CLONING AND EXPRESSION OF THE *Pseudomonas* sp KE38 EXTRA-CELLULAR PROTEASE GENE in *E. coli*

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ABSTRACT

CLONING AND EXPRESSION OF THE *Pseudomonas* sp KE38 EXTRA-CELULLAR PROTEASE GENE in *E.coli*

Proteases are enzymes that hydrolyze proteins into smaller pieces by breaking the peptide bonds. Protease enzyme is produced by all living things on Earth. *Pseudomonas* sp. KE-38 is a cold adapted bacterium isolated from soil at high altitude in Erciyes mountain, Kayseri. The purpose of this thesis was to clone the cold-active extracellular protease gene from *Pseudomonas* sp. KE-38, partial purification and characterization of the extracellular protease enzyme.

The partial sequence of protease gene from *Pseudomonas* sp. KE-38 was analysed. The estimated size encoded by this gene after sequence analysis was 105 kDa. However, the size of this enzyme that was purified in this thesis was found to be approximately 50 kDa as evaluated of gelatine zymography analysis. Further investigation of the proteins in the partially purified enzyme sample by Liquid Chromatography Mass Spectrophotometry, revealed the presence of a metalloprotease enzyme with a predicted mass of 50 kDa. These results showed that the purified and characterised protease enzyme was not the same enzyme of which its gene was amplified gene was amplified and sequenced. Nevertheless, the partial characterization of the extracellular metalloprotease was performed, and the optimum temperature and pH was found to be 30°C and 8.0 respectively. The enzyme showed high activity in the presence of calcium, ethanol. The enzyme showed extremely high stability up to 25°C above this temperature; the stability dropped sharply which confirmed that the protease was a cold active enzyme and can have a potential to be used in cold temperature applications.

ÖZET

Pseudomonas sp KE38 HÜCRE-DIŞI PROTEAZ GENİNİN KLONLANMASI ve *E.coli'*de İFADELENMESİ

Proteazlar proteinleri daha küçük parçalara peptid bağlarını hidroliz ederek yıkan enzimlerdir. Proteazlar hücre içine ve dışına salgılanabilirler, bu deneyde soğuk aktif hücre-dışı proteaz enzimi ile çalışılmıştır. Proteaz enzimi bitki, mantar, hayvan, virus ve bakteri gibi bir çok canlılar tarafından üretilmektedir. Mikrobiyal proteazların endüstriyel kulanımları, diğer organizmalardan üretilen proteazların kullanımından daha yaygındır. Çünkü mikrobiyal proteazların endüstriyel kullanımı, bu enzimlerin gerek kolay üretilmeleri, gerekse kolay izole edilmeleri gibi bir çok sebepten ötürü bitkisel ve hayvansal proteazlardan daha fazladır.

Pseudomonas sp. KE-38 bakterisinden izole edilen hücre-dışı proteaz enziminin kısmi sekansı elde edilmiştir. Sekans analizinden sonra bu gen tarafından kodlanan tahmini boyut 105 kDa olarak belirlenmiştir. Ancak bu tezde saflaştırılan bu enzimin boyutu, SDS-PAGE ve jelatin zimografi analizi sonucunda yaklaşık 50 kDa olarak bulunmuştur. Kısmen saflaştırılmış enzim numunesindeki proteinlerin Sıvı Kromatografi Kütle Spektrofotometrisi ile daha detaylı araştırılmıştır. SDS ve Zymography analizine uygun olarak tahmini 50 kDa kütleye sahip bir metaloproteaz enziminin varlığını ortaya çıkarmıştır. Bu sonuçlar, saflaştırılan ve karakterize edilen proteaz enziminin, geni amplifiye edilen ve sekanslanan enzimle aynı olmadığını göstermiştir. Yine de hücre dışı metaloproteazın kısmi karakterizasyonu yapılmıştır ve optimum sıcaklık ve pH'ın sırasıyla 30°C ve 8 olduğu bulunmuştur. Enzim ayrıca kalsiyum ve etanol ve metanol gibi alkollerin varlığında da yüksek aktivite göstermiştir. Enzim, 25°C sıcaklığa kadar son derece yüksek stabilite göstermiştir; bu sıcaklığın üzerinde ise kararlılık keskin bir şekilde düşmüştür ve bu da proteazın soğukta aktif bir enzim olduğunu ve soğuk sıcaklık uygulamalarında kullanılma potansiyeline sahip olabileceğini doğrulamıştır.

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ABBREVIATIONS

LB Luria-Bertani

SM Skimmed Milk

rpm Revolutions per minute

PCR Polymerase chain reaction

OD Optic density

DNA Deoxyribonucleic acid

ng Nanogram

µg Microgram

Taq Thermus aquaticus (DNA polymerase)

µl Microliter

ml Milliliter

µM Micromolar

mM Millimolar

BLASTn Basic local alignment search tool for nucleic acids

min Minute

sec Second

h Hour

w/v Weight per volume

µm Micrometer

nm Nanometer

sp. species (singular)

bp Base pair

kb Kilobase pairs

kDa Kilodalton

CHAPTER 1

INTRODUCTION

1.1. Industrial Enzymes

Cosmetic, detergent, food and pharmaceutical industries make use of chemicals and enzymes both in their manufacturing processes, as catalysts and biocatalysts, and in their product function. As industrialization expands, and the variety of chemical processes required by society has rapidly increased, the negative impacts resulting from overuse and contamination is very noticeable (Zhu et al. 2022); from carcinogenic effects (Calderon 2000) to global warming (Jegannathan and Nielsen 2013), the impacts resulting from the overuse of chemicals are of concern. Jegannathan and Nielsen completed a scoping review of 28 comparative environmental assessments, which presented a clear consensus that the implementation of enzymatic processes in place of chemical processes generally reduces contributions to global warming, acidification, eutrophication, photochemical ozone formation, and energy consumption (Jegannathan and Nielsen 2013). The overuse of chlorinated aromatics, sodium salts, sulphides, chlorides across industrial processes is known to result in contamination to the environment (Durga et al. 2016), which has implications on both ecologies and human health (Zhu et al. 2022). For example, in the leather industry where wetting and pickling processes are applied, chemicals such as lime and sulphate have a toxic effect on human health (Durga et al. 2016). It is a timely focus to reduce society's reliance upon unnecessary chemicals, where enzymatic processes offer a safer and more efficient alternative (Sánchez and Olson 2005).

Microorganisms are indispensable sources for industrial enzymes as they have adapted to survive in almost any environment found on Earth, from the extreme ends of pH, a high or low atmospheric pressure, high or cold temperatures, and even dynamically changing conditions(Sánchez and Olson 2005)). Microorganisms can break down energy sources and produce special enzymes capable of converting them into a variety of vital products: amino acids, nucleotides, vitamins, carbohydrates, and fatty acids (Sánchez and Olson 2005). These enzymes tend to be more stable, cheap, specific, and effective than chemical counterparts that have been used abundantly in industry

processes (Furhan 2020). Microbial enzymes have high-impact potential when adopted as replacements, especially the hydrolase groups; lipases, proteases, carbohydrases and pectinases (Furhan 2020). Application of microbial enzymes often eliminates the implications of chemicals with harmful effects on the environment. Industrial enzymes can increase the specificity of the reaction, and purity of the product, increasing yields and economy, while avoiding chemicals that often end up as harmful contaminants to nature (Furhan 2020). Industrial enzymes have been successfully adopted across sectors including the pharmaceutical, biofuel, textile, food, paper, leather, cleaning, and other industries (Hassan et al. 2013). There is a big momentum behind searching for enzymes capable of extreme activities and improved stability across a variety of conditions, increasing their scope of application (Raddadi et al. 2015).

1.2. Cold Active Enzymes

The majority of the biosphere on Earth experiences cold temperatures, which are typically below 5 °C. Despite the low temperature, where biochemical functions are often limited -as is the case with mesophilic and thermophilic groups (Furhan, Awasthi, and Sharma 2019)- the low-temperature-metabolically-active microorganisms, Psychrophiles can secrete cold active enzymes and these enzymes have higher specific activity at low temperature and require very low activation energy, in other words they function with less free energy available than mesophilic and thermophilic enzymes (Feller 2013).

Psychrophilic enzymes present high specific activity, and so offer industry a higher degree of control over product (Hasan, Shah, and Hameed 2006), improving yields in a cost-effective manner. What's more, they have an easy inactivation, preventing unwanted protein degradation, and a minimal requirement for a particular reaction. Cold-active enzymes have many possible biotechnology applications (leather, cleaning, food, bioremediation etc.) (Al-Ghanayem and Joseph 2020).

1.3. Protease

Proteases are hydrolytic enzymes that break down proteins into free amino acids by hydrolysing peptide bones (Mamo and Assefa 2018). They have an important role in all life forms for physiological and metabolic processes. Proteases regulate the localizations and activities of many proteins, generate molecular signals, modulates protein-protein interactions, and influence DNA replication and transcription (López-Otín and Bond 2008). Generally, proteases operate in the context of complex networks containing substrates, cofactors, inhibitors, adapters, receptors, and binding proteins (López-Otín and Bond 2008). That is why they are significant for not only animals but also all living things on Earth. Therefore, proteases found in plants, animals, fungi, and prokaryotes (López-Otín and Bond 2008).

Since proteases present a wide variety of actions and structures, their classification is also diverse (López-Otín and Bond 2008) (Ahangari et al. 2021). This diversity in protease enzymes is the result of evolutionary changes in a large number of enzymes exhibiting a variety of sizes and shapes (López-Otín and Bond 2008). Proteases are categorised simply into two classes, based on their action sides: exopeptidases and endopeptidases. Exopeptidases target the terminal ends of proteins, specifically the NH_2 and COOH termini of their corresponding substrates. Exopeptidases are also classified into carboxypeptidases and aminopeptidases (Ahangari et al. 2021). On the other hand, endopeptidases target sites within inner-middle peptide bonds, not the termini peptide bonds of substrate. Endopeptidases, that are classified by their catalytic activity, are divided into 4 groups: aspartic, cysteine, metillo and serine protease (Mamo and Assefa 2018). Meanwhile, Endoproteases classified by the pH are divided as acidic, neutral, and basic proteases (Ahangari et al. 2021).

Proteases were discovered in the 1800’s (López-Otín and Bond 2008) (Figure 1.1) and and research on proteases has continued since then. This is because their diversity makes them an interminable field of research (Bond 2019).

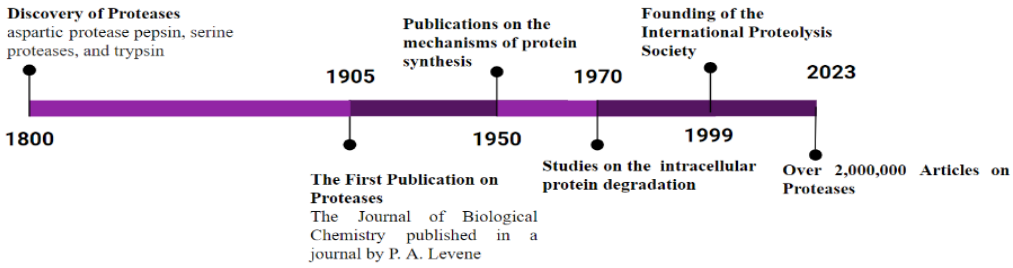


Figure 1.1. The timeline of the proteases discovery. (Bond 2019) (Levene 1905)

1.4. Bacterial Proteases

Producing different types of extracellular enzymes helps microorganisms to maintain their own living abilities like, defence, metabolism (Banerjee and Ray 2017). As mentioned, proteases are significant enzymes for all life forms (1.3. Protease). Hence bacteria can reproduce and do so faster than the other organisms, with nutrition of the organism easier to achieve than other more demanding organisms (Banerjee and Ray 2017). Therefore, the use of bacterial proteases precludes the use of proteases obtained from other living species (Banerjee and Ray 2017). Due to the ability of bacteria to adapt to a wide range of conditions of temperature, pH, and this enzyme can be supplied continuously, with the same quality and standard, regardless of the environment (Banerjee and Ray 2017) (Sharma et al. 2019). Thanks to microorganisms, the protease enzymes have become a competent and inexpensive source (Sharma et al. 2019)(Dumorné.et.al..2017).

Both intracellular and extracellular proteases can be produced by bacteria. Metabolic and cellular processes are more likely to be processed by intracellular proteases. (Razzaq et al. 2019). Protein turnover, maintenance of the cellular protein pool can be a good example for these processes (Razzaq et al. 2019). Extracellular proteases, which are secreted from the cell yet are crucial for hydrolysis in processes including cheese preparation, (Introduction 1.5.2.) or enzymatic synthesis on cleaning products (Razzaq et al. 2019). The ease of isolation makes supply of extracellular protease achievable and cost-effective for industry (Razzaq et al. 2019)(Solanki et al. 2021) (Kumar and Takagi 1999).

