BIOREMEDIATION OF 2,4,6-TRINITROTOLUENE BY NOVEL STRAINS OF AEROBIC BACTERIA

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By

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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

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ABSTRACT

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M.S. in Materials Science and Nanotechnology

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2,4,6-Trinitrotoluene (TNT) has been used extensively for military purposes since its invention in the late 19th century. TNT is a highly toxic and recalcitrant substance due to its multiple nitrated molecular configuration. TNT residues can enter biological systems and constitute a significant risk to human health. To this end, biological approaches promise great potential for removal of TNT in both aqueous and terrestrial environments by the usage of microorganisms. However, remediation capabilities of these organisms are limited due to their inadequate survival and degradation capacity in the environment. To address these issues, we investigated and demonstrated high degradation performance of novel bacterial strains isolated from TNT-contaminated sites, *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13, for an enhanced remediation process. In the first part of this thesis, we developed a novel HPLC method on a Diol-functionalized

chromatography column for accurate, rapid, economic, and environmentally friendly detection of nitroaromatic compounds. Data obtained from chromatography measurements clearly verified that the minimum limit of detection values for TNT and TNT metabolites (0.78-1.17 µg/L) were lower than the values obtained by previous reports and the widely used EPA method.

In the second part, for the first time, we achieved rapid (less than 20 h) TNT decontamination using novel bacterial strains under aerobic conditions at 99.9% efficiency. We showed that TNT was transformed into less toxic and highly reactive metabolites. The data obtained from elemental analysis and HPLC measurements together with FT-IR results indicated that approximately 71.42% of nitrogen from TNT is accumulated in the biomass.

In the third part, we designed laboratory-scale compost system for optimization of maximum TNT degradation efficiency. In this study, we observed a strong correlation between degradation capacity of microorganisms and carbon/nitrogen (C/N) ratio, air flow, and TNT amounts. We accomplished a complete TNT degradation (100%) by an in-vessel compost system in 15 days, the shortest period ever reported. These bioremediation approaches hold great promise for efficient and sustainable removal of TNT for safe environment.

Keywords: 2,4,6-Trinitrotoluene, explosives, bacteria, composting, high performance liquid chromatography, biodegradation, sustainability.

ÖZET

TRİNİTROTOLUEN'İN BİYOLOJİK DEGRADASYON YÖNTEMİ İLE YENİ AEROBİK BAKTERİLER KULLANILARAK TEMİZLENMESİ

Burcu Gümüşcü

Malzeme Bilimi ve Nanoteknoloji Programı, Yüksek Lisans

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2,4,6-Trinitrotoluen (TNT), 19. yüzyılda icat edilmesinden sonra askeri amaçlar için yaygın olarak kullanılmıştır. TNT'nin toksik olması ve doğada uzun süre bozunmadan kalabilmesi, moleküler yapısında bulunan çok sayıdaki azot bileşiklerinden kaynaklanmaktadır. Dolayısıyla, TNT kalıntıları biyolojik sistemlere girebilmekte ve insan hayatı için önemli bir risk arz etmektedir. Söz konusu kirliliğin biyolojik yollarla temizlenmesi, TNT'nin hem su içinde hem de toprakta yol açtığı kirliliği yok etmek için oldukça önemlidir. Ancak biyolojik yollarla temizleme işleminde kullanılan mikroorganizmalar, gerek TNT'yi uzun zamanlarda parçalamaları gerekse doğada hayatta kalma oranları göz önüne alındığında beklenen etkiyi gösterememektedir. Bu sorunlar göz önüne alınarak yapılan degradasyon çalışmalarında, literatürde ilk kez yüksek TNT-parçalama kapasitesi gösteren *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera*

cryocrescens STE 12, ve *Enterobacter amnigenus* STE 13 suşları TNT kirliliği olan alanlardan izole edilmiştir. Bu tezin ilk bölümünde, ilk kez, HPLC yönteminde Diol kolon kullanımıyla daha kesin, hızlı, ekonomik ve çevreye duyarlı sonuçlar elde edilmiştir. Elde edilen sonuçlar, TNT ve metabolitleri için belirlenen alt limitlerin (0.78-1.17 μg/L) literatürde kullanılanlardan daha düşük olduğunu göstermektedir. İkinci bölümde ise yukarda adı geçen bakteriler kullanılarak literatürde ilk kez 20 saat içinde TNT'nin %95 ila 99.9'u yok edilmiştir. Transformasyon sürecinde, 4-aminodinitrotoluen (4-ADNT), 2-aminodinitrotoluen (2-ADNT), 2,4-dinitrotoluen (2,4-DNT) ve 2,6-dinitrotoluen (2,6-DNT) gibi parçalanma ürünleri tespit edilmiştir. Yapılan element analizi ve FT-IR ölçümleri, TNT'den gelen azotun %71.42'sinin hücrelerin biyokütlesine katıldığını doğrulamaktadır. Üçüncü kısımda, maksimum TNT degradasyon performansını elde edebilmek için optimizasyon yapılmak üzere laboratuar ölçekli bir kompost sistemi dizayn edilmiştir. Kompost çalışmalarında karbon-azot oranları ve havalandırma süresi gibi değişkenlerin TNT degradasyonuna etkileri incelemiş, bu parametreler optimize edilmiş ve TNT'nin tümünün, literatürde kaydedilen en kısa zamanda, 15 gün içinde yok edilebildiği gösterilmiştir. Bu tezde geliştirilen yöntemler, TNT'nin hızlı bir şekilde yok edilmesi için önem arzetmektedir.

Anahtar Kelimeler: Trinitrotoluen, patlatıcılar, bakteri, kompostlaşma, yüksek performanslı sıvı kromatografisi, biyolojik yollarla parçalama, sürdürülebilir teknolojiler.

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To my wonderful family and my love…

NOMENCLATURE

2-ADNT: 2-aminodinitrotoluene

2,4-DNT: 2,4-dinitrotoluene

2,6-DNT: 2,6-dinitrotoluene

4-ADNT: 4-aminodinitrotoluene

ACN: Acetonitrile

Aerobe: An organism that requires the presence of oxygen in its environment.

Anaerobe: An organism that does not require free oxygen for its redox metabolism.

Azoxy: A compound having the general structure of R-N=N(O)-R' formed by the condensation of nitroso and hydroxylaminodinitrotoluene compounds.

CFU: Colony forming unit. A measuring unit of viable microorganism numbers in a culture. The results are expressed as CFU/mL.

Cometabolic degradation: A process in which a substance may be degraded only in the presence of a primary source of carbon.

C/N: Carbon to nitrogen ratio

DANT: Diaminonitrotoluene

DNTs: Dinitrotoluenes

FT-IR: Fourier Transform Infrared

HADNT: Hydroxylaminodinitrotoluene

HPLC: High performance liquid chromatography

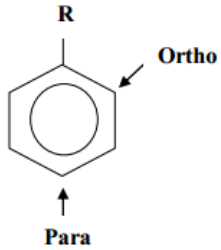
Isomer: A chemical compound that has the same molecular formula but different structural formula.

LB: Luria Bertani medium. It is generally used for enrichment of microorganisms.

NAD(P)H: Nicotinamide adenine dinucleotide phosphate. A reducing agent that is active in anabolic reactions.

Ortho: Substituents occupy positions next to each other

(R and ortho in figure)



Para: Substituents occupy positions opposite each other

(R and para in figure)

Recalcitrance: Compounds which are difficult to degrade under natural conditions.

SEM: Scanning electron microscopy

S/N: Signal to noise ratio. The S/N is a ratio of signal power of the chromatogram to the noise power and used for comparison of background noise level and the level of a desired signal.

TAT: 2,4,6-Triaminonitrotoluene

TATD: TNT and TNT derivatives

TNT: 2,4,6-Trinitrotoluene

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CHAPTER 1

SUMMARY

Nitroaromatics are organic contaminants with harmful side effects on a broad range of organisms, as underlined by the U.S. Environmental Protection Agency (EPA) [1]. 2,4,6-Trinitrotoluene (TNT), one of the most common nitroaromatic compounds, has enjoyed considerable popularity in military and industry applications since its invention in the late 19^{th} century. Widespread utilization of TNT can be attributed to its high stability, low melting point, high explosion temperature, low sensitivity to impact, and relatively safer manufacturing process compared to other nitroaromatic compounds [1].

The extensive use of TNT has resulted in considerable environmental damage, especially in biological systems, and the recalcitrant character of this contaminant resulted in an irremediable environment [2]. The reasons of this recalcitrance are given as follows:

(i) As nitroaromatics are comparatively new to the biosphere, extant species do not have any functional enzymes on TNT metabolism.

(ii) Organisms have not had enough time to evolve mechanisms for dealing with newly encountered nitroaromatics.

Consequently, TNT constitutes a significant risk to human health and other living organisms [3]. Induced oxidative stress is considered to be the main cause of toxic effects of nitroaromatics [4]. Occupational or incidental exposure of TNT may lead to irritation of the skin, disturbances of liver functions and erythrocytes, aplastic anemia, hemolytic anemia, methemoglobinemia, cataract or spermatozoa malformancy [4]. TNT contamination of groundwater has become a significant environmental hazard and today, large amounts of TNT and TNT derivatives (TATD) are still present in the environment [5].

An increasing awareness of the necessity to save the environment has directed scientific research to develop cost effective and environmentally friendly remediation strategies. Bioremediation, the utilization of microorganisms to decompose hazardous organic materials into their non-toxic constituents, is one of the leading research topics of environmental biotechnology. Bioremediation is on its way to earn an irreplaceable position for use in TNT removal in place of thermal and physicochemical approaches; since excavation, disposal, or destruction of soil does not occur during the bioremediation processes [5]. As such, bioremediation has emerged as an alternative method for treating most types of environmental

contamination. Although there are many advantages of bioremediation, there are also drawbacks related to the use of microorganisms. One such limitation is the slow microbial activity, which results in long degradation periods [6]. Since several microbes, which are functionalized in laboratory-scale studies, cannot survive in ambient conditions, it is difficult to apply them in a real-life situation. These problems necessitate the development of new approaches for effective TNT bioremediation.

In this thesis, the overarching objective is to expand the idea of TNT bioremediation by novel bacterial strains, using composting approach as an effective real-life application. We isolate and describe four new isolates capable of degrading TNT completely in 20 h, which is the fastest rate reported to date. Here we demonstrate that bacterial growth rates depend on TNT metabolism and evaluate the potential factors influencing TNT biodegradation, such as temperature and pH changes.

We designed and operated an in-vessel reactor system to increase the TNT degradation rate using fast TNT-degrading novel bacterial strains. Our results suggest that our in-vessel compost system exhibits a substantial enhancement in bioremediation performance, particularly under aerobic conditions. The results of this study indicate that even high levels of TNT contamination (100 g/kg) can be removed in 15 days.

In addition, for the first time we used Diol functionalized column for detection of TNT and related compounds from munitions-contaminated zones. To this end,

we optimized a novel high performance liquid chromatography method on a Diol chromatography column for accurate, rapid, economic, and environmentally friendly detection of nitroaromatic compounds. We present shorter analysis times (below 13 min) and lower detection limits (0.03-0.07 µg/L) than the HPLC conditions outlined in the commonly utilized EPA method 8330. In our study, the solvent consumption is reduced down to 58.3% by the Diol column, compared to the C-18 and Phenyl-3 columns.

This thesis is divided into six parts. Chapter 1begins with a brief introduction on problems faced in TNT removal and explains the motivation for undertaking this study. Existing remediation approaches in the literature are discussed to overcome these problems. A brief discussion is also provided on potential solutions to the problems of TNT remediation. Chapter 2 explains reduction mechanisms, bacterial degradation pathways and detection methods of TNT. Concerns on environmental contamination and hazards of TNT together with the remediation approaches in the literature for degradation are also introduced. In addition, an introduction is made to the high performance liquid chromatography method. In Chapter 3, we introduce the development of a HPLC method for accurate and sensitive detection of TNT and its relatives. We demonstrate a significant improvement in the detection capacity of nitroaromatic compounds compared to previous efforts. In Chapter 4, we focus on TNT biodegradation by novel bacterial strains and their proof-of-concept demonstrations of TNT bioremediation. We detail our results, which show a significant increase in degradation capacity by the newly isolated bacteria.

Chapter 5 is based on the use of the in-vessel compost system for the improved TNT bioconversion efficiency. We present our optimization data for the integration of the novel strains on a composting platform in this chapter. Finally, in Chapter 6, we conclude our studies and make remarks on our contributions to the related fields which render the importance of this work.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

In 1863, Julius Wilbrand, a German scientist, invented TNT to be used as a yellow dye (Figure 2.1). TNT was not utilized as an explosive throughout the world until German and British Militaries realized the destructive potential of the chemical in 1902. TNT was favored in both World Wars as the explosive of choice due to its relatively safer production, storage and usage compared to picric acid and nitrocellulose. For almost 100 years, TNT has been utilized in the manufacture of dyes and underwater blasting processes. As a result of extensive TNT usage, today over one million cubic yards of soils and 10 billion gallons of water in the U.S. alone are estimated to be contaminated by TNT and its derivatives [1].

Figure 2. 1. TNT flakes from the TNT manufacturing facility in Elmadag, Turkey

To date, incineration, which is the removal of nitroaromatic compounds by combustion method, has been preferred for removal of TNT from contaminated regions. This technique, however, is not only expensive but also environmentally hazardous due to the release of toxic combustion compounds. In the last few decades, the idea of remediation has become increasingly attractive for its ease in use and cost efficiency. In particular, biological methods hold promise as more effective and less costly treatment approaches compared to physical and chemical methods of TNT removal [7-9] (Figure 2.2).

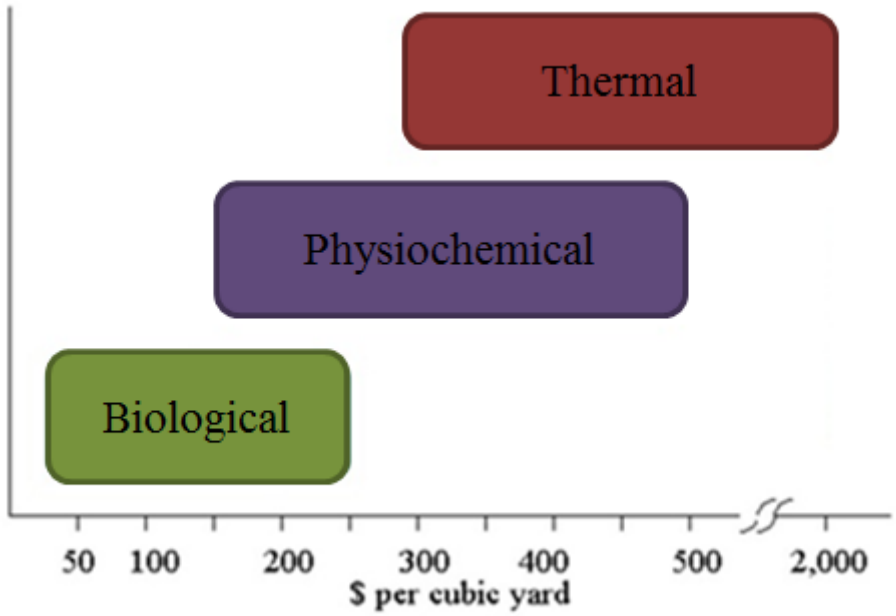


Figure 2. 2. Remediation costs of biological, physiochemical, and thermal approaches for TNT contaminated sites

This chapter is organized into five sections. In the first section, the chemical properties and synthesis of TNT are introduced alongside the molecular basis of TNT degradation. In the second section, environmental contamination and hazardous effects of TNT are discussed. The third section gives information about TNT detection methods; followed by a fourth section focusing on common bioremediation methods and the associated challenges are mentioned. Finally, in the last section, bacterial TNT degradation pathways are detailed.

2.1. Characteristics of 2,4,6-Trinitrotoluene

Aromatic compounds are composed of atoms forming a conjugated ring shape, where the atoms generally have one or two bonds between them. Ion pairs, unsaturated bonds and empty orbital of atoms provide higher durability and stability to the ring. Nitroaromatics are nitrated forms of aromatic compounds, generally with six carbon atoms merged as a circle and conjugated with a methyl group. TNT is a nitroaromatic compound comprising a methyl group present at the 1 position and 3 nitro groups located on a carbon ring at the 2, 4 and 6 positions.

TNT is a yellow, odorless solid that has an explosive character, and it is relatively unresponsive to impact, friction, or shock compared to other nitroaromatic compounds [10]. The chemical structure and properties of TNT are summarized in Table 2.1 and Table 2.2.

Property	Information	Reference
Molecular weight	227.13	[11]
Color	Yellow	[11]
Physical state	Monoclinic needles	[11]
Melting point	80.1^{o}C	[11]
Boiling point	240^{o}C	[12]
Specific gravity	1.654	[11]
Odor	Odorless	[11]
Solubility		
Water at 20^{o}C	130 mg/L	[12]
Organic solvent(s)	Soluble in acetone, acetonitrile and benzene	[11]
	Soluble in alcohol and ether	[11]
Vapor pressure at 20^{o}C	1.99x10^{-7} atm m^{3}/mole	[12]
Flashpoint	Explodes	[13]
Flammability and reactivity	4.4	[14]
Explosive temperature	240^{o}C	[14]

Table 2. 1. Chemical and physical properties of TNT

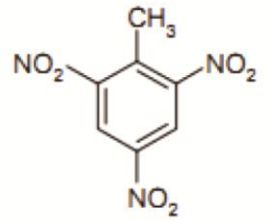
Characteristic	Information	Reference
Chemical name	2,4,6-Trinitrotoluene	[12]
Synonyms	sym-trinitrotoluene, 1-methyl-2,4,5-trinitrobenzene, 2-methyl-1,3,5-trinitrobenzene, TNT, tolit, trotyl oil, trilit, tritol	[12]
Chemical formula	$C_7H_5N_3O_6$	[11]
Chemical structure		[15]
Identification numbers:		
CAS registry	118-96-7	[11]
HIOSH RTESCS	XUO175000	[12]
HSDB	1146	[12]
NCI	C56155	[12]

Table 2. 2. Chemical identity of TNT

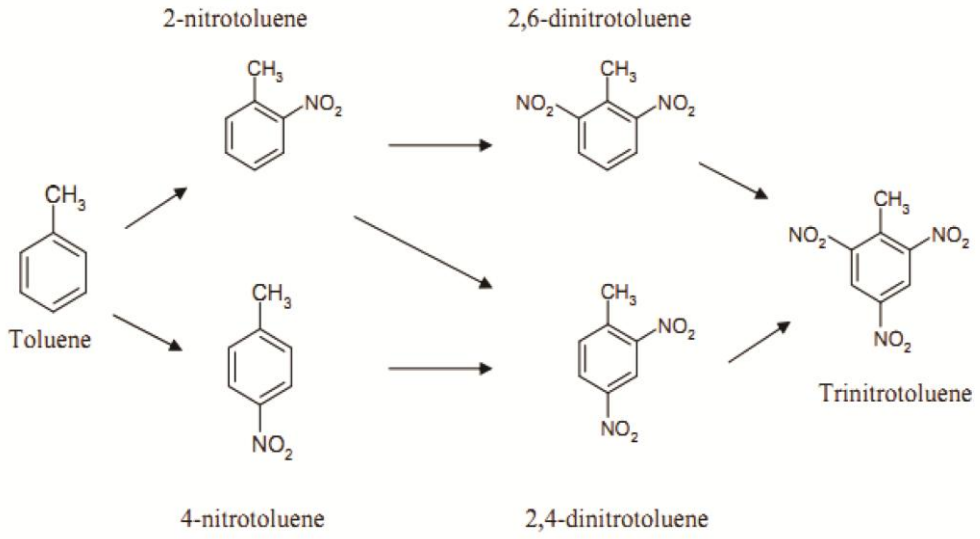


Figure 2. 3. Synthesis of TNT [16]

Synthesis of TNT is conducted through a stepwise nitration of the toluene ring, as depicted in Figure 2.3. 2-nitrotoluene and 4-nitrotoluene isomers are the first nitration products, which are followed by the isomers 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) as nitration products.

TNT can be metabolized biologically via enzymatic reduction [5]. In the biodegradation process, some enzymes, which reduce one-electron of the nitro group, are called “oxygen sensitive” due to production of radicals such as superoxides. The two-electron reduction of the nitro group is conducted by “oxygen insensitive” enzymes and no radicals are formed in this type of reduction [17,18].

While anaerobic bacteria and plants generally have oxygen sensitive enzymes, aerobic bacteria utilize oxygen insensitive enzymes for the reduction of TNT.

In the mechanism of biodegradation, a stepwise process is facilitated by microbial enzymes and results in formation of less toxic and more degradable products. As aryl nitro groups of TNT are in resonance, the nitrogen-oxygen bond therein is polarized. Since oxygen atoms are more electronegative than nitrogen atoms, partial positive charges (electrophilic character) are carried via nitrogen atoms. The electrophilic character of nitrogen atoms leads to polarization in the TNT molecule and allows the reduction of nitro groups. More particularly, the reduction of TNT molecule occurs predominantly by aryl nitro group reduction, because oxidative attacks are precluded by the electron withdrawing properties of aryl nitro groups in TNT [19]. The primary route of TNT reduction involves the conversion of nitro groups into amino, nitroso, and hydroxylamino groups [19].

