Removal of Ammonium and 4-Methylphenol from Synthetic Wastewater by Cell-Free Extracts of Nitrifying Sludge

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Abstract The feasibility of using C•ell-free extracts of nitrifying sludge to treat synthetic wastewater containing 4-methylphenol and ammonium was ex-amined. Nitrifying C•ells were broken by sonication and encapsulated into calcium alginate. Cell-free extracts (CFE) of nitrifying sludge oxidized 4- methylphenol threefold faster than whole-cells, but CFE were not able to oxidize ammonium. The CFE encapsulated into calcium alginate (CFEA) displayed partial nitrification and 4- methylphenol oxidation. Five hours was enough to oxidize 100 % of ammonium and 4-methylphenol, at volumetric rates of 20.80 mg N/L h and 42.86 mg C/L h, respectively. It isinferred that an interaction between the CFE and calcium alginate resulted in the protection of the enzymes.

Keywords 4-Methylphenol - Ammonium•Alginate•Kinetic

1 Introduction

The removal of industrial waste with high ammonium and organic matter content was considered as a major problem for waste purification technologies. Phenols and nitrogen compounds are important industrial chemicals of environmental concern since they are in-volved in many industries such as coke, refineries, man-ufacturers of resin, pharmaceuticals, pesticides, dyes, plastics, explosives, and herbicides and can also occur in their wastewaters (EI-Ashtoukhy et al. 2013). The nitrogen cycle has been modified through increases of fossil fuel combustion from vehicles and industry, fer-tilizer production, and application to the land surfac.e and livestock operations (Sheibley et al. 2014). For instance, ammonium is part of this cycle and can cause eutrophication and other health troubles (G,tsiorowski and Sienkiewicz 2013). The major part of wastewaters polluted with phenolic compounds such as 4-methylphenol ***(4-mp)*** derives from petrochemical indus-try. For example, in sour water it is common to find cresols above 0.06 and 0.71 g/L of ammonium nitrogen (Olmos et al. 2004). 4-Methylphenol is extremely toxic and corrosive and causes nervous system depression (Schepers et al. 2007). Therefore, ammonium and phe-nolic compounds need to be eliminated from wastewater before the water is discharged into the aquatic environ-ment. Physicochemical methods for phenol removal from industrial wastewaters include membrane filtra-tion, coagulation/ flocculation, ion exchange, electroly-sis, and advanced oxidation processes, among others. All these methods suffer from serious shortcomings due to limited eff ectiveness (Radushev et al. 2008; Qayyum

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et al. 2009). Biological processes use the natural metab-olism to break down and remove pollutants from wastewaters. Phenolic compounds and ammonium can be removed via these biological processes (Hanaki et al. 1990; Beristain-Cardoso et al. 2009; Racz et al. 2010; Silva et al. 2011); however, the main drawbacks are the inhibitors present in the wastewaters which can suppress the growth of the culture and the expression of enzymes involved in the degradation of these compounds (Qayyum et al. 2009).

The enzymatic treatments have been proposed as another strategy to remove phenolic compounds from wastewaters. For instance, peroxidases have strong po-tential utility for decontamination of phenol-polluted wastewater; however, large-scale use of the enzymes for phenol removal requires a source of cheap, abundant, and easily accessible peroxidase-containing mate-rial (Kurnik et al. 2015). Ammonium can be removed by cell-free extracts of *Nitrosomonas eutropha* (Schmidt and Bock 1998). Ammonium oxidation is initiated by the ammonia monooxygenase (AMO), which oxidizes ammonia to hydroxylamine. The resulting hydroxyl-amine is subsequently oxidized to nitrite by the hydrox-ylamine oxidoreductase (HAO). The AMO has not been purified in the active state, and many attempts were performed to prepare active cell-free extracts of *Nitrosomonas europaea* under oxic conditions (Susuki et al. 1981).

The "enzyme encapsulation" has shown to be a feasi-ble technology to treat wastewaters, due to the protection of the enzymes. The main advantages according to Arroyo et al. (1998) are as follows: (a) higher enzymatic stability, (b) enzymatic reuse, and (c) to design enzymatic reactors of easy operation. Karam and Nicell (1997) noted other potential advantages: (a) application to a broad range of compounds, (b) high concentrations of contam-inants, (c) no shock loading effects, (d) no delays associ-ated with biomass acclimatization, and (e) reduction in sludge volume. It is important to note that there is scarce information evaluating the kinetic behavior of cell-free extracts to remove simultaneously phenolic compounds and ammonium. Nevertheless, further work is required to kinetically characterize the ability of the enzymes obtain-ed from nitrifying culture for oxidizing inhibitory com-pounds such as 4-methylphenol and ammonium and eval-uate the accumulation of intermediates. In this context, the goal of this paper was to evaluate the capacity of cell-free extracts encapsulated in calcium alginate to oxidize simul-taneously ammonium and 4-methylphenol.

