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Degradation of 2,4,6-Trinitrotoluene (TNT)by Soil Bacteria Isolated From TNT Contaminated Soil

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Abstract: Twenty bacterial strains isolated from TNT contaminated soil. Among those strains only five were highly efficient for their abilities to grow in basal salt medium containing TNT as sole nitrogen source. From these isolates only two strains were more potent for TNT degradation in aerobic condition. These strains were identified as *Clavibacter agropyi* (Corynebacterium) (R.L1) and *Sphingomonas sanguinis* (R.L2).These strains had shown good growth with disappearance of TNT and concomitantly release of nitrite over the period of incubation time. TNT and its metabolites were analyzed by gas chromatography (GC) and results were confirmed and identified by gas chromatography/Mass spectrometer (GC/MS). The biodegradation of TNT was initially similar regardless of the microorganism. Generally, the initial degradation involved the reduction or removal of the nitro substitute giving way to an amino derivative or free nitrite. The identified amino derivatives were, P-toluidine,3,5-dinitro and Benzenamine,2-methyl-3,5-dinitro.which are known as 2-amino-4,6-dinitrotoluene(2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) respectively. Nitrite liberation was consistently found coincided with TNT disappearance from the medium. The disappearance of TNT was completely within 7days by the two isolates and/or their mixture. This is encouraging, and may indicate their potential in bioremediation of TNT contaminated soils. This is the first report on *Clavibacterium agropyri* (Corynebacterium) and *Sphingomonas sanguinis* for degradation of TNT with nitrite release into the medium.

Key words: Degradation, TNT, Contaminants,Biodegradation,Environment,GC and GC/MS analysis.

INTRODUCTION

A variety of synthetic chemicals are present in inland, rivers and marine waters and in agricultural and other soils. Some of these compounds are toxic or may be converted to hazardous products in nature as pesticides, textile dyes and explosives compounds. These toxic compounds arrive to soil through out their industrial waste water.

Nitroaromatic compounds are widely used as pesticides, explosives, and precursors for dyes, pharmaceuticals, and plastics (Higson,1992), but their toxicity, mutagenicity, and persistence make them a serious environmental problem. The explosive 2,4,6-trinitrotoluene (TNT), for example, is a common contaminant of soils and groundwater at many military and production sites, (Kaplan, 1990). Martinetz, and Rippen, (1993/1994). TNT is well known to have both toxic and mutagenic effects on various organisms, including humans (Chaudhry, 1994 and Funk *et al*., 1996). Exposure to TNT is known to cause rashes, skin hemmorages, and mucus and blood disorders (including pancytopenia, a disorder of blood-forming tissues) (Chaudhry, 1994) and Kirk, 1993). Toxic effects including liver damage (toxic hepetitis) and anemia have been reported by workers engaged in large-scale manufacturing and handling of TNT (Chaudhry,1994 and Kirk,1993). TNT has been shown to be a potent mutagen as assayed by the Ames test (Won *et al*., 1976).

TNT contaminated soils have traditionally been recovered by incineration, but its high cost and the fact that ash from incineration must be treated as hazardous waste has led to a search for other methods (Funk *et al*., 1996 and EPA, 1997). The problems with incineration have lead to further research on bioremediation of TNT (Rittmann, 1994). Bioremediation uses microorganisms and plants to transform these hazardous materials into more benign substances (Rittmann 1994).

Much work has been done to characterize specific bacteria or fungal strains for their effectiveness in biodegrading TNT (Scheibner *et al*., 1997; Boopathy, 1994 and Funk *et al*.,1996). Strains of microorganisms have been isolated to try to provide the fastest, most complete degradation of TNT (Chaudlury 1994).

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Despite the toxicity of nitroaromatic compounds, many microorganisms are able to degrade them. Such organisms are candidates for use in bioremediation (Crawford 1995a and b; Kaplan, 1990 and Marvin-Sikkema *et al*., 1994). Significant progress has recently been made in studies of aerobic and anaerobic biodegradation of nitroaromatic compounds, making bioremediation with TNT-degrading microorganisms a feasible method for the restoration of sites contaminated with these compounds.

The present work aims to isolate and identify some microorganisms from the contaminated soil sites with TNT which are able to degrade or transform it to non-toxic byproducts. This will be assure by their successful tolerance, sustain, removal, and growth in TNT enriched, such organisms will be candidate for use in bioremediation and restoration of contaminated sites with nitro-aromatic compounds.

MATERIALS AND METHODS

Soil Samples:

Soil samples were collected from the vicinity ammunition plant in Abo-zaabal military industry region, Kaliobeia, governorate, Egypt.

