T.R.
AKDENİZ UNIVERSITY
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TRANSFORMATION OF *Stenotrophomonas maltophilia* KB2 WITH eGFP PLASMIDS AND ITS INTRODUCTION INTO *Raphanus sativus*

Egem ÖZBUDAK

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AKDENİZ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

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Bu tez 18/07/2017 tarihinde aşağıdaki jüri tarafından Oybirliği ile kabul edilmiştir.

Yrd. Doç. Dr. M. Aydın AKBUDAK
Yrd. Doç. Dr. Hatice İKTEN
Doç. Dr. Ertuğrul FİLİZ

ÖZET

Stenotrophomonas maltophilia KB2 VARYANTININ eGFP PLASMİDİ İLE TRANSFORMASYONU VE *Raphanus sativus* BİTKİSİNE AKTARIMI

Egem ÖZBUDAK

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı
Danışman: Yrd. Doç. Dr. M. Aydın AKBUDAK
Haziran 2017, 28 sayfa

Bu çalışmada, bitki hücrelerindeki görüntülenmesini kolaylaştırmak amacıyla markör gen ile işaretlenmiş, non-steroidal anti-inflamatuar ilaçları parçalayabilme özelliğine sahip bakteri varyantı bitki tohumlarına aktarılmıştır.

Naproxeni parçalayabilme özelliğine sahip olduğu bilinen *Stenotrophomonas maltophilia* KB2 varyantı, eGFP markör geni ile modifiye edilmiş ve bu varyantın *Raphanus sativus* tohumlarına aktarılmasının sonucu olarak bu varyantın bitki dokularında yaşayabildiği belirlenmiştir. *S. maltophilia* KB2, kontamine olmuş topraklardan non-steroidal anti-inflamatuvar ilaçları arındırmak için fitoremediasyon amacıyla kullanılabilme potansiyeline sahiptir. Ayrıca, *S. maltophilia* bakterisinin bazı varyantlarının "endofit" olarak kayda geçmiş olması, bitkilerde patojenlere karşı arttırılmış direnç veya gelişmiş büyüme gibi endofitlerde bulunan birçok özelliğin *S. maltophilia* KB2 varyantının geniş bir kullanım alanına sahip olmasına imkan tanımaktadır.

ANAHTAR KELİMELER: Antibiyotik degradasyonu, bakteri transformasyonu, markör protein

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ABSTRACT

TRANSFORMATION OF *Stenotrophomonas maltophilia* KB2 WITH eGFP PLASMIDS AND ITS INTRODUCTION INTO *Raphanus sativus*

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MSc Thesis in Agricultural Biotechnology
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In this study, a bacterial strain which is able to degrade non-steroidal anti-inflammatory drugs (NSAIDs) was labeled with the marker gene to enable monitoring of the bacterial cells in plants and the strain was introduced into plant seeds.

As a result of the modification of *Stenotrophomonas maltophilia* strain KB2, which had been found to degrade naproxen, with the eGFP marker gene, and its introduction into plant seeds, it was determined that the strain can live in plant tissues. Therefore, in the future, this strain can be used for phytoremediation to remove NSAIDs from contaminated soils. Also, in the light of the evidence that some strains of *S. maltophilia* had been recorded as "endophytes", the general features of endophytes would be used to apply *S. maltophilia* for wide range of purposes including promoted growth or increased resistance against pathogens or biotic stress factors in plants.

KEYWORDS: Antibiotic biodegradation, strain labelling, bacterial transformation, marker protein

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

µg	Microgram
µL	Microliter
∞	Infinity Symbol
bp	Base pair
g	Gram
h	Hour
kb	Kilobase
kg	Kilogram
L	Litre
M	Molar
Mg	Miligram
min	Minute
NaCl	Sodium Cloride
OD	Optical Density
rpm	Rotate per minute
sec	Second

Abbreviations

DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
TfbI	Transformation Buffer I
TfbII	Transformation Buffer II
RT	Room Temperature

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1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceuticals the most frequently used for wide range of treatments of inflammation, fever or pain across the globe (Ghosh et al 2015). However, overuse of these drugs is a rising threat for human health and various ecosystems as they would eventually be released into environmental matrices like soil, sediments or aquatic environments including surface waters and even drinking water (Eslami et al 2015). In these environments, the concentration of NSAIDs is measured with nanograms, however it may up to micrograms per liter (Lloret et al 2010). Although toxic effects of these compounds are not expected at low concentrations, negative effects have recently been reported for some bird and aquatic species (He et al 2017). Moreover, any of the current treatment systems used in sewage plants is not specified for the effective removal of NSAIDs. Because of the persistence and hydrophilicity, they may still remain in the sewage effluents (Gentili 2007).

The absence of processes which are capable of completely removing NSAIDs in the sewage systems and the harshness of physicochemical processes that leads to the production of secondary pollutants and high cost operation, made it crucial to use bioremediation processes (Zhang et al 2013). These processes are cost effective and free of harmful secondary products. Also, there are various types of microorganisms that have specific enzymes for the degradation of drugs (Wu et al 2012). However, only limited numbers of microorganisms, which are mostly fungi, had been found to degrade or transform NSAIDs. There are even less numbers of microorganisms to degrade or transform naproxen, one of the most used NSAID class drugs. Yet, *Stenotrophomonas maltophilia* KB2 was found to degrade it effectively (Wojciesznska et al 2014).

Stenotrophomonas maltophilia is a Gram-negative bacterium that belongs to gamma proteobacteria whose members have been known to be adapted for wide range of environments with distinct niches. Just like other members, *S.maltophilia* can be isolated from contaminated waters or aqueous environments to animal and human tissues or rhizospheres of plants (Mahdi et al 2014). Moreover, with the environments where they have been adapted to live, their activities also change. Although it might be associated with various human diseases, the beneficial functions of the bacteria are also well known for producing anti-pathogenic compounds, promoting growth hormones in plants and degradation of natural or unnatural pollutants. These functions can constitute core features of phytoremediation to clean up the contaminated soils from the hazardous substances, such as NSAIDs, by using plants (Brooke 2012; Zhu et al 2012; RP et al 2009). Moreover, some strains of *S. maltophilia* can also be classified as an endophyte, which means literally "living in the plant". Endophytes are commonly found in all of the plant species, silently residing or forming colonies in tissues of plant without harming it. In fact, anti-pathogenic and plant growth-promoting properties of *S. maltophilia* are quite prevalent within endophytic species as well (Taghavi et al 2005). Therefore, they are widely used in many biotechnological applications with their specific metabolic activities. However, their interactions with their plant hosts, and also the other endogenous microorganism, are still not well known and remains to be discovered (Taghavi et al 2009).

This research has been performed to label *Stenotrophomonas maltophilia* KB2 which was found to degrade non-steroidal anti-inflammatory drugs (NSAIDs), with the *gfp* gene that will enable monitoring of the bacterial cells within plants. After the introduction of strain into seeds of *Raphanus sativus,* the possible colonization of bacteria was observed. Hence, the modification of the strain with a marker protein is a crucial step for further implementations about phytoremediation.