In both anaerobic and aerobic biodegradation pathways, the first step is reduction of aryl nitro groups to amino groups. Generally, para-positioned nitro groups are more likely to be reduced in biological systems. Subsequent reduction takes place in ortho-position, which leads to the formation of dinitrotoluene isomers. Further reduction of nitro groups yields highly reactive nitroso and hydroxylamino intermediates. After this conversion process, 2,4-DNT; 2,6-DNT; 2-aminodinitrotoluene (2-ADNT); and 4-aminodinitrotoluene (4-ADNT) are formed as common reduction products (Figure 2.4) [20].

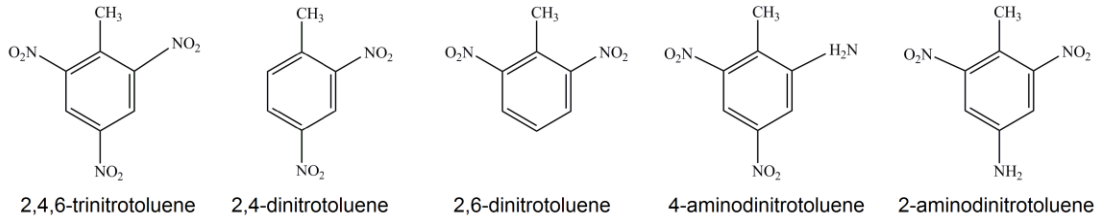


Figure 2. 4. Molecular structures of TNT and TNT derivatives

The number of nitro groups proportionally affects the likelihood of reduction because increasing numbers of nitro groups prevent electrophilic attacks from nitrotoluenes. Therefore, reduction rate of TNT is higher than that of DNTs and ADNTs [15]. In addition, degradation rates of TNT are generally higher in aerobic conditions compared to anaerobic conditions because the former involves fewer steps and relatively rapid reduction processes [21-23]. In aerobic and anaerobic conditions, further degradation products of TNT such as dinitroaminotoluenes, diaminonitrotoluenes (DANT), and triaminonitrotoluenes (TAT) are highly unstable compounds capable of irreversibly binding to soil. Finally, sequential bioconversion becomes unfeasible due to covalent interactions between organic matter in the soil and degradation products. Thus, negative effects of TNT are mitigated as a result of the mineralization process in the soil.

2.2. Environmental Contamination and Hazardous Effects of TNT

TNT has been used as a dye and explosive for almost 150 years. Large-scale manufacturing, use, and disposal of TNT resulted in widespread TNT contamination of aquatic and terrestrial systems [19]. TNT has severe negative impacts on environment due to its mutagenic properties and persistence [19]. In recent years, up to 200 g/kg of TNT were found in contaminated soils in Europe and USA [5]. TNT may persist in the environment for many decades without degradation and TNT contamination is primarily found in soils at depths of 1.5-3.4 m in open burning sites [24,25]. A previous study on environmental contamination by TNT has demonstrated that after a burning process, soil depths of 3.9 to 4.6 m were contaminated by TNT and its residues [5,19]. Since TNT is a mobile and water-soluble compound, contaminated surface water and aquifers take a pinkish color and are called "pink water" [26]. The formation of "pink water" relies on the photolysis of dissolved TNT and accumulation of complex dye-like molecules [27-29]. (Figure 2.5). Agricultural irrigation wells are under considerable risk due to the possibility of contamination by pink water [30]. The US Environmental Protection Agency (EPA) classifies TNT and its metabolites as major contaminants in class C mutagen and carcinogen compounds [1]. As TNT intermediates are highly reactive, they can rapidly undergo reactions with a variety of biological materials. The EPA discharge limits in water are 2 ng/L for TNT, 0.32 mg/L for 2,4-DNT and 0.55 mg/L for 2,6-DNT [1].

Figure 2. 5. Pink water appearance in TNT manufacturing and mine explosion sites, Elmadag, Turkey

In addition, TNT disposal processes such as incineration open burning, and open detonation cause substantial contamination of the atmosphere. The approximate half-life of TNT is between 18-184 days in the atmosphere, where particulates and tiny crystals of TNT pose serious toxic, mutagenic and carcinogenic effects in case of inhalation [12,14].

TNT can be absorbed by the human body by skin, respiratory system and gastrointestinal system. In human body, liver, kidneys, lungs and urinary tract are the most affected organs by TNT. Occupational or incidental exposure to TNT (between 0.01-1.49 mg/m^3 concentrations) may cause several detrimental effects in humans, such as anemia, abnormality in liver functions, skin irritation, cataracts, spleen enlargement, as well as immune and reproductive system defects [3,6,31-33]. Similar effects have been demonstrated in animals that were exposed to TNT

by inhalation and direct contact. Previous studies have shown that exposure to TNT elevates the risk of cancer [3,6,31]. As such, remediation of TNT is of substantial importance in order to mitigate the negative long-term effect on living organisms and remove the detrimental effects of this contaminant on the environment.

2.3. TNT Detection Techniques

Nitroaromatics can be detected by a variety of methods. The most preferred methods are high performance liquid chromatography (HPLC), capillary electrophoresis (CE), thin-layer chromatography (TLC), and gas chromatography with mass spectrometry (GC-MS). In the past few decades, different separation tools have been applied for more sensitive analysis of nitroaromatics including CE [34]. The CE method is based on sensing the molecular charges, frictional forces and hydrodynamic radii of molecules. Further alternatives include TLC [35]—in which a layer of adsorbent material is used—and GC-MS [36]—based on sensing the adsorption of the vaporized sample—and are used as alternative cost-effective methods. The chromatographic separation is the most preferred technique for providing an accurate determination and quantification of TATD in biotransformation studies and environmental measurements [37]. HPLC is a powerful analytical technique for detection of sub-ppb range concentrations of TATD. Recently, efforts on identifying and analyzing minute amounts of TNT have been demonstrated via advanced analytic methods [38,39]. To this end, prior HPLC

studies focused on increasing detection capability and reducing retention time, sample preparation and cost [40-42].

2.4. Bioremediation Approaches for TNT

TNT remediation is of substantial importance, as it is vital to combat the negative long-term effect of TNT on living organisms and remove the detrimental effects of this contaminant on the environment.

In the literature, efforts on degradation of TNT contaminated areas were demonstrated via *ex situ* and *in situ* techniques. Previously studied *ex situ* techniques are mainly incineration, wet air oxidation [43], and photocatalytic degradation [44]. Incineration, the removal of nitroaromatic compounds by combustion, is one of the long-established *ex situ* methods for reducing contamination. Further, other *ex situ* methods include wet air oxidation [43]., in which soluble complex organic compounds are oxidized by the use of air in an aqueous solution under high temperature and pressure conditions. The photocatalytic degradation [44] is an alternative *ex situ* method based on exposing photons to excite electrons from the valence to the conduction band to generate free electrons and oxidize nitroaromatics. However, relatively high implementation costs and the formation of toxic end products limit the overall effectiveness of these techniques. Thus, current studies are focused on bioremediation by *in situ* methods. Fungi [45], plants [20], anaerobic bacteria [46], and aerobic bacteria [47] have been

extensively studied for more efficient *in situ* remediation of TNT. Fungi can tolerate high concentrations of nitroaromatic explosives; however, they cannot survive in harsh environmental conditions such as extreme of temperatures and pH [5]. Genetically modified plants have been also used for bioremediation of nitroaromatics. On the other hand, this approach is unfavorable since genetically modified organisms are subject to ethical and environmental concerns [48]. Even though it is known that anaerobic bacteria are capable of transforming TNT to the environmentally benign degradation product TAT [49], anoxic conditions are required for this process.

Bioremediation by aerobic bacteria is a promising alternative to current techniques because of high TNT reduction rates, environmentally friendly degradation products, low-operation costs and widespread presence of those microorganisms in the environment [5,44]. Bioremediation is a branch of biotechnology which uses microorganisms to decompose toxic materials into various metabolic products. Transformation of hazardous compounds to CO_2, water, energy, and several metabolites occurs through numerous different pathways by enzymatic reactions in bioremediation processes [50].

2.4.1. Bacterial Degradation of TNT

Bacterial degradation refers to the complete catabolism of a compound to its constituent components by a microbial system. Early surveys indicated that TNT

was amenable to biodegradation under appropriate conditions [51-53]. In this case, two types of reactions are possible. In one type of reaction, TNT is transformed into its metabolites by reduction of nitro groups and ADNTs are formed in the presence of oxygen [6,7,9,10]. In the second, TNT is reduced completely to TAT in anoxic environments [4,5,54].

2.4.1.1. Aerobic Pathways

TNT may participate in a variety of reactions under oxygenated conditions. The most commonly reported pathway involves the reduction of nitro groups. In the initial reduction mechanism, a nucleophilic attack facilitated by microbial enzymes occurs at the aromatic ring, leading to the formation of Meisenheimer complex. Subsequently, the nitrite group is released to the environment, and Meisenheimer complex is transformed to 2,4-DNT molecules. Further down the catabolic pathway, 2-ADNT, 4-ADNT, or 2,4-DANT is formed as a result of microbial activity. Figure 2.6 summarizes the pathway for the aerobic metabolism of TNT.

In most pathways described to date, aerobic bacteria transform TNT by reducing one or two nitro groups to amino or hydroxylamino groups resulting in the irreversible bonding of these molecules with soil. Thus, degradation of TNT necessarily involves the formation of DNTs. In many studies, certain bacteria, such as *Pseudomonas* sp. strains, were shown to metabolize DNTs by dioxygenase attack that was followed by oxidation, nitrite release, and mineralization [16,56-58].

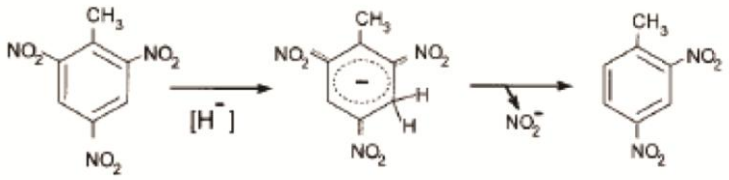


Figure 2. 6. Meisenheimer complex formation. The hydride ion can be donated by NAD(P)H, results in the formation of Meisenheimer complex [34,55]

In addition, pentaerytrhritol tetranitrate reductase enzymes, purified from *Enterobacter cloacae* strains and transferred to *Escherichia coli*, were shown to catalyze denitration reactions in TNT [59]. Likewise, the NADH dependent flavoprotein nitroreductase activity was reported to degrade TNT and reduce its nitro groups. Moreover, nitrobenzene reductase (from *Pseudomonas pseudoalcaligenes*) was demonstrated to produce 2-HADNT and 2,4-HADNT in the presence of oxygen. The reduction of TNT to amino intermediates is widely accomplished by many species such as, *Pseudomonas* sp., *Mycobacterium* sp., *Rhodococcus* sp. and *Burkholderia* sp. [4,5,16,58,60]. Several degradation mechanisms were reported to date (Figure 2.7).

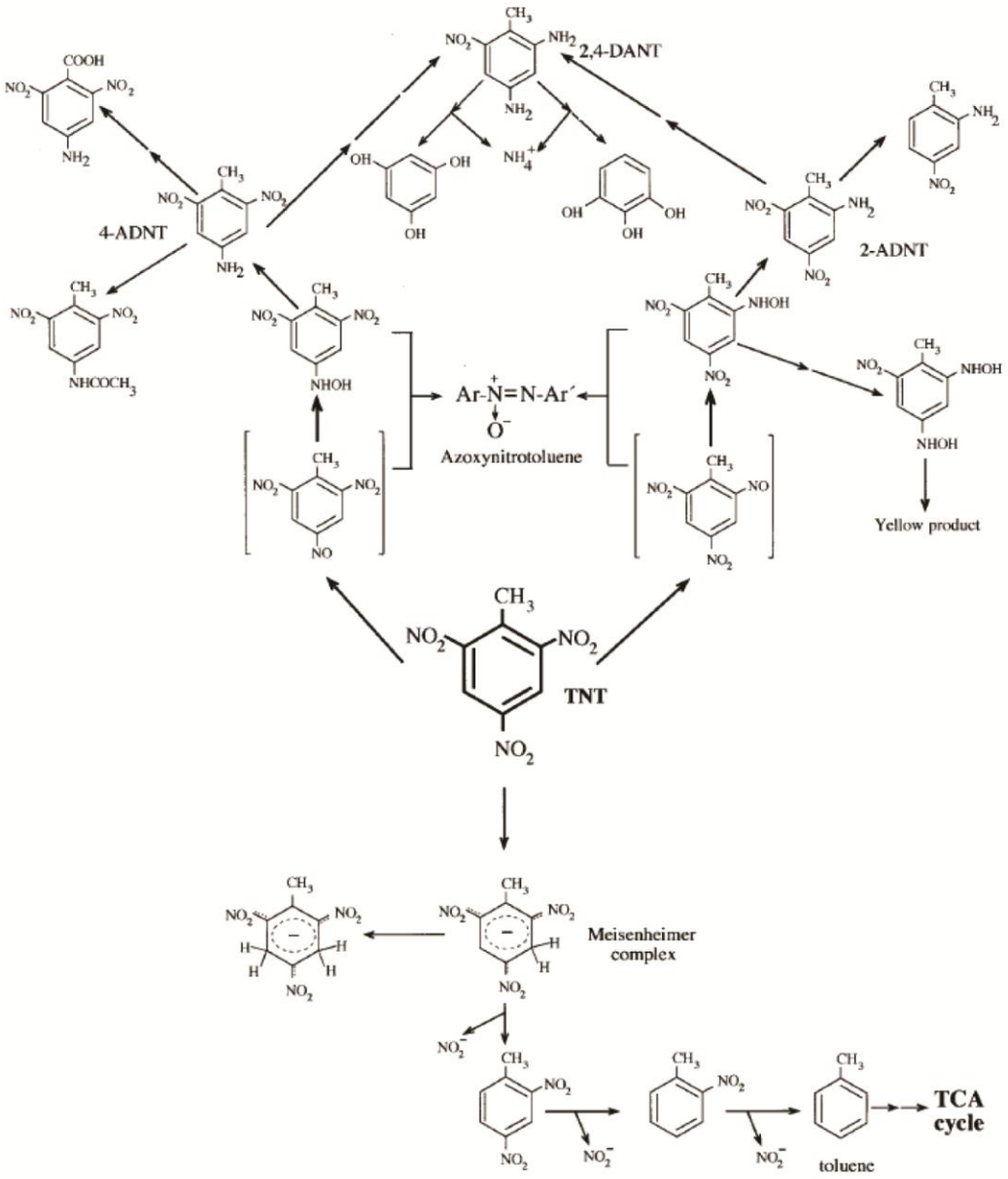


Figure 2. 7. Pathways for the aerobic metabolism of TNT. Two consecutive arrows show undefined series of intermediates between main steps. TCA cycle: Tricarboxylic acid cycle which is used for energy production [55]

Oxidative reactions are potential alternatives to reductive pathways for TNT degradation. Oxidation reactions may only occur after a second amino group is formed on the aromatic ring [61,62]. In this type of aerobic degradation, mineralization does not occur because oxidation of methyl groups is followed by decarboxylation of ADNTs. Besides dioxygenase and monooxygenase enzymes, oxidative deaminases play an important role for explanation of oxidative reactions. In these reactions, TNT was found to be metabolized slowly and transformed to DANTs with trace amounts of azoxy dimers [19,63].

2.4.1.2. Anaerobic Pathways

Anaerobic degradation processes have low redox potential because of the absence of oxygen. Anaerobic degradation of TNT was first shown in *Veillonella alkalescens* [59]. Two strains, *Clostridium* sp., and *Desulfovibrio* sp., were extensively studied for determination of anaerobic degradation pathways [5,53,64,65]. Reduction of nitro group results in the formation of TAT, which is a non-toxic, unstable and electron rich intermediate. Recent studies suggest that TAT formation is mediated by CO dehydrogenase enzymes [66]. In aerobic reactions, TAT accumulation does not occur because the intermediate undergoes rapid auto oxidation in the presence of O_2 [61].

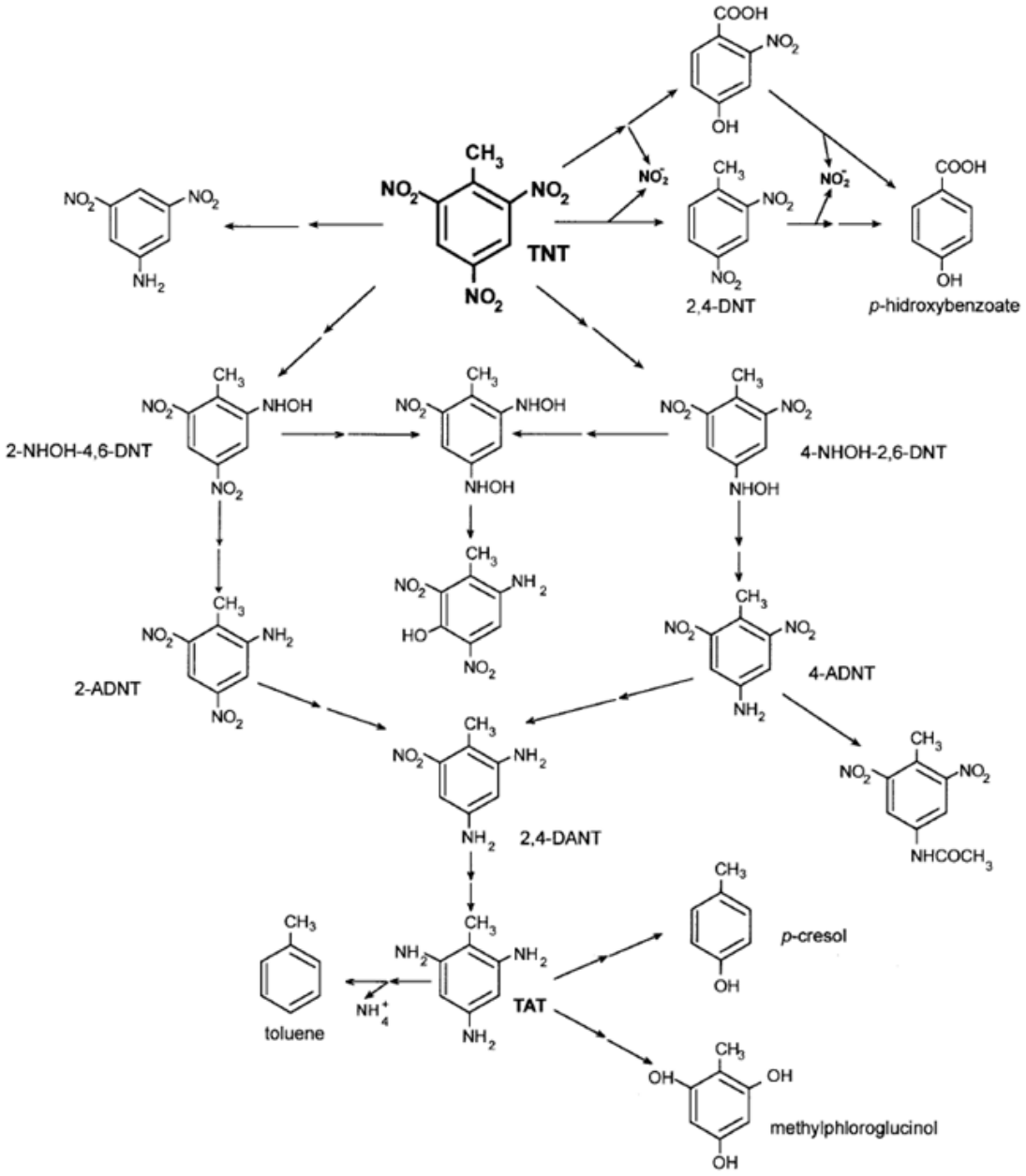


Figure 2. 8. Pathways for the aerobic metabolism of TNT. Two consecutive arrows show undefined series of intermediates between main steps [55]

2.4.1.3. Composting

Composting approach can serve as an effective bioremediation system because of its environmentally friendly end-products and low implementation costs. Additionally, composting has been documented previously to be effective in treatment of hazardous wastes, such as TNT [67]. Composting was first utilized for TNT degradation in 1978 by Osmon *et al.* [68]. In a recent study, native microbial colonies from surface soils and groundwater materials were demonstrated to degrade TNT effectively by composting method [69] and another study suggested that 92% of initial TNT can be removed by two different aerated compost systems [69]. Likewise, the biotransformation of [^{14}C]-ring-labeled TNT was tracked in a compost system [70] and another work highlighted the degradation of both TNT and its transformation products (such as 2-ADNT; 4-ADNT; 2,4-DNT; and 2,6-DNT) within 65 days [8]. Microbial transformation and mineralization of TNT in surface soils and aquifer materials was also investigated by compost systems [5,71]. Most of the microbial transformation studies focused on fungal [72], and anaerobic degradation systems [73]. However, relatively high implementation costs and long degradation periods limit the overall effectiveness of these techniques.

