Soybean Peroxidase-Catalyzed Removal of an Aromatic Thiol, 2-Mercaptobenzothiazole, from Water

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ABSTRACT: This paper demonstrates, for the first time, the capability of soybean peroxidase (SBP), an enzyme, for catalyzing the removal of an aromatic thiol, 2-mercaptobenzothiazole (MBT), from aqueous solution.

The optimum pH for enzymatic conversion of MBT in aqueous buffer was found to be in the range 6.0 to 9.0. The optimum hydrogen peroxide (H_2O_2):MBT stoichiometry was 0.6. In terms of standard units (U) of catalytic activity, the minimum SBP concentration required for 95% conversion of 1.0 mM MBT in 3 hours was found to be 0.9 U/mL. The presence of polyethylene glycol at 50 mg/L can reduce the enzyme concentration required for the same conversion by 3-fold.

It is proposed that these findings should be the basis for viable and cost-effective treatment of MBT in industrial wastewater and/or process water. *Water Environ. Res.*, **82** (2010).

KEYWORDS: soybean peroxidase, 2-mercaptobenzothiazole, polyethylene glycol, wastewater, hazardous waste.

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Introduction

2-Mercaptobenzothiazole (MBT) is a heterocyclic aromatic compound produced in large amounts for various industries. It is toxic (TOXNET, 2009); therefore, its release to the environment is regulated. The U.S. Environmental Protection Agency's (Washington, D.C.) Toxics Release Inventory (U.S. EPA TRI) reported that 85 407 kg (188 121 lb) of MBT were disposed on-site and off-site in 2008, excluding fugitive air emissions, point-source air emissions, and surface-water discharges (U.S. EPA, 2010). These on- and off-site disposals, to the extent that they occur in industrial wastewater and process water, are the eventual target of the current investigation.

Studies have been conducted to find treatment methods for the removal of MBT, taking into account that low concentrations of MBT can hamper conventional wastewater treatment and inhibit degradation of easily degradable organics and wastewater nitrification (Reemtsma et al. 1995). Various waste treatment methods have been proposed for removal of MBT, such as activated sludge (Gaja and Knapp, 1998), ozone oxidation (Fiehn et al., 1998), iron oxides (Liu et al., 2006), and photocatalytic oxidation (Habibi et al., 2001; Li et al., 2004, 2006; Malouki et al., 2004). Because none of these is perfect, it is suggested here that enzyme-based oxidative polymerization also be considered.

Such enzymatic treatment is efficient in removing various aromatic group classes, such as phenols and aromatic amines, from wastewater (Al-Kassim et al., 1994; Caza et al., 1999; Ibrahim et al., 2001; Klibanov et al., 1980; Modaressi et al., 2005; Patapas et al., 2007; Steevensz et al., 2009). Enzymatic treatment is potentially applicable to an aromatic thiol, such as MBT, because conversion of thiophenols by laccase-like enzymes has been reported (Bocks, 1966; Xu, 1996). An enzyme-based method has several advantages over other treatment methods, as a result of the enzyme active-site's high specificity for the target pollutant, high efficiency in pollutant removal, lower cost, and ease of handling and storage of the enzyme (Ibrahim et al., 2001; Steevensz et al., 2009).

Oxidoreductases of relevance here catalyze the oxidization of phenols and anilines into aromatic radicals in the presence of hydrogen peroxide for peroxidases or oxygen for laccases. Those radicals diffuse from the active site of the enzyme into solution, where they couple non-enzymatically to form dimers (Dunford, 1999). If the dimers are soluble and still phenolic or anilino, then they become substrates of the enzyme for another enzymatic cycle, forming higher oligomers. The cycle continues until the polymer generated reaches its solubility limit and precipitates out of solution, to be removed later by filtration or sedimentation. Thus, the process is a remediation, but the opposite of a degradation or mineralization. Here, the extent to which an aromatic thiol, MBT, behaves analogously to phenols and anilines under peroxidase catalysis is being studied.

Soybean peroxidase (SBP) is an oxidoreductase extracted from the soybean seed coat (Gillikin and Graham, 1991), which has been found to be effective in removing various phenols and aromatic amines from water, as outlined and cited above. This paper reports, for the first time, the capability of soybean peroxidase to remove an aromatic thiol, MBT, from buffered aqueous solution.