In the following parts of the thesis, further information and related previous research about the subject will be mentioned, materials and methods applied for this work will be explained, and the data obtained in the direction of the analyzes will be determined.

2. THEORETICAL INFORMATION AND LITERATURE REVIEW

2.1. The Consumption of Naproxen and Other NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are prevalently used as treatments for pain and inflammatory conditions. At the beginning of the last decade, huge amounts of NSAIDs consumption was recorded (Conaghan 2012). For instance, in 2001, 622 tonnes of acetylsalicylic acid, also known as aspirin, which is an important NSAID, and 345 tonnes of ibuprofen, another NSAID, were used in Germany alone. In 2000, a different member of NSAIDs, called naproxen, had a consumption amount of 35 tonnes, in England, (Nikolaou et al 2007) while it was announced that the same drug was produced around 500 tonnes annually by Albemarle Company (Wojcieszynka et al 2014).

In the global scale, NSAIDs have been prescribed greatly. Prescription for NSAIDs is written more than 70 million times in the United States per year. Nonetheless, when "over the counter" (OTC) use of the drugs, which means without prescription, is considered, 30 billion doses are consumed every year, just in the United States (Oregel et al 2013).

The overuse of NSAIDs is still continuing threat in the world as massive amount of consumption has been recorded in this decade. It was calculated that 200 tonnes of ibuprofen were consumed by just Spanish population in 2012 (Holt 2016). Meanwhile, on a worldwide scale, 35,000 metric tonnes of aspirin, which would correspond to 100 billion standard tables, were consumed every year according to the Aspirin Foundation (Møller et al 2010).

2.2. Biodegradation of Naproxen

The overuse of NSAIDs is a nascent problem as they would be eventually released into environment *via* many ways like excretion through the urinary system of humans and animals, direct disposal of the products from aquafarms, transmission during fabrication or inaccurate disposal of expired drugs (Gentili 2007). Where NSAIDs are released, their concentration may range from nanograms to micrograms per liter. Although, toxic effects of these compounds are not expected at low concentrations, the accumulation of them in an organism may be negatively resulted (Lloret et al 2010). Besides, the adverse effects on some birds and aquatic species have been already announced by Wu et al. (2012) and He et al. (2017). The effects include the damage in reproductive system, cell division inhibitions, tissue accumulation and behavioral changes. The data demonstrated the importance of removal of NSAIDs to protect the environments and the organisms from the destructive effects of these drugs.

Despite the presence of many physicochemical processes to remove NSAIDs, a lot of drawbacks also come along with these processes such as high operational cost, generation of secondary pollutants or free radicals and severe reaction conditions (Zhang et al 2013). These drawbacks, with the fact that they are hard to remove in aqueous phase because of their stability and hydrophilicity, made it crucial to apply bioremediation processes that are cost effective and free of pollutants. Also, there are various types of microorganisms which have specific enzymes for the degradation of

drugs (Wu et al 2012). However, only limited numbers of microorganisms, which are mostly fungi, (*Penicillium* sp., *Trametes versicolor, Cunninghamella elegans, C. echinulata, C. blakesleeana, Beauveria bassiana, Phanerochaete chrysosporium, P. sordida, Actinoplanes sp., Bjerkandera sp. R1, B. adusta, Irpex lacteus, Ganoderma lucida*) had been found to degrade or transform NSAIDs. *T. versicolor* transforms naproxen, one of the most consumed NSAID class of drug, to 2-(6-hydroxynaphthalen-2-yl) propionicacid and 1-(6-methoxynaphthalen-2-yl) ethanone by possibly applying, cytochrome P-450 and laccase. Degradation of naproxen was also shown by laccase isolated from *Myceliophthora thermophile* while a redox mediator was present. (Lloret et al 2010; Wojciesznska et al 2014).

Degradation or transformation of naproxen by bacteria is even less known. Although some bacterial strains, mainly from genera *Pseudomonas, Sphingomonas, Patulibacter, Nocardia, Rhodococcus and Stenotrophomonas*, able to degrade NSAIDs had been isolated, only few numbers of bacteria have been known for degradation or transformation of naproxen. *Stenotrophomonas maltophilia* KB2, which is the gram negative bacterium, was the first bacterial strain found to degrade it effectively (Wojciesznska et al 2014). More recently, other bacterial strains like *Planococcus* sp. Strain S5 (Domaradzka et al 2015) and *Bacillus thuringiensis* B1 (2015b) (Marchlewicz et al 2016) have also been found for degradation of naproxen.

2.3. *Stenotrophomonas maltophilia* KB2

KB2 strain was isolated from activated sludge and based on its morphological and physiochemical features, also in 16S ribosomal RNA analysis, it was classified as *Stenotrophomonas maltophilia*. Different from other strains of *S. maltophilia,* it is unique as having three types of dioxygenase activities (catechol 1,2-dioxygenase, 2,3-dioxygenase and protocatechuate 3,4-dioxygenase activities) which depends on the inducer. The wide range of oxygenase activities provides an ability for degradation of aromatic compounds and makes the strain perfect candidate for biotreatment of the contaminated soils and wastewaters (Urszula et al 2009). Moreover, utilization of wide range of aromatic compounds by KB2 strain and aromatic structure of naproxen suggest the strain as an applicant for possible transformation or biodegradation of the drug. Wojciesznska *et al.* (2014) proposed the mechanism for degradation of naproxen by *S. maltophilia* KB2 under cometabolic conditions (Figure 2.1).

Cometabolism is a process for the biodegradation of organic compounds which are difficult to degrade, in the presence of secondary carbon sources. Introduction of these sources to the culture results with a synthesis of cofactors required for degradation and increased biomass production. After 35 days of incubation, the strain was able to degrade approximately 28% of naproxen. However, when additional carbon and energy sources, such as glucose, were supplied along with naproxen, the degradation was boosted to the rate of almost 78%. Moreover, it was determined that the biodegradation process of naproxen could be further increased with the help of cometabolic systems (Wojciesznska et al 2014).

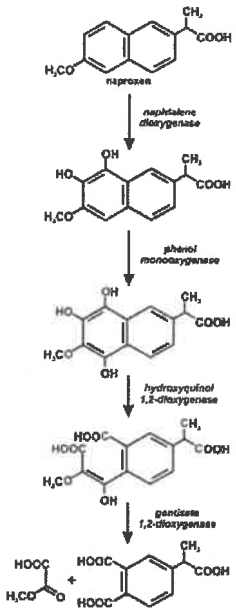

Figure 2.1. Degradation pathway of naproxen under cometabolic conditions (Wojciesznska et al 2014).

2.4. Endophytic Characteristics of *Stenotrophomonas maltophilia*

S. maltophilia, which is distributed worldwide, has been known for its versatility and ability to adapt into different environments, such as soil, water, hospital setting, plants, food or even human tissues (Youenou et al 2009). Because of its great differentiation at species level, it may show various functions, with regard to different habitats. These functions associated with the bacteria varies from the degradation of numerous compounds, including pollutants, to production of secondary metabolites or some pathogenic traits in humans (Figure 2.2) (Ryan et al 2009).

