# CARBON NANOTUBES AND THEIR EFFECT ON E. COLI VIABILITY

**N. Vlachou, A. F. Trompeta, D. Perivoliotis, A. Skarmoutsou, C.A. Charitidis**
Research Unit of Advanced, Composite, Nano Materials & Nanotechnology, School of Chemical Engineering, National Technical University of Athens, 9 Heroon Polytechniou St., Zografos, 15780, Athens, Greece

**D. Mamma, D. Kekos**
Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Heroon Polytechniou St., Zografos, 15780, Athens, Greece

## ABSTRACT
Carbon nanotubes (CNTs) are one of the most appealing nanomaterials with unique physicochemical, mechanical, and electrical properties that find application in many different fields, e.g. biology, medicine and mechanics. Moreover, they have a tremendous increase in commercial interest and subsequent mass production. Hence, it is considered crucial to study and understand the effect of CNTs on living organisms and environment for their proper future use. It has been indicated that CNTs size, surface area, purity and surface chemistry are considered important parameters for the toxicological effect on living organisms. The differences in CNTs properties could be the first reason that explains why the results of their toxic effect on various types of cells are diverse, controversial and sometimes conflicting. Another reason could be the different cell culture media and the different cell types. It is obvious that more studies are needed in order to conclude to specific results about the effect of CNTs on cells.

## INTRODUCTION
CNTs are graphitic filaments with diameters ranging from 0.4 to 500 nm and are available in varying lengths, up to several hundred micrometers, depending on the production method [1]. SWCNTs are composed of a single cylindrical sheet of graphene and MWCNTs several concentric, coaxical rolled up graphene sheets [2]. Specifically, the CNTs diameters are typically 0.4 to 2 nm and up to 200 nm for single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), respectively [3]. After the discovery of CNTs by Iijima in 1991 [4], CNTs have attracted the scientific interest owing to their excellent physicochemical, electrical and mechanical properties. Their applications in the area of polymer composites as fillers, microelectronics, energy storage and sensors have been widely reported [5]. CNTs are usually obtained by CVD method from the decomposition of gaseous hydrocarbon over transition metal-catalyst particles [6]. The CVD method is the most dominant technique for mass production of CNTs due to its easy scaling-up [7].

*Escherichia Coli* (*E. Coli – Gram – negative facultative anaerobic rods* [8]*/ Family of Enterobacteriaceae*) was isolated from faeces in 1885 by T. Escherich. *E. Coli* is a common inhabitant of the large intestine of humans and mammals. It is also found in the guts of birds, reptiles, amphibians and insects. The bacteria are excreted in great numbers with the faeces and are always present in the external environment (soil, water, foodstuffs and other objects). *E. Coli* are straight rods measuring 1.02 – 1.78 cm breadth and 2.54 – 7.62 cm length. They occur as individual organisms or in pairs and are marked by polymorphism. There are motile and non-motile types. The G+C content in DNA is 50 – 51 %. The cell surface has pili on which certain phages are absorbed. The microcapsule is not always clearly defined. *E. Coli* is a facultative anaerobe. The optimum temperature for growth is 30 – 37 $^{o}$C and the optimum pH value of medium is 7.2 – 7.5. The organism also grows readily on ordinary media at room temperature and at 10 and 45 $^{o}$C, growth becomes visible in the first two days. *E. Coli* from cold-blooded animals grows at 22 – 37 $^{o}$C but not at 42 – 43 $^{o}$C. On meat-peptone agar *E. Coli* produces slightly convex semi-transparent, greyish colonies and in meat broth it forms diffuse turbidity and a precipitate. *E. Coli* survives in the external environment for months. It is more resistant to physical and chemical factors of the external environment than the typhoid and dysentery bacteria. *E. Coli* is killed comparatively rapidly by all methods and preparations used for disinfection. At 55 $^{o}$C the organism perishes in 1 h and at the 60 $^{o}$C in 15 min. *E. Coli* is used as a test microbe in the assay of disinfectants and methods of disinfection and also in titration of certain antibiotics [9].

