CLONING AND EXPRESSION OF A LIPASE GENE FROM *PSEUDOMONAS AERUGINOSA* INTO *E.coli*

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ABSTRACT

Fifteen local isolates of *Pseudomonas* were obtained from several sources such as soil, water and some high-fat foods (Meat, olives, coconuts, etc.). The ability of isolates to produce lipase was measured by the size of clear zone on Tween 20 solid medium and by measuring the enzymatic activity and specific activity. Isolate M3 (as named in this study) was found to be the most efficient for the production of the lipase with enzymatic activity reached 56.6 U/ml and specific activity of 305.94 U/mg. This isolate was identified through genetic analysis of the 16S rRNA gene. and it was shown that the isolate M3 belongs to *Pseudomonas aeruginosa* with 99% similarity. The DNA of isolate M3 was extracted and lipase gene was amplified through PCR technique, then purified and cloned into E.coli DH5α cells first using pTG19-T plasmid, and expressed in *E.coli* Bl21 with expression vector pet-28a. The activity of lipase from transformed *E.coli* Bl21 was 196.6 U/ml and the specific activity 618.2 U/mg.

Keywords: PCR; Lipase gene; 16S rRNA gene; Plasmid; Identification

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الكلونة و التعبير لجين اللايبيز من بكتريا *Pseudomonas aeruginosa* في *E.coli*

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المستخلص

تم الحصول على 15 عزلة محلية من بكتريا *Pseudomonas* من مصادر مختلفة شملت المياه والتربة وبعض الأغذية الدهنية (لحوم، ثمار الزيتون، ثمار جوز الهند وغيرها). اختبرت قابلية العزلات على انتاج اللايبيز بطريقة الاطباق الصلبة وملاحظة الهالة المتكونة باستعمال وسط Tween 20 بالإضافة الى قياس الفعالية الانزيمية والفعالية النوعية. أعطت العزلة التي رمز لها M3 في هذه الدراسة اعلى فعالية لانزيم اللايبيز والتي بلغت 56.6 وحدة/مل وفعالية نوعية 305.94 وحدة/ملغم. اخضعت العزلة المذكورة الى التشخيص الجيني بوساطة جين 16S rRNA وقد بينت النتائج ان العزلة M3 تعود الى *Pseudomonas aeruginosa* وبنسبة تطابق بلغت 99%. استخلص الحامض النووي من العزلة M3 وتم تضخيم جين اللايبيز بتقنية تفاعل البلمرة المتسلسل وباستعمال بوادئ صممت لهذا الغرض ثم تمت تنقيته وكلونته الى خلايا *E.coli* DH5α باستعمال الناقل البلازميدي pTG19-T وثم كلونته والتعبير عنه في خلايا *E.coli* Bl21 باستعمال الناقل التعبيري pet-28a. بلغت الفعالية الانزيمية للايبيز من العزلة *E.coli* Bl21 المكونله 196.6 وحدة/مل والفعالية النوعية 618.2 وحدة/ملغم.

كلمات مفتاحية: تفاعل البلمرة المتسلسل،جين اللايبيز ، جين 16S rRNA، بلازميد، تشخيص

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INTRODUCTION

Lipases or (triacylglycerol acylhydrolases, EC 3.1.1.3) are a group of enzymes which catalyze the hydrolysis of long-chain triglycerides into fatty acids, diacylglycerol, monoacylglycerol, and glycerol (7). Lipases are a large group of industrial enzymes, which used in various industrial like food pharmaceuticals, detergents, beverages, cosmetics, degreasing formulations, biofuel, and paper (14). In the field of food industry, Lipases has been widely used in improving the flavor by producing esters of short fatty acids with alcohols. It has been used in the development of flavor in the dairy industry such as cheese, butter, margarine and others. Lipases were also used to produce low-fat meat (lean meat) by removing fat from meat and fish products. Moreover, in bakery lipases can improve dough rheology, increase in volume, dough strength and stability (2, 3. 12) Lipases are produced by many organisms like fungi, bacteria, yeast, animal, and plant (19). Microbes are an excellent source of lipases compared to other organisms because of their fast growth, small space for cultivation, with standing various temperatures and easy genetic manipulation to generate high yields desirable for diverse applications (18). In the past few years, recombinant DNA technology has allowed scientists to produce a large number of varied proteins in microorganisms, that were unavailable, relatively expensive, or difficult to produce in large quantity (5). The production of proteins using recombinant techniques is exponentially increasing as the demand increased (1). *Escherichia coli* is the most expression system that used for the production of recombinant proteins due to its inexpensive medium, short generation time, well-known genetics, easy genetic manipulation and the availability of a large number of cloning vectors . All these advantages enable *E. coli* to offer a rapid, high yield, and economical production of recombinant proteins (6, 15, 16). The present study aimed to find high lipase production *Pseudomonas* isolate and cloning the gene into *E.coli*.

