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A novel strategy for acetonitrile wastewater treatment by using a recombinant bacterium with biofilm-forming and nitrile-degrading capability

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HIGHLIGHTS

- A recombinant bacterium, *Bacillus subtilis* N4-pHT01-*nit* was established.
- Strain N4-pHT01-*nit* has two functions: biofilm formation and nitrile degradation.
- Strain N4-pHT01-*nit* had a strong resistance to loading shock from acetonitrile.
- Strain N4-pHT01-*nit* improved the degradation of acetonitrile in wastewater treatment.
- Strain N4-pHT01-*nit* was successfully immobilized in the reactor.

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ABSTRACT

There is a great need for efficient acetonitrile removal technology in wastewater treatment to reduce the discharge of this pollutant in untreated wastewater. In this study, a nitrilase gene (*nit*) isolated from a nitrile-degrading bacterium (*Rhodococcus rhodochrous* BX2) was cloned and transformed into a biofilm-forming bacterium (*Bacillus subtilis* N4) that expressed the recombinant protein upon isopropylthio-β-galactoside (IPTG) induction. The recombinant bacterium (*B. subtilis* N4-pHT01-*nit*) formed strong biofilms and had nitrile-degrading capability. Further testing demonstrated that biofilms formed by *B. subtilis* N4-pHT01-*nit* were highly resistant to loading shock from acetonitrile and almost completely degraded the initial concentration of acetonitrile (800 mg L^{-1}) within 24 h in a moving bed biofilm reactor (MBBR) after operation for 35 d. The bacterial composition of the biofilm, identified by high-throughput sequencing, in a reactor in which the *B. subtilis* N4-pHT01-*nit* bacterium was introduced indicated that the engineered bacterium was successfully immobilized in the reactor and became dominant genus. This work demonstrates that an engineered bacterium with nitrile-degrading and biofilm-forming capacity can improve the degradation of contaminants in wastewater. This approach offers a novel strategy for enhancing the biological oxidation of toxic pollutants in wastewater.

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1. Introduction

Acetonitrile is a water-soluble toxic substance that is often discharged in industrial and laboratory wastewater. Acetonitrile can be converted into toxic hydrogen cyanide and acetaldehyde in living organisms and is a significant threat to the health of both humans and livestock (Wexler, 2014; Greenberg, 1999). Therefore, untreated acetonitrile-containing wastewater discharged into a natural body of water can cause the death of aquatic organisms and damage to the ecological environment (Zhang and Jin, 1995; Pollak et al., 1991; Boadas-Vaello et al., 2007). A great deal of attention has been paid to microbial oxidation of toxic pollutants strategies for acetonitrile contamination through biodegradation, which is considered a low-cost, promising new technology due to microbe with high specific surface area, ubiquity and tolerance of a wide range of unfavorable environmental conditions (Sun et al., 2012;

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Kohyama et al., 2006). The microbial degradation of acetonitrile proceeds through two enzymatic pathways. One pathway is a combination of nitrile hydratase (NHase, EC 4.2.1.84) and amidase (EC 3.5.1.4). The other pathway is that of nitrilase (EC 3.5.5.1), in which nitrilase directly hydrolyzes acetonitrile to the corresponding carboxylic acids and ammonia (Banerjee et al., 2002).

In recent years, several acetonitrile-degrading microorganisms have been used to enhance the degradation of acetonitrile in wastewater treatment systems (Li et al., 2013; Kohyama et al., 2006). For example, the use of species such as *Rhodococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp. and *Nocardia* spp. has provided an effective approach for treating cyanide-containing wastewater (Dvořák et al., 2014; Li et al., 2013; Sorokin et al., 2007; Yamamoto and Komatsu, 1991; Okamoto and Eltis, 2007). However, because of the repeated replacement of wastewater, the high degradation rate of pollutants in wastewater treatment systems often cannot be maintained for extended periods due to the loss of the degrading microorganisms, which are washed out of the system or grazed on by protozoa. Therefore, it has been suggested that the immobilization of degrading microorganisms in wastewater treatment systems can be used as an efficient way to maintain high pollutant degradation rates. Different methods for immobilizing degrading microorganisms to improve the effectiveness of wastewater treatment have been developed. Wang et al. (2002) found that immobilizing *Burkholderia pickettii* in polyvinyl alcohol gauze (PVA-gauze) hybrid carriers enhances the biodegradation of quinoline in a membrane bio-reactor (MBR). Cruz et al. (2013) found an increase in degradation due to polymer Ca-alginate beads containing the microalgae *Chlorella vulgaris* jointly immobilized with *Azospirillum brasilense* in the tertiary treatment of municipal wastewater. However, this system is expensive, and operation of these methods is complex.