In the current study, *E. coli* was used as a microorganism model and unfunctionalized and functionalized CNTs were tested regarding the viability effect. Generally, there are different experimental procedures of CNTs' synthesis in a laboratory scale. In our study, MWCNTs were synthesized via thermal chemical deposition method (T-CVD) and the approach used was the supported catalyst. Subsequently, purification with hydrochloric acid and functionalizion of the surface of the obtained CNTs' took place, to compare the toxicity effect of

pristine and functionalized CNTs on *E. coli* cells. Finally, the effect of different CNTs concentrations on bacteria's viability was determined.

## EXPERIMENTAL PROCEDURE

- **Synthesis of Carbon Nanotubes**

A T-CVD reactor was used to synthesize MWCNTs. The reactor consists of a horizontal quartz tube with 3.4 cm inner diameter and 100 cm long housed in a three-zone cylindrical furnace 80 cm long. Synthesis of CNTs was performed by the supported catalyst approach according to which, acetylene is used as carbon source and iron particles supported entangled on $Al_2O_3$ are used as catalyst [10]were used. For the preparation of the samples, the catalyst particles were placed on a silicon substrate, which is located inside the quartz tube, in the middle of the isothermal zone of the reactor. Firstly, a constant nitrogen flow rate is passed through the quartz tube to remove the air from the system and then the reactor is heated at the desired temperature (ranging 700 – 800 $^oC$) under nitrogen flow. Subsequently, a mixture of acetylene/nitrogen replaced the nitrogen. When the reaction was completed, the raw products were cooled down to room temperature in nitrogen atmosphere.

- **Carbon Nanotubes Purification and Functionalization Process**

After the synthesis, the raw products were milled and exposed at atmospheric air flow at 400 $^oC$ for 1 h, aiming at the removal of amorphous carbon. Afterwards, they were purified with constant boiling 5M HCl in a Soxhlet extractor in order to remove the remaining metal particles. Finally, the purified CNTs were washed with distilled water and dried in an oven. To activate the CNTs surface with –COOH groups, an acid solution mixture of 6M $HNO_3$:$H_2SO_4$ 1:3 was used. Then, the CNT/acid mixture (0.15 g CNTs/10 ml acid solution) was stirred for 48 h at 80 $^oC$. The suspension was filtered and the black powder deposited on the filter was washed with distilled water, then with ethanol and acetone and dried in oven.

- **Characterization Techniques**

X-ray diffraction: The measurements were performed at room temperature with Bruker D8 Advance Twin X-ray diffractometer equipped with a Cu $K_a$ radiation source (wavelength = 1.5418 Å).
Thermogravimetric analysis: The TGA experiments were conducted in oxidative atmosphere (atmospheric air flow: 120mL/min, heating rate: 5$^oC$/min) at a Netzsch 409 EP instrument.
Scanning electron microscopy: The morphology of CNTs was determined by SEM using Nova NanoSEM 230 (FEI company) microscope with W (tungsten) filament.
Transmission electron microscopy: TEM measurements were performed with a Tecnai G2 Spirit Twin 12 microscope (FEI) after the dispersion of CNTs in distilled water.
Fourier transform infrared spectroscopy: FT-IR analysis was performed by using a ThermoScientific Nicolet 6700 Fourier Transform Infrared Spectrometer.

- **Bactericidal test – Meat Broth**

After the synthesis and characterization of CNTs, their antibacterial activity was evaluated. *E. coli* was used as a microorganism model. The bacteria were propagated in Luria-Bertani (LB) medium at 37 ◦C with shaking at 120 rpm until the $OD_{600}$ reached 0.5. Measurements of $OD_{600}$ were performed in an ultraviolet-visible (UV-Vis) spectrometer V-630iRM (JASCO) at 600 nm wavelength. Then, 2.5 % v/v of the bacterial culture was transferred into a 150 ml conical flask, mixed with MWCNTs to various final concentrations in order to estimate the bacterial behavior in different CNTs concentrations. During incubation at temperature 37 ◦C with shaking at 120 rpm, $OD_{600}$ was measured every 30 min in order to observe the growing procedure.

- **Bactericidal test – Meat peptone agar**

Moreover, pristine and functionalized MWCNTs were added, to a final concentration of 0.02 % v/v in 2.5 % v/v of the bacterial culture. During incubation at temperature 37 ◦C with shaking at 120 rpm, $OD_{600}$ was measured as well, to observe the growing procedure. Then, when the $OD_{600}$ reached the value 0.5, a diluted amount of *E. Coli* were plated on LB agar while extra 0.03 g of CNTs powder, both pristine and functionalized, to be in direct contact with the bacteria. The incubation time of the plates was about to 16 h. Experiments were repeated three times.