Materials, Analytical Equipment, and Methods

Materials. Crude dry solid SBP (E.C. 1.11.7, industrial grade lot #18541NX; Rz value of 0.75 ± 0.10; specific activity 5 U/mg, with catalytic units, U, as defined and determined below) was obtained from Organic Technologies (Coshocton, Ohio). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot #120H7060, 19 900 U/mg

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solid, as defined by the supplier) was purchased from Sigma Chemical Company Inc. (St. Louis, Missouri). Polyethylene glycol (PEG), with an average molecular mass of 3350 g/mole, was obtained from Sigma Chemical Company Inc. The MBT (purity ≥98.0%) was obtained from Aldrich Chemical Corporation (Milwaukee, Wisconsin). All other chemicals were of analytical-grade and purchased from Sigma, Aldrich, or BDH Inc. (Toronto, Ontario, Canada).

Analytical Equipment. The MBT concentrations were analyzed with a high-performance liquid chromatography (HPLC) system obtained from Waters Corporation (Milford, Massachusetts). The system consisted of binary HPLC pump, autosampler, dual wavelength absorbance detector, and C_{18} reverse-phase column (5 μM, 4.6 ×150 mm) operated by Breeze software (Waters Corporation).

A diode array spectrometer (model 8452A), obtained from Hewlett Packard Canada (London, Ontario, Canada), with a wavelength range of 190 to 820 nm and 2-nm resolution and controlled by a Hewlett Packard Vectra ES/12 computer, was used to measure sample absorbances. Quartz semi-micro cuvettes, with a 1-cm optical path length, were supplied by Hellma Canada Limited (Concord, Ontario, Canada). Syringe filters (0.2-μm, non-sterile) were from Gelman Laboratories (Mississauga, Ontario, Canada).

Analytical Methods. *Colorimetric Assay for Soybean Peroxidase Activity.* A unit of enzyme catalytic activity (U) is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 23°C. The enzyme activity was determined by monitoring the initial rate of color formation at 510 nm resulting from the oxidative coupling of phenol and 4-aminoantipyrine in the presence of hydrogen peroxide when using SBP as a catalyst (Ibrahim et al., 2001).

Solution Preparation. The buffers used were prepared according to Gomori (1955). Acetic acid–sodium acetate buffer was used in the pH range 3.0 to 5.8, and monobasic-dibasic sodium phosphate buffer was used in the pH range 5.6 to 9.0. pH values higher than 9.0 were achieved by adjusting phosphate buffer with sodium hydroxide (NaOH).

The MBT stock solution was prepared fresh daily at 5 mM in 20-mM aqueous sodium hydroxide (because it would not dissolve readily at this concentration in water alone).

High-Performance Liquid Chromatography Analysis. All batch reactor samples for HPLC analysis of MBT concentration were first filtered through 0.2-μm syringe filters. The filters were pre-conditioned by passage of 2 to 3 mL of sample to waste and then collecting an aliquot for analysis. Elutions of a C_{18} reverse-phase column (5 μM, 4.6 × 150 mm) were isocratic, with a mobile phase consisting of acetonitrile and 0.1% aqueous acetic acid (60:40; monitored at 310 nm). The injection volume was 10 μL, and the flowrate was 1.0 mL/min (MBT retention time was 2.85 minutes).

Experimental Protocol

To optimize the reaction conditions for the removal of MBT in the presence of SBP and hydrogen peroxide, the experiments were carried out in 30-mL batch reactors at room temperature (approximately 23°C). The components of the sample mixture were added in the following order to a total volume of 20.0 mL: water (distilled or tap wastewater), acetate or phosphate buffer to 40 mM, MBT to 1.0 mM, SBP, and hydrogen peroxide to appropriate concentrations to initiate the reaction. The batch reactors, open to the atmosphere, were stirred gently for 3 hours, unless noted. After that, the samples were quenched with excess catalase to a concentration of 62.5 U/mL to quickly consume any residual hydrogen peroxide, microfiltered (0.2 μm), and then tested for the residual concentration by using HPLC. All batch reactions were carried out in triplicate; standard deviations are shown with error bars on the plots. The study was designed to achieve at least 95% conversion of MBT by optimizing the following parameters: pH (3.8 to 9.8), SBP concentration (0 to 1.5 U/mL), hydrogen peroxide-to-substrate concentration ratio (0 to 2.0), and PEG concentration (0 to 150 mg/L). Experiments also were conducted to monitor the substrate consumption over time. Removal of solids without filtering could be effected with alum (2.7 mM in aluminum ion).

