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Isolation and Identification of Xylene Degrading Microorganisms from Biofilter

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Abstract: Mixed microorganisms in a biofilter medium were acclimatized with xylene vapor continuously for 8 h day^{-1} for 30 and 60 days in laboratory. Biofilter medium was coconut husk: manure compost: wastewater sludge at 75: 20: 5 (v/v). The initial moisture content of the biofilter medium was adjusted to 50% of dry weight. Two bacteria and 4 fungi from biofilter were identified at the end of experiment in enriched medium. All dominant species were fungi which were subsequently isolated and identified as *Aspergillus flavus* (EF592170), *Aspergillus terreus* (EF592171), *Penicillium glabrum* (EF592172) and *Aspergillus niger* (EF592173), respectively. The result indicates that the fungi last longer in biofilter with xylene environment and thus, may be suitable to be cultured for use in biofilter for xylene removal in industrial applications.

Key words: Biofilter, xylene, coconut husk, *Aspergillus*, *Penicillium*

INTRODUCTION

Conventional Volatile Organic Compounds (VOCs) control techniques such as adsorption and absorption, scrubbing, condensation, thermal and catalytic incineration have been used to treat VOCs in polluted air stream. Although, conventional VOCs treatment technologies were useful for reducing emissions, they can generate undesirable byproducts and are less cost-effective when treating high flow air streams containing low concentrations of pollutants (Rene *et al.*, 2005).

Biofiltration, as one method for VOC treatment, has significant advantage over conventional techniques as it has relatively low capital and operating costs and is more convenient for treating low concentration or intermittent emissions. The performance of a biofiltration system depends on the biodegradation of pollutants by microorganisms in the media and its effectiveness depends on the types and number of microorganism.

The mixed culture is commonly used in biofilter to remove pollutants. Mixed cultures were used for removal of Benzene, Toluene and Xylene (BTX) and other VOCs (Du Plessis *et al.*, 2001; Veiga *et al.*, 1999). Activated sludge from wastewater treatment plant was a popular initial microorganism source (Singh *et al.*, 2006). Bacteria such as *Bacillus* and *Pseudomonas* were shown as dominant species (Veiga *et al.*, 1999; Andreoni *et al.*, 1996).

Fungi have been found to be effective in biofiltration for removal of volatile organic compounds. Pure culture of the fungus *Cladosporium sphaerospermum* was also used for VOCs mixture treatment (Qi *et al.*, 2005). *Exophiala oligosperma or Paecilomyces variotii* were also used to remove toluene (Estevez *et al.*, 2005) and *Aspergillus niger* was successfully applied to remove hexane from contaminated air streams in biofilter (Spigno *et al.*, 2003). The effectiveness of fungi to remove xylene was found to be variable. García-Peña *et al.* (2008) used the filamentous fungus *Paecilomyces variotii* CBS115145 in a trial to remove xylene, but only succeeded in partial removal. Hasnaa *et al.* (2009) used *Phanerochaete chrysosporium* ATCC20696 and *Cladosporium sphaerospermum* ATCC12092 to remove xylene and found that the fungi system had a higher maximum elimination capacity than a bacterial system.

The knowledge on the dominant type of microorganism that can degrade certain type of VOCs is

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useful in VOCs treatment by biofiltration technique. In this study, we isolated and identified the dominant species of microorganism that can degrade xylene from biofilter media of coconut hush and manure compost which is a common media used in biofiltration in Thailand.

MATERIALS AND METHODS

The study was performed during January-June 2006 in the Laboratory at the Faculty of Public Health Burapha University, Chonburi Province, Thailand.

Biofilter media preparation: Packing materials were coconut husk pieces (1.5×1.0 cm), manure compost and wastewater sludge. The wastewater sludge was collected from a municipal waste water treatment plant in Bangkok, Thailand and was used as the initial culture. Commercial manure compost was used. The ratio of packing materials of coconut husk: manure compost: sludge was 75: 20: 5 (v/v). This ratio was reported to be the optimum ratio of packing media for biofiltration process from another study in Thailand (Pollution Control Department, 2001). The initial moisture contents of coconut husk, manure compost and sludge were 14.5, 18.1, 70.5, respectively. Gravel was put into the columns (5 cm ID×200 cm long) to the height of 20 cm and 350 g of Biofilter media was packed into column, then distilled water was added until the overall moisture content reaches 50% of dry weight approximately.