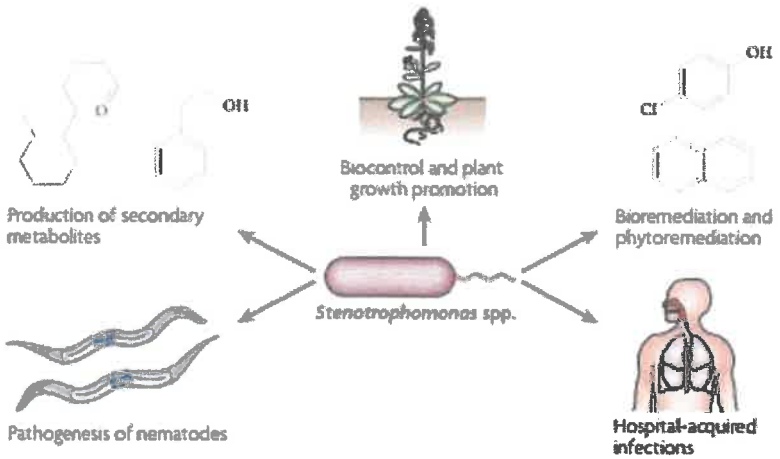

Figure 2.2. Various functions of *Stenotrophomonas* spp. (Ryan et al 2009).

Besides those functions, some strains have been found to possess endophytic features as different strains of *S. maltophilia* have been isolated from diverse plant species including plant species, including cucumber (*Cucumis sativus*) (Ryan et al 2009), oilseed rape (*Brassica napus*), potato (*Solanum tuberosum*), strawberry (*Fragaria x ananassa*) (Berg et al 2002), alfalfa (*Medicago sativa*), sunflower (*Helianthus annuus*) (Schwieger and Tebbe, 2009), various weeds (Sturz et al 2001). Furthermore, a strain called *S. maltophilia* R551-3 was found in poplar trees and might have been involved in enhanced plant growth through the synthesis of the plant hormone auxin (IAA) (Taghavi et al 2009).

Endophytes, microorganisms living within a plant without harming it, have many beneficial effects for the plants *via* direct or indirect mechanisms (Figure 2.3) (Ryan et al 2009). From these effects, production of antibiotics and volatile organic compounds against pathogens to protect plants, increased plant resistance against heavy metals, and promotion of plant growth by the acquisition of required resources and generation of growth factors, have been recorded for *S. maltophilia* until now (Zhu et al 2011). Still, the molecular mechanisms of these effects or interactions of strains with their plant hosts, and also the other endogenous microorganism, are still not well known, and remains to be discovered (Ryan et al 2009).

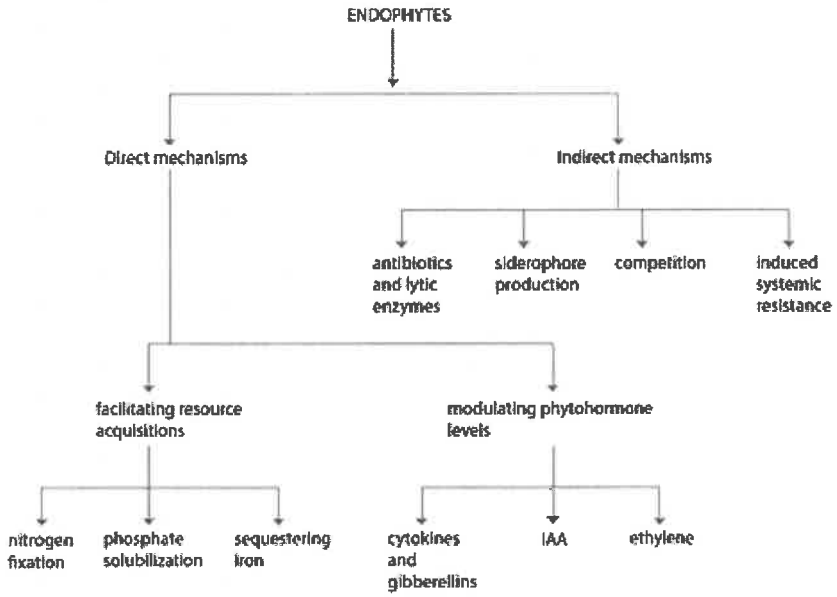

Figure 2.3. Direct and indirect mechanism of endophytes, affecting the host plants (Chaturvedi et al 2016).

2.5. Green Fluorescent Protein

In order to visualize and determine colonization of *S. maltophilia* in plant tissues and their interactions with other bacteria and their plant hosts, modification in *S. maltophilia* with a marker protein is required. By virtue of advances in molecular biology, it is possible to visualize microorganisms, by monitoring their unique activities, such as biodegradation of pollutants, determining the intracellular localization

of their proteins or detecting their presence in complex systems like activated sludge with the help of molecular markers (Chen et al 2005; Nelson et al 2007; Rosochacki and Matejczyk 2002). Commonly used markers involve genes: *lacZ* (for beta-galactosidase, *lux* (for luciferase), resistance markers for antibiotics or heavy metals, and *gfp* (green fluorescent protein). While further requirements like additional substrates, media and valuable materials would mostly be necessary for markers, GFP can produce its green fluorescence in the absence of exogenous accessory substrates and visualization would be performed without expensive equipment. Furthermore, this protein is highly stable, non-invasive and has nominal toxicity. For all these reasons, since its discovery, *gfp* has been an important tool and it has been widely accepted in various biotechnological approaches, and still promise for determination of unknown biological processes (Torres et al 2013; Rosochacki and Matejczyk, 2002).

3. MATERIALS AND METHODS

The project has been carried out at the Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Poland (Figure 3.1). There is enough materials and equipment for DNA isolation, PCR, heat shock transformation, electroporation, bacterial culturing, cell imaging and visualization, plant seeding and inoculation with bacteria.

Figure 3.1. One of the laboratories at the Department of Biochemistry in Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Poland.

3.1. Cloning of eGFP Plasmid

pMP4655 plasmid harboring *gfp* gene and resistance gene for tetracycline (Figure 3.2) was cloned by using ready-to-use competent *E. coli* DH5 alpha strain. To do that, the plasmid was transformed into *E. coli* cells with the heat shock method and after transformation, plasmid isolation was performed to obtain pMP4655.

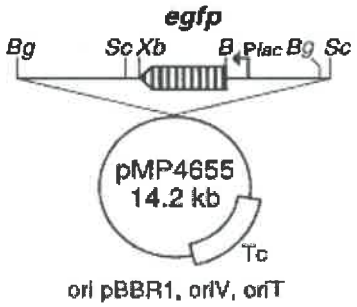

Figure 3.2. Schematic representation of pMP4655 plasmid. Xb: *Xba*I cut site, B: *BamHI* cut site, Tc: Tetracycline resistance gene (Bloemberg et al. 2000).