Results and Discussion

The goal of this study was to develop baseline operating data on the enzymatic process, which could serve, if warranted, as a starting point for later design of an in-plant specialty treatment unit. Batch reactions of MBT in the presence of SBP and hydrogen peroxide were conducted under various conditions described below. An MBT concentration of 1.0 mM (167 mg/L) was chosen for all runs to facilitate comparison with earlier work on peroxidase-catalyzed removal of phenols and anilines cited above and to represent a concentration that might arise in industrial wastewater or process water. The aqueous solubility of MBT is reported as 1.0 g/L (approximately 6 mM; International Programme on Chemical Safety [2004]), but we were unable to make solutions near that concentration without using a base. Process parameters were manipulated to achieve an arbitrary benchmark—95% conversion of MBT in 3 hours at room temperature (approximately 23°C), again for ease of comparison with previous work. In general, the reaction mixtures changed in the enzymatic reaction from clear, colorless to a white suspension that partially settled. The solids could be removed by microfiltration or by settling with alum. The optimum parameters for treating 1.0 mM MBT were determined as presented in the following sections.

Optimum pH. The optimum pH for the enzymatic conversion of MBT was investigated in defined buffers over the pH range 3.8 to 9.8, when the hydrogen peroxide and MBT were equimolar. The experiments were conducted using two SBP concentrations and a reaction period of 3.0 hours (Figure 1). Under "stringent" conditions, with respect to SBP concentration (insufficient SBP to effect full conversion, to provide easier discerning of the optimum pH), the optimum value was found to be in the range 8.0 to 9.0 (upper curve in Figure 1). Previous work with SBP and 2,4-dimethylphenol showed a similarly high optimum pH (Dutta, 2008). As the SBP concentration was increased, still under mildly stringent conditions, the degree of conversion became relatively invariant over the pH range 6.0 to 8.8 (lower curve in Figure 1). A wider range is more convenient for an enzyme that might encounter a variety of waste streams. Thus, further studies were conducted in phosphate buffer at pH 7.1.

Optimum Hydrogen Peroxide-to-Substrate Ratio. The MBT is known to be oxidized by hydrogen peroxide alone (Repkina et al., 1984). Hence, a control experiment was conducted first to investigate the effect of various hydrogen peroxide

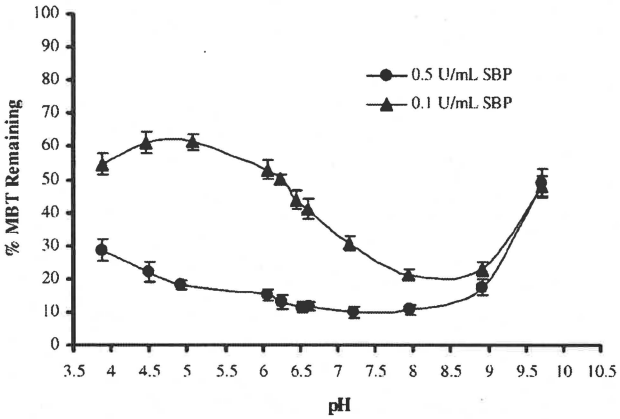

Figure 1—pH optimization for enzymatic conversion of MBT. Conditions as follows: 1.0-mM MBT, 1.0-mM H_2O_2, and 40-mM acetate or phosphate buffer; 3 hours; and HPLC analysis.

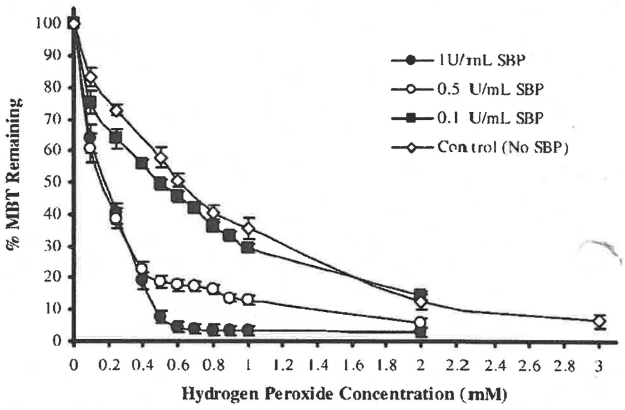

Figure 2—Effect of hydrogen peroxide concentration on the chemical and enzymatic conversion of MBT. Conditions as follows: 1.0-mM MBT and 40-mM phosphate buffer; pH 7.1; 3 hours; and HPLC analysis.