2 Materials and Methods

2.1 Reactor Performance and Culture Medium Composition

A microbial consortium coming from a laboratory-scale nitrifying sludge reactor was used for inoculating the continuous stirred tank reactor (CSTR) of 5 L. The reactor was operated over a period of 3 months at temperature of 25±0.2 °C, with hydraulic retention time of 2.5 days and 3.5 g VSS/L. The pH was automatically controlled (acid-base) at 7.0±0.3. The dissolved oxygen concentration was kept inside the reactor at 4.0±0.3 mg/L. The bioreactor was fed in average at loading rates of 137±16 NH_4^+-N/L day. The basal mineral medium was composed of (g/L) K_2HPO_4 (1.2), KH_2PO_4 (0.8), and $NaHCO_3$ (2.0), and trace element solution was supplied at 1 mL/L. The trace element solution contained (g/L) EDTA (0.05), $CuSO_4 \cdot 5H_2O$ (0.015), $CaCl_2 \cdot 2H_2O$ (0.07), $MnCl_2$ (0.03), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.015), $FeCl_3$ (0.015), and $MgCl_2$ (0.02). Sludge samples taken from the CSTR were washed with saline solution to remove fine particles before using as inoculum in the batch assays.

2.2 Effect of Sonication Time and Sample Volume over Protein Releasing

A volume of 210 mL of nitrifying sludge was taken from CSTR at steady state, and it was washed three times with mineral medium. After that, the cell wall was broken by sonication (Sonicor Instrument Corporation-Copiague, N.Y., model number SC-100; volts 110/ 120 V; cycles 50/60 Hz, AMPS). Two sample volumes (50 and 20 mL) were evaluated at 60, 30, and 13 s of sonication time. Liquid sample was centrifuged at 4500 rpm for 6 min (Solbat Centrifuge). One milliliter of supernatant was taken in order to quantify the soluble protein by the Lowry method.

2.3 Batch Assays with Whole-Cells and Cell-Free Extracts

Batch assays were conducted in 250 mL Erlenmeyer flasks containing 200 mL of mineral medium, with the same culture medium and environmental conditions above pointed out. The flask was spiked with 70 mg/L of NH_4^+-N and 100 mg/L of 4-methylphenol-C. The batch assays were inoculated approximately with

500 mg protein/L (approx. 0.6 g VSS/L) of nitrifying sludge. The liquid medium was continuously aerated, achieving dissolved oxygen (DO) concentration of 3.5± 0.3 mg/L. The initial and final pH for all batch assays was of 7.0±0.5. For the experiments using the cell-free extracts, Erlenmeyer flasks of 250 mL were inoculated with 50 mL of the CFE obtained at different sonication times (60, 30, and 13 s) and then were filled up to 200 mL of mineral medium. The initial concentrations tested were 100 mg 4-*mp*-C/L and 70 mg NH_4^+-N/L. The batch assays were evaluated for a period of 8 h, and under these environmental conditions, DO was of 3.0± 0.5 mg/L, temperature of 25±3 °C, and pH of 7±0.2. The Gompertz model was used in order to calculate the volumetric rates ((q=0.368 Ak/(g VSS/L)) (González-Blanco et al. 2012). The coefficient of determination was higher than 0.94 for all cases.

2.4 Batch Assays with Cell-Free Extracts Encapsulated in Calcium Alginate

The cell-free extract encapsulation was carried out ac-cording to the methodology proposed by Nam Sun Wang (2010). Calcium alginate was dissolved in water at a temperature of 60 °C. Palette knife was used to homogenize the calcium alginate in the water. The dis-solution process was slow, so it took around 3 h to prepare a solution of alginate gel (6 % *w*/*v*). The gel was placed in an ice bath for later use. The cell-free extracts (supernatant) were mixed with calcium alginate, and the beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess (200 mL) of stirred 0.1 M $CaCl_2$ solution, by using a syringe and needle at room temperature. Fifty milliliters of the sodium alginate beads was taken and inoculated into 250-mL Erlenmeyer flasks. The initial substrate concentrations tested were 100 mg/L of 4-*mp*-C and 70 mg/L of NH_4^+-N. The reaction time was 8 h, DO of 4±0.5 mg/L, temperature of 25±0.3 °C, and pH of 6.8 ±0.2.

