8-2018

Effects of Carbon Nanotubes on Microbial Growth and Horizontal Gene Transfer

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EFFECTS OF CARBON NANOTUBES ON MICROBIAL GROWTH AND HORIZONTAL GENE TRANSFER

A Thesis

Submitted to the Faculty

of

Purdue University

by

Ran Chen

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science in Civil Engineering

August 2018

Purdue University

West Lafayette, Indiana

THE PURDUE UNIVERSITY GRADUATE SCHOOL

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ACKNOWLEDGEMENTS

I am grateful to my parents who have been supporting my study from the start till now and support me unconditionally whenever I'm lost. And thanks to my grandmother who gave me spiritual encouragement all the time. Meanwhile, I am grateful to my uncles, aunts and cousins, who all encouraged me to study abroad.

I would like to show my appreciation to my advisor Dr. Zhi Zhou for massive support and advice through every part of the research. And can always give constructive opinions to solve the problems. I thank Dr. Chad Jafvert for giving me a lot of advice to guide my graduate program at Purdue and teaching me practical skills on the course. And thank Dr. Loring Nies for giving me a course that full of fun and share the instruments with me for my study.

I thank Zhe and Mian who had taught me almost all experimental skills in these two years and helped me to optimize experimental conditions. Thank my friends Boting, Xianzhen, Yifan and Zhenglyu who cheered me all the time.

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LIST OF ABBREVIATIONS

ARG	Antibiotic Resistant Gene
ARB	Antibiotic Resistant Bacteria
CNT	Carbon Nanotube
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
LB	Lysogeny Broth
MIC	Minimum Inhibitory Concentration
MWCNT	Multi-Walled Carbon Nanotubes
PCR	Polymerase Chain Reaction
WWTP	Wastewater Treatment Plant

ABSTRACT

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Degree Received: August 2018
Title: Effects of Carbon Nanotubes on Microbial Growth and Horizontal Gene Transfer
Committee Chair: Zhi, Zhou

Carbon nanotubes (CNTs) have widely studied in various fields since its discovery. The wide research and application of CNTs may result in the occurrence of CNTs in natural environment and their nanotoxicity has been widely recognized. Moreover, a less frequently studied effect of CNTs is the promotion of spread of antibiotic resistance. CNTs may function as a reservoir of antibiotic resistance genes (ARGs) and pose additional health risks associated with the spread of ARGs in natural environment, but no previous studies have been reported on the effects of CNTs on horizontal gene transfer. The objective of this study was to study the effects of CNTs on microbial growth and horizontal gene transfer. Two species of indigenous bacteria (erythromycin-resistant *Escherichia coli* (*E. coli*) and erythromycin-sensitive *Bacillus cereus* (*B. cereus*)) were isolated from Wabash River in West Lafayette, IN and the effects of CNTs and antibiotics on these bacteria were further evaluated. No effects were observed on growth of *E. coli* under the exposure of 500 mg/L CNTs, while growth of *B. cereus* was delayed with 500 mg/L CNTs. The minimum inhibition concentration (MIC) of *B. cereus* to erythromycin was identified

as 0.4 mg/L. When *B. cereus* was cultivated under 0.4 mg/L (1MIC) erythromycin for 24 hours, its resistance to erythromycin increased to 2 mg/L. When *B. cereus* was cultivated under 0.4 mg/L erythromycin and 500 mg/L CNTs for 24 hours, its resistance to erythromycin increased to 10 mg/L. Finally, erythromycin-resistant *E. coli*—which harbors erythromycin resistance methylase (*erm*) gene *erm*80—and erythromycin-sensitive *B. cereus*—which does not harbor gene *erm*80—were co-cultivated with CNTs and erythromycin. The results showed that horizontal gene transfer efficiency was highest under 0.4 mg/L erythromycin and 500 mg/L CNTs. The results suggest that CNTs may create additional selective pressure for the spread of antibiotic resistance genes and their effects of horizontal gene transfer should be further investigated.

CHAPTER 1. INTRODUCTION

1.1 Antibiotic Resistance and Antibiotic Resistance Genes

In 1928, Alexander Fleming discovered penicillin from *Penicillium notatum*, which was the first-time people had a visible understanding of the most powerful drugs to kill or inhibit the growth of bacteria (Andersson and Hughes 2014). In the following decades, antibiotics have been widely used on a large scale and saved millions of lives. For example, the first discovered antibiotic—penicillin—has saved at least 200 million lives since its first medical use in 1942 (The National 2016). Antibiotics have been also used in animal husbandry, crop farming and aquaculture for disease control and prevention and growth promotion (Prescott 2008, Love, Davis et al. 2011, Udo, Aklilu et al. 2011).

However, the widespread use of antibiotics has also resulted in antibiotic resistance. Every year, almost 100,000 – 200,000 tons of antibiotics are manufactured in the world (Andersson and Hughes 2014), but most of antibiotics cannot be absorbed by human or animals and eventually released to the environment. It is estimated that up to 90% of administrated antibiotics are excreted in urine or feces and end up in wastewater treatment plants (WWTPs) (Berkner, Konradi et al. 2014). Different types of antibiotics have been detected in wastewater, surface waters, and soils (Boxall, Blackwell et al. 2002, Riesenfeld,

Goodman et al. 2004, Jiang, Hu et al. 2013). However, the concentration of antibiotics in wastewater and environmental samples are relatively low. A recent study in China detected 29 antibiotics in 12 WWTPs with the total concentration ranging from 64 to 2,245 ng/L (Zhang, Zhao et al. 2017), while the total antibiotic concentrations in a few WWTPs in the US ranged from 0 to 14,200 ng/L (Mohapatra, Huang et al. 2016), from 90 to 3,730 ng/L (Karthikeyan and Meyer 2006), and from 670 to 12,890 ng/L (Batt, Bruce et al. 2006). These concentrations were significantly lower than the typical minimal inhibitory concentrations (MIC) of susceptible wide type strains that are in the mg/L (ppm) to μg/L (ppb) range (Li, Yang et al. 2009, Alam, Aqil et al. 2013, Bengtsson-Palme and Larsson 2016). Similar to the concept of semi-lethal dose in toxicology, MIC was defined as a lowest concentration of drugs that can efficiently kill or inhibit growth to the certain bacterial (McKenzie 2011). Antibiotic concentrations above MICs are required to kill pathogens to cure bacterial infections. Antibiotics occurring at sub-MIC levels in wastewater and environmental samples could not kill bacteria but instead may create selective pressure for the development of antibiotic resistance (Alonso, Sanchez et al. 2001). One previous study showed a strong correlation between antibiotic usage and increased levels of antibiotic resistance (Goossens, Ferech et al. 2005).

The development of antibiotic resistance may be related with the formation of biofilm when bacteria are exposed to antibiotics. Most bacteria cannot maintain their

normal metabolic activities in extreme environments, such as high temperature, strong acid and also included the existence of antibiotics, and some bacteria form spores to survive in a harsh environment and recover in the future (Russell, Hammond et al. 1986). In some situations where bacteria are exposed under low levels of antibiotics, biofilm can be formed as a mechanism for survival (Donlan 2001, Stewart and Costerton 2001, Drenkard and Ausubel 2002). Biofilm is an extracellular exopolysaccharide matrix that contains protein, lipid, and even DNA (Mah and O'toole 2001). Biofilm can prevent bacteria from contacting with antibiotics and works as a physical or chemical diffusion barrier. Although antibiotic resistance developed in biofilm may not change bacterial genotypes, they can still help bacteria survive under the exposure of antibiotics and improve chances for bacterial reproduction (Furuya and Lowy 2006). Antibiotic resistance in biofilm could be hard to treat, but phenotypic antibiotic resistance developed in biofilm cannot be passed into the next generation and therefore will not affect other bacteria and make them resistant.

In addition, the overuse of antibiotics accelerated the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) and increase their potential health risks to humans. ARGs are specific genes that are coded in quasi-nuclear or plasmid in ARB and transfer among different bacteria. Unlike the previously discussed antibiotic resistance mechanisms in biofilm, ARGs can persist in the environment even when the selective pressure of antibiotics disappear and be passed onto the next generation (Levy and Marshall 2004).