concentrations (without SBP) on MBT conversion (Figure 2, diamond symbols). The results showed that 95% of MBT can be converted chemically with 3.0-mM hydrogen peroxide in 3.0 hours. The appearance of these reaction mixtures was the same (white suspension) as the enzymatic ones, and HPLC analysis showed the filtrates to be identical (see below).

Treatment in the presence of SBP was conducted analogously (Figure 2, remaining symbols). With sufficient SBP (1.0 U/mL), >95% conversion of MBT was achieved with 0.6-mM hydrogen peroxide. Thus, this conversion in the presence of SBP requires approximately 5 times less hydrogen peroxide than the non-enzymatic one. With lower (stringent) concentrations of SBP, the initial steep dependence of MBT conversion on hydrogen peroxide concentration gives way to a more gradual one, and at progressively lower peroxide concentrations with lower SBP concentrations. The breakpoints in the curves are interpreted as indicating the end of the dominant SBP-catalyzed reaction superseded by dominance of the chemical reaction (Repkina et al., 1984) as the enzyme became inactivated.

According to the peroxidase reaction mechanism, for every mole of hydrogen peroxide being consumed, 2 moles of the aromatic functional group are converted to aromatic radicals that couple non-enzymatically to form dimers and then polymers (Dunford, 1999). The peroxide-to-MBT stoichiometry found that, for the enzymatic reaction (Figure 2, filled circles), 0.50 to 0.60 is close to the theoretical peroxidase stoichiometry. A slight increase in hydrogen peroxide demand (towards 0.60 moles of hydrogen peroxide per mole of MBT converted instead of 0.50) could be the result of the trace catalase activity of the peroxidase, converting a portion of the hydrogen peroxide available for reaction to water and oxygen and/or minor amounts of higher sulfur-oxidation-state products (see below).

No direct evidence was collected for the nature of the product(s), but insolubility of the preponderance of the products and peroxide consumption stoichiometry are consistent with the disulfide, an S-S-linked oxidative dimer (a 1-electron oxidation product), suggested in the literature (Bocks, 1966; Repkina et al., 1984; Xu, 1996), rather than any of the monomer-level sulfur acids (sulfenic, sulfinic, and sulfonic; 2-, 4-, and 6-electron oxidation products, respectively). The HPLC analysis of the final reaction filtrates shows only the residual MBT (<5%) plus a comparable amount (by HPLC peak area) of a more polar compound (retention time = 1.23 minutes)—plausibly one or more of the sulfur acid(s) mentioned. Based only on peroxide stoichiometry, occurrence of the sulfenic acid was considered a possible minor product in the laccase-catalyzed oxidation of 1,2-benzenedithiol (Xu, 1996).

Thus, although phenomenologically the MBT–peroxidase reaction manifests like the phenol–peroxidase reaction, in that a precipitate is separated, it is different in the nature of the product (heteroatom coupling rather than heteroatom–ring carbon coupling) (Yu et al., 1994) and in the peroxide stoichiometry (closer to 0.5 than 1.0, because polymerization stops at the dimer).