In-vessel composting is the preferred method for treatment of munitions-contaminated soils within the scope of sustainable bioremediation technologies [73]. In contrast to other techniques, in-vessel composting is more efficient and better controlled. Moreover, aerobic bacteria were utilized in bioremediation processes because of the widespread presence of those microorganisms in the

environment [5,46]. Thus, a combination of bacterial TNT degradation metabolism and in-vessel composting is a promising alternative for new generation bioremediation systems.

CHAPTER 3

DEVELOPMENT OF AN EFFICIENT HPLC METHOD FOR DETECTION OF TNT AND ITS RELATIVES

3.1. Overview

TNT contamination in the environment has become a significant environmental hazard [28,29] because of the negative effects. As large amounts of TNT and TNT derivatives (TATD) are still present in the environment [1], improved techniques for the detection and analysis of TATD are of substantial importance. We investigated a rapid and effective system for detecting TNT and its relatives in order to follow the performance of bacterial degradation and composting studies.

In this chapter, we describe a highly efficient high performance liquid chromatography technique with UV detector (HPLC-UV). We showed that the Diol functionalized column is more effective compared to C-18 and Phenyl-3 columns, in detection and separation of TNT; 2,4-DNT; 2,6-DNT; 2-ADNT and 4- 4-ADNT. Separation efficiency of the Diol column for nitroaromatics can be attributed to hydroxyl groups on the diol layer providing weaker interactions with nitroaromatic compounds, resulting in shorter retention times. Using the Diol column, total analysis time was below 13 min including column re-equilibration between the runs, the minimum resolution for the separation of TNT and TNT derivatives (TATD) was 2.6 between 2,4-DNT and 2,6-DNT. Also, the Diol column reduced solvent consumption up to 58.3% compared to C-18 and Phenyl-3 columns. The detection limit was in the range of 0.78-1.17 µg/L for TNT and its derivatives for the Diol column. Compared to the analysis outlined in the method 8330 of the U.S. Environmental Protection Agency, these results show a better detection of explosives by the proposed HPLC method. This approach can be useful for robust measurement of nitroaromatic compounds.

3.2. Materials

3.2.1. Liquid Chromatography: Device Properties

The HPLC method was invented in 1970s for enhanced separation and detection of components in a mixture. The idea of this method is to solve

inadequate and inefficient performance of existing chromatography techniques. High pressures, up to 400 atmospheres, are applied to a smaller-particle-sized column to obtain extremely sensitive qualification and quantification of compounds rapidly.

3.2.1.1. Chromatography Tools

The standards of TNT; 2,6-DNT; 4-ADNT; and 2-ADNT (1000 μg/mL in acetonitrile, purity >99.0%) were purchased from SupelCo (USA). An intermediate stock solution of 2,4-DNT was prepared by dissolving powder 2,4-DNT (SupelCo, USA) in acetonitrile at the concentration of 1000 μg/mL. Double distilled water (dH_2O) (Millipore, USA), HPLC-grade acetonitrile and methanol (Sigma-Aldrich, USA) were used throughout the experiments. Solvents were degassed prior to use. Specimens were stored in glass vials covered with light-blocking plastic bands in order to prevent degradation by the exposure to the sunlight. Stock aliquots and intermediate standard solutions were kept at +4°C in a dark room. Each solution was used for a maximum of 20 days. Calibration solutions of each reagent were prepared by diluting the standard solutions to 1000 μg/mL, 500 μg/mL, 300 μg/mL, 100 μg/mL, and 10 μg/mL in acetonitrile. Stock solutions were diluted to 1:10 ratio with acetonitrile. A mix solution was prepared by blending 150 μL of acetonitrile and 10 μL of each standard samples. All samples were prepared fresh on the day of measurement. All stocks were analyzed immediately after preparation.

All measurements were performed on an Agilent 1200 Series HPLC system (Waldbronn, Germany) equipped with 1200 series binary pump (G1310A), micro vacuum degasser (G1322A), standard auto sampler (G13229A), thermostatted column compartment (G1316A), and multi-wavelength detector (G1365D). Ruggedness and reproducibility of the suggested method was evaluated by Accurate Q-TOF LC/MS 6530 (Agilent Technologies, USA) (System properties: Degasser-G1379B, binary pump-G1312B, VWD-G1314B) and TOF LC/MS 6224 (Agilent Technologies, USA) (System properties: Degasser-G1379B, binary pump-G1312A, ASS-G1329A, TCC-G1316A, VWD-G1314B) systems. HPLC analyses were carried out with three different types of columns; Zorbax Eclipse XDB-C18 (4.6 mm x 150 mm, 5 µm), Inertsil Phenyl-3 (4.6 mm x 150 mm, 5 µm) and Inertsil Diol (4.6 mm x 150 mm, 3 µm). Each column was treated with acetonitrile-water gradients. For all measurements, room temperature was used as the system temperature. Bandwidth of the detector was set to 16 nm. Injected sample volume was 5 µL and kept constant for every run. The system was controlled by HP Chemstation software.

3.2.1.2. Mobile Phase and Gradients

The detection of reagents was facilitated by measuring the ultraviolet absorption with a multi wavelength detector at 254 nm wavelength [42,74]. Double distilled water, methanol, and acetonitrile were used as mobile phases. All

experiments were replicated at least three times. The final assigned values indicate an average value of each result.

We applied four different methods for the separation of nitroaromatics on the aforementioned columns. In the first method, the mobile phase consisted of a blend of 90% water and 10% acetonitrile. Conditions were the same in all columns. Initial acetonitrile ratio was raised from 10% to 100% in 2-26 min time range. From 26 to 28 min, acetonitrile ratio was gradually reduced to 10%. The total runtime was 28 min. The same program was employed with methanol instead of acetonitrile in order to evaluate the efficiency of an alternative solvent in the second method. Gradient programs of the third method for aforementioned columns were described in Table 3.1. As the fourth separation technique, the procedure shown in Table 3.1 was employed with addition of 0.1% formic acid to both mobile phases.

After each injection, the system was rinsed by injection of pure acetonitrile by applying method 1. Flow rate of the mobile phases was 0.7 mL/min for all techniques and column types, except for the methods 2 and 3 of the Phenyl-3 column (flow rate: 1.4 mL/min) and the method 2 of the C-18 column (flow rate: 1.2 mL/min). A flow rate of 0.7 mL/min was used in order to prevent potential damage of Diol column caused by high pressure. Higher flow rates were used for optimization-oriented methods (2 and 3) of columns with larger particle sizes. Total runtimes listed include re-equilibration time of the detector. Column and technique efficiencies were compared at four levels: 1, evaluation of retention time and separation; 2, assessment of a different mobile phase; 3, effect of formic acid

addition to the mobile phase; 4, evaluation of best performances of aforementioned columns.

Diol column (Flow rate: 0.7 mL/min)		
Time step (min)	Eluent 1: dH_2O (90%)	Eluent 2: MeCN (10%)
0	90%	10%
2	90%	10%
18	40%	60%
20	90%	10%
25	90%	10%
C-18 column (Flow rate: 1.2 mL/min)		
Time step (min)	Eluent 1: dH_2O (90%)	Eluent 2: MeCN (10%)
0	50%	50%
5	50%	50%
30	0%	100%
32	50%	50%
35	50%	50%
Phenyl-3 column (Flow rate: 1.4 mL/min)		
Time step (min)	Eluent 1: dH_2O (90%)	Eluent 2: MeCN (10%)
0	80%	20%
2	80%	20%
25	30%	60%
28	80%	20%

Table 3. 1. Gradient method for three different types of columns

3.2.1.3. Evaluation of Chromatograms

Retention times *(t_R)* and elapsed times of the elution phases *(t_M)* were obtained from chromatograms. The capacity factors *(k')* were calculated for each reagent using the following formula.

An optimal capacity factor rate is between 1 and 5; where the capacity factors of less than 1 indicate a particularly powerful separation capacity. Theoretical plate (*N*) model was calculated by using the following formula t_R refers retention time and t_w defines the bottom width of the peak of the reagent.

$$N = 16\left(\frac{t_R}{t_{w1/2}}\right)^2$$

According to the formula seen above, columns with higher *N* values are considered to be more effective. Resolution (R_s) between two desired analytes (A and B) is defined by separation value calculated with the formula below. Separation occurs only if R_s value is greater than 1 [75].

Limit of Determination (LOD) and limit of quantification (LOQ) values are calculated according to International Conference on Harmonization (ICH) guidelines [75, 76] with the formulas below. **σ** presents the standard deviation of calibration curve.

$$LOD = \frac{3\sigma}{S/N} \qquad LOQ = \frac{10\sigma}{S/N}$$

3.3. Results and Discussion

3.3.1. Column Properties

Preliminary investigations were carried out in order to appraise the convenient gradient phases for the separation of nitroaromatics. The separation capacities of C-18, Phenyl-3, and Diol columns were evaluated under the same initial conditions and runtime. Filling material of the Diol column consists of a dihydroxypropyl bonded phase on silica surface. The increased separation performance of this column is due to hydrogen bonding interactions between the analyte and diol groups. The Diol is proved to be the optimal column; it displays shorter runtime compared to the other two columns as seen in Table 3.1. Phenyl-3

column is composed of beads which include phenyl groups attached to the short hydrocarbon ends of silica. Phenyl groups on beads interact with the analyte by the π-π interactions as well as hydrophobic interactions. The aromatic rings are π-rich due to their electron donating properties, thus we were able to observe an enhanced separation of nitroaromatics. There have been a significant number of reports on the separation of nitroaromatics due to hydrophobic interactions via the filling material of C-18 column [76]. The Diol and Phenyl-3 columns have higher separation capacities compared to C-18 (Table 3.1).

3.3.2. Column- Specific Optimization

Column efficiency is determined by the theoretical plates, while the performance of separation is characterized by the resolution between adjacent peaks. In quantitative measurements, higher signal-to-noise ratio (S/N) values correspond to lower detection thresholds for the concentration of samples [76].

The separation of DNT isomers, ADNT isomers, and TNT are accomplished by analyzing the vector resultants of the interactions between nitro groups and hydrogen bonds. TNT and the column material interact weakly with each other due to the hydrogen bonds. The hydrogen bonds between the filling material and DNT isomers are relatively stronger than TNT; hence DNT is detected after TNT isomers. 2,6-DNT binds to the filling material more strongly compared to 2,4-DNT, since the hydrogen bonds are in the same direction in 2,6-DNT. Consequently, 2,6-

DNT appears in the chromatogram after 2,4-DNT molecule. ADNT isomers are the last molecules detected. The strength of the hydrogen bond supplied by the amino group is greater than the force of the hydrogen bonds in nitro groups of ADNT molecules. 4-ADNT molecules have more stable hydrogen bonds and the amino group is in a free position, therefore the molecule is retained for greater periods of time in the chromatogram. Thus, 4-ADNT is observed after the 2-ADNT molecule (Figure 3.1).

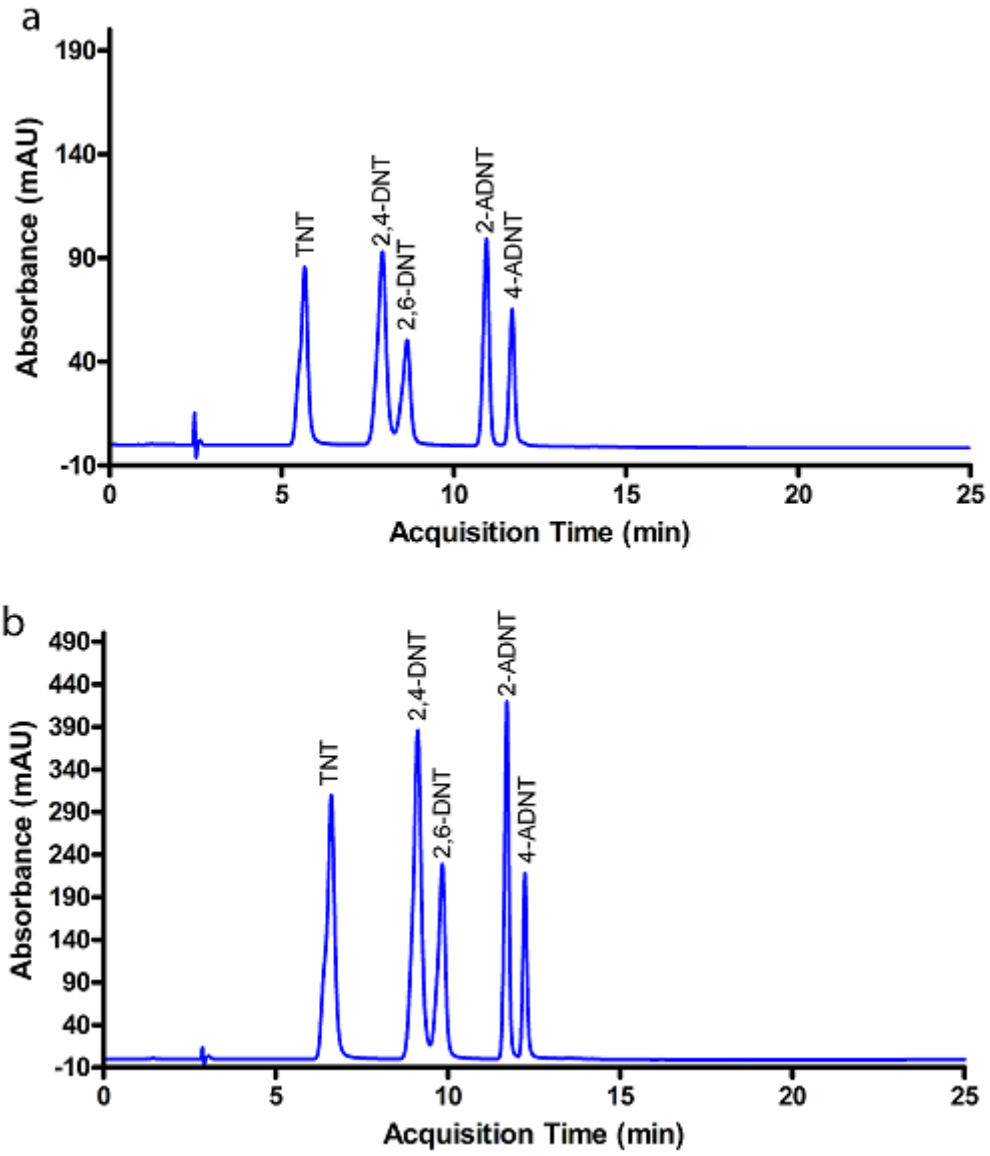


Figure 3. 1. Separation of nitroaromatics by Diol column (a) self-optimization performance, (b) with the method presented in Table 1

We also observed that the Diol column exhibits the greatest resolution rates of the isomers. Table 3.2 and 3.3 summarizes the analytical data of three columns. Even though the flow rate was lower for the Diol column, peaks of analytes were observed earlier than the other two columns (Table 3.2). The separation rates of isomers have high resolution values of the analytes as depicted in Table 3.3. Showing the highest S/N values, the Diol column is capable of detecting µg/L amounts of TATD [21]. The selectivity factor of ADNT isomers is close to 1 and shows that the analysis is robust (Table 3.3). LOD values for Diol column are as follows: 0.88 µg/L for TNT; 0.78 µg/L for 2,4-DNT; 1.17 µg/L for 2,6-DNT; 0.84 µg/L for 2-ADNT; and 0.80 µg/L for 4-ADNT (Table 3.2). In addition, the best recovery rates and LOQ values were obtained by the Diol column (Table 3.2).

(a) Diol column					
Compound	**TNT**	**2,4-DNT**	**2,6-DNT**	**2-ADNT**	**4-ADNT**
Retention time (min)	6.39	9.05	9.84	12.13	12.61
Retention time % RSD	0.96	0.24	0.08	0.26	0.04
Peak area % RSD	1.64	1.82	1.42	7.33	0.81
Recovery (%)	105±3	101±2	102±2	101±3	104±4
LOD, S/N=3 (µg/L)	0.88	0.78	1.17	0.84	0.80
LOQ, S/N=10 (µg/L)	2.92	2.60	3.90	2.80	2.67
N	7245	9762	9669	34662	137612
S/N	1989	2413	1099.5	2146	2317.2
k'	3.27	4.87	5.40	6.91	7.35

(b) C-18 column					
Compound	**TNT**	**2-ADNT**	**4-ADNT**	**2,4-DNT**	**2,6-DNT**
Retention time (min)	10.69	13.58	13.94	14.44	14.61
Retention time % RSD	0.75	0.10	0.07	0.36	0.07
Peak area % RSD	16.72	4.71	7.53	2.72	3.14
Recovery (%)	96±5	92±3	96±1	91±2	95±4
LOD, S/N=3 (µg/L)	2.14	3.05	2.21	3.95	2.01
LOQ, S/N=10 (µg/L)	7.16	10.00	7.38	10.00	6.71
N	25803	47524	42752	71943	53019
S/N	349	245.6	338.7	189.6	372.5
k'	4.09	5.65	5.63	5.83	5.96
(c) Phenyl-3 column					
Compound	**2-ADNT**	**4-ADNT**	**2,4-DNT**	**2,6-DNT**	**TNT**
Retention time (min)	14.57	15.14	15.75	16.08	17.43
Retention time % RSD	0.51	0.72	0.41	0.09	0.05
Peak area % RSD	1.48	4.62	1.70	2.38	0.41
Recovery (%)	97±3	94±2	98±4	95±3	96±2
LOD, S/N=3 (µg/L)	0.81	1.32	0.62	1.31	0.83
LOQ, S/N=10 (µg/L)	2.70	4.42	2.05	4.37	2.77
N	50045	54005	54560	57132	61797
S/N	926.2	565.5	1216.5	570.9	901.8
k'	4.39	4.60	4.83	4.95	5.45

Table 3. 2. HPLC-UV characteristics of TATD on Diol, C-18, and Phenyl-3 columns

Compound	TNT	2,4-DNT	2,6-DNT	2-ADNT	4-ADNT
Diol column					
Selectivity factor (α)	-	1.11		1.06	
Resolution ($R_{S)}$	-	2.06		2.42	
C-18 column					
Selectivity factor (α)	-	1.02		1.03	
Resolution ($R_{S)}$	-	0.74		1.36	
Phenyl-3 column					
Selectivity factor (α)	-	1.03		1.05	
Resolution ($R_{S)}$	-	1.23		2.17	

Table 3. 3. HPLC-UV characteristics of TATD on Diol, C-18, and Phenyl-3 columns

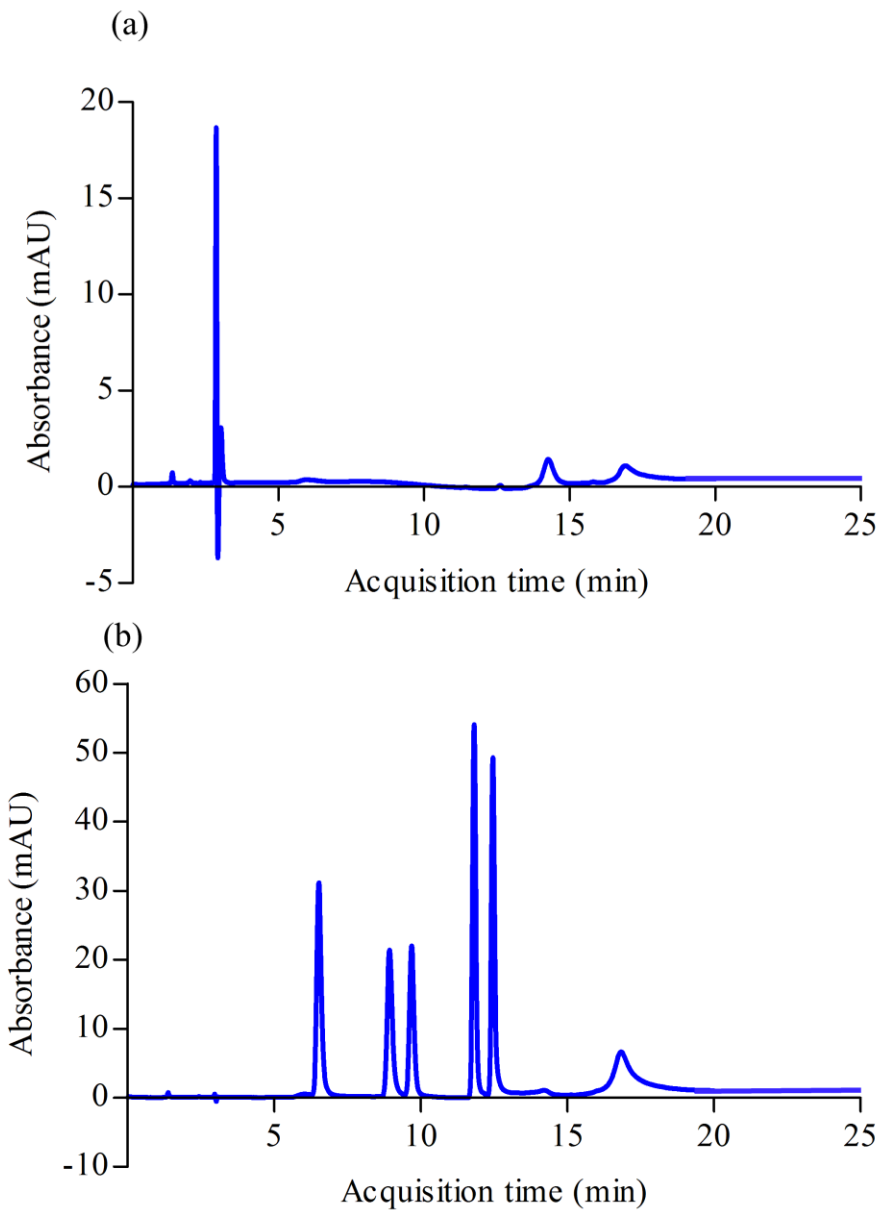


Figure 3. 2. Chromatograms obtained from the application of the proposed method to (a) blank sample (ACN) and (b) a standard addition solution spiked at LOD value

