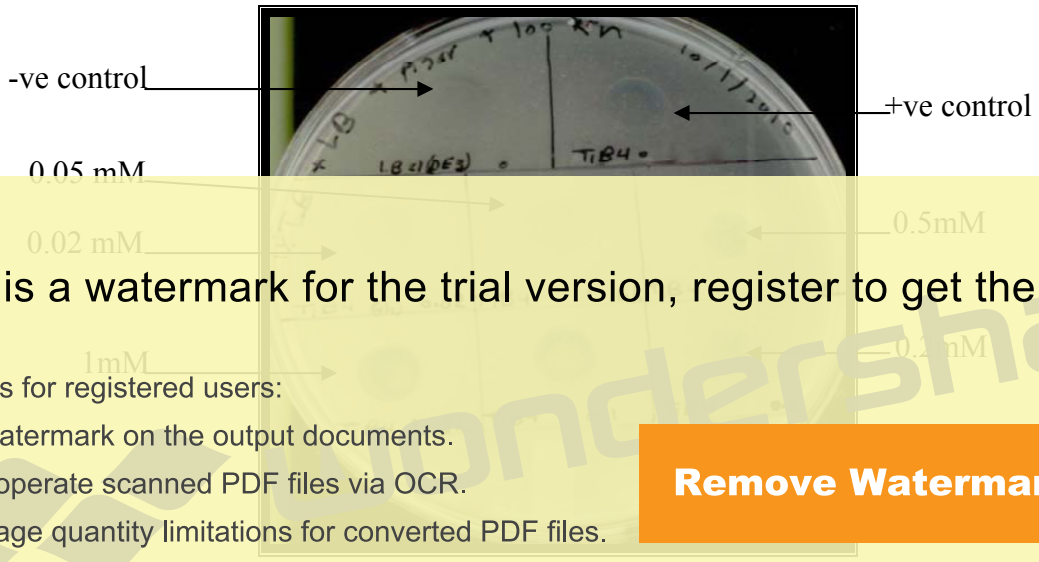


Figure (4-15): Halo of hydrolysis on LB-tributyrin agar medium after induction of lipase production by transformed *E. coli* BL21 (DE3) with different concentration of IPTG.
-ve control: wild type of *E. coli* BL21(DE3)
+ve control: transformed *E. coli* BL21(DE3) harboring recombinant pET20b in absence of IPTG.

From this figure and figure (4-16) it was found that the maximum production or maximum expression of lipase gene was achieved when transformed *E.coli* BL21(DE3) was induced with 1mM IPTG, then the expression was decreased with the decrease of IPTG concentration. Due to the hydrolysis of tributyrin and release of fatty acids on LB-tributyrin agar medium as a consequence of the effect of lipase enzyme leaked from periplasmic space

into the culture medium possibly because of an increased permeability of cell membrane during a lengthy incubation period as explained by Choi and Lee (2004). Small proteins secreted into periplasm are frequently released into the culture medium (Tong *et al*., 2000).

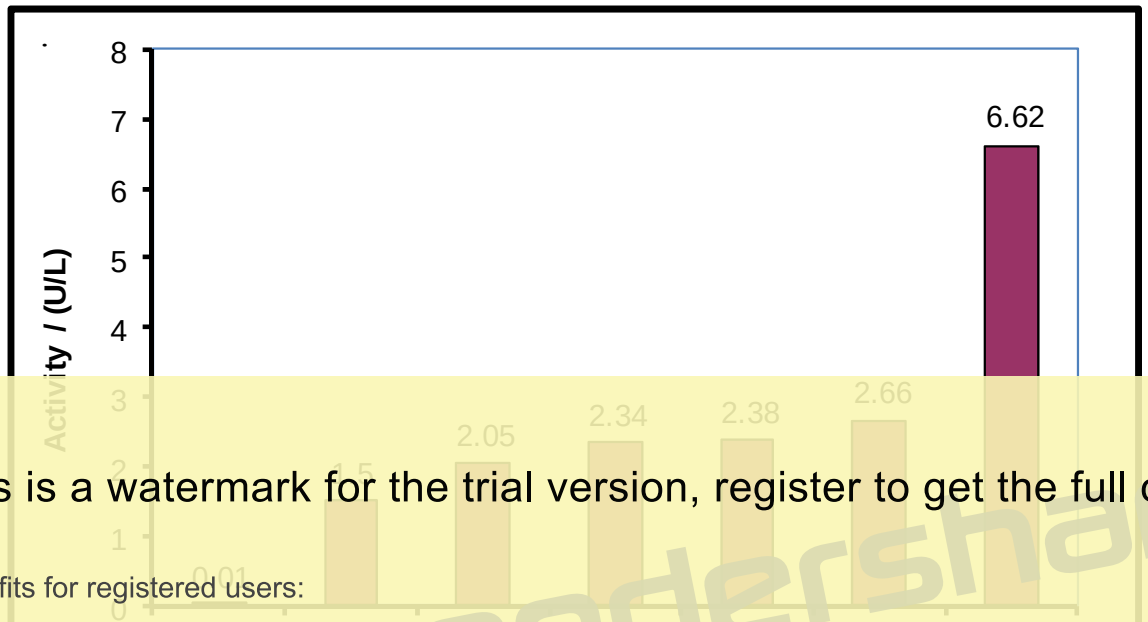


Figure (4-16): Lipase activity produced by transformed *E coli* BL21(DE3) after induction with different concentrations of IPTG. for 4 hours at 28C^{o}.S

