Application of quantitative real-time PCR for quantification of
Rhodococcus sp. EH831 in a polyurethane biofilter

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(Received: March 07, 2008; Revised received: June 25, 2008; Accepted: July 15, 2008)

Abstract: Rhodococcus sp. EH831 is a microbial species that can degrade volatile organic compounds. We optimized a method for monitoring quantitative real-time PCR (qRT-PCR) of EH831 that was incorporated into a polyurethane (PU) biofilter. When the genomic DNA of EH831 was directly extracted from a PU sample with immobilized EH831, the recovery efficiency was very low due to DNA absorption into the PU. DNA amplification during PCR was also inhibited by PU impurities. Therefore, a pre-treatment step was necessary. We successfully recovered cells from the PU by squeezing the matrix, adding sterilized water, and vortexing. The recovery efficiency ranged from 105 to 144%, and there was no statistically significant difference. We designed a novel TaqMan probe for EH831 and demonstrated its high specificity for EH831. The detection range for EH831 was 10⁵-10⁶ CFU ml⁻¹. The method described in this study can be used to investigate the relationship between quantitative analysis of Rhodococcus sp. EH831 and PU biofilter performance.

Key words: Biofilter, Rhodococcus sp., Polyurethane, Quantitative real-time PCR, Monitoring

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Introduction

Contaminated air streams can cause serious health and environmental problems (Arriga et al., 2006). Thus, it is important to remove toxic and malodorous volatile organic compounds (VOCs) from the air (Card, 1998). For treatment of flows with high volumetric flow rates that contain low concentrations of VOCs, biological techniques, rather than conventional techniques (incineration, condensation, sorption, and absorption) can be effective and economical (Shareefdeen and Singh, 2005). Among biological techniques, biofilters, which are composed of an active microbial medium, are considered environmentally friendly, economical, and effective for removal of VOCs (Gunsch et al., 2007). In particular, a polyurethane (PU) biofilter, in which microbial cultures are packed in PU, has been shown to be a promising method for the removal of VOCs (Kim et al., 2005; Kown et al., 2003; Lee et al., 2006; Shim et al., 2006).

Although many biofiltration techniques have been successfully applied in the previous two decades, the mechanism of microbial biofiltration has not been fully examined (Qi and Moe, 2006). To date, most of studies on biofiltration have monitored operating parameters, such as biomass accumulation, nutrient levels, pH, and water content (Gunsch et al., 2007). The amount of microbial biomass in a biofilter clearly influences the efficacy of VOC removal (Devinnry et al., 1996), as well as in water (Chitanand et al., 2008; Pandey, 2008). In particular, over accumulation of biomass can clog biofilters and thereby inhibit flow through the biofilter bed, resulting in reduced removal of contaminants (Deihomerie et al., 2003; Kim and Sorial, 2007; Kown et al., 2003). Thus, control of the biomass in a microbial biofilter is critical for maintaining its long-term operation (Kown et al., 2003; Kim and Sorial, 2007). In addition, it is necessary to monitor the activity of microorganisms in microbial biofilters in order to evaluate efficacy over time (Kim and Sorial, 2007). Consequently, a detailed study of microbial activity and biofilter performance is necessary for the successful implementation of a potential microbial biofilter.

Garcia-Peña et al. (2005) demonstrated a close relationship between toluene oxygenase activity and toluene degradability. Many methods have been proposed for characterization of microbial process in biofilters (Gunsch et al., 2007), but little is known about the quantitative and qualitative aspects of filtration employed by different microbial species in biofilters.

Most studies that have examined microbial biofilters have been performed using selective-culture methods that allowed culturing of microbes with specific metabolic characteristics (Malhautier et al., 2005). However, only 1-10% of known microorganisms can be successfully cultured (Juteau et al., 1999). New molecular biology techniques that use culture-independent methods make it possible to perform more detailed analyses of microbial community composition (Friedrich et al., 2002). These new techniques have also contributed to a better understanding of the evolutionary relationships of microorganisms and of their different ecological roles (Saleh-Lakha et al., 2005).

