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EC개념을 사용하여 환경 표본안에 항생제 저항 군집에 관한 연구

김 성 표*+ / 김 이 형**

The investigation of antibiotic resistance community in environment samples using EC (effective concentration) concept

Sungpyo Kim*+ / Leehyung Kim**

요약 : 본 연구의 전체적인 목적은 공학 분야 사람들이 쉽게 환경 미생물군집내의 항생제 내성균의 거동을 조사할 수 있는 방법론을 개발하는 것이다. 이 목적을 위해서, 공학에 널리 사용되는 배양 기법을 이용한 유효농도(EC)기법을 사용될 수 있는지를 본 연구에서 알아보았다. 활성슬러지내 중에 테트라싸이클린이 주입되어(존재)하여 항생제 내성균이 자라기에 좋은 조건이 되었을 때, 테트라싸이클린에 대한 50% 및 90% 유효값 (EC_{50}, EC_{90})은 대조군(테트라싸이클린 항생제가 포함되지 않은 것)과 비교하였을 때, 통계적으로 증가하였다. 특히 미생물 성장률과 유기물 농도가 높게 유지 돼서 운전된 SBR 반응조에서 그 경향이 뚜렷하였다. 그러므로, 이러한 결과는 환경미생물내에서의 지속적인 유효농도(EC)의 관찰은 환경 미생물시료에서의 테트라싸이클린 내성 군집거동의 변화를 추적하는데 실질적인 기법으로 사용될 수 있을 것으로 사료된다.

핵심용어 : 항생제 내성, 미생물 군집, 유효농도, 테트라싸이클린

Abstract : The overall objective of this study is to develop the engineering-friendly-methodology which can investigate the fate of antibiotic resistance in environment microbial community. For this purpose, effective concentration (EC) concept was adopted with cultural based method which is currently used in engineering practice. When a tetracyline antibiotic was present as selective pressure agent among microbial community, activated sludge, the EC_{50} and/or EC_{90} of tetracycline in microbial community were statistically increased compared to control, especially higher growth rate and organic loading conditions of SBRs. Therefore, these results strongly suggested that the continuous monitoring of EC in microbial community can be used for characterizing the fate of tetracycline resistance community in environmental samples.

Keywords : *Antibiotic Resistance, Microbial cimmunity, Effective Concentration, Tetracycline*

1. Introduction

Over the past several decades, a rapid emergence of antibiotic resistance in pathogens has been observed and the number of microorganisms resistant to any available antibiotic has increased (Pillai et al., 2001). Many public health officers believe that antibiotic resistant pathogens are becoming a major public health threat (Hirsch et al., 1999; Pillai et al., 2001; Guardabassi and Dalsgaard, 2002). The continuous usage of antibiotics in modern society might increase the antibiotic resistance in environment microbial community and might contribute the increase of antibiotic resistance increase in pathogen (Levy, 2002).

+ Corresponding author : ub1905ub@korea.ac.kr
* 정회원 · 고려대학교 환경시스템 공학과 · 교수, E-mail : ub1905ub@korea.ac.kr
** 정회원 · 공주대학교 건설환경공학부 · 교수, E-mail : leehyung@kongju.ac.kr

In Korea, about 1,500 tons of antibiotics are used in livestock industry and the use of antibiotics is about 0.911 kg per meat production which is more than 6 times that of USA (PSPD, 2005). Therefore, it is required to develop the protocols which can assess the fate of antibiotic resistant microbial community in environment as regular basis. Recently introduced molecular biotechnology such as DGGE (Aminov et al., 2001) can be used for analyzing antibiotic resistance community. However, these genotyping techniques might not be practical since the technique is quite sophisticated. The overall objective of this study is to develop the engineering-friendly-methodology which can investigate the fate of antibiotic resistance in environment microbial community. For this purpose, effective concentration (EC) concept was adopted with cultural based method which is currently used in engineering practice.

