Characterization, optimization and industrial applicability potential of recombinant thermoalkalophilic lipase

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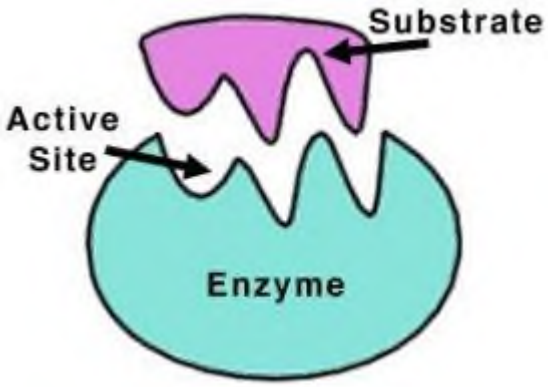
Introduction

Enzymes catalyze chemical reactions and are biological catalysts in the structure of proteins.



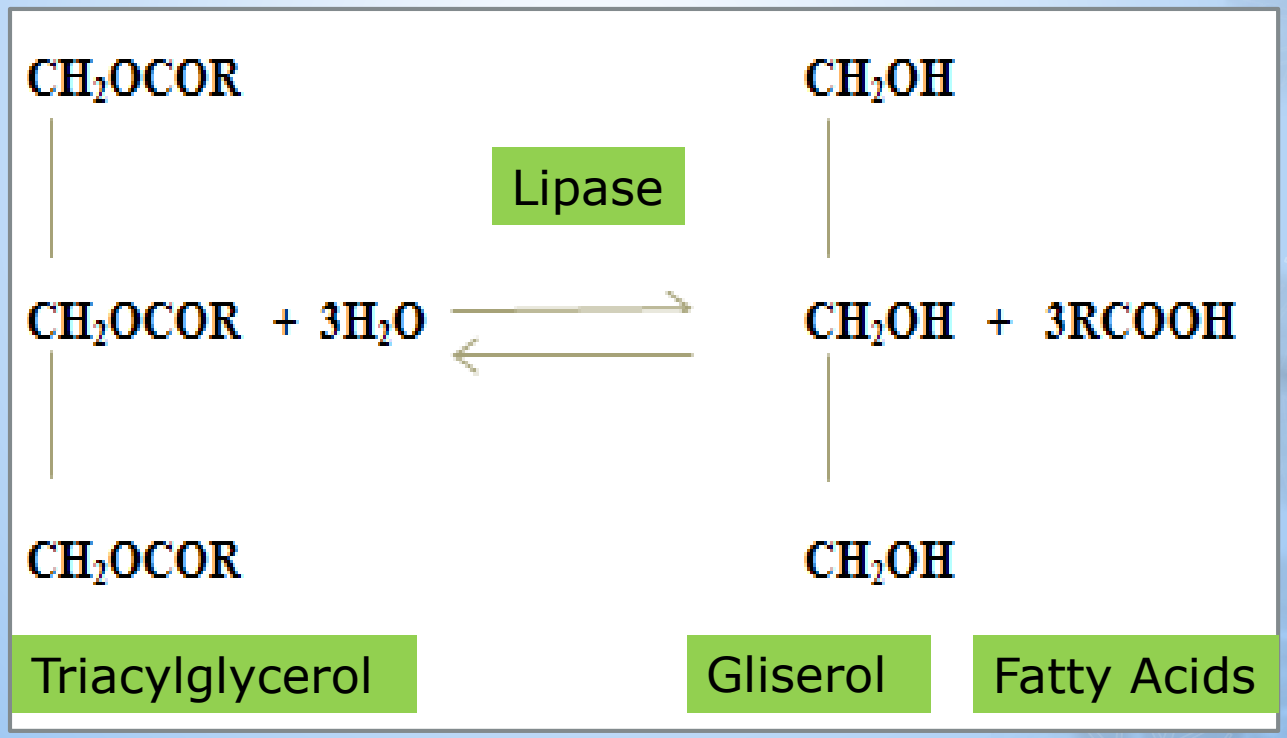
While enzymes can be active in vivo, their ability to perform in vitro conditions has enabled them to be used in non-living conditions.

Today, about 4000 enzymes are known and about 200 of them are used for commercial purposes.

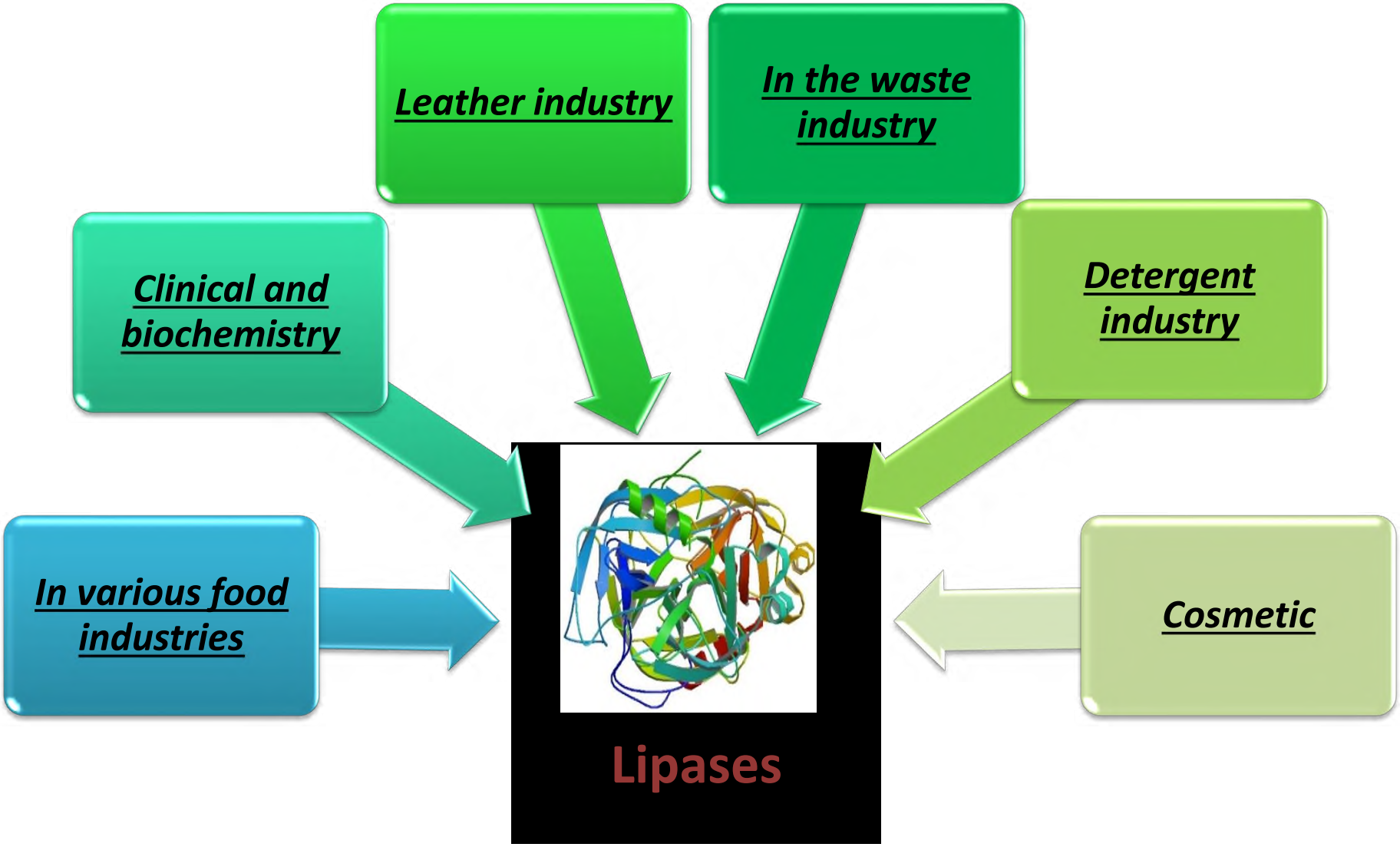
The world's enzyme demands are met by only 12 large producers and 400 small manufacturers. Approximately 60% of the total enzyme production in the world is provided from European countries.