## RESULTS AND DISCUSSION

After the synthesis, purification and functionalization of CNTs, the structure, chemical composition and purity degree was investigated. In Figure 1, TEM images of the tested CNT samples are depicted and hollow filamentous structures are revealed. Especially, MWCNTs produced via supported catalyst method present diameters of 20 – 40 nm (depending on the experimental conditions). Additionally, iron particles mostly could be seen encapsulated within the core of the MWCNTs, so their interaction with the culture medium can be considered negligible. In all cases, the length of CNTs exceeds 10μm.

Representative SEM image, EDS analysis, XRD diagram and TGA graph of CNTs produced are presented in the following figures. In Figure 2a and b, the SEM image and corresponding EDS analysis, respectively, of the produced carbonaceous materials are presented reveal a uniform diameter distribution and high purity. The main

features of XRD patterns of CNTs are close to those of graphite (Figure 2c); a typical XRD pattern consists of bands located near the (002), (100) and (110) reflections of graphite. The first peak at $2\theta \sim 26^{o}$ can be attributed to the (002) reflection of graphite while an asymmetric diffraction peak at $2\theta \sim 43^{o}$ is assigned to (100) reflection of graphite, which is typically observed for MWCNTs. TGA was used in order to evaluate the thermal stability and the purity degree of the produced CNTs (Figure 2d). The initial weight loss of 1.5% observed at temperatures up to 480 $^{o}$C could be assigned to the burning of amorphous carbon material. The residual weight % at the end of the thermal oxidative curve was 5.7 and corresponds to the iron catalyst particles. The differential thermogravimetric analysis (DTA) curve showed only one narrow peak at 560 $^{o}$C indicating the high thermal stability in air atmosphere and the uniform graphitized structure of the CNTs produced. The overall purity of the produced MWCNTs is around 92.8 wt. %.