3.1.1. eGFP plasmid transformation into *E. coli*

5 µl pMP4655 plasmid was mixed with 100 µl of competent *E. coli* DH5 alpha cells in 1.5 mL Eppendorf tube and incubated on ice for 20 minutes. After incubation the cells were heat shocked for 40 seconds at 42°C by using mini dry bath and immediately transferred to the ice for 5 minutes. Then 900 µl of liquid LB medium was added into the cells for their recovery and they are left for 2 hours of incubation at 37°C and 130 rpm. Upon incubation, centrifugation at 3000 rpm for a minute was applied to precipitate the cells. Then, 800 µl of the supernatant was removed. After resuspension, 100 µl of inoculum was spread onto agar plates with tetracycline (50 µg/ml) by using sterile L-shaped spreader. Therefore, only the bacteria carrying the plasmid and expressing tetracycline resistance, could be survived on the plate and selected. After overnight incubation at 37°C, two different colonies were picked up from the plate for inoculation into liquid LB medium with tetracycline (50 µg/ml) and incubated overnight at 37°C with shaking at 130 rpm. From the grown bacteria, plasmid isolation was performed to obtain pMP4655.

3.1.2. Plasmid isolation

Plasmid isolation culture was performed by plasmid mini kit (A&A Biotechnology). 1.5 mL of overnight culture was taken and put into sterile Eppendorf tube. It was centrifuged at 13.000 rpm for 5 minutes, supernatant was removed and 200 µl L1 Resuspension Buffer was added to resuspend the pelleted cells. Then 200 µl L2 NaOH/SDS Lysis Solution was added, the tube was inverted 6-8 times and incubated at room temperature for 3 minutes to break down the cell walls. To neutralize the mixture, 400 µl Neutralization Solution GL3 was added, the tube was inverted several times and centrifuged at 13.000 rpm for 5 minutes. Then, the supernatant was transferred to the minicolumn and centrifuged at 13.000 rpm for 1 minute. Mini-column was taken out, and the supernatant was recovered and centrifuged again at 13.000 rpm for 1 minute. For primary washing, 500 µl Primary Washing Buffer W was added to the column, centrifuged for 1 minute at 13.000 rpm and the flow-through was discarded. For secondary washing, 700 µl Secondary Washing Buffer A1 was added to the column, centrifuged at 13.000 rpm for 2 minutes. For elution of the plasmid, the column was transferred to new sterile 1.5 mL Eppendorf tube, 60 µl DNAse free water was added into the center of the column and incubated at room temperature for 3 minutes. To obtain the plasmid, centrifuge was done at 13.000 rpm for 1 minute and after that, it was stored at -4°C.

3.2. Preparation of Competent *S. maltophilia* KB2 Cells and Their Transformation with eGFP Plasmid

Both chemically competent and electrocompetent cells of *S. maltophilia* KB2 were prepared to transform with pMP4655 plasmid for expression of *egfp* gene. These competent cells, transformed with pMP4655 plasmid, were inoculated onto tetracycline plates (50 µg/ml). Therefore, only the ones that were successfully transformed and expressing tetracycline resistance gene from the plasmid would be selected with the help of the tetracycline resistance genes within the plasmid. After screening, single colonies from the plates were chosen and used for further studies.

3.2.1. Chemically competent *S. maltophilia* KB2 cells and heat shock transformation

For applying heat shock transformation process to *S. maltophilia* KB2, chemically competent cells of the strain were required. To generate these cells, single colonies were selected from an agar plate inoculated with *S. maltophilia* KB2, grown in 10 mL liquid LB medium with tetracycline (50 µg/ml) and incubated overnight. Next day, 10 mL culture was transferred into 250 mL autoclaved LB medium and grown until reaching OD 0.5. When the culture reached OD 0.5, it was kept on ice for 15 minutes, then transferred to 400 mL centrifuge bottles and centrifuged for 10 minutes at 4000 rpm, 4°C. Supernatant was discarded and the pellets were resuspended with 25 mL TfbI buffer (pH 5.7) and prepared with 100 mM RbCl, 50 mM $MnCl_2$, 30 mM Ch_3COOK, 10 mM $CaCl_2x2H_2O$ and glycerol 15%, and kept on ice for 15 minutes. After incubation on ice, samples were centrifuged at 4000 rpm, 4°C for 5 minutes. Then pellets were resuspended with TfbII buffer (pH 6.8) and prepared with 10 mM MOPS, 10 mM RbCl, 73 mM $CaCl_2x2H_2O$ and glycerol 15%. Upon resuspension, they were kept on ice for 15 minutes and then aliquoted into 0.5 mL Eppendorf tubes in the amount of 100 µl to be further used in heat shock transformation.

The fresh chemically competent cells were directly used for heat shock transformation. 5 µl of pMP4655 plasmid was added into freshly prepared competent cells, which have an amount of 100 µl, and incubated on ice for 20 minutes. Then the cells were heat shocked within mini dry bath for 40 seconds at 42°C and immediately returned to the ice for 5 minutes. 900 µl of liquid LB medium was added onto the cells and they are left for 2 hours of incubation at 30° C and 130 rpm. When incubation was finished, the cells were centrifuged at 3000 rpm for a minute. 800 µL from the supernatant was removed and the pellet was resuspended. 100 µl of inoculum from the resuspension was spread onto agar plates with tetracycline (50 µg/ml) by using sterile L-shaped spreader and incubated overnight at 30° C (Figure 3.2).

Figure 3.3. KB2-4FJK transformant cells on agar plate.

3.2.2 Electrocompetent *S. maltophilia* KB2 cells and electroporation

For electroporation, electrocompetent cells of *S. maltophilia* the strain were required. To obtain these cells, single colonies were selected from agar plate inoculated

with *S. maltophilia,* grown in 10 mL liquid LB medium with tetracycline (50 µg/ml) and incubated overnight. The following day, 1 mL was taken from the overnight culture, added into 100 mL liquid LB and grown until reaching the mid-logarithmic phase (OD value between 0.4-0.6). When the culture reached the OD interval between 0.4-0.6, it was incubated on ice for half an hour, then transferred to 400 mL centrifuge bottles and centrifuged for 10 minutes at 5000 rpm, 4°C. Supernatant was discarded and the pelleted cells were resuspended with the same volume, 100 mL, of ice cold distilled water. The centrifuge was repeated at 5000 rpm, 4°C for 10 minutes. After supernatant removal, resuspension was done with half volume of ice cold distilled water, which was 50 mL. Subsequently, it was centrifuged for 10 minutes, at 6000 rpm, 4°C and resuspended with 1 mL of 10% glycerol upon removing supernatant. Final centrifuge was done for 10 minutes, at 6000 rpm, 4° C. When the pelleted cells were resuspended with 1 mL of 10% glycerol, they were aliquoted into sterile 0.5 mL Eppendorf tubes in the amount of 100 µl to be further used in electroporation.

The fresh electrocompetent cells were directly used for electroporation. 10 µl of pMP4655 plasmid was mixed with freshly prepared competent cells, which has an amount of 100 µl. They were carefully transferred into pre-chilled electroporation cuvettes without the formation of bubbles. Upon incubation of the cuvettes on ice for 5 minutes, they were placed into holder of electroporation apparatus and pulse button was pressed. After pulsing, 1 mL of liquid LB medium was added, immediately, and mixed thoroughly. Bacteria within the medium were pipetted back to their tubes and incubated for 2 hours at 130 rpm, 30° C. When incubation was finished, the cells were centrifuged at 3000 rpm for a minute. After centrifuge, the bacteria were precipitated, 800 µl from the supernatant was removed and the pellet was resuspended. 100 µl of inoculum from the resuspension was spread onto agar plates by using sterile L-shaped spreader. The plate used for the cells transformed with pMP4655 was with tetracycline (50 µg/ml) and these prepared plates were held overnight at 30° C.