To monitor microbial communities in the environment, most techniques have quantified or detected phylogenetically specific microorganisms based on the sequences of 16S rDNA genes or of
relevant structural genes (Hristova et al., 2001). Amplification techniques based on PCR are ideally suited to these studies because many microorganisms are present in low numbers in natural mixed microbial communities. Conventional quantitative PCR method cannot provide meaningful data with regard to the population density of different microorganisms, so a quantitative PCR method is necessary (Schneegurt and Kulpa, 1998).

Quantitative real-time PCR (qRT-PCR) has been used for detection of bacterial abundance (Kolb et al., 2003). This method allows identification and quantitation of specific microorganisms or specific genes and is commonly used to detect specific species or specific enzymes in contaminated soils and groundwater. For example, Hristova et al. (2001) used the qRT TaqMan PCR method to quantify MTBE degrading-P1 strain in contaminated soils and groundwater; Baldwin et al. (2003) detected and enumerated aromatic oxygenase genes using qRT-PCR; Nyssön et al. (2006) used qRT-PCR to monitor naphthalene degradation during bioremediation; and Yu et al. (2005) used qRT-PCR to detect and quantify three groups of tetracycline resistance genes.

Thus, qRT-PCR is a useful tool for surveying specific microbial communities and for identification of enzymes that degrade contaminants. However, most of these studies have been applied to groundwater and soils (Baldwin et al., 2003; Da Silva and Alvarez, 2004; Hristova et al., 2001; Labrenz et al., 2004; Nyssön et al., 2006; Yu et al., 2005). It is necessary to optimize the qRT-PCR method so it can be used for assessment of the efficacy of microbial biofilters.

In previous study (Lee et al., 2007), Rhodococcus sp. EH831 had been isolated from oil-contaminated soil. This bacterium can metabolize a variety of hydrocarbons (hexane, cyclohexane, benzene, toluene, ethylbenzene, m, p, o-xylene, methyl tert-butyl ether (MTBE), dichloromethane (DCM), methanol, ethanol, acetone, pyrene, diesel, and lubricant oil). Therefore, EH831 is an available microorganism for VOCs removal in biofiltration system (Lee et al. 2007). A detailed study related to microbial activity and biofilter performance is important to the successful operation of a potential microbial biofilter. To clarify the relationship between microbial activity and biofilter performance, the quantitative monitoring method of microorganisms in biofilter is necessary. In this study, we optimized a method for monitoring quantitative real-time PCR (qRT-PCR) of EH831 that was incorporated into a polyurethane (PU) biofilter.

**Materials and Methods**

**Microorganism and growth conditions:** Rhodococcus sp. EH831 (KCCM-10657P), which was isolated from petroleum contaminated soil, was used as an inoculum (Lee et al., 2007). EH831 was precultured in 1.2 l serum bottles that contained 50 ml Bushnell-Hass (BH) medium (0.409 g·l⁻¹ MgSO₄·7H₂O, 0.0265 g·l⁻¹ CaCl₂·2H₂O, 1 g·l⁻¹ KH₂PO₄, 1 g·l⁻¹ NH₄NO₃, 6 g·l⁻¹ Na₂HPO₄·12H₂O, 0.0833 g·l⁻¹ FeCl₃·6H₂O) and 10 ml·l⁻¹ n-hexane (99.5%, Duksan Pure Chemical Co., Korea) as the sole carbon source for 5 day at 30°C and 180 rpm. Culture bottles were sealed with butyl rubber and an aluminum cap.

<table>
<thead>
<tr>
<th>Table - 1: Efficiency of recovery of EH831 cells from PU by adding water, vortexing, and squeezing</th>
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<tbody>
<tr>
<td><strong>Test No.</strong></td>
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<td>1</td>
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*PU, polyurethane (0.3×0.3×03 cm)*

<table>
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<tr>
<th>Bacterium</th>
<th>Threshold cycle (C) value</th>
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<tbody>
<tr>
<td>Rhodococcus sp. EH831</td>
<td>9.70*</td>
</tr>
<tr>
<td>Escherichia coli DH5-a</td>
<td>undetected</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>23.4*</td>
</tr>
<tr>
<td>Pseudomonas sp. JN4</td>
<td>30.8*</td>
</tr>
<tr>
<td>Pseudomonas sp. OX1</td>
<td>undetected</td>
</tr>
<tr>
<td>Rhodococcus sp. 412</td>
<td>10.6*</td>
</tr>
<tr>
<td>Gordonia sp. SD8</td>
<td>undetected</td>
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<tr>
<td>Rhodococcus sp. EC1</td>
<td>15.3*</td>
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<tr>
<td>Rhodococcus sp. YH1T-2</td>
<td>35.0*</td>
</tr>
<tr>
<td>Stenotrophomonas sp. T3-c</td>
<td>34.6*</td>
</tr>
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a,b Different alphabet means statistically significant (p < 0.05)