Of the commercially used enzymes, 59% are proteases, 28% are carbohydrases, 3-5% are lipases and 10% are other enzymes.

The general role of lipases is to catalyze the hydrolysis of triglycerides to fatty acids and glycerol.

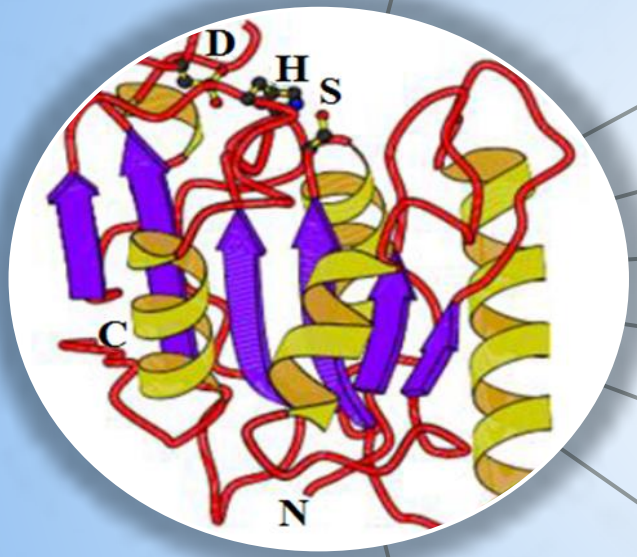


Usage Areas


Leather industry
In the waste industry
Clinical and biochemistry
Detergent industry
In various food industries
Cosmetic
Lipases

Lipases include a large number of plants, animals and microorganisms.

Microbial lipases have a wide range of applications compared to plant and animal lipases.



The reason of this,

Easy and fast reproduction of microorganisms

Genetically modified

Very high catalytic activities

More stable and cheaper

Not to create undesirable by-products,

[redacted]

[redacted]

In addition, Bacillus species are easy to grow, safe and able to secrete the amount of protein expressed in grams per liter into the medium in which they are grown, which allows them to be used for the production of heterog proteins in gene expression studies.

Bacillus pumilus Lipase

Bacillus pumilus lipases were classified as I.4 subfamily with 74-77% sequence similarity.

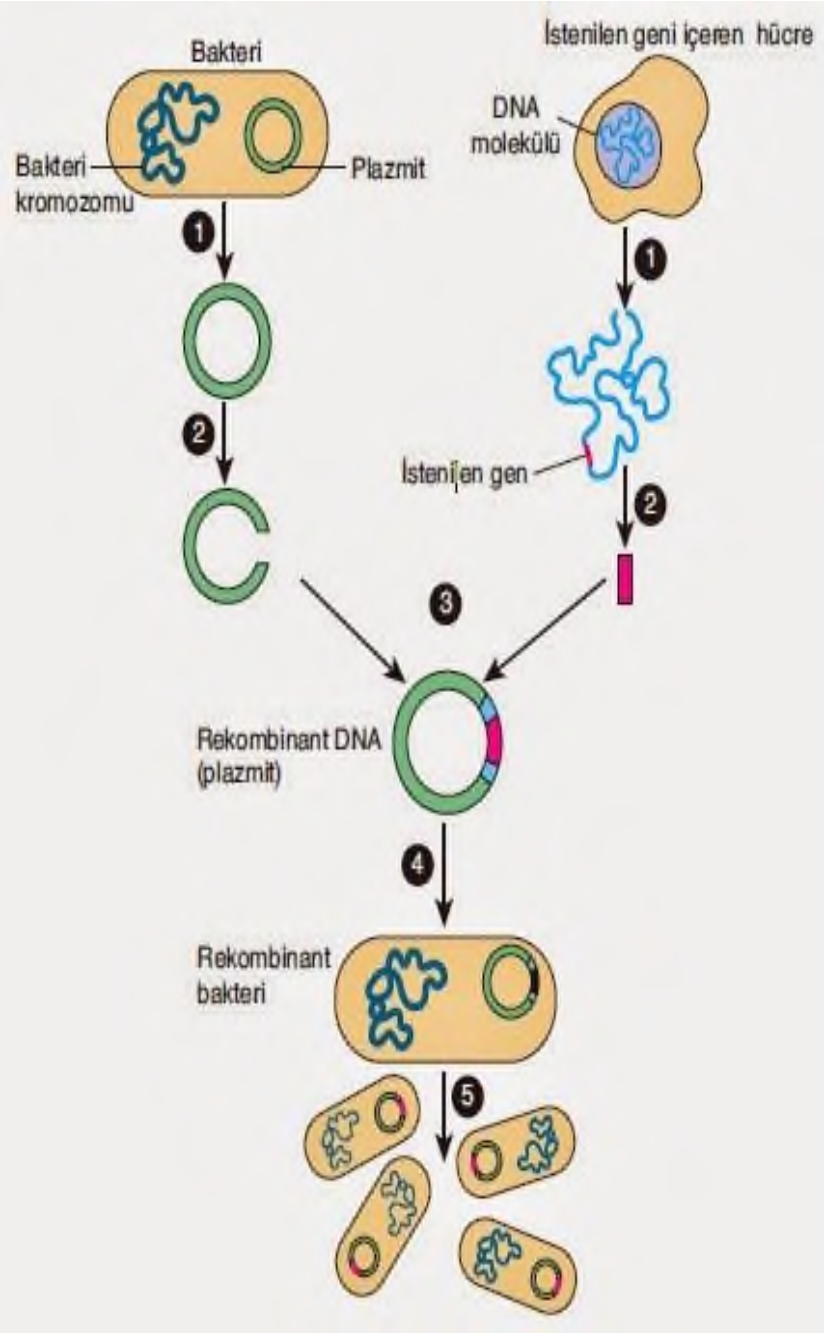
The subfamily I.4 lipases have 181 amino acid residues and all lipases have the lowest protein with a molecular weight of about 19 kDa.

Bacillus pumilus strains (GRAS) are generally safe microorganisms. Therefore, there are no restrictions on the use of these microorganism lipases in various sectors, such as food and raw materials.

Recombinant DNA technology makes it possible to isolate, identify and generate a single gene from thousands of genes in a genome and produce large amounts of this gene as the cloned DNA molecule.

Some proteins and peptides cannot be obtained from humans and animals in sufficient purity and high efficiency.

Thus, recombinant DNA technology has allowed proteins to be produced in bacteria that can be characterized as biological factories.



PURPOSE OF THE STUDY

In this study, it is aimed to produce the lipase enzyme, which is widely used in various industrial applications, as recombinant.