Minimum Soybean Peroxidase Concentrations and Polyethylene Glycol Effect. The PEG has been shown to extend the catalytic lifetime of peroxidases in reactions of the type considered here (Nakamoto and Machida, 1992). According to the "sacrificial polymer" hypothesis, it provides a shielding effect to the enzyme by adsorbing onto the insoluble polymers formed from the aromatic monomer like phenol, thereby preventing them from adsorbing to the free enzyme (Nakamoto and Machida, 1992) and removing it from solution. Thus, PEG has been proposed as an additive to enhance enzyme-based water treatment applications, because it is "generally recognized as safe" for human exposure (Harris, 1992), and it precipitates with the enzyme-generated polymeric products (Wu et al., 1998). From the current study, plots of MBT percent remaining versus the SBP concentrations, in the absence and presence of PEG, are shown in Figure 3. The four control reaction data points in the absence of SBP give evidence of substantial chemical conversion of MBT in the 3-hour time period—51% and 64% for 0.6- and 1.0-mM peroxide, respectively. Furthermore, the three runs started with 0.6-mM peroxide are identical in the absence of SBP, indicating that the presence of PEG has no effect on the chemical conversion of MBT. The minimum SBP concentration required for 95% conversion of MBT is shown to be 0.9 U/mL in the absence of PEG, while the presence of 50 mg/L of PEG reduced the enzyme concentration required for the same substrate conversion to 0.3 U/mL. Increasing the PEG concentration to 150 mg/L showed no additional reduction in the enzyme requirement. The "PEG

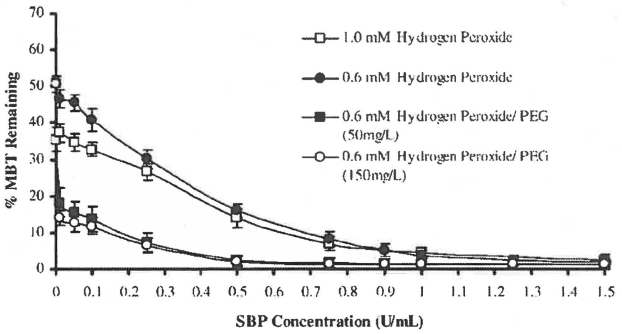

Figure 3—Effect of SBP concentration on conversion of MBT. Conditions as follows: 1.0-mM MBT, 40-mM phosphate buffer; pH 7.1; 3 hours; and HPLC analysis.

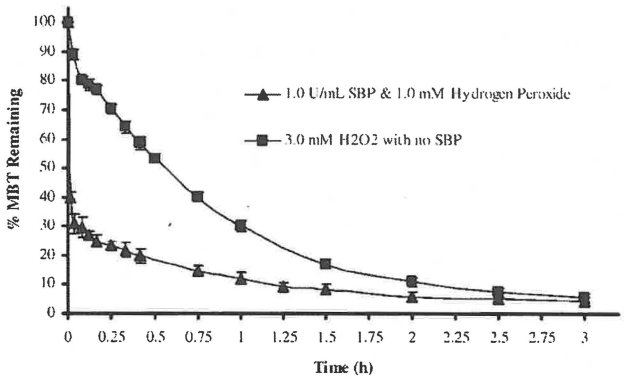

Figure 4—Time course of MBT conversion. Conditions as follows: 1.0-mM MBT and 40-mM phosphate buffer; pH 7.1; and HPLC analysis.

effect" observed here is comparable with that observed previously with peroxidase-catalyzed removal of phenols (Caza et al., 1999; Steevensz et al., 2009; Wu et al., 1998). Surprisingly, the three runs with 0.6-mM peroxide showed the greatest influence on conversion at the smallest increment of enzyme; from 0 to 0.01 U/mL, conversion jumped 33 to 35% in the presence of PEG; in the absence of PEG, the apparent "burst" of conversion was only 4% at 0.6-mM peroxide and non-existent at 1.0-mM peroxide. These differences are suggestive of enzyme inactivation—less protected in the absence of PEG and in the presence of higher peroxide concentration and, also, proportionally less protected at higher SBP concentrations (because, at 0.6-mM peroxide, it takes 50 times more enzyme to get from the burst at 0.01 U/mL to 95% conversion of MBT). To lend some credence to the foregoing inference, the following brief kinetic study was undertaken.

Time Course of 2-Mercaptobenzothiazole Conversion. The conversion of MBT was monitored for 3 hours. Control experiments in the absence of SBP showed that 95% of the MBT was converted with 3.0-mM hydrogen peroxide during this period (Figure 4, upper curve). In the presence of 1.0 U/mL SBP and 1.0 mM hydrogen peroxide, 70.0% of the MBT was converted within 3 minutes (Figure 4, lower curve). The conversion of the substrate continues at a slower rate after that time. In fact, the exponential decay rate calculated for the "slow phase" of the enzymatic reaction (0.90 $hour^{-1}$) is comparable with the decay rate for the full time course of the non-enzymatic one (0.98 $hour^{-1}$). While these rate constants derived under a pseudo-first-order assumption may not be compared closely because of the different starting peroxide concentrations, the fact that they are of the same order of magnitude suggests that, after 3 minutes, the enzymatic conversion was halted by inactivation, and chemical conversion by hydrogen peroxide completed the conversion of MBT.