MATERIALS AND METHODS

Collection of samples and identification for lipase producing isolates: Bacterial isolates were obtained from various sources such as soil, water and some fatty foods (meat, olive fruits, coconut fruits, etc.) and placed in sterilized plastic tubes. After the serial dilution were done, 0.1 ml was transferred to Pseudomonas agar plates and incubated at 37C for 24h and lipase producer colonies were picked up to a new culture.

Primary screening for lipase producing isolates

The ability of isolates to lipase production was measured by grown the bacterial colonies on the Tween 20 agar and incubated at 37C˚ for 24h. Tween 20 was used in the medium as a sole source of carbon (10). The ability of the bacterial isolates to produced lipase was scanned by measuring the diameters of the clear zones formed around the grown colonies.

Lipase production

Lipase production medium was prepared from (Arabic gum, 1% olive oil 1%, 0.5% sodium chloride, 1% pipton) according to (2). The pH of the medium was adjusted to 7.0. 100 mL of medium was added to 250 mL Erlenmeyer flasks incubated with a volume of (1×10^8 cell) of inoculum culture. The inoculated flasks were incubated at 37C on a incubator shaker at 200 rpm for 48 h. The supernatant was used as crude enzyme

Lipase assay (Secondary screening)

Lipase activity was measured by using modified titrimetric method as described by (10). 1 ml of crude enzyme solution was added to the reaction mixture containing 10 ml of 10% homogenized olive oil in 10% arabic gum, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol/L Phosphate buffer, pH 7.0. The enzyme - substrate was incubated on an orbital shaker at 150 rpm at 37ºC for 30min. The reaction was stopped by adding 20 ml ethanol-acetone (1:1). The free fatty acids were titrated with 0.1 mol/L NaOH using phenolphthalein as indicator. The reaction mixture without the enzyme was titrated in the same way and used as a blank. The lipase activity was calculated using a particular formula

Lipase activity = Vol. of NaOH (mL) × Molarity of NaoH × 1000 × df/ Vol. of Lipase (mL) × Reaction Time (min).

One unit of lipase activity was defined as the amount of enzyme that liberated 1µmol fatty acid per minute under assay conditions

Isolate identification

The isolate that produced the highest enzym was identified by analysis of the 16S rRNA gene. The genetic identification was performed by extract the bacterial DNA using Geneaid DNA extraction kit according to the manufacturer's recommendation, after that, the 16S rRNA was amplified by PCR technique using primers designed for this purpose, Forward (5′- GACGGGTGAGTAATGCCTA-3′), Reverse (5′-CACTGGTGTTCCTTCCTATA-3′), as described by (6) and PCR premix. The reaction mixture (Table1) was added to the eppendorf tube and placed in the PCR thermocycler, which was programmed as shown in Table2. The PCR product was migrated in 1% agarose gel along with 2 µl of 2000 bp DNA ladder for 45 min at 90V, stained with ethidium bromide, then agarose gel electrophoresis was visualized by UV-transilluminator. After that, the amplified produt was send to Macrogen Korean Company to analyze of the 16S rRNA nucleotides sequence.

Table 1. reaction mixture of PCR

Component	Volume (microlet
DNA	4
Forward primer 10 pmol	2
Reverse primer 10 pmol	2
PCR premix	2
Free nuclease water	10

Table 2. PCR thermocycler program

No.	stage	Temperature (C°)	Time (min)	Number of cycles
1	Denaturation	94	5	1
2	Denaturation	94	0:45	35
3	Annealing	50	0:45	35
4	Extension	72	0:45	35
5	Final extension	72	10	1
6	Cooling	4	∞	-

Lipase gene amplification

The DNA of *Pseudomonas* M3 strain was extracted using Geneaid DNA extraction kit as described by manufactures. The lipase gene was amplified by PCR technique using specific primers, with forward (5′ ATGAAGAAGAAGTCTCTGCTCCCCC 3′) and reverse (5′ CTACAGGCTGGCGTTCTTCAGGC 3′) that was designed by Primer-BLAST program from NCBI and The reaction mixture as shown in (Table.3) was used for amplified. The PCR machine was programmed as shown in (Table.4), PCR product was migrated in 1% agarose gel along with 2 µl of 2000 bp DNA ladder for 45 min at 90V, stained with ethidium bromide, then agarose gel electrophoresis was visualized by UV-transilluminator