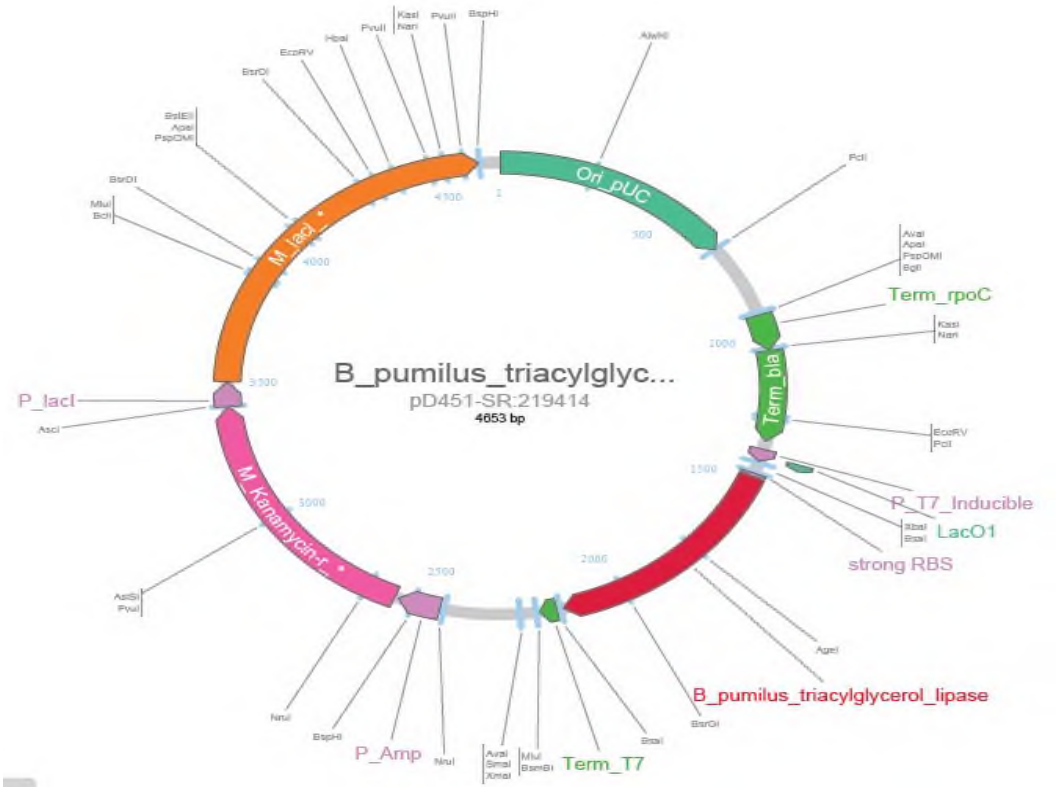
For this purpose

(NCBI no.215414329: 121-768) The cloning, recombinant production, purification and biochemical characterization of the lipase enzyme gene encoded in Bacillus pumilus MBB03 was investigated and the potential for application to the industry was investigated.

Materials and Methods

Bacterial strains, plasmid and growth media

	► *Bacillus pumilus MBB03 (Leibniz Institute, Germany)* ► *Escherichia coli BL21 (DE3) (Leibniz Institute, Germany)* ► *pD451-SR: 219414 (Newark, US)* ► *Bacterial strains were routinely grown in Luria-Bertani broth (LB) medium at 37◦C.*



ATGAAAGTGATGTTTGTTAAGAAAAGGAGTTTGCAAATTCTC
ATTGCGCTTGCATTGGTGATTGGTTCAATGGCTTTTATCCAGC
CGAAAGAGGTGAAGGCGGCTGAGCATAATCCGGTTGTGATG
GTACACGGCATTGGCGGTGCCTCTTATAACTTTTTTTCTATTA
AAAGTTATTTGGCCACACAAGGCTGGGATCGAAACCAATTGT
ATGCTATCGATTTCATAGACAAAACAGGAAATAACCGCAACA
ATGGTCCGCGTCTATCCAGATTCGTCAAAGATGTGTTAGACA
AAACGGGTGCCAAAAAAGTAGATATTGTGGCTCATAGTATGG
GCGGAGCGAACACATTATACTATATTAAGAATCTAGATGGCG
GCGATAAAATTGAAAACGTTGTCACAATTGGTGGAGCAAACG
GACTCGTTTCAAGCAGAGCATTACCAGGCACAGATCCAAATC
AAAAAATTCTTTACACATCTGTCTACAGCTCAGCTGATCTCAT
CGTCGTCAACAGCCTCTCTCGTTTAATTGGCGCAAGAAACGT
TCTGATCCATGGCGTTGGCCATATCGGTCTATTAACCTCAAGC
CAAGTGAAAGGCTACATTAAAGAAGGACTGAACGGCGGA
GGACAGAATACGAATTAA

Materials and Methods

►In this study, the lipase gene from Bacillus pumilus MBB03 (NCBI no.215414329: 121-768) pD451-SR: 219414 was cloned into vector and supplemented in E. coli BL21 (DE3) complement cells.

►The process was performed in a single step by Ni-NTA resin and His60 Ni Gravity Column.

► Protein concentrations of crude and pure enzyme were determined according to the Bradford method using bovine serum albumin (BSA) as standard.

► The approximate molecular weight of the substrate was determined by SDS-PAGE.

B.pumilus MBB03 Replication and Purification of Lipase Gene

Table 1. B. pumilus MBB03 Primer DNA sequences used in PCR amplification of the candidate lipase gene.

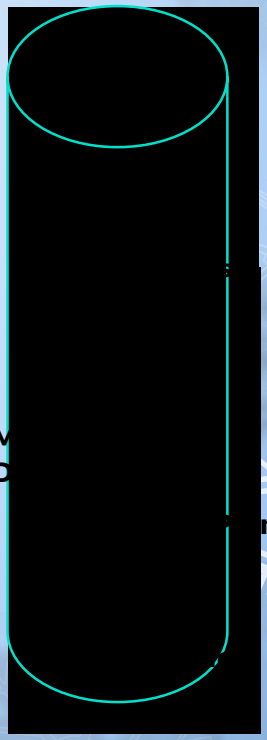
SapI

Tip IIs

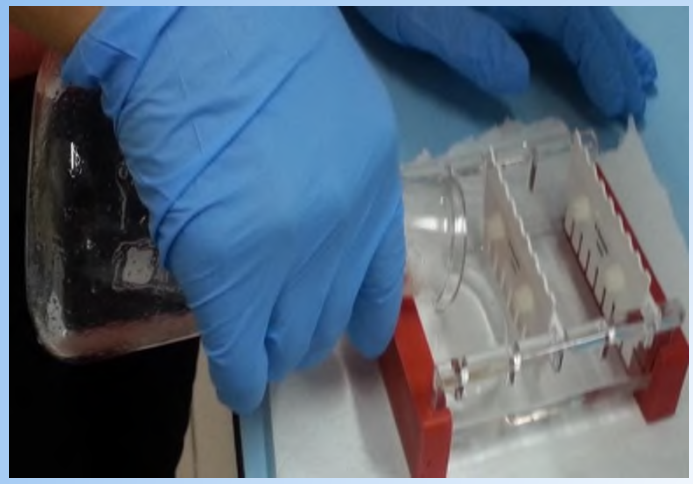
Table 2. B. pumilus MBB03 and Negative Control PCR Reaction Installation.

(- GCTGAAGCTCTT C

T’ATGAAAGTGATGTTTG -3 GCTCTTC: SapI



DNA QUANTITY DETERMINATION: AGAROSIS GEL ELECTROPHORESIS



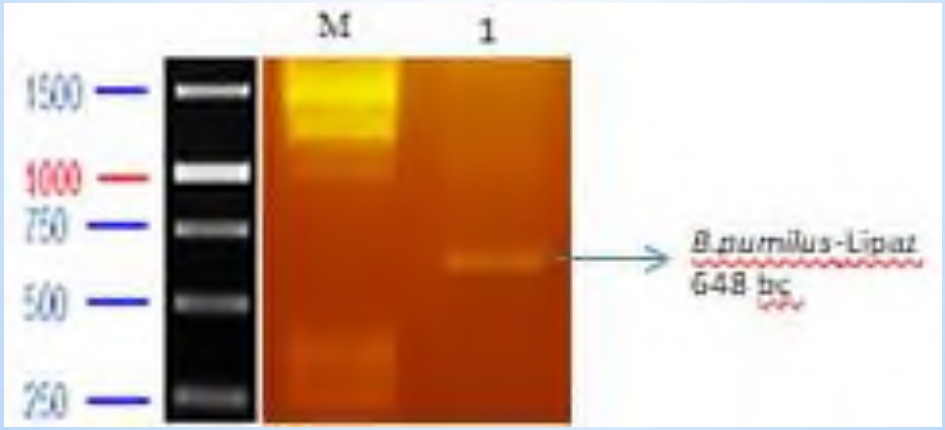


Figure 1. 1% Agarose Gel Electrophoresis Image of B. pumilus MBB03 Lipase Genes Amplified by PCR.