4.12.3: Effect of induction period on lipase production by genetically engineered *E. coli*

Production of lipase by genetic engineering *E. coli* BL21(DE3) which was studied after induction with IPTG for different periods (4, 6, and 8 hours). Results mentioned in figure (4-17) showed that production of lipase was increased with the increase of induction period due to the increase of enzyme activity in cell fractions.

Enzyme activity was 6.62, 10, and 16.8 U/ L/ 20 O.D. U when cell suspension was induced with 1mM of IPTG for 4, 6, and 8 hours respectively.

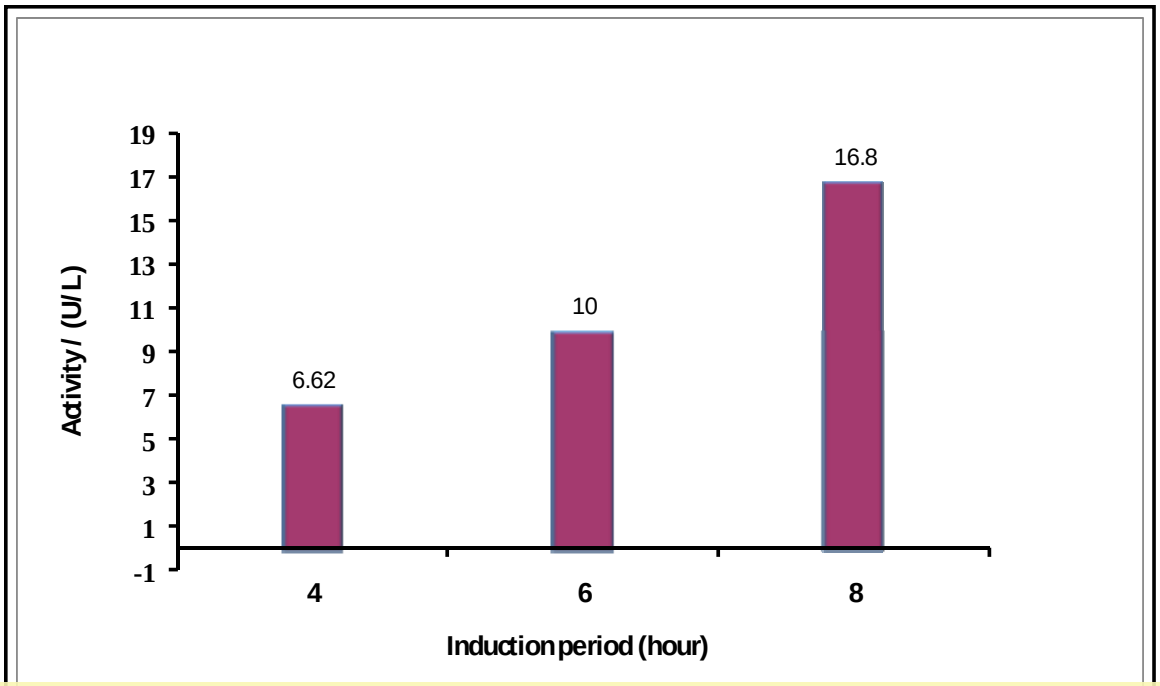


Figure (4-17): Lipase activity produced by genetically engineering *E. coli* BL21(DE3) after induction with 1mM of IPTG for different time periods.

Results indicated in figure (4-18) showed different protein bands appearing in cell fraction after electrophoresis on SDS-PAGE, among them lipase band was detected with a molecular mass of ~ 25 KD in comparison with the molecular mass of protein ladder (10-250 KD).

This figure also showed that lipase produced by genetically engineered *E. coli* BL21(DE3) without induction with IPTG gives faint protein band on polyacrylamide gel under the same conditions of preparation of cell fractions and electrophoresis on SDS-PAGE. The same results were obtained by Leow et *al*., (2004) who found that the molecular mass of lipase enzyme cloned in *E. coli* BL21(DE3) harbouring recombinant pGEX/T1 was 26KD after electrophoresis on 12% SDS-PAGE.

