Quantification of Viable *Pseudomonas* species

based on DNA Amplification

By

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Dedication

This work is dedicated to my brother Dr. Iboni Richard and Mum and Dad, Mr & Mrs Eyum all of blessed memories. You all will forever remain in my heart. They were my inspiration and thought me a lot in this life. Also, my wife Immaculate Arrey epse Eyum who has devoted her life to be partners with me in every step I make till death and I thank God for giving such a wonderful wife. To my brother Govina Eyum for his support and elderly advise. To all my siblings back home in Africa and friends and lab colleagues who has been a great source of encouragement to this work.

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ABSTRACT

PCR techniques have significantly improved the detection and quantification of bacterial pathogens on foods. However, there are still limitations in the use of PCR-based diagnostics; the lack of discrimination between DNA derived from viable and dead microorganisms is a major obstacle of the PCR method. Ethidium bromide monoazide is a DNA binding dye that differentiate viable and dead cells. However, there are reports that EMA at higher concentration can equally reduce or inhibit DNA amplification of viable cells. The aim of this study was to determine the concentration of EMA that will inhibit DNA amplification from dead *Pseudomonas* species but allow amplification of only viable *Pseudomonas* species by using EMA in conjunction with the conventional PCR.

In this study, we have developed the EMA-PCR assay for the detection and quantification of viable *Pseudomonas* species. This study shows that 1.0 μg/ml EMA inhibit DNA amplification derived from dead *Pseudomonas* species cell with no significant ($P<0.05$) inhibition from viable cells. The EMA-PCR assay can detect as few as 1CFU/PCR viable cells target DNA in a mixed ratio of viable-dead cells suspensions. This EMA-PCR assay offers the advantage of obtaining results in 2 to 4 h compared to 48 h from conventional plate counts and is sensitive and reliable for comprehensive detection and quantification of *Pseudomonas*

species that could be used in private sectors and food industries for monitoring food products to identify potential risk.

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CHAPTER 1

GENERAL INTRODUCTION

Pseudomonas is an immensely diverse genus showing a great variety of metabolic abilities and a broad ecological distribution. They are found to be adaptable to a wide range of environmental niches. The taxonomy of these group has undergone much revision over recent years and more than 200 species have been described. Species within the genus can be plant pathogens in contaminated soils and human pathogens in diverse foodstuffs. This ecological diversity means they are a common contaminant of food materials. Their metabolic diversity and a psychrotrophic growth ability allows them to grow on a wide range of foodstuffs even under refrigeration conditions and are key spoilage agents of meat, poultry, milk, fish, and eggs. (Dodd, 1997). Several traditional methods have been applied to isolate and characterize *Pseudomonas* spp. strains from foodstuffs. These traditional microbiological methods offered standardized procedures for microbial detection. However, they are time-consuming (take approximately 4–7 days) and not always compatible with short-time-to-result demand. Recently these approaches have been replaced by more exhaustive and effective nucleic acid-based investigation methods (De Jonghe et al., 2011). Food microbiology aims for the supplementation of this classical method with molecular techniques based on detection of the microbial nucleic acids which shorten the analysis time and lower the limit of detection. PCR was invented in the mid-1980s and can quickly amplify a targeted nucleic acid sequence and within a matter of hours to create billions of copies of that sequence.

PCR techniques significantly improves the detection and identification of bacterial pathogens in foods. However, there are still limitations in the use of nucleic acid-based diagnostics. These molecular assays cannot discriminate between DNA from viable and dead

Pseudomonas spp. which can lead to false-positive results as well as to the overestimation of cell numbers when evaluating food products (Josephson et al., 1993; Nogva et al., 2003). The lack of discrimination between DNA derived from viable and dead microorganisms is a major obstacle of the PCR method (Herman, 1997; Sheridan et al., 1998). The DNA extracted from cells destroyed by heating or other treatments can serve as a template for PCR after cell viability has been lost (Chaiyanan et al., 2001; Nogva et al., 2003). Detection of only live microorganisms in foods is critical in assessing the potential for foodborne infections. Several authors have attempted to detect only viable cells by mRNA using reverse transcriptase PCR (Novak and Juneja, 2001; Bentsink *et al*., 2002; Van Beckhoven *et al*., 2002). However, due to its intrinsic instability and its reproducibility in determining viable and dead cell counts accurately, using mRNA as a target has the limitation (McKillip *et al*., 1998; Sheridan *et al*., 1998; Norton and Batt, 1999). Other currently applied viable and dead cell methods are based on microscopy and flow cytometry involving Syto 9 and BacLight staining (Michel *et al*., 1999; Burnett and Beuchat, 2002; Rudi *et al*., 2005). The main difficulty with these methods is that the observation of viable cells spans a narrower detection range than PCR methodology or plate counts (Rudi *et al*., 2005). There is, therefore, a need to develop a methodology that allows PCR to discriminate DNA derived from live and dead cells to fully exploit the potential of PCR in food safety.

Ethidium bromide monoazide (EMA) has been used as a DNA binding dye to differentiate viable and dead cells (Nogva *et al*., 2003; Rudi *et al*., 2005; Lee and Levin, 2006). Viable and dead differentiation is obtained by covalent binding of EMA to DNA in dead cells by photo-activation. EMA penetrates only dead cells with compromised membrane/cell wall systems. DNA covalently bound to EMA cannot be PCR amplified (Hixon *et al*., 1975). Thus, only DNA from viable cells can be detected. However, there are reports that EMA at higher concentration can equally reduce or inhibit DNA amplification of viable cells (Nocker et al.,

2006; Nogva et al., 2003; Rudi, Moen, Drømtorp, & Holck, 2005; Pan and Breidt, 2007). Recently, EMA has been used in conjunction with real-time PCR (EMA real-time PCR) for detection and quantification of specific DNA sequence products by using SYBR® Green (Lee and Levin, 2006). This present study is designed to develop a PCR method for quantifying and discriminating between viable and dead *Pseudomonas* spp. In order to establish this protocol to quantify only viable *Pseudomonas* spp., several critical steps in the PCR was optimized. Thus, this study was not only to discriminate viable from nonviable *Pseudomonas* spp., but was equally to optimize the PCR protocol to detect *Pseudomonas* spp. using conventional PCR with *Pseudomonas* universal primers. Therefore, the aim of this study was to; i) Optimize the PCR protocol using *Pseudomonas* universal primers, ii) Determine the optimum concentration of EMA amplifying viable *Pseudomonas* spp., iii) Apply the optimized EMA-PCR to mixed viable and dead cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Food safety and microbial detection

There are at least four major food safety challenges that scientific community has been facing for the last three decades. It is well known that traditional identification and culture-based methods for pathogens or food spoilage microbes are time-consuming. Therefore, developing techniques for the rapid and early detection of viable bacteria in food is a necessary step that may enable the scientific community to better control this food safety issue. The detection and characterization of microorganisms in foods are the foundations for understanding challenges in maintaining a safe, appealing, and nutritious food supply. The development of culture-dependent methodleads to some individual microorganisms to be isolated and grown in the laboratory. Since then, a vast number of different growth media have been formulated and, along with specific culture conditions, a wide array of microorganisms can now be cultured from food. There is, however, no universally successful method for culturing microorganisms from foods. This lead to a vast underestimation of the number of culture confirmed food-borne illnesses (Kirk et al., 2015) and eventually increase the need for better methods of detection.

2.2 *Pseudomonas species.*

The genus *Pseudomonas* is composed of a heterogeneous group of bacteria characterized by important ecological significance (de Oliveira et al., 2015). The strains ascribed to the genus *Pseudomonas* are Gram-negative, straight or slightly curved rods, 0.5 to 1.0 μm by 1.5 to 5.0 μm in length. They are catalase positive and are motile by means of one or several polar flagella. Pseudomonas species have been characterized as having an obligate respiratory

metabolism, with a positive oxidase test and do not produce gas from glucose. It has been reported that they can grow under anoxic conditions in the presence of nitrate, fumarate or other electron acceptors although they are considered as aerobic bacteria (Palleroni, 1984). They are equally mesophilic bacteria, their optimal growth temperature is about 25-35°C; however, most of them are psychrotrophic microorganisms, which means they can grow at refrigeration temperatures and do not tolerate high temperatures. Cold adaptation of *Pseudomonas* spp. is linked to high levels of unsaturated lipid in the cell membrane and to a wide range of mechanisms that allow the tolerance of cold-induced stresses (Fonseca et al., 2011; Samarzija et al., 2012; Moreno and Rojo, 2014). The optimum pH environment for *Pseudomonas* strains is above 5.8: lower pH values slow down bacterial growth. *Pseudomonas* species also have a very simple nutritional requirement and have the ability to use different organic compounds as carbon and energy sources. For these reasons, *Pseudomonas* can be defined as a ubiquitous bacterium and can be found in a wide range of different environments such as organic material under decomposition, atmospheric dust, and vegetation, and have various animal and plant hosts (Anzai et al., 2000; Frapolli et al., 2007). Some strains can also be found in soil and water and some species have been documented as human pathogens, such as the well-known *P. aeruginosa*, and *P. paucimobilis, P. putida, P. fluorescens,* and *P. acidovorans* (Tummler et al., 2014). Other species have been reported as plant pathogens, e.g., *P. pseudoalcaligenes, P. savastanoi, P. syringae*, and others as animal pathogens, mainly against fishes and birds (*P. anguilliseptica, P. chlororaphis, P. psychrophila, P. aeruginosa;* Peix et al., 2009). It has equally been reported that *Pseudomonas* are well-known food spoilers, such as *P. fluorescens* which is responsible for the spoilage of dairy products, raw fish, raw meat, and eggs, as a result of their psychrotolerant character (Jay, 2003; Palleroni, 2010). Several traditional methods have been applied to isolate and characterize *Pseudomonas* spp. strains from foodstuffs; (de Jonghe et al., 2011). The ISO standards are available for the

enumeration of *Pseudomonas* species. ISO/TS 11059:2009 and ISO 13720:2010 are official methods of isolating and counting *Pseudomonas* spp. in milk and dairy products and in meat and meat products, respectively. ISO 13720:2010 reports the use of *Pseudomonas* Agar Base, a selective agar medium containing cetrimide, fucidin, and cephalosporin (CFC supplement), three antibiotics selective for other non-*Pseudomonas* strains, and incubation at 25°C for 44 hours (Tryfinopoulou et al., 2001). On the other hand, ISO 11059:2009 suggests incubation in a medium containing penicillin and pimaricin at 25°C for 48 h. Routinely applied tests for *Pseudomonas* are based on the identification of morphological, phenotypic, and biochemical characteristics of the strains. However, recently these approaches have been replaced by more exhaustive and effective nucleic acid-based investigation methods.

The recent increasing interest in molecular approaches led to the development of several clustering and typing methods that avoid the lack of information inherent in classical methods. These new approaches allowed in some cases not only the identification of the species within the genus, but also the description of phylogenetic relationships among species belonging to specific bacterial clusters. Examples are the application of REP-PCR (Repetitive Extragenic Palindromic PCR; Johnsen et al., 1996), RFLP (Restriction Fragment Length Polymorphism; Franzetti and Scarpellini, 2007), DGGE (Denaturing Gradient Gel Electrophoresis, De Jonghe et al., 2011), and PFGE (Pulsed Field Gel Electrophoresi) (Nogarol et al., 2013) to the identification of foodborne *Pseudomonas* species. Sequencing of 16S rRNA genes has been widely used to identify and categorize several species of the genus isolated from food or other environments (Laguerre et al., 1994; Moore et al., 1996; Anzai et al., 2000). Some studies have focused on the phylogeny of the whole genus (Yamamoto et al., 2000; Hilario et al., 2004; Mulet et al., 2010), while others have concentrated on subgroups of the genus, such as the investigation of blue-pigmenting strains isolated from food products and belonging to the *Pseudomonas fluorescens* group by Andreani and collaborators (2014). Both classical

microbiological and biomolecular approaches require the isolation of the strains from their original environments (culture-dependent methods). For this reason, these techniques have to take into account the problem of viable but nonculturable bacteria (VBNC). VBNC are bacteria whose metabolic activity is too low to allow reproduction. Sometimes, the induction of VBNC forms is due to stresses to which vital cells are subjected within the food chain for example, refrigeration of fresh products, use of sanitizers, or heating treatments.

The most recent detection method of *Pseudomonas* spp. in food is based on immunological or nucleic acid-based procedures. Immunological methods employ antibodies that are raised to react to surface antigens of specific microorganisms (Betts, 1999; Jay, 1996). The most common form of these methods is the enzyme-linked immunosorbent assays (ELISAs) and these are based on the use of an enzyme label. Nucleic acid-based procedures utilize probes and primers that are small segments of single-stranded complementary nucleic acid that are used to detect specific genetic sequences. Nucleic acid probes can be used to detect either DNA or RNA sequences in order to identify accurately a specific microorganism (Alexandre, Prado, Ulloa, Arellano, and Rios, 2001; Venkitanarayanan, Khan, & Faustman, 1996)

2.3 Polymerase Chain Reaction

The most widely applied nucleic acid detection method at present utilizes the polymerase chain reaction (PCR) (Mullis & Faloona,1987). This method uses primers, short segment of the known sequence to be detected and has been reported to allow for rapid identification and/or detection of microorganisms in different food matrices by amplifying specific gene fragments in an automated machine called a thermocycler and confirming the PCR amplicons by gel electrophoresis (Cloak, Duffy, Sheridan, Blair, & McDowell, 2001; Gutierrez et al., 1998; Scheu, Berghof, & Stahl, 1998; Yost & Nattress, 2000). Thus, like for

nucleic acid probes, the DNA sequence of the target organism must be known prior to the analysis. The PCR cycle consists of three steps: i) denaturation of the double-stranded DNA; this step is necessary to unwind the DNA double helix to expose the complementary nucleotide sequence for the primers to bind. It is achieved by using high temperature between 94-95°C, ii) annealing of short DNA fragments (primers) to single DNA strands; this step allows the gene of interest to be identified and amplified, without which there will be no product. For proper annealing of the primers, temperature ranges between 54-65°C is needed, iii) extension of the primer with the key enzyme, a thermostable DNA- polymerase (Taq polymerase); the enzyme recognizes both forward and reverse primers as the start point for the addition of dNTPs to synthesis new strands of the parent DNA. Following the completion of one cycle, the sample is denatured for the next annealing and extension steps during which not only the original target region is amplified, but also the amplification product of the first cycle. DNA from non-viable microorganisms can lead to false positive results being obtained. The is therefore a need to develop better methods to improve the PCR technique and to be able to discriminate DNA from viable cells and non-viable cells.

The definition of "bacterial viability" is still a subject of controversy. The most usual one is: when a sample is plated on an appropriate medium, dead bacteria are unable to produce colony forming unit (CFU) whereas viable bacteria are able to form CFU (Trevors, 2012). In this definition, the cell wall/membrane integrity of the different states is not taken into account. Another approach is based on cultivability, metabolic activity and membrane integrity has been described (Nocker and Camper, 2009). In this model, four different states are postulated. The "living" bacteria are defined as culturable, metabolically active and with an intact cell wall/ membrane. The "viable but non-culturable" (VBNC) bacteria also known as "ghost" bacteria are defined as metabolically active, with an intact cell-wall/ membrane but non-culturable. These bacteria have an intact cell-wall/membrane but are metabolically inactive and

nonculturable. Finally, the "membrane compromised" bacteria cells that have a compromised cell-wall/membrane and are non-culturable and have no metabolic activity. There are two known molecules that can be used to quantify bacterial viability: ethidium monoazide (EMA) and propidium monoazide (PMA) which are derivatives from ethidium bromide and propidium iodide, respectively. EMA has been demonstrated to have more effective penetration into damaged cells and greater PCR inhibition ability than the alternative dye propidium monoazide (PMA) (Lee & Levin, 2008). In the recent years, several studies have been reported using ethidium monoazide to discriminate DNA derived from viable and non-viable bacterial cells in PCR (Nocker et al., 2006; Nogva et al., 2003; Rudi, Moen, Drømtorp, & Holck, 2005; Lee & Levin, 2008).