1.2 Horizontal Gene Transfer

Unlike the gene transfer from paternal cells to progeny cells, horizontal gene transfer (HGT) is a process that genetic information moves between two or more types of organisms (Furuya and Lowy 2006). A pathogen that is sensitive to antibiotics can gain the corresponding ARGs through HGT and it is an irreversible process. Therefore, the spread of ARGs among pathogenic bacteria and other bacteria is becoming a severe problem to human health. Genetic exchange in HGT occurs in transformation, transduction, or conjugation. Their difference is that the medium of conduction is different. For transformation, donor cells release naked DNA into external environment, and recipient cells gain DNA by endocytosis and combine it in their own DNA. Transformation is a random process and its efficiency is related to DNA abundance and recipient cell concentration. For the transduction, phages assembly in donor cells and gain ARGs. Then phages infect recipient cells and integrate ARGs into chromosomes in recipient cells. This is an efficient way because the phages can easy position and attach to cell surface for transduction. For conjugation, both donor cells and recipient cells contact directly and ARGs in the plasmid in donor cells transfer across to recipient cells with the plasmid. Sometimes transposons are also involved in conjugation (Guo, Yuan et al. 2015). As physical contact between donor cells and recipients is needed for conjugation, any process that helps cells in direct contact can help improve the conjugation efficiency.

1.3 Carbon Nanotube

Since their discovery in the 1990s, Carbon Nanotubes (CNTs) have been widely used in many fields as a new kind of nanomaterial (Iijima 1991). Their small sizes, high mechanical strength, high surface area, and good electrical and thermal conductivity all make them a star in industrial applications (Gupta and Sharma 2002, Terrones 2003, Pop, Mann et al. 2006). In 1997, Dillon used single-walled carbon nanotubes for storage of hydrogen (Dillon, Jones et al. 1997). His results showed that hydrogen can be compressed to a high density and adsorbed inside the pores of CNTs. Such a process can achieve a higher hydrogen uptake rate than that of activated carbon and shows good potential for applications in the H_2-drived machine. CNTs are also good carriers for drugs and other materials, such as catalysts and even genes (Planeix, Coustel et al. 1994, Ahmadi, Ramezani et al. 2017, Li, Li et al. 2017). Since the carbon atom in CNTs is sp^2 bonding with each other, there is one more extra electron for each carbon atom, but all these extra electrons form a big Π-bond which make this area full of electrons and hard to be attacked by other compound.

The most common application of CNTs is to use them as adsorbents, and many studies have been done to study their adsorption capabilities. With the large surface area and excellent chemical stability, CNTs are considered as a first-class adsorbent for the removal of heavy metals and organic pollutants (Agarwal and Singh 2016). CNTs can be modified with some oxidant to achieve a higher adsorption capacity (Li, Wang et al. 2003). Many studies have shown that CNTs can adsorb lead, copper, cadmium and other acute heavy metals (Li, Wang et al. 2002, Li, Ding et al. 2003, Atieh 2011). Almost 90% of chromium

removal efficiency with the pH 6 and 60 min contact time has been achieved (Gupta, Agarwal et al. 2011). Lu and Chiu compared the adsorption capacities of Zinc (II) with single-walled CNTs, multi-walled CNTs and powdered activated carbon (PAC), and the maximum adsorption capacities were 43.66, 32.68, and 13.04 mg/g, respectively (Lu and Chiu 2006). Besides heavy metals, Li et al. applied CNTs for the adsorption of fluoride from wastewater at a pH range from 5.0 to 9.0 and compared with amorphous alumina and the results showed that the adsorption capacity of this complex was 13.5 times higher than that of activated carbon in the same condition (Li, Wang et al. 2001). Additionally, the highly toxic and stable organic pollutants like dioxin, 1,2-dochlorobenzene, perfluoroalkyl and trihalomethanes were effectively removed from water with CNTs (Long and Yang 2001, Peng, Li et al. 2003, Lu, Chung et al. 2005, Merino, Qu et al. 2016).

However, the wide application of CNTs has resulted in wide occurrence of CNTs in surface waters and soils and CNTs could be an important reservoir of adsorbed contaminant it absorbed including ARGs, which increases the potential risk of the spread of ARGs among environmental bacteria (Guo and Zhang 2017).

The effects of CNTs on microorganisms are multifaceted and highly correlated with their concentrations. For instance, 30 μg/mL could be toxic for bone cells while for osteoblast cells can tolerate high doses of CNTs at around 100 μg/mL before they showed cytotoxic effect (Mahmood, Villagarcia et al. 2013, Constanda, Stan et al. 2016). Other studies showed that low concentrations of CNTs can stimulate cell growth and proliferation (Ricci, Leite et al. 2017). CNTs can also affect other physiological activities of microorganisms. Salvador *et al*. observed that methane production rate by pure methanogens culture can increase 17 times with the existence of 5 g/L CNTs (Salvador,

Martins et al. 2017). However, the generation of extracellular vesicles were inhibited during this period. There is still a knowledge gap on how CNTs affect metabolic process and HGT of bacteria, but limited studies have been done on this topic.

1.4 Research Objectives

The objectives of this study were to:

1) evaluate the effects of CNTs on microbial growth of pure and mixed cultures of *E. coli* and *B. cereus* that were isolated from the environment;
2) evaluate the effects of CNTs on HGT.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacterial Strains

Two bacterial strains were isolated from Wabash River, West Lafayette, IN and sequenced and identified with their 16S rRNA genes at the Purdue Genomic Facility.

The pure culture of Gram-negative bacterium *Escherichia coli (E. coli)* was selected as donor bacteria in this study. The isolated strain of *E. coli* contained one type of erythromycin resistant gene (*erm*80) in plasmid pTE80. The plasmid pTE80 was also sequenced at the Purdue Genomic Facility and its identify was confirmed with BLAST analysis in NCBI. Gram-negative bacteria are intrinsically resistant to low levels of antibiotics since their cellular outer membranes are partially impermeable. The minimum inhibition concentrations (MICs) of erythromycin—a macrolide antibiotic—for the strain of *E. coli* was detected as 64 mg/L, which is likely due to the existence of *erm*80.

Additionally, we also isolated a Gram-positive bacterium *Bacillus cereus (B. cereus)* as an erythromycin sensitive recipient strain in this study, whose MIC to erythromycin was identified as 0.4 mg/L.

2.2 Erythromycin

Erythromycin was prepared as a stock solution with a concentration of 1000 mg/L and then diluted to target concentrations with nanopure water. Solid erythromycin was dissolved by pure alcohol (ACROS Organics, Ethyl Alcohol, 200proof, 99.5+%, A.C.S. Reagent). For 20 ml 1000 mg/L erythromycin stock solution, we used the following equation to calculate the required weight:

$$w_{erythromycin} = \frac{20ml * 1000\ mg/L}{850\ ug/mg} = 0.0235g$$

HPLC-PDA (SHIMADZU LC-2030C 3D) was used to monitor erythromycin concentrations. A packed column (Kinetex 2.6u C18 100A, 100 x 4.6 mm) was selected to isolate erythromycin. Mobil phase A for HPLC analysis was pure acetonitrile, and phase B was 10mM ammonium formate (0.6306 g ammonium formate was dissolved into 1 L nanopure water and sonicated for 40 min). Two phases were mixed with a ratio of 50% to 50%. Flow rate was 0.7 mL/min and running time was 4 min for each sample. Erythromycin peak was appeared at 3.25 min, and maximum peak value appeared at 288 nm. Detection limit was at 10mg/L. A standard curve was developed.

2.3 Bacterial Cultivation and Quantification

The pure cultures of *E. coli* and *B. cereus* were grown in Luria-Bertani (LB) medium in a shaking incubator at a shaking rate of 140 rpm under 37 ℃ for 12 hours until they reached 10^8 /mL. Then we diluted bacterial broth to an concentration of 10^6 number/ml and inoculated them into different treatment groups (LB only, LB with erythromycin, LB with CNTs, LB with CNTs and erythromycin).

