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High-rate biodegradation and metabolic pathways of 4-chloroaniline by aerobic granules

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ABSTRACT

A high-rate degradation of 4-chloroaniline (4-ClA) was achieved by developing mixed-culture microbial granules under aerobic conditions in a sequencing airlift bioreactor (SABR). An essential step in this process was the enrichment of the biomass with improved setting characteristics and high 4-ClA degradation activity by manipulating combined hydraulic and microbial selection pressures via stepwise increases in 4-ClA loading. The seed sludge was conditioned over a 15-day acclimation period to allow the biomass to adapt to the presence of 4-ClA and the preferred short-settling time. Over the subsequent two months, aerobic granular sludge was developed by decreasing the settling time and gradually increasing the 4-ClA loading, and glucose/CH_3COONa was used to facilitate the growth of the 4-ClA-fed biomass. High specific degradation rates of more than 0.27 g gVSS^{-1} d^{-1} were sustained for 4-ClA concentrations above 400 mg L^{-1}. At concentrations as high as (8.18 ± 0.06) g L^{-1}, 4-ClA was completely removed, and the biomass concentration was maintained. Degradation kinetics for all tests was described well by the typical substrate-inhibition pattern predicted by the Haldane equation. The aerobic granule primarily degraded 4-ClA via the meta-cleavage pathway.

1. Introduction

Since the 1980s, environmental pollution from the uncontrolled synthesis of xenobiotic compounds has become a serious threat to the biosphere. Because microorganisms present in the natural environment are not able to easily catalyze and biodegrade xenobiotics, they progressively accumulate in the water, air and soil [1]. Conventional industrial wastewater treatment relies on chemical and physical processes such as adsorption and chemical oxidation/reduction, both of which achieve high removal efficiencies but fail to achieve full degradation without the production of intermediate substances. An alternative, promising approach is the application of biological treatments that could achieve complete mineralization of the compounds with low investment and operation costs. Nevertheless, biological plants are extremely complex systems that can be difficult to operate. Microorganisms are highly sensitive to reaction conditions, and the capability of the biomass to degrade new compounds depends on the survival and activity of the metabolically specialized microbes in the bioreactor [2].

Chloroanilines are important intermediates formed during the production of a wide range of synthetic organic chemicals and polymers, including polyurethanes, rubber additives, dyes, pharmaceuticals, pesticides and herbicides. They are highly toxic, readily absorbed through the skin in dangerous amounts, and fatal if swallowed or inhaled. The conventional continuously activated sludge systems that have been used for chloroaniline removal from wastewater for many years are sensitive to fluctuations in organic loading and fail to effectively degrade xenobiotic compounds because of their toxicity to the metabolizing bacteria [3–5].

Aerobic granules are self-immobilized, mixed-culture microbial aggregates that form without any substratum or supporting carrier material for biofilm attachment. Thus, they are suspended biofilms, and carrier materials and bulky settling devices are therefore unnecessary. Most aerobic granules have been cultivated on simple and relatively biogenic substrates such as glucose, acetate and alcohol, and the ability of these granules to degrade toxic chemicals at high rates remains poorly understood [6–12]. The major objective of the present work was to cultivate aerobic granules and evaluate their ability to degrade recalcitrant synthetic compounds such as 4-chloroaniline (4-ClA). An essential step in the development of the aerobic 4-ClA-degrading granules was to condition the activated sludge seed by enriching it in the desirable biomass with improved settleability and high 4-ClA-degradation activity. This study showed that the aerobic 4-ClA-degrading granules could be cultivated from conditioned sludge at a 4-ClA loading rate of 0.8 kg m^{-3} day^{-1} by using biogenic substrates to promote the rapid growth of active biomass. The results will be useful in understanding application of aerobic granule for the effective treatment of 4-ClA and other challenging organic compounds.

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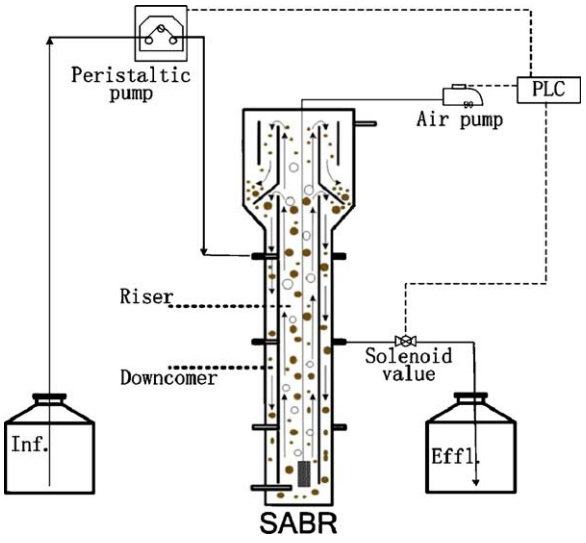

Fig. 1. Schematic diagram of the sequencing airlift bioreactor (SABR).