2.4 Ethidium Monoazide.

Ethidium monoazide bromide (EMA) is a DNA-intercalating dye that can only penetrate cells with compromised membranes and covalently binds to DNA through photoactivation and consequently inhibiting PCR (Waring, 1965). The photolysis of EMA with visible light produces a nitrene (De Traglia et al., 1978) that forms stable monoadducts when bound to DNA (Coffman, et al.,1982; Hixon et al.,1975). The free EMA in solution is photolyzed simultaneously and converted to hydroxylamine (De Traglia et al., 1978) and is no longer capable of covalent attachment (Kell et al., 1998). EMA is said to intercalate DNA every 4–5 nucleotides (Waring, 1965). It is a positively charged molecules and therefore excluded by intact, negatively charged, bacterial cell-walls but can enter bacteria with damaged cell-wall/membranes (Nocker et al., 2006). The is also a school of thought that EMA may act by cleaving the DNA and thus making it unavailable for PCR (Soejima et al., 2007). EMA method has been described as a promising method to discriminate viable and non-viable cells (Nocker, Cheung, & Camper, 2006; Nogva, Drømtorp, Nissen, & Rudi, 2003; Shi et al., 2011). EMA

was first reported to be useful to quantify viable bacteria by v-PCR (Nocker and Camper, 2006; Rudi et al., 2005) but was noticed that it can penetrate and be toxic for viable bacteria as well at high concentrations and may cause false negative results (Nocker et al., 2006; Nogva et al., 2003; Rudi, Moen, Drømtorp, & Holck, 2005; Pan and Breidt, 2007). Therefore, EMA concentration must be optimized to achieve accurate results. To the best of our knowledge, little or no studies have been performed using EMA-PCR on *Pseudomonas* species as an individual genus. Therefore, this study is aimed at developing a EMA-PCR protocol to quantify only viable *Pseudomonas* species in food that may otherwise be a food safety issue to many consumers.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions.

Five *Pseudomonas* spp. were used in this study and include *P.aeruginosa* (ATTC 10145), *P.fluorescens* (Carolina biological), *P. putida* (Carolina biological), *P.lurida* p20 and *P. psychrophila* p123. The bacterial species were grown regularly in Tryptic Soy Broth (TSB; Carolina Biological Supply) and on Tryptic Soy Agar (TSA; Carolina Biological Supply) supplemented with 1% NaCl. Routine cultures were incubated at 27^{o} C during this study

3.2 Bacterial enumeration and standard plate curve

3.2.1a Bacterial sample preparation and inoculation.

Each bacterial species and strains were stored at -80°C. A sterile wire loop was used to transfer a loop of the sample unto TSA and the plate streaked. The plate was incubated at 27°C for 18hrs plus to obtain pure bacterial colonies. One colony was then transferred using a sterile wire loop into TSB to obtain the broth culture used for further analysis.

3.2.1b Obtaining Optical density

After incubation of each species in TSB, optical density of each *Pseudomonas species* was measured and bacterial number fixed using (GENESYS 10S UV-Vis spectrophotometer - Thermo Scientific™). The optical density of the sample cuvette was adjusted to 0.2. The mixed culture sample was obtained by transferring 200 µl each of all the bacteria species already measured at 0.2 to a fresh cuvette to obtain a mixed culture.

3.2.1c Serial dilutions and plating

Seven 10-fold decimal dilutions were made from the original samples in TSB and were smeared onto Plate Count Agar (TSA). These plates were then incubated aerobically at 27°C for

determination of colony forming units. All plating were performed in triplicate. The CFU of each plate were counted for the different dilutions and recorded and values were used to obtain a standard count curve that was used for quantification of our bacterial sample in further analysis.

3.3 Optimization of PCR assay for the detection of *Pseudomonas* species using genus specific primers

3.3.1 Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.3.2 Obtaining Optical density

Refer to previous method (3.2.1b) as nothing changed prior to this method

3.3.3 DNA Extraction

Cell suspensions in tubes were centrifuged at 13,000 rpm for 10 min to obtain cell pellets. The supernatants were discarded and the cells resuspended in saline solution. Cell suspensions in saline were mixed with TZ solution in microcentrifuge tubes followed by mixing. Cells were lysed according to Abolmatty et al. (2000). The crude extracts were added directly to PCR mixtures.

3.3.4 Gradient PCR for Annealing temperature optimization using genus primers for Pseudomonas spp.

3.3.4a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.3.4b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.3.4c DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.3.4d PCR and Primers

The gradient PCR amplification was performed using a thermal cycler (Bio-Rad™ My Cycler™). PCR assay consisted of 12.5 µl of 2X reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl pH 8.8, 0.1 Tween 20, 2.5 mM $MgCl_2$, and 0.75 units of Taq polymerase (Bio21042, Midwest Scientific), 200 µM of each dNTP (TAK4030, Fisher Scientific), 1 µl of both forward and reverse primers, and 2 µl of DNA template. PCR mixture was adjusted with PCR water to a final volume of 25 µl. The PCR was performed as follows; initial denaturation at 95°C for 4 min, 35 cycles of DNA denaturation at 95°C for 35s, annealing at temperature range (54 ~ 65°C) for 35s, extension at 72°C at 50s and final extension at 72°C for 4 min and holding at 4°C.

The primers used to amplify our target gene (16S rDNA) was 10 µmol universal primers (Sigma Genosys).

3.3.4e Gel Electrophoresis and Visualization of PCR products

Following amplification, gel loading solution (G-7654, Sigma–Aldrich) were added to the PCR product and the mixtures were loaded on 1.3% high-melting agarose gels stained with Ethidium Bromide (EB, cat. no. E-8751, Sigma–Aldrich). The PCR samples were separated using a horizontal electrophoresis chamber in 1X TBE. DNA fragments in the gels were finally visualized with an ultraviolet transilluminator (TR-302, Spectronics corporation). Gel images were obtained using a Canon Powershot G3 digital camera with an orange optical lens filter. NIH ImageJ software were used for relative quantitation of fluorescent DNA bands derived from the gel result. The mean values of the fluorescence of bands were derived from three independent experiments.

3.3.5 Optimization of PCR Extension Time

3.3.5a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.3.5b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.3.5c DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.3.5d PCR

The PCR was performed as follows; initial denaturation at 95°C for 4minutes, 35 cycles of DNA denaturation at 95°C for 35s, annealing at 59°C for 35s, extention at 72°C at 30-60s and final extension at 72°C for 4 min and holding at 4°C.

3.3.5e Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

3.3.6 PCR Assay and Primers Detection sensitivity

3.3.6a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.3.6b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.2.6c Serial dilutions

After measuring the OD of each species and fixing the bacterial number to 10^8 CFU/ml, serial dilutions were made to obtain different bacterial CFU/ml. Each decimal dilution and the original sample were used for DNA extraction and detection of our target gene.

3.3.6d DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.3.6e PCR

The PCR was performed as follows; initial denaturation at 95°C for 4 min, 35 cycles of DNA denaturation at 95°C for 35s, annealing at 59°C for 35seconds, extension at 72°C at 50s and final extension at 72°C for 4 min and holding at 4°C.

3.3.6e Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

3.3.7 Optimization of PCR cycle number

3.3.7a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.3.7b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.2.7c Serial dilutions

After measuring cell concentration of 10^8 CFU/ml, the decimal dilution was used for DNA extraction and detection of our target gene. This was used because our detection sensitivity showed positive even at this dilution (1CFU/PCR assay). The PCR was run at 32 – 36 cycles.

3.3.7d DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.3.6e PCR

The PCR was performed as follows; initial denaturation at 95°C for 4 min, 31-35 cycles of DNA denaturation at 95°C for 35 s, annealing at 59°C for 35 s, extension at 72°C at 50 s and final extension at 72°C for 4 min and holding at 4°C.

3.3.7e Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

3.4 Optimization of Ethidium Monoazide - PCR Assay

3.4.1 Determination of the maximum concentration of EMA not inhibiting PCR from viable cells.

3.4.1a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.4.1b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method. Varying quantities of EMA (1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 μg) were added to the cell suspensions and the tubes placed in the dark at room temperature. The tubes were then set into chipped ice, with their lids off, and exposed to the light from a type T-halogen lamp to activate and photolyze the EMA.

3.4.1c DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.4.1d PCR

The PCR was performed as follows; initial denaturation at 95°C for 4 min, 35 cycles of DNA denaturation at 95°C for 35 s, annealing at 59°C for 35 s, extension at 72°C at 50 s and final extension at 72°C for 4 min and holding at 4°C.

3.4.1e Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

3.4.2 Determination of the minimum concentration of EMA inhibiting amplification of DNA from dead cells

3.4.2a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.4.2b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.4.2c Heat treatment of bacterial cells

Microcentrifuge tubes containing cell suspensions were heated at 95 °C in a water bath for 5 min. After heat treatment, different quantities of EMA (0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, and 5 μg) were added into corresponding tubes. Post-step followed the method described above.

3.4.2d DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.4.2e PCR

Refer to previous method (3.4.1d) as nothing changed prior to this method

3.4.2f Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

3.4.3. DNA amplification of the viable cells within a mixture of viable and dead cells by PCR.

3.4.3a Bacterial sample preparation and inoculation.

Refer to previous method (3.2.1a) as nothing changed prior to this method

3.4.3b Obtaining Optical density

Refer to previous method (3.3.2) as nothing changed prior to this method

3.4.3c Serial dilutions and EMA treatment

Suspension of viable cells in microcentrifuge tubes containing 1 x 10^2, 3.2 x 10^2, 1 x 10^3, 3.2 x 10^3, 1 x 10^4, 3.2 x 10^4, and 1 x 10^5 CFU respectively were mixed with suspension of dead cells. The total number of viable plus dead cells was kept constant at 1 x 10^5 CFU. The viable-dead cell mixtures were then treated with EMA at a final concentration of 1.0 μg/ml.

3.4.3d DNA Extraction

Refer to previous method (3.3.3) as nothing changed prior to this method

3.4.3e PCR

Refer to previous method (3.4.1d) as nothing changed prior to this method

3.4.3f Gel Electrophoresis and Visualization of PCR products

Refer to previous method (3.3.4d) as nothing changed prior to this method

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CHAPTER 4

RESULTS AND DISCUSIONS

RESULTS

4.1 Optimization of PCR Assay for the Detection of *Pseudomonas* spp using Genus Universal Primers

4.1.1 Gradient PCR for Annealing temperature optimization.

Each of the five *Pseudomonas* species were run at different annealing temperatures. *Pseudomonas aeruginosa* (figure 1), *P. fluorescens* (figure 2) *P. lurida* (figure 3) *P. putida* (figure 4), *P. psychrophila* (figure 5) *and* mixed culture (figure 6) The primers results showed positive bands at temperatures 54- 63°C. Some species (*P. aeruginosa* and mixed culture) had positive band detection at even 64°C and 65°C (no band) 59°C had the brightest relative fluorescence band compared to all other bands.

4.1.2 Optimization of PCR Extension Time.

Results for the different extension time as seen in all five species *Pseudomonas aeruginosa* (figure 7), *P. fluorescens* (Figure 8) *P. lurida* (figure 9) *P. putida* (figure 10), *P. psychrophila* (figure 11) and mixed culture (figure 12) showed positive bands at 60 to 30 s. 45 and 50 s that had the brightest fluorescence intensity and were considered to be the optimum primer extension time of the Taq polymerase enzyme and was used throughout the rest of this study.

4.1.3 PCR Assay Detection sensitivity

For the detection sensitivity, different DNA concentration were used as the PCR detection sensitivity of our gene target ranging from 10^5 – 1 CFU/PCR. Our results of all five species and the mixed culture shows a detection of up to 1 CFU/PCR assay. Figures 13-18 (*P. aeruginosa, P. fluorescens, P. lurida, P. putida, P. psychrophila,* and *P.* mixed culture)

respectively shows positive bands at 10^5 to 10^1 CFU/PCR (bright bands) and 10^0 CFU/PCR (faint band).

4.1.4 Optimization of PCR cycle number

The optimization of cycle number was determine using a DNA detection sensitivity of 1CFU/PCR for all five species and the mixed culture. The PCR was run at 32 – 36 cycles. Results shows DNA amplification of our target gene only at higher cycles. When the PCR was run at 32, 33 and 34 cycles, the was no PCR product seen (Figure 19, 20 and 21, respectively). Positive but faint bands could only be seen at 35 cycles (figure 22) and prominent and distinct bands seen at 36 cycles (figure 23).

4.2 Optimization of EMA - PCR Assay

4.2.1 Determination of the maximum concentration of EMA not inhibiting PCR from viable cells.

The maximum concentration of EMA that will not prevent the DNA amplification of viable cells was determined by treating the viable cells with a range of EMA concentration (1 ~ 50 µg/ml). We observe that when viable cells of four out of the five species (*P. aeruginosa, P. fluorescens, P. lurida, P. putida, P. psychrophila,*) and the mixed culture were treated with the EMA at concentration of 10 µg/ml or less, the was no significant inhibition of amplification of the target DNA coming from the viable cells (figures 24, 25, 26, 27 28, and 29, respectively). The was significant ($P < 0.05$) reduction in the amplification of the target DNA of viable cells when the concentration of EMA was above 30 µg/ml and increasing the concentration of EMA above 30 µg/ml caused a proportional decrease in the amplification of target DNA of viable cells. Distinct inhibition of target DNA amplification could be seen at concentration of 40 and 50 µg/ml for *P. aeruginosa, P. fluorescens, P. lurida, P. putida* and mixed culture. The was

complete inhibition of target DNA amplification from viable cells of *P. psychrophila* at concentration of 20 µg/ml and above (figure 28).

4.2.2 Determination of the minimum concentration of EMA inhibiting amplification of DNA from dead cells.

The amplification of our target DNA from heat killed cells was completely inhibited when the cells were treated with EMA concentration of 1 µg/ml or higher as seen in figures 30-35. We observed that the target DNA from the heat killed cells were amplified when the EMA concentration was 0.2 to 0.8 µg/ml.

4.2.3 DNA amplification of viable cells within a mixture of viable and dead cells by PCR

Suspension of viable cells of all five species of *Pseudomonas* in microcentrifuge tubes containing 1.0×10^2, 3.2×10^2, 1.0×10^3, 3.2×10^3, 1.0×10^4, 3.2×10^4, and 1.0×10^5 CFU/PCR respectively were mixed with suspension of dead cells. The total number of viable plus dead cells was kept constant at 1.0×10^5 CFU. At different ratio of viable and dead cells using 1 µg/ml EMA concentration, the was a steady decrease in the fluorescence band intensity, an indication of decrease amplification of our DNA target gene (Figure 36). At live cells concentration of 1.0×10^5 CFU, we saw the brightest band. As the live ratio in the live-dead mixture decrease from 32% (figure 36, lane 2) to 0.1% (figure 36, lane 7), the was a steady decrease in the DNA amplification of our target gene as more DNA templates were deriving from the dead cells.