OD600 (Eppendorf, BioPhotometer) was used to monitor bacterial concentration. We collected 1 mL bacterial sample for each sampling point. LB only solution was used as a blank sample. We diluted the same sample from 1 to 100 times to count bacterial numbers in a cell counting plate. We measured 10 μL sample and counted the number of bacteria in 5 locations and calculated the mean value with the following equation:

$$Bacteria\ concentration = \frac{Mean\ value/(4 * 10^{-6})}{Dilution\ number}$$

2.4 MIC Test

To measure MIC, we tested microbial growth of *B. cereus* under erythromycin concentrations of 0.4, 2, 5, 10, 15, 20 mg/L, which were prepared from the erythromycin stock solution with a concentration of 1000 mg/L. Then *B. cereus* was diluted to 10^6 number/ml and mixed with erythromycin solutions and cultured in a shaking incubator at

140 rpm and 37 ℃ for 12 hours. The lowest concentration that no *B. cereus* can survive in 12 hours at this condition was selected as the MIC in this test.

2.5 Transconjugation Experiment

Pure cultures of *E. coli* and *B. cereus* were grown in Luria-Bertani (LB) medium in a shaking incubator at 140 rpm and 37 ℃ separately for 12 hours until they reached 10^8 number/mL. Then we inoculated *E. coli* and *B. cereus* at the ratio 10^5/mL : 10^6 mL in 120 mL LB broth. The LB broth was supplemented with erythromycin concentrations of 0 mg/L (0 MIC of recipient strain), 0.04 mg/L (0.1 MIC), 0.4 mg/L (1 MIC), and 0.8mg/L (2 MIC). These treatment groups were cultured in a shaking incubator at 140 rpm and 37 ℃.

To identify recipient cells that gained erythromycin resistance, we periodically collected mixed bacterial solution for plate counting. Solution was serial diluted in 1X PBS buffer and spread with 100 μL for each LB-selective agar that contained 100000 IU/L polymyxin B and 0.8 mg/L erythromycin. Polymyxin B was used to inhibit growth of *E. coli* in mixed culture and high concentration of erythromycin was used to prevent the growth of erythromycin-sensitive *B. cereus*. These plates were incubated at 37 ℃ overnight and then the number of colonies in each plate was counted.

2.6 DNA Extraction and PCR

Every colony or enriched bacterial liquid was collected into an autoclaved 1.5 mL centrifuge tube and add 50 μL 1x TE buffer. Then the tube was heated into 105 ℃ for 5 minutes and centrifuged for 2 minutes at 3200 rpm to separate the solid waste. DNA samples were stored in -20 ℃ refrigerator.

A set of primers was designed to quantify *erm*80 gene and their sequences are listed as follows:

Forward primer :5'-ACTTACCCGCCATACCACAG-3'

Reverse primer: 5'-AGGGAGTTGTTTCCTCCCGT-3'

The amplicon size is 223 bp and melting temperature Tm is 60℃.

PCR amplification of DNA from individual colonies was conducted in a 25 μL reaction system. For a 15 reactions group, we added 88 μL GoTaq Buffer (Promega, Madison, WI), 279.4 μL sterile nanopure water, 8.8 μL *erm*80 f-primers and 8.8 μL *erm*80 r-primers, 8.8 μL dNTP, and 2.2 μL DNA polymerase into a 1.5 mL autoclaved centrifuge tube and mixed well. Then the solution was separated to each PCR tube with a volume of 23.5 μL. Then 1.5 μL DNA template was added in each PCR tube.

All reactions were performed in a CFX96 Real-time PCR detection system (Bio-rad, Hercules, CA) with the following thermal conditions: an initial denaturation at 95 ºC for 3 min, followed by 40 cycles of 30 sec at 95 ºC, 30 seconds of annealing at 60 ºC, and 30 seconds at 72 ºC.

2.7 Gel electrophoresis

Gel electrophoresis was used to separate the target DNA bands. Gel was prepared with 1% agarose gel and 0.01% SYBR green (SYBR Safe DNA gel stain) and dissolved in microwave oven for 1 minute. DNA Ladder (0'GeneRuler 1kb Plus DNA Ladder) was loaded for the first lane. The gel electrophoresis was conducted under 140V for 27 min. Finally, Gel images were for captured with an integrated digital camera.

2.8 Carbon Nanotube

C-grade Multiwalled CNT (NTL-12111) was used in the experiments. CNTs were added into nanopore water and Tween80 and shaked at 140 rpm for 6-8 hours and sonicated in for 30 min. Then the solution was filtered and rinsed by nanopore water and dried under room temperature.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Adsorption of Erythromycin by CNTs

Erythromycin was dissoved by pure alcohol (99.5%) to make a 1000 mg/L stock solution and diluted into 0, 10, 20, 40, 60, 80 and 100 mg/L with nanopure water. The detection limitation of HPLC-PDA was around 10 mg/L, so we used the value of 0, 20, 40, 60, 80 and 100 mg/L to develop a standarf curve (Figure 1). The regression coefficient (R^2) is 0.9931.

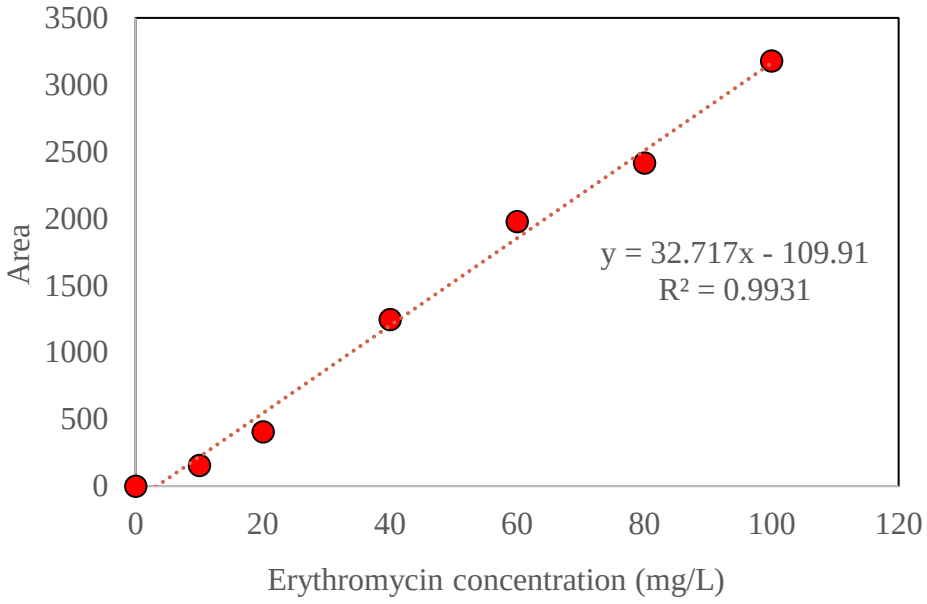

Figure 1. Erythromycin standard curve

Erythromycin with an initial concentration of 100 mg/L was added into 500 mg/L CNTs to evalute whether erythromycin was absorbed by CNTs and how effective concentrion of erythromycin changed over time. The result is shown in Figure 2. After one hour, the effective concentrion dropped to 82.16 mg/L form 100.74 mg/L and the concentration was still 82.20 ±1.22 mg/L in 24 hour, suggesting that the najory of erythromycin was still available in the liquid phase.

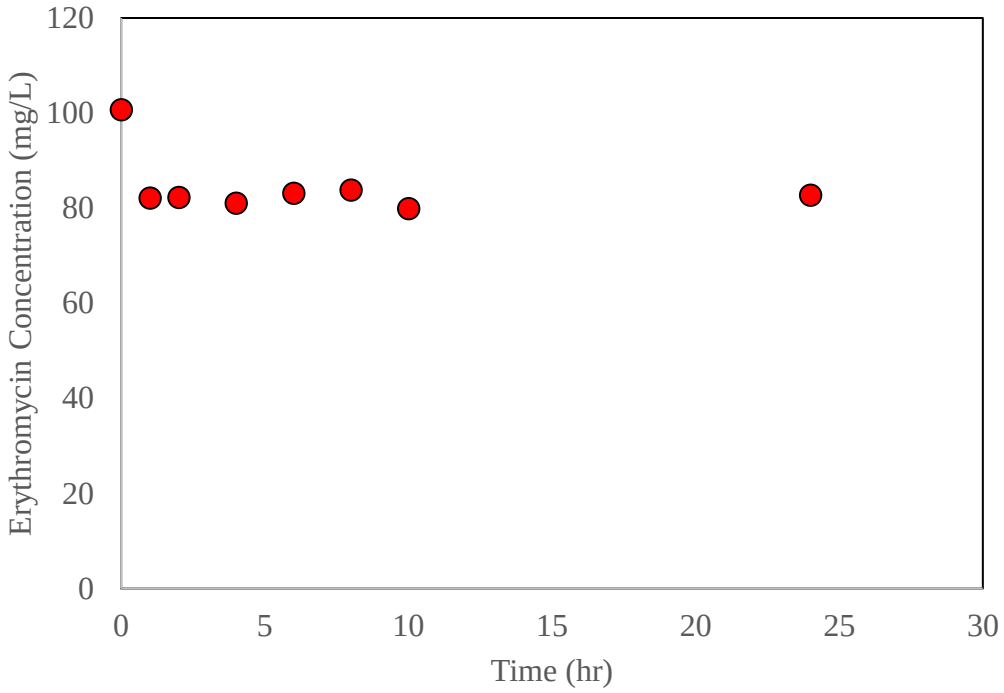

Figure 2. Erythromycin absorption by CNTs
(100 mg/L erythromycin initial concentration and 500 mg/L CNTs)