Substance	Nominal concentration (mg/L)				
TNT	0.05	0.10	0.30	0.50	1.00
Series	**Back-calculated concentration (mg/L)**				
1	0.051	0.12	0.34	0.59	0.97
2	0.059	0.11	0.37	0.5	1.19
3	0.053	0.18	0.33	0.56	1.02
AVG	0.05	0.14	0.35	0.55	1.06
SD	0.004	0.038	0.021	0.046	0.115

Substance	Nominal concentration (mg/L)				
2-ADNT	0.05	0.10	0.30	0.50	1.00
Series	**Back-calculated concentration (mg/L)**				
1	0.049	0.17	0.32	0.55	1.04
2	0.042	0.13	0.34	0.47	1.00
3	0.056	0.094	0.31	0.52	1.09
AVG	0.05	0.13	0.32	0.51	1.04
STD	0.007	0.038	0.015	0.040	0.045

Substance	Nominal concentration (mg/L)				
4-ADNT	0.05	0.10	0.30	0.50	1.00
Series	**Back-calculated concentration (mg/L)**				
1	0.057	0.11	0.28	0.50	0.99
2	0,054	0.14	0.27	0.53	1.03
3	0,059	0.17	0.,35	0.54	1.07
AVG	0,060	0.14	0.30	0.52	1.03
STD	0.003	0.030	0.044	0.021	0.040

Substance	Nominal concentration (mg/L)				
2,4-DNT	0.05	0.10	0.30	0.50	1.00
Series	**Back-calculated concentration (mg/L)**				
1	0.05	0.1	0.36	0.58	1.07
2	0.045	0.14	0.34	0.51	0.99
3	0.056	0.12	0.26	0.56	1.1
AVG	0.05	0.12	0.32	0.55	1.05
STD	0.006	0.020	0.053	0.036	0.057

Substance	Nominal concentration (mg/L)				
2,6-DNT	0.05	0.10	0.30	0.50	1.00
Series	**Back-calculated concentration (mg/L)**				
1	0.043	0.13	0.31	0.55	1.05
2	0.052	0.09	0.25	0.54	1.07
3	0.048	0.12	0.39	0.51	0.93
AVG	0.05	0.11	0.32	0.53	1.02
STD	0.005	0.021	0.070	0.021	0.076

Table 3. 4. Comparison of the nominal and back-calculated concentrations for Diol column

Accuracy of the Diol column was evaluated by applying the proposed method to the analysis of reagents obtained from SupelCo (Table 3.4 and Figure 3.2). All samples were spiked at known concentrations. Figure 3.3 shows the results obtained for the reagents by using the proposed procedure were compared with the different concentrations by means of a linear regression model. The results suggest

that the Diol column is the best choice among the columns tested, because of its optimal resolution capacity, economic advantages and lower toxicity.

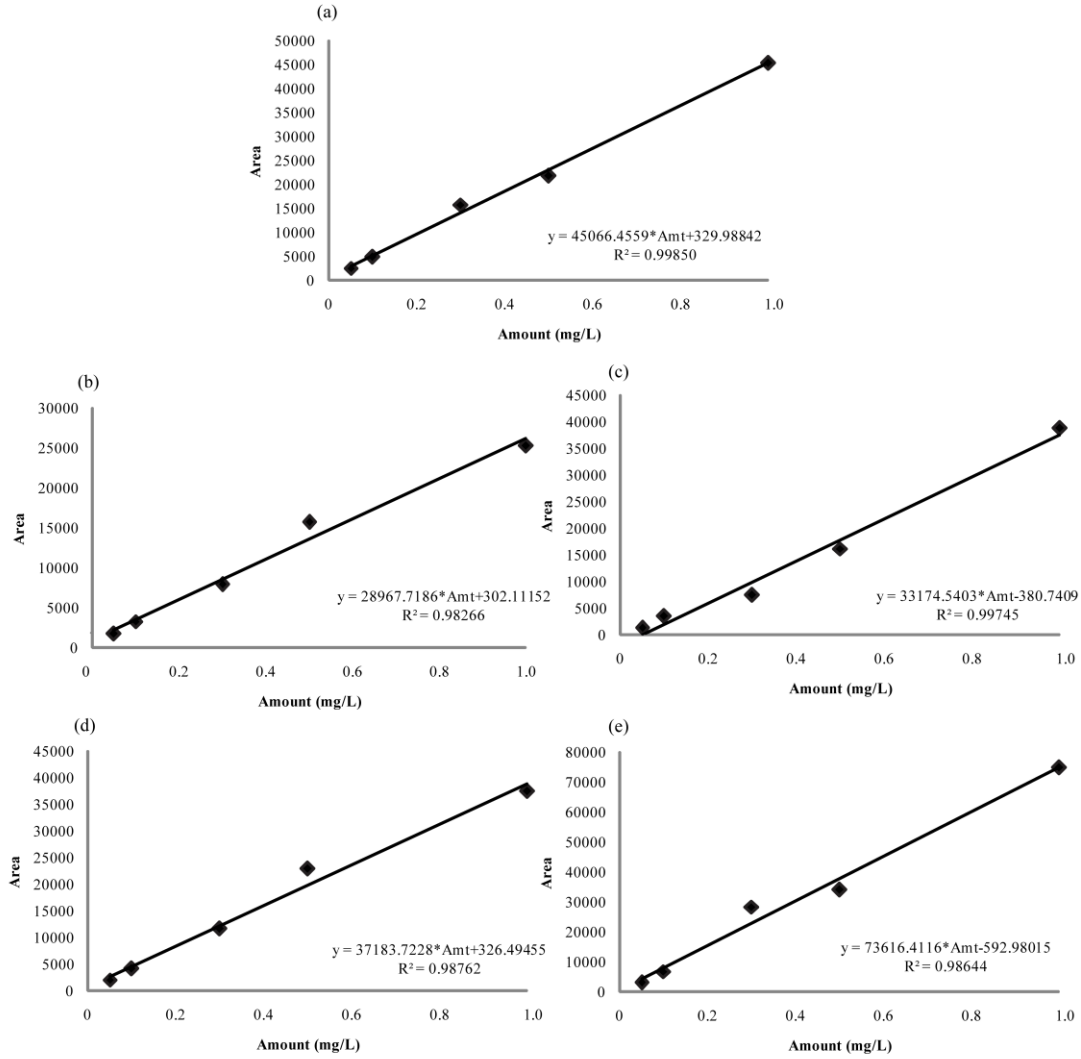


Figure 3. 3. Calibration curves of (a) TNT; (b) 2-ADNT; (c) 4-ADNT; (d) 2,4-DNT; and (e) 2,6-DNT for Diol column

In this work, an alternative HPLC-UV method for the C-18 column is suggested for the detection of nitroaromatics. This method is superior with low retention times of reagents but inferior with higher detection limits of explosives to the method 8330 recommended by the EPA [1]. In the separation chromatogram of the C-18 column, peaks of the reagents displayed a minor overlap with the method presented in Table 3.1. Each reagent eluted in the predicted order under the reversed phase conditions as follows: TNT (10.69 min); 2-ADNT (13.58 min); 4-ADNT (13.93 min); 2,4-DNT (14.43 min); and 2,6-DNT (14.61 min). The separation occurs via hydrophobic interactions. TNT is the most hydrophobic molecule among the other tested reagents, thus the TNT peak was observed earlier than the other metabolites. The amino group on the 4-ADNT molecule is less polar than the nitro group on TNT. As a result of that, 4-ADNT was observed after TNT molecule. 2-ADNT was detected after 4-ADNT because of the attraction between the methyl group of 2-ADNT with the nitro and amino groups. DNT isomers have two polar groups on the aromatic ring therefore, DNT isomers are eluted last. Less prominent steric effects present more powerful hydrophobic interactions. The interaction of methyl group on 2,4-DNT with the filling material is stronger than that of the 2,6-DNT isomer, since it has only one neighboring nitro group (Figure 3.4).

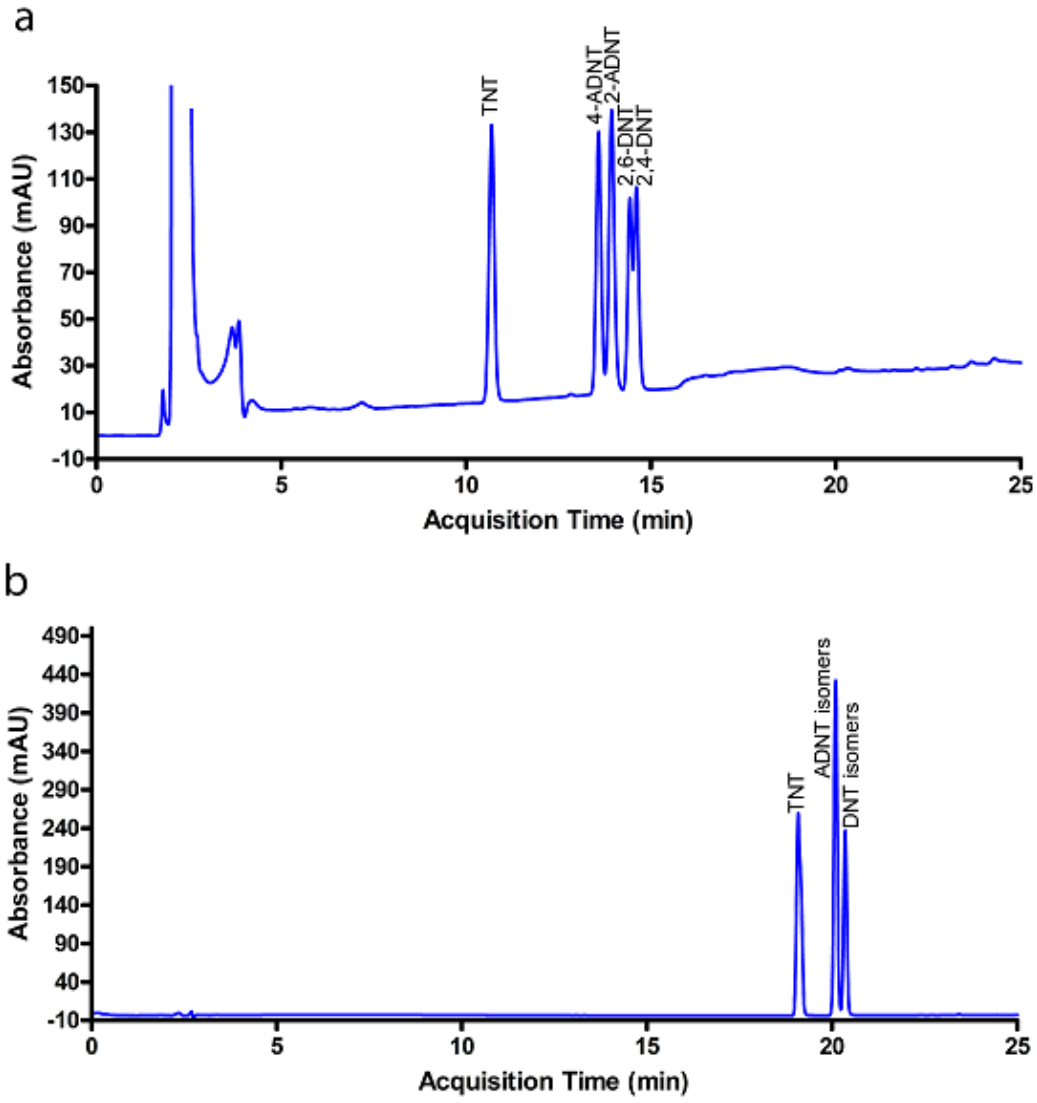


Figure 3. 4. Separation of nitroaromatics by C-18 column (a) self-optimization performance, (b) with the method presented in Table 1

The resolution values of the C-18 column were 1.36 for ADNT isomers and 0.74 for DNT isomers. From these results, it is clear that elution of the C-18 column with the method described is not efficient for the analysis of TATD. We observed a relatively poor separation of TATD by the C-18 column, with low S/N values for TNT (349.00); followed by 2-ADNT (245.60); 4-ADNT (338.70); 2,4-DNT (189.60); and 2,6-DNT (372.50) (Table 3.2). Even though the selectivity factor of C-18 column is high (1.03); low resolution rates were noted (Table 3.3). For the C-18 column, LOD was between 2.01 and 3.95 µg/L with the method described in Table 3.1. In accordance to our work, this column is less convenient for the analysis of TATD.

The reagent peaks were separated completely on the Phenyl-3 column while the retention times of the molecules were higher than that of the Diol column. The interaction between the phenyl groups of the column and the aromatic rings of 2-ADNT and 4-ADNT is comparatively weak. As expected, ADNT isomers were eluted first. In addition, peaks of DNT isomers were detected after the ADNT isomers because of the absence of electron donating groups on the DNT isomers and the stronger adherence to the filling material; π interactions are not present in TNT molecules due to the location of the three nitro groups on the aromatic ring of TNT. Our observations suggest that the interaction between the filling material and the nitro groups of TNT is stronger than that of the other analytes. Therefore, TNT was the last molecule to pass through the column (Figure 3.5).

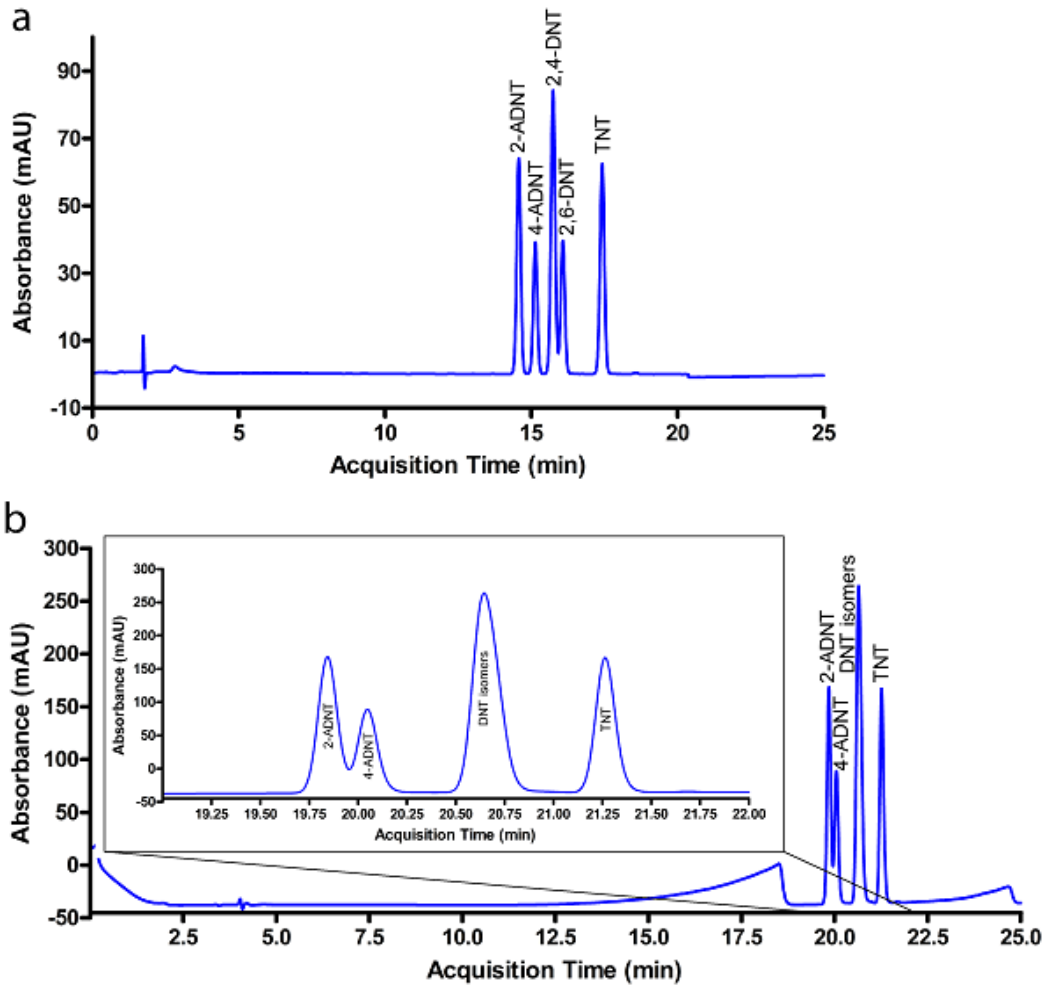


Figure 3. 5. Separation of nitroaromatics by Phenyl-3 column (a) self-optimization performance, (b) with the method presented in Table 1

Phenyl-3 column provided sensitive separation rates for isomers (Table 3.2). Detection limits of the Phenyl-3 column were between 0.62-1.32 µg/L. The separation rate of molecules was very high for Phenyl-3 column; hence, peaks of DNT isomers overlapped approximately 2% of their resolution values. It is interesting to note that S/N of the Phenyl-3 column is lower than that of the C-18 column. On the other hand, the Phenyl-3 column is not efficient as the Diol column when retention times are considered. The selectivity factor is approximately 1.04 for Phenyl-3 column. High resolution values suggest that this column can be utilized for quantitative analysis of TATD. In contrast, for an effective analysis and quick handle on the rate of separation, Phenyl-3 column was inferior to the Diol column.

3.3.3. Effects of Mobile Phase and Different Solvents on the Performance of the Columns

We evaluated the effect of formic acid at 0.1% (v/v) in both mobile phases-water and acetonitrile-by the methods proposed in Table 3.1, in order to obtain enhancement in elution over the normal mobile phases. As expected, introducing formic acid to the system caused a decrease in retention time, on the other hand the reagent peaks for Diol and Phenyl-3 columns overlapped with each other. It is important to note that the C-18 column presents a high separation efficiency. Figure 3.4-a and Table 3.2-b depict the separation results for C-18 column. Therefore, the

addition of formic acid enhances the separation performance of C-18 column for the separation of nitroaromatics. The separation capacity of the C-18 column increases with the addition of formic acid, although it is still lower than the Diol and Phenyl-3 columns without introducing formic acid. One can envision a scenario in which formic acid protonates the polar amino groups; as a result more durable interactions between the non-end-capped silica surface and the polar groups are observed. Thus, an increased elution capacity could be provided by the C-18 column.

For mobile phase optimization, methanol was substituted with acetonitrile as a cheaper alternative. Neither hydrophilic interactions nor hydrogen bonds are formed between column filling materials and the eluent phase (methanol-water), hence shorter retention times were observed for the Diol and the Phenyl-3 columns. In addition, methanol was thought to be inferior to acetonitrile because of lower compound resolutions for the C-18 column. Methanol was less convenient as a solvent phase in separation of nitroaromatics despite its economic advantage and lower toxicity.

The main difference between TNT and its derivatives is the location of nitro groups on the benzene ring. The vectorial net forces among hydrogen bonds of the Diol column between amine and nitro groups of the nitroaromatics enable a high-quality separation of nitroaromatics. The data obtained for the Diol column (Table 3.2) suggested that the best separation was obtained by this column due to the high selectivity of its hydrogen bonds.

CHAPTER 4

BIODEGRADATION OF TNT BY NOVEL BACTERIAL STRAINS

4.1. Overview

Rapid microbial-mediated degradation of TNT contaminated soils was demonstrated by four novel strains, *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13, in a real-sample based study. Complete removal of 100 mg/L TNT was achieved within 20 h under aerobic conditions by the isolates. In this bio-conversion process, TNT was primarily transformed to 2,4-DNT and 2,6-DNT. In addition, 4-ADNT and 2-ADNT were observed as side products of the degradation process. TNT degradation occurs between pH 4.0-8.0 and 4-43^{o}C, the most efficient degradation was observed at pH 6.7 and 30^{o}C. Elemental analysis results indicated

that 24.77% of nitrogen from TNT accumulated in the biomass. TNT molecule was used as a sole nitrogen source.

4.2. Materials

4.2.1. Organisms, Culture Conditions, and Degradation

M8 medium supplemented with 100 mg/L TNT was used to assess the TNT degradation capabilities of the isolates [77]. The effect of an additional nitrogen source on TNT degradation was investigated by using M9 medium [78]. Bacterial cultures were also maintained by Luria Bertani (LB) medium for FT-IR studies. All media were autoclaved after addition of components. All incubations were performed in dark at 30 ºC. All aqueous cultures were agitated on a rotary shaker operating at 125 rpm. Bacterial growth was monitored by measuring turbidity at 600 nm with SpectraMax Microplate Reader M5/M5^{e} (Molecular Devices, USA).