Chemical, reagents, and media:

The stock solution of TNT prepared in acetone, was filtered through (0.22 um, Satorius, Germany) sterilized and used as 100 mg /L basal solt medium (BSM) as the sole source of nitrogen. The (BSM) was used for evaluation of tolerance and degradation of TNT. Composition of media was as mentioned by Litake *et al*., (2005); its compositions are, D-glucose (5mM), $MgSO_4$; $7H_2O$ (0.7%), K_2HPO_4 (0.2%), sodium acetate (0.1%), tri-sodium citrate (0.04%) and $(NH_4)_2SO_4$was replaced with TNT. All chemicals and solvents were of analytical grade.

Isolation of Bacterial Strains:

The random sample of soil was collected from the vicinity of ammunition plant. Inocculum was prepared by suspending 1g.of soil in 9 ml sterile distilled water, mixed thoroughly and allowed to settle down. A loopful of the supernatant was streaked on nutrient agar plates and incubated at 30^{o}C. The microbial colonies obtained were used to prepare secondary culture. The single isolated colonies from purified secondary culture plates were inoculated individually into 30 ml of nutrient broth to grow over night. One ml. of this over night grown culture ($1x10^6$cell/ml) was then inoculated into 30 ml mineral medium containing TNT (100 ug/ml) as nitrogen source in 100 ml flat bottom conical flask and incubated on rotary shaker at 150 rpm.and 30^{o}C. The microbial activity of the liquid cultures was determined by Fluorescein diacetate (FDA) hydrolysis method. The incubation time for TNT experiments were up to 7days. The persistence of TNT amount was determined after 1, 3 and 7days by gas chromatography (GC) and its transformed compounds were identified by gas chromatography mass spectroscopy (GC/MS). Experiments were repeated minimum three times to reproduce the results.

Identification of Isolates:

The identification of bacterial isolates was achieved by program Biolog Microlog 34.2 base and software Biolog highward, California, USA, Muller and Ehlers, (2005) by Microbial Identification Unit, Plant Pathology Institute, Agricultural Research Center, Giza.

Tolerance assessment:

The isolated strains were tested for their tolerance to TNT by growing in basal media contained different concentrations of TNT (50, 100, 200, 400, 600, and 1000 ppm) as sole nitrogen source and determined their growth under the different TNT concentrations.

Analysis of TNT and its Transformed Compounds:

The disappearance, transformation and strains tolerant for TNT were monitored, and quantified by the extraction of culture supernatants (obtained after centrifugation of culture at 8000 rpm for 20 min.) by ethyl acetate and the analysis were done with Gas chromatography/Flame ionization detector (GC-FID) Hewlett Packard 5890 series II plus, equipped with capillary column Ultra-1(cross linked silicone gum) 25m x 0.32 mm x 0.52um.film thickness. The oven temperature was maintained at 130^{o}C.initially for 2.5 min. and ramped 7^{o}C.rise per minute of 240^{o}C and held for 2 minutes final hold. The nitrogen flow rate was 4.3 ml./min. Final confirmation, and identification of TNT and its transformed compounds were done by Aglint Gas

chromatography-mass spectrometer (GC/MS) at conditions mentioned above. The GC/MS was equipped with a capillary 5% phenylmethyl silicone column (30 m by 0.025mm film thickness).

Total Microbial Activity:

Spectrophotometric determination of the hydrolysis of fluorescein diacetate (FDA) was shown to be a simple and rapid method for determining microbial activity in soil and liquid cultures, Schnurer and Rosswall (1982). FDA hydrolysis was determined as a measure of total microbial activity in liquid culture amended with TNT as sole source of nitrogen, activity was calculated as ug. ml^{-1} produced fluorescein.

Estimation of Nitrite (NO_2^-):

A demineralization of TNT in the form of release of nitrites during growth of organisms was assayed at 1, 3 and 7 days of incubation by withdrawn 10 ml of the culture and centrifugated at 8000 rpm.for 20 min. the supernatant was filtered through 0.25mM microbial filter, and the filtrate was used for analysis of nitrite. Nitrite was estimated colorometrically as mentioned by Geetanjali etal. (2005).

Estimation of Ammonium (NH_3^-):

The reduced nitrite to ammonium in the cultures containing TNT during growth of organisms was assayed at 1, 3 and 7 days of incubation. The analysis of ammonium was carried out colorometrically according to the method of Fowcett and Scott (1960).