2. Experimental

2.1. Experimental setup

As shown in Fig. 1, the 5-L sequencing airlift bioreactor (SABR) used in this study (8 cm down-comer I.D., 64 cm riser height, 5 cm riser I.D.) was housed in a temperature-controlled room at 25 ± 2 °C and operated in 12-h cycles consisting of 20 min of influent filling, 865–870 min of aeration, 5–10 min of settling, and 5 min of effluent withdrawal. For aeration, fine air bubbles were supplied through a dispenser at the reactor bottom at a superficial gas velocity of 2.4 cm s^{-1}. Effluent was discharged at a volumetric exchange ratio of 70%, equivalent to a hydraulic retention time (HRT) of 17.2 h. The abiotic loss of 4-ClA in the SABR was negligible under identical operational conditions.

The SABR was first inoculated with 2.5 L of fresh activated sludge (equivalent to 7.8 g L^{-1} suspended solids) that was taken from a municipal wastewater treatment plant in Hangzhou, which treats conventional urban sewage. The initial reactor biomass concentration was about 4.0 g L^{-1}. The seed sludge was conditioned over a 15-day acclimation period to allow the biomass to adapt to the 4-ClA and to develop the short settling time required for successful granulation. During this period, the reactor was fed with synthetic wastewater containing 800 mg COD L^{-1} of glucose and CH_3COONa, and 5–10 mg L^{-1} of 4-ClA. After the 15-day acclimation period, the settling time in each cycle was reduced from the original 10 min to 5 min, and the 4-ClA concentration was increased stepwise to 400 mg L^{-1} by the end of the study.

The synthetic wastewater consisted of a buffered mineral salt medium (pH 7.5 ± 0.3) with the following composition (in mg L^{-1}): yeast, 0.0006; NH_4Cl, 120 (30 as N); KH_2PO_4, 44 (10 as P); $K_2HPO_4{\cdot}3H_2O$, 59; $CaCl_2{\cdot}2H_2O$, 23; $MgSO_4{\cdot}7H_2O$, 25; $FeSO_4{\cdot}7H_2O$, 20; $NaHCO_3$, 200 (120 as $CaCO_3$) and a trace mineral solution, 1.0 mL. The trace mineral solution was composed according to the method reported by Zhu et al. [13].

2.2. Analytical methods

During the aerobic granule-cultivation phase, raw samples from the SABR were periodically analyzed for COD, pH, free chloride, biomass concentration (mixed liquor suspended solids [MLSS] and volatile suspended solids [VSS]), sludge volume index (SVI), minimal settling velocity, and specific oxygen utilization rate (SOUR) in accordance with standard methods [14].

In order to quantify the 4-ClA, raw samples were centrifuged for 10 min at 12,000 × *g*, after which the supernatant liquid was filtered using 0.22-μm filter paper and analyzed by high-performance liquid chromatography (HPLC) using a Waters1525 binary HPLC pump, a Waters2487 dual λ-absorbance detector, and a Waters717 Plus autosampler (Waters, Milford, MA, USA). A ZORBAX Eclipse XDB-C18 column (4.6 mm × 150 mm I.D., 5 μm particle size; Agilent, USA) was used for the separation. Reversed-phase separations were performed using a mobile phase of water and methanol (30:70, v/v) flowing at 1.0 mL min^{-1}. The detection wavelength was set at 240 nm.

Freshly harvested granules were washed in a 50 mM phosphate buffer (pH 7.6) at least twice and recovered by centrifuging (2500 × *g*, 10 min) until a final concentration close to 5 g L^{-1} was reached in a 20-mL phosphate buffer. The sample was then placed in an ice bath and sonicated at 200 W (20 kHz) for 30, 3-s periods alternating with short, 1-s resting periods. Crude cell extracts were recovered by centrifuging (14,000 × *g*, 20 min) at 4 °C. Catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) activities were measured spectrophotometrically according to Nakazawa and Nakazawa [15]. C12O activity was measured by following the formation of cis,cis-muconate at 260 nm (silica cuvettes, 5 mm path length), and C12O activity was represented by the formation of hydroxymuconic semialdehyde at 375 nm. Reaction mixtures initially contained 33 mM Tris/HCl (pH 8.0), 3.9 mM Na-EDTA, and an appropriate amount of enzyme. After a 5-min incubation period at 30 °C, the reaction was started by adding catechol or 4-chlorocatechol to reach a final concentration of 0.5 mM. Protein was measured as described previously [16], using bovine serum albumin (BSA) as the standard. All assays were conducted in triplicate.