M) DNA marker 1) B. pumilus MBB03 lipase gene.

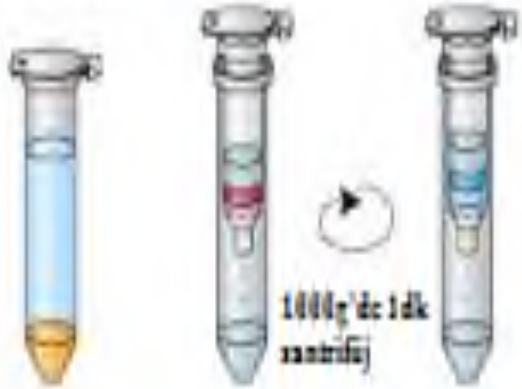
Purification of PCR Product

After the image in the gel

PCR product purification kit and

PCR product and PCR buffer

The mixture is loaded on the column.



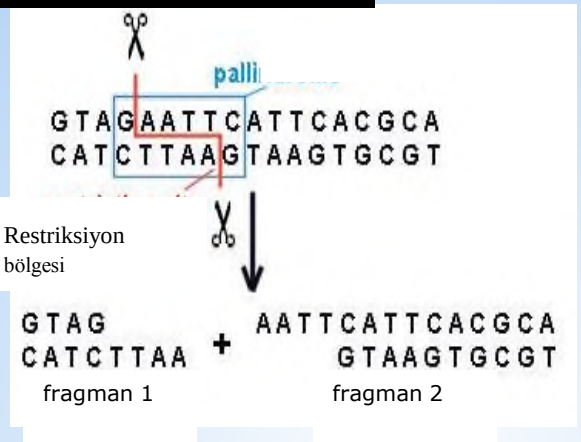
silica DNA column used.

The liquid formed at the bottom is discarded.

Restriction Enzyme Cutting and Purification of Plasmid DNA and PCR Products

[redacted]

Plasmid Vector



B.pumilus MBB03

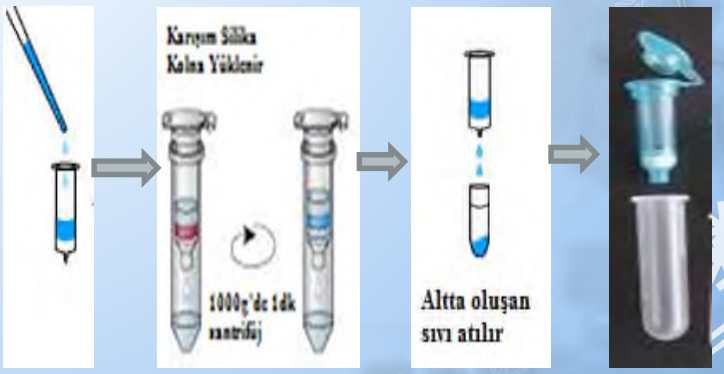
The reaction was incubated for 2 hours at 37 ° C.

The reactions were incubated at 65 ° C for 3 min and terminated.

[redacted]

SapI

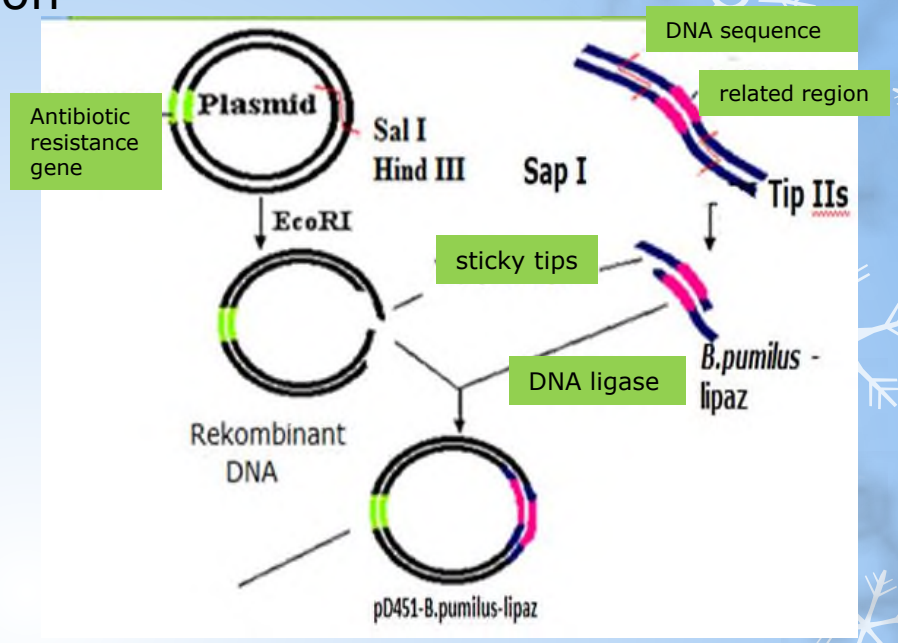
Tip IIs

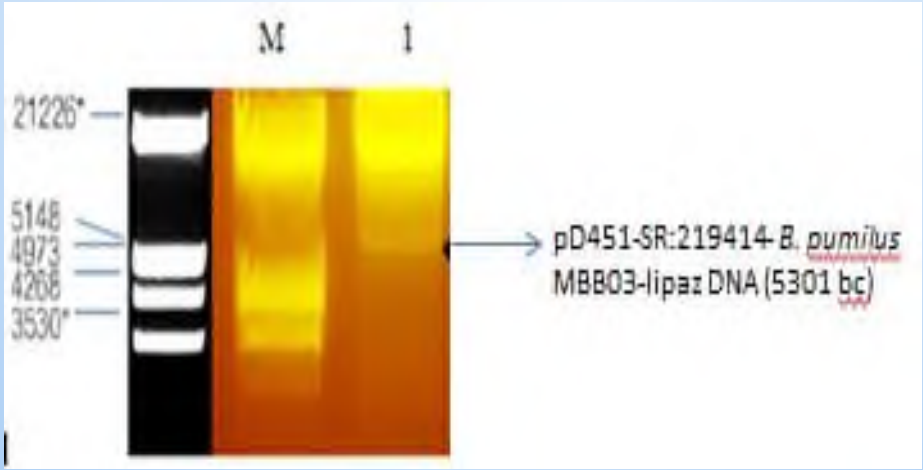


Transfer of the B.pumilus MBB03 lipase Gene to pD451-SR: 219414 Vector

the ligation

Component	Volume for 1 reaction
10x T4 DNA Ligaz Tamponu (0,5 M Tris-HCl,0,1 M $MgCl_2$, 10 mM ATP, 0,1 M DTT, pH 7.5)	2 µL
Cut vector	5 Ml
B.pumilus MBB03 *lipaz* geni	5 µL
T4 DNA Ligaz (400 U/µL) (cat no: M0202S, New England Biolabs, Amerika)	2 µL
Water (Deionized Water)	6 Ml
Total Volum	20L





B.pumilus-gene:648 bç

Vektör:4653 bç

Figure 3. pD451-SR: 219414-B.pumilus MBB03-lipase DNA 1% Agarose Gel Image.