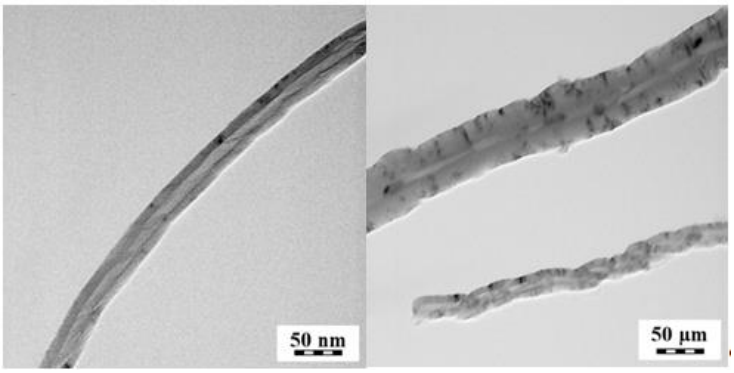


**Figure 1.** TEM images of pristine (left) and functionalized (right) MWCNTs

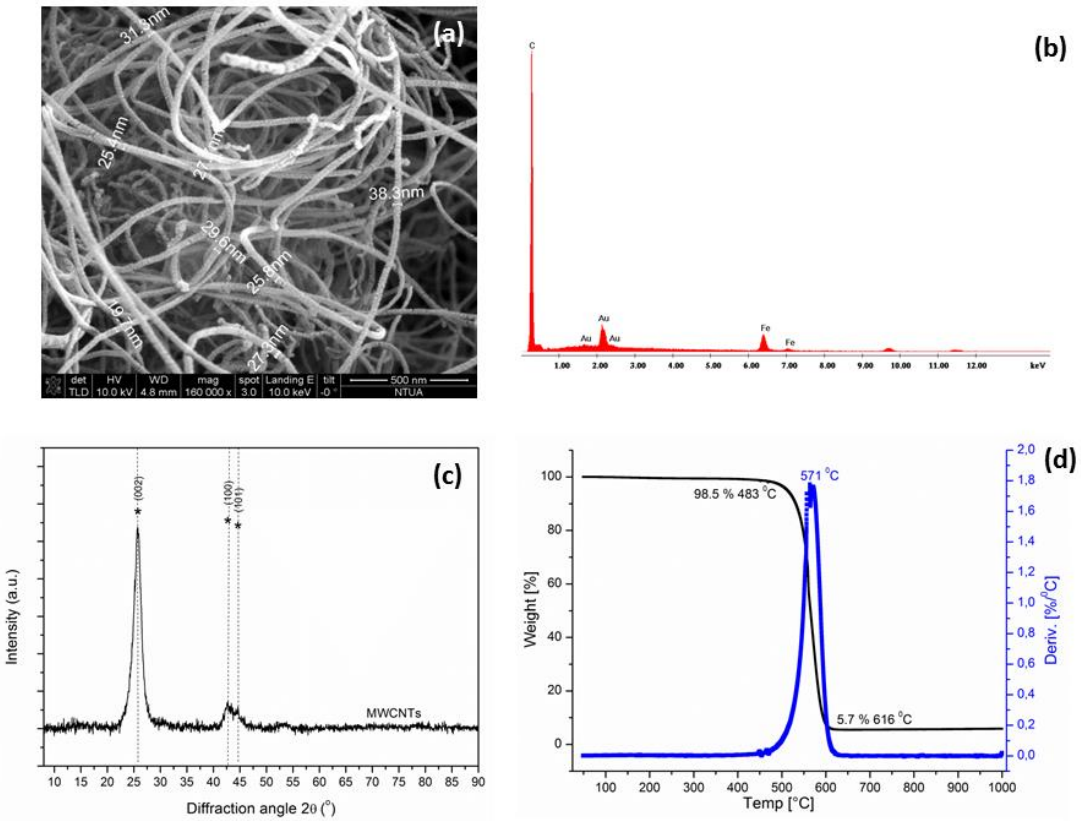


**Figure 2.** (a) SEM image at 160000 magnification, (b) EDS analysis, (c) XRD diagram and (d) TGA graph of sample PS2.

**Table 1.** XPS analysis of pristine and functionalized with oxygen containing groups.

| Chemical Bond | Pristine CNTs | Functionalized CNTs with -COOH |
|---|---|---|
| C-C (sp3) | 7.6 | 15.9 |
| C=C (sp2) | 81.5 | 66.2 |
| C-O | 2.8 | 6.3 |
| C=O | 1.6 | 2.6 |
| COOH | - | 4.5 |

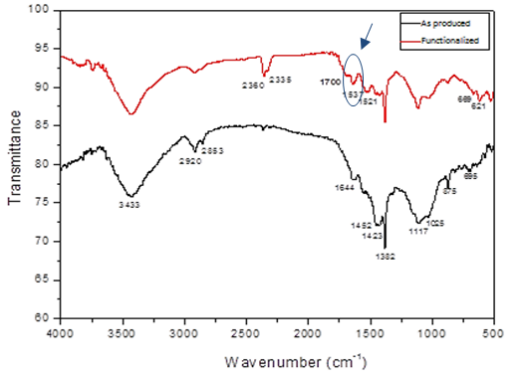


**Figure 3.** FT-IR spectra for pristine and functionalized CNTs.

X-Ray Photoelectron Spectroscopy (XPS) and FT-IR analysis were used to study the effectiveness of the functionalization process. Table 1 presents that oxygen containing groups were successfully introduced (13.4% at.) in the CNTs' sidewalls. This result is also confirmed by the FT-IR spectra, where the characteristic peak of C=O bond at ~1700 $cm^{-1}$ is observed for the functionalized CNTs (Figure 3).

After the synthesis and characterization of CNTs via T-CVD method, bactericidal test was conducted. Initially, various concentrations of the pristine CNTs were compared to commercial ones, as it is shown in Figure 4. The optical density (OD) evolution pattern is mostly unaffected by all the tested concentrations of pristine CNTs in contrast to commercial CNTs. For the commercial CNTs, Figure 4a shows that when the concentration of CNTs reaches 0.02 % w/v, the bacterial growth is substantially affected (red line). Contrariwise, it is not observed the same for the pristine ones (Figure 4b). This could be explained by the fact that commercial available CNTs are subjected to surface treatment to improve their dispersibility [11]; whereas, the pristine CNTs that we synthesized had not been subjected to any surface treatment. As a result, agglomerates were formed resulting in lower contact area with the bacteria compared to the commercial CNTs that showed greater dispersion (Figure 5).