Estimation of Nitrate (NO_3^-):

The oxidation of released nitrite to nitrate in the cultures containing TNT during growth of organisms was assayed at 1, 3 and 7 days of incubation. The analysis for nitrate was carried out colorometrically in the centrifugated and filtered cultures by the nitration of salicylic acid method according to Cataldo *et al*. (1975). Potassium nitrate was used as standard.

Nitrate Reductase Test:

Nitrate reductase activity in the broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also, testes the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. The test of enzyme activity was determined colorometrically as described by Dvorakova etal. (1998).

RESULTS AND DISCUSSION

Identification of Isolates:

The two bacterial isolates (strain R.L1 and R.L2) were capable to grow on the BSM-TNT as sole nitrogen source and transformed it to amino derivatives and release of nitrite in culture were identified as *Clavibacterium agropyri* (corynebacterium) and *Sphingomonstainas sanguinis* respectively. This result is confermed by Maeda etal. (2006) who, isolated five *pseudomonas sp*. strains and *Sphingomonas sp*. Strain from soils in Japan have been polluted by TNT. These six strains converted TNT into reduced products, Leys (2004) isolated *Microbacterium* and *Sphingomonas* strains from contaminated soil with polycyclic aromatic hydrocarbons (PAHs) which used these compounds as sole source of carbon and energy. Also, Michael *et al*. (2007) have isolated *Clavibacterium agropyri* (Corynebacterium) from engine oil contaminated soil which was capable of degrading organic compounds.

TNT as sole nitrogen source during growth of isolates:

The organisms were inoculated in mineral medium contained TNT to observe their activity patterns and utilization of TNT nitrogen by aerobic pathway. The initial inoculi for all different organisms was about$1.0x10^6$ cells.ml^{-1}. The growth activity was monitored spectrometrically and measured as FDA hydrolysis. The obtained results are presented in (Table1) and graphically in (Fig.1). The data indicated that the activities of *Sphingomonas sanguinis* and the mixture of *Sphingomonas sanguinis+ Clavibacterium agropyri*(corynebacterium),were similary and high slightly than that of *Clavibacterium agropyri* (corynebacterium) activity.

Generally, it is observed that, TNT removal was associated with increasing the activity of microorganisms since, the increasing in microbial activity was accompanied with decreasing of TNT amounts in the culture. The most promising activity was observed with the organism *Sphingomonas sanguinis*. This result indicates

that these microorganisms are able to use the molecular nitrogen of TNT in building their cells. This result is in agreement with those obtained by many investigators (Duque *et al*. 1993; Boopathy and Kulpa 1994; Jones *et al*. 1995; Fuller and Manning 1997; Stolpmann *et al*.1999; Fritsche *et al*. 2000; Ibraham *et al*. 2001) who noticed that, certain of *Pseudomonas* sp. and fungi can use TNT as a nitrogen source through the removal of nitrogen nitrite from TNT under aerobic condition.

Table 1: FDA hydrolysis of *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixture cultures amended with TNT as sole nitrogen source, determined as ug Fluorescein.ml^{-1}.

Treatment	Ug Fluorescein.ml^{-1}		
	1	3	7 days
Un-inoculated media +TNT	0.1	0.3	0.2
Clavibacterium agropyri	10	11.3	11
Sphingomonas sanguinis	12	14	12
S. sanguinis + c. agropyri	12	12	13

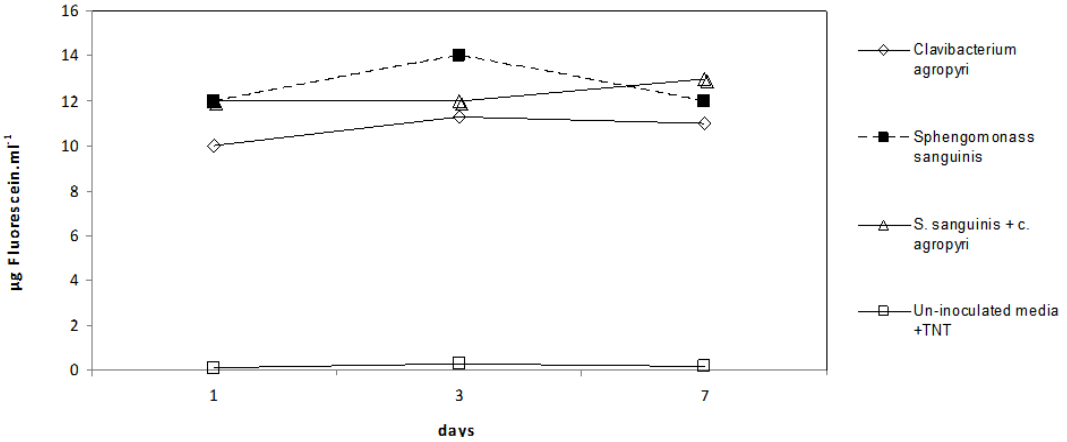

Fig. 1: FDA hydrolysis of *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixed cultures amended with TNT as sole nitrogen source.

