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Microaerobic degradation of 2-Mercaptobenzothiazole present in industrial wastewater

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HIGHLIGHTS

- The isolated *strain CSMB1* utilizes benzothiazole and its derivatives as sole carbon and energy.
- Benzene ring opening of benzothiazoles is through meta cleavage under microaerobic condition.
- A microaerobic degradative pathway was proposed for 2-Mercaptobenzothiazole.
- The strain *CSMB1* is an ideal candidate for leather industrial wastewater treatment.

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ABSTRACT

Microaerobic degradation of 2-Mercaptobenzothiazole (2-MBT) was investigated using an isolated bacterial strain *CSMB1*. It was identified as *Alcaligenes* sp. *MH146* by genomic analysis. The isolate degraded 50 mg/L concentration of 2-MBT which was measured in terms of Total organic carbon (TOC) (700 mg/L). A maximum degradation of 86% with a residual TOC concentration of 101 mg/L was obtained after 72 h, with the biomass growth of 290 mg/L. The presence of specific activity of catechol 2, 3 oxygenase was observed in all the tested derivatives of benzothiazoles and the benzene ring opening was observed through meta cleavage. By analyzing the 72 h incubated culture supernatant, 2-MBT, and all its biotransformed products were degraded into polar compounds. With the analytical results obtained, a possible microaerobic degradative pathway was proposed and illustrated for 2-MBT. It is concluded that microaerophilic isolate *CSMB1* was able to degrade 2-MBT and its intermediates by utilizing them as sole carbon and energy.
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1. Introduction

Benzothiazoles are heterocyclic aromatic compounds, useful in multiple applications due to their biological and pharmacological properties with their bicyclic ring system. 2-Mercaptobenzothiazole (2-MBT) is used as a vulcanization accelerator in the rubber processing industry [1,2] and corrosion inhibition agent in cooling systems. 2-Thiocyanomethylthiobenzothiazole (TCMTB), one of the derivatives of 2-MBT [3] is used as a preservative to prevent hides and skins from deterioration during transportation and storage [4,5]. Direct discharges of 2-MBT occur in effluents from industries producing rubber products [6] and in leather industrial wastewater [7]. 2-MBT is non-volatile and has low water solubility. It is a toxic xenobiotic and a persistent substance in soil hence becomes, highly resistant to biological treatment [8]. Depending on soil conditions, the half-life of 2-MBT ranges from 92 to 248 days. 2-MBT and its derivatives are toxic to living forms including human beings [9]. According to the standardized Microtox test performed [10], for an exposure time of 15 min, the 50% toxic concentration was 1.5 μM for 2-MBT. Recently, the occurrence of benzothiazoles has been detected in exhaled human breath [11].

The advanced oxidation process (AOP) is one of the pretreatment options suggested, but the residuals cannot be decomposed biologically, and thus alternative methods need to be used [12]. In activated sludge systems, 2-MBT was resistant and degraded only at concentrations around 20 mg/L [13–15]. In a two-stage anaerobic/aerobic biological treatment, 2-MBT was found to be refractory to anaerobic treatment. Brownlee and Drotar [16,17] observed that 2-MBT was transformed into methylated metabolite (MTBT) by *Corynebacterium* sp., *Pseudomonas* sp., *and Escherichia*

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coli. In recent times, there is growing interest in the bioremediation of toxic compounds under microaerobic conditions. The organisms utilize C and N sources by different metabolic strategies based on the availability of O_2, switching between growth modes [18]. The microaerophilic microorganisms were found to be provided with metabolic versatility [19]. Less energy requirement for blower operation, minimum sludge production, recovery from organic shock loads are some of the possible advantages of microaerobic treatment systems [20–22]. It overcomes the major limitation of high operational costs of the aerobic process [23,24].

The possibility of the biodegradation of 2-MBT by a pure isolated strain *Alcaligenes* sp. *MH146 strain CSMB1* has been investigated under the microaerobic condition without any external supplement of carbon and nitrogen. The mechanism of degradation was studied using UV–vis spectroscopy, enzymatic assays, HPLC and GC–MS analysis. With the results obtained a microaerobic degradative pathway for 2-MBT was proposed.

2. Materials and methods

2.1. Chemicals

Catechol, Benzothiazole, 2-Hydroxybenzothiazole, 2-Methyl benzothiazole, 2-Mercaptobenzothiazole 2-Methyl thiobenzothiazoles and 2-amino benzothiazole were procured from Sigma-Aldrich, India. 2-(thiocyanomethylthio)-benzothiazole, (TCMTB) was provided by a tannery in Chennai, India. The pesticide 2-MBT is a light yellow powder with a faint odor. The molecular weight and molecular formula are 167.2513 g/mol and $C_7H_5NS_2$ respectively. The culture medium used for the study is a mineral salt medium containing (g/l): K_2HPO_4, 1.2; KH_2PO_4, 0.3; $MgCl_2 \cdot 7H_2O$, 0.5; NaCl 1.0; $CaCl_2 \cdot 2H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; and 10 ml of trace elements. All medium components of analytical grade were procured from E.Merck Mumbai (India). All the solutions were prepared in Milli-Q water.