2.5 Analytical Methods

Nitrate and nitrite were determined by ion chromatog-raphy (Beckman Coulter, ProteomeLab PA 800). The buffer was prepared with 5 mL of Na_2SO_4 (0.1 M), NaCl (10 mM), and a commercial solution CIA Pak OFM anion-BT (Waters) plus 35 mL of deionizer water. A microcapillary of melted silica (60 cm long and

75 mm internal diameter) was used. The absorbance was measured in the ultraviolet region using a mercury lamp at 254 nm and 25 °C. Ammonium was determined by selective ammonium electrode (pHoenix electrode Co., model NH331501). The 4-methyphenol, benzalde-hyde, *p*-hydroxybenzoate, and *p*-hydroxybenzaldehyde were analyzed by HPLC with a C-18 reverse-phase column (Sigma-Aldrich, USA), column size 300 mm× 3.90 mm. A mixture of acetonitrile/deionized water (60:40, *v*/*v*) was used as the solvent, and the flow rate was maintained at 1.5 mL/min (Perkin Elmer Series 200, USA). For soluble protein, biomass sample was centrifuged (1200*g*) for 3 min and supernatant was analyzed (Lowry et al. 1951).

3 Results and Discussion

3.1 Reactor Performance

Experimental results showed that CSTR achieved grad-ually high ammonium conversion to nitrate, being the ammonium removal higher than 95 % at steady state (Fig. 1). Ammonium was oxidized to nitrate, with Y_{NO3} of 1.0 ±0.03 mg NO_3 -N/mg NH_4^+-N consumed. At steady state, nitrate yield value did not significantly change (variation coefficient below 10 %). Thus, the profile was clearly nitrifying. Therefore, aerobic granu-lar sludge was metabolically stable and suitable to be used as an inoculum source for the enzymatic studies.

3.2 Process of Cell-Free Extraction

Table 1 shows the released protein based on the sample volumes (20 and 50 mL) and three sonication times (60, 30 and 13 s). In 60 s, the released protein was lower than 30 s of sonication time; it might be due to the long time of sonication which damaged the released protein. Turkey's statistical test (α=0.05) was used to analyze the different treatments. Statistical analysis indicated a significant difference between both volumes, showing that 50 mL was the better treatment to separate the protein from the biomass. On the other hand, prelimi-nary controls with CFE sonicated at 13, 30, and 60 s were performed in order to evaluate the enzymatic ca-pacity to oxidize 4-*mp*. At 13 and 30 s of sonication time, CFE did not oxidize 4-*mp*, whereas with 60 s of sonication, the CFE oxidized 4-*mp*. Therefore, the en-zymatic capacity of cell-free extracts for oxidizing 4-

methylphenol and ammonium was evaluated with 50 mL of sample previously sonicated at 60 s.

3.3 Performance of Cell-Free Extracts on Ammonium and 4-*mp* Removal

The kinetic behavior of nitrifying sludge (whole-cells) produced in steady state was evaluated for 10 h in batch cultures (Fig. 2). Ammonium was oxidized to nitrate, with consumption efficiency of 99±2 % and Y_{NO3} of 1.02 mg NO_3 -N produced/mg NH_4^+-N consumed. Nitrite was transitorily formed, but it was totally oxi-dized to nitrate at the end of batch cultures. The ammo-nium consumption volumetric rate was of 12.03± 1.2 mg NH_4^+-N/L h. The cell-free extracts did not show enzymatic activity for ammonium oxidation since it did not change in the evaluated period (Fig. 3a). AMO is the protein that catalyzes the oxidation of ammonium to hydroxylamine, the first step of nitrification. However, this enzyme has not been purified in active state, and one of the most serious problems associated with the characterization of this enzyme in cell-free extracts has been the chemical instability (Ensign et al. 1993; Moir et al. 1996).

The phenolic compound (4-*mp*) was oxidized in the evaluated period by the whole-cells, detecting *p*-OH-benzoate as the transitory intermediate (Fig 3b). 4-*mp* was completely mineralized since soluble organic car-bon was not detected as residual at the end of the batch assays. The cell-free extracts also oxidized 4-*mp*, but the intermediate detected was *p*-OH-benzylalcohol. For in-stance, in heterotrophs, 4-methylphenol is oxidized to *p*-OH-benzylalcohol by the "*p*-cresolmethyhydroxylase" enzyme, which degrades 4-*mp* via protocatechuate ortho pathway (Joesaar et al. 2010). This enzyme consists of two subunits to form an $\alpha_2\beta_2$ complex: the α subunits contain an active site FAD covalently linked to a tyro-sine residue, while the β subunit is a *c* type cytochrome (Mclntire et al. 1981).