4.2.1.1. Bacteria Isolation from TNT Contaminated Soil

Soil samples were collected from a TNT manufacturing and mine explosion site in Elmadag, Turkey (+39° 49' 56.58", +33° 31' 5.57"). Collection sites have been contaminated by TNT for 50 years. Samples were collected from visible soil discoloration sections, bulky regions, pink waters, and bottom lines of pink water regions. Soil was removed using spatulas to a depth of 5-10 cm and enclosed in

separate sterile 50 mL polypropylene conical tubes. TNT concentration amounts of the samples were between 20-245 mg/kg. The measured moisture level was around 20-35% for soil samples and 60-78% for bulk samples.

Figure 4. 1. TNT sampling in munitions-explosion sites, Kırıkkale, Turkey

4.2.1.2. Isolation and Identification Methods

Collected samples were immediately inoculated in M8 media (0.1%, w/v or v/v). On the 4th day, the inocula were transferred to secondary cultures. The resulting bacterial enrichments were introduced into M8 medium (0.1%, v/v) and incubated overnight. Single colonies were isolated by plating enrichments onto M8 medium agar. In total 120 different colonies were isolated from collected samples (Figure 4.3 and 4.4). The *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains, which showed the highest TNT degradation capacities among the 120

isolates in our study, was identified by 16S intergenic spacer ribosomal DNA analysis. The results were compared to the National Center for Biotechnology Information (NCBI) BLAST database for species identification (Figure 4.2).

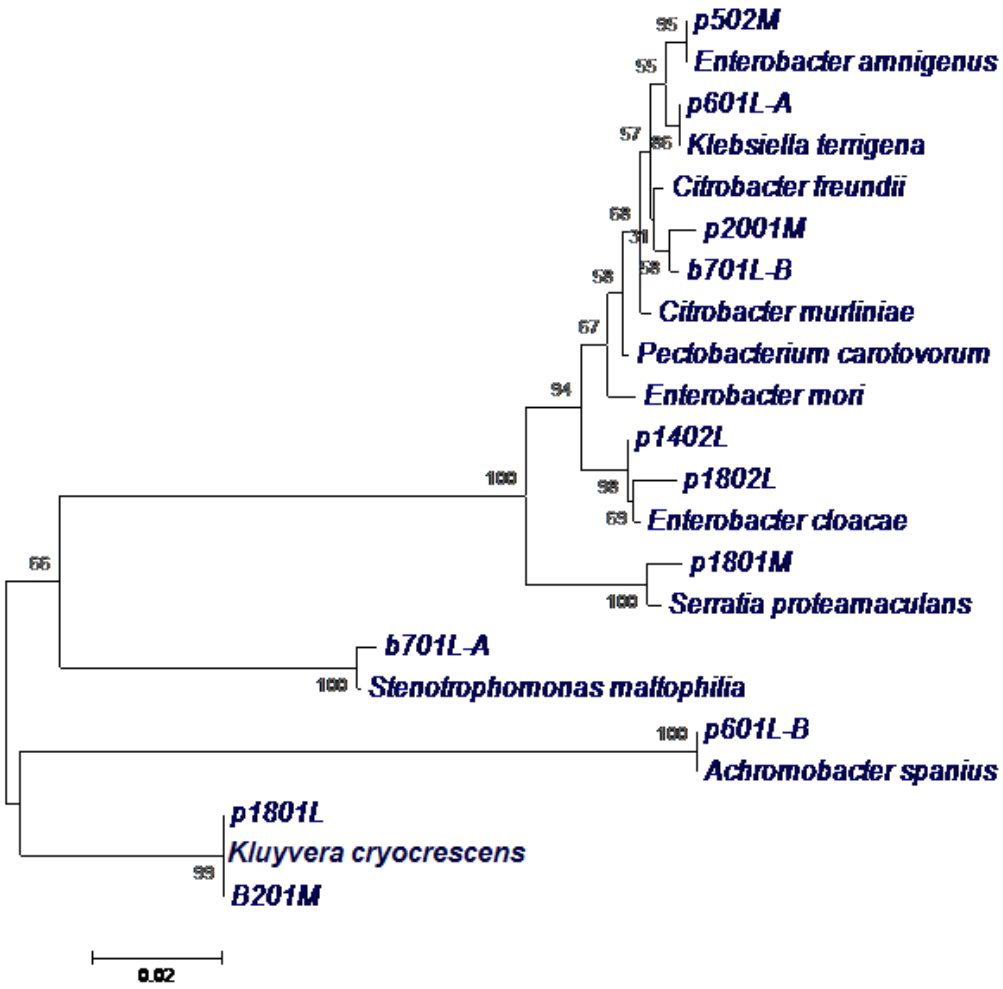


Figure 4. 2. Phylogenetic tree of isolates *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains

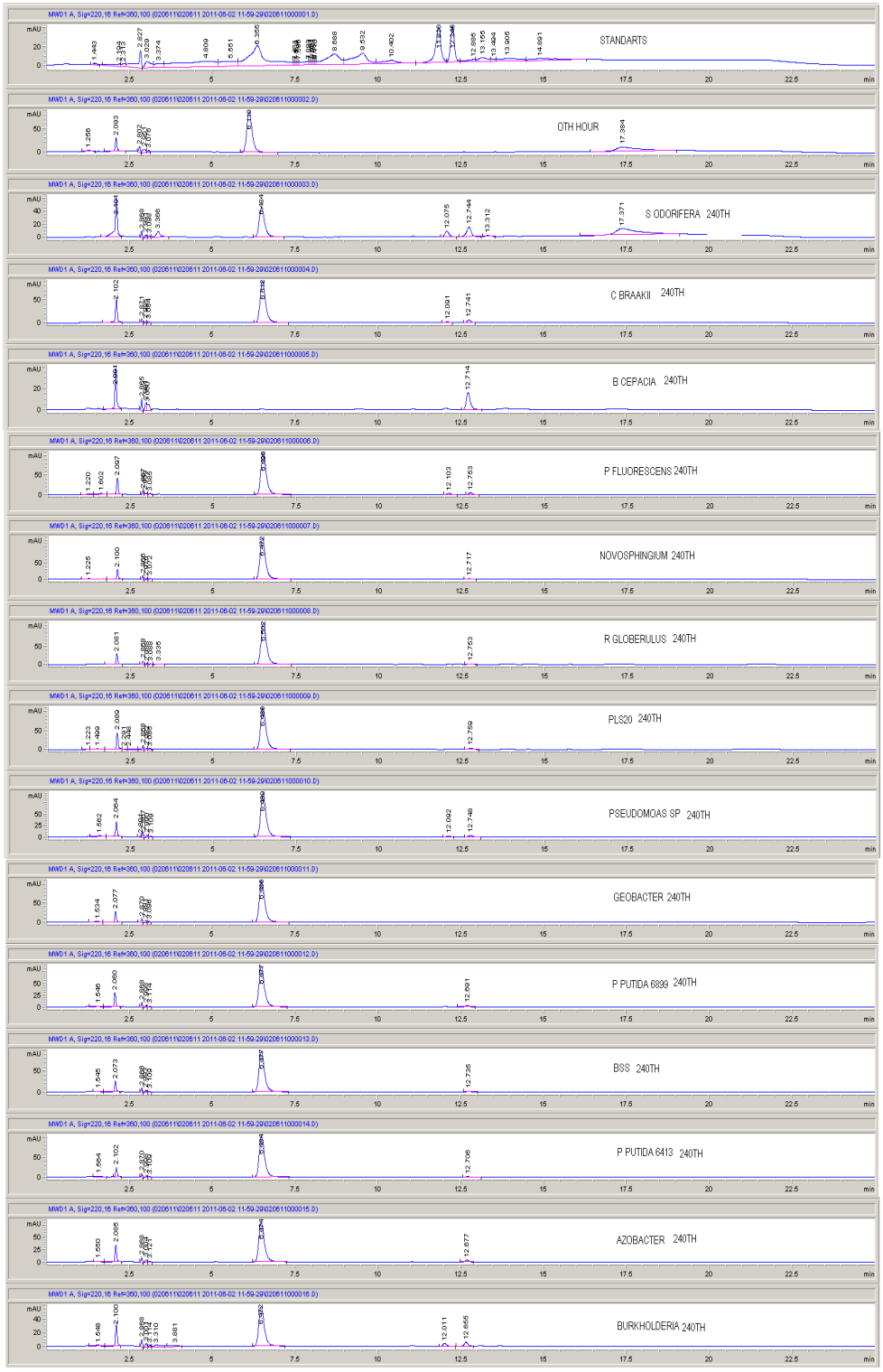


Figure 4. 3. Selection of strains according to their TNT degradation capacity (1)

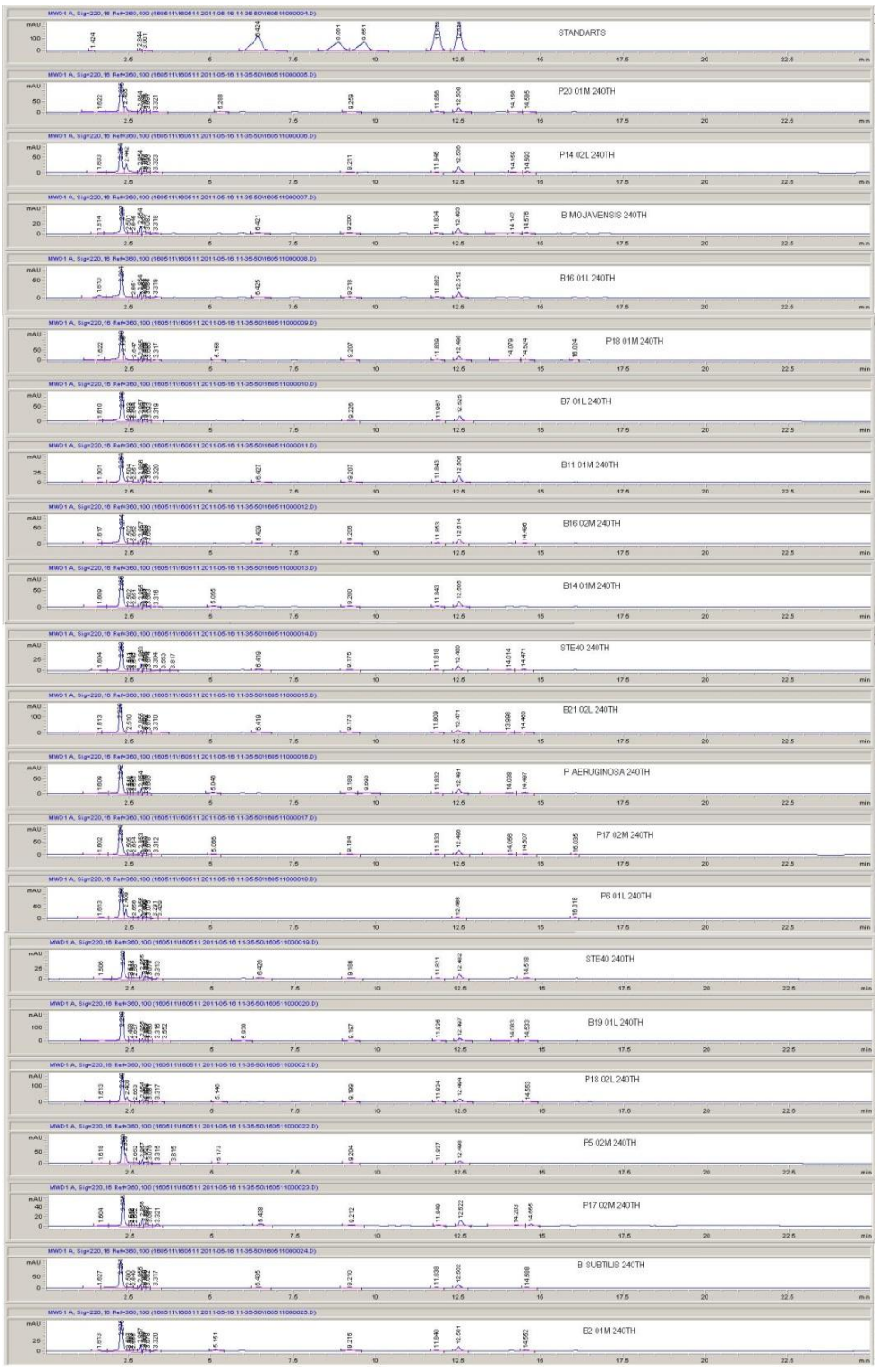


Figure 4. 4. Selection of strains according to their TNT degradation capacity (2)

4.2.1.3. TNT Biodegradation Analysis

In order to identify TNT catabolism products, 10^8 cells per mL were inoculated in M8 medium (0.1% v/v) and kept for 20 h at 30 ºC on a rotary shaker (125 rpm) in aerobic conditions. Aliquots were taken from the inocula at 1 h intervals to determine growth rates and remaining TNT concentrations. Growth rates of the strains were obtained by either measuring the optical density at 600 nm or determining colony forming units in M8 medium agar. TNT reduction was traced by colorimetric nitrite (NO_2), nitrate (NO_3), and ammonia (NH_4) measurements as well as HPLC and FT-IR measurements. Formations of NO2, NO3, and NH4 during growth were assayed hourly by Spectroquant test kits (Merck, Germany). Triplicate set of experiments were performed for the evaluation of the selected bacterial strains.

In addition, elemental analysis measurements were carried out for the detection of nitrogen amount introduced to the cell biomass. 30 mL of cultured M8 media was centrifuged at 14,000 rpm. The remaining pellets were washed twice with distilled water and dried overnight at 45ºC. Control measurements were conducted in non-inoculated media under the same conditions.

Elemental analysis technique is first introduced in 1980s to investigate carbon, hydrogen, nitrogen, oxygen and sulphur contents of a sample. The elements are determined by absorption of SO_2 or hydrohalogenides by release of CO_2, H_2O and N_2 gases from samples because of the nitric/nitrous oxide reduction in high

temperatures. This technique is currently used in biological studies to perform advanced calculations of carbon, hydrogen, nitrogen, oxygen and sulphur elements of a sample.

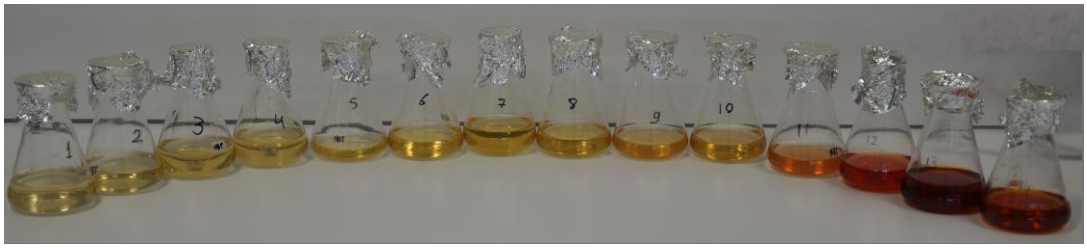


Figure 4. 5. M8 medium with 100 mg/L TNT with different pH levels

The pH tolerances of the isolates were identified by modified M8 and LB media (inoculation amount: 0.1%, v/v) with pH values between 1.0 and 14.0 (Figure 4.5). The pH of the media was adjusted using 1N HCl and 1N NaOH. Bacterial growth and TNT levels were monitored hourly using HPLC. M8 and LB cultures were incubated between the range of 0 and 50 ºC and growth was monitored over 24 h in order to examine the temperature reliability of the strains. All experiments were performed in duplicate and autoclaved cultures served as the control group.

Analysis of metabolites from both M8 and M9 media was performed using HPLC as described in Chapter 3.

4.2.1.3.1. Fourier Transform-Infrared Spectroscopy (FT-IR)

The FT-IR method is an infrared radiation approach for determining the semi-quantitative analysis of components in a mixture. The method has been used for 70 years for qualitative analysis of unknown materials. In this method, an infrared radiation is sent to the sample. A molecular fingerprint is monitored depending on the transmission and absorption of infrared radiation passing through the sample. The FT-IR method is widely used for determining the effects of explosive materials, such as TNT, on any type of cell [79-82].

Samples were inoculated in both LB and M8 media and 1 mL aliquots were drawn from each culture. Supernatants were removed after centrifugation at 14,000 rpm for 5 min. Cell pellets were washed twice with physiological saline (0.9% w/v of NaCl) and stirred with distilled water. 20 µL of the final solution was dried at 45^{o}C for 1 h [83]. FT-IR spectra were collected over the wavenumber range 4000-400 cm^{-1} using a Nicolet 6700 FT-IR Spectrometer (Thermo-Scientific, USA). The ground corrections of CO_2 and water were applied automatically before measurements. The system was controlled by OMNICTM software. The FT-IR of M8 medium was used as reference spectrum in order to determine the differences caused by TNT. Samples were analyzed in triplicate.

4.2.3.1.2. Scanning Electron Microscopy

This tool is widely utilized for imaging of a sample by scanning process with electron beams. The first SEM image was obtained in 1935. The method became popular for visualizing surface of samples utilizing the interaction between the beams and sample surface.

Samples were prepared for scanning electron microscopy via incubating the culture overnight. 4 µl aliquot of the strain was drop-casted to silicon wafer surface and dried overnight at 45ºC. For fixation, solid specimen was kept in 2.5% gluteraldehyte in phosphate buffer solution (PBS) for 1 h. The fixed specimen was rinsed in PBS for 10 min, after that serial dehydration with 30%, 50%, 70%, and 80% ethanol (each rinse 10 min) was employed. The specimen was left in absolute ethanol for 25 min and then coated with 3 nm thick Au-Pd film for SEM.

4.3. Results and Discussion

4.3.1. Identification and Imaging

Based on TNT degradation capacity analysis, *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains exhibited the highest and the fastest degradation activity among the 120 different strains, which were isolated from TNT-contaminated soil samples from Turkey. These strains have the following physiological and

biochemical characteristics: gram-negative (G^-), rod shape, non-spore forming, and aerobic (Table 4.1). The colonies of strains *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 were smooth and yellow after an overnight incubation. The 16S intergenic spacer ribosomal DNA analyses of the strains show 97.9-99.8% similarity with *Citrobacter murliniae*, *Achromobacter spanius*, *Kluyvera cryocrescens*, and *Enterobacter amnigenus* in the similarity search based on the BLAST database at NCBI (Figure 4.1), respectively.

Bacteria isolated from TNT-contaminated and TNT-free media were also investigated by SEM. Figure 4.6 showed that cells grown in LB medium had smooth surface. On the other hand, the membranes of the cells grown in M8 medium had many structural defects such as partial uneven zones and irregular rod shapes.

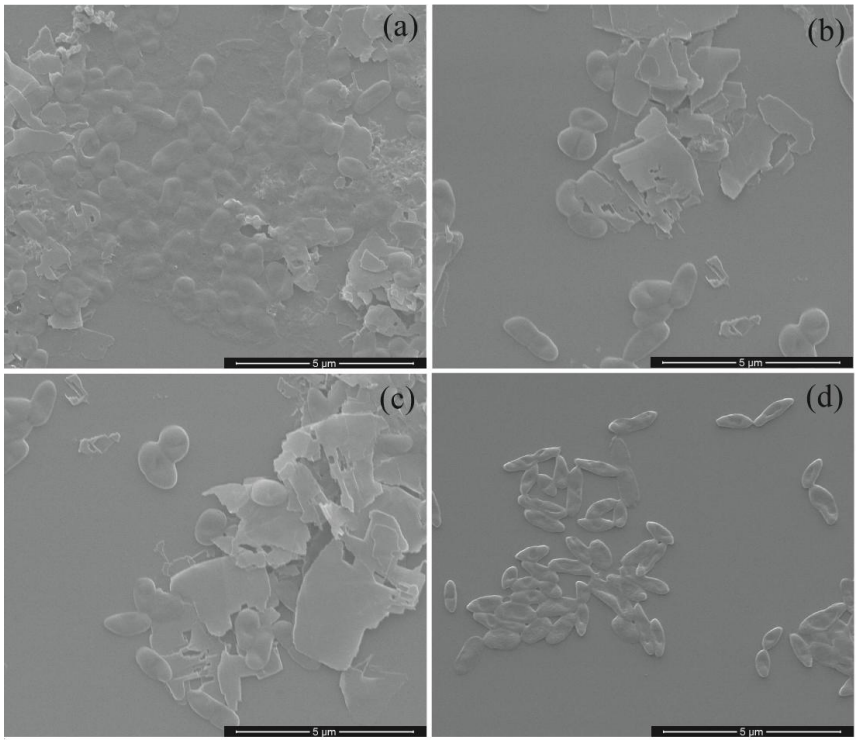


Figure 4. 6. SEM imaging of the strains. (a) *Citrobacter murliniae* STE 10, (b) *Achromobacter spanius* STE 11, (c) *Kluyvera cryocrescens* STE 12, (d) *Enterobacter amnigenus* STE 13

Strain name	Species	TNT degradation rate	2,4DNT formation	2,6DNT formation	2ADNT formation	4ADNT formation	Gram staining	Spore Formation	Biosafety level	Form
STE 10	*Citrobacter murliniae*	++	++	-	+	++	Negative	Non-spore forming	1	Cocco-bacillus
STE 11	*Achromobacter spanius*	+++	+++	+	++	++	Negative	Non-spore forming	1	Bacillus
STE 12	*Kluyvera cryocrescens*	+++	-	-	-	+++	Negative	Non-spore forming	1	Bacillus
STE 13	*Enterobacter amnigenus*	++	-	-	-	+++	Negative	Non-spore forming	2	Bacillus

+	0.001- 0.003mM
++	0.003- 0.004mM
+++	< 0.005mM

Table 4. 1. Characteristics of strains isolated from TNT-contaminated sites

4.3.2. Degradation of TNT by Newly Isolated Strains

The TNT degradation efficiencies of the strains were evaluated by HPLC. Chromatography results demonstrated that 99.7-99.9% of the initial TNT amount was degraded after 20 h of incubation in M8 medium. Figure 4.7 depicts the growth of the strains in M8 medium with 100 mg/L TNT as the sole nitrogen source.