2.2. Microorganism

The bacterial strain used in this study was isolated from microaerobic reactor present in our laboratory, fed with phenol as sole carbon. For biodegradation of 2-MBT, a microaerophilic *Alcaligenes* sp. *MH146 strain CSMB1* was selected since it is capable of utilizing 2-MBT (50 mg/L) as the sole source of carbon and nitrogen even after repeated purification. It was identified by 16S rRNA gene analysis as *Alcaligenes* sp. *MH146* with an NCBI accession number GenBank ID: FJ626617.1. It is one among the six bacterial isolates capable of degrading a mixture of heterocyclic compounds in the consortium. The isolated bacterial strains were deposited in MTCC, IMTECH, India for patent filing [25]. For enrichment of 2-MBT and for degradation studies, a specially designed laboratory scale bioreactor (3.5 l) equipped with biosensors for automatic control to maintain microaerobic condition was used.

2.3. Analytical methods

For growth determination, the cells in the cultures (50 ml) were harvested by centrifugation (12,000*g*) at 4 °C for 10 min and centrifuged after washing with milli-Q water. The cells were then dried at 60 °C overnight for 24 h until a constant weight was obtained. The analytical methods pertaining to the degradation studies were estimated as per Standard Methods [26], namely, Total Organic Carbon (TOC) (5310-B), Nitrogen Ammonia, (4500-NH3-C) and Sulfate, (4500-SO4-E).

2.4. Degradation assay

Studies were conducted with 50 mg/L concentration of 2-MBT. This concentration corresponds to the concentration that commonly present in the leather industrial effluents. 2-MBT biodegradation was monitored by estimating in terms of TOC and the growth of the isolated *strain CSMB1* was also measured. The degradation profile of 2-MBT was observed using UV–vis spectrophotometer (Shimadzu UV2450) by wavelength scan spectra. Identification of intermediate metabolites was inferred based on the spectrum of pure compounds that was read against buffer blanks at their respective (λ_{max}) wavelengths (2-MBT, 310 nm 2-MeBT, 249 nm 2-MTBT, 279 nm 2-BT, 251 nm 2-ABT, 260 nm 2-OHBT 278 nm and BTSA 310 nm). The inhibitory effect on the growth of *Alcaligenes* sp. *MH146 strain CSMB1* at different initial concentrations of 75, 100 and 125 mg/L of 2-MBT was evaluated. For tests on the metabolic versatility, the utilization of the various benzothiazole derivatives such as TCMTB, 2-BT, 2-MTBT, 2-ABT 2-OHBT and 2-MeBT by *Alcaligenes* sp. *MH146 strain CSMB1* was evaluated. In this case, the mineral medium was substituted with the respective substrate as sole carbon and nitrogen source at 50 mg/L concentration.

2.5. Enzyme activity assays

Cells of the strain *Alcaligenes* sp. *MH146 strain CSMB1* were grown in mineral medium supplemented with 2-MBT and harvested at mid-log phase. The cells were washed twice with phosphate buffer (pH 7.0) and sonicated on ice using a Digital Sonifier Model 250 (Branson, USA). The lysate was centrifuged at 12,000 × *g* for 30 min. The cell-free extract thus obtained was either stored at −40 °C or used immediately for enzyme assays. Catechol 1, 2 oxygenase (EC 1.13.11.2) and Catechol 2,3 Oxygenase (EC 1.14.13.1) activity was measured by monitoring the formation of either cis, *cis*-muconic acid or 2-HMSA at 260 or 375 nm respectively [27]. The reaction mixture (3 ml) contained a Tris-HCl buffer (50 mM, pH 8.0), catechol (1 mmol) and cell extract (100 μl). Readings were taken at 30 s intervals for 5 min at room temperature. Controls without substrate or cell extract were prepared for each assay. One enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per minute or consumption of the substrate. Protein concentration was determined according to the method of Bradford [28].

2.6. HPLC analysis

The 2-MBT culture supernatant was taken after every 12 h of incubation and centrifuged at 12,000 × *g* for 20 min. The metabolites formed after 2-MBT degradation was extracted two times using an equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and concentrated in a rotary vacuum evaporator. The ethyl acetate extract in the flask was dissolved in methanol. The culture supernatant was analyzed by HPLC (Shimadzu VP series Model LC-10ADVP) on Phenomenex Luna C18 reversed-phase column, 5 μm particle size, equipped with Photodiode Array UV–vis Spectrophotometric Detector (200–500 nm). The mobile phase was 80:20 acetonitrile/buffered aqueous solutions. The buffered aqueous solution was prepared with 4 ml phosphoric acid, 25 ml methanol in 1-l milli-Q water operated with a flow rate of 1.0 ml/min and 15 min run time at a maximum absorption wavelength of 310 nm to detect 2-MBT. Samples of 10 μl were injected into the Rheodyne injector port.

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