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CLONING AND EXPRESSION OF A PROTEASE GENE FROM *PSEUDOMONAS AERUGINOSA* INTO *E. COLI*

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ABSTRACT

Ten local isolates of *Pseudomonas* were obtained from several sources such as remnants of massacres, soil and some high-protein foods (Meat, legumes, etc.). The ability of isolates to produce protease was measured by the size of clear zone on Skim milk agar and by measuring the enzymatic activity and specific activity. Isolate Y7 (as named in this study) was found to be the most efficient for the production of the protease with enzymatic activity reached 82.1 U/ml and specific activity of 451.09 U/mg. This isolate was identified through genetic analysis of the 16S rRNA gene. And it was shown that the isolate Y7 belongs to *Pseudomonas aeruginosa* with 100% similarity. The DNA of isolate Y7 was extracted and protease 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 protease from transformed *E.coli* Bl21 was 195.3 U/ml and the specific activity 582.9 U/mg.
***Keywords*:** PCR; Protease gene; 16S rRNA gene; Plasmid; Identification

Introduction

Protease enzymes EC.3.4 are classified under the hydrolysis enzymes and consist of a large group of enzymes that hydrolyze proteins into peptides and amino acids, which are widely distributed enzymes in nature as it is found in animals, plants andmicro-organisms (4), Due to their wide specialization with regard to the reaction materials, these enzymes have been distinguished by many applications, especially in the food, medical, pharmaceutical, industrial and other fields of applications. Proteases occupy great industrial importance and constitute 60% of the total sales of various enzymes in the sectors of the global market for enzymes. The production of protease enzymes from micro-organisms reaches hundreds of tons, especially those extracted from bacterial species (11), Alkaline protease enzymes were first discovered in 1963 and constitute about 25% of the enzymes marketed commercially at the global level, it has been used with detergents, in the manufacture of protein decomposers with high nutritional value from slaughterhouse residues, fish and cheap protein materials, and in the leather industry (leather tanning)(1). Studies indicate that most of the commercial cleaning enzymes are alkaline proteases which are produced from most microorganisms that are secreted outside the cell in one step and directly (9).

Microbes are an excellent source of Protease 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 (14). 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 (3). The production of proteins using recombinant techniques is exponentially increasing as the demand increased (10). *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 (3,12,8). The present study aimed to find high Protease production *Pseudomonas* isolate and cloning the gene into *E.coli*.

Material and Methods

Collection of samples and identification for protease producing isolates

Bacterial isolates were obtained from various sources such as remnants of massacres, soil and some high-protein foods (Meat, legumes, 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 protease producer colonies were picked up to a new culture.

Primary screening for protease producing isolates

The ability of isolates to protease production was measured by grown the bacterial colonies on the Skim milk agar and incubated at 37C° for 24h. Skim milk was used in the medium as a sole source of protein (9). The ability of the bacterial isolates to produced protease was scanned by measuring the diameters of the clear zones formed around the grown colonies.

Protease production

protease production medium was prepared from (Casein 0.5% , Yeast extract 0.5%, 1% glucose, 0.1% magnesium sulfate) 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) f 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.

Protease assay (Secondary screening)

The activity of the protease enzyme was estimated by (7). 1.8 of the substrate was transferred to a test tube and in two replications, and placed in a water bath at a temperature of 37 ° C for 10 minutes and adding 0.2 ml of the enzyme solution to it, the tubes were incubated at the same temperature for 30 minutes, the reaction was stopped by adding 3 ml From (TCA) Trichloro acetic acid at a concentration of 5%. The Blank control solution was prepared in the same way, with the exception of adding the enzyme solution after adding (TCA). The reaction solution was filtered through a filter paper No.1 Whatman eliminates the precipitate. The filtrate absorbance was measured at a wavelength of 280 in a spectrophotometer. The unit of activity of the enzyme was defined as the amount of enzyme in milliliters that gives a 0.001 increase in absorbance at a wavelength of 280 nm per minute and under experimental conditions. The enzymatic activity was measured in all stages of the study through the following equation:-

$$\text{Enzymatic activity (U/ml)} = \frac{\text{A280nm}}{0.001\times30\times0.2}$$