1.5. Industrial Applications of Microbial Proteases

Industrial applications are mainly dependent on stability and consistency of product quality between batches, to ensure repeatability. For an effective biocatalyst, it must show resistance across various pH, temperature and aeration factors that are important for industrial processes (Dumorné et al. 2017). Microbial enzymes are perfect fit for these requirements because they are able to produce enzymes to maintain their own metabolism and normal physiological condition (Banerjee and Ray 2017). Since various microorganisms can produce a wide variety of enzymes, the usage areas of enzymes have also diversified. For example, acid-tolerant enzymes, cold/heat tolerant enzymes, salt- tolerant enzymes are the enzymes that are widely used in industry (Cavicchioli et al. 2002).

1.5.1. Protease in Detergent Industry

A long shelf life, thermostability, effective stain removal ability, and compatibility with ingredients such as perfumes, other enzymes, non-ionic/anionic surfactants, and oxidising agents, are the optimum characteristics for a detergent (Al-Ghanayem and Joseph 2020) The following properties are sought from proteases used in detergents 1) Optimum activity at alkaline pH; 2) Activity at low wash temperatures; 3) Stability at high temperatures; 4) Stability with other components; and 5) Broad enough specificity to make it possible to clean a variety of stains (Abrar and Hamza 2017).

After extensive research, it emerged that the production of proteases with high activity at low temperatures is possible, after certain limitations were found in thermostable proteases. These low temperature proteases were introduced commercially in 1985 as third generation proteases and have been used ever since (Preiss et al. 2015), conserving energy and time (Furhan 2020).

1.5.2. Protease in Food Industry

Proteolytic enzymes are widely used in the dairy industry because of their ability to hydrolyse proteins and to coagulate milk proteins in the cheese making process

(Kieliszek.et.al..2021).

Bacterial strains with proteolytic activity play an important role in the degradation of proteins in meat and meat products by improving the taste and colour of the meat (Bekhit et al. 2014). Similarly, in baking, proteolytic enzymes enhance the quality and flexibility of bread, which also contains proteins (Kieliszek et al. 2021). Furthermore, proteolytic enzymes reduce allergenicity in bakery food substances for people affected by Celiac disease. Proteases can digest gluten allowing for gluten free foods (bread, pasta etc.), suitable for consumption by people with Celiac disease (M'hir et.al..2014).

Additionally, the brewing business makes use of proteases to improve texture. Beer's constituents cause it to become unclear when cooled, which is not what consumers wanted. Using proteases, the proteins are dissolved, lowering cloudiness (Kieliszek et al. 2021). Also, serine proteases are used to remove cells that form biofilms on food (Lequette et al. 2010). Microbial proteases have been significant in the creation of conventional fermented foods for centuries. (dos Santos Aguilar and Sato 2018).

1.5.3. Protease in Textile Industry

They can enhance the life of woollen and silk fabrics by retaining the quality of cloth fibres after washing at lower temperatures (Furhan 2020). The protease enzyme has largely replaced chemicals in textiles, for example it is used in various textile processing applications such as desizing, biopolishing, fading of denim and non-denim fabrics, bleach cleaning, biological cleaning (Alan et al. 2022). By hydrolyzing the polyamide surface with protease, fabrics can be processed without the use of chemicals, facilitating effective textile production. (Alan et al. 2022).

1.5.4. Protease in Bioremediation

The use of proteases is very important for improving biological remediation because proteases are also a good tool for cleaving bonds in organic compounds and catalysing their conversion to less toxic and more biodegradable forms (Karn and Kumar 2015). An example of bioremediation is activated sludge. Proteases increase the

rate of degradation of biodegradable materials such as activated sludge, resulting in more efficient treatment processes (Whiteley et al. 2002).

1.5.5. Protease in Polymer Degradation

Waste plastic can be broken down faster by bacteria-containing soil in the presence of water (Mohan Pathak 2017). The degradability of the polymer depends not only on the materials but also on the organisms; because the enzymes produced by microorganisms are specific to target different molecular structures (or sections of polymeric structures) (Mohan Pathak 2017). As they are broken into monomeric subsections, the microbes are digesting and feeding upon them, safely decomposing and removing them from the environment (Mohan Pathak 2017); the biodegradation of polyethylene succinate (a type of aliphatic synthetic polyester) by a microbial protease occurs as follows (Figure 1.2.) :

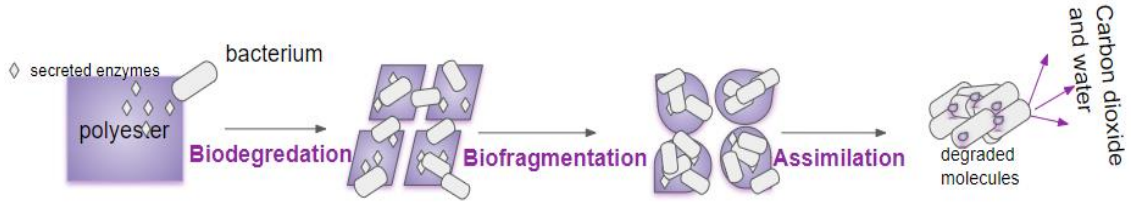


Figure 1.2. Biodegradation process of Polyethylene Succinate by microorganism. (Mohan Pathak 2017)

1.5.6. Protease in Pharmacology

Proteases have important roles in bacterial physiology, biochemistry and pathogenicity. Bacterial proteases are a new drug gateway for new antimicrobial agents (Culp and Wright 2017). One of the most important tasks of proteins is to help maintain homeostasis, proteases that break down proteins are therefore the most important regulators of physiological processes. Many pathological conditions in eukaryotic organisms are associated with the inability of the protease enzyme to perform its

function. Therefore, this enzyme is seen as attractive targets for drug discovery (Agbowuro et al. 2018). Therefore, by developing and applying new research understandings on the mechanisms of these enzymes, new drugs with different mechanisms of action specific to an organism can be sought and solutions to diseases can be found (Agbowuro et al. 2018). For example, proteases belonging to the subtilisin group were discovered to be effective in the treatment of burns and deep abscesses, producing an anti-inflammatory response and accelerating the healing process (Abrar and Hamza 2017).

1.6. *Pseudomonas* sp KE38

Pseudomonas sp. KE-38 is a bacteria, which was identified as a strain of *Pseudomonas fluorescens* by 16s rRNA gene analysis. It was isolated from soil samples taken from the high altitudes of the Erciyes Mountain in Kayseri (Karakaş and Arslanoğlu 2020). It is a gram negative bacteria, which can grow at temperatures between 5°C to 30°C. In previous studies in our laboratory, *Pseudomonas* sp KE-38 has been shown to produce an extracellular lipase enzyme, and the lipase gene was cloned, expressed and sequenced. (Karakaş and Arslanoğlu 2020). While sequencing the lipase gene, a partial sequence with a high homology to *Pseudomonas* proteases was discovered next to the lipase gene (Figure 1.3). These findings suggested that bacteria was also potentially producing extracellular protease enzyme. In this study we aimed to determine if *Pseudomonas* sp. KE-38 was producing an extracellular protease and if yes, then clone, isolate and characterize the enzyme.

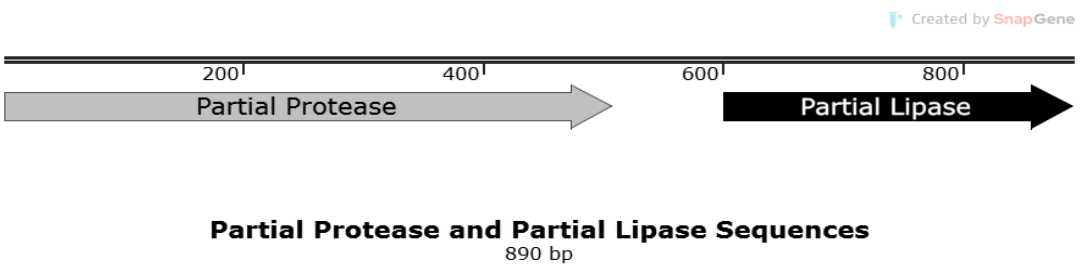


Figure 1. 3. Partial Lipase (black arrow) and Protease Partial (grey arrow) Sequences of *Pseudomonas* sp. KE-38 from previous studies (Adan Gökbulut Alper Arslanoğlu et al. 2013). Numbers represent nucleotides.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used buffers, solutions, chemicals, their compositions, and measurements are presented in Appendix A.

2.1.1. Commercial Kits

Table 2.1. Commercial Kits

Kits	**Supplier Company**
GeneJET Genomic DNA Purification Kit	Thermo Fisher
Gel/PCR DNA Fragments Extraction Kit	Geneaid
Mini Plasmid Isolation Kit	Geneaid

2.1.2. Plasmid

The PTZ57R/T cloning vector was used. (Thermo Scientific) shown in figure 2.1.

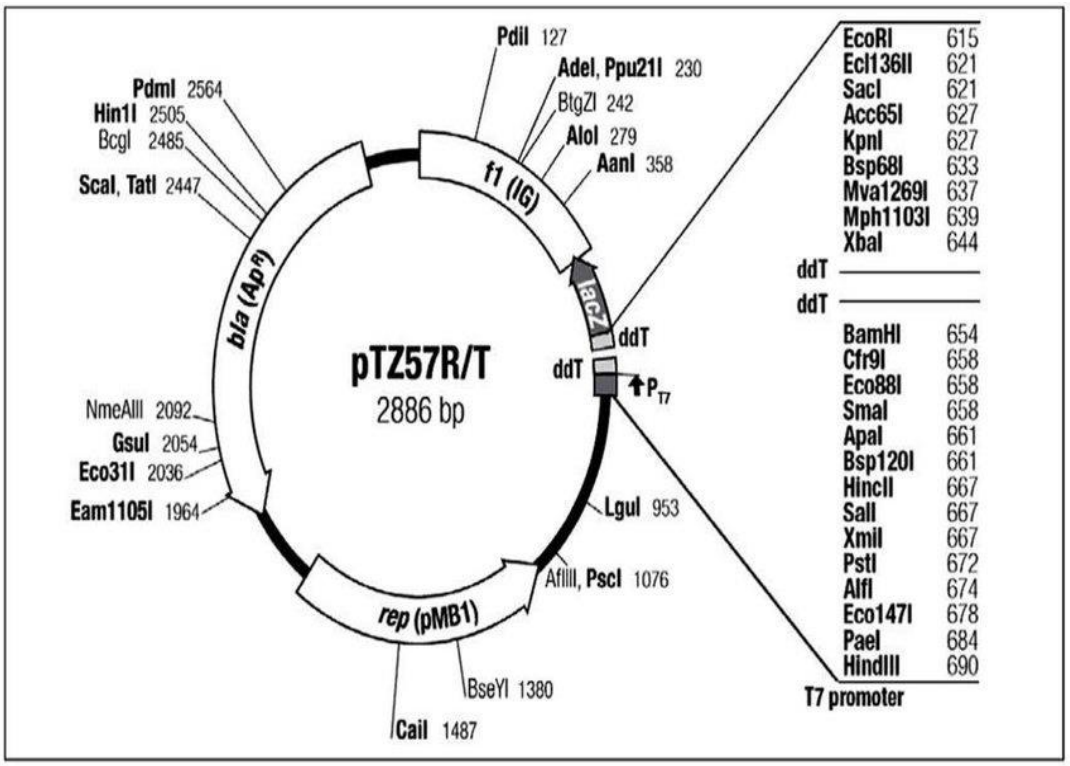


Figure 2.1. Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated. (Thermo Scientific)

2.1.3. Primers

Primers were designed and ordered from Sentebiolab. The sequences of primers used in this study are presented in table 2.2.

Table 2.2. Primer Sequences Used for PCR

Primer Name	Sequence
Es_Prot_Stop	5’-TCA GAA CCG CCA GTC CAG C-3’
Es_Prot_TolC	5’-CAA CTA TTT ACC ACC CGC-3’
KE38_Prot_F	5’-CAT ATG AGT AAC AAG CAC AAC ACA CGT G-3’

2.2. Methods

2.2.1. Cultivation of Bacteria

Routine cultivation of both *Escherichia coli (E. coli)* and *Pseudomonas sp.* KE-38 were performed in Luria-Bertani (LB) broth with constant shaking at 200 rpm, medium at 37 °C and room temperature 25 °C, respectively.

Pseudomonas sp. KE-38 was also cultivated in 10% skimmed milk in dH2O where needed.

15% agar was added to the growth media for solid cultures.