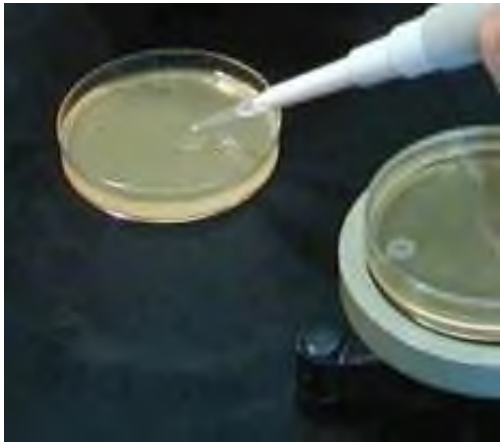
M) DNA marker 1) pD451-SR: 219414-B.pumilus MBB03-lipase plasmid DNA

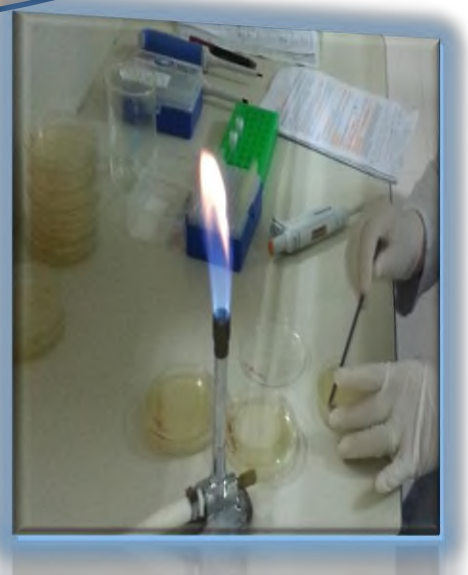
pD451-SR: 219414 cloning of the lipase gene in the expression vector

The lipase gene from Bacillus pumilus MBB03 was directly synthesized and cloned with the His-tag using the p1451-SD expression vector in the restriction sites SapI and Tip IIs.

► Recombinant vectors were transformed into E. coli BL21 (DE3) compund cells.

► Positive colonies were selected after overnight incubation in a medium of 40 μg / ml kanamycin..



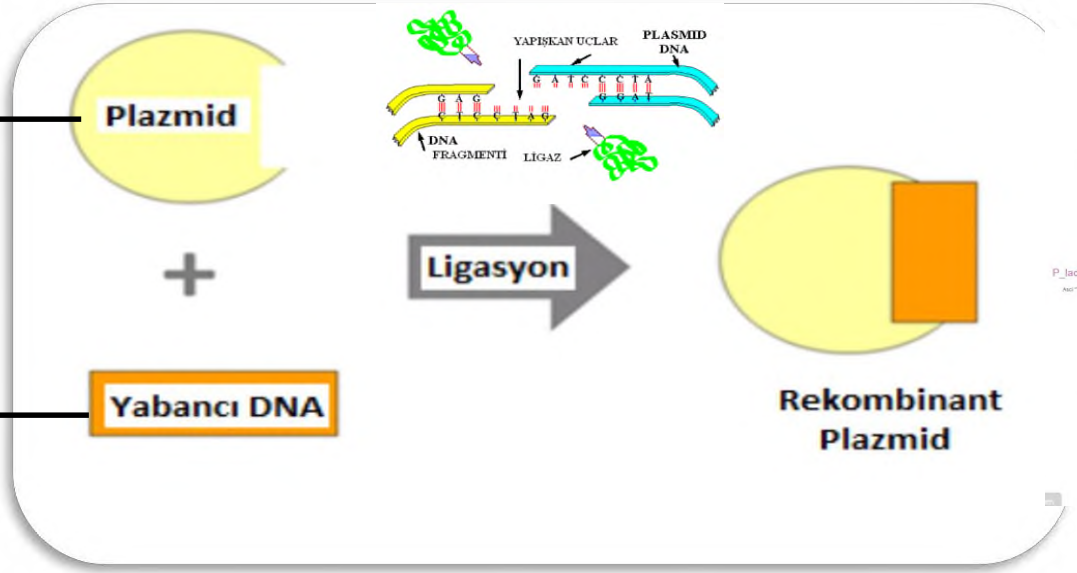


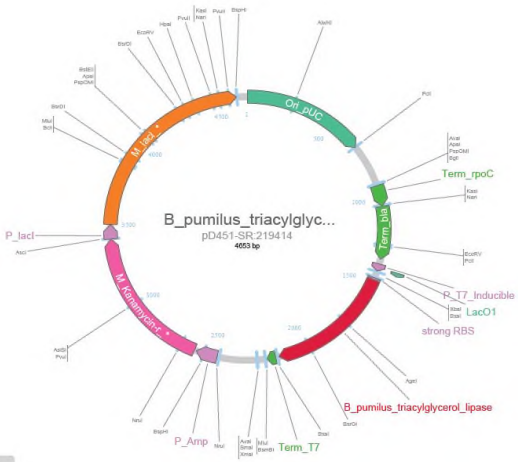
Recombinant Lipase Expression

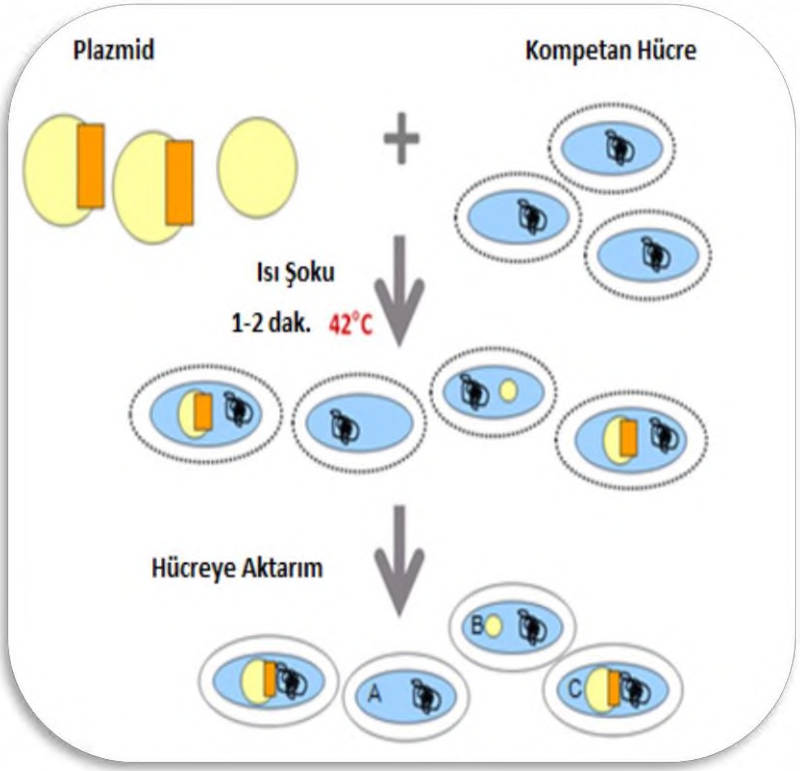
- *1 ml of positive culture was taken in 100 ml LB medium and shaken in a 250 ml bottle at 37 ° C.*
- *The culture was amplified until A600 reached to 0.6-0.8, followed by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, GeneON, Germany), and incubation continued for 5 hours at 37 ° C by shaking.*
- *The culture was centrifuged at 3000 g, + 4 ° C for 15 min and the supernatant discarded. The pellet was stored at -20 ° C until protein analysis.*
- *1 ml samples were collected and the pellets were used for subsequent analyzes.*