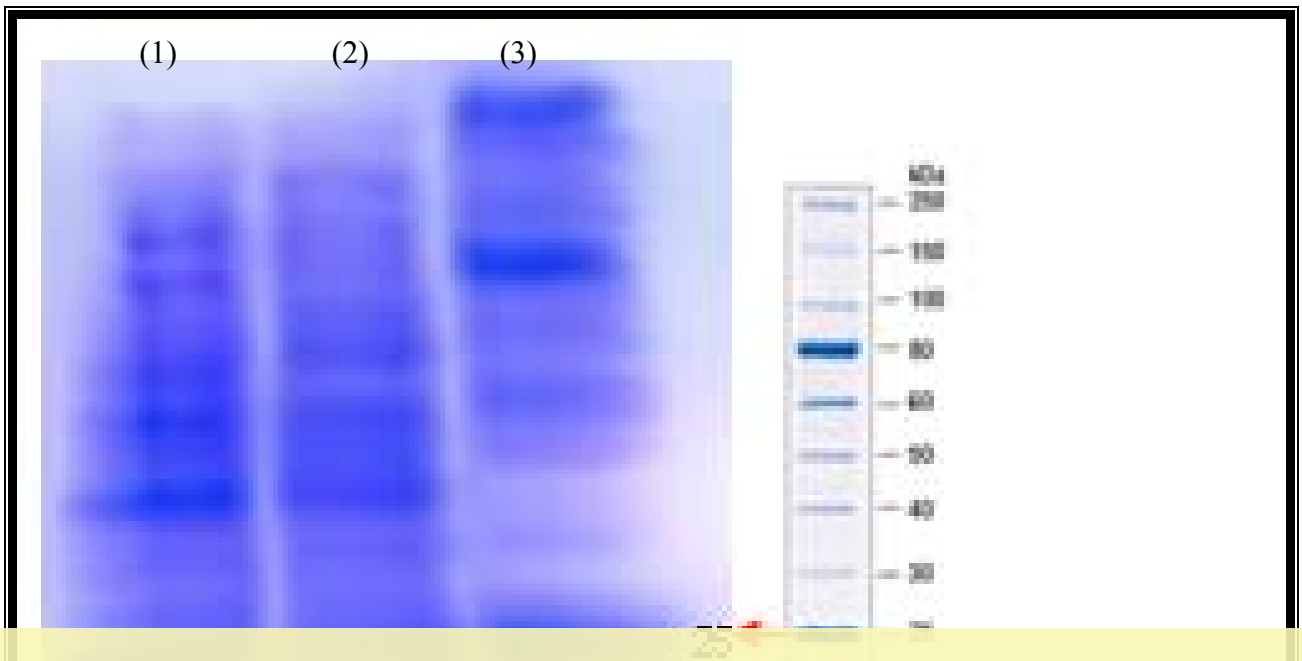


Figure (4-18): protein profile of soluble cell fraction of genetically engineered *E. coli* BL21(DE3) after electrophoresis on SDS-PAGE (12%) for 45 minutes

Lane (1): protein profile of cell fraction after induction with 1mM IPTG for 8 hours.

Lane (2): protein profile of cell fraction without induction with IPTG

Lane (3): protein Ladder (10-250KD).

The activity of lipase is highly pH dependent, because of the influence on the structure of proteins and hence governs their catalytic activity (Chou *et al.*, 1995).

4.12.4: Effect of medium pH on lipase production

Effect of medium pH on lipase production by genetically engineered *E. coli* BL21(DE3) was studied on LB-tributyrin agar medium at different pH values (6,7and 8) and induction with 1mM IPTG for four hours.

Results mentioned in figure (4-19) showed that production of lipase in soluble cell fraction of genetically engineered *E.coli* Bl21(DE3) was increased by the increase of medium pH from pH6 to 8.

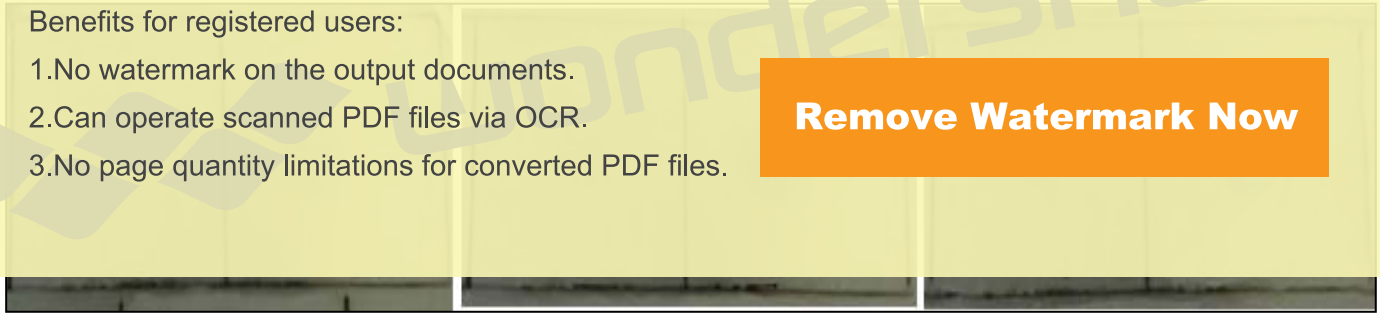


Figure (4-19): Effect of medium pH expressed by zone of hydrolysis on lipase production on LB- tributyrin agar medium at 37ºC for 16 hours.

For this figure, maximum production of lipase was achieved at pH8 expressed by the degree of transparent zones of hydrolysis on LB-tributyrin agar medium.