Table 1. Five *Pseudomonas* species used in this Study

Pseudomonas species	Designation
Pseudomonas aeruginosa	ATCC 10145
Pseudomonas fluorescens	Carolina Bio. 155255
Pseudomonas lurida	P20 (wild type)
Pseudomonas putida	Carolina Bio. 135525
Pseudomonas psychrophila	P123 (wild type)

Table 2. Optimized PCR Protocol using the Pseudomonas universal Primers

PCR Steps	Temperature (°C)	Period	
Initial Denaturation	95	4 Min	
Denaturation	95	35 s	35 Cycles
Annealing	59	35 s	
Extension	72	50 s	
Final extension	72	4 Min	
Holding	4	∞	

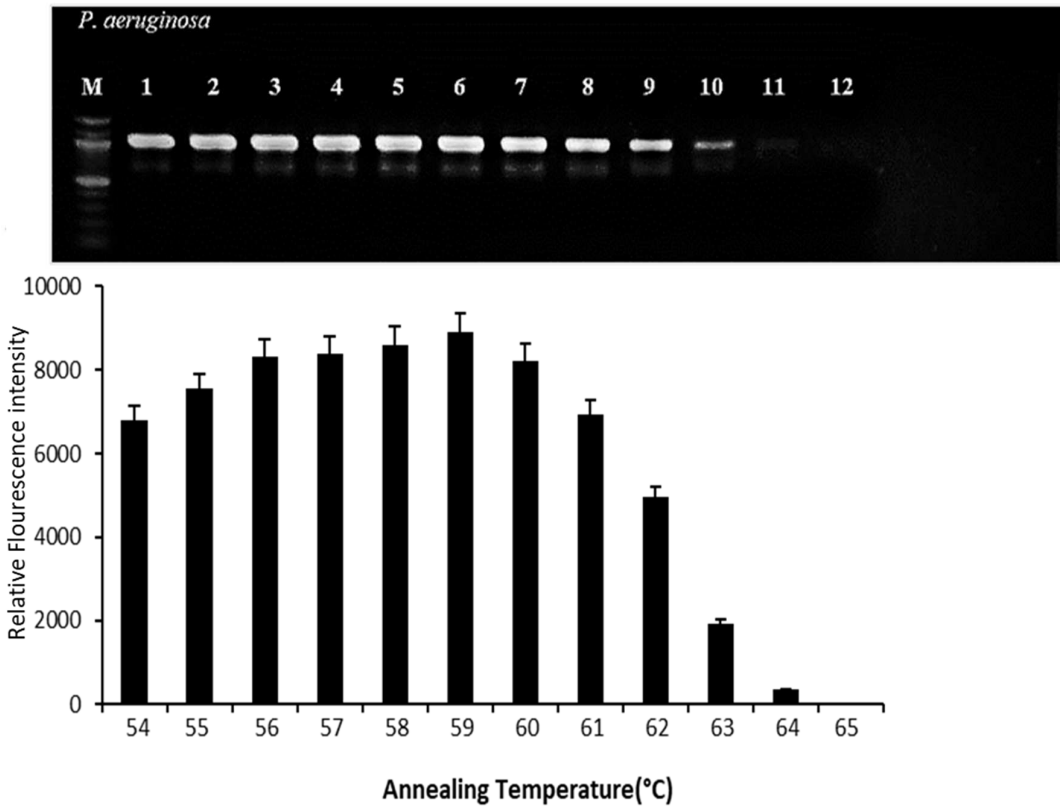

Figure 1. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas aeruginosa.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the temperature at 54- 65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

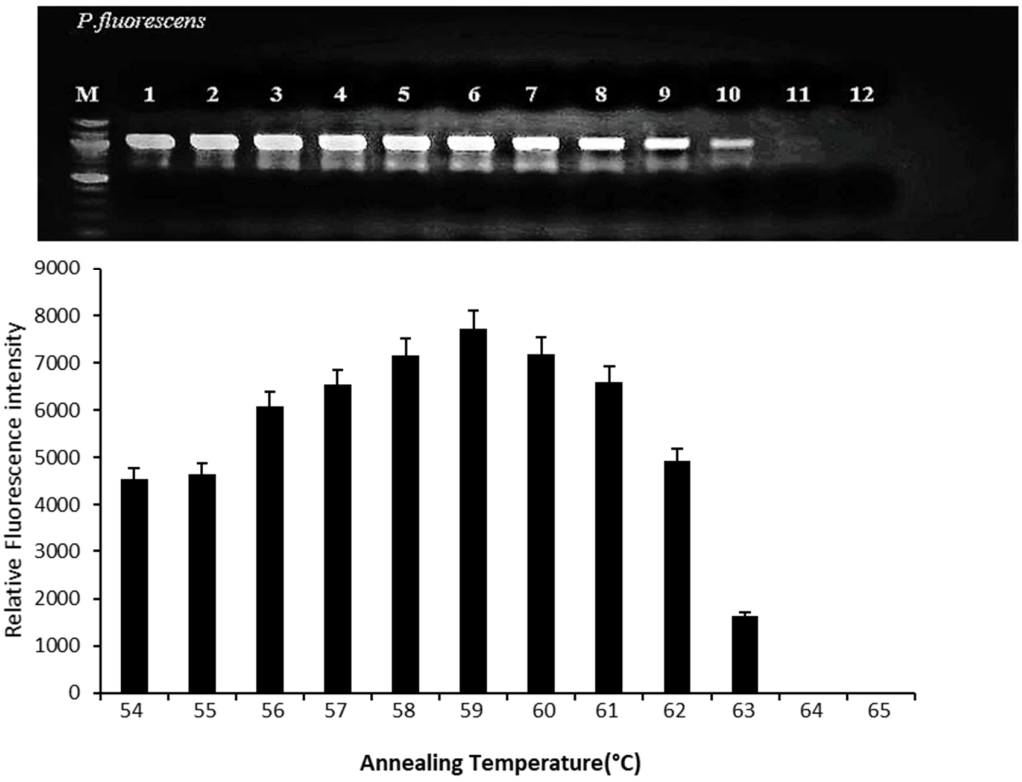

Figure 2. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas fluorescens.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the temperature at 54-65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

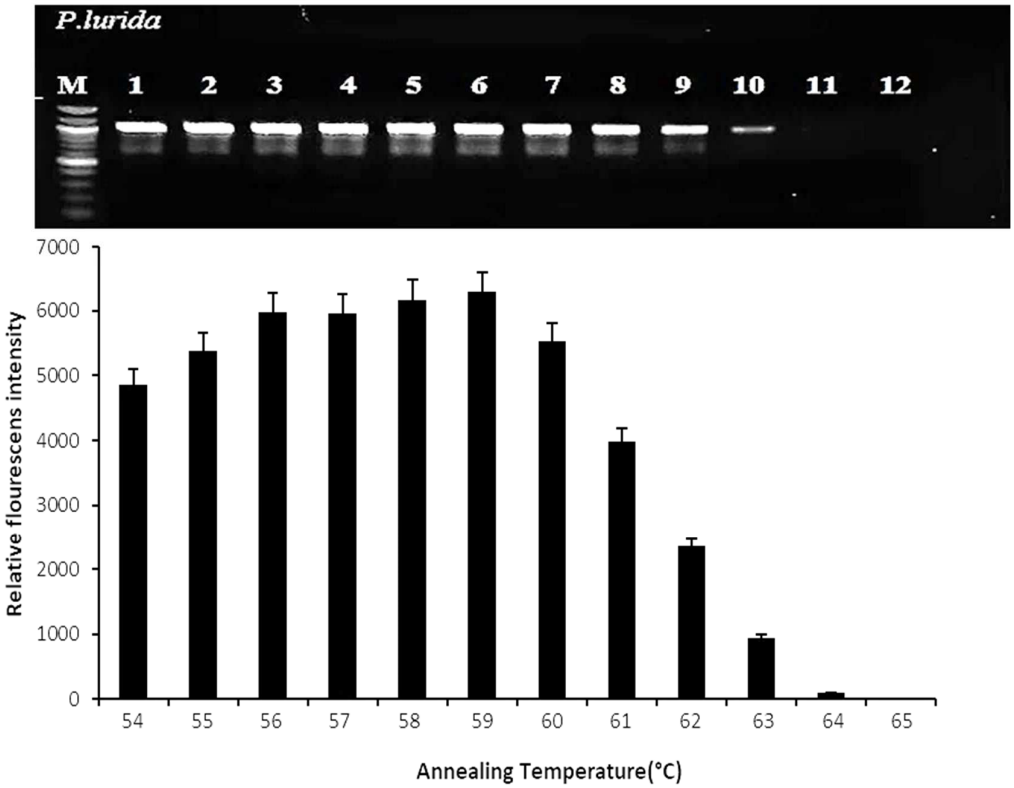

Figure 3. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas lurida.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the temperature at 54-65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

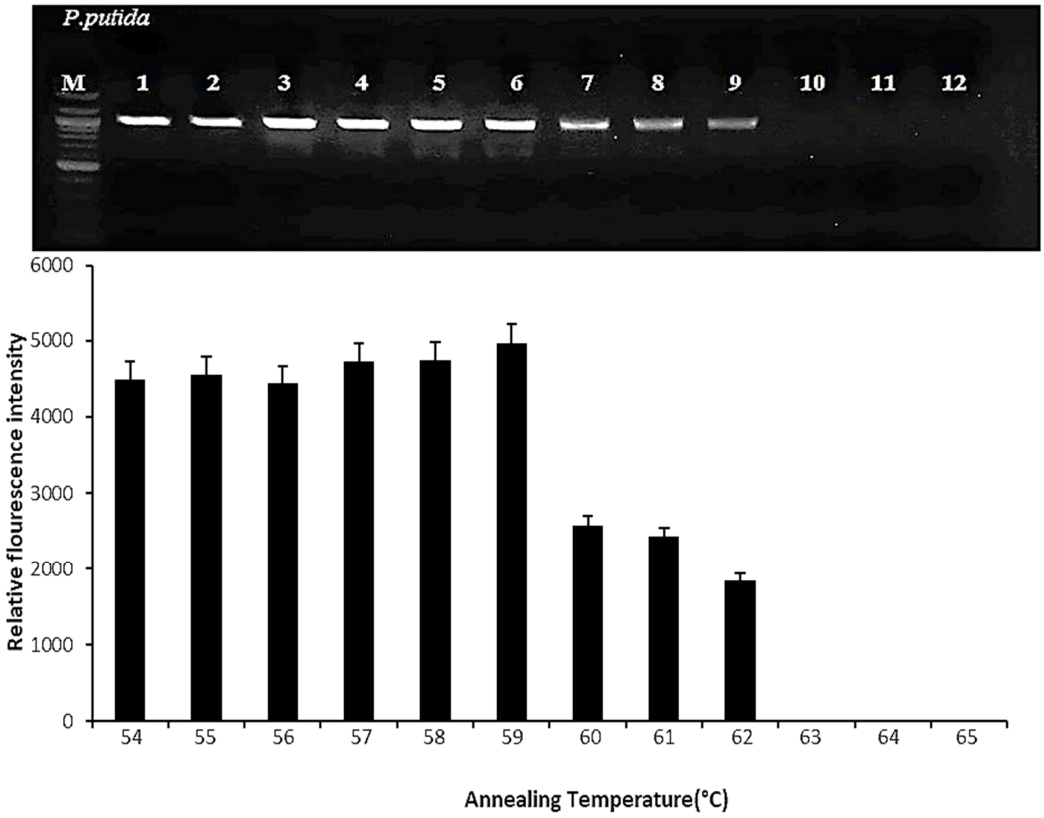

Figure 4. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas putida.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the temperature at 54-65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

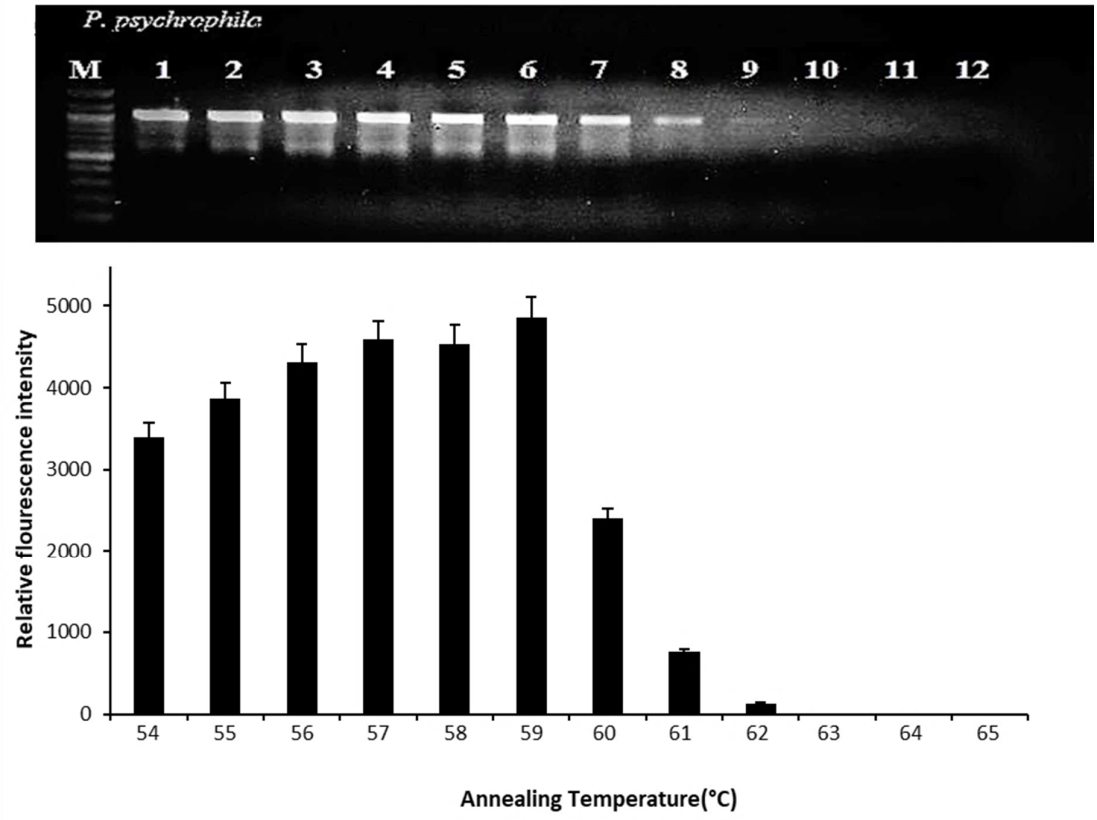

Figure 5. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas psychrophila.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the temperature at54-65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

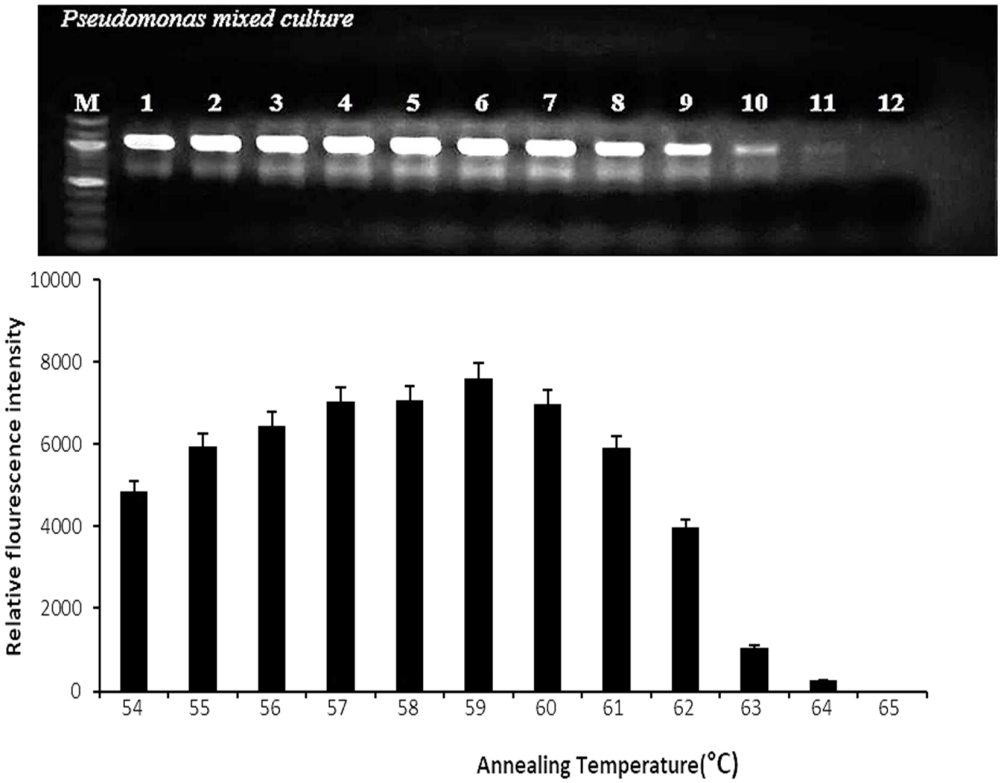

Figure 6. Gradient PCR for optimization of PsUN-F/R annealing temperature using *Pseudomonas mixed culture.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-12 corresponds to the annealing temperature at 54- 65°C. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding annealing temperatures.

