Water Research 102 (2016) 90–95

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

The integration of cyanide hydratase and tyrosinase catalysts enables effective degradation of cyanide and phenol in coking wastewaters

Ludmila Martínková*, Martin Chmátal

Laboratory of Biotransformation, Institute of Microbiology of the Czech Academy of Sciences, CZ-142 20 Prague 4, Czech Republic

ARTICLE INFO

Article history:
Received 8 February 2016
Received in revised form
31 May 2016
Accepted 6 June 2016
Available online 8 June 2016

Keywords:
Cyanide hydratase
Tyrosinase
Cyanide
Phenol
Cresols
Coking wastewater

ABSTRACT

The aim of this study was to design an effective method for the bioremediation of coking wastewaters, specifically for the concurrent elimination of their highly toxic components – cyanide and phenols. Almost full degradation of free cyanide (0.32–20 mM; 8.3–520 mg L^{-1}) in the model and the real coking wastewaters was achieved by using a recombinant cyanide hydratase in the first step. The removal of cyanide, a strong inhibitor of tyrosinase, enabled an effective degradation of phenols by this enzyme in the second step. Phenol (16.5 mM, 1,552 mg L^{-1}) was completely removed from a real coking wastewater within 20 h and cresols (5.0 mM, 540 mg L^{-1}) were removed by 66% under the same conditions. The integration of cyanide hydratase and tyrosinase open up new possibilities for the bioremediation of wastewaters with complex pollution.

1. Introduction

The release of cyanide from e.g. mining industries and coal coking presents a challenge for the development of new environmental technologies. The treatment of cyanide-containing wastewaters has been examined using various physical, chemical and biological means or combinations of them (Pal and Kumar, 2014; Papadimitriou et al., 2009). Chemical oxidation with chlorine or hypochlorite is one of those most widely used. However, this method has significant disadvantages such as the special requirements for waste disposal, high consumption of the noxious chemical agent and potential formation of chlorinated organic compounds (Huertas et al., 2010). The use of activated sludge (Papadimitriou et al., 2006, 2009) or other microbes (Ebbs, 2004) is more eco-friendly. However, the complex pollution of the wastewaters, primarily high concentrations of cyanide and phenol in coking effluents, may impair the viability and activity of microorganisms (Papadimitriou et al., 2009; Sharma and Philip, 2014). Therefore, cyanide-hydrolyzing enzymes, which are environmentally benign, selective and resistant to high concentrations of cyanide, are an interesting alternative. Cyanide hydratases (CHTs; EC 4.2.1.66) are especially attractive due to their extraordinarily high specific activities (V_{max} in order of 10^3 U mg^{-1}), robustness and no need for cofactors (for reviews, see Gupta et al., 2010; Martínková et al., 2015). These enzymes originate from filamentous fungi but can be easily expressed in *Escherichia coli* in high yields (Basile et al., 2008; Rinágelová et al., 2014). Some of them were examined in the elimination of cyanide from industrial electroplating-bath samples (Basile et al., 2008). However, as far as we know, CHTs have not been used for eliminating cyanide from coking effluents. Therefore, a CHT which was recently overproduced in *E. coli* (Rinágelová et al., 2014) was examined in this work for its potential to remove cyanide from these wastewaters.

Phenols are additional coking wastewater components with a strong negative environmental impact. Tyrosinase (TYR; EC 1.14.18.1), which has been mostly obtained from the common button mushroom, or laccase (LAC; EC 1.10.3.2), were examined for the degradation of various phenolic pollutants (for a review see Martínková et al., 2016). The use of immobilized LAC to remove phenols from coking wastewaters was demonstrated by Wang et al. (2012). TYR was used, e.g., to eliminate phenols from olive oil production wastewaters (Pigatto et al., 2013) or BPA from river waters (Kampmann et al., 2014). The possibility of using TYR to dephenolize two different industrial wastewaters (from the production of triarylphosphates and coke) was briefly mentioned by Atlow et al. (1984) but the application of this method to the

* Corresponding author.
E-mail address: martinko@biomed.cas.cz (L. Martínková).

http://dx.doi.org/10.1016/j.watres.2016.06.016
0043-1354/

remediation of coking wastewaters has not been studied further. Therefore, the potential of TYR for phenol degradation in this type of wastewaters was examined in this study.

Moreover, CHT and TYR were combined in this work, aiming to design an effective two-step method for a complex treatment of coking wastewaters. The first step consists of the enzymatic removal of cyanide by CHT. The elimination of cyanide was expected to alleviate the inhibition of TYR - a metalloenzyme containing two Cu atoms - by cyanide, which is a strong inhibitor of this enzyme with a K_i of ca. 0.14 mM (Gasparetti et al., 2012) due to the complexation of Cu by cyanide. Actually, in this way an effective degradation of phenols by TYR was enabled in the second step. Thus two of the most toxic components of coking wastewaters are almost fully removed by this bienzymatic process.