2.2.2. Competent *E. coli* Cell Preparation

The *E. coli* DH5α cells were used or competent cells. *E. coli* DH5α cells were inoculated into LB broth, and incubated at 37°C overnight for 16 hours with constant shaking. The next day, 1 mL of these overnight cultures was added into 99 mL of fresh LB broth (1/100 dilution). The incubation was set at 37°C, 200 rpm, and 4 hours. Optical density (OD) of the incubated culture medium was measured at 600 nm using a spectrophotometer (Biochrom S2100 Diode Array spectrophotometer) with regular intervals until it reached 0.4. The culture medium was then transferred to 50 ml falcon tubes and kept on ice for 20 minutes. The supernatant was then discarded and centrifuged at 4000 rpm for 10 minutes at 4°C, each pellet resuspended in 20 mL of ice-cold 0.1M CaCl2, incubated on ice for 30 minutes, and spun at 4000 rpm at 4°C. It was centrifuged for 10 minutes. The supernatant was discarded and the pellets were combined by resuspending in 5 mL of ice-cold 0.1M CaCl2 in 15% glycerol. 50 µl of cells were placed in each tube, labelled and stored at -80 °C.

2.2.3. Detection of Extracellular Protease Production by Agar Plate Assay

Bacteria were inoculated on the center of the plates prepared with 10% skimmed milk, 15% agar, and water. Protease-producing bacteria were detected a few days after incubation at 25 °C.

2.2.4. Agarose Gel Electrophoresis

One gram of agarose was measured and mixed with 100 mL 1x Tris-base, Acetate, EDTA (TAE) buffer. 0.2 ug/mL Ethidium bromide was added to the mixture and agarose gel was poured into the well comb in place.

2.2.5. Genomic DNA Extraction from *Pseudomonas sp.* KE-38:

A single colony of *Pseudomonas sp*. KE-38 was inoculated to 10 ml Luria-Bertani (LB) broth and incubated at room temperature (25 ℃) with overnight shaking, and 3ml was used to purify genomic DNA by using Genejet Genomic DNA Purification Kit according to the manufacturer's instructions. The integrity and concentration of the isolated genomic DNA was determined visually by comparing the DNA band width with the marker DNA (6x DNA Loading Dye, Thermo Scientific) after electrophoresis on 1% agarose gel.

2.2.6. Plasmid DNA Extraction from *E. coli* Dh5alpha:

One loop of *E. coli* Dh5alpha cells were inoculated into 10 ml LB with 100 mg/ml ampicillin and incubated overnight. Three ml of that culture was used to purify plasmids by Geneaid Mini Plasmid Isolation kit according to manufacturer's instructions. Isolated plasmids were visualised by electrophoresis on 1% agarose gel.

2.2.7. Amplification of the Extracellular Protease Gene from *Pseudomonas* sp. KE-38 Genome by PCR

PCR amplification was performed using Q5 Hot Start High-Fidelity Master Mix (New England Biolabs) with 1 ug of *Pseudomonas sp.* KE-38 genomic DNA, 2.5 uM of each primers, (Es_Prot_TolC 5'-CAA CTA TTT ACC ACC CGC-3'- and Es_Prot_Stop 5'-TCA GAA CCG CCA GTC CAG C-3'-), 25 ul of 2x enzyme Master Mix, and 0.5 ul of Q5 Hot DNA polymerase in a total volume of 50 ul. Thermal cycling conditions were as follows, initial denaturation at 98 ℃ for 1 minute, followed by 30 cycles of denaturation at 98℃ for 10 seconds, primer annealing at 55℃ for 30 seconds, and primer extension at 72℃ for 2 minutes. The amplified PCR products were visualised by agarose gel electrophoresis.

2.2.8. Cloning and Partial Sequencing of the *Pseudomonas sp.* KE38 Extracellular Protease Gene

2.2.8.1. Ligation

PCR amplified protease gene (165 ng/ul) was ligated with pTZ57R/T TA (Thermo Scientific) cloning vector (55ng/ul) which contains an ampicillin resistance gene in a reaction containing 10 ul of 10x Buffer T4 DNA Ligase with 10mM ATP Ligase Reaction Buffer (New England Biolabs), 1 ul of T4 DNA Ligase (New England Biolabs). The reaction mixture was incubated at room temperature for 1 hour.

2.2.8.2. Transformation

E. coli DH5α cells from -80°C were thawed on ice for 5 minutes. 2 ul of ligation reaction mixture was added onto the thawed cells, and incubated on ice for 25 minutes. Cells were heat shocked, for 30 seconds in a water bath at 42 °C, followed by and incubation on ice for 2 minutes. After the heat shock 250 ul of fresh LB broth was added onto cells and incubated at 37°C for 1 hour with shaking at 200 rpm. In order to select the transformants, cells were spread on LB agar plates supplemented with 100 mg/ml ampicillin. Cells containing inserts, which are resistant to ampicillin, formed colonies on the agar plate and these colonies were selected.

2.2.8.3. Identification of Recombinant *E.coli* Transformants by PCR

The recombinant plasmids were purified with the Mini Plasmid Isolation Kit and PCR were performed with Q5 Hot Start High-Fidelity Master Mix for 10 ul, forward primer Es_Prot_TolC for 0.5 µl, Es_Prot_Stop reverse primer for 0.5 µl, nuclease-free water for 9,2 to make the mixture up to 20 µl. Thermal cycling conditions were as follows, initial denaturation at 98 °C for 1 minute, followed by 30 cycles of denaturation at 98°C for 10 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 72°C for 2 minutes. The amplified PCR products were visualised by agarose gel electrophoresis.

2.2.8.4. Sequencing of Pseudomonas *sp.* KE-38 Extracellular Protease Gene

The purified recombinant plasmids were sequences with primers, (Es_Prot_TolC 5'-CAA CTA TTT ACC ACC CGC-3'- and Es_Prot_Stop 5'-TCA GAA CCG CCA GTC CAG C-3'-), were performed at the Biotechnology and Bioengineering Central Research Laboratories (CFB), Izmir Institute of Technology. Homology analysis using BLAST was performed on the obtained partial sequence.

2.2.9. Preparation of Extracellular Enzyme

The preparation of extracellular enzymes performed in LB media. Firstly, KE-38 bacteria inoculated to LB agar and incubated at room temperature (RT) overnight. Secondly, 1 loop of bacteria inoculated 100 ml LB media, at RT overnight. Finally, 1% sample inoculated into fresh 200 mL LB media, at 25^{o} C for 48h. Then a centrifuge performed a several times, at 4000 G for 20 minutes at 4^{o} C. The supernatant was collected and filtered through a 0.2-µm cellulose acetate membrane to obtain cell-free-supernatant (CFS), which was used as extracellular enzyme for the determination of extracellular proteolytic activity. After this samples were concentrated in centrifuge at 4000 g for 10 min at 4^{o} C by using 3K filtered falcons and the samples were washed several times with 100mM TrisHCl-2mM CaCl2.

2.2.10. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular mass of protease enzyme was determined by SDS-PAGE on 12 % resolving gel (30% acrylamide/bis 6.0 ml, 0.5M Tris-HCl, ph 8.8 3.75 ml, 10% SDS 150 µl, dH2O 5.03 ml, TEMED 10 µl and 10% APS 75 µl) used together with 7.5 % stacking gel ((30% acrylamide/bis 3.75 ml, 0.5M Tris-HCl, ph 6.8 3.75 ml, 10% SDS 150 µl, dH2O 7.8 ml, TEMED 10 µl and 10% APS 75 µl)) and stained with Coomassie Brilliant Blue, as described by Laemmli (1970). 10 µl of samples were loaded into gel with 2µl 5x SDS loading dye (β-Mercaptoethanol 5%, Bromophenol blue 0.02%,

Glycerol 30%). After loading the 12 µl of samples electrophoresis was performed at 60 volts for 20 minutes and then 120 volts for 2 hours. Gel was stained overnight with Coomassie Brilliant Blue dye (1 g of Coomassie R250 to 300 ml of methanol, 650 ml of MQ water and 50 ml of acetic acid.). IV Protein Ladder (Thermo Scientific) was used as a molecular mass marker.

2.2.11. Determination of the Activity And Molecular Weight of Protease by Zymogram Analysis on PAGE

12% polyacrylamide gel was prepared with 3% gelatine as the substrate for the protease enzyme. 10 µl of each sample was mixed with 2 µl of 5x non-reduce loading dye (1.25ml 1M Tris, pH 6.8, 2.0ml 20% SDS, 3.75ml ddH2O, 2.0ml Glycerol) and loaded on gelatine-acrylamide gel, and zymography was performed at 150 volts for 2 hours. The gel was initially washed in water and then in washing solution 25 ml 1M Tris, pH 7.6, 5ml 1M CaCl2, 12.5ml Triton X, ddH2O to 1L). Both washings were performed at room temperature with shaking for 30 min. The gel was then rinsed for 5–10 min in incubation buffer (25 ml 1M Tris, pH 7.6, 5ml 1M CaCl2, 75ml 5M NaCl, ddH2O to 1L) at 25°C with agitation. Incubation buffer was refreshed and incubated for the protease reaction at 25°C for 24 hours. The gel was stained with the staining solution (40% MeOH, 10% Acetic Acid, 0.1% Coomassie Brilliant Blue) for 1 hour and rinsed with H2O for 2 times and destained with destaining solution (40% MeOH, 10% Acetic Acid) 30 minutes. IV Protein Ladder (Thermo Scientific) was used as a molecular mass marker. The enzyme activity was determined by observing a clear zone on the gelatine containing dark blue gel.

2.2.12. Liquid Chromatography Mass Spectrometry

The purified extra-cellular protease enzyme was analysed by Liquid Chromatography Mass Spectrometry. Analysis performed at National Mass Spectrometry Application and Research Center (NMSC) at Izmir Institute of Technology.

2.2.13. Enzyme Assay

2.2.13.1. Quantification of Extracellular Proteolytic Activity

The activity of protease enzyme measured by Azocasein assay according to the method described by Iversen-Jorgensen with modifications. The reaction mixture and control sample contained 530 µl of Azocasein (4 mg/ml Azocasein in 50mM Tris HCl pH:8.0 and 0.5mM CaCl2) 130 µl of reaction buffer (50mM Tris HCl and 0.5mM CaCl2 pH :0.8), 13 µl protease enzyme, and 530 µl Azocasein, 117 µl reaction buffer respectively. The enzyme activity assayed at 25°C for 30 minutes. The reactions were stopped by adding 250 µl TCA (10%.) and incubated for 30 min at +4°C and samples were immediately centrifuged for 20 min at 10,000 x G. The absorbance of the supernatant was determined at 340 nm.

2.2.13.2. Determination of Temperature and pH Optimal of the Protease

The extracellular protease activity was quantified under 6 different temperatures (10°C, 20°C, 25°C, 30°C, 40°C) for 1 hour at 10 min intervals and the resulting enzyme activity was measured spectrophotometrically at 340 nm.

The effect of pH was also determined by using different pH range (sodium acetate buffer (pH 5), potassium phosphate buffer (pH 5-7), tris-HCL buffer (pH 7-9) and glycine-NaOH buffer (pH 9-12)) to observe active pH range of the extracellular proteases produced by the KE 38. All buffers were at 50 mM concentration. The assay mixture (117 µl each buffer, 530 µl 4mg/ml azocasein and 13 µl enzyme solution) was incubated at 25°C for 1 hours in different pH values of above buffers and the enzyme activity was determined by spectrophotometric protease assay.

2.2.13.3. Determination of Temperature and pH Stability of the Protease Enzyme

The thermostability was carried out by pre- incubating the enzyme solution at a temperature range of 0°C to 50°C for 1 hr. The residual activity was measured with standard enzyme reaction. The control is enzyme reacted at zero time and consider as 100%.

The pH stability was determined by pre- incubating the enzyme solution in buffers with different pH values (5–12, described above) at room temperature for 1 hour. The residual activity was measured with standard enzyme reaction. The control is enzyme reacted at zero time and considered as 100%.

2.2.13.4. Effect of Various Metal Ions and Organic Solvents on Protease Activity

The effect of metal ions on protease activity was determined by using 50 mM metal ions (CaCl2, CuCl2.2H2O, MgCl2, ZnO4.7H2O, MnCl2 and EDTA) The assay mixture (530 µl 4mg/ml azocasein, 66 µl each metal ion buffer, 51 µl TrisHCl and 13 µl protease enzyme) was incubated at 25°C for 1h the enzyme activity was determined by spectrophotometric protease assay.

The purified enzyme incubated at different solvents (30% Methanol, 30% Ethanol, 30% Triton-X 100, 30% Tween-20 and 30% Acetone). The reaction mixture (265 µl 8 mg/ml azocasein, 198 µl TrisHCl, 184 µl each solvent and 13 µl enzyme) was incubated in room temperature for 1 hour and the enzyme activity was determined by spectrophotometric protease assay.

CHAPTER 3

RESULTS

3.1. Confirmation of Protease Activity by Plate Assay

Pseudomonas fluorescens KE-38 was isolated from the soils of Erciyes Mountain in Kayseri at high altitudes. In the past years, this bacterium was studied in our laboratory, and it was shown to produce extracellular cold active lipase enzyme (Adan Gökbulut Alper Arslanoğlu et al. 2013).