In Figure 3, the adsoprtion capability of CNTs was tested. When the CNT concentration was set at 500 mg/L, antibiotic absorption was raised for 17.94 to 21.52 mg/L while initial concentration dropped from 100 to 60 mg/L. Meanwhile, when CNT concentration was set as 50 mg/L, antibiotic absorption dropped to between 0.61 to 7.15 mg/L. This means that the absorption ability of CNTs were not linear. Under the same concnetration of CNTs, the absorbed value of erythromycin by CNTs decreased when the initial concentrition was higher.

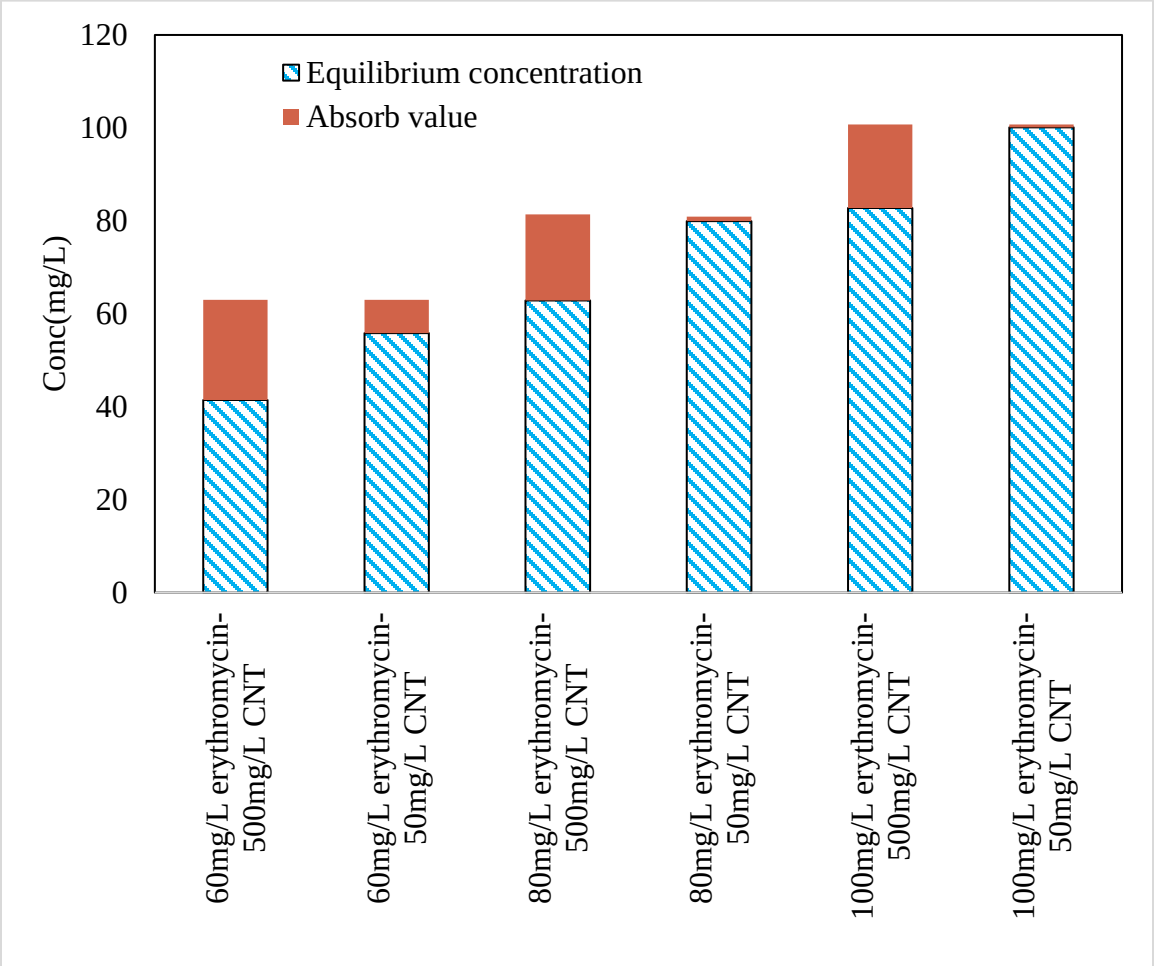

Figure 3. Adsorption ability of CNTs in different erythromycin concentrations.

3.2 Effects of CNTs on microbial growth of *B. cereus* and MIC test

The effects of CNTs on bacterial growth were evaluated. Four different groups were set to evaluate the effect of CNTs and erythromycin on recipient (*B. cereus*) as following:

I): Recipient

II): Recipient + 0.4 mg/L erythromycin

III): Recipient + 500 mg/L CNT

IV): Recipient + 0.4 mg/L erythromycin + 500 mg/L CNT

Figure 4 shows the growth curve of *B. cereus*. Figure 4A shows the growth curve of *B. cereus* measured with OD600 values. The logarithmic growth period started at 2-hour once *B. cereus* was inoculated and lasted for about 3 hours. When 500 mg/L CNTs was added (Curve III), the growth of *B. cereus* was slightly delayed, and the lag time was 1 hour. However, when cultured under 1 MIC (0.4 mg/L) erythromycin, the growth of *B. cereus* was almost inhibited from the beginning (Curve II & IV). But after 7 hours, group *B. cereus* with 0.4 mg/L erythromycin started growth again and entered logarithmic growth period. Interestingly, group IV, which had an additional 500 mg/L CNTs as compared to group II, also entered logarithmic growth period 1 hour later. The growth curve based on plate counting (Figure 4B) showed similar trend of the result from OD600 test.