3.3. Confirmation of *egfp* Gene in Transformed Cells

Single colonies chosen from the plates of the transformant *S. maltophilia* KB2 cells were inoculated into liquid LB medium with tetracycline (50 µg/ml) and incubated overnight at 30°C, 130 rpm. Upon overnight incubation, DNA isolation was performed from the culture by using plasmid mini kit (A&A Biotechnology). The presence of *egfp* gene was confirmed with double digestion and Polymerase Chain Reaction (PCR) by using primers specifically designed for the gene. Digestion and PCR products were loaded onto the 1% agarose gel to check the presence of target bands representing regions of the e*gfp* gene that were the cut or amplified. When the presence of the gene was determined in the samples, visualization of the bacterial cells under fluorescent microscope was performed. Glowing of the cells with a green color under the microscope demonstrated the expression from *egfp* gene.

3.3.1. Double digestion of isolated DNA with *BamHI* and *Xba*I

The sequence including e*gfp* gene in pMP465 plasmid has unique cut sites for *BamHI* and *Xba*I restriction enzymes as it is flanked by the recognition sites of the enzymes. Therefore, plasmid DNA isolated from the transformant cells, was used as a template for the digestion reaction to check the presence of the plasmid. After adding

the ingredients into 1.5 ml Eppendorf tube, it was incubated within mini dry bath for 1.5 hours at 37°C. The ingredients of the reaction are represented in Table 3.1.

Table 3.1. Ingredients of double digestion reaction

Ingredients	**Amount**
Template DNA	2 µl
BamHI Buffer[1]	2 µl
*Xba*I	1 µl
BamHI	0.5 µl
H_2O	14.5 µl
Total	**20 µl**

3.3.2. Amplification of *egfp* gene fragment using polymerase chain reaction

Polymerase Chain Reaction (PCR) was applied for isolated DNA from the transformant cells by using specific primers targeted for ~240 bp region in the eGFP plasmid. The nucleotide sequences of forward and reverse primers are shown in Table 3.2, the ingredients of the reaction are shown in Table 3.3, and the reaction conditions are represented in Table 3.4 and Table 3.5.

Table 3.2. Nucleotide sequences of forward and reverse primers

Primers	5' - 3' nucleotide sequences
Forward Primer	AGCTGAAGGGCATCGAC
Reverse Primer	TGGTTGTCGGGCAGCAC

Table 3.3. Ingredients of Polymerase Chain Reaction

Ingredients	**Amount**
Template DNA	1 µl
10X *Taq* Buffer	2.5 µl

[1] Occasionaly, Tango Buffer was used.

Table 3.3. (Cont.)

10 mM dNTP	0.5 µl
$MgCl_2$	2 µl
F Primer	1 µl
R Primer	1 µl
Taq Polymerase	0.5 µl
H_2O	16.5
Total	**25 µl**

Table 3.4. Conditions of Polymerase Chain Reaction

Temperature	**Time**	**Number of cycles**
94 °C	5 min	
94 °C	45 s	30
45 °C	30 s	30
72 °C	2 min	30
72 °C	5 min	
4 °C	∞	

Table 3.5. Conditions of Polymerase Chain Reaction with different annealing temperatures

Temperature	**Time**	**Number of cycles**
94 °C	5 min	
94 °C	45 s	10
43 °C	30 s	10
72 °C	2 min	10

Table 3.5. (Cont.)

94 °C	45 s	10
45 °C	30 s	10
72 °C	2 min	10
94 °C	45 s	10
47 °C	30s	10
72 °C	2 min	10
94 °C	45 s	10
49 °C	30 s	10
72 °C	2 min	10
72 °C	5 min	
4 °C	∞	

3.3.3. Agarose gel loading

To visualize the products of plasmid DNA isolation, double digestion and PCR under UV light, 1% (w/v) agarose gel was prepared with 50 ml TA Buffer, 0.5 gr agarose and 2.5 µl ethidium bromide. 10 µl from each sample was mixed with 2 µl 6X Loading dye before loading into the wells and to the last well 2 µl of 1 kb DNA Ladder (Thermo, Gene Ruler) was added. Gel running was performed at 110V for 45 minutes. Double digestion and PCR products were run on 1% agarose gel images were captured by BioScreen Gel Imaging System.

3.3.4. Fluorescent microscopy imaging

Next step of confirmation for the transformation was done with fluorescent microscopy. For the visualization under the microscope, inoculation was done by using sterile loop, from the plate of transformant and non-transformant *S. maltophilia* KB2 cells into new plates with tetracycline (50 µg/ml). After overnight incubation, several colonies were selected from the plates and resuspended within 0.9% NaCl. Then a drop of sample was taken from the solutions with Pasteur pipette, spread onto the glass slides and covered with coverslips. The slide of the transformant cells was used for the analysis with fluorescent microscope to check the expression of the eGFP plasmid, while the slide of non-transformant cells was used to control autofluorescence properties of *S. maltophilia* KB2 strain.

The analysis was performed with Nikon Eclipse Ni Microscope which is suitable for fluorescence microscopic visualization (Figure 3.3). The excitation and emission

filters were preferred at 480 nm and 510 nm, in order, as these spectra are used for viewing eGFP proteins.

Figure 3.4. Microscope used for fluorescent microscopy imaging

3.4. Introduction of Transformed Bacteria into Plant Seeds

Once the introduction of pMP4655 into the *S. maltophilia* KB2 cells had been confirmed, strain was ready to introduce into plant seeds according to protocol used for endophytic bacteria introduction. As a plant host, commercial seeds of *Raphanus sativus* within the packets that are ready to sow were used (Figure 3.4). After introduction, the final step was identification of bacteria in plant tissues. It was performed by both applying DNA isolated from plant tissues to further analysis with double digestion and PCR, and observation with scanning electron microscopy (SEM).

Figure 3.5. Commercial seeds of *Raphanus sativus*

For the introduction into plant seeds transformants of *S. maltophilia* KB2 cells were inoculated into 10 mL liquid LB medium with tetracycline (50 µg/ml) and incubated overnight. Next day, the culture was transferred into a 50 mL Falcon tube to centrifuge for 5 minutes at 3000 rpm, room temperature. After centrifuge, pelleted cells were resuspended with isotonic solution of NaCl (0.9%). Meanwhile, the seeds were unpacked and placed in a detergent solution (20% Ace) for 3 minutes for sterilization. Sterilized seeds were transferred into the bacterial solution and incubated for half an hour at 80 rpm, 25° C. Upon incubation, seeds were washed with sterile water 3 times, then spread onto the plates prepared with water agar including 1.5% agar content (Figure 3.5). To another plate with water agar, unprocessed seeds directly added without the introduction of the bacteria, for being used as negative control group. Both plates were left at room temperature for germination of the seeds.