Isolate identification

The isolate that produced the highest enzyme 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`-AGAGTTTGATCCTGGCTCAG-3`), Reverse (5`- TACGGTTACCTTGTTACGACTT 3`),as described by (5) and PCR premix. The reaction mixture (Table1) was added to the eppendorf tube and placed in the PCR thermo cycler, 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 (microletter)
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	95	05:00	1
2	Denaturation	95	0:30	30
3	Annealing	60	0:30	30
4	Extension	72	01:00	30
5	Final extension	72	07:00	1
6	Cooling	4	∞	-

Protease gene amplification

The DNA of *Pseudomonas* Y7 strain was extracted using Geneaid DNA extraction kit as described by manufactures. The protease gene was amplified by PCR technique using specific primers, with forward (5′-ATGAAACTGAGCCTGGGACCG -3′) and reverse (5′-TCAGCATAGCCCCGCCTCCT -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- trans illuminator.

Table 3 : Reaction mixture of PCR

Component	Volume(microliter)
DNA template	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	65	45	35
4	Extension	72	45	35
5	Final extension	72	10	1
6	Cooling	4	∞	-

Insertion of protease gene into pTG19-T vector

The protease 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
Protease 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.

Protease 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. Protease 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 Discussion

Isolating and identifying of protease production isolate

From 10 isolates obtained in this study, it was found that the isolate Y7 had highest activity 82.1 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 1395 bp and the isolate belongs to *Pseudomonas aeruginosa* with 100% similarity (Table 6).

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

No.	Strain	Identity	Accession
1	*Pseudomonas aeruginosa PBI*	100%	MF777034.1
2	*Pseudomonas aeruginosa CLN1*	100%	MN830401.1
3	*Pseudomonas aeruginosa AT5*	100%	MN636767.1
4	*Pseudomonas aeruginosa PA4*	100%	MN636761.1
5	*Pseudomonas aeruginosa CIFRI-ONKPA1*	100%	MN481050.1
6	*Pseudomonas aeruginosa PA06*	100%	MN326719.1
7	*Pseudomonas aeruginosa ST11*	100%	MK894869.1
8	*Pseudomonas aeruginosa 4-36*	100%	MK205174.1
9	*Pseudomonas aeruginosa YB01*	100%	MF872727.1
10	*Pseudomonas aeruginosa DQ-1*	100%	MH997455.1

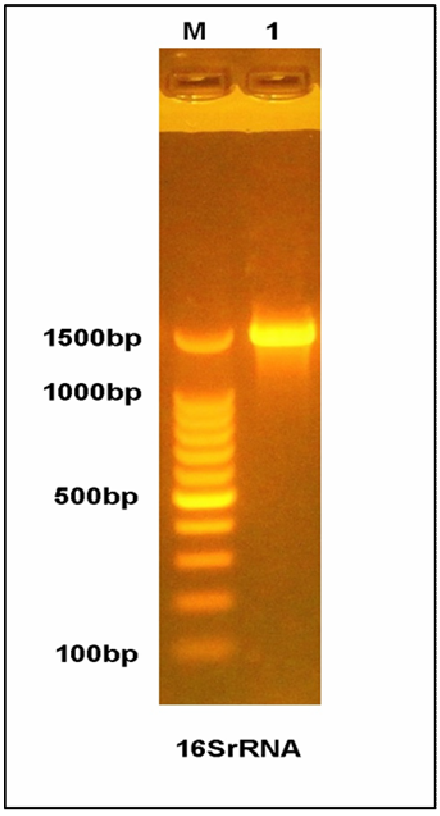

Fig. 1 : Amplified 16S rRNA gene electrophoresis results of Y7 isolate

Protease gene cloning and expression

The protease gene was amplified through PCR technique; the result showed a single band with a size ≈990bp. 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* DH5□, 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 protease gene. These result confirmed insertion of protease gene to *E. coli* DH5□ (fig.3).