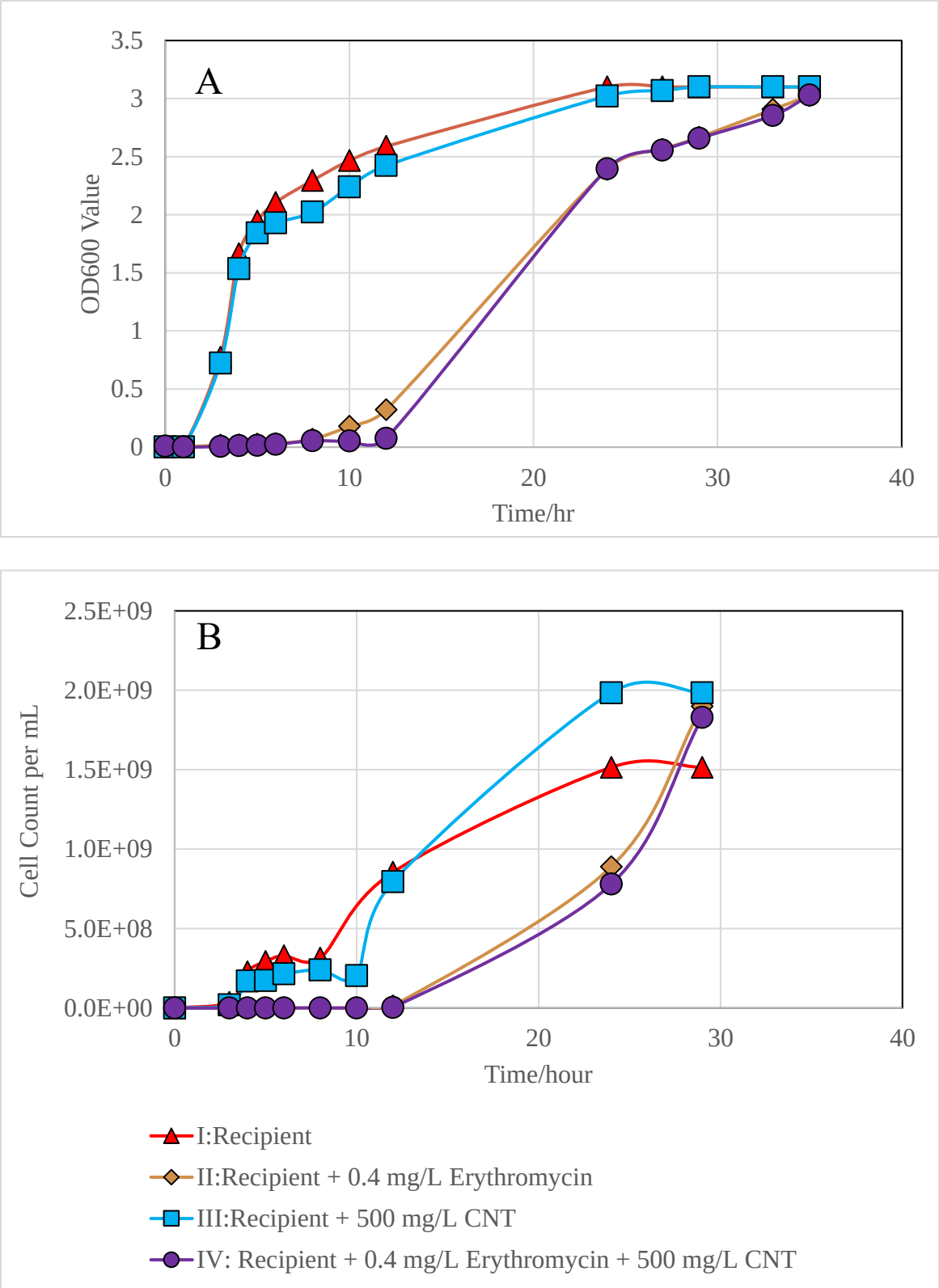

Figure 4. Growth curves of *B. cereus*

As some bacteria can gain antibiotic resistance by multiple routes (Donlan 2001, Stewart and Costerton 2001), MIC test was conducted in different growth periods. Four groups of bacterial samples in different period were diluted to 10^6 CFU/mL and mixed with erythromycin solution with a volume ratio of 1:1 and cultivated in a shaking incubator at 140 rpm and 37 ℃ for 12 hours.

Figure 5 shows bacterial growth after 8-hour incubation. The group with *B. cereus* only and groups with *B. cereus* and 500 mg/L CNTs or 0.4 mg/L erythromycin all became turbid under 0.4 mg/L erythromycin. However, the group of *B. cereus* with both 500 mg/L CNTs and 0.4 mg/L erythromycin were turbid at 5 mg/L, suggesting that *B. cereus* under the pressure by both antibiotic and CNTs can become resistant to 5 mg/L erythromycin in 8-hour. Table 1 shows the results of the MIC test.

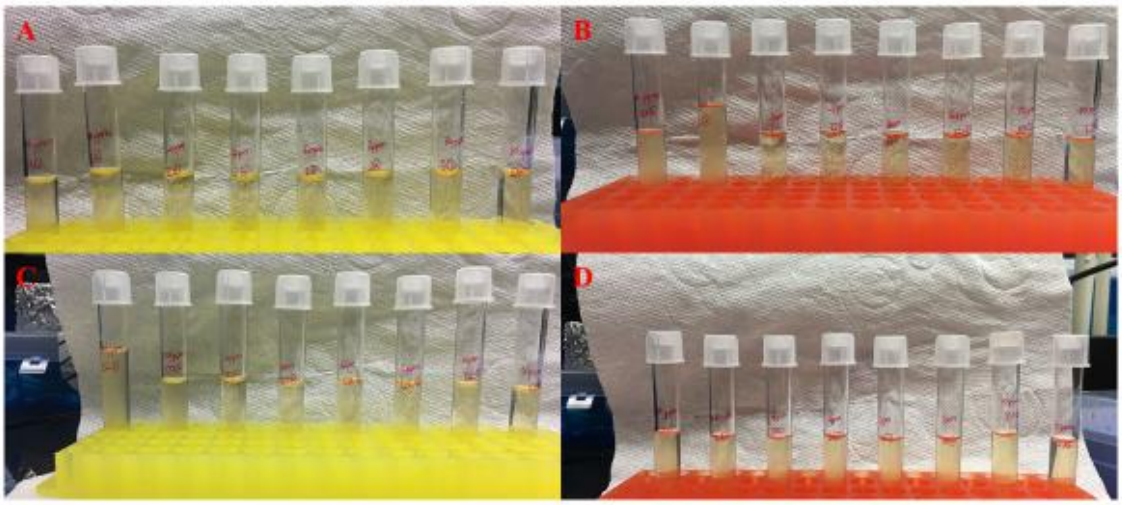

Figure 5. MIC Test in 8-hour
(A: Group I in 8-hour; B: Group II in 8-hour; C: Group III in 8-hour; D: Group IV in 8-hour)

Table 1. Result of MIC test in 8-hour

Concentration		0.4 mg/L		2 mg/L		5 mg/L		10 mg/L	
8hr	I	+	+	-	-	-	-	-	-
	II	+	+	-	-	-	-	-	-
	III	+	+	-	-	-	-	-	-
	IV	+	+	+	+	+	+	-	-

'+' means turbid liquid and '-' means clear liquid

Similar to the 8-hour MIC test, the group with *B. cereus* only and group with *B. cereus* with 500 mg/L CNTs were turbid at 0.4 mg/L. For group of *B. cereus* under 0.4 mg/L erythromycin, the resistance increased up to 2 mg/L.

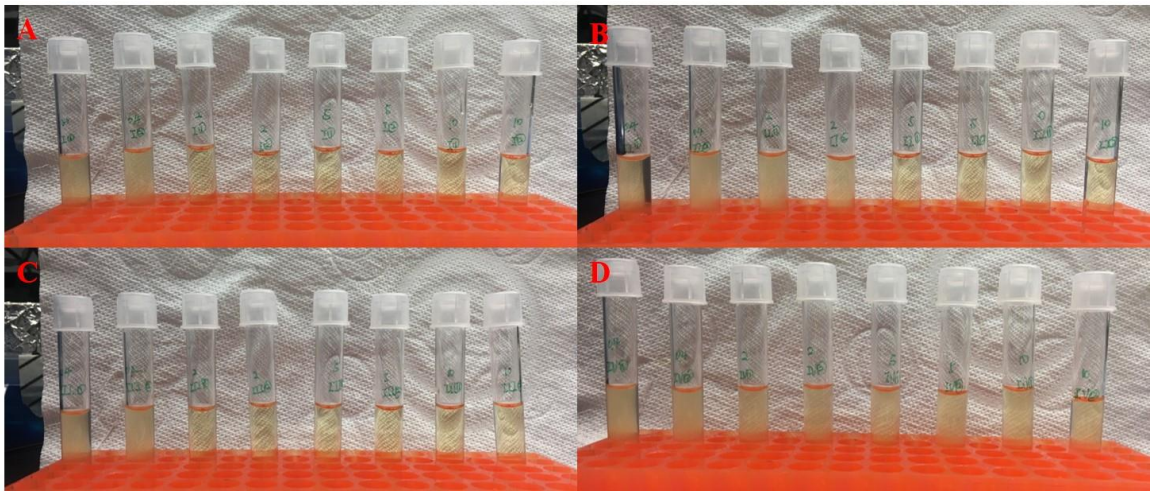

Figure 6. MIC Test in 24-hour

(A: Group I in 24-hour; B: Group II in 24-hour; C: Group III in 24-hour; D: Group IV in 24-hour)

Unexpectedly, Group IV gained resistance that was higher than 10 mg/L. To determine the upper limit, we extended detection range for Group IV up to 20 mg/L. As shown in Figure 7, the resistance was between 10 mg/L and 15 mg/L. Table 2 shows the results of MIC test in 24-hour.

