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Improved biodegradation potential of chlorobenzene by a mixed fungal-bacterial consortium

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A defined consortium of *Ralstonia pickettii* L2 (bacterium) and *Trichoderma viride* LW-1 (fungus) was selected to assess its potential for the enhanced biodegradation of mono-chlorobenzene (CB). At an initial concentration of 220 mg L^{-1} CB, the developed consortium showed an enhanced degradation rate of 0.50 mg $CB \cdot g^{-1}{}_{protein} \cdot h^{-1}$, while the individual *Ralstonia* sp. L2 and *Trichoderma* sp. LW-1 showed average degradation rates of 0.34 and 0.32 mg $CB \cdot g^{-1}{}_{protein} \cdot h^{-1}$, respectively. A CO_2 conversion level of up to 86.3% reflected a possible high mineralization extent of CB by the co-culture. The estimated μ_{max} and v_{max} values were 0.36 h^{-1} and 0.41 h^{-1} for the consortium, which were much higher than the values obtained by each strain individually. 2-Chlorophenol (2-CP) accumulated in the growth medium of strain L2 and inhibited its growth, but it could be consumed quickly by the fungus LW-1, providing a possibility to reach complete biodegradation of CB in a short time. Real-time PCR revealed that bacterium L2 played a major role in the initial stage, and that fungus LW-1 grew well if 2-CP was generated. These results suggest that the fungal-bacterial consortium might be effectively applied for complete biodegradation of CB and have a potential environmental implication in purification of CB-contaminated environments.
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1. Introduction

Volatile organic compounds (VOC) are important emerging atmospheric pollutants because of their effect on ozone build-up, global warming potential and toxicity. VOCs are mainly emitted by industrial processes and automobile exhaust gases and, to a minor extent, by some other daily activities (Kennes and Veiga, 2013). Chlorinated VOCs are of prime concern because of their severe impact on ecosystems (Guo et al., 2016; Braeckevelt et al., 2011). Mono-chlorobenzene (CB), one of the most common products among the chlorinated aromatic VOCs, is used as a solvent, degreaser and intermediate in the synthesis of various pesticides and dyes (Zilli and Nicolella, 2012). The accumulation of CB in the human body can affect the function of the central nervous system and even cause cancer, teratogenesis, or mutagenesis. Because CB emissions have a strong negative impact on air quality and human health, increasing attention is being paid to its elimination. Consequently, there is a need for development of an effective approach for the removal of that pollutant.

Several physico-chemical techniques have been reported to effectively remove CB. He et al. (2016) reported that the conversion of CB over a Mn3/KIT-6 catalyst could reach 90% or more for 1000 min. However, the catalytic combustion process would need a large amount of heat from fuels burning in the reacting furnace. Another study claimed that the removal level of CB over Pd/Fe particles reached up to 90% within 120 min using an electrochemical method (Luo et al., 2014). However, the higher operational costs and greater energy consumption characterizing conventional waste gas treatment processes converts biological treatment processes into a competitive alternative. Among the various technologies for CB removal, biopurification is widely considered to be a cost-effective technology because of its low operating costs and easy maintenance (Mudliar et al., 2010; Kennes and Veiga, 2013). However, CB is a recalcitrant substrate, and there

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are few specific efficient strains for CB removal. Several studies have described bioreactors that could remove CB from waste gases. However, the removal efficiencies reached to date are often relatively low. Wang et al. (2008a) used a biofilter for the removal of CB with an initial concentration of 500 mg m^{-3}, and the removal efficiency was 60% when there was no UV as the pretreatment. Zhou et al. (2016) found that the removal efficiency of CB by biotrickling filters was relative lower (−40% during the first 30 d) and decreased obviously as the residence time decreased. Therefore, exploring the development of a successful and reliable biotreatment system, especially one inoculated with efficient bio-degraders, is necessary.

Several microorganisms belonging to different taxonomic groups of bacteria have been found to be capable of removing pollutants through biotransformation or biodegradation. The co-culture of different bacterial species was also shown to have the potential to be more efficient for the biodegradation of recalcitrant compounds because of the synergistic interactions between different pure strains (Khalid et al., 2009; Cerqueira et al., 2011). Zhang et al. (2013) described a consortium, consisting of three isolates that led to significantly improved degradation and mineralization of paracetamol, although each strain could degrade paracetamol to different extents individually. The reason for the observed synergistic effect might be that these strains may each exhibit different degradation abilities toward paracetamol and its major metabolites (4-aminophenol and hydroquinone), by acting together they allow a faster and more complete degradation process.

Several researchers recently found that bioreactors containing a microbial population principally composed of bacteria show poor performance for the treatment of highly hydrophobic VOCs (Dorado et al., 2008) because of the limited mass transfer between gas and liquid phases. In contrast, fungi generally perform better for the removal of hydrophobic VOCs (e.g., α-pinene, benzene and CB) under low pH values or low relative humidity (Kennes and Veiga, 2004; Hassan and Sorial, 2010; Cheng et al., 2015) because of hostile growth conditions and the large hyphal surfaces of the fungal strains (Vergara-Fernández et al., 2008; Bordel et al., 2010). In a previous studies, *Paecilomyces variotii* had rather good ability to remove a mixture of hydrophobic VOCs that contained propanal, toluene and hexanol (Estrada et al., 2013), and *Exophiala* sp. Showed higher removal rates for gaseous benzene at a low pH of 4.9 than the one obtained at neutral pH value (Rene et al., 2012). However, the lower growth rate, long degradation time and other disadvantages limit the potential use of fungi for the biodegradation of such compounds.