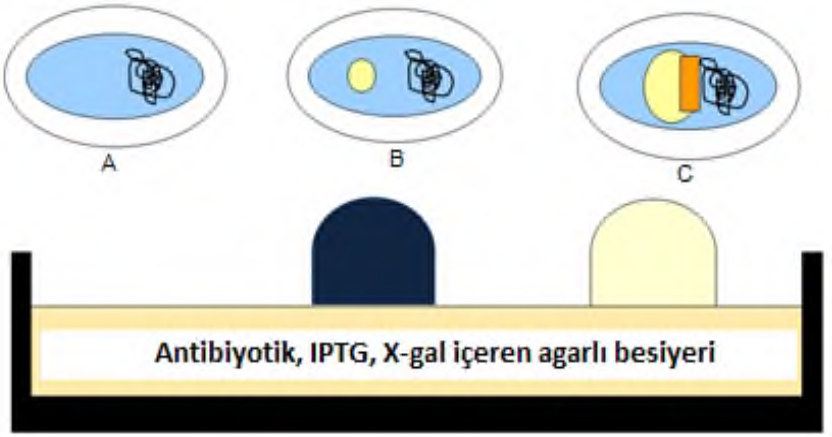
pD451

Lipase gene



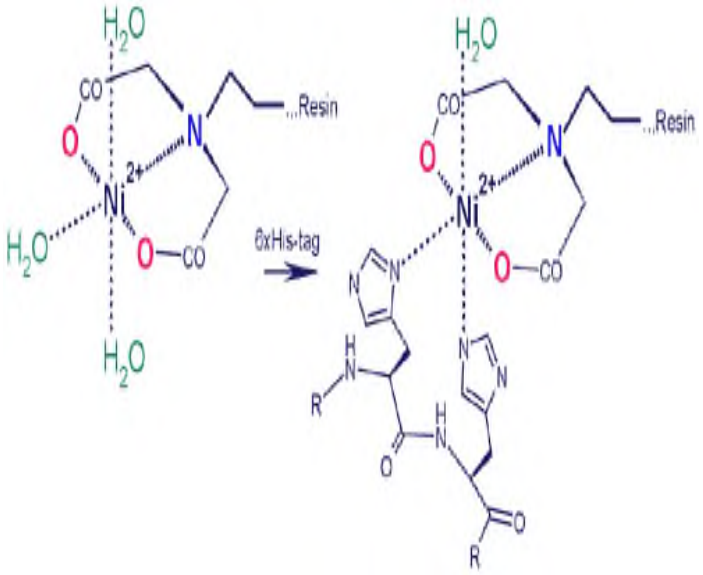






Purification of recombinant enzyme

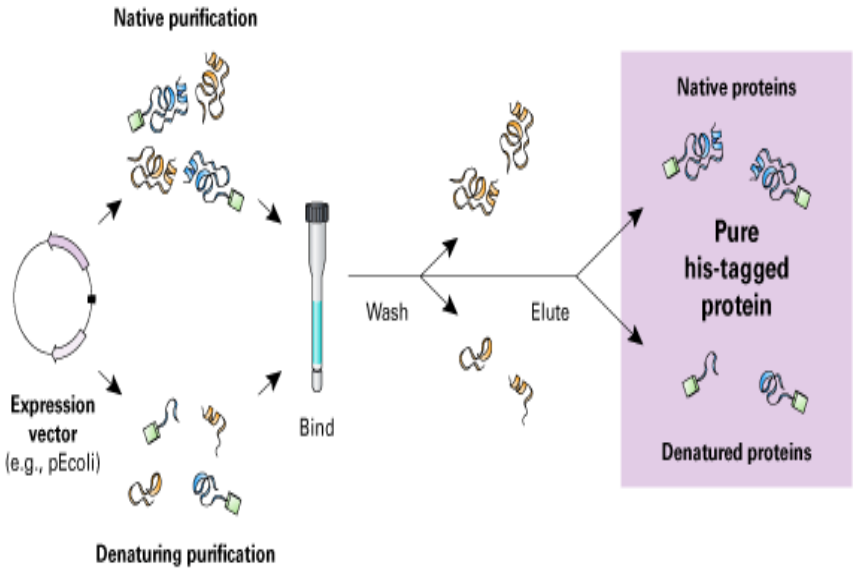
The His-tagged enzyme was purified by affinity chromatography using a one-step procedure using Ni-NTA agarose resin (50% filler Qiagen in 30% ethanol).



Protein Purification with His60 Ni Superflow Resin

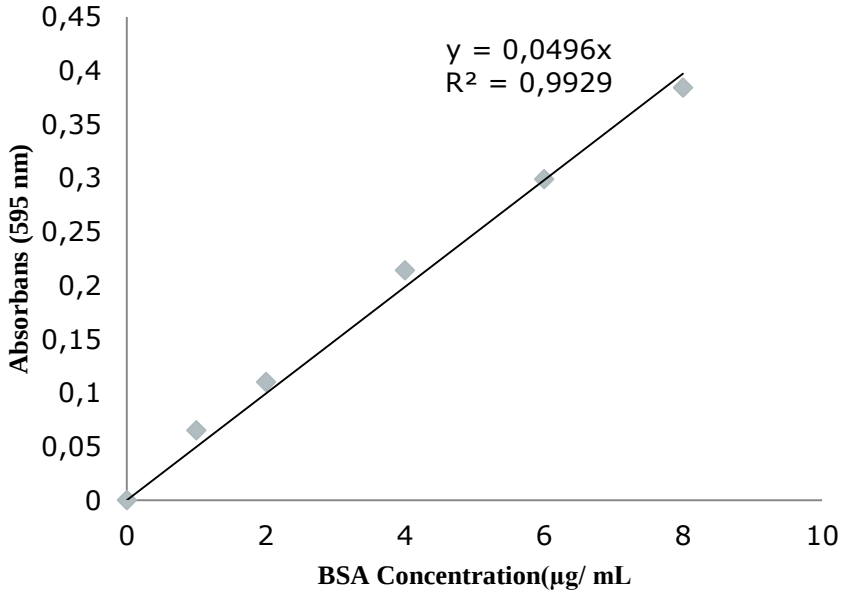
Equilibration & Wash Buffer and Elution Buffer were used in the purification process.

The collected fractions were visualized with 10% SDS-PAGE.

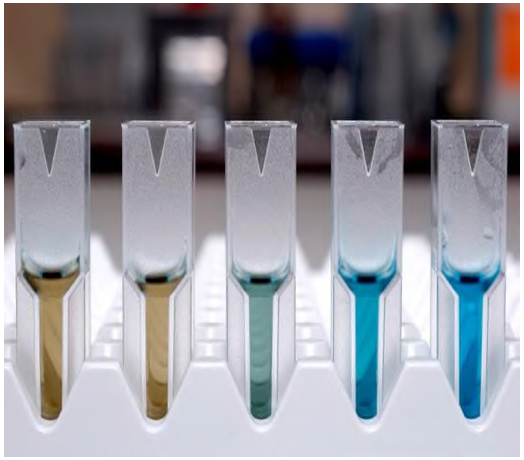


Protein determination

Protein concentrations of crude and pure enzyme were determined according to the Bradford method using bovine serum albumin (BSA) as standard.



Test Example	0.2mg / ml BSA Stock Solution	Water Volume, µl	Coomassie Reagent Volume, µl
Blank	0	800	200
BSA Standart-1µg/ml	5	795	200
BSA Standart-2µg/ml	10	790	200
BSA Standart-4µg/ml	20	780	200
BSA Standart-6µg/ml	30	770	200
BSA Standart-8µg/ml	40	760	200
Protein Örneği	50	750	200



Electrophoresis

The molecular weight of the purified enzyme was carried out in a Mini Protean 4-gel system (Biorad, USA) according to the method of SDS-PAGE Laemmli.