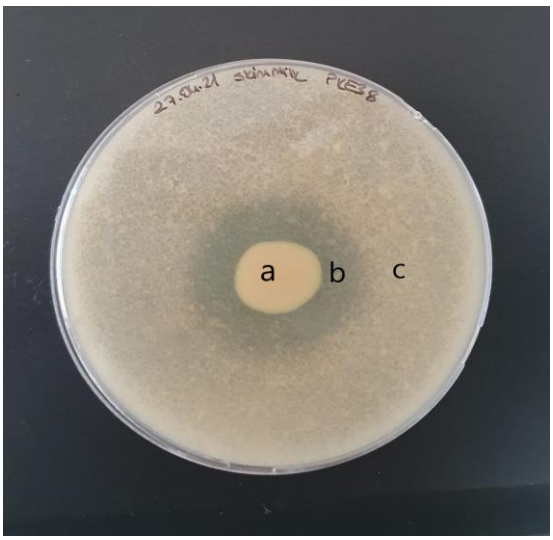


Figure 3.1. Protease Plate Assay showing proteolytic activity of protease enzyme (Budak 2016) (a. *Pseudomonas fluorescens* KE-38, b. transparent zone caused by hydrolysed peptide bonds, c. White Zone Skimmed milk media with proteins)

Pseudomonas fluorescens KE-38 (*P fl* KE-38) was first examined in the skimmed milk agar plate test by dropping 5 µl of liquid culture sample onto the plate (Figure 3.1.). Then, the enzyme was isolated from liquid bacterial culture and protease enzyme activity was compared with acetic acid on the skimmed milk agar plate and gelatine agar plate. Plates were divided into two and 5 µl of acetic acid was dropped on

the left half and 5 µl enzyme sample was dropped on the right half and these plates were incubated at room temperature for 48 hours. While clear zone formation was observed in both halves of SM, in the gelatine agar plate only zone formation was observed on the half where the enzyme was dripped. (Figure 3.2.A-B)

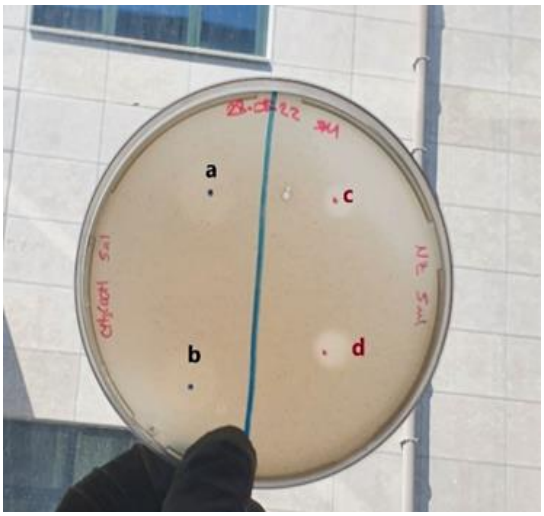


Figure 3.2. A. Comparison of acid and enzyme activity in SM plate. **a-b.** 5 µl acidic acid. **c-d.** 5 µl *P fl* KE-38 Protease enzyme, clear zone format on after 48h 25°C incubation.

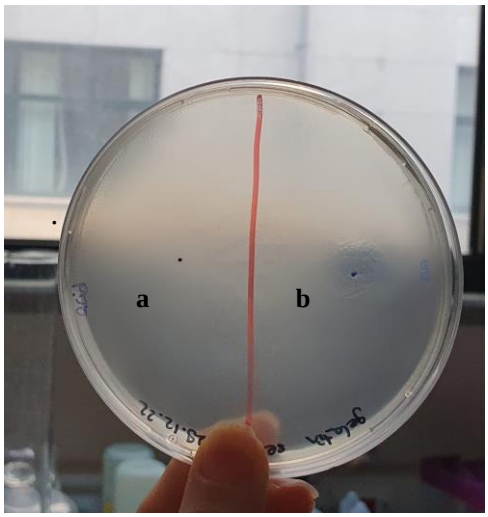


Figure 3.2.B. Comparison of acid and enzyme activity in Gelatine plate **a.** A 5 µl of acetic acid, no zone formation after 48h 25°C incubation. **b**. 5 µl protease enzyme, clear zone formation after 48h 25°C incubation.

3.2. Amplification and Partial Sequencing of the Protease Gene

The DNA sequence of the end part of the protease enzyme was obtained from a previous study conducted in our laboratory (Adan Gökbulut Alper Arslanoğlu et al. 2013). (Figure 3.3. A.) Using this sequence, the sequence of the protease gene, and its previous parts (Tol C) in the chromosome region of the closest organism to which it was matched, was determined in the National Center for Biotechnology Information (NCBI) blast. (Figure 3.3. B.) (Appendix B) Other *Pseudomonas* genomes showing the highest homology to these chromosomal regions were determined by the blast, and the 7 *Pseudomonas sp.* phylogenetically closely related ones were selected. The aforementioned sequences of the selected genomes were aligned by the Clustal Omega

software (www.clustal.org) and the region with high homology was determined and a primer was designed to bind to this region. (Es_Prot_TolC, and Es_Prot_Stop) (Table 2.2) (Figure 3.4.A and Figure 3.4.B.)

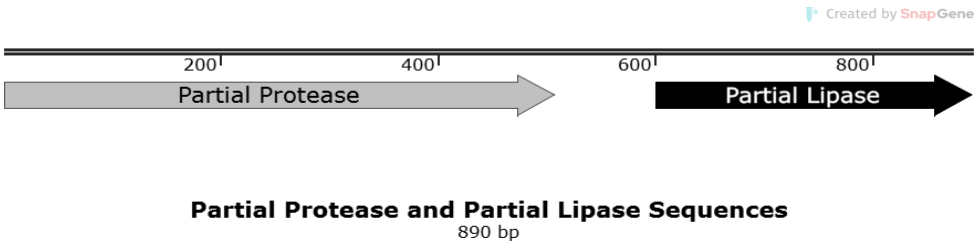


Figure 3.3.A. Partial Lipase (black arrow) and Partial Protease Sequences (grey arrow) of *P fl* KE-38 from previous studies (Adan Gökbulut Alper Arslanoğlu et al. 2013). Numbers represent nucleotides. This image was created by SnapGene.

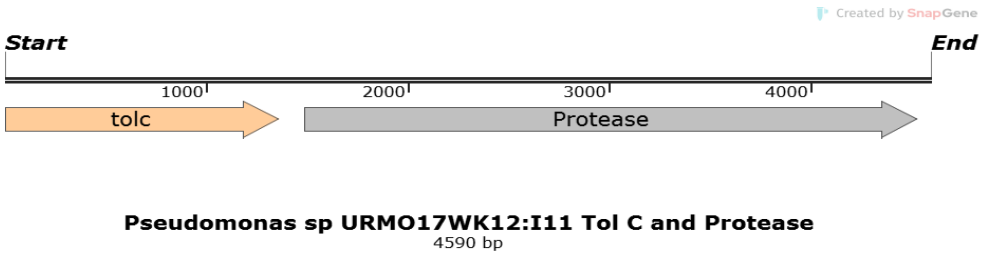


Figure 3.3. B. Tol C (orange arrow) and Protease (grey arrow) demonstration on the chromosomes of *Pseudomonas sp.* URMO (the closest relative to *P fl* KE-38). Numbers represent nucleotides. Start point is where the Tol C sequence begins, and End point is where the Protease gene ends. This image was created by SnapGene.

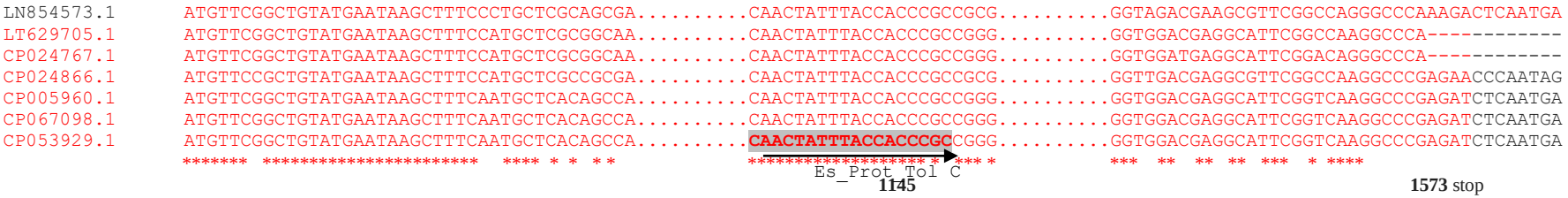


Figure 3.4.A. Multiple alignments of 7 different *Pseudomonas fluorescens* sp. Tol C sequences. Only 5',3', and middle sections of the Tol C sequences are shown. Binding side of the Tol C pirmer are shown by the arrows. LN854573.1 (*Pseudomonas* sp. URMO17WK12:I11), LT629705.1 (*Pseudomonas arsenicoxydans)*, CP024767.1 (*Pseudomonas arsenicoxydans* sp), CP024866.1 (*Pseudomonas* sp. ACM7), CP005960.1(*Pseudomonas mandelii* JR-1), CP067098.1(*Pseudomonas* sp. SW-3), CP053929.1(*Pseudomonas* sp. B14-6). the grey highlight is the Es_Prot_Tol C (Tol C) primer sequence.

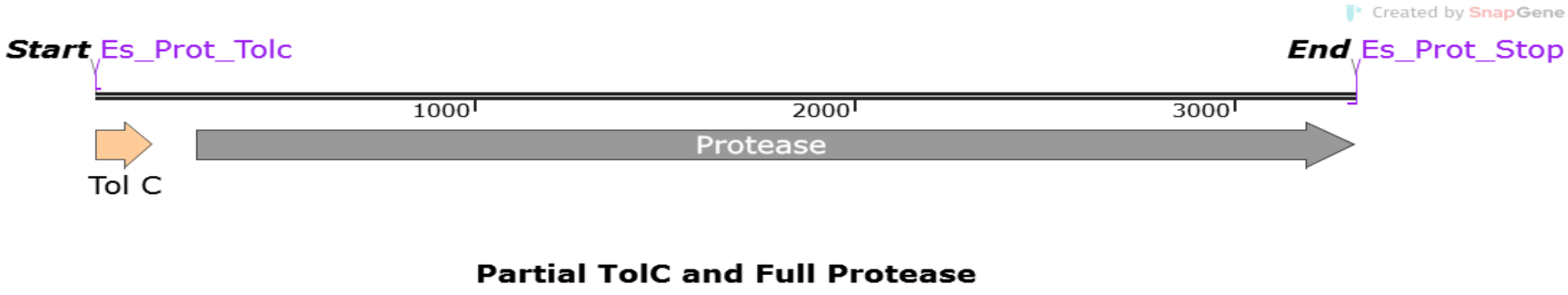


Figure 3.4. B: Display of the region to be amplified by PCR, partial Tol C (orange arrow) and full protease gene (grey arrow) together with the Es_Prot_Tol C and Es_Prot_Stop primer binding sites. Start is where Tol C primer binds and End is where protease gene ends. This image was created by SnapGene.

A fragment including the extracellular protease open reading frame of about 3.3 kbp in length was amplified by PCR using Q5 Hot Polymerase due to its high proofreading activity using primers Es_Prot_Tol C and Es_Prot_Stop. Gradient (55°C/70°C) PCR was performed due to the degeneracy of the primer pair. The primer pair amplified the target sequence at all temperatures between 55°C and 61.5°C. (Figure 3.5.) 3.3 kbp PCR fragment amplified at 55°C was cut from agarose gel and purified for cloning purposes.

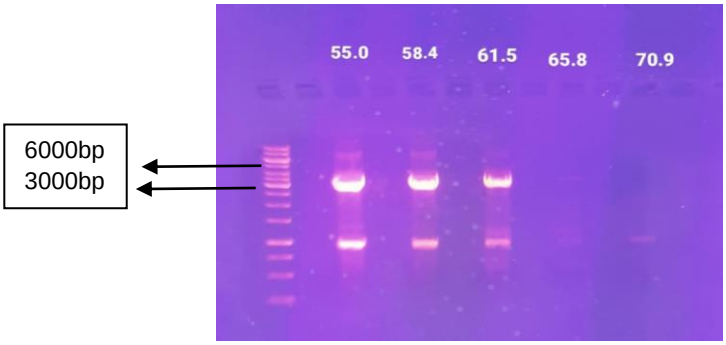


Figure 3.5. The Protease gene fragment amplified by temperature gradient PCR using Tol C and Es_Prot_Stop primers, from 55°C to 70°C,. Line 1: 1 kb DNA size marker (Thermo Scientific) (250, 500, 750, 1000, 1500 bp….).

After obtaining the purified protease fragment (3.3 kbp), it was cloned into pTZR57r/t TA cloning vector. Plasmid DNA isolated from 8 transformant colonies were analysed by PCR and 6 colonies were found to contain the protease gene. (Figure 3.6) The isolated plasmids were also confirmed to contain the insert by restriction digestion with Xbal and BamHI enzymes. (3.7.A.)