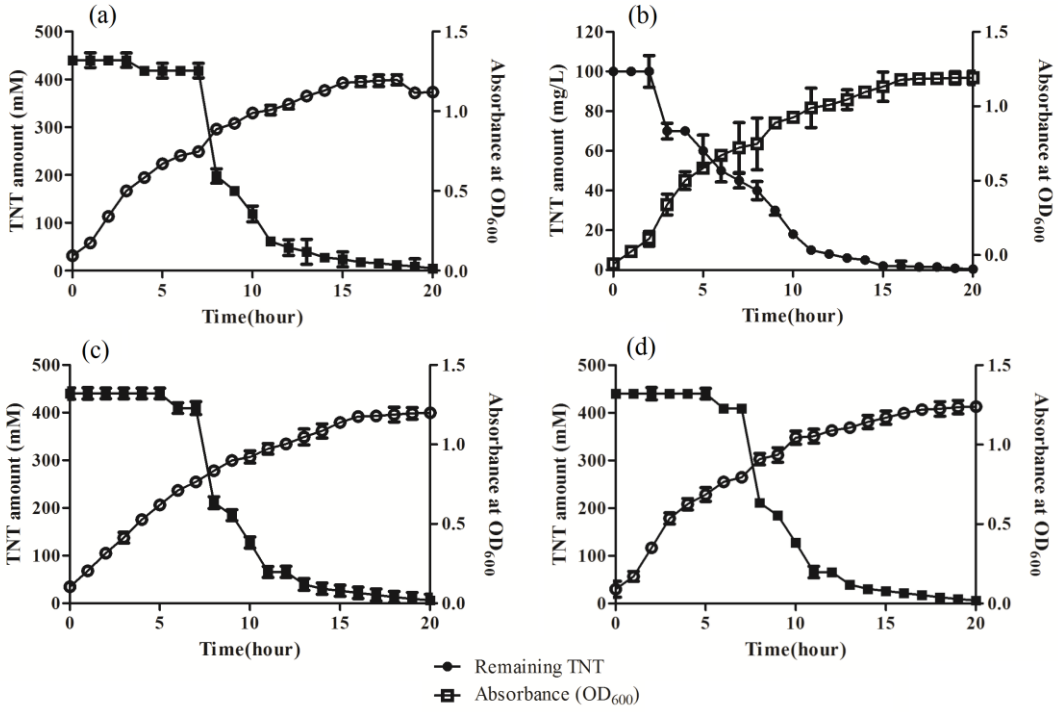


Figure 4. 7. TNT degradation rate vs. bacterial growth (a) *Citrobacter murliniae* STE 10, (b) *Achromobacter spanius* STE 11, (c) *Kluyvera cryocrescens* STE 12, (d) *Enterobacter amnigenus* STE 13

As biomass increased, the remaining TNT amount decreased during the growth period. The formation of 2,4-DNT and 2,6-DNT were observed only in the first 4 h. The 2,4-DNT formation reached a maximum level of 7 mg/L at 4^{th} h. The amount of 2,6-DNT in the active culture was at the highest point at 4^{th} h (3 mg/L). 2-ADNT and 4-ADNT concentrations increased exponentially starting from the 4^{th} h of the growth until the end of the experiment (Figure 4.8). The maximum concentrations of 2-ADNT and 4-ADNT were 19 and 59 mg/L, respectively. Two adjacent peaks of metabolites were detected between 15^{th} and 20^{th} h, correlating with previous reports [20,78,84-86].

The TNT degradation capacity of strains was tested at different temperature and pH values to determine optimal conditions for remediation. The results showed that the strains had higher degradation activity at pH 6.0-7.0 and 30^{o}C, correlating with previous observations [5]. Besides, effective TNT degradation rate (100 mg/L) reached >80% and >60% for pH 4.0-8.0 and 4-43^{o}C, respectively (Figure 4.9). Typical growth and TNT degradation curves of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains were depicted in Figure 4.7. The OD_{600} value increase and TNT concentration decrease were parallel to each other.

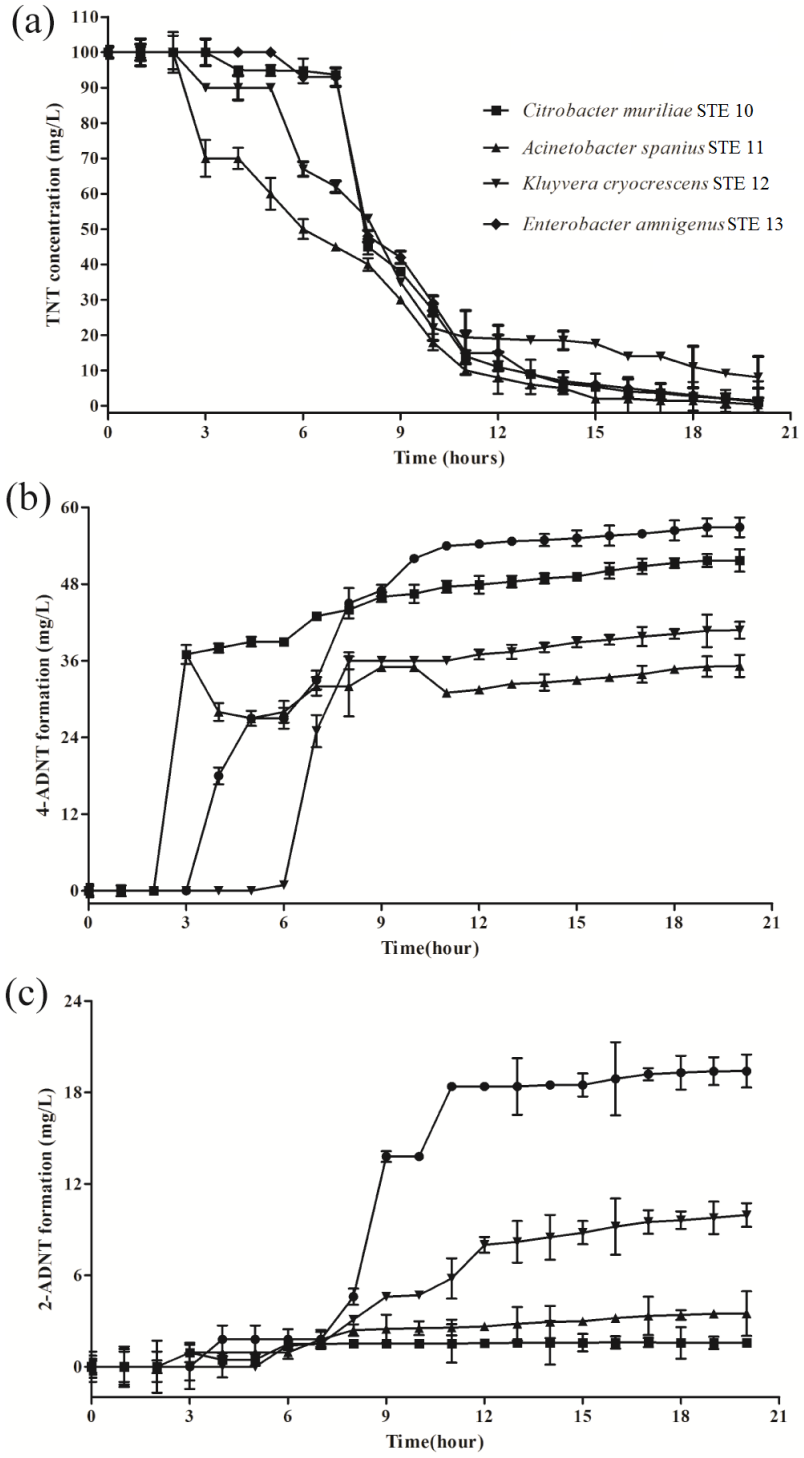


Figure 4. 8. (a) TNT degradation, (b) 2-ADNT and (c) 4-ADNT formation rates of the proposed strains

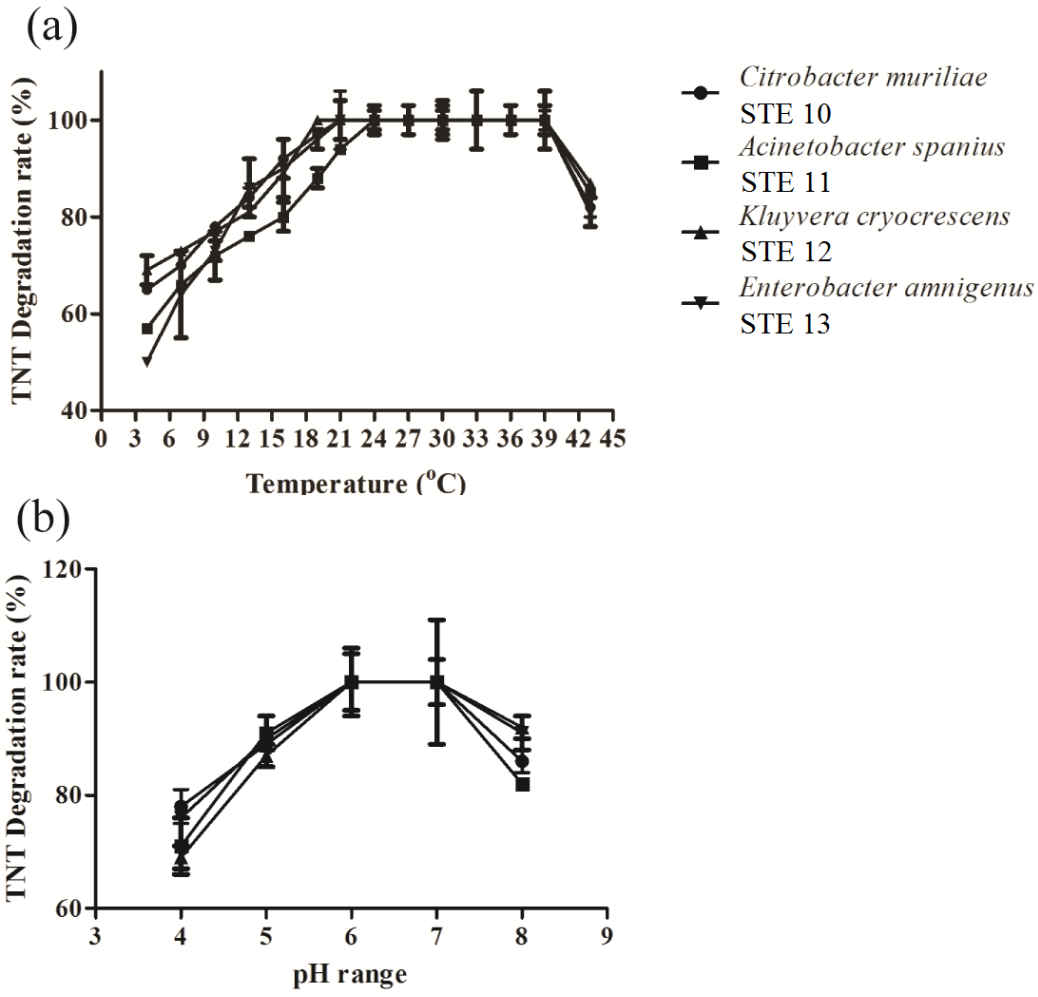


Figure 4. 9. Impact of temperature and pH changes on the proposed strains

4.3.3. Nitrogen Balance during the TNT Degradation

The amount of TNT incorporated into the cell biomass was determined via nitrogenous compounds released into the medium by the strains. Concentrations of

NO_2, NO_3, and NH_4 compounds were traced for 48 h and the strains showed an active nitrogen metabolism when TNT is provided as the sole nitrogen source. The isolated strains were divided into three major groups on the basis of differences in their nitrogen metabolism. *Citrobacter murliniae* STE 10 and *Enterobacter amnigenus* STE 13 constituted the first group and revealed similar results (Figure 4.10-a, 4.10-d). NO_3 amounts did not change significantly in the first 24 h. A strong NO_3 peak was observed between 24^{th} and 48^{th} h. After that, NO_3 levels became undetectable. The concentrations of NO_2 fluctuated between 0.06-0.03 mg/L and the minimum occurred at 0.02 mg/L. NH_4 concentrations remained the same until the 18^{th} h and then increased exponentially. It is important to note that the final NH_4 formation in the *Citrobacter murliniae* STE 10 culture was 1.5 fold greater than that of the *Enterobacter amnigenus* STE 13 culture at the end of the experiment. The second group, consisting of *Kluyvera cryocrescens* STE 12, showed a different NO_3 formation profile (Figure 4.10-c). After reaching a peak value, NO_3 concentration gradually decreased to undetectable levels. Additionally, NO_2 levels reach the minimum and maximum values of 0.02 mg/L and 0.07 mg/L, respectively. The highest NH_4 concentration was 2.74 mg/L. The third group contained *Achromobacter spanius* strain STE 11. In this case, NO_3 level increased sharply between 0-6 h and remained stable until 18^{th} h, then slowly decreased to undetectable levels (Figure 4.10-b). 0.02 mg/L and 0.07 mg/L became the minimum and the maximum NO_2 concentrations, whereas NH_4 levels rose up to 2.58 mg/L during growth.

The nitrogen balance analysis revealed that approximately 13.22% of TNT ended up in the cell biomass while 58.25% of TNT was converted to DNTs and ADNTs. In addition, 12% of TNT was converted into unknown metabolites. The total NO_2, NO_3, and NH_4 accumulation during growth was about 4.59%. The remaining 11.94% of nitrogen was not detected and might be released as nitrogen gas in the degradation process (Table 4.2). However, 96.52% of TNT was degraded during growth. The remaining amount might disappear because of solvent losses or artifacts.

We focused on bacterial growth and TNT degradation in M9 medium to understand the effects of an additional nitrogen source (NH_4Cl) on the strains. TNT degradation in M8 medium turned out to be higher compared to that of M9 medium, which was confirmed by HPLC results and growth rates. The bacterial growths were stimulated by the presence of 100 mg/L TNT in M9 medium.

Strain	Initial nitrogen (mg/L)	Final nitrogen (mg/L)
Citrobacter murliniae STE 10	9.69	15.19
Achromobacter spanius STE 11	3.34	24.77
Kluyvera cryocrescens STE 12	3.70	12.05
Enterobacter amnigenus STE 13	5.04	22.63

Table 4. 2. Changes in nitrogen amounts during TNT degradation

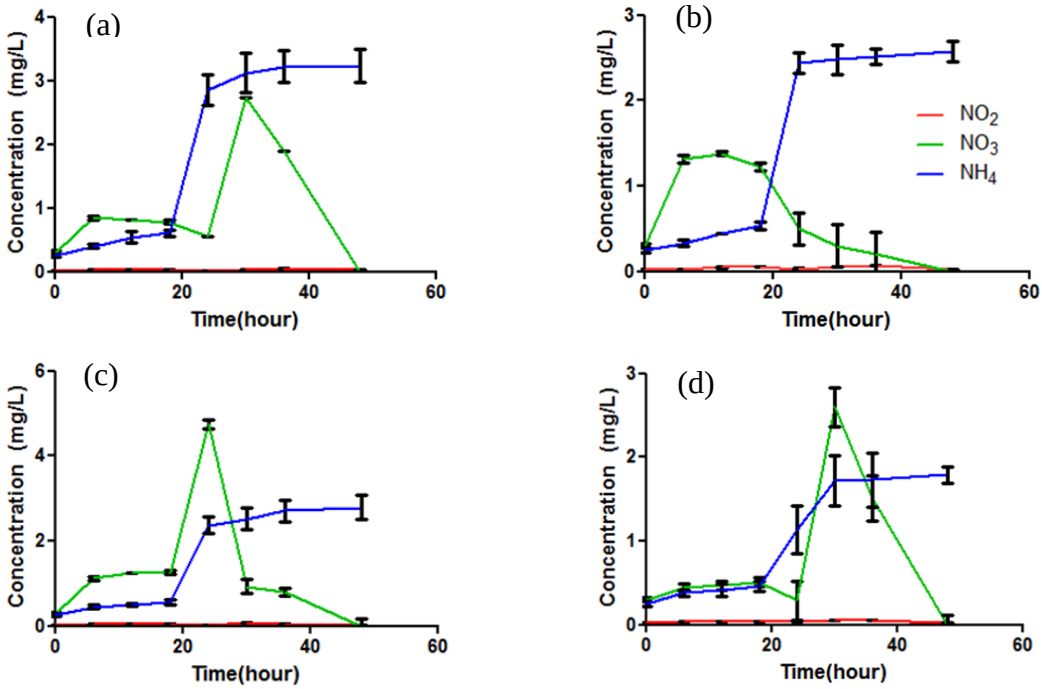


Figure 4. 10. Changes in NO_2, NO_3, and NH_4 amounts. (a) *Citrobacter murliniae* STE 10, (b) *Achromobacter spanius* STE 11, (c) *Kluyvera cryocrescens* STE 12, (d) *Enterobacter amnigenus* STE 13

On the other hand, TNT degradation capacities of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 turned out to be 45%, 70%, 59%, and 42% of the initial amount of TNT (100 mg/L) within the first 24 h in M9 medium (Figure 4.11).

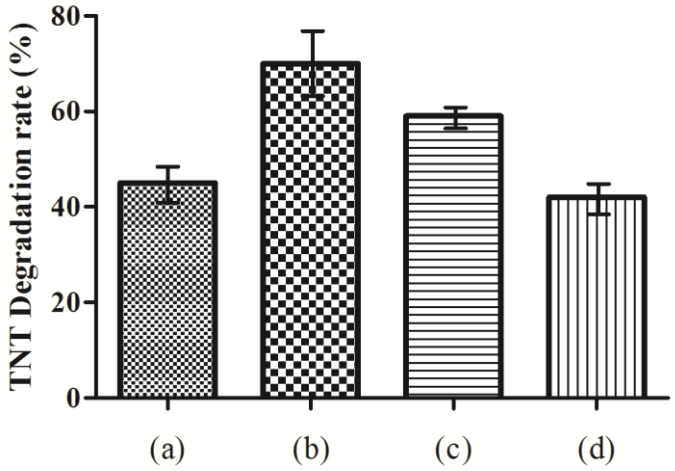


Figure 4. 11. TNT degradation rates of the proposed strains in M9 medium (a) *Citrobacter murliniae* STE 10, (b) *Achromobacter spanius* STE 11, (c) *Kluyvera cryocrescens* STE 12, (d) *Enterobacter amnigenus* STE 13

TNT degradation by the strains was confirmed using FT-IR spectroscopy. The differences in chemical attractions of nitrogenous compounds in the presence of TNT were shown. The results of Naumann *et al*. [83] were used to determine the corresponding nitrogen bonds. Considerable surface reflection spectra of TNT detected around 1530 cm^{-1}, as stated before by Gomez *et al*. [80]. The IR spectroscopic signatures revealed TNT-related changes in TNT-free and TNT-containing samples during the growth period. 1600 cm^{-1} region was determined as the marker of the bacteria and indicated differences in spectra intensities of N_2, C, and glucose changes.