Reaction Components	5% Yarning Gel Components (mL)	12% Separation Gel Components (mL)
Pure water	2.7	4.0
%30 Akrilamid/bisakrilamid	0.67	3.3
1.0 M Tris (pH 6.8)	0.5	---
1.5 M Tris (pH 8.8)	---	2.5
%10 SDS	0.04	0.1
% 10 APS	0.04	0.1
TEMED	0.004	0.004

Determination of Enzyme Activity

Lipase activity was determined spectropometrically according to Winkler and Stuckman (1979) in the presence of pNPP-p-nitrophenyl palmitate (C16). The substrate mixture was prepared by mixing the solution with a concentration of 10 mM in acetonitrile at a concentration of 1: 4: 95 (v / v / v) with ethyl alcohol and 50 mM phosphate buffer (pH 7.5). 980 dalgaL of this mixture was taken and 20 ansL of the enzyme solution was added to it, after which the absorbance changes at room temperature were measured at 405 nm. The activity was determined as the amount of enzyme required to elicit 1 mgmol of p-nitrophenol at room temperature per minute and the specific activity was calculated as activity per mg protein.

Activity Calculation

- Activity [(U) and (µmol dak^{-1})] $= \frac{\Delta A}{\varepsilon \cdot t}$

- **Spesific Activity** [(U/mg prt) veya (µmol dak^{-1} mg prt^{-1})]= $\frac{\text{Activity}}{V_{Enzym} \cdot C_{Enzym}}$
- ΔA: $A_{Last} - A_{First}$
- t (time): minute
- ε : 14.8 µmol^{-1}cm^2
- V_{Enzym}: mL
- C_{Enzym}: (mg/mL) protein species

Results

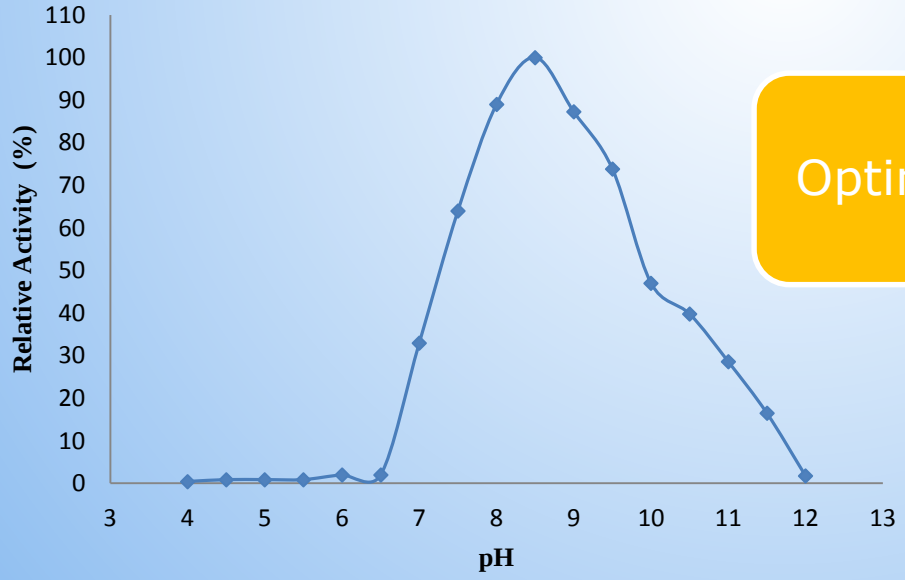
B. pumilus MBB03 lipase SDS-PAGE electrophoresis.

M) Protein marker 1) SDS-PAGE image after purification. 2) B. pumilus MBB03-lipase before Ni-NTA column purification

Purification Table for Purified Lipase Enzyme from B.pumilus MBB03

Effect of pH on Lipase Activity

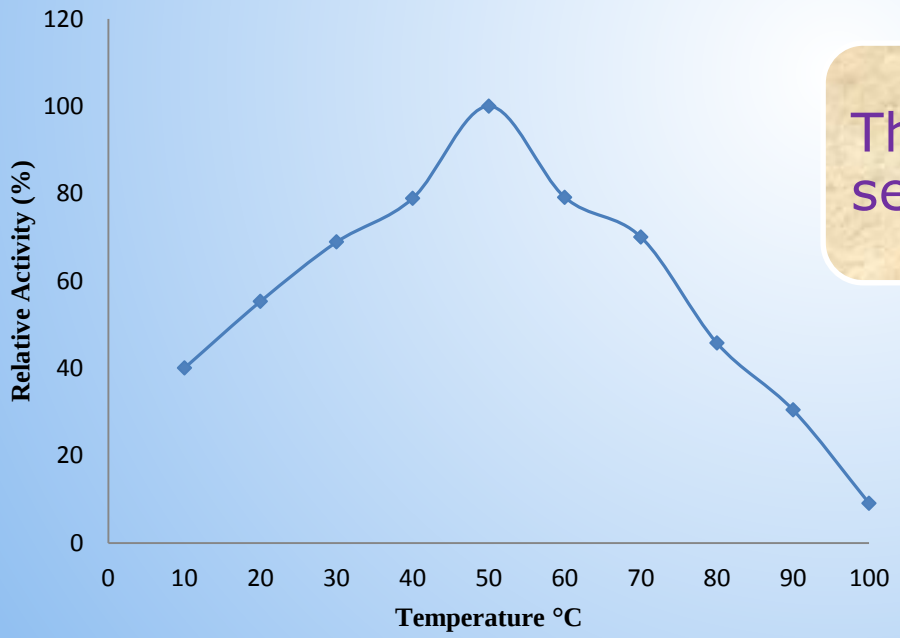
In order to determine the effects of pH on the activity of B. pumilus MBB03 lipase, the optimum pH values of the p-nitrophenyl octane as the substrate were determined using the buffer solutions prepared at different pHs. For this, prepared at a concentration of 50 mM; pH 4.0-5.0 Acetate buffer, pH 6.0-7.0 KH2PO4 buffer, pH 8.0-9.0 Tris-HCl buffer and pH 10.0-12.0 Glycine-NaOH buffers were used. The effect of substrate concentration, protein content and other parameters on enzyme activity was investigated by using the optimum pH value.



Optimum pH is set to 8.0.