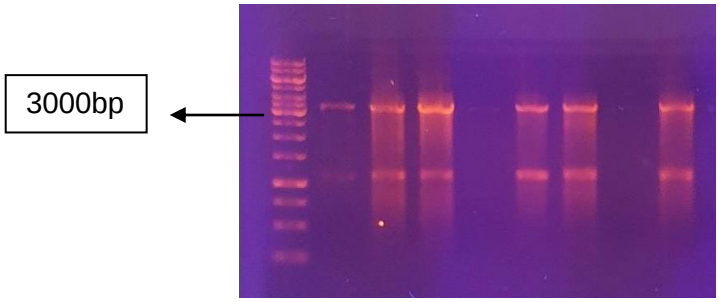


Figure 3.6. Amplified protease fragment in pTZR57r/t TA cloning vector. Line 1: 1 kb DNA size marker (Thermo Scientific) Line 2,3,4,6,7,9: ~3450 bp insert (Protease gene) 2886 bp plasmid

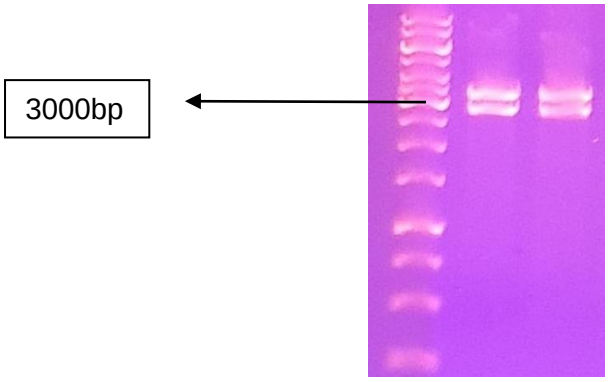


Figure 3.7.A. Restriction enzyme Xbal/BamHI. Line 1: 1 kb DNA size marker (Thermo Scientific) Line 2: ~3450 bp insert (Protease gene) 2886 bp plasmid

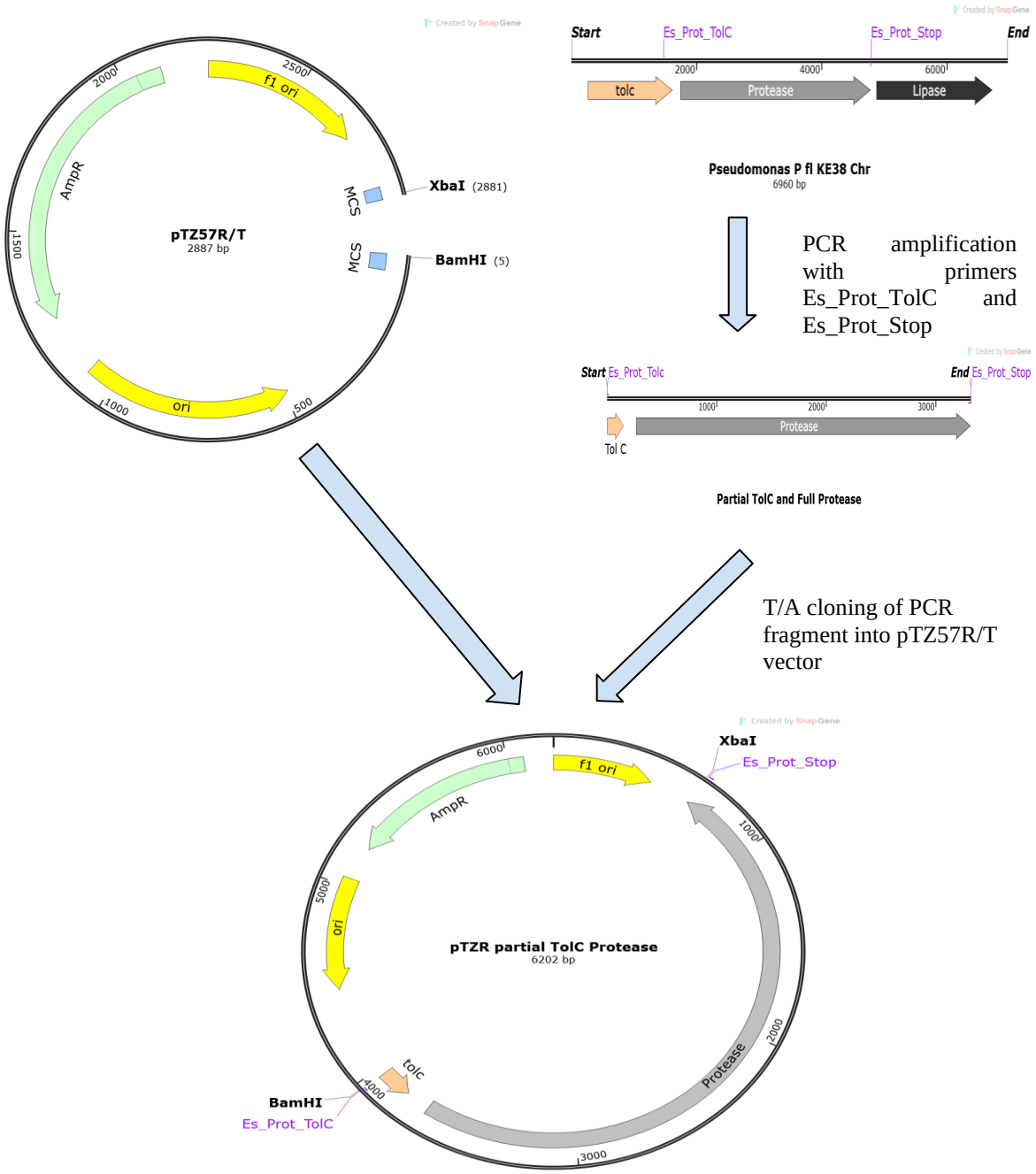


Figure 3.7. B. T/A cloning demonstration, pTZR plasmid, KE-38 chromosome, PCR amplification, T/A cloning of PCR fragment into vector, restriction enzymes were shown bold. Amplification of the protease gene was successfully achieved by using Es_Prot_TolC and Es_Prot_Stop primers. The amplified 3.3 kbp fragment was then cloned into pTZ57RT/A cloning vector and sequenced by using the same primers. These images were created by SnapGene.

In order to obtain the sequence of the extracellular protease gene of *Pseudomonas* sp. KE-38, plasmid pTZR57 with insert was carefully sequenced in BIYOMER with primers Es_Prot_Tol C and Es_Prot_Stop. (Appendix B) The result of the sequence was then used for designing a new primer, KE-38_Prot_F 5'-CAT ATG AGT AAC AAG CAC AAC ACA CGT G-3' -29 bp, for the amplification PCR performed by using KE-38_Prot_F and Es_Prot_Stop primers under the same conditions as described above. (Method 2.2.7.) After 1% agarose gel electrophoresis specific band around 3400 bp was observed. (Figure 3.8.)

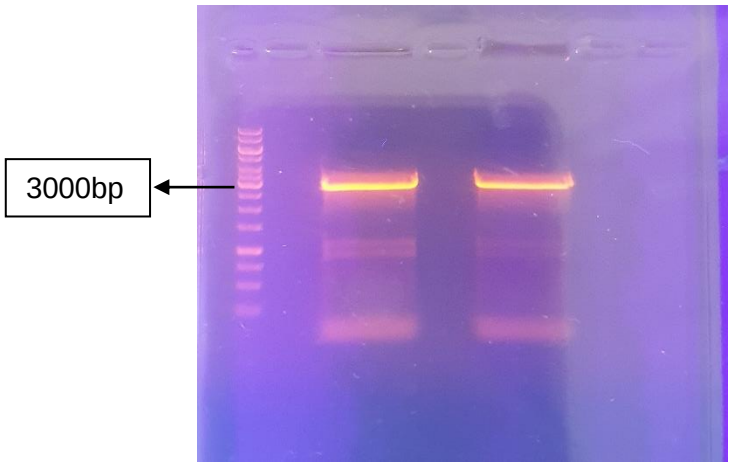


Figure 3.8. Amplified Protease gene using primers KE_Prot and Es_Prot_Stop Line 1: 1 kb DNA size marker (Thermo Scientific) Line 3,5: Amplified protease gene band around 3400 bp.

3.3. Qualitative Determination of Extracellular Proteolytic Activity

The proteolytic activity of *P fl* KE-38 was observed at different temperatures and pH conditions and the bacteria is capable of producing extracellular protease enzyme that create a clear zone around the protein-containing media (Skimmed Milk).

First to decide at the optimal time for the collection supernatant, bacteria were incubated in a skimmed milk media at room temperature for 72 hours. (Method 2.2.9)

Supernatants were collected every 24h and the maximum proteolytic activity was observed at 48h. Experiments were conducted for the 48h enzyme samples.

3.4. SDS Page and Substrate Specificity

SDS page is a method which shows molecular weights of the protein and separates them by their weight (Nowakowski, Wobig, and Petering 2014). The concentration of the gel was described above. (Method 2.2.10.) The activity of the enzyme was observed in the SDS page as the enzyme used the substrate and substrates molecular weights decreased. 450 µl/ml BSA, 1% alpha casein and gelatine 3 mg/ml were used as a substrate, enzyme assay was performed after one hour of incubation. The protease showed the highest activity against alpha casein and the lowest against BSA. (Figure 3.9.A.) Gelatine caused smear formation in the SDS gel. However, a decrease in smear was observed in the sample incubated with the enzyme for 1 hour. (Figure 3.9. B.)

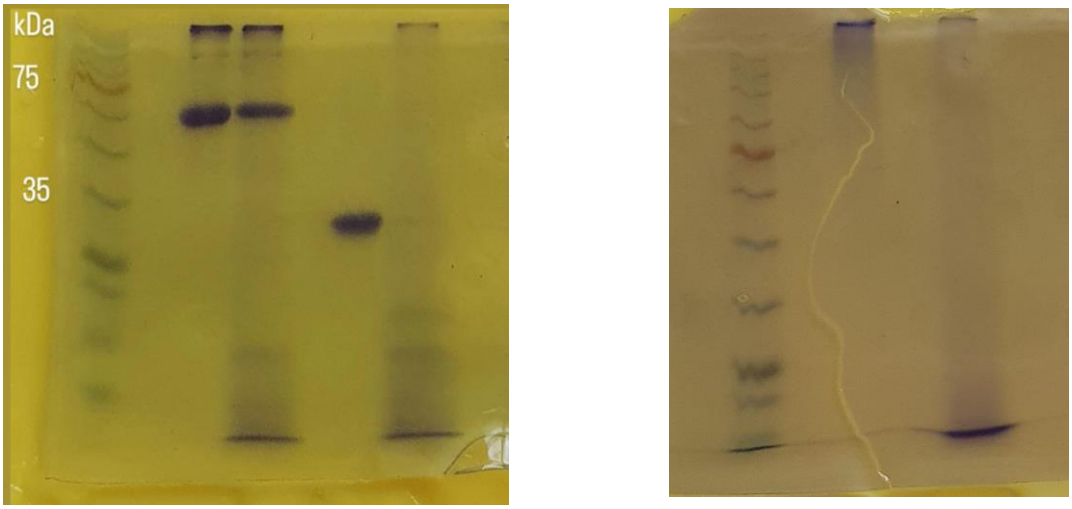


Figure 3.9. Substrate specificity SDS-PAGE results A and B respectively.
Line 1 1Kb Protein marker, red represents 75 kDa. A. Line 3, BSA 450mg, Line 4, 1h incubated BSA (450 mg/ml) and protease enzyme Line 6, Alpha casein 1% Line 7, 1h incubated sample Alpha casein and enzyme. B. Line 3, Gelatine 3 mg/ml, Line 4, 1h incubated Gelatine and protease enzyme.