Nitrite release from TNT in the liquid culture:

Nitrite assays were performed to detect the release of (NO_2^-) group from TNT, a positive evidence of TNT demineralization. The results of nitrite assays are presented in (Table 2) and graphically in (fig.2). The findings of nitrite release was corresponded to disappearance of TNT from culture media with incubation time. The absence of nitrite in un-inoculated media + TNT (control) demonstrated that, the presence of nitrite in medium was a result of bacterial transformation of TNT. From the data illustrated in (table2) and (fig.2) can observe that, the nitrite released from TNT by *Clavibacterium agropyri* (Corynebacterium) and *Sphingomonas sanguinis* cultures was higher through the first three days of incubation and thereafter was decreased, while the reverse was occurred with the mixing mixtures of them, this indicate that *Clavibacterium agropyri* (Corynebacterium) and *Sphingomonas sanguinis* are more efficient and rapid for TNT transformation. Un-inoculated media+TNT treatment showed negligible amounts of nitrite, this may be due to water hydrolysis. This result is accordance with that obtainned by Geetanjali *et al*., (2005) who noticed that, nitrite liberation was consistently found coincided with TNT disappearance from the medium of *Salmonella typhimurium*. Also, Duque *et al*.(1993); Boopathy and Kulpa.(1994); Jones *et al*.(1995); Fuller and Manning (1997);Stolpmann *et al*.(1999); Fritsche *et al*. (2000) and Ibraham *et al*. (2001) demonstrated that, certain of *Pseudomonas* sp. and fungi can use TNT as a nitrogen source through the removal of nitrogen nitrite from TNT under aerobic conditions and the further reduction of the released nitrite to ammonium which is incorporated into carbon skeleton. Wittch etal.(2009) noted that unstable reduced derivatives of TNT produced by microorganisms have been found to release nitrite by rearomatization and/or condensation.

Table 2: Detection of Nitrite in *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixture cultures amended with TNT as sole nitrogen source.

Treatment	Ug $NO_2^-.ml^{-1}$		
	1	3	7days
Un-inoculated media + TNT	8.0	4,0	4.0
Clavibacterium agropyri	226.0	267.0	122.0
Sphingomonas sanguinis	149.0	247.0	127.0
S. sanguinis + c. agropyri	93.0	141.0	146.0

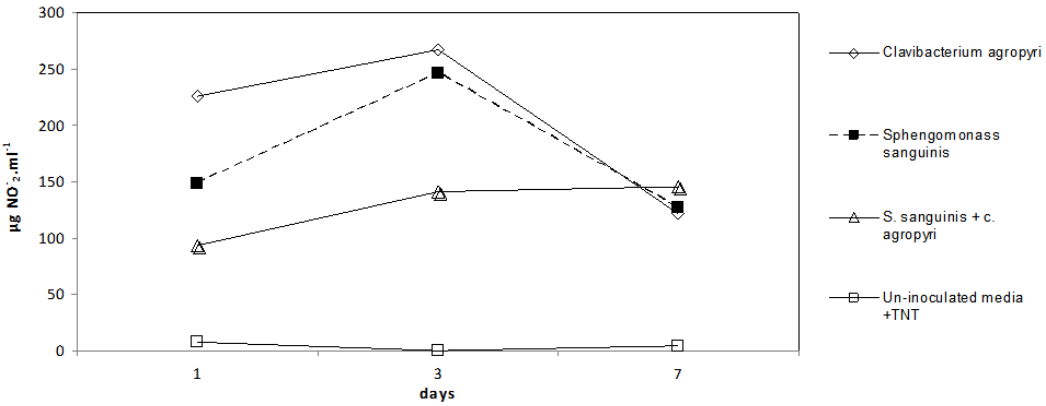

Fig. 2: A graph shows Nitrite release during TNT degradation by *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixed cultures.