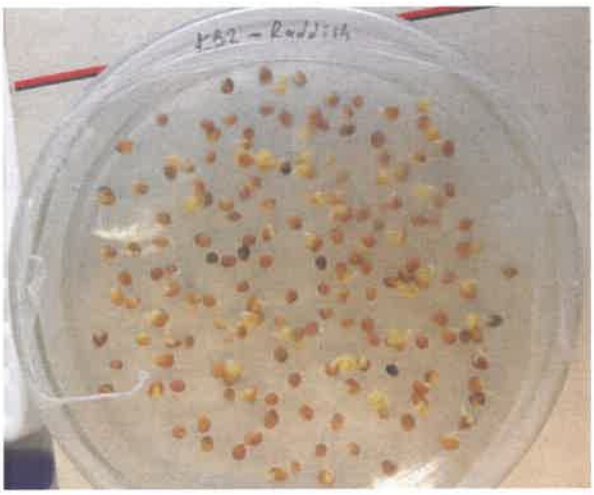

Figure 3.6. Plate of radish seeds transformant *S. maltophilia* KB2 strain introduced

3.5. Extraction of Endophytes from Plant Tissues

9 days after preparation of the plates, both groups of radish plants, germinated from the *S. maltophilia* KB2-introduced seeds and non-processed seeds, were taken and used for extraction of endophytes living in their tissues. For this purpose, root (Figure 3.5) and stem parts (Figure 3.6) were separately split up, afterwards placed on mortars. 100 µl of isotonic NaCl solution (0.9 %) was added and the plant samples were ground using pestle. The ground samples were placed into 1.5 mL Eppendorf tubes, 900 µl of liquid LB medium was added and incubated for an hour, at 130 rpm, 30° C. Upon incubation, the cells were centrifuged at 3000 rpm for a minute. Following centrifugation, 800 µl supernatant was removed and the pellets were resuspended. 100 µl of resuspended cells was taken and spread onto agar plates with tetracycline (50 µg/ml), using sterile L-shaped spreader. Prepared plates were held overnight at 30° C. Next day, liquid LB medium with tetracycline (50 µg/ml) was inoculated by single colonies chosen from the plates and incubated overnight at 30°C, 130 rpm. Upon overnight incubation, plasmid DNA was isolated from the culture by using plasmid mini kit (A&A Biotechnology). From the isolated DNA, double digestion and Polymerase Chain Reaction (PCR) were applied to confirm the presence of eGFP plasmid in *S.*

maltophilia KB2. Digestion and PCR products were load on the 1% agarose gel to check the presence of target bands representing regions of the e*gfp* gene that were the cut or amplified, in order.

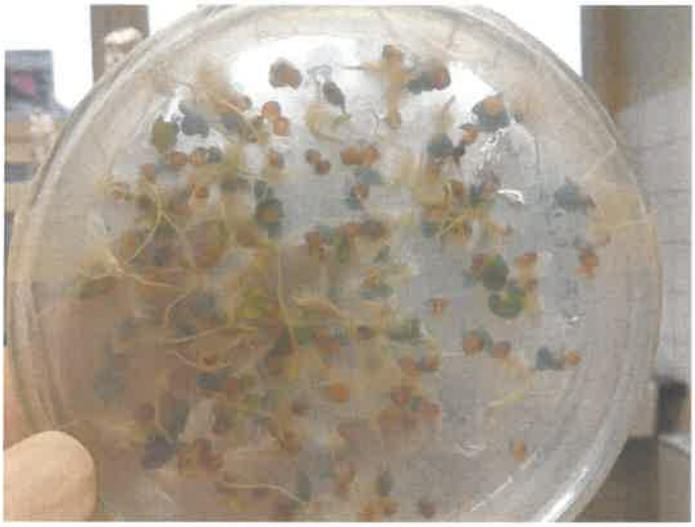

Figure 3.7. Roots of radish plants used for endophyte extraction

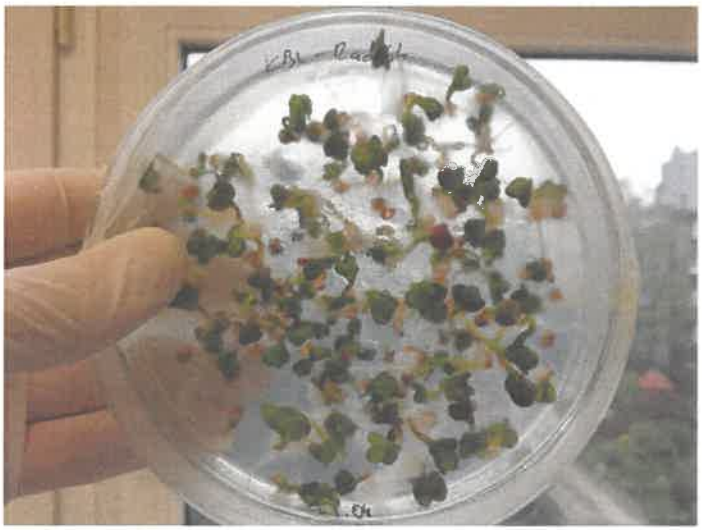

Figure 3.8. Stems of radish plants used for endophyte extraction

4. RESULTS AND DISCUSSION

The aim of our study was to label *Stenotrophomonas maltophilia* strain KB2 which is able to degrade non-steroidal anti-inflammatory drugs (NSAIDs), with the *egfp* gene that would enable monitoring of the bacterial cells within plant tissues. Therefore, after the introduction of *S. maltophilia* strain KB2 into seeds of *Raphanus sativus* and examination of the plant tissues grown from the seeds, potential endophytic behaviors of the strain would be investigated.

The scope of the study included multiplication of eGFP plasmid, preparation of competent cells of *S. maltophilia* strain KB2, transformation of eGFP plasmid into the strain and its confirmation, and lastly introducing the transformants into radish plant seeds.

4.1. Isolation of eGFP Plasmid from *E. coli*

Although the degradation mechanism of *S. maltophilia* strain KB2 was revealed, the knowledge about the strain is still limited. In order to determine its characteristic about potential for colonization and interaction with plants or other microorganisms within them, a marker protein supplementation is required. eGFP protein, being an ideal marker, is crucial for investigation of the unknown features of this strain, especially for its functions within plant tissues or complex systems like activated sludge. pMP4655 plasmid, harboring *egfp* gene and resistance gene for tetracycline, is designed for the visualization of Gram-negative bacteria in plants. The plasmid can be used for the analysis of microorganisms for the processes like biocontrol, biofertilization, biostimulation, competition for niches, colonization, and biofilm formation. Therefore, the plasmid was ideally suited for determining and the visualization of *S. maltophilia* strain KB2 within plant tissues.

The isolation of plasmid pMP4655 encoding e*gfp* gene was performed by using ready-to-use competent *E. coli* cells. After introduction of the plasmid into the competent cells, plasmid isolation was performed. According to gel loading results of the isolated plasmids, that were captured by BioScreen Gel Imaging System, the presence of pMP4655, having the size of approximately 14 kb, were confirmed (Figure 4.1).