3. 5. Gelatin Zymography

Zymography was used to measure the proteolytic activity and the size of the enzyme (Ren, Chen, and Khalil n.d.). The transparent bands show that the enzyme breaks down the gelatine. In other words, enzymes used gelatine as a substrate. Proteolytic bands were exhibited with a molecular mass of nearly ~48 kDa (Figure 3.10)

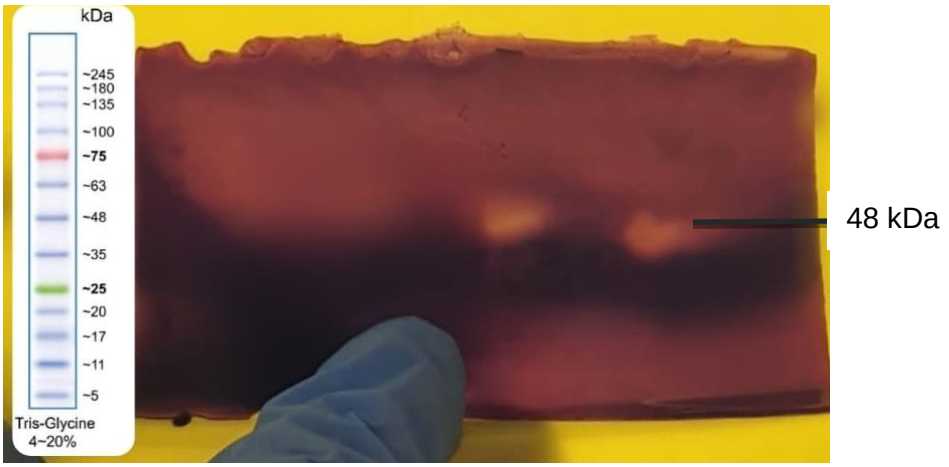
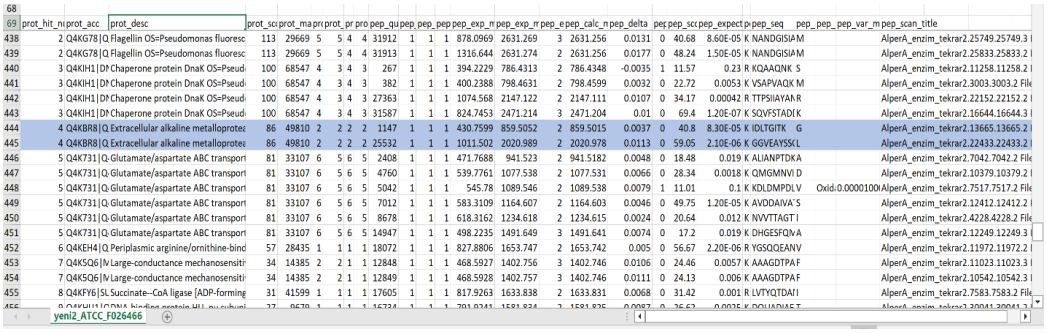


Figure 3.10. Gelatine Zymography Line 1, 1 kDa protein marker, Line 6, Protease enzyme Line 8, 48h Protease enzyme. Clear zone around ~48 kDa indicates enzyme activity.

3.6. Liquid Chromatography Mass Spectrometry

Extracellular protease enzyme was partially purified from the LB medium in which the bacteria grew was filtered and concentrated (Method 2.2.9). Proteomic analysis (Liquid Chromatography Mass Spectrometry) was performed on this concentrated extractant. Peptides identified by the MS were compared to the MASCOT database. (www.matrixscience.com) (4.1.A.) The peptide sequence (**NPDLIENFTSGTDKIEVSGVLK**) was obtained. This sequence was given a match to the serralysin family of metalloprotease, *Pseudomonas sp*. (4.1.B.) When the sequence was compared with the protein blast in NCBI it was shown that *Pseudomonas*

sp. URMO serralysin family metalloprotease has the matching sequence with the matching molecular weight ~48 kDa (4.1.D.)



prot_hit_nu	prot_acc	prot_desc	prot_sco	prot_ma	prot_pr	pro	pep_qu	pep_pep	pep_exp_	pep_exp_m	pep_e	pep_calc_m	pep_delta	pep	pep_sco	pep_expect	p	pep_seq	pep_pep_var_m	pep_scan_title
2	Q4KG78	Q.Flagellin OS=Pseudomonas fluoresc	113	29669	5	4	4	31912	1	1	878.0969	2631.269	3	2631.256	0.0131	0	40.68	8.60E-05	K	NANDGISIAM		AlperA_enzim_tekrar2.25749.25749.3
2	Q4KG78	Q.Flagellin OS=Pseudomonas fluoresc	113	29669	5	4	4	31913	1	1	1316.644	2631.274	2	2631.256	0.0177	0	48.24	1.50E-05	K	NANDGISIAM		AlperA_enzim_tekrar2.25833.25833.2
3	Q4KIH1	D.Chaperone protein DnaK OS=Pseud	100	68547	4	3	4	3	267	1	1	394.2229	786.4313	2	786.4348	-0.0035	1	11.57	0.23	R	KQAAQNK	S	AlperA_enzim_tekrar2.11258.11258.2
3	Q4KIH1	D.Chaperone protein DnaK OS=Pseud	100	68547	4	3	4	3	382	1	1	400.2388	798.4631	2	798.4599	0.0032	0	22.72	0.0053	K	VSAPVAQK	M	AlperA_enzim_tekrar2.3003.3003.2 File
3	Q4KIH1	D.Chaperone protein DnaK OS=Pseud	100	68547	4	3	4	3	27363	1	1	1074.568	2147.122	2	2147.111	0.0107	0	34.17	0.00042	R	TTPSIIAYAN	R	AlperA_enzim_tekrar2.22152.22152.2
3	Q4KIH1	D.Chaperone protein DnaK OS=Pseud	100	68547	4	3	4	3	31587	1	1	824.7453	2471.214	3	2471.204	0.01	0	69.4	1.20E-07	K	SQVFSTADIK		AlperA_enzim_tekrar2.16644.16644.3
4	Q4KBR8	Q.Extracellular alkaline metalloprotea	86	49810	2	2	2	1147	1	1	430.7599	859.5052	2	859.5015	0.0037	0	40.8	8.30E-05	K	IDLTGITK	G	AlperA_enzim_tekrar2.13665.13665.2
4	Q4KBR8	Q.Extracellular alkaline metalloprotea	86	49810	2	2	2	25532	1	1	1011.502	2020.989	2	2020.978	0.0113	0	59.05	2.10E-06	K	GGVEAYSSCL		AlperA_enzim_tekrar2.22433.22433.2
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	2408	1	1	471.7688	941.523	2	941.5182	0.0048	0	18.48	0.019	K	ALIANPTDKA		AlperA_enzim_tekrar2.7042.7042.2 File
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	4760	1	1	539.7761	1077.538	2	1077.531	0.0066	0	28.34	0.0018	K	QMGMNVIDA		AlperA_enzim_tekrar2.10379.10379.2
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	5042	1	1	545.78	1089.546	2	1089.538	0.0079	1	11.01	0.1	K	KDLDMPDLV	Oxidi	0.0000100(	AlperA_enzim_tekrar2.7517.7517.2 File
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	7012	1	1	583.3109	1164.607	2	1164.603	0.0046	0	49.75	1.20E-05	K	AVDDAIVAS		AlperA_enzim_tekrar2.12412.12412.2
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	8678	1	1	618.3162	1234.618	2	1234.615	0.0024	0	20.64	0.012	K	NVVTTAGTI		AlperA_enzim_tekrar2.4228.4228.2 File
5	Q4K731	Q.Glutamate/aspartate ABC transport	81	33107	6	5	6	5	14947	1	1	498.2235	1491.649	3	1491.641	0.0074	0	17.2	0.019	K	DHGESFQIVA		AlperA_enzim_tekrar2.12249.12249.3
6	Q4KEH4	Q.Periplasmic arginine/ornithine-bind	57	28435	1	1	1	1	18072	1	1	827.8806	1653.747	2	1653.742	0.005	0	56.67	2.20E-06	R	YGSQQEANV		AlperA_enzim_tekrar2.11972.11972.2
7	Q4K5Q6	V.Large-conductance mechanosensiti	34	14385	2	2	1	1	12848	1	1	468.5927	1402.756	3	1402.746	0.0106	0	24.46	0.0057	K	AAAGDTPAF		AlperA_enzim_tekrar2.11023.11023.3
7	Q4K5Q6	V.Large-conductance mechanosensiti	34	14385	2	2	1	1	12849	1	1	468.5928	1402.757	3	1402.746	0.0111	0	24.13	0.006	K	AAAGDTPAF		AlperA_enzim_tekrar2.10542.10542.3
8	Q4KFY6	SL.Succinate--CoA ligase [ADP-forming	31	41599	1	1	1	1	17605	1	1	817.9263	1633.838	2	1633.831	0.0068	0	31.42	0.001	R	LVTYQTDAI		AlperA_enzim_tekrar2.7583.7583.2 File

Figure 3.11. A. Liquid Chromatography - Mass Spectrophotometry (LC-MS) Analysis, MASCOT Database peptide sequences. Blue highlight represents Metalloprotease peptide sequence.

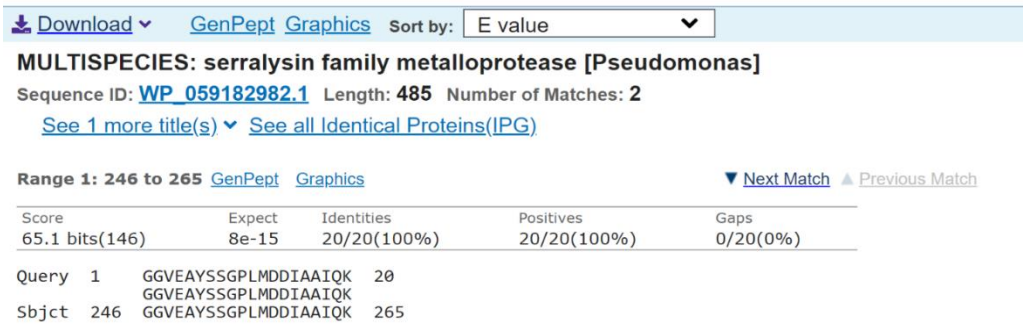


Figure 3.11.B. Peptide blast analysis (selected species *Pseudomonas sp*.), shows 100% identities with the peptide sequence.

```
  1 mskvknnaid laelafapsh alaapssafs qidsfshqyd rggnltvngk psysvdqaat
 61 qllrdgaayq dkdgsgkiel tytfltsass stmnkhgitg fsqfsaqqka qavlamqswa
121 dvanvtfaek atggdghmtf gnysggqdga aafaylpgtg agydgtswyl tnssytpnkt
181 pdlnnygrqt ltheightlg lahpgdynag egaptyndat ygqdtrgysv msywsesntn
241 qnfskggvea yssgplmddi aaiqklygan tttrtgdtty gfnsnagrdf lsassssdkv
301 vfsvwdaggk dtldfsgftq nqkinlneas fsdvgglvgn vsiakgaiie naiggsgndl
361 lignqvsnel kggagndily gaggadklwg gagsdtfvfa assdskpgva dqildfvsgl
421 dkidltgitk gaglhfvssf tgavgdavlt nsggnsllsv dfsghgvadf lvstvgqaaf
481 sdiva
```

Figure 3.11.C. The protein sequence of serralysin family metalloprotease [*Pseudomonas* sp.] Molecular weight 50.0175 kDa.

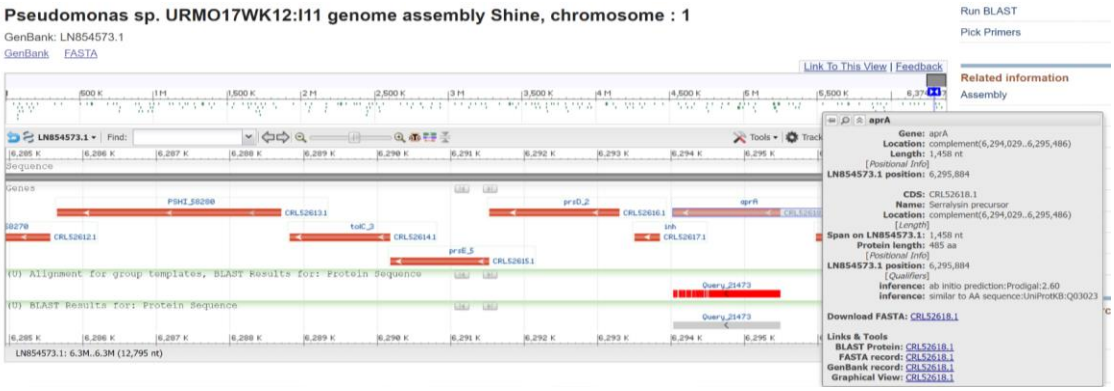


Figure 3.11.D. Sequences of genes on *Pseudomonas sp.* URMO chromosome. Grey box on the right is the explanation of AprA.

3.7. Partial Characterisation of Protease Enzyme

3.7.1. Optimum Temperature and Temperature Stability

The temperature activity profile of the protease was examined over the temperature range from 10 to 40°C, and the maximum activity was observed at 30°C (Figure 3.12). Even though enzyme activity was observed at 10°C, the activity of the enzyme was 95.2% lower than maximum activity. At 40°C the protease enzyme lost its activity by 28.6% compared to the maximum activity.

The thermal stability of the recombinant protease was also measured by incubating an aliquot of the enzyme at different temperatures for 60 min and then assaying the residual activity under optimal temperature conditions. The enzyme tends to be stable, with activities over 97%, at temperatures 10°C, 20°C and 25°C. The activity drops significantly at temperatures above 25°C. No enzyme activity is observed at 60°C.