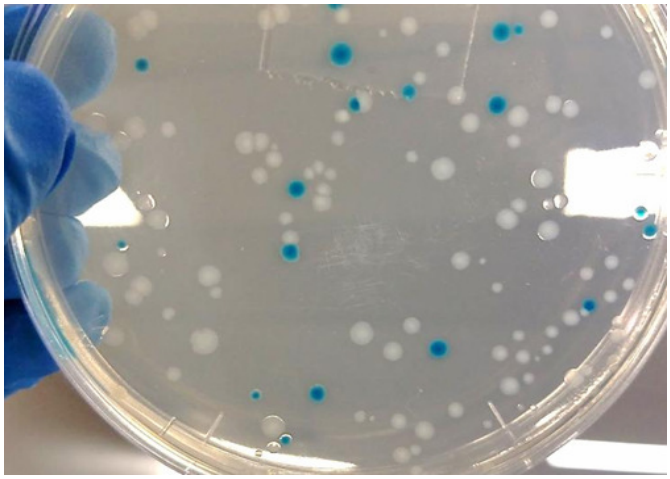

Fig. 2 : Blue-white screening of transformed *E.coli* DH5α cells

The prptease 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 protease

gene expression was inducted by IPTG. protease production by transformed *E. coli* BL21 (DE3) was assayed in LB medium included IPTG; the result showed that the protease activity reached 195.3 U/ml. Several studies reported the cloning of protease gene Omran *et al* (6) Cloned protease gene from *pseudomonas aeruginosa* into *E. coli* HB101 cells usng pACYC184 vector. Draper et al (13) Reported that they cloned elastase gene from *Pseudomonas aeruginosa* using pET28b vector and the restriction enzymes *Nco*I and *Bam*HI into *E. coli BL21* cells, and the specific activity of protease from transformed cells was 6.480 U/mg. Hsin et al (15) Transformed keratinase gene from *Pseudomonas aeruginosa* into *E.coli AD494(DE3)pLysS* using pET-43b(+) vector and, and the specific activity of protease from the *E.coli AD494(DE3)pLysS* transformed cells was 146U/mg.