Microorganisms acclimatization system: Moisture saturated air and xylene vapor were pumped into the mixing chamber. Then xylene vapor from mixing chamber with concentration of 2 g m^{-3} was pumped through the biofilter media in the columns continuously for 8 h day^{-1} for 30 and 60 days at constant flow rate of 0.06 m^3 h^{-1} to acclimatize xylene degrading microorganisms. The room temperature was 28-33 °C. Gas samples for xylene analysis were collected from inlet and outlet of the column by Solid Phase Micro Extraction (SPME) Holder with SPME fiber (Supelco-57330-U). The samples were analyzed by a Gas Chromatography with a flame-ionization detector (Unicham, G 610 Series) with capillary column (EQUITY™ -5; Supelco-20750-01B). The xylene removal efficiencies at the 30th day and 60th day were 88 and 86%, respectively.

Microbial cell count: Viable cell number in the biofilter media was determined by the following procedure. Ten gram wet weight of biofilter media was put into 90 mL of sterilized distilled water in flask and shaking at 200 rpm for 5 min. One milliliter of suspension was transferred for 10 fold serial dilutions and plated on Plate Count Agar (PCA). The plated were incubated at room temperature (28-33°C) for 1-7 days. After incubation, the grown colonies of microbial were counted and expressed as colony forming unit (cfu) per gram of wet biofilter media.

Isolation of dominant species: After 30 and 60 days, the biofilter media was sampled for 10 g and put into flasks containing 50 mL of various enriching liquid media such as malt extract medium, nutrient, heart brain infusion or peptone broth to enhance the growth of microorganisms. The flasks were shaken at 200 rpm for 10 h. After that 0.1 mL of supernatant was transferred to nutrient agar, malt extract agar or potato dextrose agar to enhance the growth of microorganisms. The observation on microbial growth was performed in 1-7 days. The microorganisms were purified by consecutive subcultures until all single colonies were isolated.

All pure colonies were cultivated on the mineral medium agars (Verdin *et al*., 2004) that were added with 0.3, 0.5 and 1 mL of xylene as sole carbon source. After 7 days the microbial growths was observed from 0.3 and 0.5 mL xylene agar plates. The identification of bacteria was performed by morphology, gram staining and biochemical test and the identification of fungus were performed by phenotype technique and DNA sequencing method.

DNA sequencing method: Fungal DNA was extracted using the method of Makimura *et al.* (1994). The Internal Transcribed Spacer (ITS) was amplified using the primer pairs ITS-1 (5' TCC GTA GGT GAA CCT GCG G) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC), GeneElute™ PCR Clean-up Kit (Sigma, Saint Louis, Missouri) according to the manufacturer's instructions. All cycle sequencing reactions were performed with an ABI 3130 XL automated sequencer (Hitachi) according to the manufacturer's instructions (PE Applied Biosystems, Foster City, California). Each sequence were aligned and analyzed to ensure a high quality of sequence data. All DNA sequences were submitted to Gen Bank.

RESULTS AND DISCUSSION

Two strains of bacteria (B1 and B2) and 4 strains (*Aspergillus flavus* (EF592170), denoted as M1; *Aspergillus terreus* (EF592171), denoted as M2; *Penicillium glabrum* (EF592172), denoted as M3 and *Aspergillus niger* (EF592173), denoted as M4 of fungi were found from 30 day acclimatization system. The same 4 types of fungi were found from 60 day acclimatization system and there was no bacteria or other microbial growth in the enrich media.

Table 1: Amount of microorganisms (%) isolated from biofilter

	Total plate count (cfu g^{-1})	
	9.0×10^{7} to 8.2×10^{8}	8.5×10^{6} to 8.0×10^{7}
Strains	30 days	60 days
M1 *Aspergillus flavus*	22	22
M2 *Aspergillus terreus*	23	33
M3 *Penicillium glabrum*	23	33
M4 *Aspergillus niger*	20	11
B1	5	Not found
B2	6	Not found

Table 2: Morphology and type of microorganisms isolated from biofilter

Details	Morphology	Type of microorganisms
30 days		
Bacteria	White to yellow colonies, size 1-2 mm Gram negative rod	B1: Glucose non_fermentative bacteria (GNB)
	White colonies size 2-3 mm Gram negative rod	B2: Glucose non_fermentative bacteria (GNB)
Fungi	Dark green colonies	M1: *Aspergillus flavus*
	Yellow to brown colonies	M2: *Aspergillus terreus*
	Gray colonies	M3: *Penicillium glabrum*
	Black colonies	M4: *Aspergillus niger*.
60 days		
Bacteria	Not found	
Fungi	Dark green colonies	M1: *Aspergillus flavus*
	Yellow to brown colonies	M2: *Aspergillus terreus*
	Gray colonies	M3: *Penicillium glabrum*
	Black colonies	M4: *Aspergillus niger*