2. Materials and Methods

Antibiotic. Tetracycline was selected as a model antibiotic in this study since it induces resistance to itself as well as other antibiotics in a broad range of bacterial species (Steinman et al., 2003). Further, tetracycline is heavily used as an animal growth promoter (Roberts, 1996) and is one of the most frequently found antibiotics in the environment (Kolpin et al., 2002).

Bioreactors. For testifying the developed method, two Sequencing Batch Reactors (SBRs labeled A and B type) which were initially inoculated with same activated sludge were operated. These two reactors were operated with same conditions but tetracycline augmentation in influent wastewater. One of SBRs received the same wastewater augmented with 250 μg/L tetracycline (labeled A type). The augmented tetracycline concentration was selected in a

Table 1. The characteristics of operated SBR A and SBR B

Reactor	Phase	pH	MLSS (mg/L)	D.O. (mg/L)	SRT (days)	OLR (kg TCOD/m^3-d)	Effluent	
							TSS (mg/L)	SCOD (mg/L)
SBRA	1	7.1 ± 0.3*	699 ± 97	4.6 ± 0.6	10	0.18	12.4 ± 7.5	25 ± 13
	2	6.9 ± 0.3	1892 ± 233	4.0 ± 0.3	10	0.77	14.5 ± 6.2	32 ± 6
	3	7.2 ± 0.2	1001 ± 173	5.0 ± 0.2	3	0.82	14.2 ± 4.5	36 ± 8
SBR B	1	7.1 ± 0.3	669 ± 119	4.6 ± 0.3	10	0.18	9.5 ± 4.5	26 ± 12
	2	6.9 ± 0.3	1994 ± 215	4.2 ± 0.3	10	0.77	13.2 ± 5.1	35 ± 10
	3	7.2 ± 0.2	1003 ± 207	5.0 ± 0.2	3	0.82	22.4 ± 7.9	46 ± 8

*Average ± Standard deviation

previous study which could induce tetracycline resistance (Kim, 2007). Therefore, we are expecting that One of SBRs served as a control with no antibiotic added (B types). It received domestic wastewater that contained background concentrations of tetracycline only. During this study, three different operating conditions (Phase 1, 2 and 3) were applied to each bioreactor. Changes in volumetric organic loading (OLR) were achieved by alteration of influent volumetric wastewater flux. Changes in growth rate were achieved by altering the solids retention time (SRT) of the reactors and were independent of organic loading rate. Detail operations of SBRs are described in Table 1 and elsewhere (Kim, 2007).

Culture cultivation. Concentrations of total heterotrophic bacteria and tetracycline resistant bacteria from two SBRs were measured by plating in triplicate on R2A agar containing 0, 0.1, 0.25, 1, 5, and 20μg/mL tetracycline, respectively. Colonies were enumerated after incubation at 28°C for seven days and translated to colony forming units per milliliter (CFU/mL) of original sample. More details of culture cultivations are also described in elsewhere (Kim, 2007).

EC_{50} and EC_{90}. In this study, EC_{50} and EC_{90} represent the concentration of tetracycline in agar that inhibits and/or kills 50% and 90% of the cultivated microorganisms, respectively. EC_{50} and EC_{90} values were calculated from the experimental data to assess changes in resistance of the biomass. For example, an increase in either EC_{50} and EC_{90} values implies that the study population is becoming more resistant to tetracycline. To calculate the EC value, the dose-response curve of the survival ratio as a percent was plotted as a function of tetracycline concentration (figure 1). In calculating the EC values, it was assumed that the survival ratio response as a function of tetracycline concentration follows a log normal distribution. This assumption is supported by previous research (Jumbe et al., 2003). The log normal distribution function provided by the EXCEL 2000 (Microsoft) program was used to calculate EC_{50} and EC_{90} values. To evaluate the hypotheses, EC_{50} and EC_{90} values were compared statistically with Wilcoxson rank sum test.