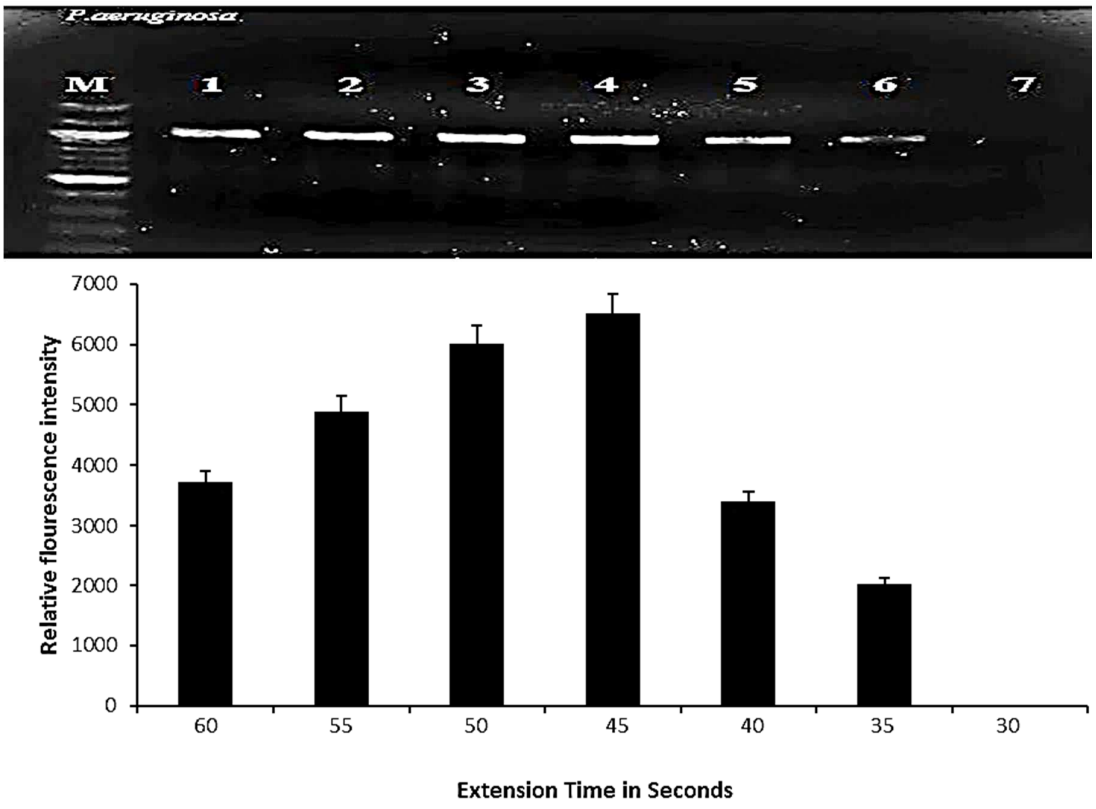

Figure 7. Optimization of PCR extension time using *Pseudomonas aeruginosa.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 60-30 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

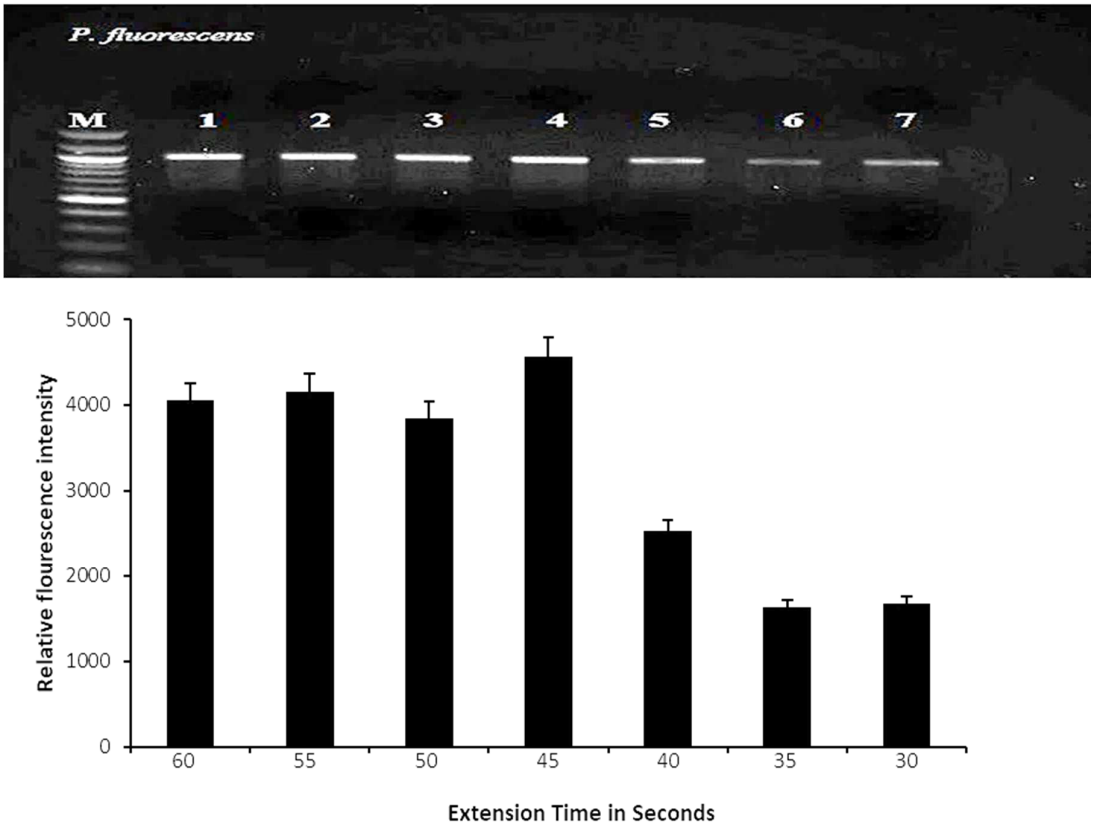

Figure 8. Optimization of PCR extension time using *Pseudomonas fluorescens.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 60-30 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

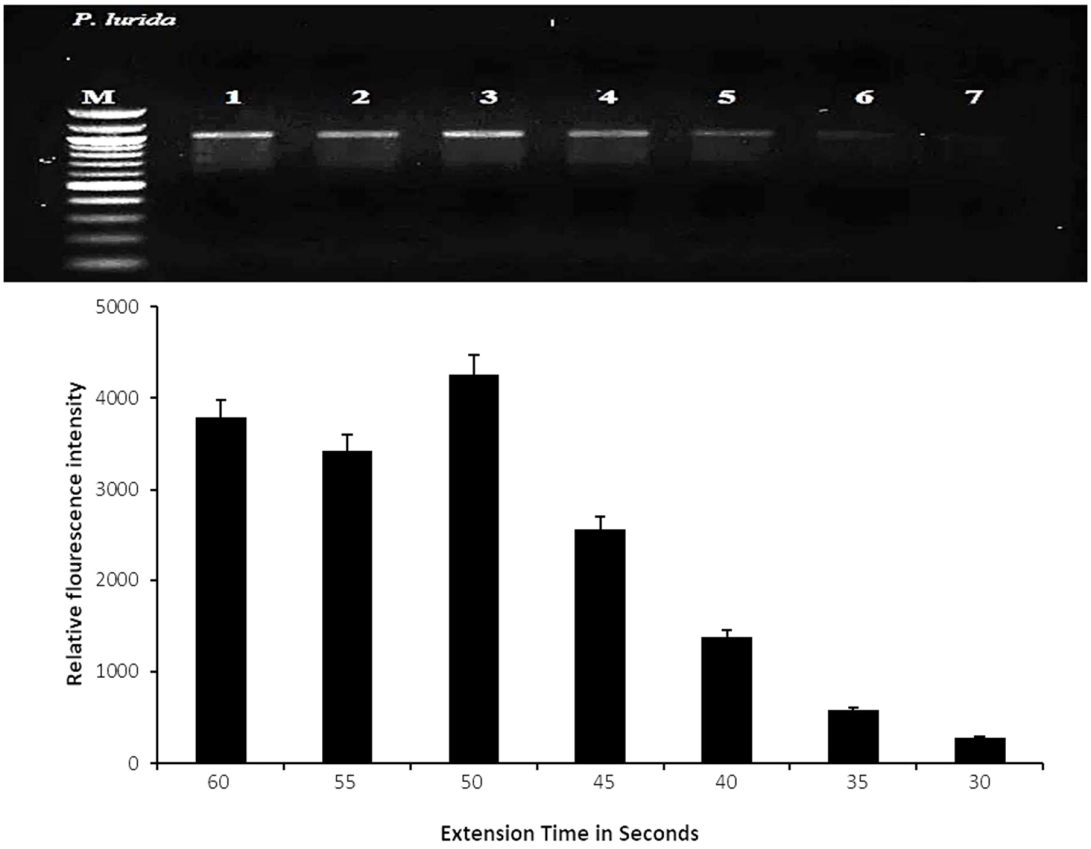

Figure 9. Optimization of PCR extension time using *Pseudomonas lurida.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 60-30 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

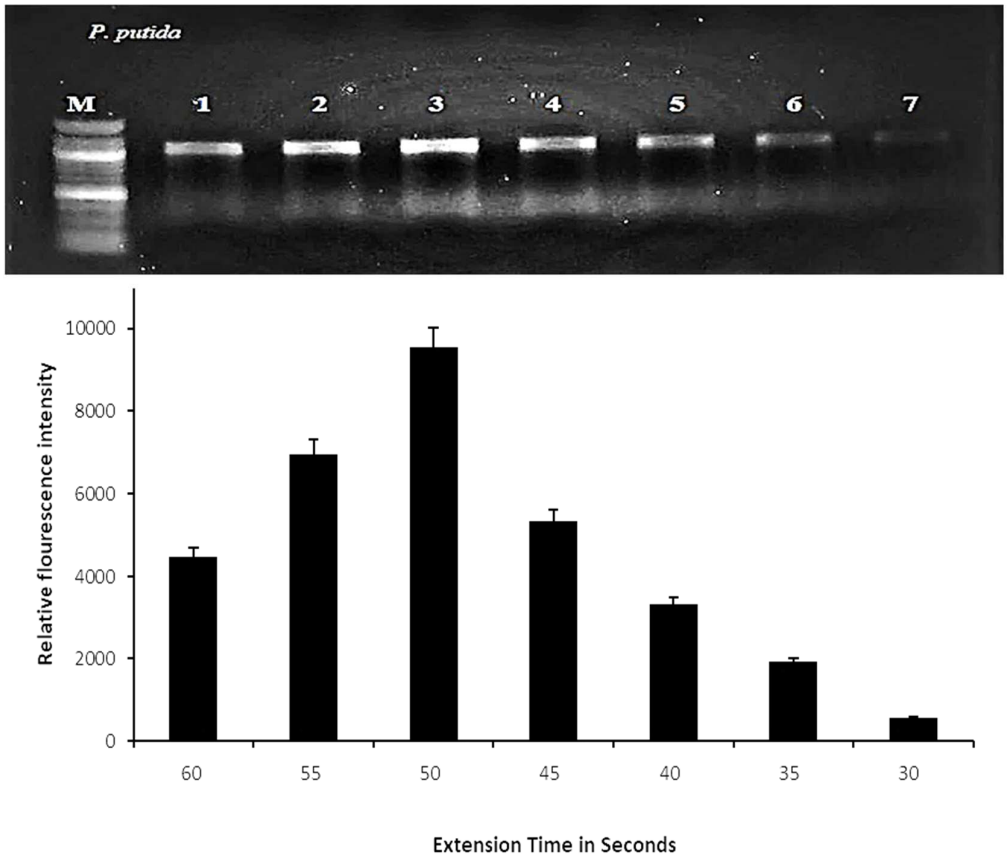

Figure 10. Optimization of PCR extension time using *Pseudomonas putida.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 6030 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

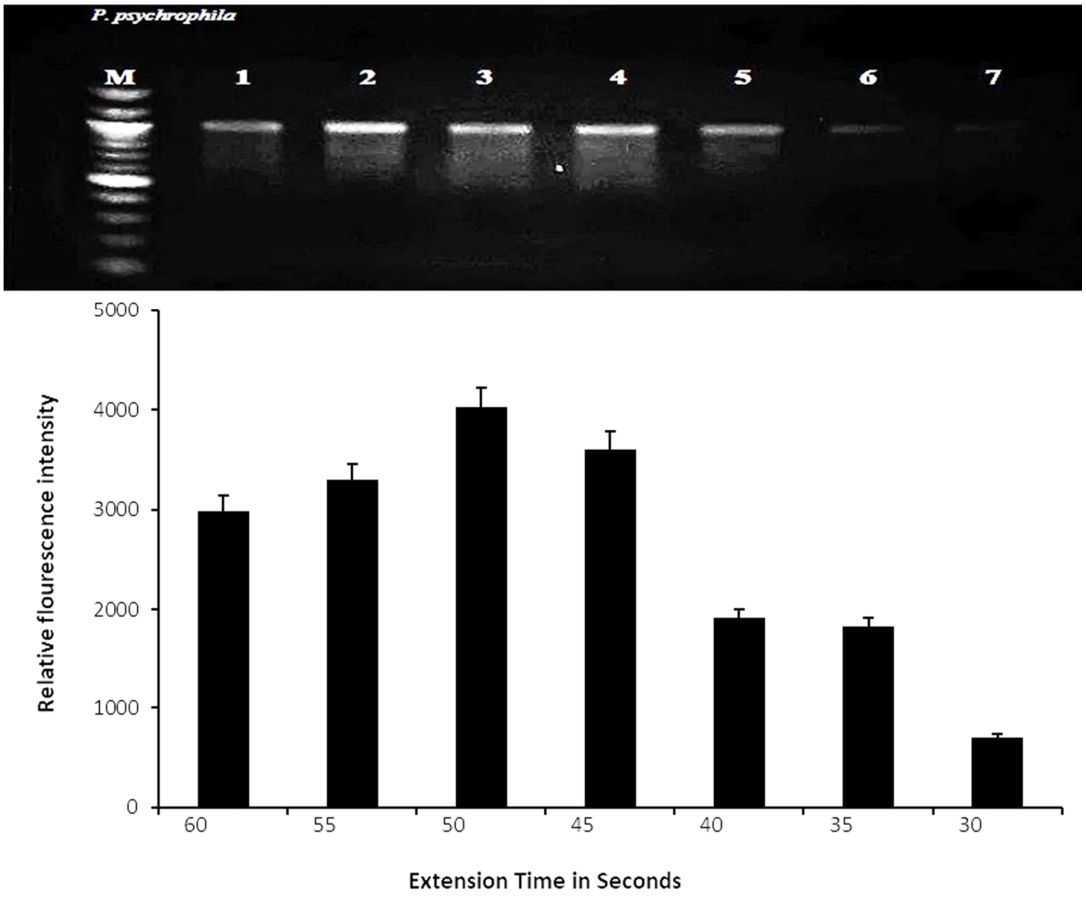

Figure 11. Optimization of PCR extension time using *Pseudomonas psychrophila.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 60-30 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

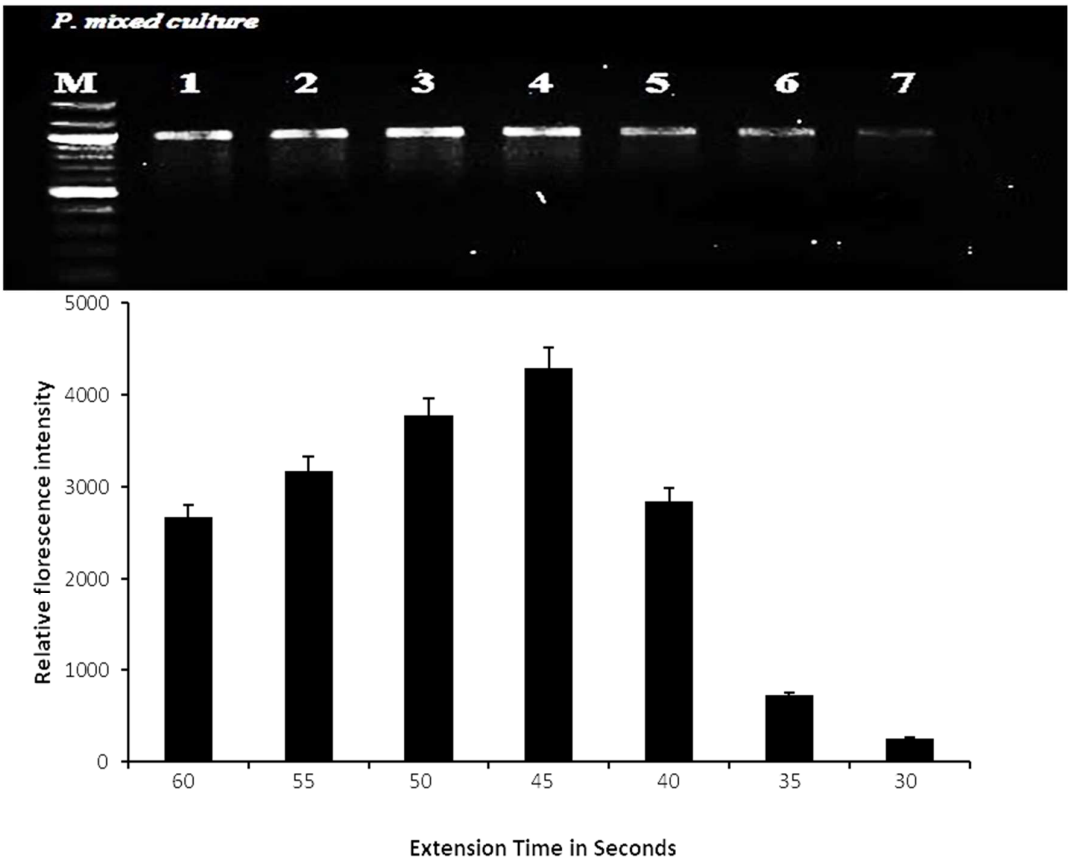

Figure 12. Optimization of PCR extension time using *Pseudomonas mixed culture.* Top: typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, the lane #1-7 corresponds to the extension time at 60-30 seconds. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding extension time.