Despite the great potential of bacteria and fungi for the biodegradation of VOCs, both are limited in their individual abilities to completely degrade and detoxify pollutants. Fungal-bacterial consortia exhibit synergistic activity that may lead to enhanced degradation of some recalcitrant compounds, thus providing an improved and alternative method for their efficient removal. Wang et al. (2012) compared the removal rates of pyrene by a fungal-bacterial consortium, a fungal consortium or a bacterial consortium. They showed that the maximal removal level (67% within 28 d) was observed for the fungal-bacterial consortium, while the others reached 39% and 56%, respectively. Cheng et al. (2015) studied the performance of fungal and bacterial biofiltration for the removal of toluene and observed a better performance for the fungal-bacterial consortium. In addition to the enhanced capture of VOCs and attachment of bacteria by fungal hyphae, the efficient utilization of intermediate metabolites by fungi may be another reason for the better performance exhibited by the mixed consortium.

It is known that removal of some specific metabolites from the media could reduce the inhibitory effects of toxic intermediates. In the past few years, our group isolated a bacterium, *Ralstonia pickettii* L2, with the ability to degrade CB (Zhang et al., 2011). CB degradation by bacterium L2 occurs via initial hydroxylation under the monooxygenase contained by bacterium L2 to yield 2-chlorophenol (2-CP), followed by monooxygenation of the aromatic ring to yield 3-chlorocatechol. A chlorine atom is then replaced by a hydroxyl group to form phenol, followed by a second adjacent hydroxylation to induce ring fission, similar to the catechol pathway, after which the molecule enters into the TCA cycle. It was found that 2-CP was a major intermediate during CB biodegradation by bacterium L2, while there was no obvious evidence of this strain utilizing 2-CP in the above paper. Other groups also found that 2-CP is a growth inhibitor and further limited the continuous degradation of CB (Vanderberg et al., 2000; Rafiee et al., 2012). However, if 2-CP was removed from the cultural medium, its biodegradation of CB would proceed more efficiently.

The degradation is faster and more efficient when performed by a fungal-bacterial co-culture that by individual cultures. The higher microorganisms' biodiversity results in a better removal capability of the biodegrading ecosystem (Festa et al., 2016). To date, most studies have used combined bacterial-fungal systems for the removal of some rather easily biodegradable-pollutants (Qu et al., 2010; Su and Lin, 2013), while very few studies have evaluated their potential for treating highly recalcitrant compounds. In the case of CB degradation, research pertaining to fungal-bacterial consortia is missing. Moreover, studies pertaining to the analysis of the microbial community structure and the in-depth reasons for the merits of the fungal-bacterial biodegradation processes are very scarce. Therefore, there is a great need for further research in such fields allowing set-up of such mixed consortium for the efficient removal of recalcitrant compounds like CB.

In this study, a bacterium designated as L2 and the fungus LW-1 (both of which isolated by our group) were used in co-culture to enhance CB biodegradation. Both strains were capable of degrading CB, and bacterium L2 had a higher ability to degrade CB than fungus LW-1. The removal efficiencies of CB by this fungal-bacterial consortium and single pure strains under batch and continuous culture conditions in shake flasks were evaluated, and the optimum initial biomass and proportion of these two strains were determined based on their CB removal. Information regarding the biodegradation kinetics of the consortium and the pure strains are also provided according to empirical models. Finally, a microbial analysis based on the polymerase chain reaction and pyrosequencing was used to provide insight into the relationship between the bacterial and fungal population during CB biodegradation. The results presented herein are expected to provide useful clues for further application in engineered biodegradation systems.

2. Materials and methods

2.1. Chemicals, nutrient media and inocula

Chlorobenzene (98%) was purchased from Huadong Medicine Group Co., Ltd. (Hangzhou, China). 2-Chlorophenol (2-CP) was purchased from Aladdin Shanghai Co., Ltd. (Shanghai, China). DNA extraction kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Other chemical agents were acquired from Sinopharm Chemical Reagent Co., Ltd. as analytical grade reagents.

The mineral inorganic medium used for biodegradation experiments had a pH of 7 and consisted of the following chemical composition (per liter distilled water): 0.5 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 4.5 g KH_2PO_4, 2 g NH_4Cl, and 2 mL of trace mineral solution. The trace mineral solution had the following chemical composition ($mg \cdot L^{-1}$): 120 $FeCl_3$, 50 H_3BO_3, 10 $CuSO_4 \cdot 5H_2O$, 10 KI, 45 $MnSO_4 \cdot 4H_2O$, 20 $Na_2MoO_4 \cdot 2H_2O$, 75 $ZnSO_4 \cdot 7H_2O$, 50

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