Effect of Temperature on Lipase Activity

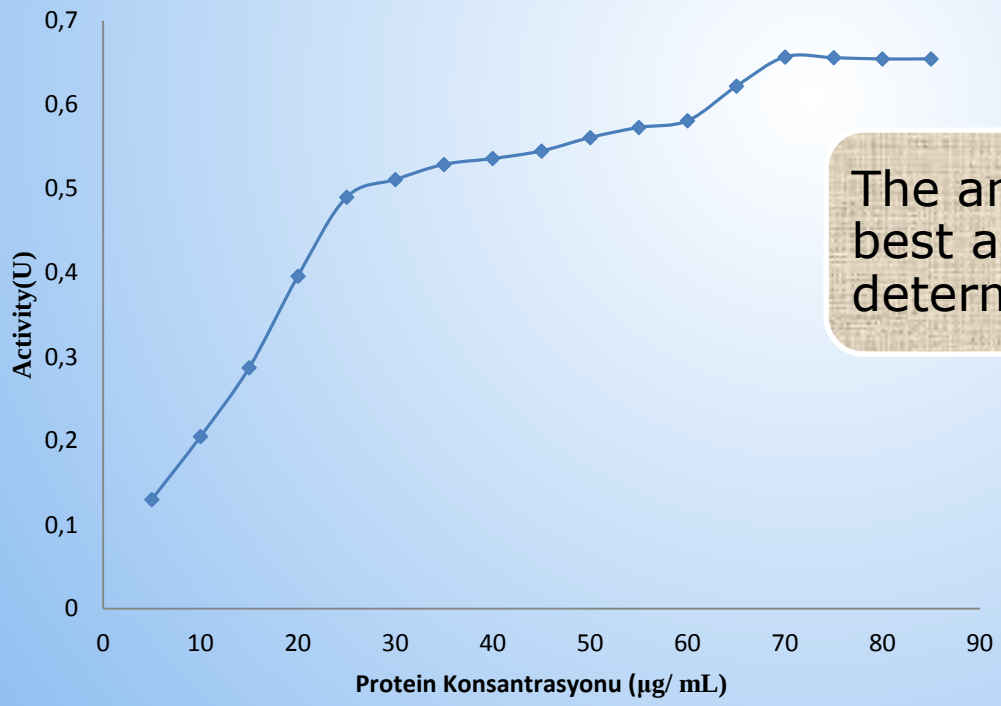
The optimum temperature for the activity of B. pumilus MBB03 lipases was determined using the p-nitrophenyl octane as the substrate, respectively. In order to determine the effect of the temperature on the enzyme activity, measurements were made in the temperature range of 10-100 ° C in increments of 10 ° C. The buffer and substrate solution mixtures were incubated for 10 minutes at the specified optimum pH and in the specified temperature range. The results were plotted as a function of temperature. The only peak observed in the graph was taken as the optimum temperature value.



The optimum temperature was set at 50 ° C.

Investigation of the Effect of Protein Concentration on Lipase Activity

In order to determine the protein concentration of B. pumilusMBB03 lipase, the lipase activity was measured in the concentration range of 0.5 aktivite100 µg / mL of enzymes in the presence of the p-nitrophenyl octane substrate for B.pumilus MBB03, respectively. In order to investigate the change in enzyme activity by varying the lipase concentration and to determine the optimum amount of protein at which the actin was highest, activity change was measured against varying amounts of protein at predetermined optimum pH and temperature and constant substrate concentration, and the effect of protein concentration on lipase activity was plotted by investigating the effect.



The amount of protein showed the best activity for the enzyme was determined as 75µg / mL.

Investigation of the Effect of Some Metal Ions on Lipase Activity

To investigate the effect of some metal ions on the B. pumilus MBB03 lipase enzymes, some CaCl2, NaCl2, CuCl2, KCl2, BaCl2, MgCl2, MnCl2, HgCl2, NiCl2, ZnSO4, CuSO4, CoSO4, FeSO4, MgSO4 was added from 100 mM stock solutions of metal ions and lipase activity was measured under optimum conditions. No metal ion-free test medium was accepted as control. The activity of the control group was considered to be 100 and the activity changes of the test medium containing metal ions were determined as% value and% relative activities were calculated.

Metal İyonları	Bağıl Aktivite (%) (*B. pumilus* Serbest enzim)		
	0.1 mM	0.5 mM	1mM
Kontrol	100	100	100
$CaCl_2$	99	105	114
$NaCl_2$	122	106	105
$CuCl_2$	108	111	90
KCl_2	94	97	103
$BaCl_2$	102	92	94
$ZnSO_4$	122	60	74
$HgCl_2$	102	70	63
$FeSO_4$	82	113	98
$CuSO_4$	72	73	75
$CoSO_4$	58	63	59
$NiCl_2$	53	122	98
$MgCl_2$	102	103	95
$MnCl_2$	80	91	72
$MgSO_4$	79	67	62

The Effect of Some Detergents, Surfactants and Chemicals on B. pumilus MBB03 Lipase Activity

as ionic detergent; sodium deoxycholate, sodium taukolate and sodium dodecyl sulfate,

as non-ionic detergent ; Tween 80, Tween 20, Triton X-100,

as a ziwitterionic detergent ; CHAPS was used.

10% stock solutions of these detergents were prepared and final concentrations were determined as 0.01-1% in reaction mixtures and activity was determined under optimum conditions. No detergent-free test environment is considered as control. The activity of the control group was considered to be 100 and activity changes of the experimental medium containing metal ion were determined according to the control and% relative activities were calculated.

as a ziwitterionic detergent; CHAPS was used..

Some Detergents, Surfactants and Chemicals	Concentration (%)	Relative Activity (%) B.pumilus Free Enzyme
Control	-	100
Triton X-100	0.1	63
	0.5	54
	1.0	36
Tween 80	0.1	45
	0.5	30
	1.0	25
Tween 20	0.1	22
	0.5	19
	1.0	14
CHAPS	0.1	127
	0.5	105
	1.0	84
Sodium deoxycholate	0.1	109
	0.5	103
	1.0	94
Sodium taurocholate	0.1	105
	0.5	96
	1.0	92
SDS	0.1	57
	0.5	56
	1.0	43

Investigation of the Effect of Some Organic Solvents on Lipase Activity

Ethanol, methanol, isopropanol, n-butanol, acetone, dimethylsulfoxide (DMSO), xylene, dimethylformamide (DMF), glycerol were used.

10% stock solutions of these organic solvents were prepared and final concentrations were determined as 0.01-1% in reaction mixtures, and activity was determined under optimum conditions. No organic solvent-free test medium was accepted as control. The activity of the control group was considered to be 100 and activity changes of the experimental medium containing metal ions were determined according to the control and% relative activities were calculated.

Some Organic Solvents and Chemicals	Concentration (%)	Relative Activity (%) B.pumilus Free enzyme
control	-	100
Ethanol	0.1	89
	0.5	82
	1.0	76
Methanol	0.1	80
	0.5	77
	1.0	72
Isopropanol (%)	0.1	82
	0.5	81
	1.0	70
n- Butanol (%)	0.1	88
	0.5	87
	1.0	80
Acetone (%)	0.1	92
	0.5	89
	1.0	89
DMSO (%)	0.1	79
	0.5	76
	1.0	75
Xylene (%)	0.1	107
	0.5	89
	1.0	77
DMF (%)	0.1	96
	0.5	80
	1.0	66
Glycerol (%)	0.1	125
	0.5	108
	1.0	70

Investigation of the Effects of Some Chemical and Inhibiting Agents on Lipase Activity

Phenyl methyl sulfonyl fluoride (PMSF),
β-mercaptoethanol,
1,4-dithiothreitol (DTT) and
EDTA

10 Mm stock solutions were prepared and final concentrations were determined as 0.01-1mM in the reaction mixtures and activity was determined under optimum conditions.

No chemical and inhibitor-free assay medium was accepted as control. The activity of the control group was considered to be 100 and activity changes of the experimental medium containing metal ions were determined according to the control and% relative activities were calculated.

Some Chemical and Inhibitory Agents	Concentration(mM)	Relative Activity (%) B.pumilus Enzyme
control	-	100
PMSF	0.1	85
	0.5	84
	1.0	64
β-mercaptoethanol	0.1	70
	0.5	62
	1.0	60
DTT	0.1	67
	0.5	57
	1.0	57
EDTA	0.1	106
	0.5	102
	1.0	100

The DNA of the B.pumilus MBB03-lipase gene (NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) lipase (Gen. ID: 1321705: 106-1374), cloned as a result of analyzes, by Kim et al. 2002) revealed the similarity in the reported B.pumilus genome.

Sequence assays were performed in ABI Prism 377 DNA Sequencer (Applied Biosystems, USA) using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit.

The quality of the resulting sequences was checked by 4peaks software (http://nucleobytes.com/index.php/4peaks). The sequences in which the sequences were most similar to the DNA data bank were determined using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) program.

This sequence is 100% similar to the Bacillus pumilus triacylglicerol lipase found in GenBank.

Result

Thank You
For Listening
To Me