Conclusions

Conclusions

- Lipase gene of locally isolated *G. stearothermophillus* BSR3 can be amplified using specific primers designed according specific regions upstream and downstream coding sequence of the gene using DNA database found in NCBI.
- Sequencing of lipase gene was successfully achieved after cloning in pPCRScript cloning vector .
- Amplified lipase gene of locally isolated *G .stearothermophillus* BSR3 can be cloned successfully using pET20b(+) expression vector and introduced to *E.coli* BL21(DE3) as a suitable host for expression of different genes from various organisms.
- Successful expression of lipase gene in *E.coli* host cells via recombinant pET20b(+) expression vector was achieved if the coding region of a gene was designed to be containing leader sequence and stop codon of the lipase gene of *G. stearothermophilla* BSR3.
- Optimum conditions for lipase production by genetically engineered *E.coli* was achieved after induction of the cell culture with 1 mM IPTG for 8 hours at 28 ◦c and medium pH8.
- SDS-PAGE is a suitable tool for analyzing the degree of gene expression and at the same time to determine the approximate molecular mass of the protein under study.

5.2 Recommendations:

- Full purification of lipase enzyme produced by genetically engineered *E.coli* using different chromatography techniques.
- Characterization of purified lipase enzyme for preparative and applicable purposes.
- Using purified lipase enzyme in different industrial and agricultural applications.
- Immobilization of purified lipase enzyme for biotransformation of substrates for different applications.
- Determining the kinetics of purified lipase enzyme produced be genetically engineered *E.coli* to compare enzyme characteristics with those of lipases from different origins.

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CTAACTCTCAAGCCAATCCACTTCCCTTTTTTCAAGTTTGCGGATCATCACTTGCTCGTC 60

CCGCTGGTTTCCTGTCGGCCCATAATAATTGAAGCCGTAGCTTAATTGCGCATAGCCTCC 120

AATCATTTGCGTCGGTTTAACATGGATGCGGGTCGGACTGTTAAATGTGCCGCCGCGCAG 180

TTTGCTAATTGGCGAGCCGGGCACGTTAATATGGCGTTCCTGCACGTGATACATAAACGC 240

GACTCCACGCGGCAGGCGATGGCTGACGACTGCCCGGGCGATGACCACCCCAATTGCGGTT 300

GTACATTTCAATCCAGTCGTTGTCAGCAATGCCGGCTTCTGCGGCATCTTCTTTGTTCAT 360

CCAGACAGTCGGCCCGCCGCGGAAAGATCGTCAACATCGGCAGCGTATCCCAATATGTGCT 420

TCCGGTTTCGCTTCCGCTAAAGACAGGGGTCGTCAACACTTGCCGCGGTTGAGCAGTGAT 720

AGCCTGAAAGGTAATATGTTCCTCGGCTCGTTCAACGACTAAATCTTTTAACGGCTGCAT 780

CGTCTTTTTCTCCATTTCTCCCCACGCTTTGAGGGCCAAGGAACCGTTCGTTGTGCTTGA 840

AAGAGCCAAAATGGCTTCTGCGGCATCCCGGTCTGTCAACAAACTCGGACAGTCTTGATA 900

CGGGCCTTCTAGCGCTGAAGAACCGAGCGTATCAAGCAGCCATTTGTCTTCTTCCTTGGC 960

ATCCCAACTGAGCCCTTTCGCCCCGATTTGTTCACGGACGATCGGGCCAAGCGCCACAAA 1020

CTTGTTGTATATTTCCTTATAATCCCGCTCTACAATATGCAGGCGCGGAAAATTCACTCC 1080

CGGAACCGGCTCTGTCGTTCCATCTTTCCAATCGTTTACCACCCCAAACGGCTGCGCGAT 1140

CTCATCGCGTGTATTATGCAACAGCGGGGTTGCGAGCACATTTGACACCGGCTTCGAAAA 1200

ATAGTGGGCTGCCAGCTCAGAAAATGTTTTGGCCAAGCCTTTAAAAAAGTCCCAATCCGA 1260

TTTTGCTTCCCACGGTGGGGCAATGGTGGGATTTGAACGGATGCACAAACGGGTGCATATC 1320

CGTGCTGCTGACATCATATTTCTCATACCACGTCGCCGCTGGAAGAACGATGTCCGAATA 1380

ATGATGAAAGGCTGCCGGGAGGACGATATCGGAATA 36

TTCGATCGCAAACGTTAAATTCCCTTGTTTTATTTCATTGACCACATATTGAATCACTTC 336

TTCATTCGTCTTCGCATTTGCCTGTTCCACTAGCGCAATACTATTGGCATTAAACTGCGG 1740
TTCGTTTGTCTTTGCGTTTGCCTTTTCCACTAACGTCATGCTGTTTGCGTTAAATTGCGG 396

ATAAGATGGAAGCCAGCCTAGCCGGGCAGCCAAATAGTTGTAATCACCACCATGTTCGTA 1800
ATAAGATGGGAGCCACCCTAAACGAACCGCTAAATAGTTATAGTCGCCGCCATGCTGATA 456

