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# Effect of organic solvents on i*n vitro* enzymatic degradation of dibenzothiophene and 4,6-dimethyl dibenzothiophene

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**ABSTRACT**

*A novel intracellular 66,500 Da monomeric enzyme was purified from the Staphylococcus sp. Strain S3/C cells cultured in Pseudomonas Mineral Salts (PMS) medium containing dibenzothiophene as sole carbon and sulfur source. The enzyme had the ability to degrade dibenzothiophene and 4,6-dimethyl dibenzothiophene in the presence of organic solvents e.g. toluene, iso-octane and n hexadecane under aqueous – hydrocarbon biphasic conditions as well as under complete non-aqueous conditions at pH 6.0 when incubated at 30°C. Dibenzothiophene was the inducer of the enzyme and NADH acted as an essential coenzyme for the oxidation of dibenzothiophene. $K_m$ of DBT for the enzyme was 1.7 $\times 10^{-5}$ M and the $V_{max}$ was 0.34 µM $min^{-1}$.*

**Key words:** Dibenzothiophene, 4,6-dimethyl dibenzothiophene, enzymatic degradation, organic solvents

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## INTRODUCTION

Dibenzothiophene degradation by microbes under aerobic conditions [1, 2, 3] has been studied extensively because of the recalcitrance of the compound and its alkyl derivatives to the hydrodesulfurization process [4]. The genes [5] as well as the enzymes [1, 6, 7] of the well known 4S pathway of DBT desulfurization have been well characterized. The approach, however, has to be taken that ensures that the enzymes involved in the degradation of DBT should be able to work in the hydrocarbon environment under *in vitro* conditions. An attempt has been made for the same by the purified enzyme from *Staphylococcus* sp. strain S3/C that has been shown to degrade DBT under hydrocarbon – aqueous biphasic conditions [8].

## MATERIALS AND METHODS

***Culturing of cells and cell lysis:***

The bacterial cells were cultured in Pseudomonas Mineral Salts medium lacking sulfur [PMS (-S)] medium with dibenzothiophene (DBT) as sole source of sulfur [8]. A thick cell suspension was prepared (80 g dry biomass $l^{-1}$ in 40 mM phosphate buffer, pH 6.8) and protease inhibitors were added to a final concentration of 5 mM EDTA, 1mM DTT and 1mM β–mercaptoethanol. The cells were lysed sequentially by repeated freeze thawing –10°C and 37°C; followed by sonication using a solid small probe assembly of tip diameter 9.5 mm; 23 kHz (MSE Soniprep 150) at 4°C in a jacketed vessel. A total of 10 sonication cycles of 30 second at an interval of 1 min were performed. The cell debris was removed by centrifugation at 4000 × *g* for 5 min and the clear supernatant was used as the crude enzyme extract.

***Salting out of proteins by ammonium sulfate precipitation:***
The crude enzyme extract was transferred to a jacketed vessel maintained at 4°C using a refrigerated circulating water bath. Appropriate amount of ammonium sulfate was weighed according to the starting volume of the crude enzyme extract and the proteins were salted out in different fractions (0-30%, 30-50%, 50-70%, 70-90% ammonium sulfate and 90% supernatant. The contents were centrifuged at 10,000 × *g* at 4°C, the supernatant decanted and the precipitate dissolved in 40 mM phosphate buffer (pH = 6.8). Enzyme activity in each fraction was determined after desalting and dialyzed against 0.1M phosphate buffer (pH = 6.8) using centricon YM 30 tubes (Millipore; molecular weight cut off value of 30 kDa) at 3000 × *g* for 1 h. The protein content in each fraction retinate was determined by Folin Lowry's method using albumin of Bovine Serum as standard.

***Size exclusion through G-200 sephadex column:***
The total volume, $V_t$ of the column was 70.7 ml; void volume, $V_o$ being 20.5 ml. The height of the bed, H was 82.5 cm and internal diameter of 1.5 cm. The flow rate of the mobile phase (0.1-1% sodium chloride gradient buffer) was maintained at 0.1 ml $min^{-1}$. 1 ml fractions were collected and the enzyme activity and protein concentration was determined in each fraction.