Summary and Proposed Treatment. The current study has shown that SBP efficiently catalyzes the oxidative dimerization and removal of MBT from aqueous solution at approximately pH 7 and that efficiency can be improved markedly in the presence of PEG. Specifically, 1.0-mM MBT can be >95% removed in 3 hours at room temperature (approximately 23 °C) by 0.9 U/mL SBP in the presence of 0.6-mM hydrogen peroxide. In the additional presence of PEG at 50 mg/L, the enzyme requirement drops to 0.3 U/mL. While the same MBT conversion and removal is facile in the presence of hydrogen peroxide alone, approximately 5 times more peroxide is required to achieve it on the same time scale. This efficiency of enzyme use, which is better than the best we have achieved in the analogous removal of phenol from refinery process water (Steevensz et al., 2009), suggests that these findings could be developed further into a viable and cost-effective specialized treatment unit within a plant, in advance of the general biological treatment system.

Conclusions

Crude SBP is a suitable enzyme for the peroxidase-catalyzed conversion of the aromatic thiol, MBT. It does this effectively over a fairly broad pH range in the neutral–basic region in the presence of near-stoichiometric amounts of hydrogen peroxide. The presence of PEG can reduce the enzyme concentration required for the same substrate conversion by 3-fold. Chemical conversion of MBT by peroxide to the same product requires 6-fold more peroxide for the same time scale.

Credits

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ILLINOIS ENVIRONMENTAL PROTECTION AGENCY
WATER POLLUTION CONTROL PERMIT

LOG NUMBERS: 3753-07 **PERMIT NO.:** 2007-EN-3753

FINAL PLANS, SPECIFICATIONS, APPLICATION AND SUPPORTING DOCUMENTS PREPARED BY: Huff and Huff, Inc.

DATE ISSUED: July 20, 2007

SCREENED

SUBJECT: EXXONMOBIL OIL CORPORATION – Construction of Hydrogen Peroxide Injection System - ExxonMobil Oil Corp. Joliet Refinery WWTP tributary to Des Plaines River - NPDES Permit No. IL0002861

PERMITTEE TO CONSTRUCT

ExxonMobil Oil Corporation
Joliet Refinery
25915 S.E. Frontage Road
Channahon, IL 60410

Permit is hereby granted to the above designated permittee(s) to construct and/or operate water pollution control facilities described as follows:

The construction of a hydrogen peroxide tank and injection system consisting of a 12,000-gallon hydrogen peroxide tank and all of the pumps, piping, indicators, meters, and appurtenances necessary to treat tank TK588 to supplement the Benzene Recovery Unit before discharging to the Joliet Refinery WWTP for treatment tributary to the Des Plaines River under NPDES Permit No. IL0002861.

This Permit is issued subject to the following Special Condition(s). If such Special Condition(s) require(s) additional or revised facilities, satisfactory engineering plan documents must be submitted to this Agency for review and approval for issuance of a Supplemental Permit.

SPECIAL CONDITION 1: This Permit is issued with the expressed understanding that there shall be no surface discharge from these facilities, except for those otherwise approved under NPDES Permit No. IL0002861. If such discharge occurs, additional or alternate facilities shall be provided. The construction of such additional or alternate facilities may not be started until a Permit for the construction is issued by this Agency.

SPECIAL CONDITION 2: The operational portion of this permit shall be governed by NPDES Permit No. IL0002861.

SPECIAL CONDITION 3: Issuance of this permit does not release the Permittees from any liability for prior violations of the Act or Rules and Regulations promulgated thereunder.

THE STANDARD CONDITIONS OF ISSUANCE INDICATED ON THE REVERSE SIDE MUST BE COMPLIED WITH IN FULL. READ ALL CONDITIONS CAREFULLY.

SAK:MEL:375307.wpd

DIVISION OF WATER POLLUTION CONTROL

cc: EPA - Des Plaines FOS
Huff and Huff, Inc.
Records - Industrial
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Alan Keller, P.E.
Manager, Permit Section

EXHIBIT
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