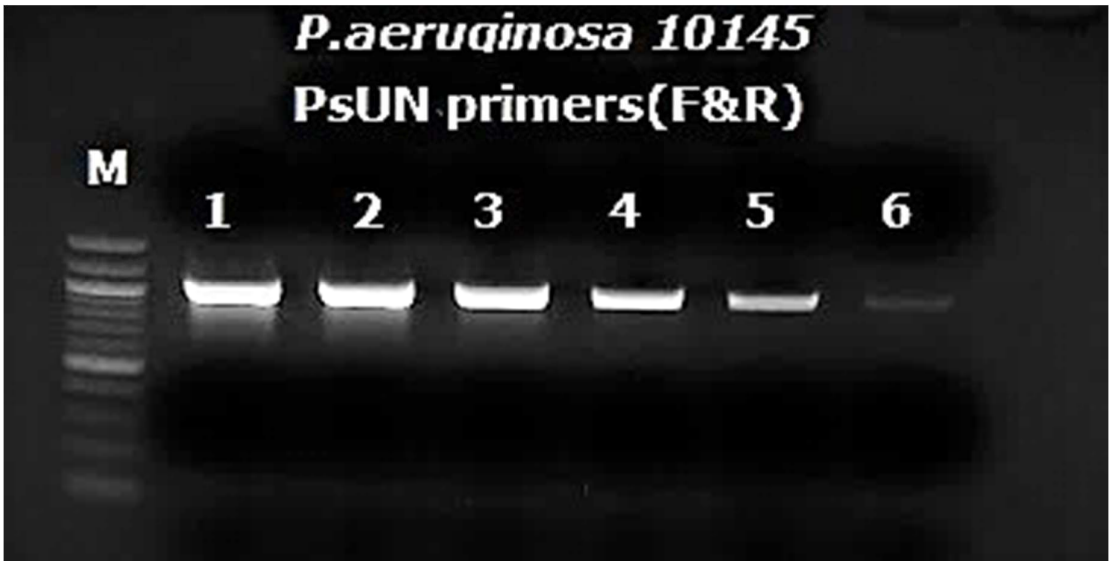

Figure 13. Determination of PCR assay detection sensitivity of *Pseudomonas aeruginosa* using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

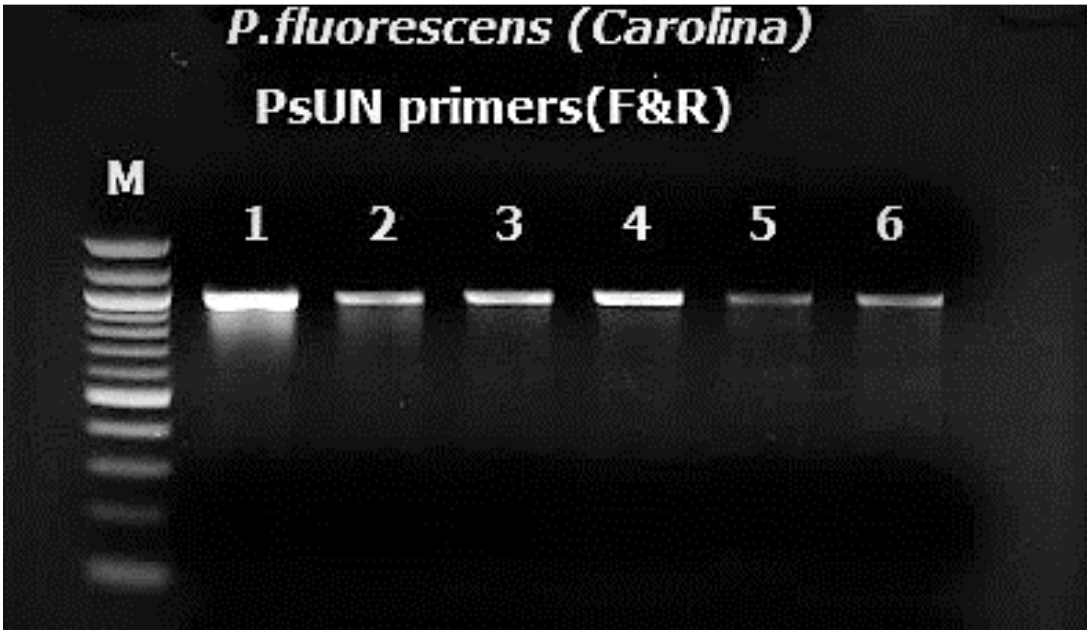

Figure 14. Determination of PCR assay detection sensitivity of *Pseudomonas fluorescens* using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

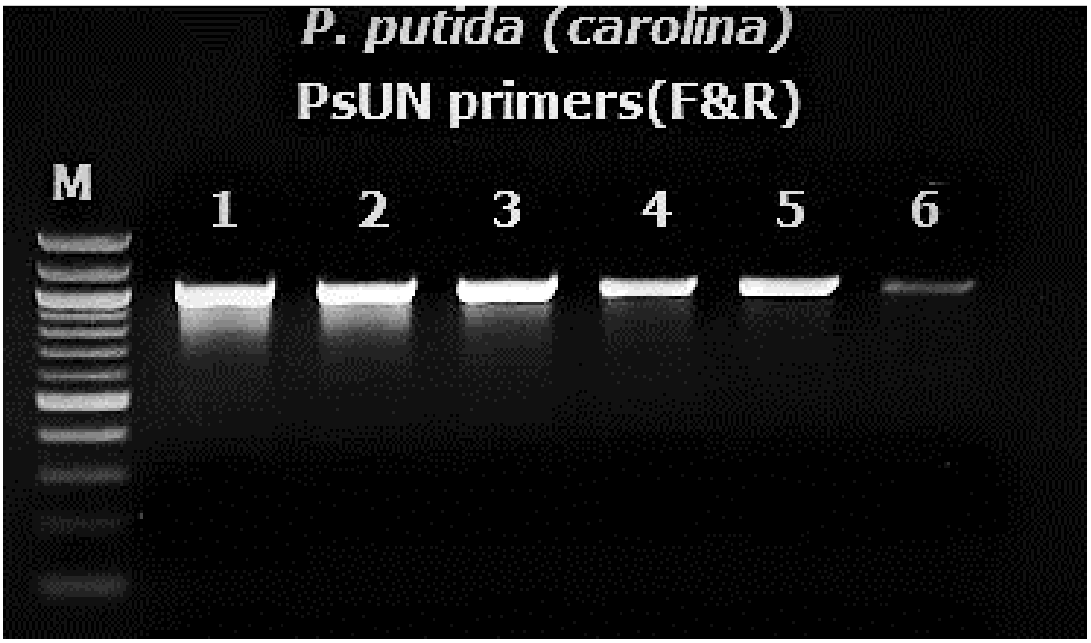

Figure 15. Determination of PCR assay detection sensitivity of *Pseudomonas putida* using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

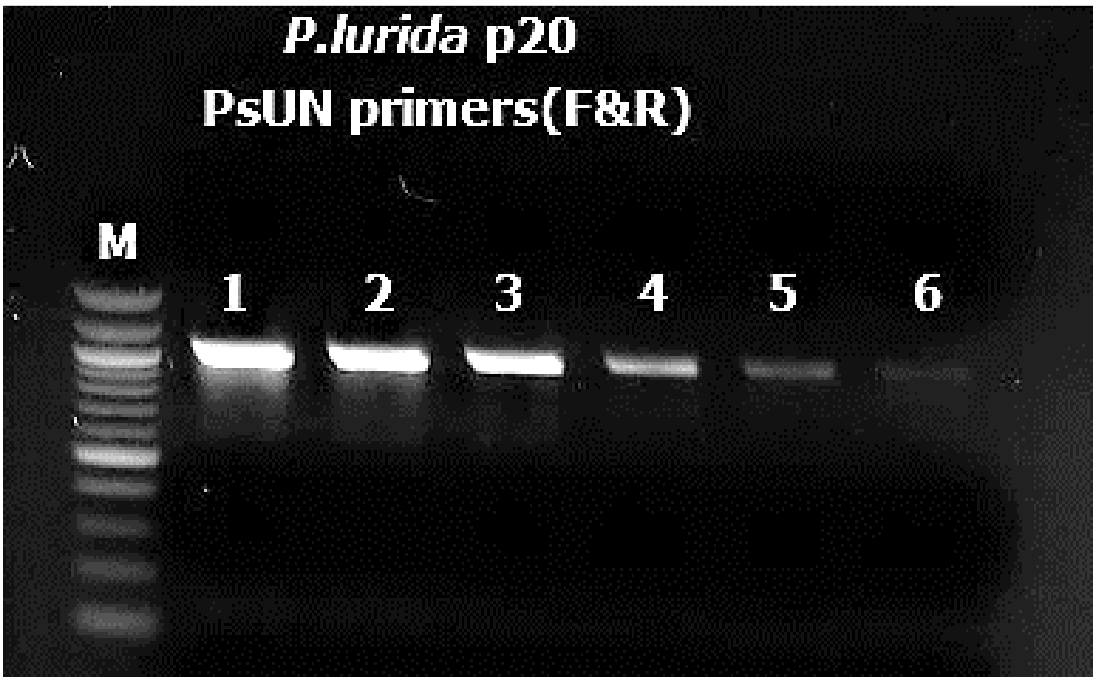

Figure 16. Determination of PCR assay detection sensitivity of *Pseudomonas lurida* using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

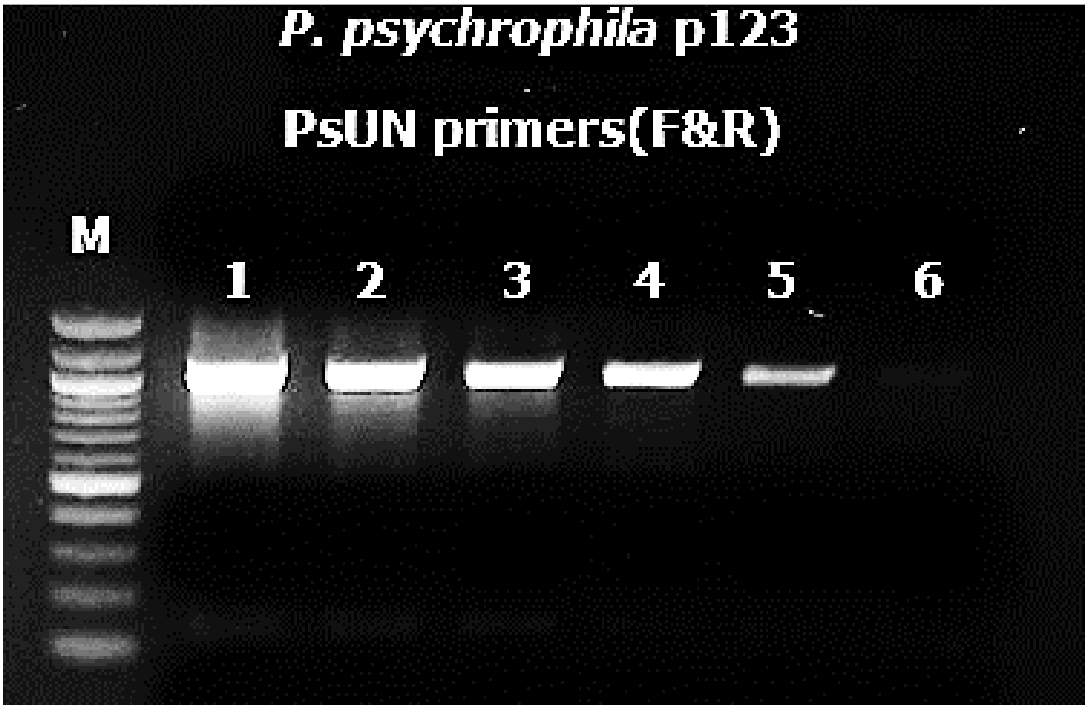

Figure 17. Determination of PCR assay detection sensitivity of *Pseudomonas psychrophila* using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

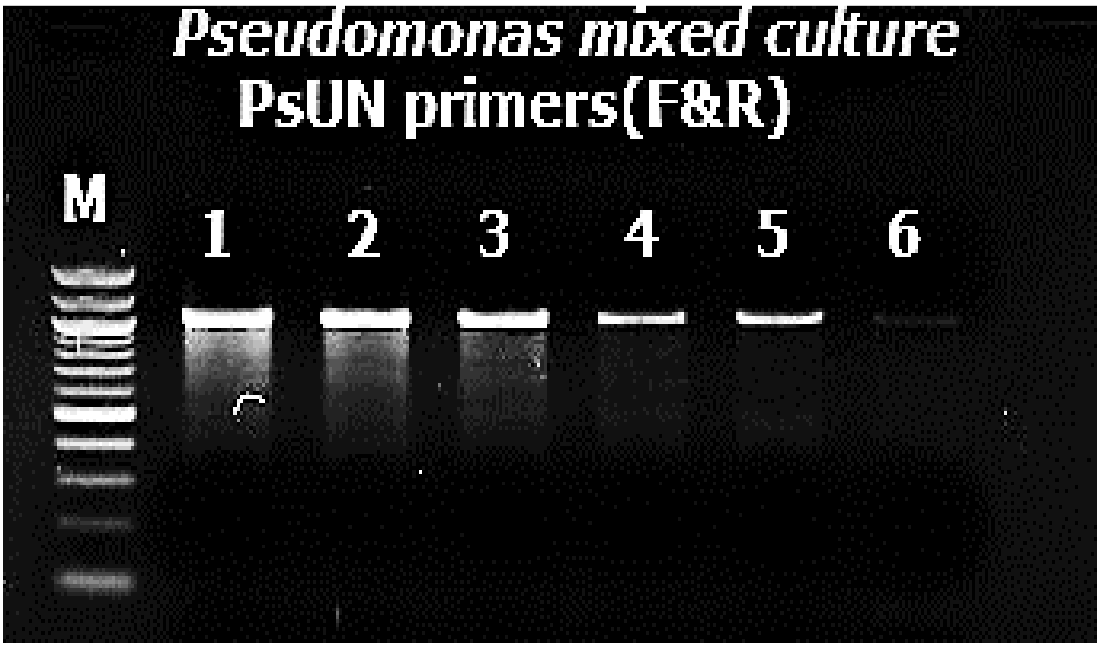

Figure 18. Determination of PCR assay detection sensitivity of *Pseudomonas* mixed culture using PsUN F/R. Typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, lane 1: 1.0 x 10^5, lane 2: 1.0 x 10^4, lane 3: 1.0 x 10^3, lane 4: 1.0 x 10^2, lane 5: 1.0 x 10^1, and lane 6: 1.0 CFU/PCR.