Table 3. Reaction mixture of PCR

Component	Volume(microliter)
DNA tamplet	4
Forward primer 10 pmol/ul	2
Reverse primer 10 pmol/ul	2
PCR premix	2
Free nuclease water	Up to 20

Table.4 Cycling conditions of gene amplification

No.	Stage	Temperature °C	Time min	Number of cycles
1	Denaturation	94	5	1
2	Denaturation	94	45	35
3	Annealing	50	45	35
4	Extension	72	45	35
5	Final extension	72	10	1
6	cooling	4	∞	-

Insertion of lipase gene into pTG19-T vector

The lipase gene was extracted and purified from agarose gel using Gel/PCR DNA extraction kit from Geneaid. The gene was ligated with pTG19-T vector using T4 DNA ligase. the ligated mix was prepared according to the manufacturer's instructions as shown in Table.5

Table.5 The ligation mix

Component	Volume(microliter)
pTG19-T vector 25 ng/ul	2
Lipase gene	2
T4 DNA ligase 200u/ul	2
Free nuclease water	Up to 10
10X Buffer ligase	1

Transformation of E.coli DH5α cells

The cloned pTG19-T vectors were inserted into *E.coli* DH5α competent cells using TA cloning kit. The transformed cells were spread on LB (Luria Bertani) plates containing 50µg/ml of ampicillin and 80µg of each X-Gal 20mg/ml and 100mM IPTG and spread on surface, and then incubated overnight at 37 °C.

Lipase gene ligation to pET-28a(+) Expression Vector

The pTG19-T cloned vectors were extracted from positive *E.coli* DH5α cells (white colonies) by BiONEER Plasmid Extraction kit and according to the kit instructions. Lipase gene was restricted from the pTG19-T vector using Bam HI restriction endonucleases and purified by migrated in 1% Agarose gel and extracted with gel DNA extraction kit. The extracted DNA fragments were ligated with Bam HI restriction site in pET-28a (+) expression vector usingT4 DNA ligase with the same ligated mix that mentioned in (Table.5).

RESULTS AND DISSCUSSION

Isolating and identifying of lipase production isolate

From 15 isolates obtained in this study, it was found that the isolate M3 had highest activity 56.6 U/ml .This one identified by genetic analysis using the 16S rRNA gene, and the results of electrophoresis showed one band with (fig.1), which is confirmed the amplification of the 16S rRNA gene. The results of sequencing was analyzed using Blast program from NCBI, and it was found that the gene has size of 637 bp and the isolate belongs to Pseudomonas aeruginosa with 99% similarity (Table.6).

Table 6. The similarity of identified isolate with other *P.aeruginosa* strains from gene bank

No.	Strain	Identifity	Accession
1	*Pseudomonas aeruginosa* FQR12	99%	MF144446.1
2	*Pseudomonas aeruginosa* RSB3	99%	LN589738.1
3	*Pseudomonas aeruginosa* DUVASU/Hs-1	99%	KY930659.1
4	*Pseudomonas aeruginosa* DUVASU/F6	99%	KY930655.1
5	*Pseudomonas aeruginosa* DUVASU/1035	99%	KY930656.1
6	*Pseudomonas aeruginosa* DUVASU/F2	99%	
7	*Pseudomonas aeruginosa* DUVASU/A6	99%	*KY930654.1*
8	*Pseudomonas aeruginosa* P6D102-476	99%	KY930648.1
9	*Pseudomonas aeruginosa* HS9	99%	EF510037.1
10	*Pseudomonas aeruginosa* BTTDD3	99%	MH000683.1
			MG648420.1