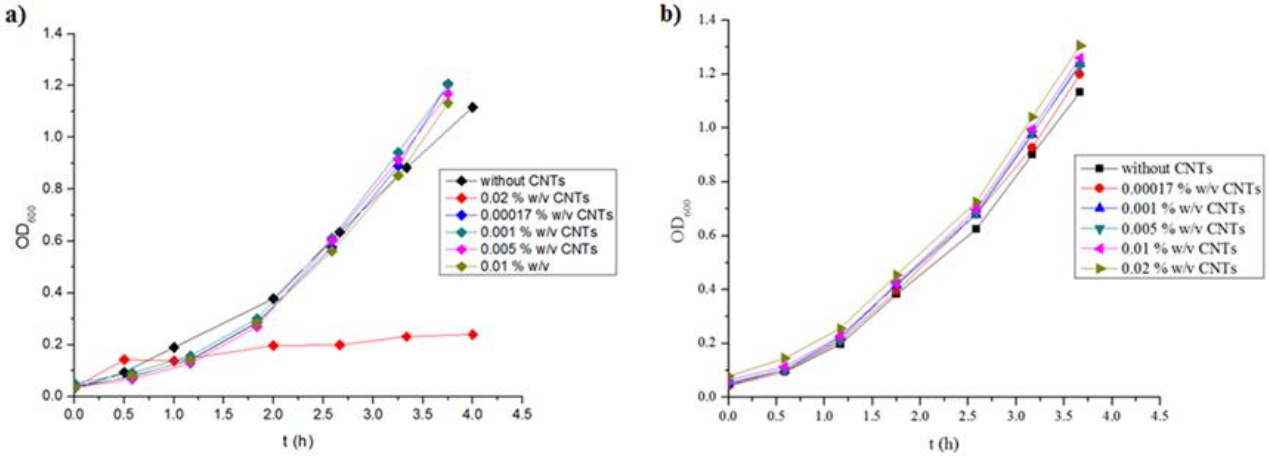


**Figure 4.** OD growth curves of *E. Coli* that were treated with a) commercial CNTs and b) pristine CNTs synthesized via CVD method.

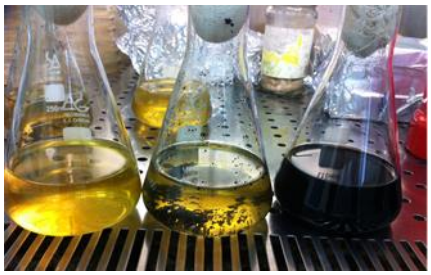

**Figure 5.** LB medium without CNTs (left), with pristine CNTs (middle) and with commercial CNTs (right).

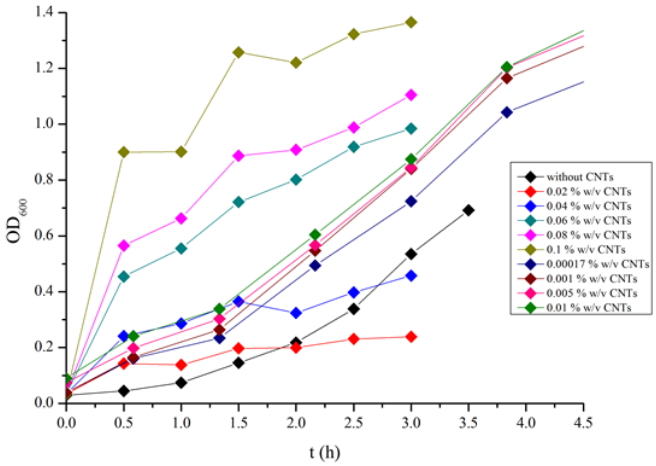


**Figure 6.** OD growth curves of E. Coli that were treated with various concentrations of commercial CNTs.

From the linear part's slope of the growth curves, the growth rate ($\mu_{max}$) was determined. Additionally, it was observed that by increasing the concentration of the commercial CNTs, the growth rates were declined (Figure 7). Moreover, it can be seen that for concentrations above 0.02 % w/v, the effect on growth rate is maximized while further increase in the CNTs concentration, results in almost constant growth rate. Specifically, the growth rate for the 0.02 % w/v CNTs is 50.8 % lower than the zero CNTs concentration, while for the 0.04 % w/v CNTs is 70.6 % lower. Further increase results in approximately 27 % reduction in growth rate. The bacterial growth is hindered at higher CNTs concentrations indicating antibacterial activity. Increasing the concentration, not only the growth rate decreases but also the doubling time increases almost five times compared with the initial one for zero CNTs concentration.