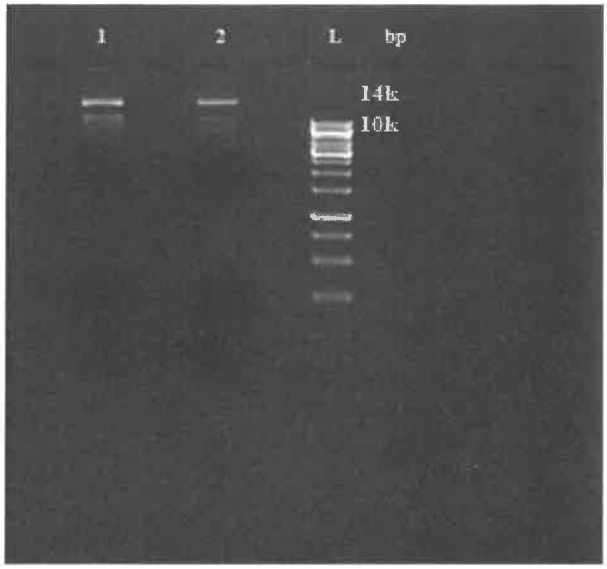

Figure 4.1. Agarose gel loading result for DNA isolated from *E. coli.* 1: Colony No.1; 2: Colony No. 2; L: Gene Ruler 1 kb DNA Ladder

4.2. Confirmation of *egfp* Gene within Transformed Cells

For the modification of *S. maltophilia* strain KB2 with the isolated plasmid pMP4655, the competent cells of the strain were required. Both chemically competent and electrocompetent cells were prepared for heat shock transformation and electroporation. Although electroporation has been stated as the most efficient method by Ye et al (2014), no growth was observed on the plates after transformation attempt with electroporation in our work. On the other hand, heat shock transformation successfully provided transformed cells of KB2 strain (Figure 3.2). Competent cells of *S. maltophilia* had been prepared for three different methods (chemical transformation, electroporation and conjugation) before method by Ye et al (2014). However, this is the first report about successful preparation of chemically competent *S. maltophilia* strain KB2 cells. These cells can be used for further transformations with different plasmid to apply in various purposes.

DNA isolated from the transformant *S. maltophilia* strain KB2 cells were examined and after confirmations for the presence of the plasmid, the cells were used for further processes. The confirmation of the transformed KB2 strain was performed by double digestion of isolated DNA by *BamHI* and *Xba*I restriction enzymes. These enzymes had unique restriction sites for the sequence involving *egfp* gene and the expected products around 10 kb and 700 bp after the digestion was observed in the gel loading result. Beside double digestion, PCR was also applied for the isolated DNA from the transformant cells. The forward and reverse primers used in the reaction, are designed for the amplification of specific region within *egfp* gene, approximately corresponding to 230 bp. Therefore, amplified product of *egfp* gene fragment represented with a band around 230 bp on the gel confirmed the presence of *egfp* gene within isolated DNA (Figure 4.2).

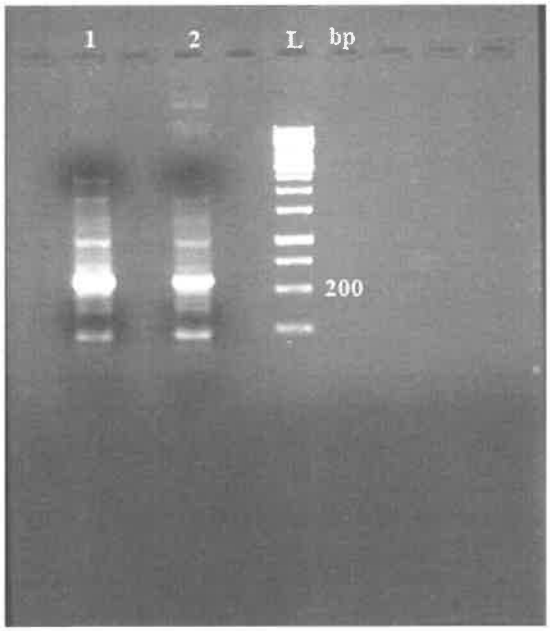

Figure 4.2. Agarose gel loading result for amplification of *egfp* gene fragment with plasmid DNA isolated from transformant *S. maltophilia* KB2 cells as a template. 1: PCR Product; 2: PCR product by using higher amount of template DNA (5 µl); L: Gene Ruler 1kb DNA Ladder

As there were also non-specific amplifications, PCR with different annealing temperatures was applied. Instead of one annealing temperature (45°C), four different temperatures (43°C, 45°C, 47°C and 49°C) were used. Though non-specific amplifications had been dramatically reduced, they could not be completely eliminated (Figure 4.3).

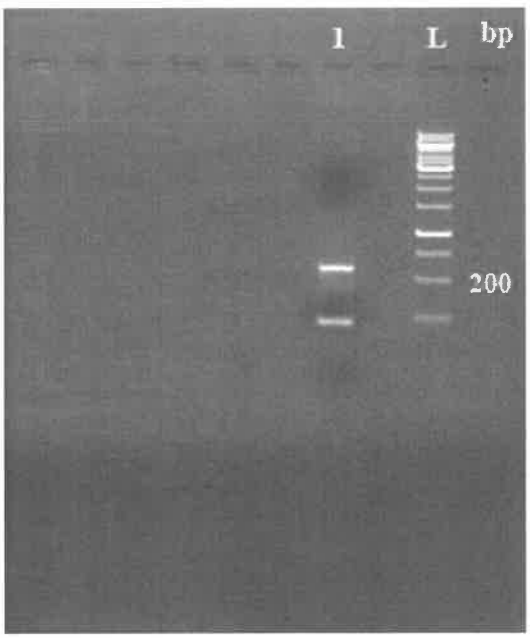

Figure 4.3. Agarose gel loading result for amplification of *egfp* gene fragment by using different annealing temperatures with plasmid DNA isolated from transformant *S. maltophilia* KB2 cells as a template. 1: PCR Product; L: Gene Ruler 1kb DNA Ladder

4.3. Fluorescence Microscopy Visualization of *S. maltophilia* KB2 Cells

After confirming the presence of *egfp* gene in the transformants, the expression of the plasmid was analyzed with fluorescent microscopy by using the excitation and emission filters at 480 nm and 510 nm. The reason for preferring these wavelengths was their characterization for the best visualization of eGFP proteins. The glowing of the samples of the transformants under the microscopy (Figure 4.4), while the non-transformant cells did not have autofluorescence, proved the expression of eGFP plasmid within the cells.