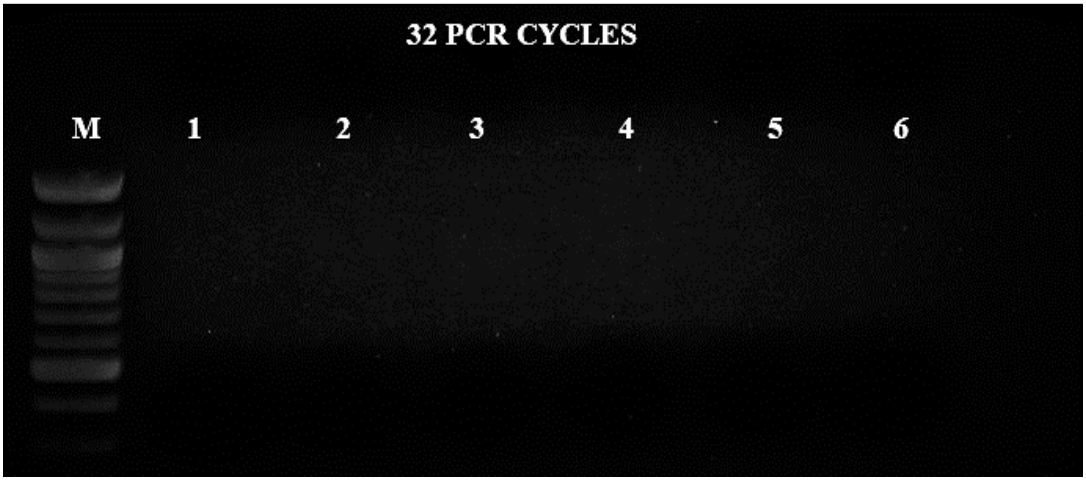

Figure 19. Optimization of PCR cycle number using DNA concentration of 1CFU/PCR as the detection sensitivity limit. The figure shows a typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, Lane 1: *Pseudomonas aeruginosa*, Lane 2: *Pseudomonas fluorescens*, Lane 3: *Pseudomonas lurida*, Lane 4: *Pseudomonas putida*, Lane 5: *Pseudomonas psychrophila*, Lane 6: *Pseudomonas* mixed culture

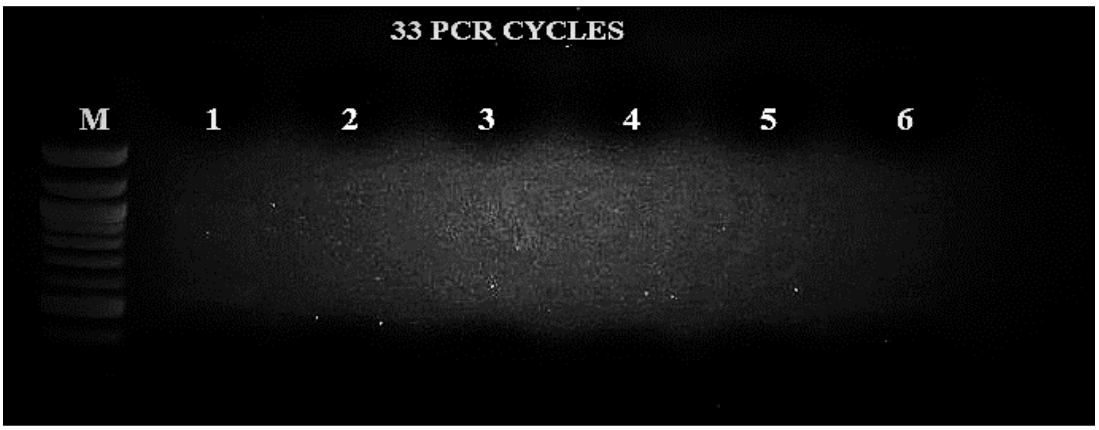

Figure 20. Optimization of PCR cycle number using DNA concentration of 1CFU/PCR as the detection sensitivity limit. The figure shows a typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, Lane 1: *Pseudomonas aeruginosa*, Lane 2: *Pseudomonas fluorescens*, Lane 3: *Pseudomonas lurida*, Lane 4: *Pseudomonas putida*, Lane 5: *Pseudomonas psychrophila*, Lane 6: *Pseudomonas* mixed culture

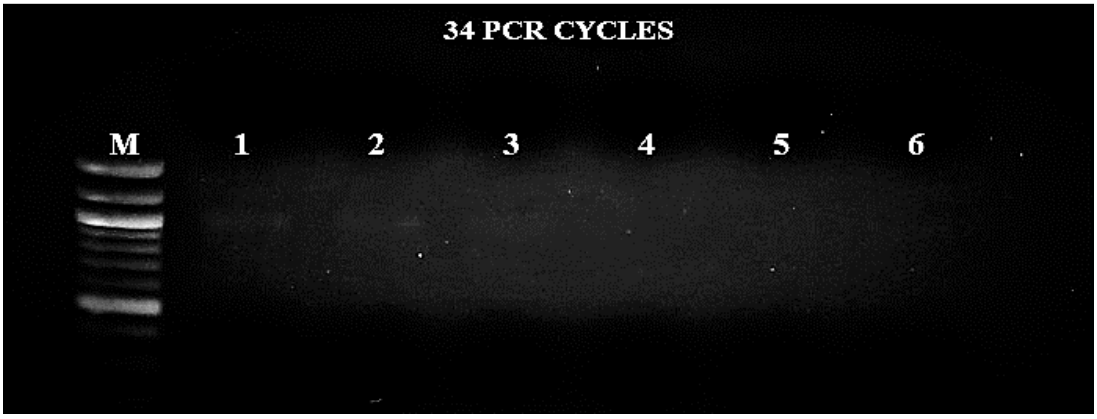

Figure 21. Optimization of PCR cycle number using DNA concentration of 1CFU/PCR as the detection sensitivity limit. The figure shows a typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, Lane 1: *Pseudomonas aeruginosa*, Lane 2: *Pseudomonas fluorescens*, Lane 3: *Pseudomonas lurida*, Lane 4: *Pseudomonas putida*, Lane 5: *Pseudomonas psychrophila*, Lane 6: *Pseudomonas* mixed culture

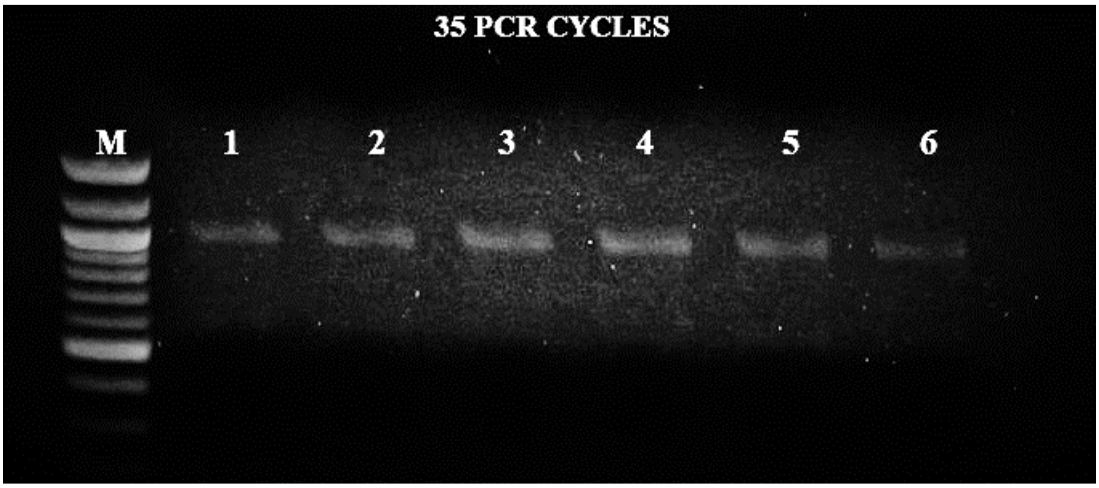

Figure 22. Optimization of PCR cycle number using DNA concentration of 1CFU/PCR as the detection sensitivity limit. The figure shows a typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, Lane 1: *Pseudomonas aeruginosa*, Lane 2: *Pseudomonas fluorescens*, Lane 3: *Pseudomonas lurida*, Lane 4: *Pseudomonas putida*, Lane 5: *Pseudomonas psychrophila*, Lane 6: *Pseudomonas* mixed culture

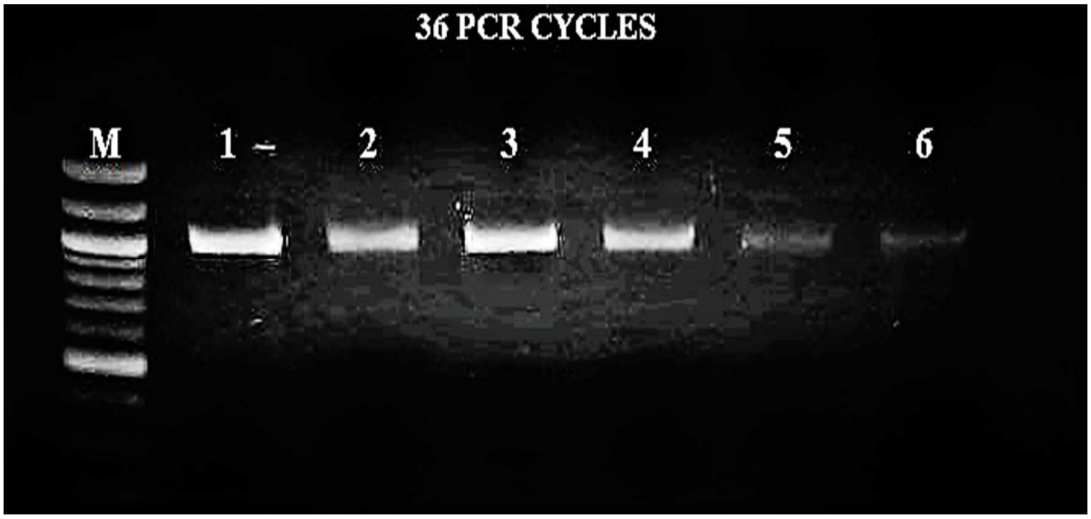

Figure 23. Optimization of PCR cycle number using DNA concentration of 1CFU/PCR as the detection sensitivity limit. The figure shows a typical agarose gel image of PCR amplified products. Lane M represents 100 bp DNA ladder, Lane 1: *Pseudomonas aeruginosa*, Lane 2: *Pseudomonas fluorescens*, Lane 3: *Pseudomonas lurida*, Lane 4: *Pseudomonas putida*, Lane 5: *Pseudomonas psychrophila*, Lane 6: *Pseudomonas* mixed culture

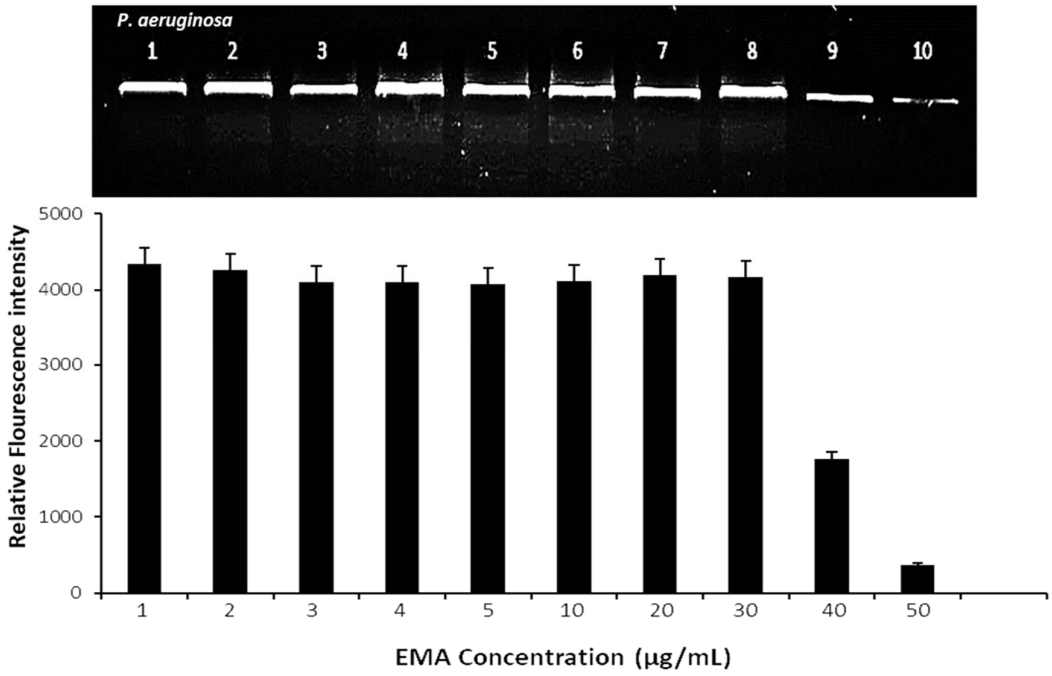

Figure 24. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas aeruginosa*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

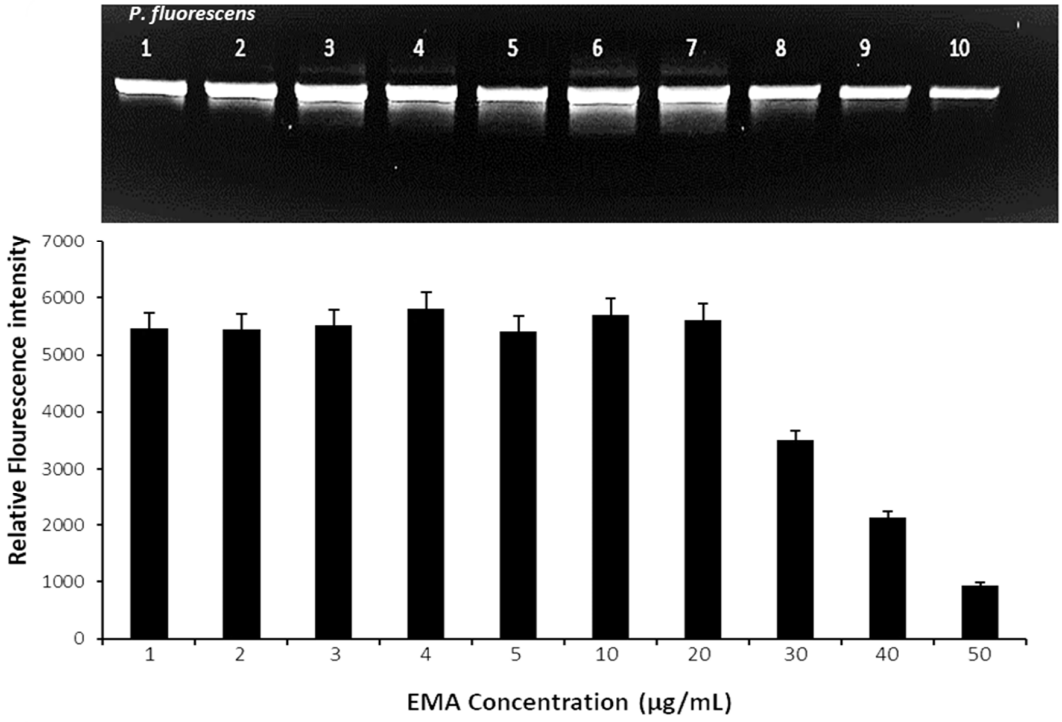

Figure 25. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas fluorescens*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

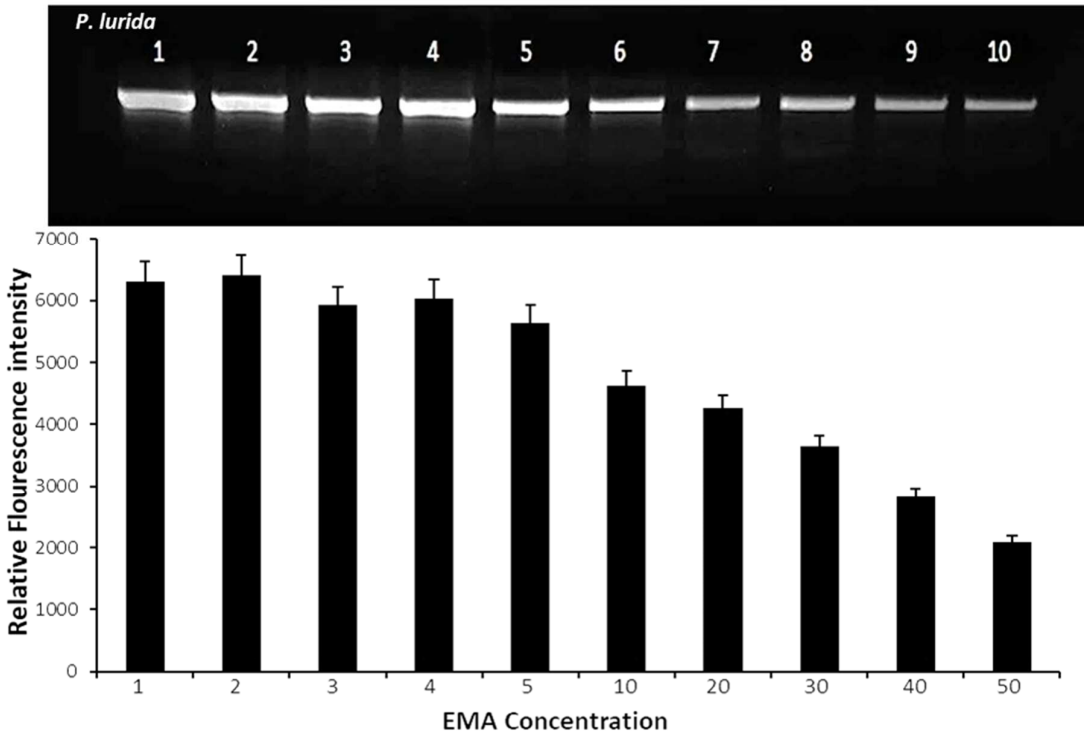

Figure 26. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas lurida*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