Reduction of Nitrite released from TNT to Ammonium in liquid culture:

Ammonium assay was performed to detect the reduction of the released NO_2^- group from TNT to ammonium. A positive evidence of nitrite reduction to ammonium was occurred. The results of ammonia assays are presented in (Table 3) and graphically in (Fig.3). The finding of ammonia was corresponded to decreasing of nitrite in cultures with incubation time. From the illustrated data can observe that the reduction of nitrite to ammonium in cultures of each *Clavibacterium agropyri* (Corynebacterium); and *Sphingomonas sanguinis* began low and increased after that, while the mixture of the two strains was more efficient and rapidly in its transformation of nitrite to ammonium. This result confirmed that obtained by Antonio *et al*. (2005) who, stated that *Psedomonas putida* JLR11 releases nitrogen from TNT ring as nitrite or ammonium. Also, Spain (1995); Bruns-Nagel *et al*., (1996|) and Gilcrease *et al*. (1995) who stated that, in most cases, microbial transformations of TNT take place through successive reduction of the nitro groups. Also, Litake *et al*. (2005) noted that the isolated microorganisms have been carried out nitrogen group reduction under aerobic conditions. Nunez *et al*. (2001) announced that, certain strains 0f pseudomonas and fungi can use TNT as sole nitrogen source through the removal of nitrogen as nitrite from TNT under aerobic conditions and the further reduction of the released nitrite to ammonium.

Table 3: Detection of ammonium in *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixture cultures amended with TNT as sole nitrogen source.

Treatment	Ug NH_3-.ml		
	1	3	7 days
Un-inoculated media +TNT	8	16	26
Clavibacterium agropyri	17	493	219
Sphingomonas sanguinis	20	458	227
S. sanguinis + c. agropyri	401	349	249

Estimation of Nitrate (NO_3^-):

Nitrate assay was performed to detect the oxidation of the released NO_2^- group from TNT to nitrate (NO_3^-). A positive evidence of nitrite oxidation to nitrate (NO_3^-) was occurred. The results of nitrate (NO_3^-) assays are presented in Table 4 and graphically in Fig.4. The finding of nitrate (NO_3-) in the cultures of *Clavibacterium agropyri* (Corynebacterium); and *Sphingomonas sanguinis* containing TNT during the growth was higher at the begaining of the experiment than that in their mixture and it was decreased after. *Sphingomonas sanguinis* was the more efficient in transformation of nitrite to nitrate while, the mixture of strains showed low transformation at the begaining of the experiment and increased thereafter.

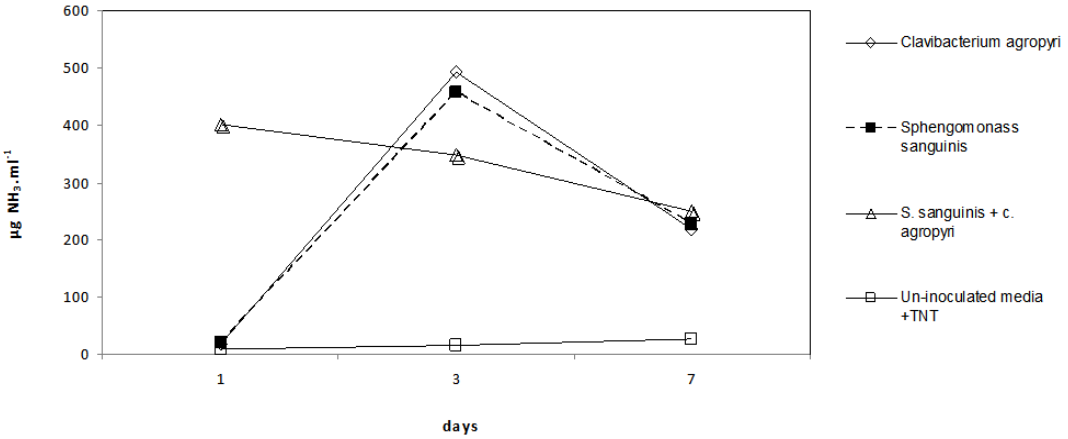

Fig. 3: Detection of ammonium in *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixed cultures amended with TNT as sole nitrogen source.

Table 4: Detection of Nitrate (NO_3^-) in *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixture cultures amended with TNT as sole nitrogen source.