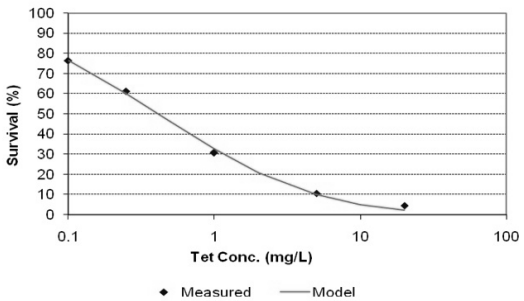

Figure 1. The Dose-Response Curve of the Survival Ratio Change (%).

In addition, the results of EC technique were compared with the percentage of tetracycline resistance, which is more common technique. The percentages of tetracycline resistance were calculated as the colony numbers (CFU/mL) on 5ppm and 20 ppm of tetracycline containing R2A agar normalized by the colony number of total bacteria with no tetracycline concentration. These ratios were statistically compared between experimental conditions using Wilcoxson Rank Sum Test.

3. Results

In Table 2, the average EC_{50} and EC_{90}

concentrations of heterotrophic microorganisms under each operating condition are summarized for both tetracycline fed SBRs (A) and non tetracycline fed SBRs (B). For each experimental condition, differences in EC_{50} and EC_{90} concentration valuated at the 95% confidence limit using Wilcoxson rank sum test. For the comparison purpose, the percentage of tetracycline resistance (%) in each biomass was also calculated (Table 3). Based on the analysis using EC_{50} and EC_{90} values (Table 2), the role of tetracycline as a selective agent for increasing resistance in SBR biomass is different in each phase. In phase 1, For SBRs under the low growth rate (SRT = 10 days) and long HRTs (HRT = 24hrs) applied, tetracycline showed weak positive influence to tetracycline resistance. The EC_{50} concentration in SBR A was not statistically different compared to that of SBR B but The EC_{90} concentration was significantly different between SBR A and B biomass. Similar trends were observed under Phase 2 condition (10 day SRT, 7.4 hr HRT). However, under Phase 3 conditions (3 day SRT, 7.4 hr HRT), the presence of tetracycline did play a significantly positive role as a selective agent for increasing EC_{50} and EC_{90} concentrations of heterotrophic biomass when compared to non tetracycline fed SBRs.

Table 2 EC_{50} and EC_{90} values in SBRA and SBRB biomass

Phase		SBR A (tetracycline augmented, mg/L)	SBR B (control, mg/L)	n (Sample numbers)	Wilcoxson rank sum test
1	EC_{50}	0.18 ± 0.12*	0.12 ±0.09	13	SBR A = SBR B
	EC_{90}	1.67 ± 1.00	0.88 ± 0.49	13	SBR A > SBR B
2	EC_{50}	0.33 ± 0.12	0.27 ± 0.13	10	SBR A = SBR B
	EC_{90}	5.68 ± 4.42	1.55 ± 0.77	10	SBR A > SBR B
3	EC_{50}	0.52 ± 0.34	0.25 ± 0.10	11	SBR A > SBR B
	EC_{90}	6.37 ± 5.84	1.80 ± 1.32	11	SBR A > SBR B

*Average ± Standard deviation

Similar trends for the positive effect of tetracycline with increased growth rates and longer HRT (increased organic loading) was observed in the percentages of tetracycline resistance (Table 3).

In this study, tetracycline resistance was likely induced with tetracycline (SBR A) under High OLR (Phase 2) and Low SRT (Phase 3) conditions but not much in low HRT and SRT (Phase 1). These observations could be explained by high substrate availability (High OLR) and high specific growth rate (low SRT) in Phase 2 and 3 conditions.