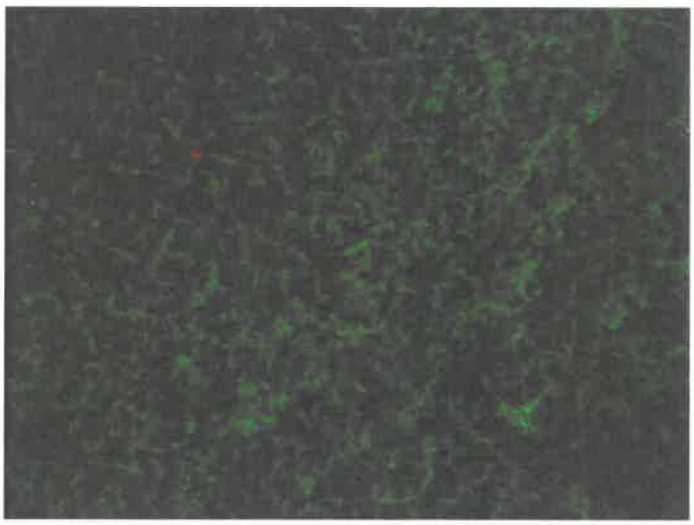

Figure 4.4. Fluorescence microscopy image of transformant *S. maltophilia* KB2 cells transformed *S. maltophilia* KB2 cells glow under fluorescence microscopy, as a result of eGFP plasmid expression

4.4. DNA Analysis of Endophytes Living within Radish Tissues

Once the presence and expression of the plasmid were confirmed within transformed KB2 strain cells, they were introduced into *Raphanus sativus* seeds (Figure 3.4). Its seeds were chosen as *R. sativus* has easy and fast germination, quick maturation and takes up little space. The seeds were placed on white agar plates where they had suitable conditions for the germination. Nine days after the germination, seedlings were used for the analysis. To do that both root (Figure 3.5) and stem parts (Figure 3.6) were selected, and endophytes living in these parts were determined. After grinding, the endophytes from both tissues were screened by using tetracycline plates (50 µg/ml). Only transformed KB2 strain cells expressing eGFP plasmid with tetracycline resistance gene would survive, while the other endophytes extracted from the plant could not. However, when the seeds which were used in negative control group were inoculated

with heat killed *S. maltophilia* KB2 cells, growth in the control plates were observed. Therefore, it was considered that a horizontal gene transfer might had been happened between heat killed *S. maltophilia* KB2 cells and other endophytes living in *Raphanus sativus* plants. Taghavi et al (2005) had shown that transfer of plasmids *via* horizontal gene transfer among endophytes were possible and this feature would further be used for spreading of the target plasmid within natural endophytic microbial communities. For this reason, in the negative control group, unprocessed seeds were used instead the seeds inoculated with heat killed *S. maltophilia* KB2 cells. Upon screening, single colonies were chosen from root and stem endophytes that were survived on the plate, and DNA isolation was performed. The isolated DNA was used for PCR and also PCR with different annealing temperatures. According to the gel loading result (Figure 4.5), *S. maltophilia* strain KB2 transformed with eGFP plasmid had successfully been introduced into the seeds and could have survived within both root and stem parts of *Raphanus sativus.*

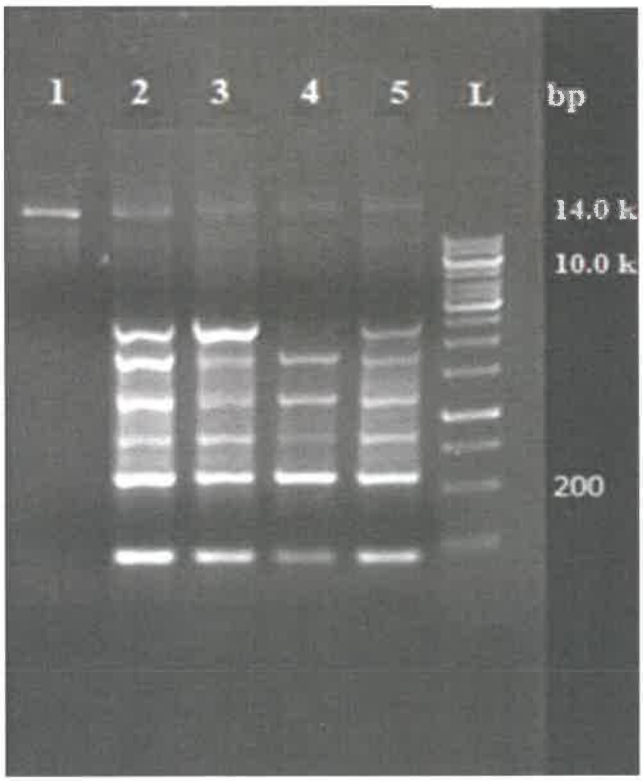

Figure 4.5. Agarose gel loading result for the analysis of endophytes within radish tissues. 1: Plasmid DNA isolated from endophytes in root tissue; 2: Amplification of *egfp* gene fragment with plasmid DNA isolated from endophytes in root tissue; 3: Amplification of *egfp* gene fragment with plasmid DNA isolated from endophytes in stem tissue; 4: Aamplification of *egfp* gene fragment at temperature by using different annealing temperatures with plasmid DNA isolated from endophytes in root tissue; 5: Amplification of *egfp* gene fragment by using different annealing temperatures with plasmid DNA isolated from endophytes in stem tissue; L: Gene Ruler 1 kb DNA Ladder

Many different strains of *Stenotrophomonas maltophilia* had been shown to be living in roots of various plant species such as, cucumber (*Cucumis sativus*), oilseed rape (*Brassica napus*), potato (*Solanum tuberosum*), strawberry (*Fragaria x ananassa*), alfalfa (*Medicago sativa*), sunflower (*Helianthus annuus*), barnyard grass (*Echinochloa crusfalli*) and poplar trees. With this study we showed that *S. maltophilia* strain KB2 can live within root and stem tissues of radish (*Raphanus sativus*), by applying the modification of strain and its introduction into radish seeds.

In the future, the strain can be used for the applications associated with phytoremediation to remove NSAIDs from the contaminated soils. Also, in the light of the evidence that some other strains of *S. maltophilia* had also been recorded as "endophytes", the general features of endophytes would be used to apply *S. maltophilia* for wide range of purposes. These features include anti-pathogenicity, plant growth promotion and increased resistance against abiotic stress factors. Thanks to the modification with plasmid pMP4655 carrying *egfp* gene, the strain and these functions would easily be investigated.

5. CONCLUSION

The overuse of non-steroidal anti-inflammatory drugs (NSAIDs) is a rising threat for various ecosystems, as these drugs can easily release into different environments including soil, sediment or aqueous matrices. Furthermore, there is no effective removal for NSAIDs in sewage plants because of their persistence and hydrophilicity. The deficiency of processes for complete removal of NSAIDs, makes it essential to apply bioremediation processes.

This work was about modification of *Stenotrophomonas maltophilia* strain KB2, able to degrade, one of the most used NSAID class drug, naproxen, with the eGFP protein. Until now, few bacterial strains that are able to degrade naproxen, such as *Planococcus* sp. Strain S5 and *Bacillus thuringiensis* B1(2015b), have been isolated. However, *S. maltophilia* strain KB2 is the first bacterial strain reported for degradation of naproxen. Moreover, having three types of dioxygenase activities (catechol 1,2-dioxygenase, 2,3-dioxygenase and protocatechuate 3,4-dioxygenase activities) make KB2 strain different from the other strain of *S. maltophilia.*

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CV

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