ATGCTAGTCAGTCGGATAATCACCAGGCAGCCCACGGTTGACGCCCATTTTGGCAAGCAA 2280

ATTATAATCGACAGGATAATCACCCGGCAGCCTTTCATCAT 917

TAAGTCAAATACTGTCGTGACGTACACCGTCTCTCCGTTTTGCTCGATCGCTTTGACCGG 2340

CACCCCTCGTTCGAGCACTGTTTTGTTTCCTGCTTCAAAGTGTGGAAACTGCACGATGAC 2400

AGTATCGTATTGCTGGCCAAGGAACGTCAAAGCCGGCTGAATTCCCATTCGTTTATTGTC 2460

TATATCGTTTAGTTGCAAATTCCAGTTCCCGTTGTTCTCCCAACGATGGCCAATCGTCCC 2520

ATTAGGAACAGCAAACGTCTGTGATTGCTCATCCCAAACGATCGTTTTCCACTCTGCATA 2580

CTGAAGCGGCATGCCAAGATCGCTCGCCCGCAAAAAGCGTCCGGCCGCATAACGATCCCC 2640

GTGCTTTTTCAGCATGACTAAAAACGGCAAATCGGTGTACTTTTTAACATAGTCAGTAAA 2700

ATAATCAGCCGATGATTGACATAAAACTCTTTCAAAATCACATGGGTCATCGCCATCGC 2760

CAGCGCTCCGTCCATCCCCGGCTGCACCGGCAGCCAGTTATCGGCGAACTTCACAAATTC 2820

AATGCGATCCGGCCCGTATTTTTGAATCGTATAAATAAGCGATGCGGCGATCATCGTATA 3140

AATGCGATCCGGCCCGTATTTTTGAATCGTATAAATAAGCGATGCGGCGATCATCGTATA 3180

AACATCATCCCATTTCGCCCGTACGAATCCTCCTTTTCCACGGGCGCGTTTGTACCGTTT 3240

CGCTTTTTCCGGGTCCTCGACAATCGACTTCCACGCAGCCACAGGGTCTCCTTCTTGTTC 3300

CAGCGCTTCTTTCCAAAGCCTCAACAGCGCCCCGCGCACATACGGATAGCGGACGCGAAG 3360

CGGACTGTATATATACCAGGAAAAACTCGCTCCCCTCGGACAGCCGCGCGGTCATATTC 3420

CGGCATATCGGGTCCTCTTCTCGGATAGTCGGTTTGCTGCGTTTCCCATGCGATAATGCC 3480

••

GTCTTTTACATGCACTTTCCAACTGCATGAGCCTGTGCAGTTGACCCCATGGGTTGTGCG 3540

••

Appendix (I) : DNA sequence alignment of *B. stearothermophillus* BSR3 lipase gene with portion of *Geobacillu*s sp. WCH70 nitrate reductase.

```
WCH70   ATGAGACGCGGTATTGTAAGCACCATCACGATTGTATCCGTGCTGGCGGGAATATTATGG   60
QUERY   ATGAGACGCGGTATTGTAAGCACCATCACGATTGTATCCGTGCTGGCGGGAATATTATGG   60
        ************************************************************

WCH70   CTTGGCGGTTTTGCCATGGGCATTCAAGATCAATTTTTTTCCGCCGCGATGCCGCCAGCA   120
QUERY   CTTGGCGGTTTTGCCATGGGCATTCAAGATCAATTTTTTTCCGCCGCGATGCCGCCAGCA   120
        ************************************************************

WCH70   GGCACGACGAAATATACAAAAGAGAAAAAAGCGGACAACCGTGAAATTTACATTGTCGCT   180
QUERY   GGCACGACGAAATATACAAAAGAGAAAAAAGCGGACAACCGTGAAATTTATATTGTCGCT   180
        ************************************************** *********

WCH70   CTTGGCGACTCGCTGACAAGGGGAACGGGAGACGAAAGCGGAAAAGGGTATATTGGCTAT   240
QUERY   CTTGGCGACTCGCTGACAAGGGGAACGGGAGACGAAAGCGGAAAAGGGTATATTGGCTAT   240
        ************************************************************

WCH70   ATGGTCGATTCTCTCCGTAAAAAAACAACGAAACCGATTCGTGTAACAAACAAGGCTATT   300
QUERY   ATGGTCGATTCTCTCCGTAAAAAAACAACGAAACCGATTCGTGTAACAAACAAGGCTATT   300
        ************************************************************

WCH70   AAAGGACAGCGATCTGACGGACTCCTTAAGCAATTAGGACAAGCTGAGATAAAGAGGCAG   360
QUERY   AAAGGACAGCGATCTGACGGACTCCTTAAGCAATTAGGACAAGCTGAGATAAAGAGGCAG   360
        ************************************************************

WCH70   CTAAAAATGGCTGACATCATCGTGATGACGATCGGCGGGAATGATTTGTTTCAAGGCGGA   420
QUERY   CTAAAAATGGCTGACATCATCGTGATGACGATCGGCGGGAATGATTTGTTTCAAGGCGGA   420
        ************************************************************

WCH70   GAAGCGCTAAAGCTTGCGCCAAAGCAAATCGAGCAGGCAAAAAACGCGTATTTACACAAC   480
QUERY   GAAGCGCTAAAGCTTGCGCCAAAGCAAATCGAGCAGGCAAAAAACGCGTATTTACACAAC   480
        ************************************************************

WCH70   TTAGACCGTATTTTTCAAACGATTCGCAGCGTCAATAAAGATGCAGTTGTTTTTTA[illegible]ATC   540
QUERY   TTAGACCGTATTTTTCAAACGATTCGCAGCGTCAATAAAGA[illegible]   540
        ************************************************************

WCH70   [illegible]
QUERY   GGGC[illegible]CGCAAAGA[illegible]
        [illegible]

WCH70   [illegible]ATTTGCTTCGGCAGAAACGGCAGCTCGCTATCCAAATATCATTGCTGTGCCG   660
QUERY   CA[illegible]GGAATTTTGCTTCGGCAGAAACGGCAGCTCGCTATCCAAATATCATTGCTGTGCCG   660
        ************************************************************

WCH70   ATATTCGATTTGTTTGAGCTTCATGTGAATGATTATTTGTACAGCGACCATTTCCATCCG   720
QUERY   ATATTCGATTTGTTTGAGCTTCATGTGAATGATTATTTGTACAGCGACCATTTCCATCCG   720
        ************************************************************

WCH70   AATAAGGAAGGCTATAAACGCATTGGCGAACGTGTCGCTTCTTTAATTACGTGGACGGAG   780
QUERY   AATAAGGAAGGCTATAAACGCATTGGCGAACGTGTCGCTTCTTTAATTACGTGGACGGAG   780
        ************************************************************

WCH70   GAGGACAAACAATGA   795
QUERY   GAGGACAAACAATGA   795
        ***************
```