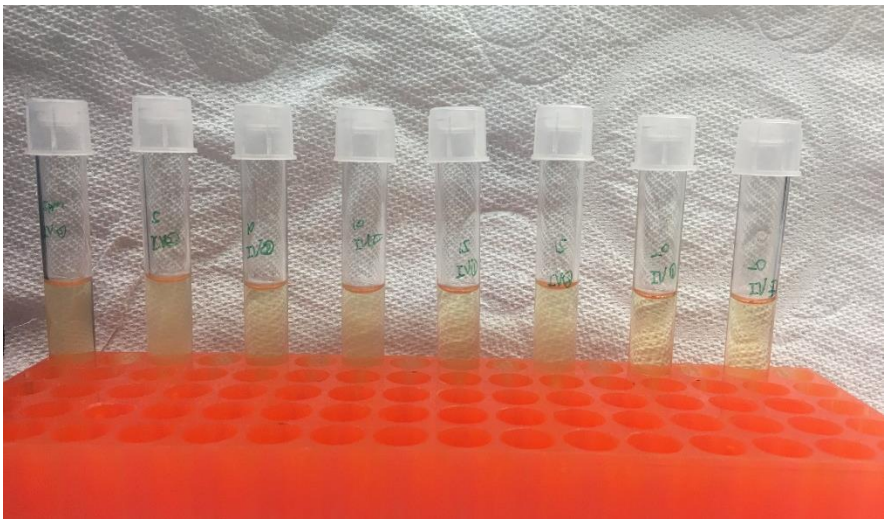

Figure 7. Group IV in 24-hour with MIC concentration from 5 to 20 mg/L

Table 2. Result of MIC test in 24-hour

Concentration		0.4 mg/L		2 mg/L		5 mg/L		10 mg/L		15 mg/L		20 mg/L	
24hr	I	+	+	-	-	-	-	-	-				
	II	+	+	+	+	-	-	-	-				
	III	+	+	-	-	-	-	-	-				
	IV	+	+	+	+	+	+	+	+	-	-	-	-

To evaluate if resistant bacteria contains *erm*80 gene, DNA samples were further tested with a gel electrophoresis test. The details of samples in each lane are shown in Table 3. The result of gel electrophoresis is shown in Figure 8.

Table 3. Gel electrophoresis in MIC test

Lane	Sample
1	Ladder
2	Positive Control (template of *erm*80 gene from *E. coli*)
3	Negative Control (nanopure water)
4	DNA from Group IV: Recipient + 0.4 mg/L erythromycin + 500 mg/L CNT in 24-hour and grow in 10 mg/L erythromycin
5	DNA from Group IV: Recipient + 0.4 mg/L erythromycin + 500 mg/L CNT in 24-hour and grow in 5 mg/L erythromycin
6	DNA from Group IV: Recipient + 0.4 mg/L erythromycin + 500 mg/L CNT in 8-hour and grow in 10 mg/L erythromycin
7	DNA from Group III: Recipient + 500 mg/L CNT in 24-hour and grow in 0.4 mg/L erythromycin – replicate 1
8	DNA from Group III: Recipient + 500 mg/L CNT in 24-hour and grow in 0.4 mg/L erythromycin - replicate 2
9	DNA from Group II: Recipient + 0.4 mg/L erythromycin in 24-hour and grow in 2 mg/L erythromycin – replicate 1
10	DNA from Group II: Recipient + 0.4 mg/L erythromycin in 24-hour and grow in 2 mg/L erythromycin – replicate 2
11	DNA from Group I: Recipient only in 24-hour and grow in 0.4 mg/L erythromycin – replicate 1
12	DNA from Group I: Recipient only in 24-hour and grow in 0.4 mg/L erythromycin – replicate 2
13	Positive Control (template of *erm*80 gene from *E. coli*)
14	Negative Control (nanopure water)

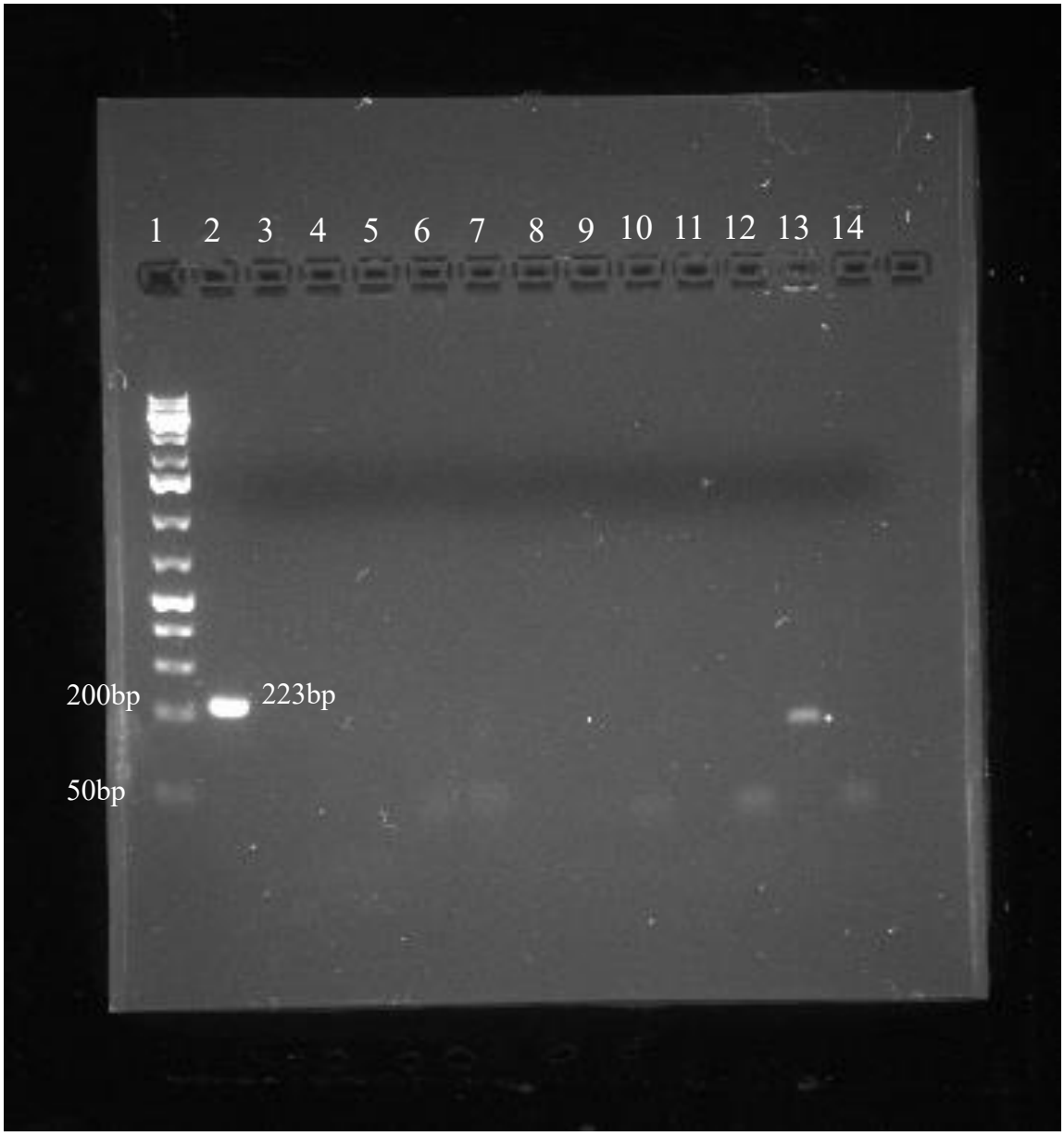

Figure 8. Result of Gel electrophoresis of MIC test

No pollution was detected during PCR and gel electrophoresis and no *erm80* gene was detected in all samples. These results indicated that the observed changes of MIC were not due to *erm*80 gene.