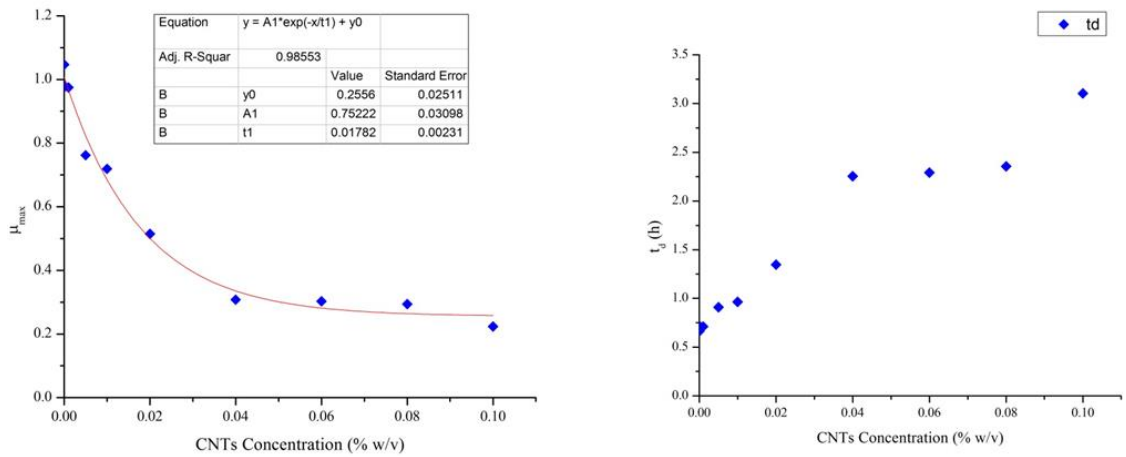


**Figure 7.** Growth rate curves as a function of CNTs concentration in % w/v.

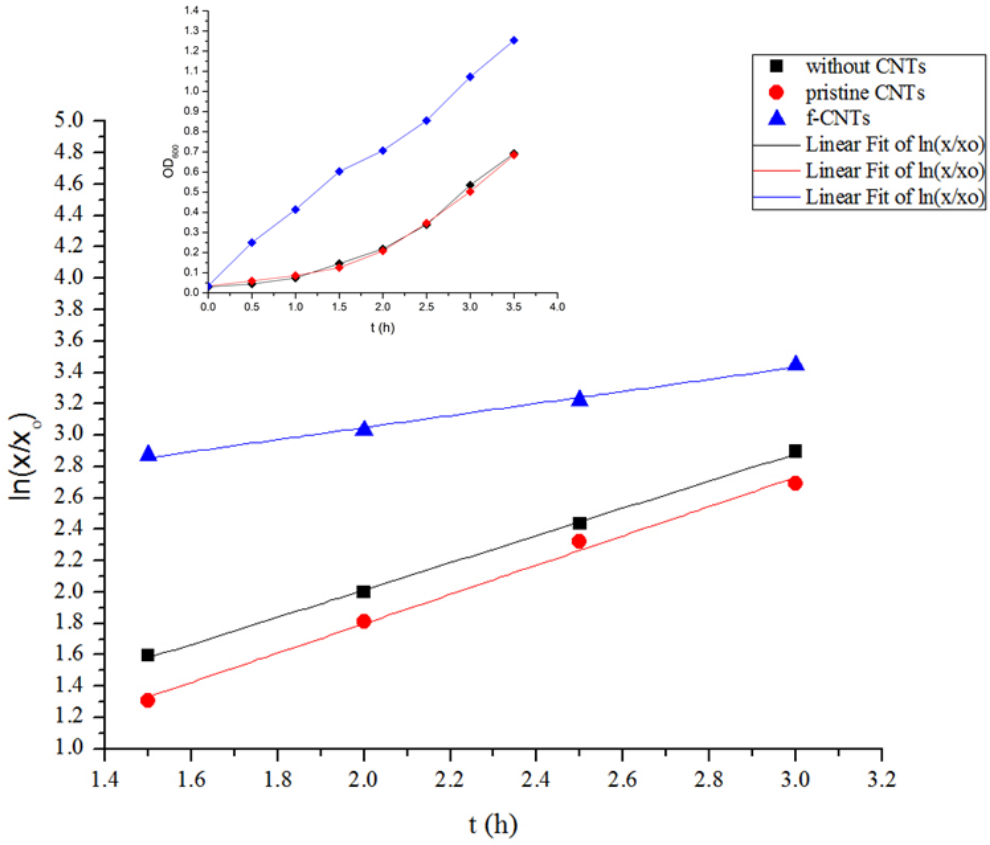


**Figure 9.** OD growth curves of E. Coli that were treated with pristine and functionalized CNTs, synthesized via CVD method.

**Table 2.** The evaluated growth rates and doubling time for *E. Coli* incubated with CNTs.

| Material | Growth rate ($\mu_{max}$) | Doubling time ($t_d$), h |
|---|---|---|
| Control (without CNTs) | 0.868 | 0.71 |
| Pristine CNTs | 0.934 | 0.75 |
| Functionalized CNTs | 0.384 | 1.89 |

Moreover, when functionalized CNTs were tested against pristine ones, the first displayed more inhibitory effects compared to the control sample (*E. Coli* without CNTs) for the same concentration, as it is shown in Figure 9. The growth rate of bacteria was 55.76 % lower than the control when incubated with functionalized CNTs and the doubling time was about 3 times longer. The difference between pristine and functionalized CNTs in antimicrobial activity may as well be explained by the fact that the functionalized CNTs exhibit greater dispersion than the pristine ones.

Finally, the effect of directly depositing CNTs onto petri dish with the addition of *E. coli* cells was investigated (Figure 10). It was observed that *E. coli* preferred to develop colonies in areas of the petri dish where there were not CNTs, indicating possible inhibition in colonies development. Moreover, the number of colonies was reduced 27 % in case of pristine CNTs deposition and 73 % in case of functionalized CNTs.

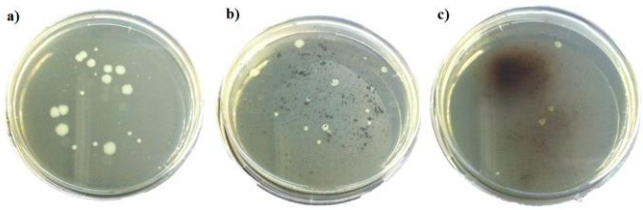


**Figure 10.** Directly depositing CNTs onto petri dish with the addition of E. coli cells.

## CONCLUSIONS

MWCNTs, with 20-40 nm diameter, were synthesized *via* CVD method and fully characterized by our laboratory. Thereafter their antibacterial activity was tested and compared to the antimicrobial activity of commercial MWCNTs.