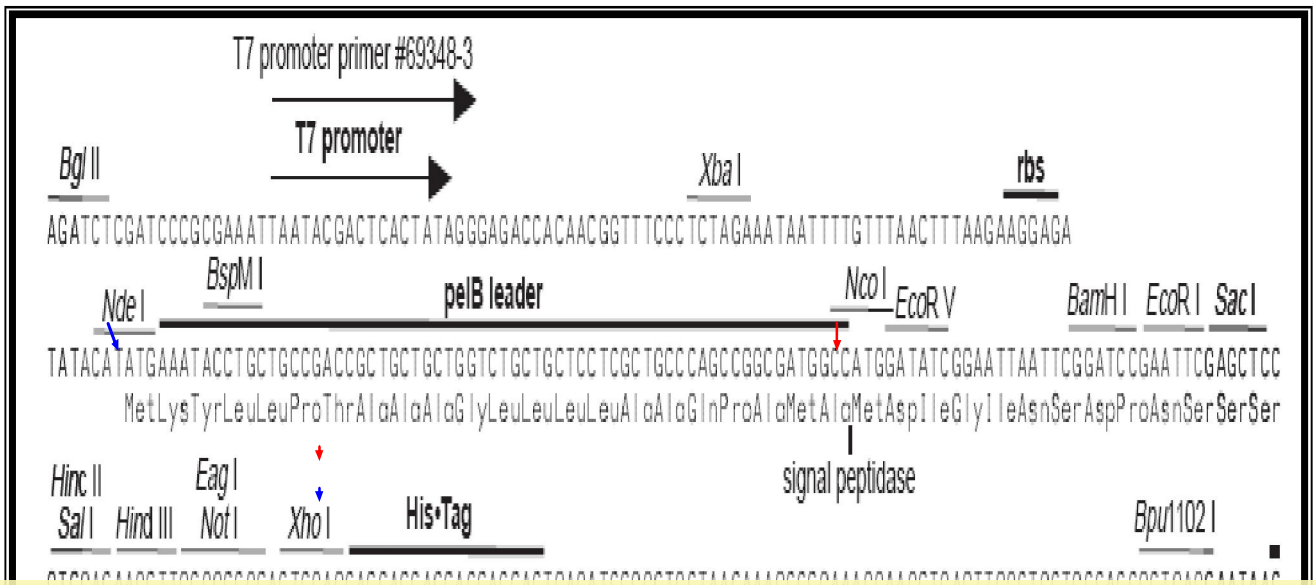
Appendix(II) : DNA sequence alignment of lipase gene of *B. stearothermophillus* BSR3(Query) and *Geobacillus* sp. WCH70 arrows represent point mutation .

```
WCH70   MRRGIVSTITIVSVLAGILWLGGFAMGIQDQFFSAAMPPAGTTKYTKEKKADNREIYIVA    60
QUERY    MRRGIVSTITIVSVLAGILWLGGFAMGIQDQFFSAAMPPAGTTKYTKEKKADNREIYIVA   60
         ************************************************************************

WCH70   LGDSLTRGTGDESGKGYIGYMVDSLRKKTTKPIRVTNLAIKGQRSDGLLKQLGQAEIKRQ  120
QUERY    LGDSLTRGTGDESGKGYIGYMVDSLRKKTTKPIRVTNLAIKGQRSDGLLKQLGQAEIKRQ 120
         ************************************************************************

WCH70   LKMADIIVMTIGGNDLFQGGEALKLAPKQIEQAKNAYLHNLDRIFQTIRSVNKDAVVFYI   180
QURY     LKMADIIVMTIGGNDLFQGGEALKLAPKQIEQAKNAYLHNLDRIFQTIRSVNKDAVVFYI  180
         ************************************************************************
WCH70   GLYNPFSDLGDAKKTSAIVRQWNFASAETAARYPNIIAVPIFDLFELHVNDYLYSDHFHP    240
QUERY    GLYNPFSDLGDAKKTSAIVRQWNFASAETAARYPNIIAVPIFDLFELHVNDYLYSDHFHP   240
         ************************************************************************

WCHlip  NKEGYKRIGERVASLITWTEEDKQ_ 264
QUERY    NKEGYKRIGERVASLITWTEEDKQ_264
         *********************************
```