**Fig. 1:** Correlation between the dilution of EH831 culture and cell numbers determined using a viable plate count method

**Packaging material:** The packing materials for the biofilter were PU foam cubes (Seilsponge, Korea), with an average size of 0.3 × 0.3 × 0.3 cm. The bulk density, water holding capacity, porosity, average pore size, and surface area of the PU were 0.205 g·cm⁻³, 57 g·H₂O g⁻¹, 98.8%, 0.8 mm, and 76.81 m²·g⁻¹ respectively (Kown et al., 2003).

**Recovery of EH831 from polyurethane:** The recovery of bacterial cells from the PU was optimized as follows. PU (0.5 g) was placed in a 100 ml beaker and sterilized for 15 min at 121°C, and
then water was evaporated at 80°C. The culture broth of EH831 was serially diluted by 10<sup>9</sup>-fold. One ml of the diluted culture broth was inoculated into each of seven 100 ml beakers that contained the sterilized and dried PU. The beakers were maintained at room temperature for 1 hr, then 9 ml of sterilized water was added to each beaker, beakers were vortexed for 3 min, and the cell suspension was recovered by squeezing from PU cubes with a sterilized stainless steel spatula. The cell suspension was then serially diluted and spread on 4 sets of Luria-Bertani (LB) agar plates (tryptone, 10 g l<sup>-1</sup>; yeast extract, 5 g l<sup>-1</sup>; sodium chloride 15 g l<sup>-1</sup>). After incubating at 30°C for 3 day, growth of colonies grown was measured. The recovery efficiency was calculated by comparing viable cell numbers in the liquid culture and the PU. The results were analyzed by ANOVA with the SPSS software.

To optimize the cell recovery method from PU cubes, the effects of surfactant addition, sonication pretreatment, and the combination of surfactant and sonication were investigated. The surfactant Pluronic F68 (PF68, Sigma, St. Louis, MO, USA) (Kastner et al., 1999) was added to the PU (final concentration, 2.5% (w/v)) at the same time that the 9 ml of sterilized water was added. PF68 does not inhibit the growth of EH831, and is not degraded by this bacterium (Lee et al., 2007). The effect of sonication was investigated using 100 ml screw type bottles instead of beakers. After adding 9 ml of sterilized water to a bottle that contained PU and EH831, the bottle was sealed with a cap and sonicated for 20 min. The effect of sonication and surfactant was studied by adding the PF68 surfactant into the bottle, followed by sonication for 20 min as described above. All experiments were performed in duplicate.

**DNA extraction and purification:** To extract DNA from cell suspensions that were recovered as described above, two methods were used: (a) Fast DNA spin kit (BIO101 kit, Qbiogene, USA) and (b) addition of 0.1 N NaOH after 1 ml of suspension was centrifuged at 14,000 rpm for 10 min. Harvested cells were suspended in 30 ml of 0.1 N NaOH and kept at 95°C for 30 min. The Fast DNA spin kit was performed according to kit protocols. Extracted DNA samples obtained by the two methods were purified using a PCR purification kit (QIAGEN GmbH, Hilden, Germany), and DNA concentration was measured using an UV/Vis spectrophotometer (Agilent 8453, Agilent technologies, USA) at 260 nm wavelength.