Treatment	Ug $NO^-_3.ml^{-1}$		
	1	3	7days
Un-inoculated media +TNT	8	4	0
Clavibacterium agropyri	191	7	9
Sphingomonas sanguinis	219	7	9
S. sanguinis + c. agropyri	9	13	26

Nitrate Reductase Assessment:

Nitrate reductase activity was determined in the two broth of *Sphingomonas sanguinis* and *Clavibacterium agropyri* (Corynebacterium) without TNT addition. The obtained results indicated that the two strains produced nitrate reductase enzyme and its activity was higher in *Sphingomonas sanguinis* than *Clavibacterium agropyri* (Corynebacterium) strain. Since, the performed amounts of nitrite (NO^-_2) and (NH^-_3) by *Sphingomonas sanguini* were more than that performed by *Clavibacterium agropyri* (Corynebacterium). This result is agree with that obtained by Oh *et al*. (2001) who, noted that TNT was rapidly degraded by nitroreductase enzyme obtained from a *Pseudomonas aeruginosa* strain isolated from TNT contaminated soil. The TNT-nitroreductase catalyzed reduction of TNT to 4-hydroxylamine-4,6-dinitrotoluedene(4HADNT) and 4-amino-2,6-dinitrotoluene (4ADNT).

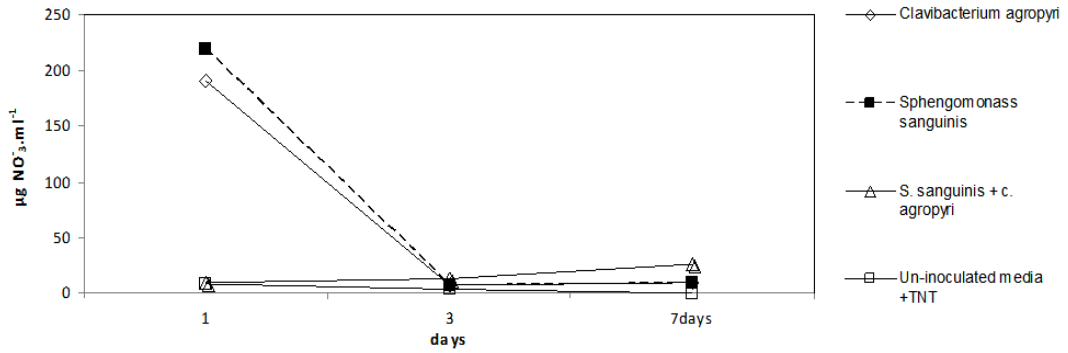

Fig. 4: A graph shows Nitrate (NO_3^-) development during TNT degradation by *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixed cultures.

Disappearance of TNT from the bacterial cultures:

The disappearance of TNT from the liquid cultures of *Sphingomonas sanguinis*, *Clavibacterium agropyri*(Corynebacterium) and their mixture were monitored, and quantified at various time points (1, 3, and 7days) by GC and GC/MS instruments.

Data from GC analysis (Table 5) and (Fig.5) showed that TNT was removed from the mineral salt medium during growth. Three prominent peaks were observed, the first one have retention time (R_t8.65 min) corresponded to TNT while, the second and third peaks had (R_t 12.37 and 14.56 min.) were identified by GC/MS as P-toluidine, 3, 5-dinitro (4ADNT) and Benzenamine, 2-methyl-3,5-dinitro (2ADNT) respectivily. The area under the curve of TNT peak was reduced as incubation time advanced and with appearance of the other two peaks.

There was a complete disappearance of TNT peak during the course of incubation days of cultures *Sphingomonas sanguinis* and the mixture of strains, whereas small amounts of TNT was observed in the culture of *Clavibacterium agropy*ri (Corynebacterium). This results indicate that, the *Sphingomonas sanguinis* strain or the mixing between the two strains is more efficient and rapid in biotransformation of TNT. Table 5 and (Fig.5 represents the results of GC analysis of TNT at a various time points (1, 3 and 7days) during *Clavibacterium agropyri* (Corynebacterium) and *Sphingomonas sanguinis* and their mixture incubation. While, the Fig.6 represents the GC chromatogram analysis of TNT and its transformations. Also, Fig.7 represent the GC/MS graphic of TNT and its two dominant metabolites obtained by the above mentioned strains and their mixture. These results indicate that TNT was transformed to aminodinitrotoluenes derivatives (ADNTs), this result is agreement with those obtained by Duque *et al*. (1993) who, noted that, the nitro groups in TNT molecule were reduced on the ring to their amino analogs via a hydroxylamine intermediate. Also, Boopathy *et al*. (1994) reported the four isolated pseudomonas spp. from contaminated soil with TNT extensively transformed TNT and the main intermediates were identified as 4-amino-2,6-dinitroyoluene and 2-amino-4,6-dinitrotoluene. Maeda *et al*.(2006) isolated six strains (5 *pseudomonas sp.* And one *Sphingomonas sp*.)from soils polluted by TNT. The six strains converted TNT into reduced products including 2ADNT and 4ADNT. The same results were published by Nunez etal. (2001) when they noted that most but not all aerobic microorganisms reduce TNT to the corresponding amino derivatives via the formation of hydroxaylamine intermediates. This result also, confirmed that the nitrate reductase was induced by the presence of TNT. Also, some investigators noted that, complete reduction of the nitro group to an amino group seems to decrease the mutagenic effect of TNT (Cash 1998, George *et al*.2001 and Tan *et al*.1992). The isolates *Sphingomonas sanguinis* and the mixture of (*Clavibacterium agropyri+ Sphingomonas sanguinis*) have removed 100% of TNT very efficiently during growth, without any addition of growth enhancer or growth promoting substances.