***Characterization of the enzyme by SDS-PAGE:***
30 µg protein samples were loaded in each well and electrophoresed on 12% SDS polyacrylamide gel along with low molecular weight (Amersham pharmacia biotech) protein marker: Phosphorylase b (94 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa). Electrophoresis was carried out at 30 mA constant current (BIO-RAD power pack 1000) and the gel was silver stained.

***In – vitro enzyme assay:***
The *in vitro* assay for enzyme was carried out under biphasic conditions maintaining the hydrocarbon to aqueous phase ratio (H/A) of 3:1. 0.1 M sodium phosphate buffer with 6 µmol NADH was present as the aqueous phase and the substrate, 0.4 µmol dibenzothiophene, was dissolved in an organic solvent (*e.g. n*- hexadecane, toluene or iso-octane). The degradation kinetics of DBT by the crude enzyme extract, the cell sonicate, and various purified fractions was determined in 15 ml screw capped round bottomed culture tubes. The tubes were incubated at 30°C at 150 rpm in INNOVA (New Brunswick, NJ) environmental shaker inclined at an angle of 20° from the horizontal plane. The reaction was carried out for 3 h and the initial rate of the reaction, $v_o$, was calculated from the slope of DBT concentration vs time (min) linear plot. The reaction was stopped by addition of 10 µl of 6 N HCl and the hydrocarbon phase was recovered by centrifugation at 4000 × *g* for 10 min.

The DBT content of the hydrocarbon phase was measured quantitatively by using DBT sulfone as internal standard by the Varian STAR 3600 CX Gas Chromatograph with sulfur specific Pulse-Flame Photometric Detector (PFPD). 0.2 µl samples were injected in a 30 m by 0.32 mm internal diameter fused silica wall coated open tube column with a 4µ methyl silicone coating as the stationary phase. The injector temperature was 340°C and the temperature of the column oven was maintained at 150°C for 5 minutes followed by a rise of 4°C/minute to 300°C. Helium was used as carrier gas and hydrogen / air as the detector gases with a flow rate of 2 ml $min^{-1}$.

The enzyme activity was measured in terms of International Units (I.U.). One unit of enzyme was defined as the amount of enzyme required degrading 1µM of dibenzothiophene per minute at 30°C. Specific enzyme activity was measured as the enzyme activity per gram of the protein present in the reaction mixture.

***Effect of pH:***
The rate of reaction for enzymatic degradation of organosulfur compounds was determined in buffers in a pH range of 4.0 to 8.0. The pH profile for the enzyme activity was obtained by plotting the initial rate of reaction against the respective pH at which the reaction was carried out.

***Effect of temperature:***
Effect of temperature for enzymatic degradation of organosulfur compounds was determined in the range 25°C to 40°C under the conditions mentioned above at optimum pH.

***Substrate specificity of the enzyme:***
The purified enzyme was reacted with dibenzothiophene, dibenzothiophene sulfone, dimethyl sulfoxide, 4,6-dimethyl dibenzothiophene and hydrodesulfurised diesel as substrates for the determination of its substrate specificity. The reactions were set under biphasic conditions with H/A 3. The enzyme activity was measured in terms of the decrease in substrate concentration by PFPD Gas Chromatography.

***Effect of organic compounds on enzymatic DBT degradation activity:***
Three solvents were tested for their effect on the enzyme activity *e.g. n*- hexadecane, toluene and iso-octane (H/A 3). Another set of tubes was also kept under complete non-aqueous conditions.