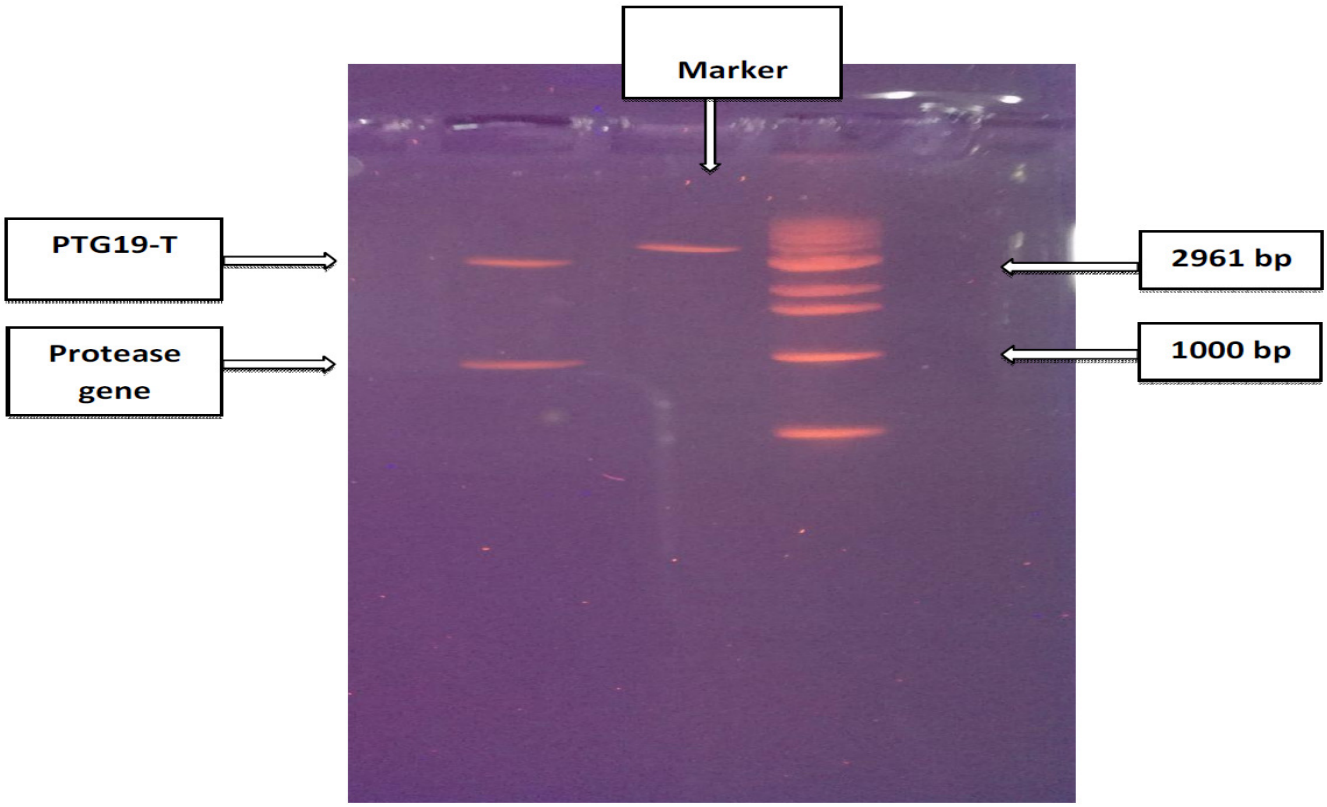

Fig. 2 : Electrophoresis product of pTG19-T vector and protease gene

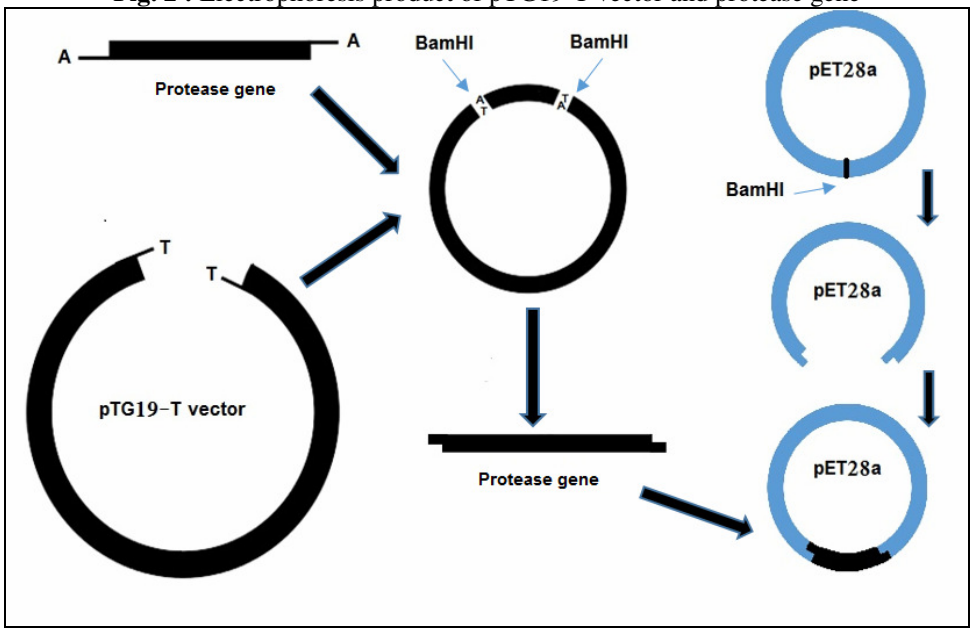

Fig. 3 : Schematic representation of the protease gene cloning

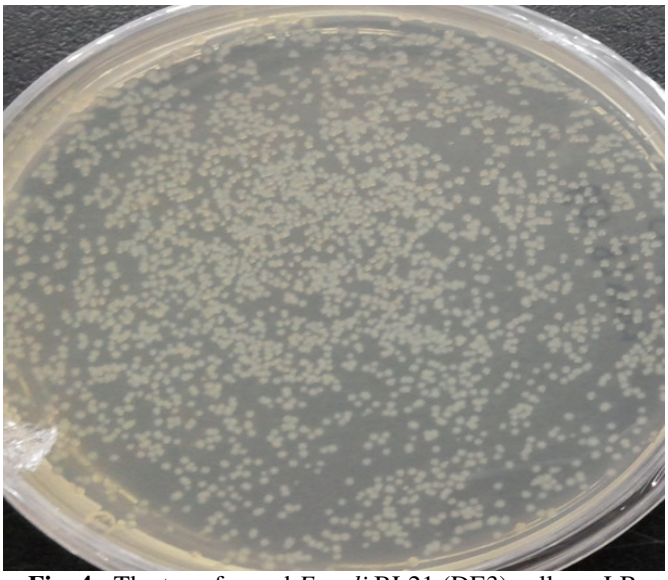

Fig. 4 : The transformed *E. coli* BL21 (DE3) cells on LB kanamycin agar

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