Biofilms are communities composed of bacterial cells adhering to inert or active entities and encased by a substrate that can be hydrated (Douterelo et al., 2014). Biofilm formation could be an ideal system for immobilizing degrading bacteria in wastewater treatment because biofilms provide a protective environment that shields bacteria from harm by toxic substances, adsorbs organic pollutants in wastewater and allows the pollutants to be degraded by the resident microorganisms. Therefore, inoculating specific contaminant-degrading and biofilm-forming bacteria into a moving bed biofilm reactor (MBBR) is an efficient method for treating pollutants in wastewater (Li et al., 2013; Yu, 2007; Qureshi et al., 2005). This approach provides a simple and inexpensive way to immobilize degrading bacteria within wastewater treatment systems. However, several issues with this approach still exist with respect to the appropriate combination of degrading bacteria and biofilm-forming bacteria. For example, biofilm-forming bacteria may not tolerate the high concentrations of pollutants in wastewater, and pollutant-degrading bacteria may not be fully immobilized in biofilms and may be washed out of the system. Furthermore, mutual growth inhibition may occur between biofilm-forming bacteria and degrading bacteria, and biofilm-forming bacteria simply mixed with degrading bacteria may not form a sufficiently large biofilm and may increase the complexity of operation (Li et al., 2008). Each of these problems reduces the efficiency of wastewater treatment. To our best knowledge, no studies have reported a single bacterium with the ability to high-efficiently form biofilms and degrade pollutants simultaneously. Therefore, constructing recombinant bacteria with the capacity for forming biofilms effectively and degrading pollutants and testing the practical application of the engineering bacterium in wastewater treatment are required.

The primary goal of this work was to clone the nitrilase gene (*nit*) from *Rhodococcus rhodochrous* BX2 (an acetonitrile-degrading bacterium isolated from soil) and transformed it into *Bacillus subtilis* N4 (Li et al., 2012, 2013), which possesses a strong biofilm-forming capacity. Nitrilase was successfully expressed in *B. subtilis* N4 upon induction. Biofilm formation and acetonitrile degradation by the engineered bacterium were investigated both in pure culture and in MBBR. The bacterial composition of the biofilm in MBBR was identified by high-throughput sequencing to determine the immobilization and the dominant acetonitrile degradation function of the engineered bacterium. The new knowledge obtained from this research provides an efficient strategy for improving pollutant removal in wastewater treatment systems.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and carrier type

R. rhodochrous BX2 was isolated from soil and found to have efficient acetonitrile-degrading capacity (Li et al., 2012). *B. subtilis* N4 was isolated from biofilms in a laboratory-scale acetonitrile wastewater treatment system and was found to have a strong biofilm-forming capacity (Li et al., 2013). An *Escherichia coli-B. subtilis* shuttle vector plasmid pHT01 was purchased from MoBiTec (Goettingen, Germany; Nguyen et al., 2007). *E. coli* DH5a competent cells were purchased from TaKaRa (Dalian, China). The acetonitrile utilization was tested in Mineral medium (mg L^{-1}): KH_2PO_4, 1700; Na_2HPO_4, 9800; $MgSO_4{\cdot}7H_2O$, 100; $CaCO_3$, 2; $ZnSO_4{\cdot}7H_2O$, 1.44; $FeSO_4{\cdot}7H_2O$, 0.9; $CuSO_4{\cdot}5H_2O$, 0.25; and H_3BO_3, 0.06. The MBBR reactors contain 10 L of synthetic wastewater (mg L^{-1}): acetonitrile, 800; glucose, 300; NH_4Cl, 40.1; KH_2PO_4, 9.2; $CaCl_2$, 10.7; $MgSO_4$, 8; and $FeSO_4$, 0.11 (Li et al., 2013). All chemicals were obtained from Aladdin Industrial Corporation (Shanghai, China). The carrier was high-density modified polyethylene (Dalian ShengYuan Water Treatment & Equipment Developing Co. Ltd., Dalian, China). The carrier was a cylinder with a diameter of 12 mm, height of 9 mm and density of 0.94–0.98 g cm^{-3}. The carrier had crossed sheets inside to divide the cylinder into four sectors. Its specific surface was approximately 600 m^2 m^{-3}. The filling volume of the carriers was 30% in the reactor.

2.2. Construction of pHT01-nit and B. subtilis N4 transformation and selection

The nitrilase gene (*nit*) was amplified from *R. rhodochrous* BX2 using the primer set *nit*-F, 5′-GCG*TCTAGA*ATGGTCGAATACACAAACAATTTC-3′ and *nit*-R, 5′-ATT*GACGTC*TCAGATGGAGGCTGTCGC-3′ (primer sequences were derived from the nitrilase gene sequence from *R. rhodochrous* BX2, GenBank accession JN255154.1; *Xba* I and *Aat* II restriction sites are marked in italics) and was ligated into pHT01. The expression vector was designated pHT01-*nit* (see Supplementary material). Clones were verified by DNA sequencing (BGL Beijing, China).

The pHT01-*nit* plasmid was electro-transformed into electro-competent *B. subtilis* N4 prepared using the method described by Biver et al. (2013). Transformants (designated *B. subtilis* N4-pHT01-*nit*) were selected on LB agar plates containing 5 μg mL^{-1} chloramphenicol after incubation at 30 °C for 2 d. The assay for plasmid stability was performed as described by Xiong et al. (2013) and Ma et al. (2011) (see Supplementary material).

2.3. Expression of nitrilase in B. subtilis N4

Recombinant *B. subtilis* N4-pHT01-*nit* cells were cultivated in LB broth supplemented with chloramphenicol (5 μg mL^{-1}) and grown at 30 °C. Recombinant protein expression was induced by the addition of 1 mM IPTG (Amresco LLC, Solon, OH, USA). Cell-free

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