2. Methods

2.1. Materials

Coking wastewater samples were kindly provided by Třinec Iron and Steel Works, Třinec, Czech Republic (Dr. S. Czudek). The sample was withdrawn after the ammonia removal step and its properties were as follows: pH 7.4, phenol 16.5 mM (1,552 mg/L), cresols 5.0 mM (540 mg/L) and free cyanide (CN^-) 0.32 mM (8.3 mg/L) as determined by the analytical methods described below (Section 2.5). Other chemicals were from standard commercial sources and of the highest grade of purity. CHT was produced and optionally purified as described previously (Rinágelová et al., 2014). Commercial TYR was obtained from Sigma. Alternatively, TYR was produced from the fruiting bodies of *Agaricus bisporus* (purchased at a local market). The method described by Zynek et al. (2010) was slightly modified. Briefly, the biomass (350 g of wet weight) was homogenized in acetone (800 mL) at 4 °C for 30 s. After removing acetone by filtration, the crude homogenate was stored at −20 °C until use.

2.2. Enzyme assays

The CHT activity was assayed as described previously (Rinágelová et al., 2014). One unit of CHT activity was defined as the amount of enzyme which catalyzed the transformation of 1 μmol of CN^- under the assay conditions (pH 8.0; 30 °C). The activity of TYR was determined with 6.7 mM phenol at pH 7.4 (Tris/HCl, 50 mM) and 25 °C. The samples were centrifuged and the substrate concentrations were monitored in the supernatants by HPLC (Section 2.5). One unit of TYR activity was defined as the amount of enzyme which catalyzed the transformation of 1 μmol of phenol under the assay conditions.

2.3. Biodegradation by cyanide hydratase

CHT (5 or 20 U, *i.e.* 3.85 and 15.4 μg of protein, respectively) was added to model mixtures (2-mL total volume) containing 50 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl and 6–20 mM KCN. Optionally, Na_2S, KSCN or phenol were each added to a 5 mM final concentration. Incubation proceeded for 5 min at 35 °C with shaking (Thermomixer Eppendorf, 750 rpm). Alternatively, the buffer was replaced with the basic synthetic wastewater (BSW; Papadimitriou et al., 2006) without glucose containing [mM] sodium acetate 24, NaCl 1.7, $CaCl_2$ 0.34, KCl 0.27, NH_4Cl 11.2 and K_2HPO_4 1.5. Optionally, mixtures of KSCN and phenol or KSCN, phenol and Na_2S were added to final concentrations of 2, 8.5 and 5 mM of KSCN, phenol and Na_2S, respectively. Incubation proceeded for 5 min under the above conditions. In real coking wastewaters (2-mL total volume), the initial concentration of free cyanide was adjusted to 10–20 mM, CHT (20 U mL^{-1}) was added and incubation proceeded for 5–20 min as described above. The samples were centrifuged and the concentrations of cyanide and optionally formamide were monitored in the supernatants by spectrophotometry and HPLC, respectively (Section 2.5). The cyanide and formamide concentrations were calculated as the means of two independent experiments with SD < 15%.

2.4. Biodegradation by cyanide hydratase and tyrosinase

CHT (20 U mL^{-1}) was added to model mixtures (2-mL total volume) containing 50 mM phosphate buffer (pH 8.0), 5 mM phenol and 0.1 or 1 mM KCN. Incubation proceeded for 10 min at 35 °C with shaking (Thermomixer Eppendorf, 750 rpm), and the concentration of cyanide was monitored (Section 2.5). Alternatively, model mixtures were replaced with coking wastewater (diluted 1:1 or undiluted) and the reactions were carried out under the same conditions. Various amounts of TYR were then added and incubation was continued at 25 or 35 °C for 2–20 h. Optionally, CHT and TYR were added at the same time. Following sample centrifugation, the phenol and cresol concentrations were monitored in the supernatants (Section 2.5) and calculated as means of two independent experiments with SD < 15%. Experiments without CHT served as controls.

2.5. Analytical methods

The concentrations of phenol and cresols were determined by HPLC under the following conditions: Chromolith Speed ROD column, RP-18e, 50 × 4.6 mm (Merck); mobile phase: 20% acetonitrile, 0.1% H_3PO_4; flow rate 2.0 ml min^{-1}; phenol: RT = 1.31 min, *o*-, *m*-, *p*-cresol: RT = 2.26 min (isomers not separated); detection at 210 nm; 34 °C. The concentration of cyanide was determined spectrophotometrically by the picric acid method (Basile et al., 2008). The concentration of formamide was determined by HPLC as described previously (Kaplan et al., 2013).

3. Results and discussion

3.1. Effect of coking wastewater components on cyanide degradation

The potential effects of coking wastewater components were tested with purified CHT in the buffer containing 20 mM (520 mg L^{-1}) cyanide. The degree of cyanide degradation was ca. 78% and 90% with 5 and 20 U of CHT mL^{-1}, respectively, after 5 min in the control experiments without additives. The examination of the individual components (5 mM sulfide, thiocyanate or phenol) indicated no substantial effects of these compounds on the degree of cyanide degradation, which was not decreased by more than ca. 7% in any experiment (*not shown*). In the model wastewater void of phenol, thiocyanate and sulfide (BSW; Section 2.3), a cyanide (6 mM, 156 mg L^{-1}) degradation of ca. 72% was achieved with 5 U of CHT mL^{-1} within 5 min. The addition of thiocyanate (2 mM) and phenol (8.5 mM) caused almost no change. The use of BSW containing sulfide (5 mM) resulted in a decrease in the cyanide degradation to ca. 60% with the same amount of CHT. However, higher concentrations of sulfide may cause erroneously increased results of cyanide concentration determined by the picric acid method (Fisher and Brown, 1952). In any case, the testing of the model mixtures suggested that CHT was functional in the presence of the typical components of coking wastewaters.