## RESULTS AND DISCUSSION:

***Physical characterization of the enzyme***
The enzymes required to degrade DBT by *Staphylococcus* sp. strain S3/C were found to be intracellular and induced in the presence of DBT ((Figure 1, Table 1), much like the other reported enzyme systems *e.g.* monooxygenases [9]. The enzyme activity was observed in the 30-50% ammonium sulfate fraction of the induced cells. This suggests that the induction of the enzyme occurs in response to the selective stress produced by the presence of DBT as the sole sulfur source in culture medium [10], which was necessary for the degradation of DBT. Alternatively, it is also possible that the expressed protein (Figure I) may assist the enzyme systems inherent to the microbial metabolic pathway for DBT degradation. This fraction when purified with YM 30 centricon membrane (Table I) retained the enzyme in the retinate depicting its molecular weight to be more than 30kDa.

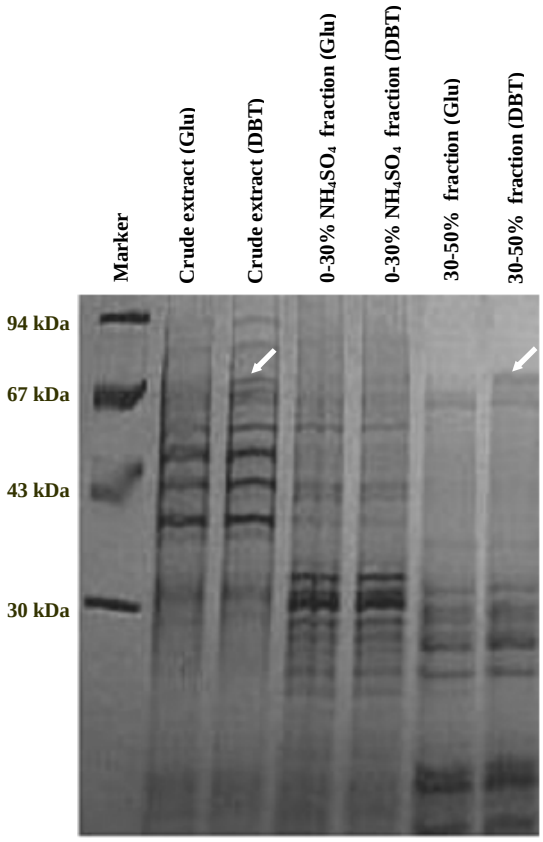


**Figure I: SDS - PAGE gel showing inducible nature of the enzyme.**

The retention time of the dialyzed 30-50% ammonium sulfate fraction eluted from the G 200 sephadex column was 5.4 h. Fivefold purification of the enzyme could be achieved although the yield of the enzyme decreased

considerably (Table I). The molecular weight of 67 kDa (Figure I) suggests that the enzyme is a monomer. The purified enzyme is colorless, which suggests that the enzyme may not require any transition metal like $Fe^{2+}$, $Fe^{3+}$, $Cu^{+}$, $Cu^{2+}$, $Co^{6+}$, $Mn^{+}$ etc. as a prosthetic group for its activity [9].

The white arrow indicates the presence of an extra protein, molecular weight (67kDa) in the cells cultured in the presence of DBT. This induced band is absent in the cells cultured in medium with glucose (Glu) as sole carbon source.

**Table I: Partial purification of the DBT degrading enzyme.**

| | Enzyme activity (U) | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U $mg^{-1}$) | Purification (Fold) | Yield (%) |
|---|---|---|---|---|---|---|---|
| Crude extract | 0.28 | 57 | 189 | 106.4 | 0.56 | 1 | 100 |
| Ammonium sulfate fractionation | 0.44 | 5 | 84.75 | 75.86 | 0.89 | 1.6 | 71.3 |
| Dialysis | 0.5 | 3 | 68.2 | 68.2 | 1 | 1.78 | 64.1 |
| G-200 sephadex | 1.44 | 1 | 8 | 23.04 | 2.88 | 5.14 | 21.6 |

***Enzyme kinetics***

The addition of NADH as a co-factor was essential for DBT degradation under *in-vitro* conditions (data not shown) suggesting that the enzyme belongs to the class oxidoreductases. Optimum pH for the maximum DBT degradation has been found to be 6.0 (Figure II) with the enzyme's preference for phosphate buffer.