3.3 Effects of CNTs on *E. coli* growth

OD600 test was used to monitor *E. coli* growth. Two conditions were tested as follows:

D: Donor (10^6 cell/mL *E. coli*)

D+C: Donor (10^6 cell/mL *E. coli*) + CNTs (500 mg/L)

Both groups entered logarithmic growth period at 2-hour and it took about 9 hours to reach maximum bacterial concentration. During the logarithmic growth period, the OD600 value of group that contained both *E. coli* and CNTs was 4.7% ± 0.8% higher than the group with only *E. coli*. The results showed that 500 mg/L CNTs slightly promoted *E. coli* growth, but the effect was not significant.

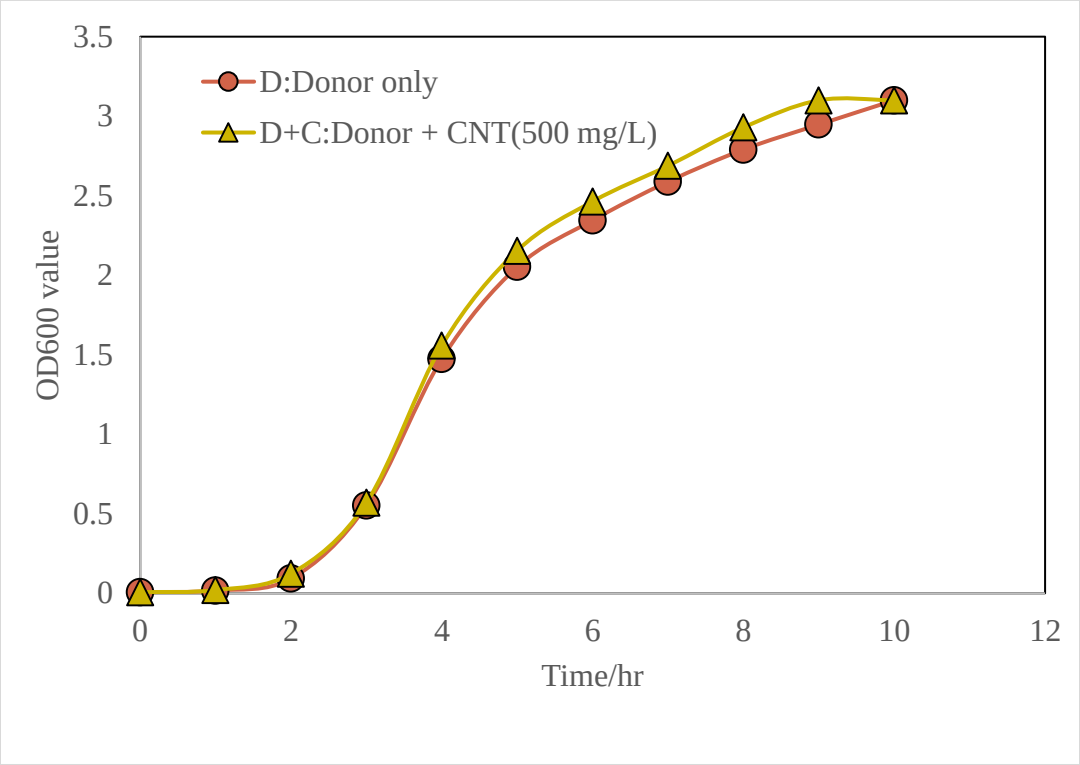

Figure 9. Growth curve of *E. coli*

3.4 Effects of CNTs on HGT

E. coli containing plasmid pTE80 was selected as donor cell in this study and erythromycin-sensitive *B. cereus* was selected as recipient cell. Growth of mixed culture under different erythromycin concentrations and CNTs was evaluated and the results are shown in Figure 10.

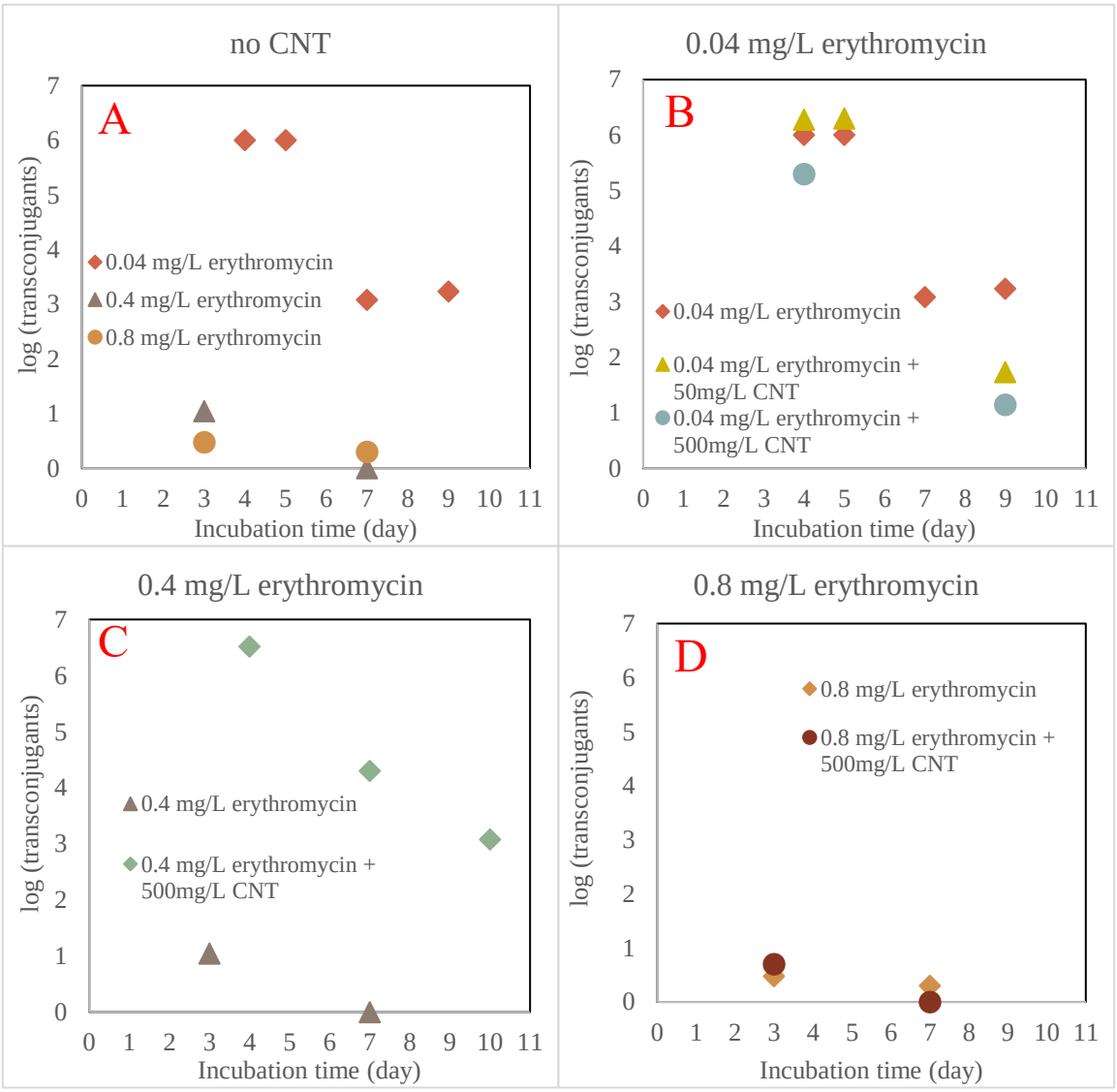

Figure 10. Result of mixed culture experiment

As shown in Figure 10A, when there was no CNTs in the medium, more transconjugants were observed under 0.04 mg/L erythromycin. As shown in Figure 10B, when 500 mg/L CNTs incubated together with 0.04 mg/L erythromycin in the mixed culture, the number of transconjugants decreased significantly. Situation of 50 mg/L CNTs with 0.04 mg/L erythromycin in mixed culture was more complex. In first several days, the number of transconjugants in mixed culture of 50 mg/L CNTs with 0.04 mg/L erythromycin had no significant difference with group that had 0.04 mg/L erythromycin only. But after a few days, the number of transconjugants with 50 mg/L CNTs decreased quickly. In Figure 10C, existence of 500 mg/L CNTs significantly increased the number of transconjugants under 0.4 mg/L erythromycin. As shown in Figure 10D, when erythromycin concentration was much higher than 0.4 mg/L, almost no transconjugants were observed and the effects of CNTs were insignificant.