The lower initial viable cell number in a sample, the longer growth time it needs to reach the exponential phase. Therefore, the appearance of the exponential growth time can be used as an indicator of initial viable cell number in the sample and thus to evaluate the antimicrobial activity of CNTs to bacterial cells. After incubation with various CNTs concentrations, it was observed that the growth rate of bacterial cells in a culture medium decreased with increasing the amount of CNTs until the concentration of 0.04 % w/v where then decreased with a much smaller rate. Additionally, the 0.02 % w/v seems to be the threshold concentration, since higher concentrations indicated drastic reduction of the growth rate. Thereafter, the effect of functionalization due to oxidation of the surface of the CNTs was tested for the threshold concentration. The functionalized CNTs displayed more inhibitory effects than the pristine CNTs, compared to the control sample (*E. Coli* without CNTs) for the same concentration. Finally, the effect of directly depositing CNTs onto petri dish with the addition of *E. coli* cells was investigated and observed possible inhibition in colonies development.

Concisely, the commercial CNTs have used in this study, seem to have antibacterial properties as well as the functionalized MWCNTs synthesized by our laboratory. The functionalized CNTs, for the same concentration, appears to affect more the bacteria's growth rate. The pristine CNTs did not show any inhibitory effect on the bacteria, probably due to the great state of agglomeration it presented.

## ACKNOWLEDGMENTS

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