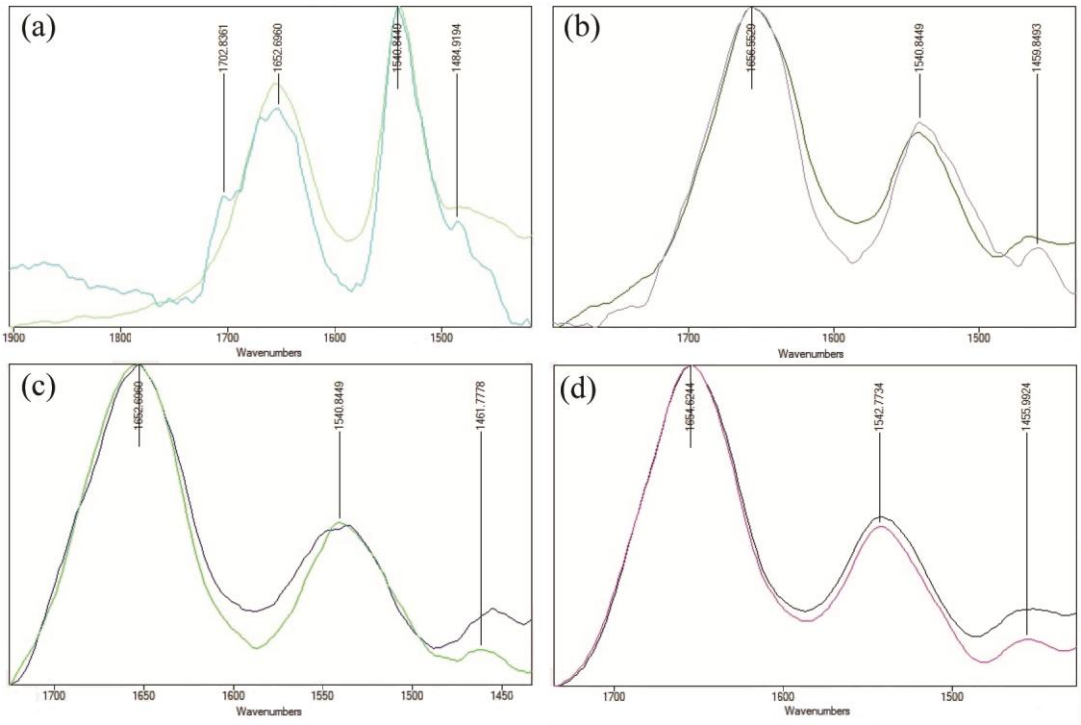


Figure 4. 12. FT-IR spectroscopy of the strains. Amide I and amide II peaks demonstrated that bacteria used TNT as a sole source of nitrogen (a) *Citrobacter murliniae* STE 10, (b) *Achromobacter spanius* STE 11, (c) *Kluyvera cryocrescens* STE 12, (d) *Enterobacter amnigenus* STE 13

Figure 4.12 depicts significant decreases in amide I (1544 cm^{-1}) and amide II (1655 cm^{-1}) peaks for the P6 01L-B strain after 20 h of incubation. Additional changes were observed at 910 cm^{-1}, 1087 cm^{-1}, 1171 cm^{-1} and 1350 cm^{-1} for 2,6-NO_2 scissors and C-H stretching, C-H ring vibration, C-C implane ring trigonal band 2,4,6-C-N and C-CH_3 stretching, and NO_2 group band symmetric stretching vibration, respectively.

4.4. TNT Degradation vs. Nitrogen Metabolism

In the present study, we discovered that the strains *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 were capable of utilizing TNT as a sole source of nitrogen [49,87-91]. The strains were isolated from munitions-contaminated regions and showed higher and more effective TNT degradation compared to that of other 120 isolated strains. The nitroreductase activities of the strains were previously reported by Kumagai *et al.* [88,91]. HPLC, FT-IR, element analyzer measurements not only proved the TNT degradation rate and effectiveness of the strains, but also indicated that TNT reduced to ADNTs.

The strains coalesced and the cell membranes had collapses in TNT contained media (Figure 4.6). This may suggest that TNT constitutes a significant stress factor to the strain, despite the active TNT metabolism of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13. Morphological changes of bacterial cell membranes in the presence of TNT were shown for the first time in our study.

The formation of TNT metabolism intermediates including the azoxynitrotoluenes, ADNTs, and diaminonitrotoluenes were described in the previous reports [4,5,87,91]. On the other hand, DNT formations were less emphasized in the literature [4,5]. In this study, the strains were shown to degrade TNT by nitro group reduction in aerobic conditions. After 3 h of TNT degradation,

ADNT traces were detected as subsequent degradation products. Furthermore, two unknown metabolites were detected as dead-end products of degradation. It is likely that these metabolites were two different conformations of stable nitroreductase products, such as isomers of hydroxylaminodinitrotoluene or diaminonitrotoluene.

Citrobacter murliniae STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 were able to be grown successfully in M8 medium (Figure 4.7), presumably because of their high TNT tolerance and utilization capacity. The strains could utilize TNT immediately after being introduced to a TNT-containing medium; it is likely that the strains constitutively expressed nitroreductase activity. Despite the toxic effects and deformations observed in cell surfaces in the presence of TNT, the strains degraded TNT completely in 20 h. Since there is no nitrogen compound other than TNT in cultured M8 medium, the detection of NO_2, NO_3 and NH_4 is a direct indicator of TNT catabolism. In general, NO_2 stayed in minute amounts because of the aerobic metabolism of the described strains [79].. The complete degradation of TNT (100%) resulted in the formation of NH_4 within 20 h [79]. NO_3 was probably produced at the first stage of TNT transformation, and subsequently converted to another form of nitrogenous compound, such as NH_4. In bacteria cultures, NO_3 was observed to be present in the supernatant immediately after TNT degradation started. This pattern suggested that nitroreductases were constitutively expressed in the strains. Similar results were reported by Rahal *et al.* [79]. In addition, the usage of an additional nitrogen source in M9 medium decreased the TNT transformation

capacity from 100% to 42%. It was clear that the strains were capable of utilizing TNT with a decreased degradation capacity even if there is an additional nitrogen source in the growth medium.

Three broad groups were observed to form on the basis of nitrogen metabolism differences. Such differences may reflect protein expression patterns of the isolated strains. TNT is not a commonly utilized nitrogen source for bacteria and the presence of TNT as the sole nitrogen source may present conditions similar to nitrogen starvation. During nitrogen starvation, bacteria are known to express proteins to facilitate the utilization of alternative nitrogen sources. As such, nitroreductases might be expressed as a response to both the presence of TNT and the absence of more commonly available sources of nitrogen.

The first group observed, comprising *Citrobacter murliniae* STE 10 and *Enterobacter amnigenus* STE, 13 were characterized by a relative low release of NO_3 for about 24 h before a prominent NO_3 peak. Likewise, the supernatant of *Kluyvera cryocrescens* STE 12 yielded little NO_3 before 18 h, where NO_3 concentration displayed a marked increase. This pattern may reflect the delayed expression of nitroreductases as a response to the nitrogen limitation caused by the use of TNT as the sole source of this element. The third group, composed of *Achromobacter spanius* STE 11, displayed a notably different pattern of release for nitrogenous products, where NO_3 was observed to be present in the supernatant immediately after exposure to TNT. This pattern suggested that nitroreductases were constitutively expressed in *Achromobacter spanius* STE 11, as opposed to the

other three strains which must have expressed this TNT-reducing enzyme after exposure to the moiety. The high cellular accumulation of nitrogen observed in elemental analysis results might also be caused by reduced nitrogen availability due to the use of TNT as the exclusive nitrogen source, which may have caused "starvation-like" effects and led to the cells accumulating nitrogen.

The results of HPLC, colorimetric tests, and elemental analysis measurements were in excellent agreement with FT-IR bands observed for the strains. The intensity of bonds at 1602 cm^{-1} (NH_2, NH_3, N-NO_2 and C=C bonds), 1410 cm^{-1} (C-N amines) and 1077 cm^{-1} (C-O-C, C=O, P=O, and PO_2 attractions) was significantly different, thus the dynamics of bacterial growth was affected by TNT presence. Changes in 1606 cm^{-1} region suggest that the strain transformed TNT during growth. In addition, the presence of TNT affected nitrogen metabolism as demonstrated by intensities of amide I (1544 cm^{-1}) and amide II (1655 cm^{-1}) spectra. FT-IR spectra results were highly similar to previous studies [79-82].

Elemental analyses were conducted to detect nitrogen amount that was dissociated from TNT and incorporated to cell biomass (Table 4.2). Nitrogen contents of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 increased gradually up to 24.77 mg/L during growth period. In 20^{th} h, the highest nitrogen level was reached (24.77 mg/L), suggesting that the bacteria can effectively utilize TNT as a sole nitrogen source and incorporate it into the cell biomass. The high cellular accumulation of nitrogen observed in elemental analysis results might also

be caused by reduced nitrogen availability due to the use of TNT as the exclusive nitrogen source. This source may have caused “starvation-like” effects and led to the bacterial cells accumulating nitrogen in the cell biomass.

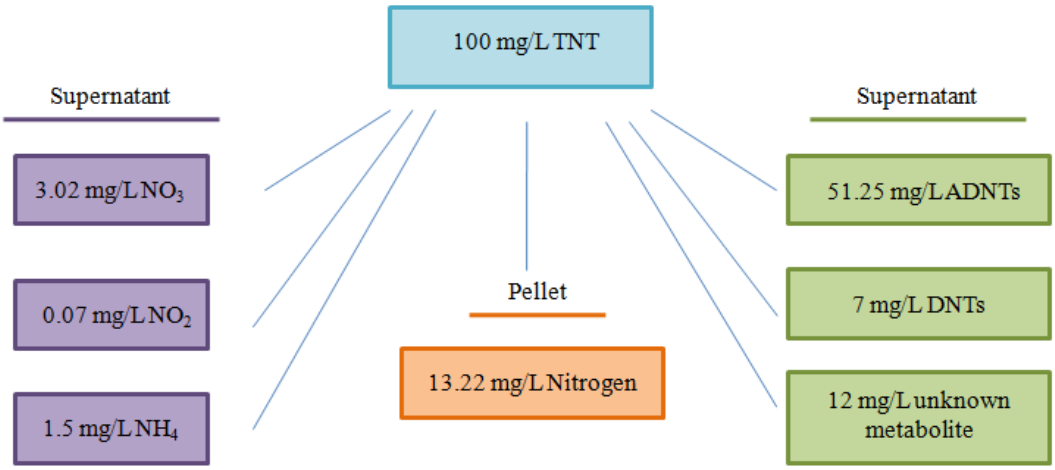


Figure 4. 13. Fate of TNT after biodegradation process by novel bacteria

At the end of 20 h, approximately 96.52% of the total TNT was degraded and 3.48% of TNT might have disappeared because of solvent losses or artifacts. In addition, the strains were able to preserve the high degradation capacity in pH and temperature ranges broader than the other TNT-degrading strains described in the literature [3,4]. (Figure 4.13). Our results suggest that these strains can be utilized safely and effectively in TNT biodegradation.

CHAPTER 5

BIOREMEDIATION OF TNT-CONTAMINATED SOILS USING IN-VESSEL COMPOSTING METHOD

5.1. Overview

This study was undertaken to increase the TNT degradation rate using fast-TNT degrading novel bacterial strains by a laboratory-scale in-vessel compost system. Optimization studies were conducted using three composting reactors operated at 2 different carbon/nitrogen ratios (20/1 or 30/1 on a dry weight basis), 2 aeration rates (5 or 7 L/min used at 5 min on per 1 h, 3 levels of TNT contamination (2 g/kg, 10 g/kg or 100 g/kg) and 2 concentrations of microbial inoculation (0.1% or 1%, w/w). The feedstock used for the optimization was food waste, cow manure,

potting soil, and wood chips. Relative changes in chemical and biological structure (moisture content, carbon/nitrogen ratio, and pH), TNT concentration, and bacterial count (CFU) were measured during composting period. Maximum temperatures observed during the runs were in the range of 50.1-60.5^{o}C. Analyses of the compost system suggested that optimum TNT degradation occurred in a mixture having carbon/nitrogen ratio=20/1 at 5 L/min aeration rate using 5 min on per hour off intermittent aeration.

Complete TNT degradation (99.9% of initial amount) of this mixture was achieved when novel isolates, *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13, (1%, w/w) used (1.1-fold increase over non-inoculated mixtures). Additionally, reduction of TNT was found to be independent of initial TNT concentration. The results of this study indicated that even high TNT contaminations can be removed in 15 days, which is the shortest period reported so far.

5.2. Materials

5.2.1. Chemicals, Reagents and Compost Ingredients

TNT was obtained from Kırıkkale Brass Factory, Turkey. Standards of TNT; 2,4-aminodinitrotoluene (2,4-DNT); 2,6-aminodinitrotoluene (2,6-DNT); 4-aminonitrotoluene (4-ADNT); 2-aminonitrotoluene (2-ADNT) (1000 μg/mL in

acetonitrile, purity <99.9%) were purchased from SupelCo (USA) for analytical experiments. HPLC-grade acetonitrile (Sigma-Aldrich) was utilized for the extraction of TNT from composting samples. In addition, double distilled water (Millipore, USA) and methanol (Sigma-Aldrich) were used throughout the HPLC measurements. Chromafil PET-20/25 membrane filters (0.2 µm, Macherey-Nagel, USA) were used for filtration of samples.

Food waste was collected from dining halls, hotels, and cafeterias at the Middle East Technical University (METU) campus. Generally, the food waste consisting of bread, red pepper, lemon, cracked wheat, rice, carrot, napkin, beans, peas, tomato, parsley, lettuce, yoghurt, etc. are used in the composting process. Cow manure and wood chips (0.8–2.7 cm long) were obtained from Farm Operations at Middle East Technical University. Soil was obtained from greenhouse in Bilkent University.

5.2.2. Experimental Design

Reactor mixtures were prepared by considering initial C/N ratios and moisture contents of individual gradients on a wet weight basis. Eleven experimental conditions were assigned in two replicates and tested with three in-vessel composting reactors. The experimental procedure involved two carbon-nitrogen ratios (C/N=20/1 and C/N=30/1), three TNT levels (2 g/kg, 10 g/kg and 100 g/kg), and two bacterial strain levels (0.1% and 1%, w/w) (Table 5.1) [5].

Different TNT and inoculum levels were used to assess the TNT degradation capacity of compost systems [5]. In addition, two intermittent aerations were used at 5 min on per hour off cycles with flow rates of 5 or 7 L/min. Table 5.1 summarizes the experimental set-up. The moisture range of the system was kept between 50-60% for all runs. Incubation periods of composts were 15 days.

TNT was dissolved in acetonitrile (1:4) and introduced to the compost amendments. Sequential runs were performed by the addition of low (0.1%, w/w) or high (1%, w/w) levels of novel bacterial strains (*Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13) to the reactor to simulate the complete *ex situ* removal of TNT from contaminated soils.

5.2.3. Reactor Design

In-vessel composting system consisted of three vertical cylinders with a lid each and 10 L capacity. The reactors were covered with glass fibre for heat insulation. Aeration was supplied by an air-compressor. Air flow was provided at the bottom using cuprous aeration tubes. Perforated stainless steel plates were placed inside the reactors at the bottom to enhance aeration in the composting mixture during runs and air was discharged from the exit line from the top. Temperatures were recorded continuously at two points as an indicator of success for composting trials. Composting amendments were mixed manually outside the reactors and then placed into the reactors. More particularly, microorganisms, soil,

manure, food waste, wood chips and TNT were mixed in the listed order. The diagram of the system is depicted in Figure 5.1.

Experiment no	TNT concentration (g/kg)	Air flow (L/min)	C/N ratio
1	2	5	20
2	2	5	30
3	2	7	20
4	2	7	30
5	10	5	20
6	10	5	30
7	10	7	20
8	10	7	30
9	100	5	20
10	100	5	20
11	100	5	20

Table 5. 1. Experimental design for in-vessel composting studies

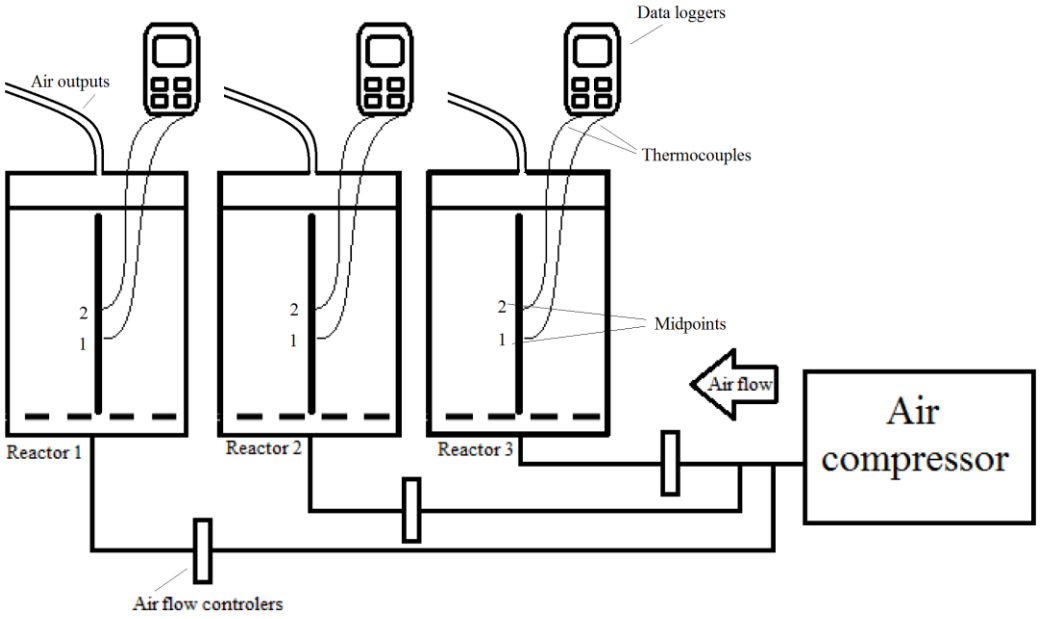


Figure 5. 1. Schematic laboratory set-up of in-vessel composting reactors. 1 and 2 express the first and second midpoints of the compost pile, respectively

5.2.4. Compost Sampling and Analysis

Every five days, samples were pooled from different areas of the composting pile and mixed to yield a representative sample. The temperature of the compost was recorded by thermocouples every 6 h. For moisture measurements, 1-3 g of sample was oven-dried (105^{o}C) for 24 h. A blend of distilled water and compost sample (1:10, w/v) was stirred for 30 min for pH determination. A Flash 2000 CHNS/O Elemental Analyzer (Thermo Scientific, USA) was utilized for carbon and nitrogen analyses. The carbon, nitrogen and moisture contents of the

ingredients were analyzed prior to experiments. Percentages of the amendments were adjusted to maintain the moisture and C/N ratio at the levels designated in Table 5.4. Samples were dried for 24 h and then pulverized via a mortar for C/N ratio analyses. Compost samples were blended with 50:50 (v/v) acetonitrile and the bulky solutions were sonicated for 6 h. After solutions were centrifuged (15,000 rpm, 15 min), 1.5 µL of supernatant was collected from each sample. Supernatants were purified with 0.2 µm membrane filters and HPLC was employed for further analyses.

All measurements were performed on Agilent 1200 Series HPLC system consisting of Agilent 1200 Degasser, 1200 isocratic pump, 1200 ALS auto sampler, and 1200 MWD multi wavelength detector. A wavelength of 254 nm was preferred as the operation wavelength of the study. Analyses and quantification of the results were obtained using HP Chemstation software. The system was calibrated with 5 different sample standards prior to the measurements. The method described in Chapter 3 was applied for detection of TNT and its metabolites.

Material	Food waste	Manure	Wood chips	Soil
Nitrogen (%)	43.39	11.58	55.40	14.54
Carbon (%)	1.27	1.25	0.30	1.01
C/N	34.16	9.26	184.66	14.4
Moisture (%)	74.59	37.70	11.18	45.86

Table 5. 2. Carbon, nitrogen and moisture values of compost ingredients

Figure 5. 2. Composting process. Top left, food waste before mixing; bottom left, compost pile after mixing all ingredients; bottom left, 0^{th} day of compost; bottom right, finished compost

Microbial content of the compost was determined using LB enrichment medium. The samples were collected in sterile polystyrene tubes on 0^{th} and 15^{th} days and immediately inoculated in LB broth overnight at 30ºC on a rotary shaker (125 rpm). 100 µl aliquots of the culture were diluted serially and inoculated onto LB agar plates. Grown colonies were counted overnight and results were compared in order to demonstrate the enhanced TNT degradation rate caused by the addition

of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains to the system. Our preliminary experiments showed that the temperature and pH tolerances of these bacteria were between 4-43°C and 4.0-8.0, respectively.

5.2.5. Statistical Analysis

The effect of different parameters on TNT degradation rates were evaluated by Minitab (Minitab Inc., USA) statistical software package. ANOVA and Tukey test were used for statistical evaluation of results. The level of significance was utilized at 5%.

5.3. Results and Discussion

5.3.1. Raw Material Analysis

The total carbon and nitrogen contents of the soil were approximately 14% and 1%, respectively. Moisture values of soil samples were between 10% and 15%. The pH value of the soil was 6.05±0.1 prior to the addition of TNT. Carbon and nitrogen amounts were measured using element analyzer. The characteristics of all ingredients were summarized in Table 5.2.

5.3.2. Evaluation of Composting Process

Two different C/N ratios (20/1 and 30/1) and aeration rates (5 and 7 L/min) were used to determine the optimal operational conditions required for complete TNT removal. The resulting conditions were then applied in all further composting experiments using three different levels of TNT concentration.

Since temperature is a primary marker of microbial activity and biodegradation rate of a compost system [92], temperatures were monitored every 6 h at two different positions within the compost pile (Figure 5.1). The variation of temperature at different C/N ratios and aeration rates are displayed in Figure 5.3. The highest temperatures recorded were 52.5°C for C/N=30/1 and 53.1 °C for C/N=20/1 aerated at 5 L/min. These results indicated no significant difference with the respect to C/N ratio ($P>0.05$). However, the peak temperature values of 48.4°C for 7 L/min air flow and 50.2°C for 5 L/min air flow rate at C/N ratio of 20/1 differed significantly ($P>0.05$). The temperature increased to a maximum (60.5°C) within 5 days and decreased after 10 days as a general feature of composts. The temperature decrease might have occurred because of the loss of degradable material in compost and the rapid changes in temperature could reflect the enhanced intensity of microbial activity. These results correlated with studies reported by Neklyudov *et al.* [92].