In Figure 11, we used gel electrophoresis to test the existence of *erm*80 gene from the transconjugants in the mixed culture experiments. The result showed that transconjugants contained *erm*80 gene and confirmed the successful transfer of *erm*80 gene from *E. coli* to *B. cereus*.

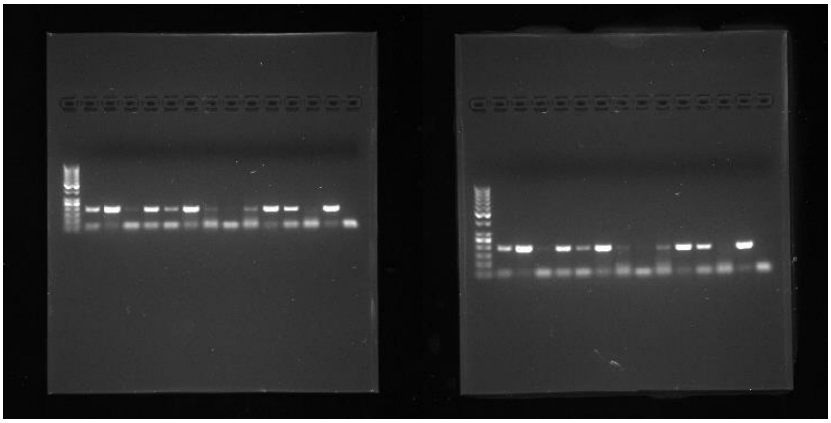

Figure 11 Gel electrophoresis result of mix culture

3.5 Mechanisms of the Effects of CNTs

In separate cultures, 500 mg/L CNTs had no significant effect on the growth of *E. coli*, but existence of 500 mg/L CNTs slightly delayed growth of *B. cereus.* The results that *B. cereus* was more sensitive to CNTs were consistent with previous studies that 5 g/L CNTs promoted the growth of methanogens (Salvador, Martins et al. 2017).

Under the exposure of both 0.4 mg/L erythromycin and 500 mg/L CNTs, *B. cereus* increased its resistance up to 10 mg/L, but no such effect was observed with *erm*80 gene. The most possible mechanism for the observed effects on *B. cereus* was the protection under CNTs and EPS, or even with other dead bacteria as previously described (Donlan 2001, Stewart and Costerton 2001). Such a phenotypic resistance change may not cause long-term health risk.

Existence of CNTs enhanced HGT efficiency at high erythromycin concentration (0.4 mg/L) while CNTs could slightly inhibit horizontal gene transfer at low erythromycin concentration (0.04 mg/L). And 0.4 mg/L erythromycin with 500 mg/L CNTs was the best group for horizontal gene transfer in the given groups. In mixed culture period, donor bacteria, recipient bacteria and CNTs composed a complex system (Figure 12). The agglomerated CNTs could be an idea support for both *E. coli* and *B. cereus,* which provided a good place for material exchange among all bacteria. Meanwhile, CNTs can work as a bridge or tunnel that could connect two individual bacteria. CNTs can absorb erythromycin from water solution and make the small and complex system under a high selective pressure of antibiotic, but on the other hand, the existence of CNTs can release the antibiotic selective pressure of outside medium. The damage of CNT to these two bacteria

and plasmid is still unknow. The contradictory effects of CNTs on HGT need to be further investigated.

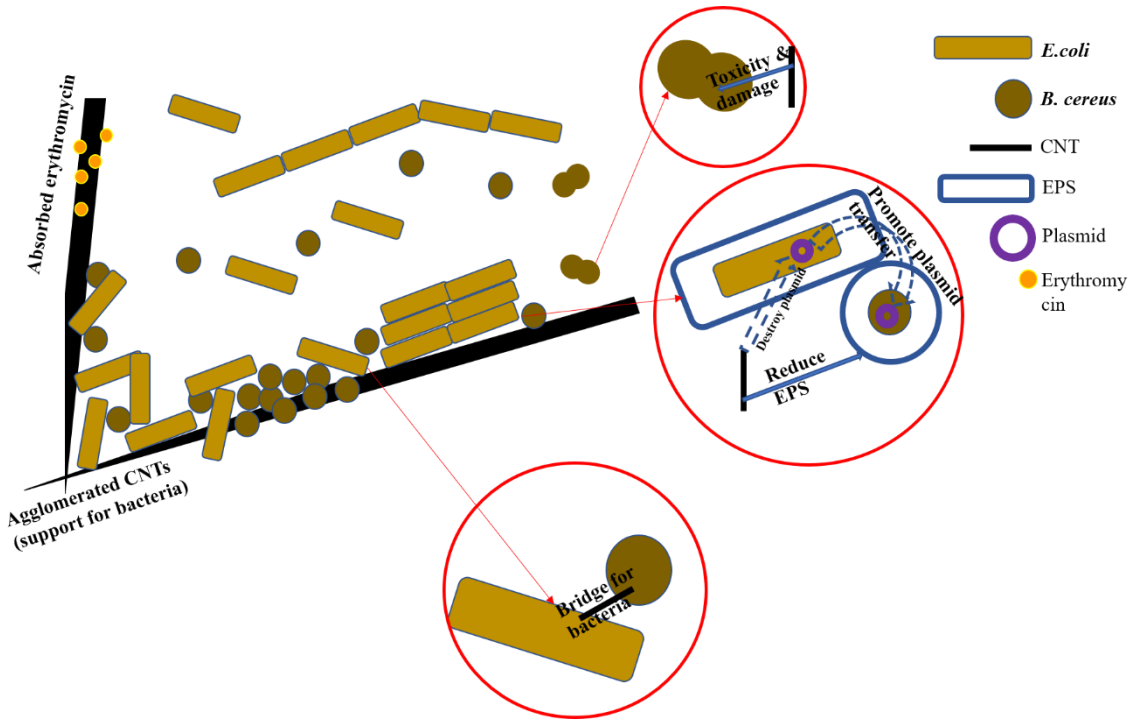

Figure 12. Possible mechanisms of HGT in mixed culture

CHAPTER 4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusion

The effects of CNTs on microbial growth and horizontal gene transfer were evaluated in this study. Two species of indigenous bacteria (erythromycin-resistant *Escherichia coli* (*E. coli*) and erythromycin-sensitive *Bacillus cereus* (*B. cereus*)) were isolated from Wabash River in West Lafayette, IN and the effects of CNTs and antibiotics on these bacteria were further evaluated. No effects were observed on growth of *E. coli* under the exposure of 500 mg/L CNTs, while growth of *B. cereus* was delayed with 500 mg/L CNTs. The minimum inhibition concentration (MIC) of *B. cereus* to erythromycin was identified as 0.4 mg/L. When *B. cereus* was cultivated under 0.4 mg/L (1MIC) erythromycin for 24 hours, its resistance to erythromycin increased to 2 mg/L. When *B. cereus* was cultivated under 0.4 mg/L erythromycin and 500 mg/L CNTs for 24 hours, its resistance to erythromycin increased to 10 mg/L. Finally, erythromycin-resistant *E. coli*—which harbors erythromycin resistance methylase (*erm*) gene *erm80*—and erythromycin-sensitive *B. cereus*—which does not harbor gene *erm80*—were co-cultivated with CNTs and erythromycin and the results showed that horizontal gene transfer efficiency was highest under 0.4 mg/L erythromycin and 500 mg/L CNTs. The results suggest that CNTs

may create additional selective pressure for the spread of antibiotic resistance genes and their effects of horizontal gene transfer should be further investigated.

4.2 Future Work

It is important to monitor the change of erythromycin concentration during the incubation period, and further analysis of erythromycin concentrations with a better detection limit is needed.

Additional experiments with different combinations of sub-MIC antibiotics and CNT concentrations will help reveal how trace levels of antibiotics and CNTs may affect microbial growth and horizontal gene transfer.

To assess potential public health of CNTs, the resistance mechanism of *erm*80 gene needs to be further evaluated. Gene expression of *erm*80 can be correlated with CNT effects and used to study the mechanism of antibiotic resistance in biofilm. It is also important to study if resistance gens can be passed on to the next generation or if transferred gene can be duplicated in non-extreme environment without the selective pressure of antibiotics.

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