The optimum temperature for maximum enzyme activity was found to be 30°C in conjunction with the mesophilic range for DBT degradation by *Staphylococcus* sp. strain S3/C whole cells [8]. Although the whole cells showed a wide range of temperature (20°C – 40°C) for DBT degradation (data not shown); under the present experimental conditions the purified enzyme showed its activity under a narrow range of temperature (Figure III).

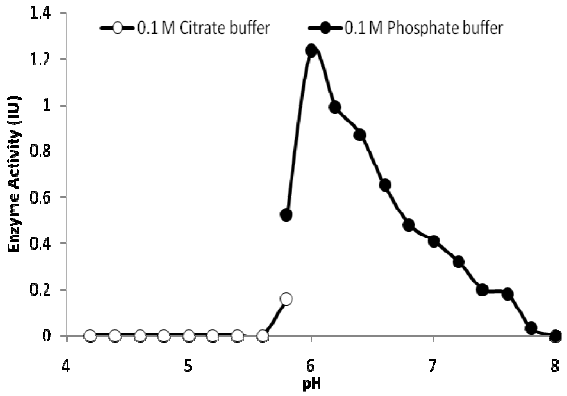


**Figure II: Effect of pH on enzymatic DBT degradation.**

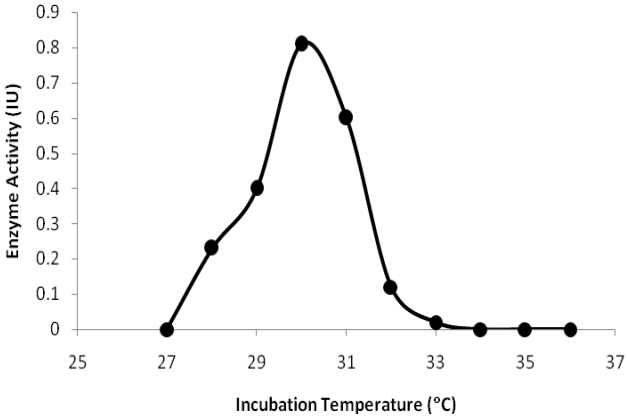


**Figure III: Effect of temperature on enzyme activity.**

Different organosulfur compounds like DBT, DBT sulfone, dimethyl sulfoxide, 4,6 dimethyl DBT and the alkyl DBTs present in the hydrodesulfurised diesel were tested for their reactivity with the purified enzyme under *in-vitro* biphasic condidtions. Non-sulfur DBT analogue, 2-hydroxybiphenyl, was also taken as a substrate for enzyme reaction. No enzymatic degradation of DBT sulfone was observed (Table II) indicating that the presence of oxygen atoms attached to the sulfur atom may be inhibitory to the enzyme attack. It was observed that the enzyme metabolizes only DBT and 4,6 dimethyl DBT.

**Table II: Substrate specificity of the purified enzyme under *in-vitro* conditions**

| Substrate | Enzyme activity (U) | Specific enzyme activity (U mg$^{-1}$) |
|---|---|---|
| *Dibenzothiophene* | 1.44 | 2.88 |
| *4,6 dimethyl DBT* | 0.5 | 1.00 |
| *2-hydroxybiphenyl* | 0.00 | 0.00 |
| *DBT sulfone* | 0.00 | 0.00 |
| *Dimethyl sulfoxide* | 0.00 | 0.00 |

$K_m$ of DBT for the enzyme was $1.9 \times 10^{-5}$ M and the $V_{max}$ was 0.37 µmol min$^{-1}$ (Figure IV). The reaction proceeded in the first order. In the case of 4,6 dimethyl DBT, the reaction was a deviation from the first order and showed the requirement for higher initial substrate concentration for the reaction to proceed (Figure V).