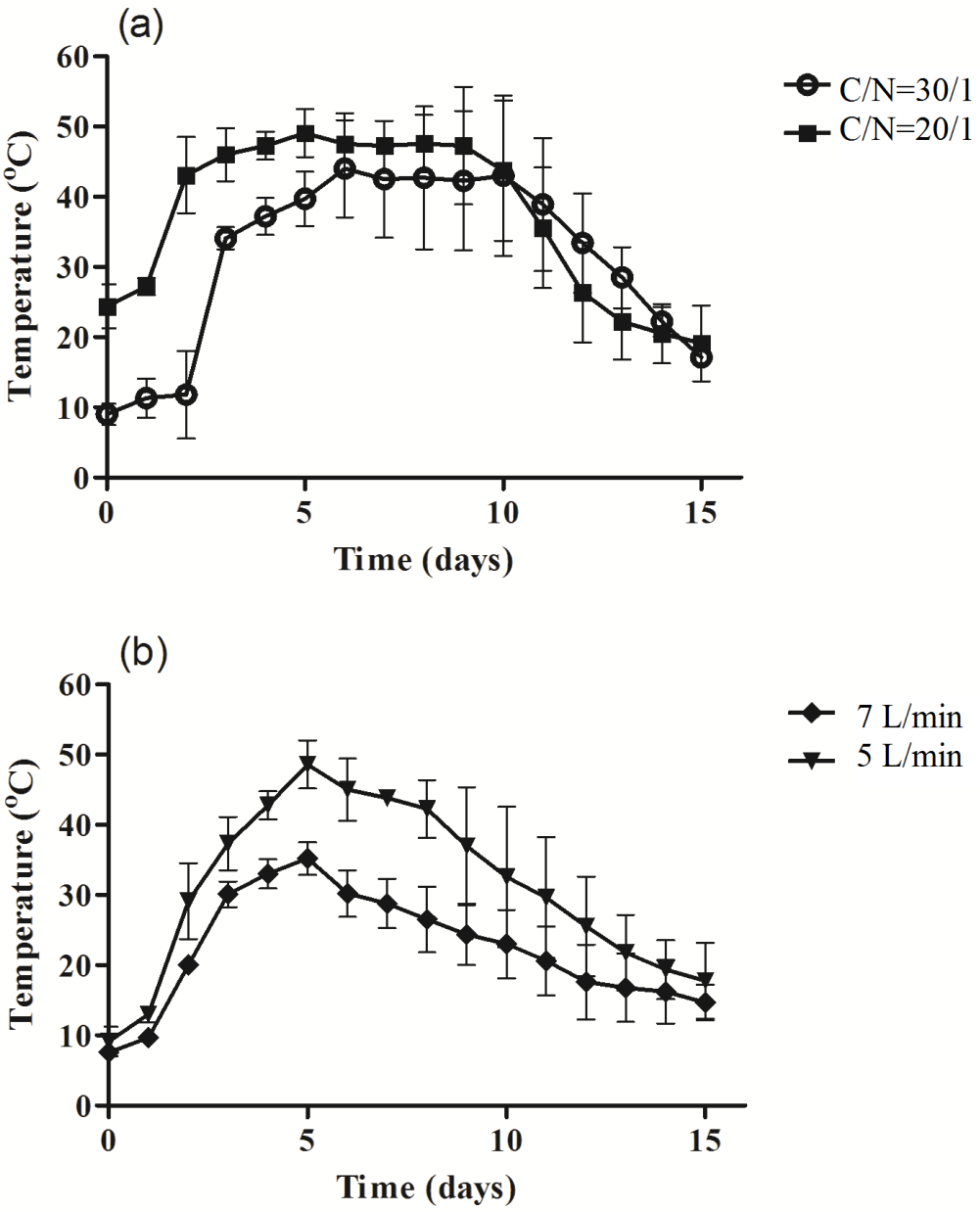


Figure 5. 3. Typical temperature changes at different (a) C/N ratios (constant air flow: 5 L/min) and (b) aeration rates (constant C/N= 20/1) during composting

Moreover, aeration rates directly affected the duration of the high temperature periods. Higher temperatures could not be maintained with a 7 L/min of aeration rate due to leading moisture and temperature decrease in the compost pile. The results demonstrated in Table 5.3 reveal that there is no need for high temperatures (>50^{o}C) to reduce TNT efficiently. The use of wood chips allowed an enhanced porosity in compost piles; thus a 5 L/min aeration rate satisfied oxygen needs of aerobic microbes [92].

TNT concentration	2 g/kg		10 g/kg		100 g/kg	
Aeration	C/N		C/N		C/N	
	20	30	20	30	20	30
5 L/min	0.024	0.024	0.024	0.024	0.022	[1]NP
7 L/min	0.022	0.029	0.018	0.031	[1]NP	[1]NP

[1]NP: Not Performed

Table 5. 3. Analytical and microbiological results for composting experiments at various conditions

In addition to aeration rate and temperature, moisture plays an important role for maintaining dynamics of biodegradation in compost system. Table 5.4 summarizes the variation of TNT removal with the tested operational conditions. The moisture content for all runs was typically between 50-60%, which allowed a healthy microbial activity during composting treatment as previously stated by

Spain *et al*. [5]. Although the conservation of moisture might depend on the water content of compost ingredients, a gradual decrease of moisture was observed during the composting process due to aeration. Another parameter for determining biodegradation rate is the measurement of compost weight. The combination of C/N= 20/1 and 5 L/min air flow parameters stimulated higher TNT degradation and higher reduction in compost weight, because of better composting conditions (Table 5.4).

	No inoculation		0.1% inoculation (w/w)		1% inoculation (w/w)	
	Initial	Final	Initial	Final	Initial	Final
TNT concentration (g/kg)	100 ± 2.9	0.006 ± 0.001	100 ± 2.9	0.048 ± 0.002	100 ± 3.1	0.0014 ± 0.0003
C/N	19.9 ± 1.1	14.3 ± 1	19.9 ± 0.2	13.1 ± 0.4	19.9 ± 0.7	12.2 ± 1.3
Weight of compost (kg)	8.5 ± 1.2	7.5 ± 0.8	8.5 ± 0.9	7.6 ± 0.3	8.5 ± 0.8	8.4 ± 0.1
Microbial content	$1.1x10^{8}$	$3.7x10^{8}$	$3.1x10^{9}$	$3.8x10^{9}$	$2.9x10^{10}$	$5.5x10^{10}$
Moisture (%)	59.1 ± 1.5	57.5 ± 1.1	53.8 ± 2.1	50.1 ± 3.4	60.5 ± 1.8	57.6± 2.5
pH	6.3 ± 0.2	7.1 ± 0.1	6.7 ± 0.3	7.3 ± 0.1	6.1 ± 0.2	7.1 ± 0.2

Table 5. 4. TNT degradation rates (day^{-1}) at various composting conditions

The pH of the compost system was altered by the mineralization of the organic matter. Typically, pH values gradually increased during the runs,

suggesting that the accumulation of ammonia and elimination of organic content in the compost mixture occurred because of microbial activity. Thus, as stated in Table 5.4, initial and final pH values of bacteria inoculated composts were higher than that of non-inoculated mixtures. While pH values were around 6.0-6.5 on the 0^{th} day compost, at the end of the composting period pH values increased to 7.0-7.5. Similar results were reported by Unmar *et al* [93].Further, the mineralization of the organic matter affects the C/N ratio differentiation which is responsible for pH, temperature, and microbial activity changes in a dynamic compost system. In the present work, C/N ratios demonstrated a gradual decline during the course of in-vessel composting. Among the operational parameters tested, C/N ratio of 20/1 was identified as the optimum value for TNT degradation, demonstrating highest TNT removal (Table 5.3).

In addition, Table 5.3 demonstrated that TNT reduce was independent of initial concentration. The results revealed that C/N ratio was not affected by TNT concentration at low air flows, while TNT concentration affects the C/N ratio at higher aeration rates (Table 5.3 and Table 5.4).

5.3.3. Assessment of TNT Degradation

Eleven different operational parameters were tested for the degradation of TNT (Table 5.1). Among them, the combination of C/N=20/1 and air flow 5 L/min parameters were observed to yield most effective TNT degradation rate (Table 5.3).

Figure 5.5 summarizes the overall TNT removal efficiencies of the tested parameters. HPLC analysis results indicated that inoculated and non-inoculated treatments presented different TNT removal rates. In the high TNT contamination setting, the difference of microbial content between initial and final measurements was lower in non-inoculated compost system than the inoculated ones. The results demonstrated in Table 5.4 are compatible with previous studies [5,20,73,74,92]. Several mechanisms were previously demonstrated to be highly effective in biodegradation process. Hydrophilic interactions or formation of coplanar electron-acceptor complexes may play a role for association of TNT derivatives to the organic mixture. Nucleophilic addition of the amino groups to carbonyl groups or aromatic carbons results in adherence to humic substances [73]. Minute amounts of 4-ADNT were observed between days 5-10, indicating that TNT was aerobically converted to more biodegradable forms and further degradation products bound the soil irreversibly. Finally, addition of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains enhanced the TNT biodegradation capacity (1.1 fold) of in-vessel compost system.

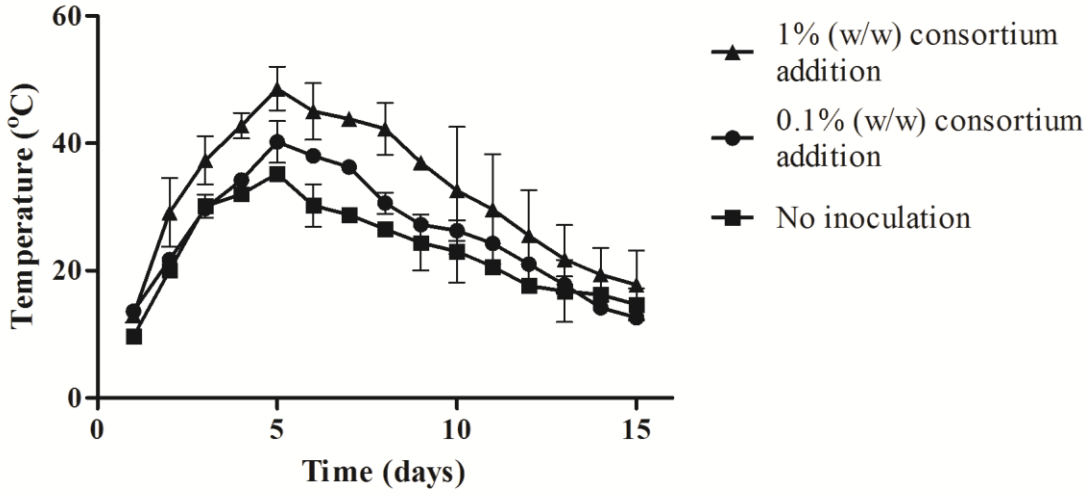


Figure 5. 4. Changes of the temperature in different inoculation levels of bacteria in the compost system at C/N=20/1 and 5 L/min air flow rate

5.3.4. Toxicity Tests of Remediated Composts

The toxicity of TNT to four representative species of higher plants, pelargonium (*Pelargonium domesticum*), corn (*Zea mays*), tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*), were determined in final composting mixtures to demonstrate TNT removal. During a 10-week period, these pants in 100 g/kg TNT contained, TNT-free and remediated compost mixtures were observed. Toxicity experiments showed that TNT was phototoxic for tested plant species based on their growth rates. Phytotoxicity of TNT varied between plant species (Figure 5.6). Corn and geranium were more sensitive to TNT compared to green pepper and tomato. While high TNT concentrations (100g/kg) inhibited the growth,

plants in remediated compost mixtures could grow healthily. In addition, our results are in accordance with previous studies [94,95]. These results suggested that TNT removal was achieved successfully by an in-vessel compost system with the addition of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains.

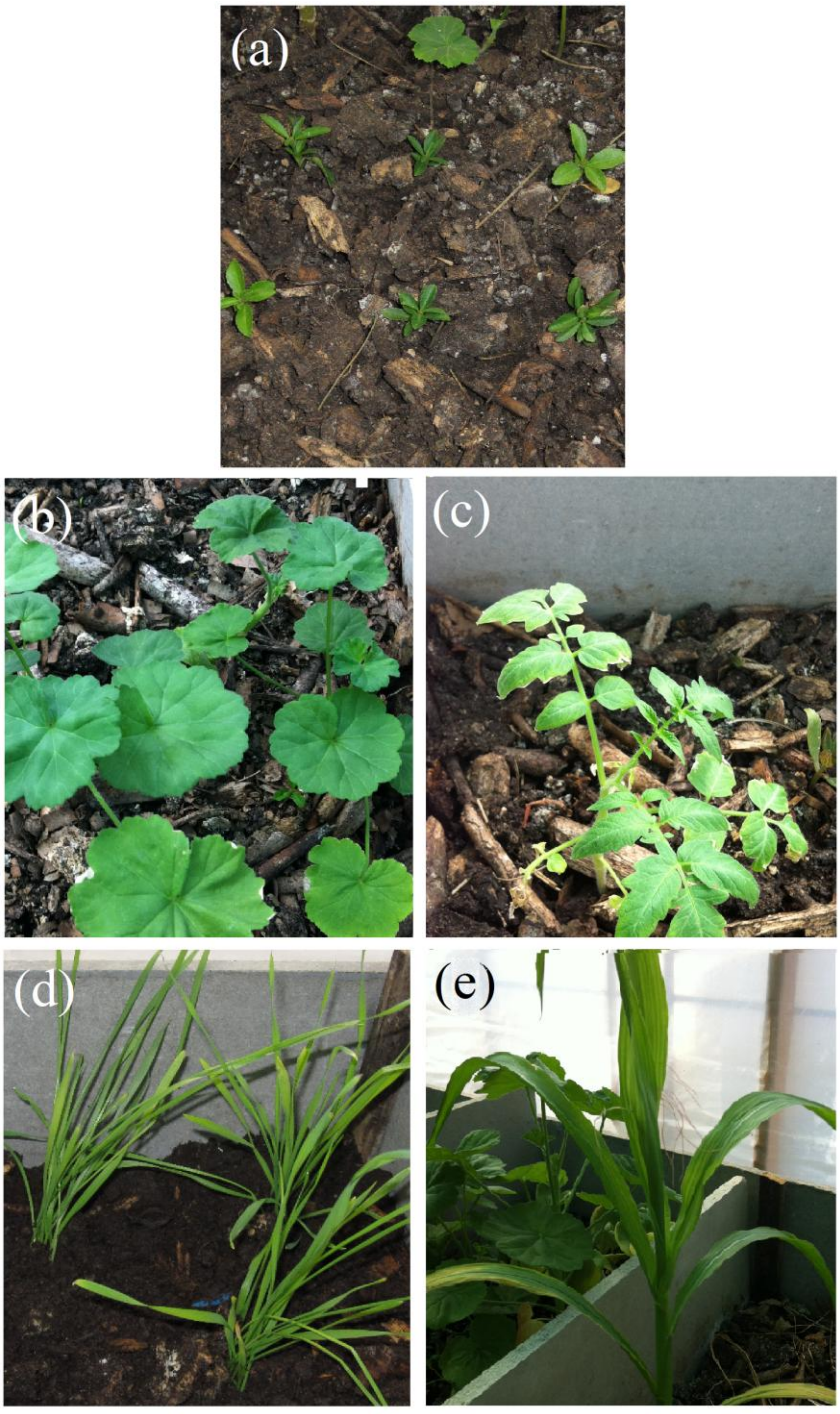


Figure 5. 5. Phytotoxicity tests in remediated composting piles. 1st week of growth for all, 10th week of (b) pelarganium, (c) tomato, (d) wheat, (e) corn

CHAPTER 6

CONCLUSIONS

In this thesis, we worked on improving the effectiveness of TNT bioremediation by enhancing the traditional methods and examining the TNT metabolism of novel bacterial strains in both *in situ* and *ex situ* treatment conditions. Conclusions are presented below divided specifically according to the chapters.

6.1. Development of an HPLC Method for Detection of TNT and Relatives

In this thesis work, we proposed and demonstrated a novel analytical method based on HPLC-UV coupled with Diol, C-18 and Phenyl-3 columns. The method was employed for identification of TNT and TNT derivatives. The Diol

column shows remarkable results especially in the subsequent separation of TATD compared to the other two columns. The results indicate that the Diol column displays an accurate baseline separation. This study details a more efficient and economic method for the detection of TATD compared to the methods presented in the literature. Decreased solvent consumption and release of non-hazardous chemical waste will make this approach highly promising for the separation of nitroaromatics. Further proofs for the effectiveness of Diol column are the reduction of flow rate and analysis time. In our study, Phenyl-3 and C-18 columns consume 55.3% and 58.3% more acetonitrile and water compared to the Diol column; as displayed in Table 3.1, Figures 3.1-a, 3.4-a and 3.5-a. Likewise, durations of the separation for the Phenyl-3 and C-18 columns are 12% and 40% more than that of the Diol column, respectively. Sensitivity of Diol column is, approximately 102.6%, higher than other two columns. Also, this column is able to detect nitroaromatics in the range of 0.78-1.17 µg/L by showing the highest S/N amounts. By means of the optimal resolution capacity, less toxicity and economic advantages, the proposed method also suggests an alternative technique to the EPA 8330 method. The Diol column was found to be the most efficient choice among the columns tested in our study. This work overcomes most of the disadvantages of previous methods for the analysis of TATD using HPLC-UV.

6.2. Biodegradation of TNT by Novel Bacterial Strains

We proposed and demonstrated that the complete biotransformation of TNT can be obtained in 20 h in a simple growth medium inoculated with *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains. In addition, these strains are able to preserve the high degradation capacity in pH and temperature ranges (pH 4.0-8.0 and 4-43^{o}C). The benign side products of TNT enable its safe biodegradation. These promising results suggest that these strains can be utilized safely and effectively in TNT biodegradation. At the end of 20 h, 96.52% of the total TNT was degraded. In addition, the strains were able to preserve the high degradation capacity in pH and temperature ranges broader than the other TNT-degrading strains described in the literature [4,5]. The proposed results suggest that these strains can be utilized safely and effectively in TNT biodegradation. Further study to gain more detailed understanding of TNT degrading enzymes from *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains are needed.

6.3. Bioremediation of TNT-Contaminated Soils Using In-Vessel Composting Method

The present in-vessel composting investigation has demonstrated that complete removal of high TNT concentrations can be achieved in 15 days with the addition of *Citrobacter murliniae* STE 10, *Achromobacter spanius* STE 11, *Kluyvera cryocrescens* STE 12, and *Enterobacter amnigenus* STE 13 strains. To our knowledge, this is the shortest TNT degradation rate observed by the use of in-vessel composting process. Specifically, the degradation rate was shown to be affected by the C/N ratio, aeration rate and presence of additional microorganisms at different TNT concentrations. TNT degradation rate was found to be independent of initial TNT concentration. This study reports an optimization approach to assess the most rapid and effective TNT bioremediation so far demonstrated in an in-vessel compost system using the novel bacteria. The operational parameters such as C/N ratio, aeration rate, TNT concentration, and microbial content had a significant impact on degradation rate.

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CONTRIBUTIONS

Journal Papers

1. Gumuscu B, Cekmecelioglu D, and Tekinay T. Enhanced Bioremediation of 2,4,6-Trinitrotoluene Contaminated Soil by Fast-TNT Degrading Bacteria in In-Vessel Compost system. *In submission.*
2. Gumuscu B and Tekinay T. Rapid and Extensive Biodegradation of 2,4,6-Trinitrotoluene by A Bacterial Strain Isolated from TNT-Contaminated Soil. *In submission.*
3. Gumuscu B, Erdogan Z, Guler MO, and Tekinay T. A Highly Sensitive Determination of Trinitrotoluene and its Metabolites by Using Diol Functionalized Column with High Performance Liquid Chromatography. *In submission.*
4. Gumuscu B and Tekinay T. (2011) TNT degradation by Novel Bacteria Strains. Current Opinion in Biotechnology 22: S66

Patent

1. Gumuscu B, Tekinay T. Isolation of Novel Bacterial Strains Providing Degradation of 2,4,6-Trinitrotoluene, patent no: 2012/01868. *Turkish Patent Institute, pending.*

Conference Presentations

1. Gumuscu B and Tekinay T. Biodegradation of TNT by Novel Bacterial Strains, NATO-29th AVT Panel Business Week (AVT-197: Munitions Related Contamination-Source Characterization, Fate and Transport), San Diego, California, USA, 16-20 April 2012.
2. Gumuscu B, Cekmecelioglu D, and Tekinay T. Bioremediation of 2, 4, 6 Trinitrotoluene Contaminated Soil Using In Vessel Composting Method. Eurasia Waste Management Symposium, Istanbul, Turkey, 14-16 November 2011.
3. Gumuscu B and Tekinay T. Environmental Effects of TNT and DNT: A study on TNT Tolerant Bacterial Strains. NATO-Munition and Propellant Disposal and its Impact on the Environment (AVT-177) Conference, Edinburgh, Scotland, 17-20 October 2011.
4. Gumuscu B and Tekinay T. Extensive Degradation of TNT by Genetically Modified TNT Tolerant Bacterial Strains. European Biotechnology Congress 2011, Istanbul, Turkey, 28 September-1 October 2011.