Microorganisms isolated from biofilters acclimatized for 30 and 60 days are shown in Table 1. The total number for the 30 day samples was higher than the 60 day samples. The ratios among the fungi were not found to shift radically between both acclimatized periods, but the absence of bacteria species in 60 day samples indicates that bacteria is not suitable for long term application of biofilters. Table 2 shows details of the 4 fungi which can grow and use xylene as sole carbon source in the media.

Bacteria and fungi are the two dominant microorganisms group presented in biofilters. But their relative abundances can vary widely. Bacteria have the advantage of rapid substrate uptake and growth. Under favorable conditions, they dominate fungi (Devinny *et al*., 1999). In our experiment, there was no additional nutrient added to the biofilter media and this made the condition less favorable for bacteria. Therefore, only two types of bacteria were found after 30 days and none after 60 days. Fungi generally grow more slowly and their large sizes give them a smaller surface-to ratio for substrate uptake. However, they can degrade a greater variety of contaminants and withstand harsher condition. Kennes and Veiga (2004) presented on fungal biocatalysts in biofiltration that fungi using xylenes as carbon and energy sources are scarce. It is noted that some strains of the white-rod fungus *P. chrysosporium* can degrade xylenes during primary metabolisms, although biodegradation is only partial (<50%) and the level of mineralization is quite limited (Kennes and Veiga, 2004). Similar result was reported by García-Peña *et al.* (2008) with the fungus *Paecilomyces variotii* (biodegradation about 30%). However, there may be more fungi which can biodegrade xylene more effectively.

Present result reveals that there are 4 species of fungi that can use xylene as carbon and energy sources. Four dominants fungi species were phenotype and DNA sequencing methods (Appendix) identified them as *Aspergillus flavus*, *Aspergillus terreus*, *Penicillium glabrum* and *Aspergillus niger*. The findings of four fungi found as dominant species in the biofilter media commonly used in industrial biofiltration application in Thailand indicate that they can last long enough in the xylene vapor environment (60 days are quite acceptable between replacement of this type of media). The number of them decreased about 10 times from 30 to 60 days period, but this is to be expected along with the degeneration of biofilter media in general.

CONCLUSION

The absence of bacteria after 60 days acclimatization may indicate that bacteria have shorter survival time than the fungi. Even though other researchers have found that both bacteria and fungi could remove VOCs, present results do not contradict those findings, but indicate that for the conditions set for this experiment the fungi were the longer-lasting species and probably are more responsible for xylene removal in the later phase of biofilter media's lifetime. This leads to better understanding of the dynamics of microorganisms in xylene removal. The media used in this experiment as well as the temperature may also be an important factor in determining the survival rate of certain microorganism species. It is expected that different media may favor different microorganisms. Further researches with different media may provide results with higher microbial counts and thus higher xylene removal efficiencies.

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APPENDIX

DNA sequencing: Dominant species were confirmed and identified by DNA sequencing method. DNA was extracted using the method of Makimura *et al*. (1994). The internal transcribed spacer (ITS) was amplified using the primer pairs ITS-1 (5' TCC GTA GGT GAA CCT GCG G) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC). Four sequences of them were indicated as *Aspergillus flavus*, *Aspergillus terreus*, *Penicillium glabrum* and *Aspergillus niger* as follow.

Aspergillus flavus (EF592170)

- Genomic DNA 422 bp *Aspergillus flavus*
- Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus

- **REFERENCE:** 1 (bases 1 to 422)
- **BASE COUNT:** 84 a 122 c 116 g 100 t
- **ORIGIN:**

1 tccgtaggtg aacctgcgga aggatcatta ccgagtgtag ggttcctagc gagcccaacc
61 tcccacccgt gtttactgta ccttagttgc ttcggcgggc ccgccattca tggccgccgg
121 gggctctcag ccccgggccc gcgcccgccg gagacaccac gaactctgtc tgatctagtg
181 aagtctgagt tgattgtatc gcaatcagtt aaaactttca acaatggatc tcttggttcc
241 ggcatcgatg aagaacgcag cgaaatgcga taactagtgt gaattgcaga attccgtgaa
301 tcatcgagtc tttgaacgca cattgcgccc cctggtattc cgggggggcat gcctgtcgag
361 cgtcatgctg cccatcaagc acggcttgtg tgttgggtcg tcgtccccca tcatcccagg
421 gg //