Table 5: Disappearance rate of TNT from the liquid cultures of *Clavibacterium agropyri* (Corynebacterium), *Sphingomonas sanguinis* and their mixture determined as ug TNT found.ml^{-1}.

Treatment	ug TNT.ml^{-1}		
	1	3	7days
Un-inoculated media +TNT	100.00	98.00	95.00
Clavibacterium agropyri	73.50	47.50	5.00
Sphingomonas sanguinis.	93.50	67.00	0.00
s sanguinis. + c. agropyri	0.53	0.13	0.00

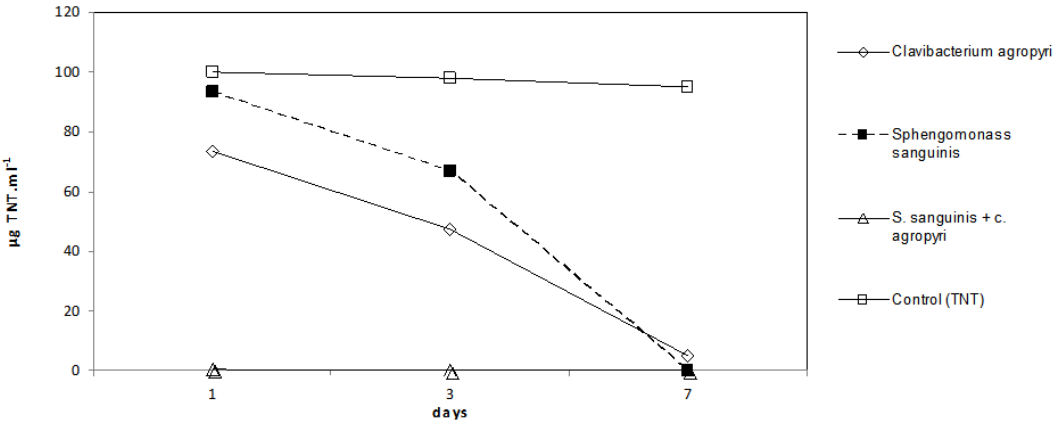

Fig. 5: Graphical presentation of GC data, showing TNT amounts presented in mineral medium during growth at given time points.

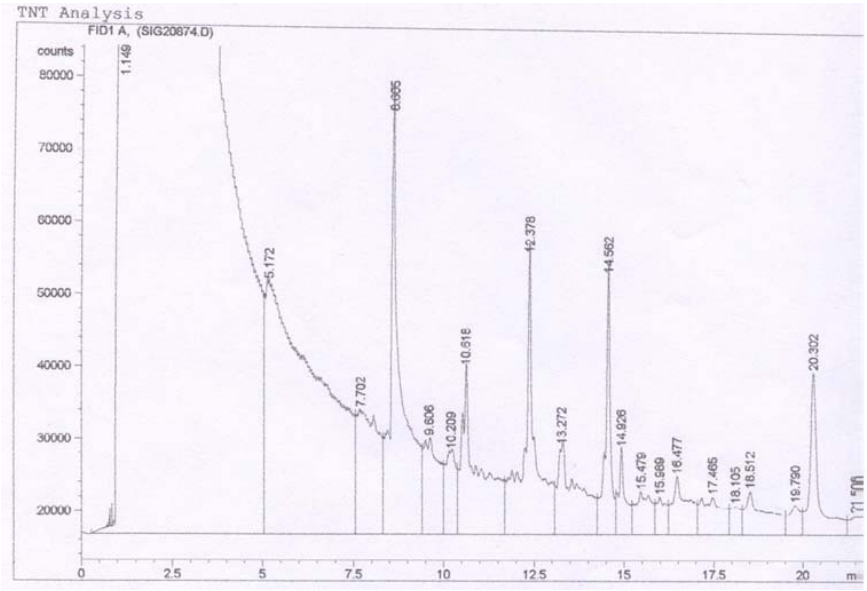

Fig. 6: GC graph, showing TNT and its transformations products during growth of the isolates in mineral medium enriched with TNT.