The morphology and grain size of the granular sludge were obtained by an image-analysis system (DMLB + QCOLite, Leica Cambridge Instruments). Prior to scanning electron microscopy (SEM), the specimens were gently washed with a phosphate buffer solution, fixed with 2.5% glutaraldehyde in the phosphate buffer (pH 7.0) for more than 4 h, and then post-fixed with 1% OsO_4 for 1 h. The fixed granules were dehydrated by a graded series of ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%). The dehydrated granules were dried with a liquid CO_2 Critical Point Dryer (HCP-2, Hitachi) and were finally observed with a Philips Model XL30 SEM.

The specific 4-ClA degradation rates for the aerobic granules were calculated from the 4-ClA degradation curves that were obtained from the experiments with a reaction volume of 500 mL and a range of 4-ClA concentrations up to 2000 mg L^{-1}. The reaction system was kept at 30 °C and shaken in an orbital shaker at 200 rpm. Target compound depletion (as measured by HPLC) and Cl^- ion liberation (measured spectrophotometrically) were used as indicators of substrate utilization and were periodically measured in duplicate to ensure data quality. A kinetic analysis of the degradation data was performed on the basis of Haldane's equation for an inhibitory substrate: $V = V_{max}{\cdot}S/[K_s + S + (S^2/K_i)]$, where V and V_{max} are the specific and the maximum specific substrate degradation rates (mgClA $gVSS^{-1}$ h^{-1}), respectively, and S, K_s and K_i are the substrate concentration, half-saturation constant, and inhibition constant (mgClA L^{-1}), respectively.

3. Results

3.1. Cultivation of aerobic 4-ClA-degrading granules

Fig. 2 shows the SABR performance during the entire acclimatization process. The seed sludge was first acclimated for 15 days to allow the biomass to adapt to both the 4-ClA with biogenic substrate and the short settling times required for successful granulation. After 15 days, stable operating conditions were achieved, including a biomass concentration of 1.82 gVSS L^{-1}, an SRT of 1.7 days, and residual 4-ClA and COD levels in the effluent below 0.5 mg L^{-1} and 150 mg L^{-1}, respectively. Microscopic observation showed that the seed sludge had a morphology consisting of fluffy, irregular and loose flocs. Immediately following inoculation, the biomass concentration decreased to 1.07 gVSS L^{-1}; after about two weeks of SBR operation, it stabilized at 1.82 gVSS L^{-1}. Aerobic granular sludge was first observed in the SABR as small spherical particles dispersed within amorphous sludge flocs (Fig. 4). The SVI decreased to 72 mL g^{-1} on the 15th day of reactor operation, indicating the transformation of seed flocs into granules. The average granule size increased from 0.14 mm to 0.38 mm in the initial sludge, the minimal settling velocity of sludge increased from less than 1 m h^{-1} to 8.1 m h^{-1}, and SOUR values increased from about 8 mg $gVSS^{-1}$ h^{-1} to 26.5 mg $gVSS^{-1}$ h^{-1} (Table 1).

In the second phase of the experiment, the biomass was acclimated to the 4-ClA. The influent 4-ClA concentration was progressively increased from 10 mg L^{-1} to 400 mg L^{-1} while the total COD was maintained at 1000 to 1800 mg L^{-1}. The sludge presented very good settleability characteristics throughout the experimental period. The granules eventually grew to become the dominant form of biomass in the reactor, as evidenced by the gradual increase in mean biomass size and the decrease in SVI after 30 days (Fig. 3). The biomass concentration in the reactor generally showed an upward trend with the exception of the week after day 16, when the settling time was reduced to 5 min. The biomass concentration plateaued at 6.42 gVSS L^{-1} beyond day 60. Despite the fact that the influent 4-ClA concentration gradually increased to 400 mg L^{-1}, the effluent concentration was less than 0.5 mg L^{-1} during the first several weeks and further declined to below 0.01 mg L^{-1} beyond day 60. The effluent COD concentration decreased to below 100 mg L^{-1}, which probably represented soluble organic matter from biomass