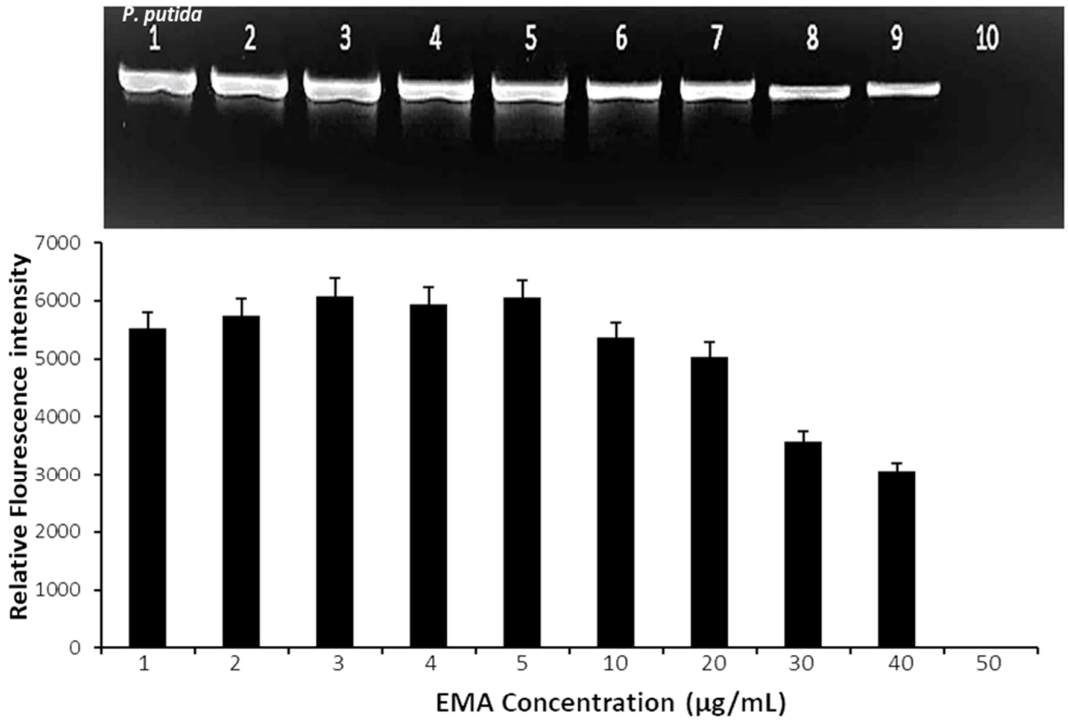

Figure 27. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas putida*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represent fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

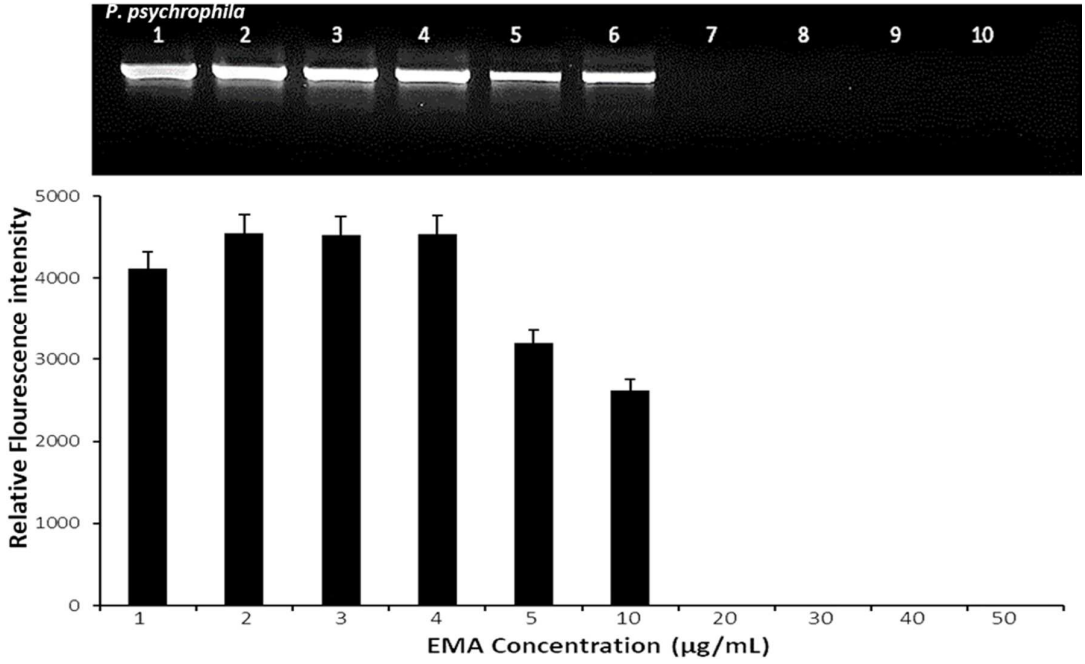

Figure 28. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas psychrophila*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

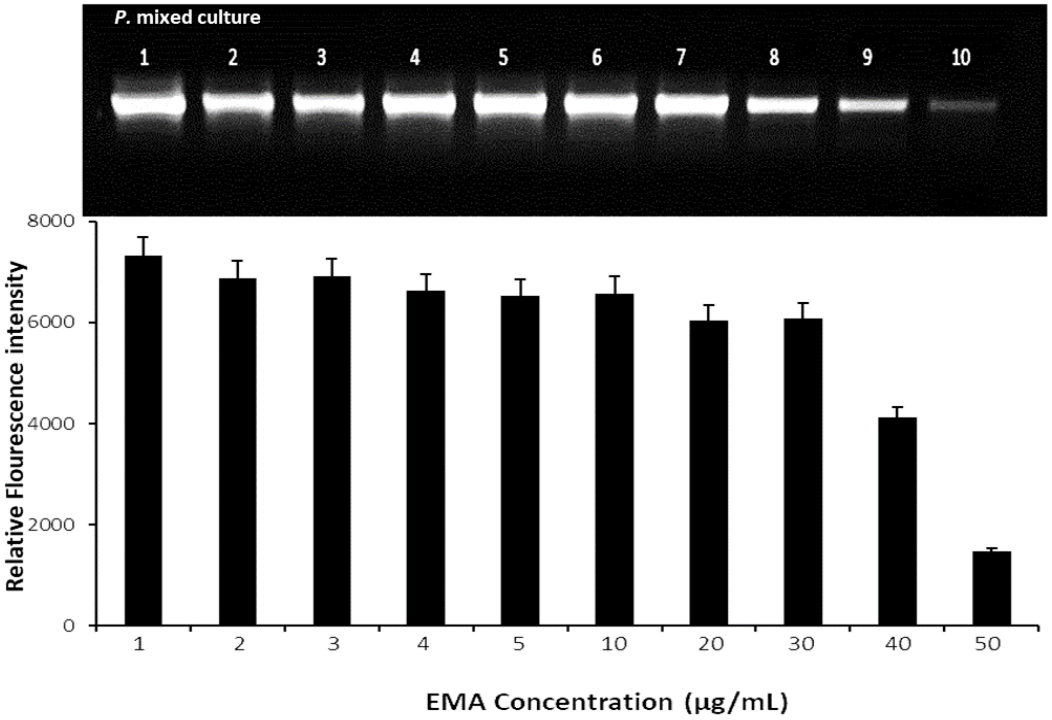

Figure 29. Optimization of the maximum concentration of EMA not inhibiting DNA amplification derived from viable cells of *Pseudomonas* mixed culture. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (1-50µg/ml respectively). Bottom: bar graph represent fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

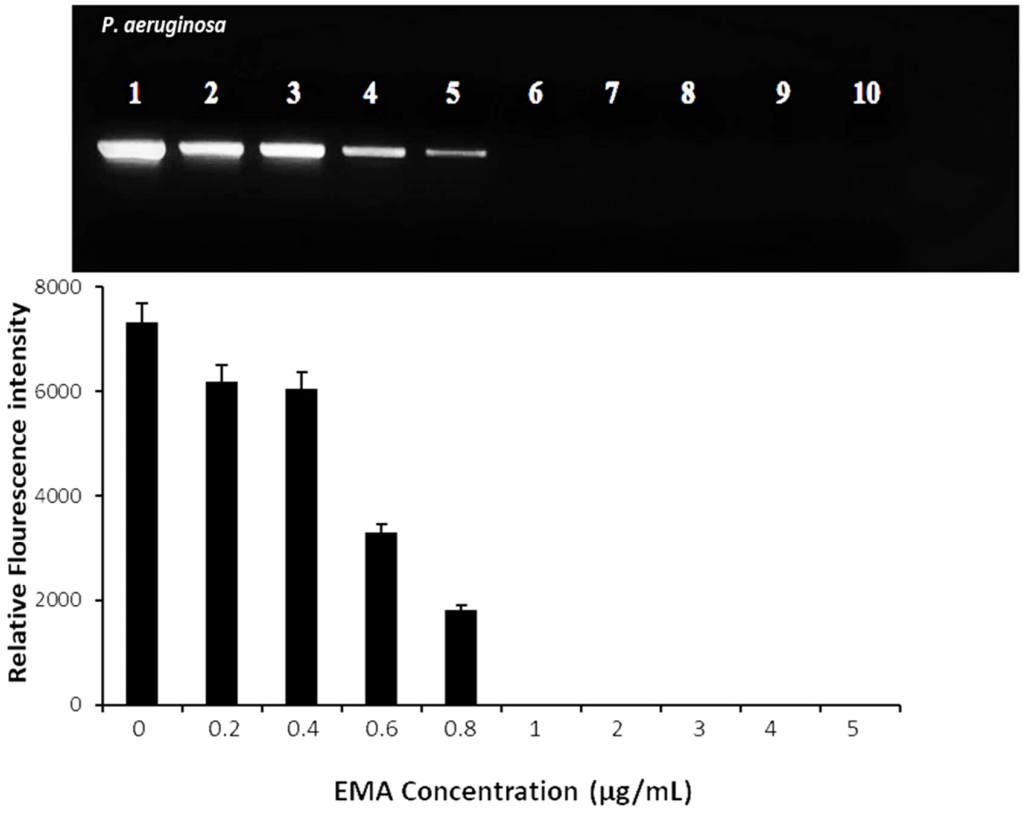

Figure 30. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas aeruginosa*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA.

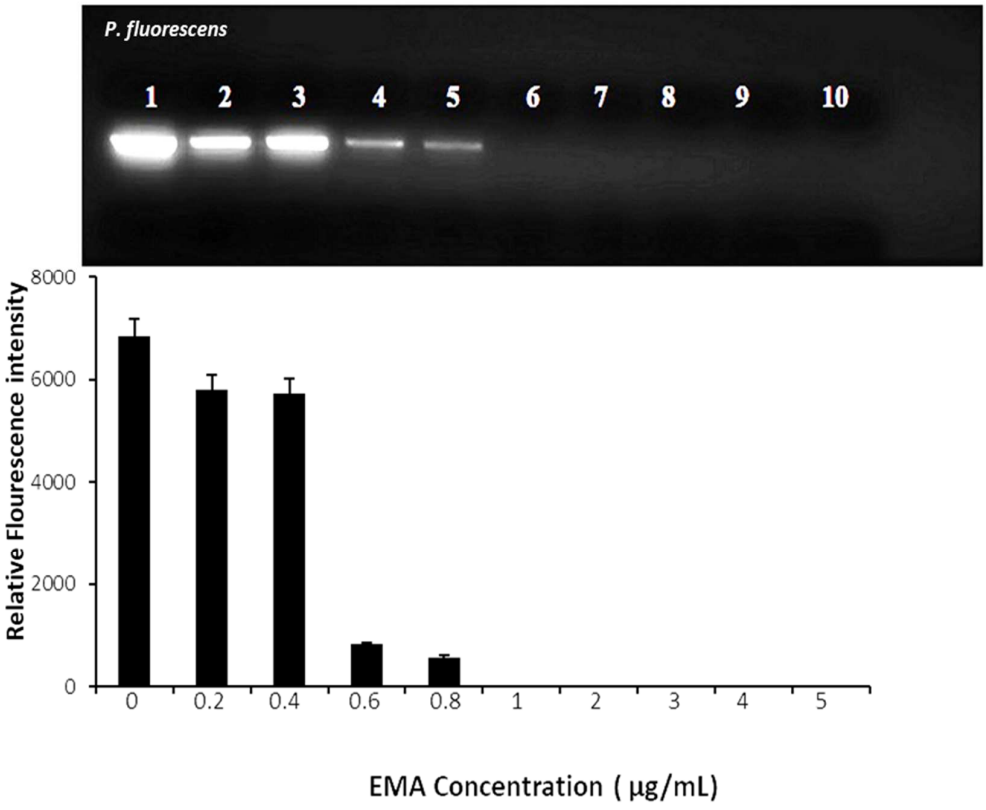

Figure 31. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas fluorescens*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA

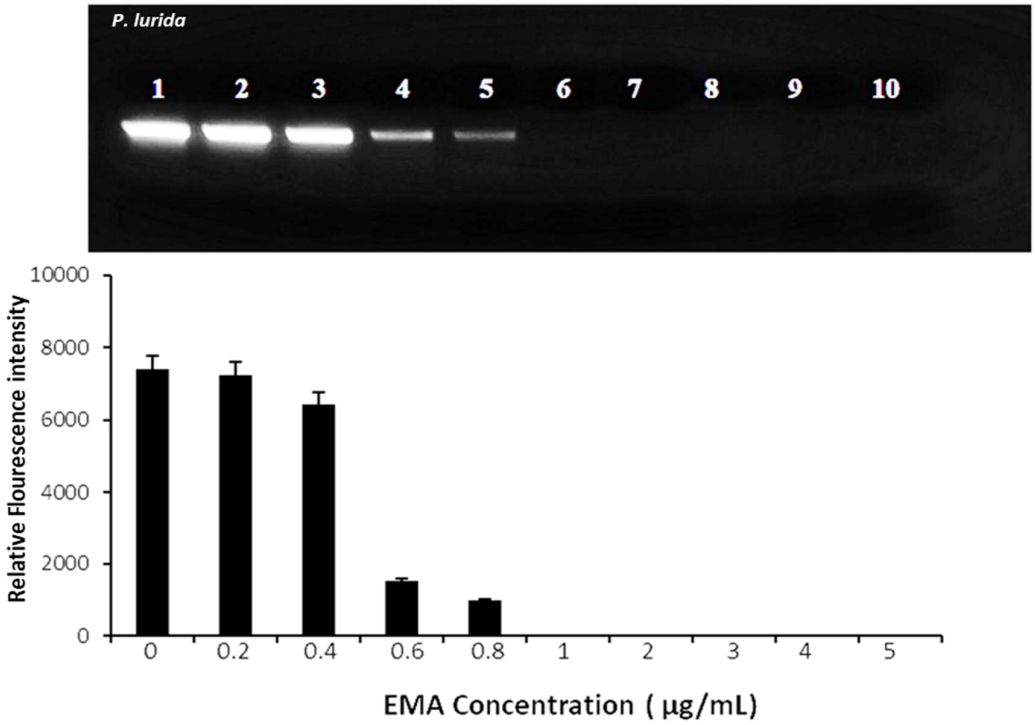

Figure 32. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas lurida*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA

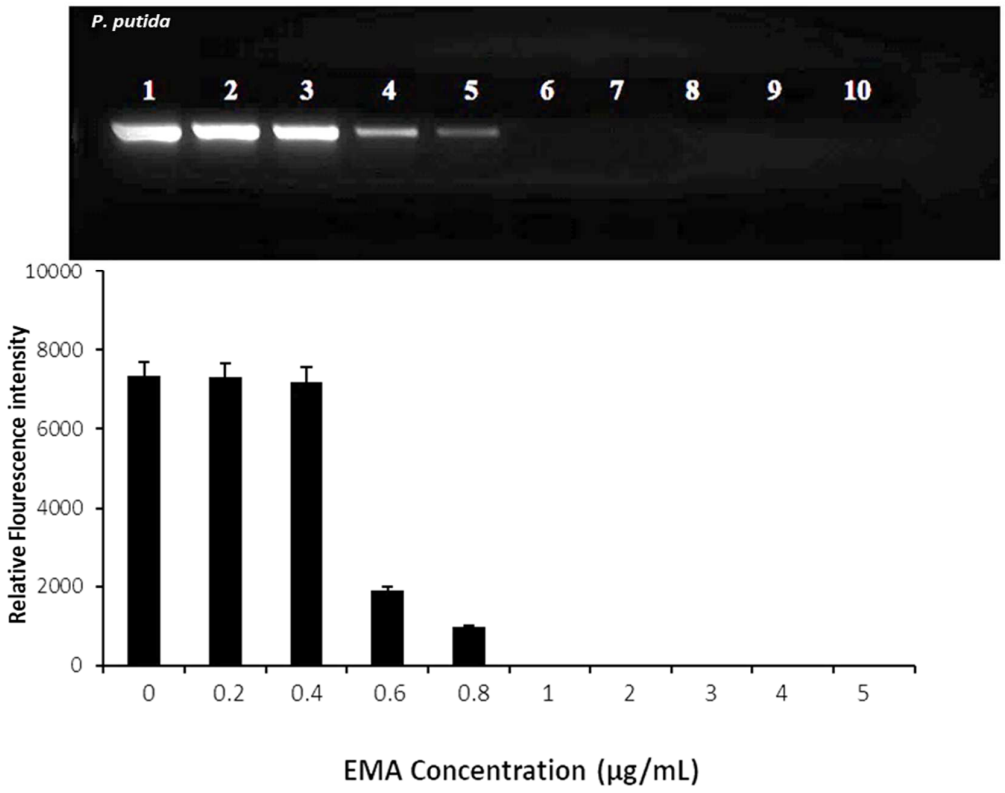

Figure 33. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas putida*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0.2-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA