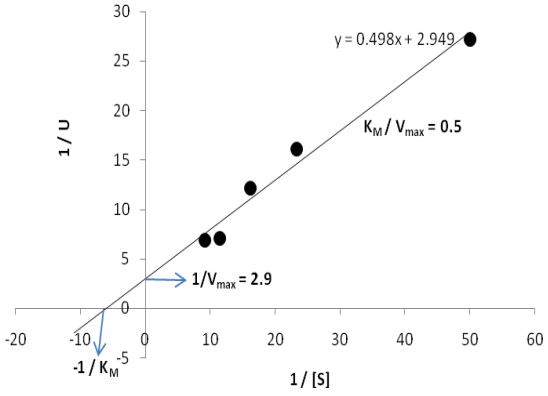


**Figure IV: Determination of $K_m$ and $V_{max}$ for DBT degrading enzyme by Lineweaver – Burk plot.**

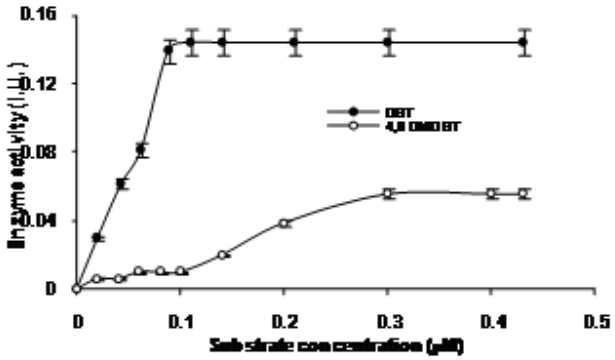


**Figure V: Effect of substrate concentration on organosulfur compound degradation.**

It has been shown that the purified enzyme preferentially degrades DBT in comparison to 4,6 DMDBT may be due to the steric hindrance from the two methyl groups present at the $C_4$ and $C_6$ carbon atoms of the molecule. Interestingly, 4,6 DMDBT also showed a phenomenon of positive co-operativity *i.e.* the enzyme showed enhanced activity on this substrate only when it was present in large quantities.

***Effect of organic compounds on degradation of DBT by purified enzyme***

The solvents iso-octane and toluene were found to enhance DBT degradation as compared to *n.* hexadecane (Table III) in relation to their polarity. This suggests that the active site of the enzyme may involve the interaction of oxygen radical with the substrate for its effective oxidation favored by the polar environment for the electron transition from substrate to the product under *in vitro* conditions [11].

**Table III: Effect of co-substrates on DBT degradation**

| Co-substrate concentration (µM) | DBT degradation enzyme activity (U) | Specific enzyme activity (U mg$^{-1}$) |
|---|---|---|
| *n. HEXADECANE* | | |
| *Non-aqueous* | $0.1^{a} \pm 0.01$ | 0.14 |
| *H/A = 3* | $0.8^{b} \pm 0.01$ | 1.33 |
| *ISO-OCTANE* | | |
| *Non-aqueous* | $0.5^{c} \pm 0.01$ | 0.71 |
| *H/A = 3* | $1.2^{d} \pm 0.03$ | 2.00 |
| *TOLUENE* | | |
| *Non-aqueous* | $0.78^{b} \pm 0.02$ | 1.11 |
| *H/A = 3* | $1.44^{e} \pm 0.03$ | 2.88 |

The data is represented as mean ± SEM (Standard error of Mean). Means bearing similar superscripted alphabets do not differ from each other at $P \leq 0.05$ (Based on Duncan's multiple-range test).

The present findings clearly indicate that the enzyme can oxidize more DBT *in vitro* (66%) in comparison to *in vivo* (57%) [8], which was because the identified enzyme was involved in the initial step of the metabolic pathway of *Staphylococcus* sp. strain S3/C multi-enzyme system. The purified enzyme has the potential to be used for oxidation of DBT and its alkyl derivatives in the presence of hydrocarbon.

## CONCLUSION

Biodesulfurization of DBT has been extensively studied in aqueous and under biphasic conditions [12]. Several studies encompass on the use of bacteria by way of metabolic engineering [13] or use of organic solvent bacteria for desulfurization of DBT. The present study highlights the possible use of purified enzyme(s) for desulfurization of dibenzothiophene *in vitro.*

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