A

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aacgcgtatttacacaacttagaccgtatttttcaaacgattcgcagcgtcaataaagatgc
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ccgcgatcgtaaggcagtggaatttgcttcggcagaaacggcagctcgctatccaaatatc
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tttccatccgaataaggaaggctataaacgcattggcgaacgtgtcgcttctttaattacgt
ggacggaggaggacaaacaatgactcgagatctttctagaagatctcctacaatattctcag
ctgccatggaaaatcgatgt

B

tgtcgcttctttaattacgtggacggaggaggacaaacaactcgaatctttctagaagatct
cctacaatattctcagctgccatggaaaatcgatgttcttcttttattctctcaagattttc
acgctgtatattaaaacttatattaagaactatgctaaccacctcatcagaaccgttgtagg
tggcgtgggttttct

C

ctcgagtcattgtttgtcctcctccgtccacgtaattaaagaagcgacacgttcgccaatgc
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D

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ggaaggctataaacgcattggcgaacgtgtcgcttctttaattacgtggacggaggaggaca
aacaactcgagatctttctagaagatctcctacaatattctcagctgccatggaaaatcgat
gttcttctttattctctcaagattttcaggctg

Appendix (IV) : Nucleotide sequence of *Geobacillus thermoleovorans* BSP3 *lip* gene

B: Lipase gene fragment without leader sequence $(ll)^-$,but without stop codon $(stp.cd)^-$ with *Nco*I/*Xho*I cohesive ends .

B: Lipase gene fragment without leader sequence $(ll)^-$,but with stop codon $(stp.cd)^+$,with *Nco*I/*Xho*I cohesive ends .

C: Lipase gene fragment with both leader sequence $(ll)^+$,and with stop codon $(stp.cd)^+$,with *Nco*I/*Xho*I cohesive ends .

D: Lipase gene fragment with leader sequence $(ll)^+$,but without stop codon $(stp.cd)^-$,with *Nco*I/*Xho*I cohesive ends .

الخلاصـــة

جـمـعـت خمســـــون عيـــنة تـــــــربة من مناطق مختلفة من جنوب العراق لغرض عزل بكتريـــا *Bacillus stearothermophilus* , وقد شملت تلك النماذج عينات تربة من محافظة البصرة , وذي قـــــار , وميسان , والمثنى , وكربلاء على التوالي , وقد تم الحصول على 45 عزلة بكتيرية من تلك العينات أخضـــعت جميعاً للتشخيص على أساس الصفات المظهرية , والزراعية , والخصائص الكيموحيوية لتلك العزلات . وقـــــد شخصت 29 عزلة من بين المجموع الكلـــــي للـــــعـزلات على أنها *B. stearothermophilus*

درست قابلية العزلات المحلية لبكتريا *B. stearothermophilus* على أنتاج أنزيم اللايبيز على أساس أقطار مناطق التحلل على الوسط الزرعي LB-tributyrin المتصلب بمادة الأكار. وقد أشارت النتائج الى وجود عزلتين تميزتا بكفائتهما العالية على أنتاج الأنزيم , هذا وقد أختيرت العزلة *B.stearothermophilus* BSR3 للدراسة الجزيئية عن أنزيم اللايبيز على أساس كفائتها في أنتاج

النكليوتيدية على أساس التعاقب النكليوتيدي الكامل لجين اللايبيز للبكتريا *Geobacillus* sp. WCH70 لتضخيم جين اللايبيز للعزلة المحلية BSR3 ليتضمن تعاقبات السيطرة أعلى وأسفل مجرى الجين , ثم كلونة القطعة المضخمة في ناقل الكلونة pPCRScriptSK(+) تم بعدها تعيين التعاقب النكليوتيدي للجين بطريقة ايقاف السلسلة (chain termination). وقد أشارت النتائج الى أن جين اللايبيز المضخم للعزلة المحلية BSR3 كان مطابقاً (بدرجة تماثل 99%) لجين اللايبيز للعزلة القياسية *Geobacillus* sp. WCH70. وعلى أساس نتائج التشخيص الكيموحيوي للعزلة BSR3 ونتائج تماثل التعاقب النكليوتيدي لهذه البكتريا مع التعاقب النكليوتيدي لجين اللايبيزللعزلة القياسية *Geobacillus* sp. WCH70 فقد أعتبرت العزلة المحلية على أنها *Geobacillus stearothermophilus* BSR3 .