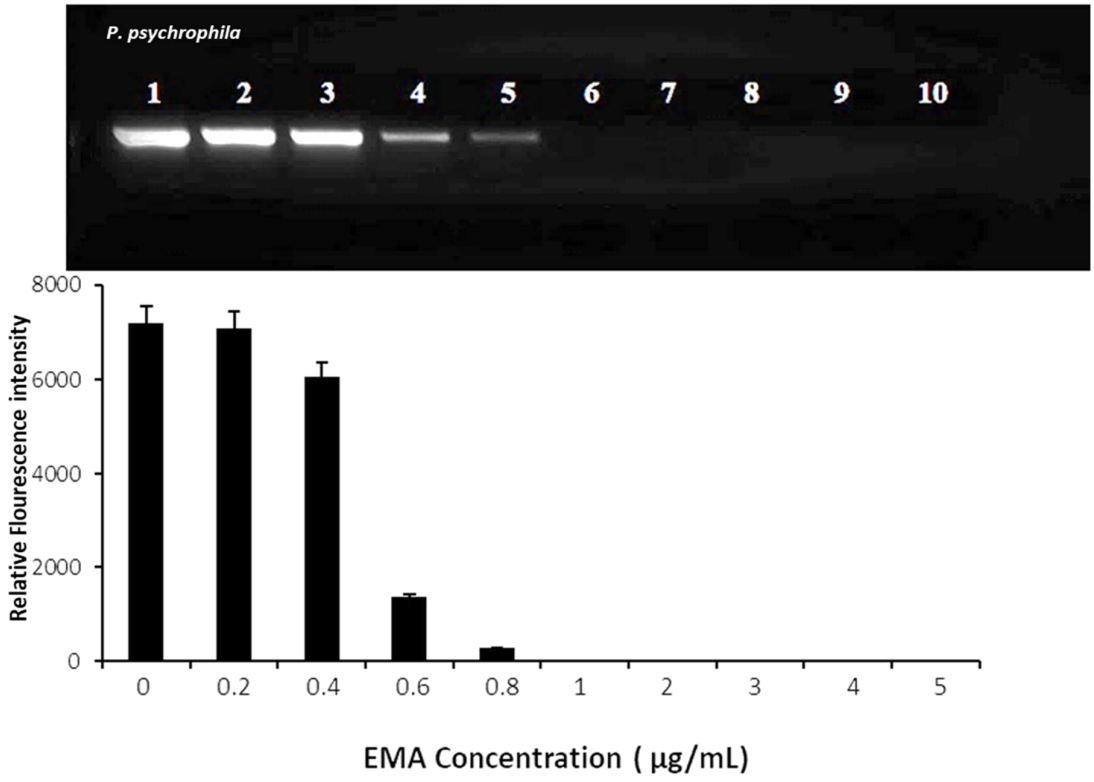

Figure 34. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas psychrophila*. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA

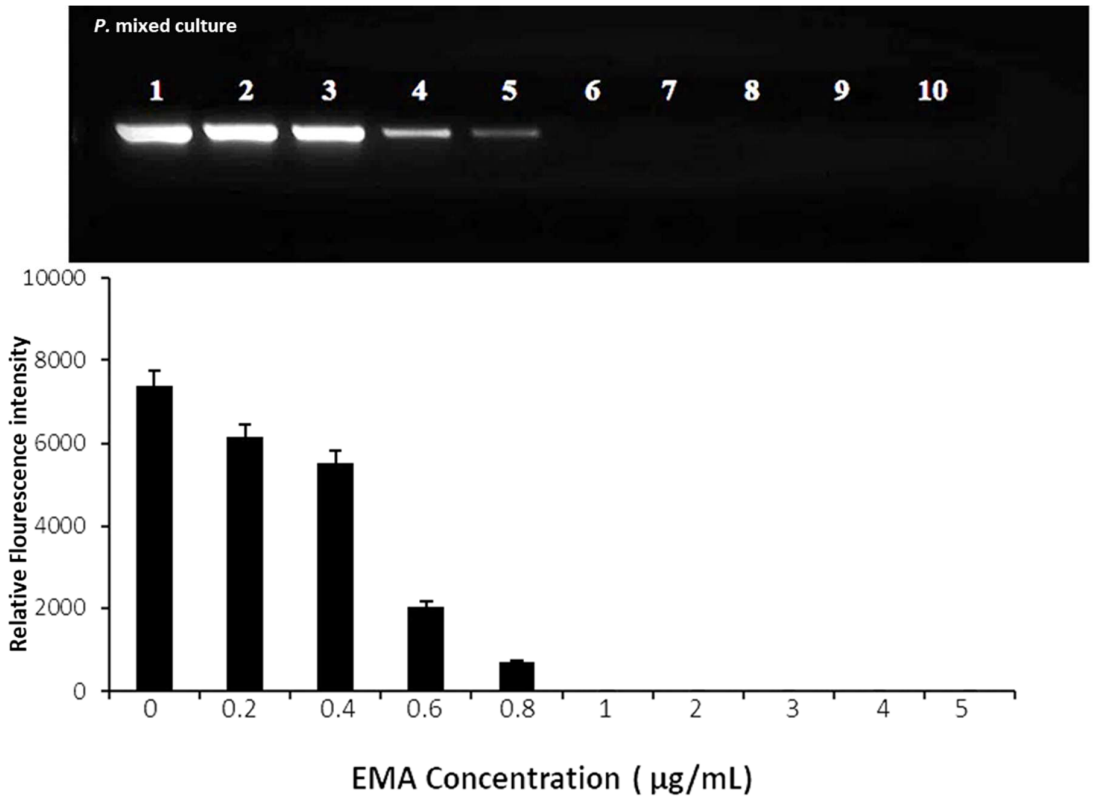

Figure 35. Optimization of the minimum concentration of EMA to inhibiting DNA amplification derived from dead cells of *Pseudomonas* mixed culture. Bacterial samples were treated with different concentration of EMA. Top: typical agarose gel image of PCR amplified target products. Lanes 1-10, varying concentration of EMA (0-5µg/ml respectively). Bottom: bar graph represents fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentration of EMA

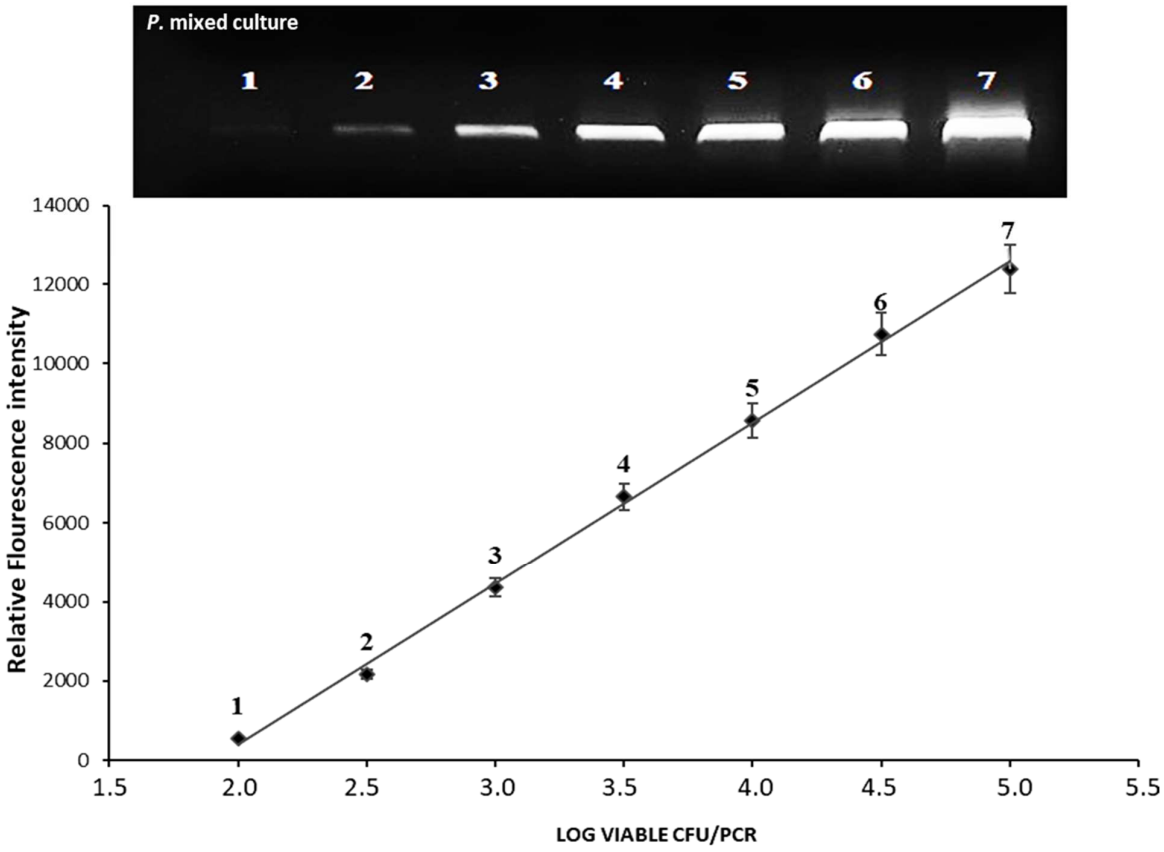

Figure 36. DNA amplification of the viable cells within a mixture of viable and dead cells by PCR. Relationship between the relative fluorescence intensity of amplified DNA bands and the log of genomic targets from viable CFU in a mixture of viable CFU and dead cells. Top: typical agarose gel image of PCR amplified products. Total number of genomic targets in live–dead cell mixtures from which target DNA was derived was kept constant CFUs. Lanes 1–7: varying numbers of CFU/PCR: 1×10^2- 1×10^5.

DISCUSSION

A Polymerase chain reaction (PCR) assay appears to offer good potential in advancing the technology of rapid microbial detection. PCR being a sensitive and specific technique need extensive optimization of the steps in the reaction. Therefore, for us to explore this technology in combination with, three major steps in the reaction were optimized; annealing temperature of the *Pseudomonas* universal primers, extension time of the primers and the PCR cycle number. We were also able to evaluate the sensitivity of the PCR based on the concentration of DNA (CFU/PCR).

The theoretical annealing temperature of the *Pseudomonas* universal primers used in this study was 55°C. This temperature was based on the calculated melting temperature (T_m) of 60°C for both forward and reverse primers. The annealing temperature in PCR is crucial because when is lower than the optimum temperature, non-specific DNA fragments will be amplified causing multiples bands on the agarose gel. If the is too high, the yield of the desired product and sometimes the purity is reduced. When gradient PCR was performed to determine the experimental optimal annealing temperature for the reaction from a range of temperatures (54 – 65°C), results from all five bacteria species shows that the primers annealed best at 59°C. At temperatures above 60°C, the was a proportional decrease in the DNA amplification. This could be due to primers being destroyed by high temperature above their melting temperature or due to non-specific binding to the other regions of the bacterial DNA.

The extension time of PCR depends upon the synthesis rate of DNA polymerase and the length of target DNA. Longer than recommended extension times can result in higher error rates, spurious banding patterns and/or reduction of amplicon yields. Our results from all five bacteria species using a range (30 – 60 seconds) of primer extension time shows that 45 – 50 seconds is sufficient for the Taq Polymerase enzyme to extend the primers to amplify 987 bp

of the target gene (16S rDNA). Results obtained from all five bacteria equally shows that the assay can detect up to 1CFU per PCR and could detect a wider range of bacteria load 1 – 10^5 CFU. Therefore 1CFU/PCR was further used to optimize the PCR cycle number. For the PCR cycle number, we observed that only higher cycles of 35 and 36 (figures 29 and 30) had positive bands. Therefore, the minimum cycle number to see a product in our PCR protocol was 35cycles and this was used for the optimization of the EMA concentration of viable and non-viable cells.

The EMA-PCR can discriminate DNA amplification from viable *Pseudomonas* species as expected. When viable cells were treated with EMA concentration of 20µg/ml and below the was no significant inhibition of DNA amplification deriving from viable bacterial cells occurred in our EMA-PCR procedure. At higher concentration of EMA, 20µg/ml and above, the was a significant ($P< 0.05$) reduction of the DNA amplification and increasing the concentration resulted in proportional decrease in the amplification of the target DNA deriving from viable cells. Noticeable inhibition of the target DNA was seen at 50 µg/ml. These results are similar that of (Levin & Lee, 2006) who also noticed that increasing the EMA concentration above 20 µg/ml resulted to a significant reduction in the target DNA deriving from viable cells. Although EMA is considered a better DNA intercalating dye and permeable to only compromised cells, our findings show that at higher concentration of EMA can become toxic and cross the membrane of intact cells. Based on our findings, EMA concentration of 20 µg/ml or less is suitable to be used for the quantification of target DNA from viable *Pseudomonas* species in future research.

For the optimization of minimum concentration of EMA required to inhibit the amplification of DNA from dead cells, when the dead cells of all five *Pseudomonas* species were subjected to treatment of EMA at a concentration of 1.0 µg/ml or higher, amplification of

target DNA was completely inhibited. Target DNA from heat killed cells was amplified when the EMA concentration was 0.2-0.8μg/ml. The 1.0 μg/ml EMA concentration that inhibited the amplification of DNA from dead cells is well below the EMA concentration of 20 μg/ml that resulted in significant inhibition of amplification of DNA from viable cells. We therefore establish that EMA at a concentration of 1.0 μg/ml is ideally suitable for discrimination of DNA from viable and non-viable mixed *Pseudomonas* species. This EMA concentration was confirmed by using different proportions of viable-dead cells mixture of all five *Pseudomonas* spp.

For the viable-dead cells mixture, when the DNA from all five bacteria species were kept constant at a total of 1.0 x 10^5 CFU/PCR deriving from different ratios of viable and dead cells, amplification of the target DNA from dead cells was effectively inhibited by 1.0 μg/ml of EMA as expected. The was a proportional decrease in the fluorescence intensity of the PCR bands as the log of genomic DNA derived from viable cells yielding target DNA decrease. Even when the heat killed cells was 99.99% of the total bacteria population, the fluorescence intensity of the PCR band was still almost undetectable.

CHAPTER 5

CONCLUSION

In this present study, we have developed an EMA-PCR assay for the detection and quantification of viable *Pseudomonas* species. We reported in this study that 1.0 µg/ml EMA can be used to inhibit the DNA amplification derived from dead *Pseudomonas*. However, when the concentration of EMA was 30µg/ml and above, the was a notable inhibition of DNA amplification derived from viable *Pseudomonas*. Therefore, we have established an EMA based conventional PCR method to differentiate and quantify viable and dead *Pseudomonas* species with a standard EMA concentration of 1.0 µg/ml. Our protocol can detect as few as 1CFU/PCR viable cells target DNA in a mixed viable-dead cells suspension and will be useful in improving the detection and quantification of *Pseudomonas* species in food. The ability of EMA to penetrate only cells that have undergone lethal membrane damage constitutes a powerful method for distinguishing between viable and dead bacterial cells by PCR. The conventional plate count for quantifying the number of viable bacterial cells in and on foods furnishes no information on the total number of dead bacteria present. The PCR is no longer restricted to the detection and enumeration of only the total amount of target DNA derived from both viable and dead *Pseudomonas* cells but will discriminate viable vs dead *Pseudomonas* species. EMA-PCR also offers the advantage of obtaining results in 2 to 4 hours compared to 48 hours from conventional plate counts

This EMA-PCR study could be useful for the selective detection of viable *Pseudomonas* species. Therefore, these microorganisms can be promptly detected, preventing contaminated food from being packaged and stored.

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