Aspergillus terreus (EF592171)

- Genomic DNA 522 bp *Aspergillus terreus*
- Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus

- **REFERENCE:**1 (bases 1 to 522)
- **BASE COUNT:** 87 a 164 c 143 g 128 t
- **ORIGIN:**

1 tccgtaggtg aacctgcgga aggatcatta ccgagtgcgg gtcctcgtgg cccaacctcc
61 cacccgtgac tattgtacct tgttgcttcg gcgggcccgc cagccctgct ggccgccggg
121 gggcgtctcg cccccgggcc cgtgcccgcc ggagacccca acatgaaccc tgttctgaaa
181 gcttgcagtc tgagtgtgat tctttgcaat cagttaaaac tttcaacaat ggatctcttg
241 gttccggcat cgatgaagaa cgcagcgaaa tgcgataact aatgtgaatt gcagaattca
301 gtgaatcatc gagtctttga acgcacattg cgcccctgg tattccgggg ggcatgcctg
361 tccgagcgtc attgctgccc tcaagcccgg cttgtgtgtt gggtcctcgt cccccggctc
421 cgggggacgg gcccgaaagg cagcggcggc accgcgtccg tctttttta ttgaaaatgg
481 ggtttgtctt ccgctccgcc tccccccccc ctttttttt cc //

Penicillium glabrum (EF592172)

- Genomic DNA 560 bp DNA *Penicillium glabrum*
- Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Penicillium

- **REFERENCE:** 1 (bases 1 to 560)
- **BASE COUNT:** 111 a 164 c 158 g 127 t
- **ORIGIN:**

1 tccgtaggtg aacctgcgga aggatcatta ctgagtgagg gccctctggg tccaacctcc
61 cacccgtgtt tattgtacct tgttgcttcg gtgcgcccgc ctcacggccg ccgggggggct
121 tctgcccccg ggtccgcgcg caccggagac actattgaac tctgtctgaa gattgcagtc
181 tgagcataaa ctaaataagt taaaactttc aacaacggat ctcttggttc cggcatcgat
241 gaagaacgca gcgaaatgcg ataactaatg tgaattgcag aattcagtga atcatcgagt
301 ctttgaacgc acattgcgcc ccctggtatt ccgggggggca tgcctgtccg agcgtcattg
361 ctgccctcaa gcacggcttg tgtgttgggc tccgtccccc cggggacggg tccgaaaggc
421 agcggcggca ccgagtccgg tcctcgagcg tatggggctt tgtcacccgc tctgtaggcc
481 cggccggcgc cagccgacaa ccaatcatcc ttttttcagg ttgacctcgg atcaggtagg
541 gatacccgct gaacttaagc//

Aspergillus niger (EF592173)

- Genomic DNA 585 bp *Aspergillus niger*
- Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus

- **REFERENCE:** 1 (bases 1 to 585)
- **BASE COUNT:** 107 a 175 c 170 g 133 t
- **ORIGIN:**

1 tccgtaggtg aacctgcgga aggatcatta ccgagtgcgg gtcctttggg cccaacctcc
61 catccgtgtc tattgtaccc tgttgcttcg gcgggcccgc cgcttgtcgg ccgccggggg
121 ggcgcctctg ccccccgggc ccgtgcccgc cggagacccc aacacgaaca ctgtctgaaa
181 gcgtgcagtc tgagttgatt gaatgcaatc agttaaaact ttcaacaatg gatctcttgg
241 ttccggcatc gatgaagaac gcagcgaaat gcgataacta atgtgaattg cagaattcag
301 tgaatcatcg agtctttgaa cgcacattgc gccccctggt attccggggg gcatgcctgt
361 ccgagcgtca ttgctgccct caagcccggc ttgtgtgttg ggtcgccgtc ccctctccg
421 gggggacggg cccgaaaggc agcggcggca ccgcgtccga tcctcgagcg tatggggctt
481 tgtcacatgc tctgtaggat tggccggcgc ctgccgacgt tttccaacca ttctttccag
541 gttgacctcg gatcaggtag ggatacccgc tgaacttaag catat //

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