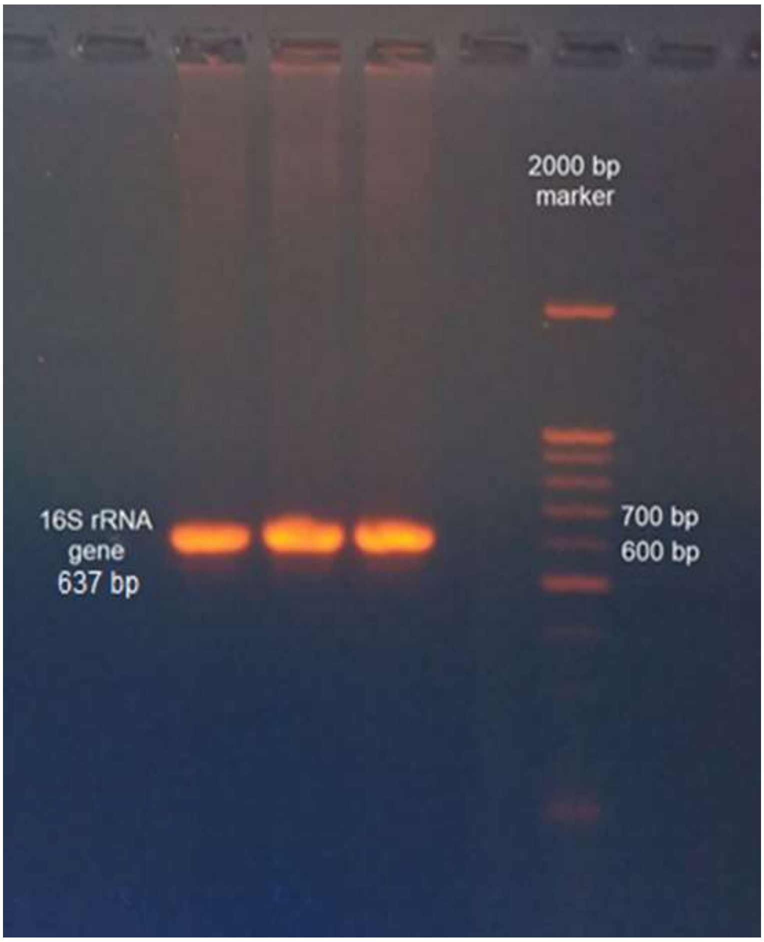

Figure1. Amplified 16S rRNA gene electrophoresis results of M3 isolate

Lipase gene cloning and expression

The lipase gene was amplified through PCR technique; the result showed a single band with a size ≈930 bp.The recombinant pTG19-T vector was used to transform *E.coli* DH5α cells. The Transformed white cells were selected on LB-agar medium containing AMP/X-gal/IPTG by blue-white screening (fig.2). The pTG19-T vector was extracted from transformed *E. coli* DH5α, digested with the restriction endonuclease *Bam*HI, and run on 1% agarose gel. The result showed two bands one with size of ≈2900 bp, which represent the vector and the other ≈1000 bp, which belongs to the lipase gene. These result confirmed insertion of lipase gene to *E. coli* DH5α (fig.3).

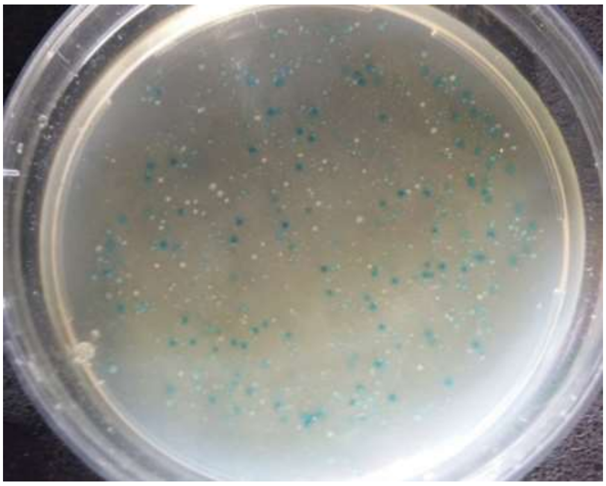

Figure 2. Blue-white screening of transformed *E.coli* DH5α cells

The lipase gene was purified and ligated with pET-28a (+) expression vector as shown in (fig.3) using T4 DNA ligase and transformed into *E. coli* BL21 (DE3) cells. The transformed cells (fig.4) were picked on LB-agar medium including kanamycin according to kanamycin resistance marker originated from pET-28a (+)vector. The lipase gene expression was inducted by IPTG. Lipase production by transformed *E. coli* BL21 (DE3) was assayed in LB medium included IPTG; the result showed that the lipase activity reached 196.6 U/ml. Several studies reported the cloning of lipase gene. Kanmani et al (9) Cloned lipase gene from *Bacillus amyloliquefaciens* into Escherichia coli DH5α cells usng pUC18 vector, and they found that the lipase activity form transformed cells was 240 U/ml. Su et al (17) Reported that they cloned lipase gene from *Pseudomonas lipolytica* using pMD19-T vector and the restriction endonucleases *Nco*I and *Xho*I into *E. coli* DH5α cells first, and then into *E. coli* BL21 (DE3) cells using pET- 22b vector and the relative restriction endonucleases and the specific activity of lipase from transformed cells was 52.6 U/mg. Zhang et al (18) Transformed lipase gene from *Psychrobacter* sp into *E. coli* BL21 using pUC118 vector and *BamH*I restriction enzyme, and the specific activity of lipase from the *E. coli* BL21 transformed cells was 31 U/mg.