**Optimization of qRT-PCR:** To design the TaqMan probe and primers, 16S rDNA of Rhodococcus sp. EH831 was subjected to PCR using 27f (AGA GTT TGA TCM TGG CTC AG; M=C:A) and 1492r (TAC GGY TAC CTT GTT ACG ACTT; Y=C:T) primers (Martin-Laurent et al., 2001). PCR conditions and reagents were described previously (Ryu et al., 2006). The amplified 16S rDNA was purified using a PCR purification kit (QIAGEN GmbH, Hilden, Germany), and then sequenced (Ryu et al., 2006). Identified sequences were analyzed for similarities with the Basic Local Alignment Search Tool (BLAST), a network service at Genbank (Hristova et al., 2001). Based on National Center for Biotechnology and Information (NCBI) Blast search results, the most unique partial sequences of EH831 were identified, and then the novel TaqMan probe and primers for EH831 were designed. TaqMan primers were 5'-CGCAACCTTTGTTCCTGTTG-3' for the forward primer and 5'-GTTGCCACCGCGCTGTCT-3' for the reverse primer. The probe sequence was 5'-CGTTAAGCCACCC-3' and was 5' labeled with FAM (6-carboxyfluorescein).

The purified genomic DNA samples that were obtained from the Fast DNA spin kit and from lysis with NaOH were diluted 10<sup>9</sup>-fold and then qRT-PCR was performed. qRT-PCR (Applied Biosystems 7300 real time PCR system, Applied Biosystems, USA) was performed using 25 µl samples in the MicroAmp Optical 96-well reaction plate and MicroAmp Optical Caps (Applied Biosystems, USA). Three µl of the purified genomic DNA was added as a template in 22 µl of the PCR master mix. The PCR reaction mixture consisted of 12.5 µl of TaqMan universal PCR master mix (Applied Biosystems, USA), 1.25 µl of TaqMan primers and probe and 8.25 µl of distilled water. At the same time that qRT-PCR was performed, the

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**Fig. 2: TaqMan qRT-PCR standard curve.** (A) C<sub>T</sub> values vs. genomic DNA concentrations of EH831. (B) Genomic DNA concentrations of EH831 vs. viable cell number of EH831.
standard samples were also subjected to PCR. DNA concentration and CFU ml\(^{-1}\) of template were calculated from the standard curve. Standard samples were performed in triplicate. PCR conditions and data analysis were described previously (Hristova et al., 2001).

To test the specificity and sensitivity of our TaqMan probe, bacterial genomic DNAs showing high or low phylogenetic similarities with Rhodococcus sp. EH831 were extracted using the Fast DNA spin for soil kit and qRT-PCR was performed as described above. The strains used in this study were Escherichia coli DH5\(\alpha\), Bacillus amyloliquefaciens (ATCC 2335), Pseudomonas sp. JN4 (AY878329), Pseudomonas sp.OX1 (Bertoni et al., 1998), Rhodococcus sp. 412 (Hong et al., 2007), Gordonia sp. SD8 (EF460468), Rhodococcus sp. EC1 (AY878707; Lee and Cho, 2008), Rhodococcus sp. YHLT2 (Ryu et al., 2006), and Stenotrophomonas sp. T3-c (Kown et al., 2003). After qRT-PCR, the standard curve (DNA concentration vs. CFU ml\(^{-1}\)) was made to determine DNA concentrations and values of threshold cycles (C\(_T\)). All experiments were performed in triplicate. The results were analyzed by ANOVA with the SPSS software.

Results and Discussion

Recovery of Rhodococcus sp. EH831 from polyurethane:
Before performing recovery experiments of EH831 cells from the PU, we verified our methods for counting EH831 using the viable plate count method. As expected, the number of colonies that developed on each plate decreased linearly according to dilution, with a slope of 1.04 and a correlation coefficient (r\(^2\)) of 0.97 (Fig. 1). As described in the Materials and Methods, the cell suspension was obtained by squeezing the PU after adding water and vortexing. Total cell number of EH831 recovered from PU was compared to that in the inoculum (Table 1). The recovery efficiency ranged from 105 to 144%, and there was no statistically significant difference between the inoculum and PU (Table 1). These results suggested that the method applied in this study sufficiently recovered EH831 cells from PU. On the other hand, the effects of surfactant addition and/or sonication pretreatment on the cell recovery from PU were not significant: the recovery efficiency of EH831 was from 88 to 16%, which was similar or even decreased for some cases.

Optimization of DNA extraction: When DNA samples were directly extracted from the PU cubes without a pre-step for cell recovery, there were some insubomitable problems. A commercial DNA extraction kit could not be used because the PU cubes were too large. DNA samples could be obtained from 0.5 g of the PU by squeezing with a sterilized spatula after adding 9 ml of 0.1 N NaOH solution and heating at 95°C for 30 min. However, DNA concentration was reduced due to adsorption onto the PU.