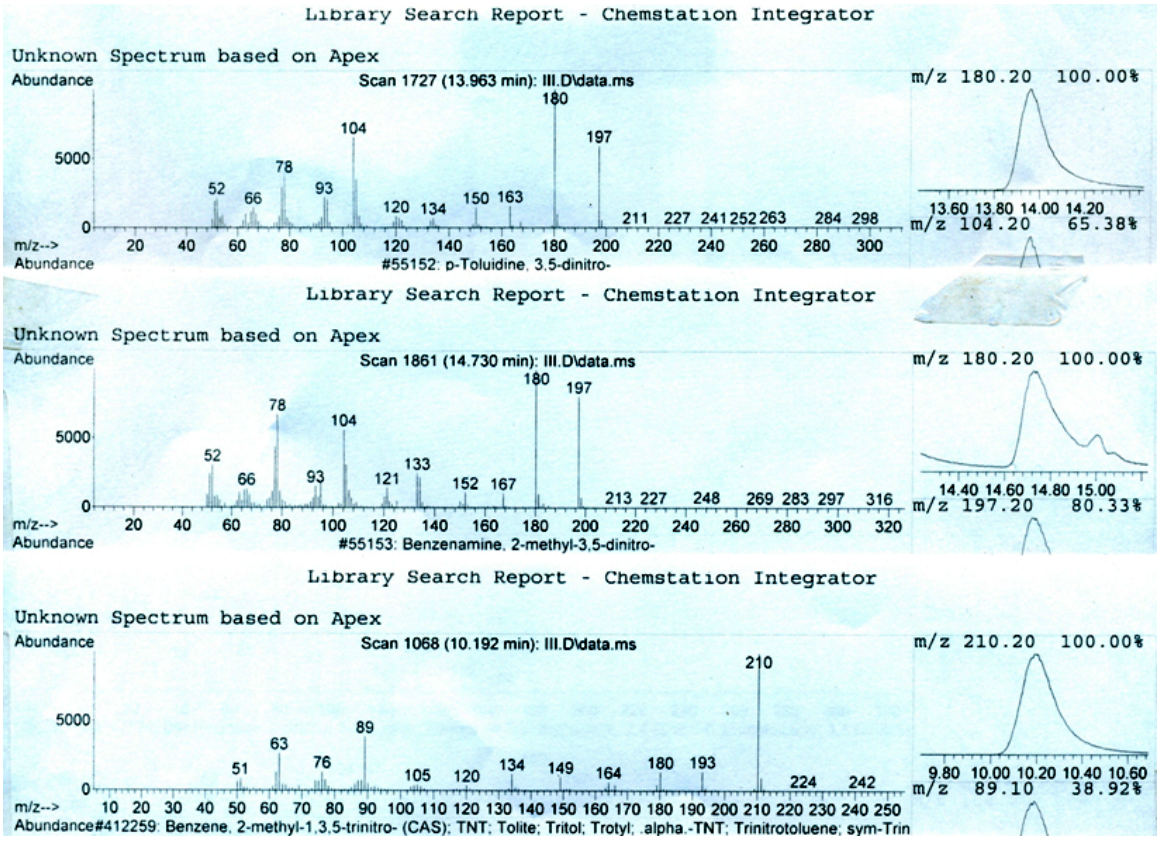

Fig. 7: GC/MS graph showing TNT and its transformation spectrum during growth of the isolates in mineral medium.

Conclusion:

TNT is an environmental toxin, which needs to be removed from contaminated soil sites to prevent its toxicity on ecosystem**.** Bioremediation is an effective and eco-friendly method of removal of TNT from natural occupational and environmental sites**.** Bacterial degradation of TNT by aerobic method is easy to maintain than anaerobic system of cultivation. The two microbial strains (*Clavibacterium agropyri* (Corynebacterium) and *Sphingomonas sanguinis*) isolated from a TNT contaminated soil transformed TNT rapidly from 100ppm to non-detectable levels in MSM medium. The cells were able to grow well in media contained TNT and they were able to release the nitro group from TNT molecule, these strains consume the TNT as sole source of nitrogen and produce nitrate reductase enzyme which convert the nitro group to amino derivates. These strains have not been reported earlier for using TNT as sole source of nitrogen with concomitant release of nitrites and therefore it is logical that these isolates may be having potential for TNT degradation.

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