لدراسة نظام التعبير لجين اللايبيز المشتق من العزلة المحلية BSR3 في بكتريا *E.coli* فقد تم تصميم أربعة أستراتيجيات لتضخيم جين اللايبيز ليتضمن تعاقبات الشفرات الوراثية (ORF) بوجود وبعدم وجود تعاقبات السيطرة الضرورية لتعبير الجين في مضيفه الجديد وكما يأتي :

1- تعاقب ORF بدون التعاقب العائد$^{-}$(ll) (Leader sequence) وبدون شفرة الأنهاء(stop codon) $^{-}$(stp. cd) ذات نهايتين لاصقتين من نوع *Nco*I/*Xho*I .

2- تعاقب ORF بدون التعاقب العائد$^{-}$(ll) , وبوجود شفرة الأنهاء $^{+}$(stp. cd) ذات نهايتين لاصقتين من نوع *Nco*I/*Xho*I.

3- تعاقب ORF بوجود التعاقب العائد $^{+}$(ll) وشفرة الانهاء $^{+}$(stp. cd) ذات نهايتين لاصقتين من نوع *Nco*I/*Xho*I.

4- تعاقب ORF بوجود التعاقب العائد $^{+}$(ll) وبدون شفرة الانهاء $^{-}$(stp. cd) ذات نهايتين لاصقتين من نوع *Nco*I/*Xho*I

بالتعبير الجيني في المضيف الجديد بالمقارنة مع تعاقب الشفرات الوراثية لجين اللايبيز للبكتريا *Geobacillus* sp. WCH70 , تم بعدها تحويل النواقل الهجينة (Recombinant vectors) في بكتريا *E.coli* DH5α . وقد أشارت النتائج الى تطابق تعاقب الشفرات الوراثية للقطع الاربعة لجين اللايبيز للعزلة BSR3 مع تعاقبات الشفرات الوراثية لجين اللايبيز للبكتريا *Geobacillus* WCH70 بدرجة تماثل 99.98% وكانت الأوزان الجزيئية للقطع الأربعة الناتجة هي 721 و 721 و 796 و 796 زوج قاعدي على التوالي.

لغرض دراسة التعبير الجيني الأمثل للقطع الأربعة لجين اللايبيز فقد تم الهضم المزدوج للنواقل الهجينة بزوج أنزيمات التقييد *Nco*I/*Xho*I و *Nde*I/*Xho*I لأستئصال القطع الأربعة وكلونتها في

ناقل التعبير pET20b(+) ثم أدخلت النواقل الهجينةالى بكتريا *E. coli* BL21(DE3) بعملية التحويل البكتيري . وقد أشارت النتائج الى أن الكلونة الحاملة للناقل الهجين pET20b(+) الحاوي على قطعة جين اللايبيز المتضمنة التعاقب القائد $^{+}$(ll) وشفرة الانهاء $^{+}$(stp.cd) الخاصة بجين اللايبيز أعطت أعلى درجة تعبير جيني وأنتاجية لأنزيم اللايبيز من بين الكلونات الاخرى لبكتريا *E. coli* المهندسة وراثيا الحاوية على الأشكال الأخرى لجين اللايبيز . وقد تم أنتقاء هذه الكلونة لدراسة الظروف المثلى لأنتاج اللايبيز .

درست الظروف المثلى لأنتاج اللايبيز من الكلونة المنتقاة من بكتريا *E. coli* BL21 المهندسة وراثياً تحت مختلف الظروف التغذوية والمزرعية . وقد أشارت النتائج الى أن أعلى أنتاجية لأنزيم اللايبيز قد تحققت بعد حث البكتريا على الأنتاج الأنزيمي بمادة IPTG بتركيز 1 ملي مولار لمدة ثمانية ساعات بر قم هيدروجيني pH8 لوسط الأنتاج بدرجة 28 درجة مئوية . كما درس التعبير الجيني تحت الظروف المثلى للأنتاج من خلال دراسة النسق البروتيني للاجزاء الخلوية (Cell fractions) مع نفس الحزمة البروتينية لانزيم اللايبيز للاجزاء الخلوية بدون حث الانتاج بمادة الـ IPTG.

صدق الله العظيم

سورة البقرة –جزء من الأية (٢٦٩)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين/كلية العلوم

قسم التقانة الأحيائية

أستنسال, تعبير و توصيف كيموحيوي للليبيز الثابت حراريا المعزول من عزلة محلية لبكتريا *Geobacillus stearothermophillus* BSR3

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