Batch assays with granular sludge (whole-cells) re-moved the phenolic compound for 8 h, at volumetric rate of 26.52 mg C/L h. In batch assays with cell-free extracts, 4-methylphenol was totally consumed for 6 h, at volumetric rate of 83.2 mg C/L h. Therefore, cell-free extracts oxidized 4-methylphenol threefold faster than whole-cells. This kinetic behavior might be associated

owing to the missing of the cell wall, improving the contact between enzyme and substrate.

3.4 Performance of Cell-Free Extract Encapsulation on Ammonium and 4-*mp* Removal

Figures 3a and 4a show the time course of nitrogen by using cell-free extracts encapsulated in calcium alginate (CFEA). Ammonium was partially oxidized to nitrite, with consumption efficiency of 100 % and nitrite yield of 0.99 ±0.02 mg NO_2 -N produced/mg NH_4^+-N con-sumed. The ammonium was oxidized at volumetric rate of 21±3.5 mg NH_4^+-N/L h, showing that CFEA oxi-dized ammonium 1.8-fold faster than whole-cells. Nitrate was not detected, suggesting that nitrite reduc-tase enzyme lost activity during the sonication proce-dure. Nonetheless, the AMO and hydroxylamine oxide-reductase kept the activity. The CFEA provided greater

hydroxylamine and (b) hydroxylamine oxide-reductase enzyme which oxidizes HA to nitrite. The batch assays were repeated for three times, obtaining the same kinetic pattern, suggesting the enzymatic stability.

The phenolic compound was oxidized in a short time, 3 h, with consumption efficiency of 100 % (Figs. 3b and 4b). 4-Methylphenol was oxidized at volumetric rate of 20.8 mg C/L h. Four intermediates such as *p*-OH-benzylalcohol, *p*-OH-benzoate, *p*-OH-benzaldehyde, and benzaldehyde were identified. The presence of these intermediates suggested several enzymes such as methylhydroxylases, dehydrogenases, and hydroxylases. According to our knowledge, this is the first time reporting "benzaldehyde" as an intermediate of the 4-*mp* oxidation pathway. The cell-free extracts displayed the best activity than whole-cells and CFEA (Fig. 5). The CFEA presented half of the activity regarding CFE; perhaps this diminishing might be due to the alginate structure, affecting the substrate transport. Nonetheless, cell-free extracts encapsulated in calcium alginate oxidized 4-methylphenol 1.6-fold faster than whole-cells.

On the other hand, the catabolism of 4-methylphenol is conducted by three biochemical pathways: protocatechuate, catechol, or gentisic acid, which can be oxidized later to compounds that enter to the citric acid cycle, being the final product CO_2 (Harwood et al. 1996). These biochemical pathways depend on the metabolic capacity of each microorganism. For instance, in the present work, with the phenolic intermediates identified in the cell-free extracts encapsulated in calcium alginate, the possible route followed was the protocatechuate acid ortho-cleavage. In this pathway, *p*-cresolmethyhydroxylase (PCMH) converts 4-*mp* to *p*-OH-benzylalcohol and later to *p*-OH-benzaldehyde, which is subsequently oxidized to *p*-OH-benzoate by *p*-OH-benzaldehyde dehydrogenase (POB) (Cunane et al. 2000; Jõesaar et al. 2010). Benzaldehyde, the phenolic intermediate identified in the present work, was includ-ed in the pathway (Fig. 6).

Finally, the cell-free extracts encapsulated in calcium alginate improved significantly the kinetic aspect than whole-cells. A packed reactor with cell-free extracts encapsulated in a polymeric matrix might be a feasible technology for the pre-treatment of wastewater polluted with ammonium and phenolic compounds, producing phenolic intermediates and nitrite, which can be re-moved in a denitrifying reactor, being the products CO_2 and N_2. Thus, the global reaction time might be diminished and the sludge production minimized.

4 Conclusions

A continuous aerobic reactor was stabilized under nitri-fying conditions with ammonium consumption efficien-cy of 100 % and nitrate yield of 1.0±0.03 mg NO_3 -N/mg NH_4^+-N consumed. In batch assays, cell-free ex-tracts encapsulated in alginate oxidized 4-methylphenol 1.6-fold faster than whole-cells. This is the first study to demonstrate that cell-free extracts encapsulated in calci-um alginate show the enzymatic capacity to carry out the ammonium oxidation linked to 4-methylphenol oxida-tion, and this research might be used as an aerobic pre-treatment coupled to a denitrifying process, in order to biotransform phenolic and nitrogen compounds into substances not toxic for the environment.

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