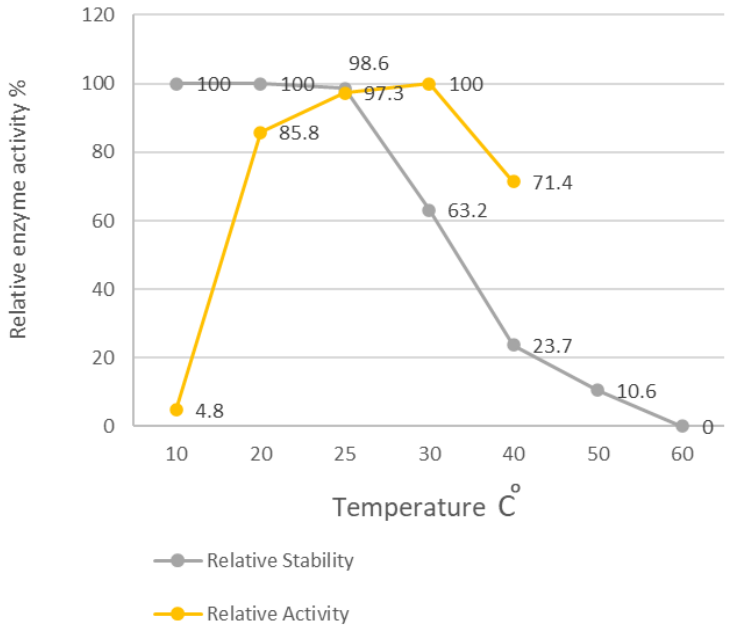


Figure 3.12. The effect of the Temperature Activity and Temperature Stability Relative activity graph Standard errors, 10°C ±2.1% , 20°C ±2.4% , 25°C ±3.4%, 30°C ±3.7%, 40°C ±5.50%. Grey line represents the stability of the enzyme, yellow line shows relative pH enzyme activity. Data are expressed as mean ± SD of triplicate experiments.

3.7.2. Optimum pH and pH Stability

Protease enzyme activity was determined at various pH ranges from 5 to 12. The enzyme showed maximum proteolytic activity at pH 8.0 and minimum activity in the highest alkaline buffer at pH 12 (Figure 3.13).

The enzyme activity at pH 9.0 and 10.0 was 92.6%, and 81% of the maximum respectively (Figure 3.13). The enzyme activity rapidly dropped down to around 70% at pH 7.0, and to less than 50% at pH 5.0 (Figure 3.12). The pH stability of the enzyme shows similarity with the pH activity. In the acidic range, the enzyme stability increases at a greater rate than the activity with increasing pH, leading up to an optimum peak in both at pH 8.0 (Figure 3.13). The enzyme shows high stability exhibits above 90% for the pH 7 to 9 range (Figure 3.13).

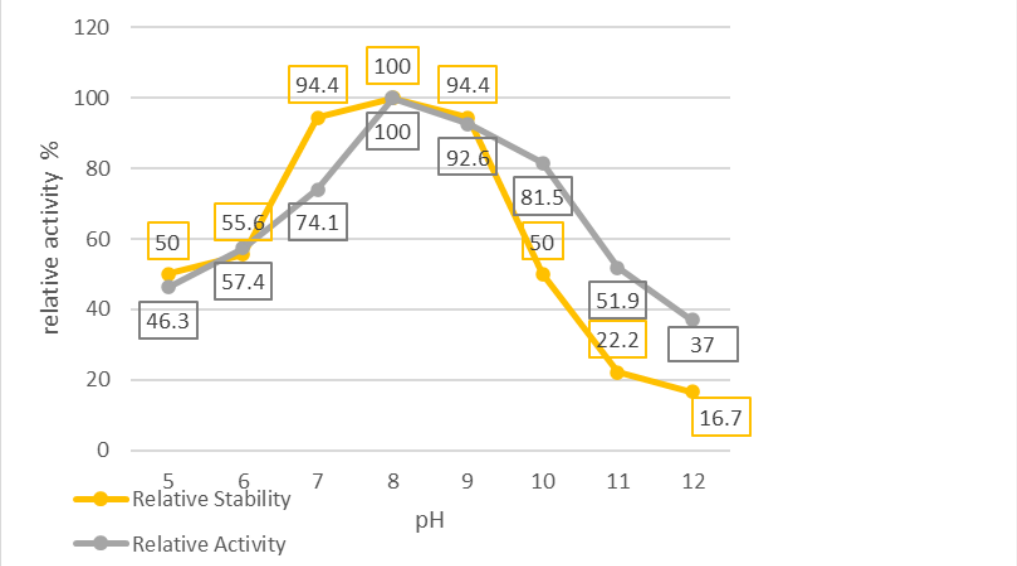


Figure 3.13. Relative enzyme activity and stability graphs at different pH values. The enzyme activity was assayed as described in the materials and methods section. Standard errors; pH 5.0 ± 7.0%, pH 6.0 ± 6.7%, pH 7.0 ± 7.3%, pH 8.0 ± 3.3%, pH 9.0 ± 3.1%, pH 10.0 ± 5.2%, pH 11.0 ± 4.6% and pH 12.0 ± 7.1%. Data are expressed as mean ± SD of triplicate experiments. Yellow line represents the stability of the enzyme, grey line shows relative pH enzyme activity.

3.7.3. Effects of Organic Solvent and Metal Ions

Acetone, Triton X-100 and Tween 20 completely abolished enzyme activity. In sharp contrast, Ethanol significantly increased protease activity up to 102.2%. (Figure 3.14. A.) Methanol showed the same activity in normal conditions.

Investigation of the effects of metal ions on protease activity can be enhanced by about 11% with 5 mM Ca^2+. Among the other metal ions shown in the figure 3.14, Cu^2+, Mg^2+, Zn^2+ decreased enzyme activity. While EDTA was observed to cause a decrease in enzyme activity, it inhibited over 85% of the protease activity. (Figure 3.14. B.)

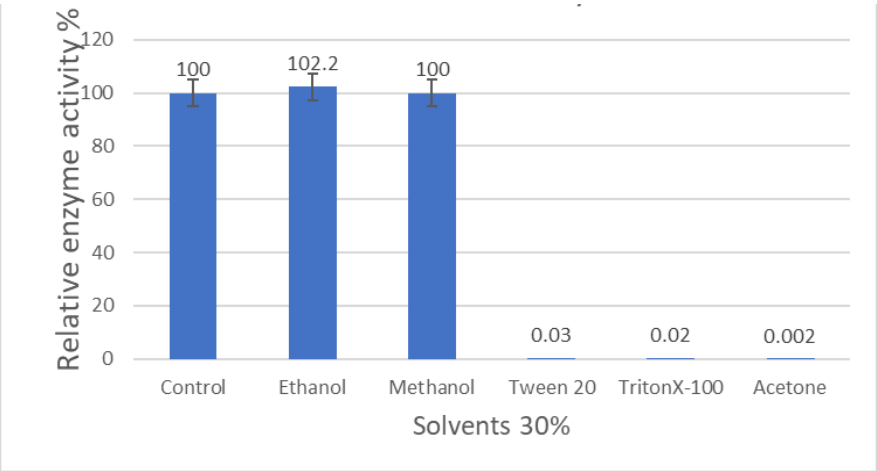


Figure 3.14.A. The effect of the solvents on protease activity graph. The effect of different organic solvents on enzyme activity. Data are expressed as mean ± SD of triplicate experiments.

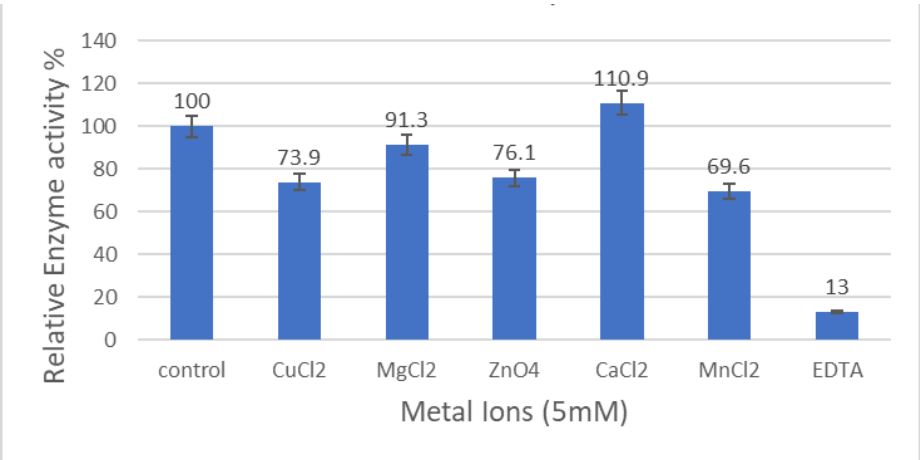


Figure 3.14. B. The effect of various metal ions and inhibitor on protease enzyme activity. The effect of different metal solutes or ions on enzyme activity. Data are expressed as mean ± SD of triplicate experiments.

CHAPTER 4

DISCUSSION/CONCLUSION

4.1. Confirmation of Protease Activity by Plate Assay

In this study, a novel protease enzyme was purified successfully from *Pseudomonas* sp. KE-38, which was isolated from soil samples of Erciyes mountain in Kayseri (Adan Gökbulut Alper Arslanoğlu et al. 2013). Protease activity was examined on two different agar plates (one Skimmed Milk and one Gelatine), in order to differentiate the production of a clear zone, as a result of lactic acid production on skimmed-milk, from the non-enzymatic proteolytic activity (Jones at al. 2007) (Figure 3.2. A.). This control avoids false positive results from non-enzymatic activity. In the half of the skimmed milk plate where the acetic acid was dropped, the clear zone was not as defined as those in the protease dropped half (Figure 3.2. A.). Meanwhile, in the gelatine the proteolytic activity was significant; with much more defined clear zones (Figure. 3.2.B). This indicates that *P fl* KE-38 successfully produces an extracellular protease enzyme.

4.2. Amplification and Partial Sequencing of the Protease Gene

The amplification of the partial Tol C and protease was successfully performed, and the amplified region was sequenced; however, non-specific bands were observed on the agarose gel (Figure 3.6.A.); in the literature, it has been observed that *Pseudomonas* sp. proteases could be found more than one copy in their genomes (Liao and Mccallus 1998). This might be an indicator explaining the non-specific binding in this study.

The protease gene was amplified, cloned into a pTZ57R/T cloning vector, and used for the transformation of *E. coli* Dh5alpha cells. The cloned protease gene was then sequenced and subjected to NCBI BLAST (blast.ncbi.nlm.nih.gov) analysis for the conformation of the amplified gene (Appendix B) and the sequence information was used to design a new primer (KE38_Prot_F) for the amplification of the protease enzyme coding region.

The protease open reading frame was amplified using the newly designed primer KE38_Prot_F and Es_Prot_Stop. However, the cloning and the expression phase of the experiment was not attempted owing to the time restriction and budget deficiency. It was concluded from the sequence analyses of the amplified region (with Es_TolC and Es_Prot_Stop primers) (Method 2.2.7.) that this gene was 3.0 kb in length (predicted to code for a protein of about 105 kDa) and showed very high homology with *Pseudomonas* sp. URMO, (Appendix B) which was determined to be an extracellular serine protease gene on the same operon with Tol C and the lipase gene.

4.3. Substrate Specificity of *P fl* KE-38‘s Protease Enzyme

The specificity of the enzyme against different substrates was determined. Proteolytic activity was observed using SDS with the substrates; skimmed milk, gelatine, alphacesein and azocasein. The enzyme presented an ability to break down gelatine (Figure (3.10 B and 3.11.)), and given its ease of production, it could pose useful for the recovery of metal ions, for example from photographic waste liquids (Dumorné et al. 2017). BSA was not a good substrate for *P fl* KE-38‘s protease enzyme, with reduced digestion of substrate compared to azocasein and gelatine. (Figure 3.10. A.) This may be due to the enzyme being a metalloprotease from the serralysin family. It may not be able to cut through the amino acid sequences in BSA. (Results 3.6)

4.4. Liquid Chromatography - Mass Spectrophotometry and Zymography

Zymography analysis showed that the protease enzyme's molecular weight is approximately 50 kDa (Figure 3.11) However, the expected molecular weight of the protease gene was 105 kDa [Protein weight) considering the PCR amplified and sequenced protease gene. In order to further assess this, proteomic analysis using Liquid Chromatography - Mass Spectrophotometry (LC-MS) was conducted. LC-MS analysis and MASCOT database search revealed the presence of a different protease enzyme than the one we anticipated for (Figure 3.11 A.) Protein BLAST analysis performed in NCBI with the peptide sequence “**GGVEAYSSGPLMDDIAAIQK**” and

"**IDLTGITK**" obtained by LC-MS analysis in the genome of *P fl* KE-38's closest relative *Pseudomonas* sp. URMO has shown that the sequence of the partially purified enzyme was most likely to be a metalloprotease enzyme of the serralysin family belonging to *Pseudomonas* sp. with 100% similarity (Figure 3.11. B.) Another BLAST analysis using the sequence of this metalloprotese (Figure 3.11. C.) revealed that the protein was coded by the *AprA* gene of *Pseudomonas* sp. URMO (Figure 3.11.D). This protease gene region is located 3 genes behind the targeted and amplified protease gene. (Figure 3.11.D) The Gelatine zymography results (Result 3.5) and the proteomic results (Figure 3.11.C) present a consistent case that the similar molecular weight sits around ~50 kDa. (www.aatbio.com) Since the protease enzyme was obtained from the medium, (Method 2.2.9) there were remnant contaminants such as protein and various medium compounds. These components were washed three times with TrisHCl buffer and could not be removed even though they were filtered. It is not surprising that the enzyme obtained was not the enzyme in the amplified region. However, the reason why the amplified protease enzyme is not visible in the zymogram may be that it was not expressed in LB, it can be produced in other media in future studies and the experiment can be conducted again. Another reason could be that the modifications in the middle parts of the gene and this may have prevented the expression of the protease as the whole sequence of the protease enzyme could not be reached in this experiment.