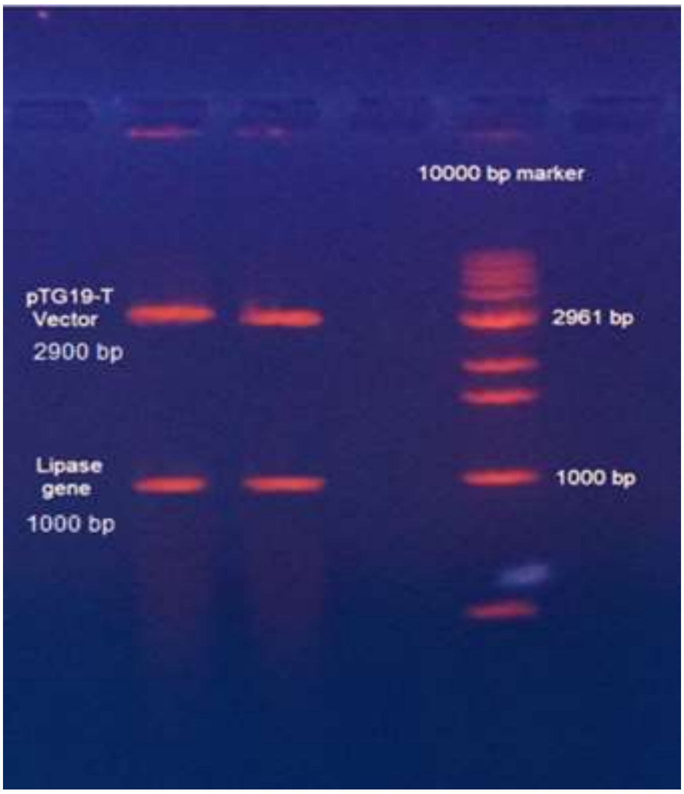

Figure.2 Electrophoresis product of pTG19-T vector and lipase gene

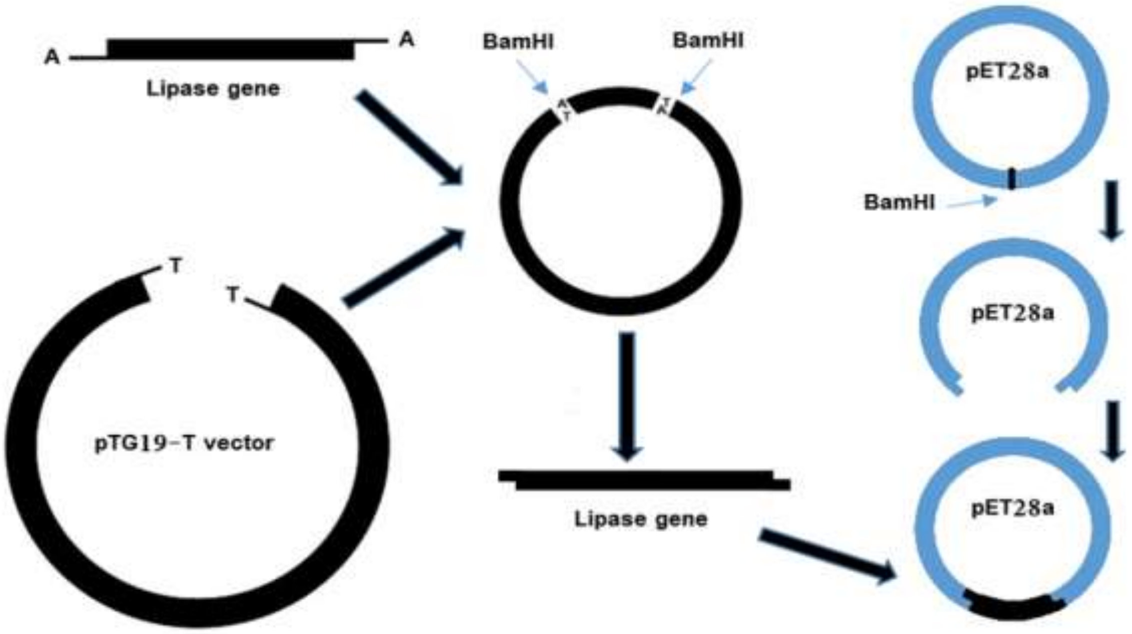

Figure 3. Schematic representation of the lipase gene cloning

Figure 4. The transformed *E. coli* BL21 (DE3) cells on LB kanamycin agar

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