There were also potential problems in the qRT-PCR procedure. It is possible that impurities might be extracted from the PU during the DNA extraction process, and these impurities might inhibit the PCR. These results suggest that a recovery step is indispensable for bacterial DNA extraction from EH831 that is immobilized onto PU.

The DNA concentration extracted from EH831 cells by the BIO101 kit was 166.6 ng µl\(^{-1}\) and that by the 0.1 N NaOH method was 52.3 ng µl\(^{-1}\). This indicated that the BIO101 kit was more effective for extraction of genomic DNA.

Quantitative monitoring of EH831 using qRT-PCR: To investigate the specificity and sensitivity of the designed probe for Rhodococcus sp. EH831, we performed qRT-PCR using various DNA templates showing high or low phylogenetic similarities to EH831 (Table 2). Our results show that C\(_T\) values ranged from 23 to "undetected" for E. coli, Bacillus amyloliquefaciens, Pseudomonas spp. JN4 and OX1, Gordonia sp. SD8, Rhodococcus sp. YHLT-2 and Stenotrophomonas sp. T3-c. Even though the probe was designed for EH831, Rhodococcus spp. 412 and EC1 had low C\(_T\) values (Table 2). We suspect that Rhodococcus spp. 412 and EC1 were phylogenetically similar to EH831 because they were isolated from the same source (Hong et al., 2007; Lee and Cho, 2008). Although strain YHLT-2 is also a Rhodococcus species, its C\(_T\) value was substantially lower, presumably because it was isolated from a different source (Ryu et al., 2006). Although some Rhodococcus spp. were detected with high sensitivity, we conclude that the probe used in this study is very useful for detection of Rhodococcus sp. EH831.

To obtain the qRT-PCR standard curve, we plotted the relationships between C\(_T\) and genomic DNA concentration of EH831 (Fig. 2a), and between viable cell number and genomic DNA concentration of EH831 (Fig. 2b). The C\(_T\) values decreased linearly with DNA concentration (r\(^2\) = 0.999; Fig. 2). The detection range of EH831 varies from 10\(^5\) to 10\(^11\) CFU ml\(^{-1}\) and the slope is -3.48. A repeat of these experiments yielded an r\(^2\) > 0.99 and slopes of -3.42 and -3.74. The slope of an ideal qRT-PCR standard curve is -3.32 and between -3.0 and -3.9 corresponding to qRT-PCR efficiencies of 80-115% (Zhang and Fang, 2006), indicating that the slope of -3.48 meant high efficiency. The standard curves resulting from our TaqMan probe for EH831 show a higher detection limit and higher viable cell numbers than previous studies (Zhang and Fang, 2006). Since cell concentrations in biofilter systems are higher than in other samples (Yun and Ohta, 2005), the ranges of our standard curve (10\(^5\)-10\(^12\) CFU ml\(^{-1}\)) are acceptable.

To confirm the accuracy of our quantitative method, the EH831 cell suspension 7.6×10\(^7\) CFU ml\(^{-1}\) were immobilized onto the PU cubes and genomic DNA was extracted from the cells by the sequential treatment of water, vortexing, and squeezing. Then, we performed qRT-PCT using the DNA sample as a template, and calculated cell number from the threshold cycle value and the standard curve (Fig. 2). The resulting cell count was 4.3±1.9×10\(^7\) CFU ml\(^{-1}\), indicating no significant difference from the original cell number immobilized onto the PU cubes.

Taken together, our results indicate that our monitoring method, which uses qRT-PCR and a novel TaqMan probe for Rhodococcus sp. EH831, has been optimized and will be useful for tracking the fate of EH831 in PU biofilters. The method optimized in
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this study can be widely applied to monitor other microorganisms as well as Rhodococcus sp. EH831 if specific primers and prove for each microorganism are used.

Acknowledgments
This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-311-D00589). Eun-Hee Lee was financially supported the Korea Science and Engineering Foundation through the Advanced Environmental Biotechnology Research Center at Pohang University of Science and Technology (R11-2003-006-06001-0), and the KOSEF NRL Program grant funded by the Korea government (MEST) (ROA-2008-000-20044-0).

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