Substrate concentration is a well known factor that affects the gene-transfer. The

R-plasmid, a known vehicle for providing antibiotic resistance, can be transferred by conjugation and requires substantial cellular energy to do so. Accordingly, it is not unreasonable that higher gene transfer frequencies have been measured in nutrient-rich high growth rate environments (Ehlers, 1997; Guardabassi and Dalsqaard, 2002). Several researchers stressed the importance of microbial growth rates for plasmid transfer frequencies (Levin *et al.*, 1979; Ehlers, 1997). Levin *et al.* (1979) reported that the fastest rate of R-plasmid conjugation is thought to occur during high growth rate. Ehlers (1997) suggests that the positive strong relationship between increased growth rate sand conjugation process because those two require replication of DNA and the fastest rate of conjugation is thought to occur during exponential growth phase.

4. Discussion

Generally, counting cultured colonies on selected media, which contain specific concentrations of an antibiotic, has been used for quantifying antibiotic resistant bacteria (Guardabassi and Dalsgaard, 2002). There are two major problems for adapting this method to an environmental sample. One is to set the antibiotic concentration to be used in a media. Each microorganism has different Minimum Inhibitory Concentration (MIC) and inevitably, the concentration used simply becomes a reference point. Sometimes, the antibiotic concentration used is determined arbitrarily (Grabow et al., 1973; Koditscheck and Guyre, 1974; Iwane et al., 2001). In other cases, published clinical laboratory guidelines are used for determining the antibiotic concentration in media (Guardabassi and Dalsgaard, 2002; Schwartz et al., 2003). In the previous studies, a single antibiotic concentration in a media was used. However, a single antibiotic concentration gives limited information on the distribution of antibiotic resistance in the bacteria population (Guardabassi and Dalsgaard, 2002).

Table 3 The percentage of tetracycline resistance (%) in SBRA and SBRB biomass

Phase (Tet conc.)		SBR A (%)	SBR B (%)	n (Sample numbers)	Wilcoxson rank sum test
1	5 ppm	6.21 ± 3.67*	3.90 ± 1.79	13	SBR A = SBR B
	20 ppm	1.76 ± 0.50	2.10 ± 0.79	13	SBR A < SBR B
2	5 ppm	11.38 ± 3.26	5.67 ± 2.40	10	SBR A > SBR B
	20 ppm	2.62 ± 0.89	1.84 ± 0.85	10	SBR A > SBR B
3	5 ppm	13.61 ± 4.87	5.06 ± 1.88	11	SBR A > SBR B
	20 ppm	4.52 ± 2.49	1.84 ± 1.04	11	SBR A > SBR B

*Average ± Standard deviation

Consequently, the protocol for assessing antibiotic resistance microbial community is quite necessary for evaluating antibiotic resistance in environmental samples.

As shown in previous session, developed antibiotic resistance community monitoring technique using effective concentration (EC) showed comparable results with the results of percentage of antibiotic resistance, which is traditional antibiotic resistance evaluation technique. In addition, newly developed technique gave us the information about the change of resistance pattern in microbial community which can not be provided by the percentage of antibiotic resistance. For example, EC_{90} concentration in Phase 1 was statistically increased after receiving tetracycline but not EC_{50}. This indicated that more tetracycline resistance community (EC_{90}) responds sensitively in addition of tetracycline rather than lesser tetracycline resistance community (EC_{50}). However, this trend can not be captured with the results of the percentage of tetracycline resistance (Table 2).

Therefore, these results strongly suggested that the continuous monitoring of EC_{50} or EC_{90} of tetracycline in microbial community is promising technique for characterizing the fate of tetracycline resistant bacteria in microbial community.

5. Conclusions

1) Adopted EC concept was successfully used for analyzing the fate of the antibiotic resistant bacterial community analysis in environmental samples.
2) Continuous tetracycline exposure increased the EC_{50} and EC_{90} concentrations of activated sludge(microbial community) under increased growth rate and organic loading rate conditions of SBRs. Similar trends for the positive effect of tetracycline with increased growth rates and longer HRT(increased organic loading) was observed in the percentages of tetracycline resistance.

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