Gram negative bacteria frequently have serralysins as a virulence factor. (Bardoel et al. 2012). The Apr gene region contains (*AprA*) and (*Apr I)* the alkaline protease gene clusters and alkaline protease inhibitor respectively. Apr is conserved among *Pseudomonas* species (Bardoel et al. 2012). Serralysin family protease could also kill insects by destroying antibacterial peptides and could be used industrially as a bioinsecticide (Lee and Lee 2022).

In future studies, cloning and expression studies can be performed with two different protease enzymes (*AprA* and Serine Protease) obtained as a result of this experiment and after the purification of recombinant enzyme, a new characterization should be performed. Also, the industrial potentials of these two enzymes can be studied.

4.5. Partial Characterization

Partial characterization was performed successfully with the protease enzyme isolated from *P fl* KE-38. According to Figure 3.14, *P fl* KE-38's protease enzyme showed a broad pH profile (pH 5.0–12.0) and an optimal pH in the alkaline domain. It is known that the cold-active protease from psychotropic bacteria - belonging to various genera - displays maximal activity at 20–30 °C (Turkiewicz et al. 2003). Likewise *P fl* KE-38's optimum temperature range fell in the range 20-30°C (Figure 3.13.). The enzyme was active and stable across a broad range of temperatures. Such thermostability is another important distinction for industrial applications (Salwan et al. 2013).

The ability of *P fl* KE-38' to be active at low temperatures, offers practical and economic industrial and biotechnological potential. Enzyme lost its activity in high temperatures; this is likely due to the heat-activated conformational changes that are known to denature enzymes. The implications of different solvents, metals ions and compounds on enzyme activity varied; with methanol and ethanol enhancing activity (Figure 3.14. A), while Tween 20 and Triton X-100 inhibited the enzyme activity. Given Tween 20 and Triton X-100 are the most commonly used non-denaturing and non-ionic surfactants in lab detergents, *P fl* KE-38 extracellular protease presents incompatibility for use in most industrial detergents. The effect of, and mechanisms by which, zwitterionic surfactants interact with *P fl* KE-38 extracellular protease remains to be explored in future studies. The enzyme presented calcium-dependence with Ca^2+ enhancing the activity. EDTA decreased enzyme activity substantially. It is known that EDTA is used as a chelating agent that binds to calcium (Mellau LS n.d.), therefore metal ions may have an important role in maintaining enzyme conformation. (Figure 3.14. B.)

4.6. Conclusion

The amplification of the extracellular protease enzyme was performed and the partial sequence of amplified region from *Pseudomonas* sp. KE-38 was analysed. Sequence analysis showed that protease encoding gene sits around 105 kDa. Subsequent analyses (SDS-PAGE and gelatine zymography) performed with the purified enzyme, showed that the enzyme size was approximately 50 kDa. Liquid Chromatography Mass Spectrophotometry analysis revealed that the purified enzyme was a metalloprotease enzyme, and the size of the enzyme was in agreement with gelatine zymography at approximately 50 kDa. Partial characterisation experiments were performed, and the enzyme showed extremely high stability until 25°C. The optimum working temperature was found to be 30 °C and an optimum pH of 8.0. The enzyme also showed high activity in the presence of ethanol and methanol and calcium.

APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Luria Bertani (LB) broth, per liter

10g tryptone, 5g yeast, 5g NaCl and dH2O up to 1 L

Luria Bertani (LB) agar, per liter

10g tryptone, 5g yeast, 5g NaCl, 15g agar, and dH2O up to 1 L

Luria Bertani (LB) agar, Ampicillin plates, per liter

10g tryptone, 5g yeast, 5g NaCl, 15g agar and dH2O up to 1 L after cooled down 1 ml of ampicillin stock solution (50 mg/ml) to a final concentration of 50 μg/ml added.

Skimmed Milk (SM) agar, per liter

100 ml skimmed milk (0.1%), 15g agar, and dH2O up to 1L

Skimmed Milk (SM) broth, per liter

100 ml skimmed milk (0.1%), and dH2O up to 1L

SOC medium, per 100 ml

2g Tryptone, 0.5g Yeast Extract, 1ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M Mg2+ Stock, 1ml 2M Glucose were dissolved in dH2o up to 100 ml

50X TAE Electrophoresis Buffer

242g Tria base and 37.2g Na2Edta (2H2O) were dissolved in 900 ml deionized water. 57.1 ml glacial acetic acid was added, the volume was adjusted to 1 liter with deionized water.

Ethidium Bromide Stock Solution (10mg/ml)

0.2 ethidium bromide (EtBr) was dissolved in 20 ml dH2O. This mixture was stored at room temperature in the dark.

Ampicillin Stock Solution (50 mg/ml)

Dissolve 2.5 g of ampicillin sodium salt in 50 ml of deionized water. This mixture was filter-sterilized (0.22 pore).

TrisHCl Buffer Solution (1M, pH 8.5)

157.6 g/mol TrisHCl was dissolved in dH2O up to 1L

PBS Stock Solution, per L (10M pH 4.7)

NaCl 8g, KCl 0.2g Na2HPO4 1.44g, KH2PO4 0.245g up to 1L

APPENDIX B

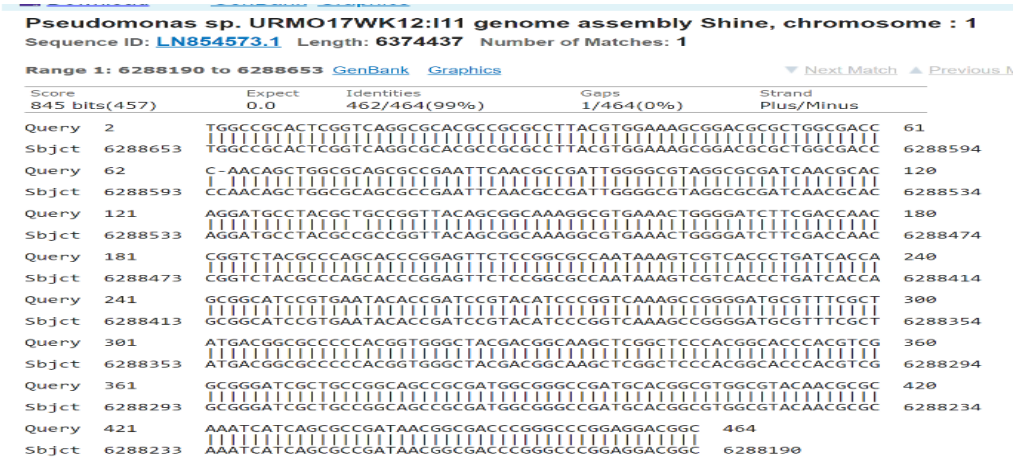
PARTIAL SEQUENCE AND NUCLEOTIDE BLAST

Partial Sequence of Protease Gene from *P fl* KE-38, Sequenced with Es_Prot_TolC Primer

GCTATGACTACTTGATGGCCTGGACCAAATTGCACTACTACGCCGGAACCTTGAGCGAGCAGGA
GGGGCCGGGGKAGACGAAGCGTTCGGCCAGGGCCCAAAGACYCAATGATGGATCTGTAGCAGC
TGCCGAAGGCTGCGTTCGGCTGCGACACGGGACGTGTGAATTTGCAGGCGCCTTTCGAGGCAAA
AAGGGATTTTGGCAACACAACTAAGGACGGTAGCTGG**ATGAATAACAAGCACAACACACTGG**
CCGTGCTCTGCGCACTGGCCGCACTCGGTCAGGCGCACGCCGCGCCTTACGTGGAAAGCGGACG
CGCTGGCGACCCCAACAGCTGGCGCAGCGCCGAATTCAACGCCGATTGGGGCGTAGGCGCGAT
CAACGCACAGGATGCCTACGCTGCCGGTTACAGCGGCAAAGGCGTGAAACTGGGGATCTTCGA
CCAACCGGTCTACGCCCAGCACCCGGAGTTCTCCGGCGCCAATAAAGTCGTCACCCTGATCACC
AGCGGCATCCGTGAATACACCGATCCGTACATCCCGGTCAAAGCCGGGGATGCGTTTCGCTATG
ACGGCGCCCCCACGGTGGGCTACGACGGCAAGCTCGGCTCCCACGGCACCCACGTCGGCGGGA
TCGCTGCCGGCAGCCGCGATGGCGGGCCGATGCACGGCGTGGCGTACAACGCGCAAATCATCA
GCGCCGATAACGGCGACCCGGGCCCGGAGGACGGCATTATTCGTGGCAACGACGGTGCGGTGT
ACAAGGCAGGTTGGGATGCCCTGATCGCCAGCGGCGCGCGGATCATCAACAACAGCTGGGGCA
TCGGCATTACCGATCGCTTCGACCTCGGCGGACGCGATCCCGGCGTACCCCGCACTTCACCGTG
CAGGATGCGCAGTTGCAGTTTCAACGARGATCCCAGCMCTGCYCGGAACCCAACCGGGCGGGT
GCCTACGACGGTGGCGATCGCGCGATCTGCAGCGGCATCGTCACGATYTTGCCGCCGGCACGAC
TACCACTGATAACTGAASCCATGCCGGACTCGGCTAWTTTC

Nucleotide Blast Results with Amplified Region

The result of the blast made after the information obtained from the sequencing. The sequence of the amplified region from *P fl* KE38 gave 99% match with *Pseudomonas sp.* URMO.



Pseudomonas sp. URMO17WK12:I11 genome assembly Shine, chromosome : 1

Sequence ID: LN854573.1 Length: 6374437 Number of Matches: 1

Range 1: 6288190 to 6288653 GenBank Graphics Next Match Previous M

Score	Expect	Identities	Gaps	Strand
845 bits(457)	0.0	462/464(99%)	1/464(0%)	Plus/Minus

```
Query  2        TGGCCGCACTCGGTCAGGCGCACGCCGCGCCTTACGTGGAAAGCGGACGCGCTGGCGACC  61
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288653  TGGCCGCACTCGGTCAGGCGCACGCCGCGCCTTACGTGGAAAGCGGACGCGCTGGCGACC  6288594

Query  62       C-AACAGCTGGCGCAGCGCCGAATTCAACGCCGATTGGGGCGTAGGCGCGATCAACGCAC  120
                | |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288593  CCAACAGCTGGCGCAGCGCCGAATTCAACGCCGATTGGGGCGTAGGCGCGATCAACGCAC  6288534

Query  121      AGGATGCCTACGCTGCCGGTTACAGCGGCAAAGGCGTGAAACTGGGGATCTTCGACCAAC  180
                ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288533  AGGATGCCTACGCCGCCGGTTACAGCGGCAAAGGCGTGAAACTGGGGATCTTCGACCAAC  6288474

Query  181      CGGTCTACGCCCAGCACCCGGAGTTCTCCGGCGCCAATAAAGTCGTCACCCTGATCACCA  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288473  CGGTCTACGCCCAGCACCCGGAGTTCTCCGGCGCCAATAAAGTCGTCACCCTGATCACCA  6288414

Query  241      GCGGCATCCGTGAATACACCGATCCGTACATCCCGGTCAAAGCCGGGGATGCGTTTCGCT  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288413  GCGGCATCCGTGAATACACCGATCCGTACATCCCGGTCAAAGCCGGGGATGCGTTTCGCT  6288354

Query  301      ATGACGGCGCCCCCCACGGTGGGCTACGACGGCAAGCTCGGCTCCCACGGCACCCACGTCG  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6288353  ATGACGGCGCCCCCCACGGTGGGCTACGACGGCAAGCTCGGCTCCCACGGCACCCACGTCG  6288294

Query  361      GCGGGATCGCTGCCGGCAGCCGCGATGGCGGGCCGATGCACGGCGTGGCGTACAACGCGC  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  421      AAATCATCAGCGCCGATAACGGCGACCCGGGCCCGGAGGACGGC  464
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Sbjct  6288233  AAATCATCAGCGCCGATAACGGCGACCCGGGCCCGGAGGACGGC  6288190
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