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Full Length Research Paper

Removal of dibenzothiophene, biphenyl and phenol from waste by *Trichosporon* sp.

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Utilization of dibenzothiophene and flocculation of this compound from environment by yeast was studied. The yeast was isolated from phenol-contaminated waste and identified as *Trichosporon* sp. This strain used phenol and dibenzothiophene as the only sources of carbon and sulfur, respectively. However it had better growth when glucose and yeast extract was added to basal medium containing dibenzothiophene. When the yeast was grown on dibenzothiophene in basal medium almost 100% of dibenzothiophene was reduced in 14 days, but in the present of glucose, only 77% was reduced.. The results showed that glucose produced more biomass for dibenzothiophene utilization without any sign of 2-hydroxybiphenyl (2-HBP) production. However the yeast utilized phenol and biphenyl such that there was no accumulation of biphenyl in media. The results showed that *Trichosporon* sp. have the capacity to transform biphenyl and dibenzothiophene, and therefore could be used for removal of these toxic compounds.

Key words: *Trichosporon*, yeast, dibenzothiophene, phenol, 2-hydroxybiphenyl, transformation, flocculation.

INTRODUCTION

The study of aromatic hydrocarbons in coastal marine environment is important, since these areas receive considerable pollutant inputs from land-based sources via coastal discharge. The carcinogenic properties of these compounds and stability during their atmospheric and aquatic transport have generated interest in studying their sources, distribution, transport mechanisms, environmental impact and fate (DouAbol, 1997). Also fossil fuels and crude oil contained significant amounts of organosulphur compounds such as alkyl- and cycloalkyl thiols, and aromatic heterocycles based on thiophene which produced sulphur dioxide in to the atmosphere during combustion and causes acid rain and air pollution (Oldfield et al., 1997; Tanaka et al., 2002; Baldi et al., 2003). Dibenzothiophene (DBT) is considered a model polycyclic sulphur compound contained in fossil fuels (Gallardo et al., 1997; Matsubara et al., 2001). It has been reported that some bacteria use DBT as a sole source of sulfur without breaking its carbon-carbon backbone (Matsubara et al., 2001). Bacteria that desulfurize organic sulfur compounds without metabolizing the carbon skeleton are less common and generally use a sulfur-selective oxidative pathway (Kilbane, 1989). Many strains of bacteria are not useful because they mineralize the carbon skeleton (Lee et al., 1995).

However, DBT has been widely used as a model compound to screen microorganisms which might be used in desulfurization of fossil fuels (Lei et al., 1996; Denis-Larose et al., 1997). Microorganisms that able to utilize DBT mainly belong to bacteria, especially gram-positive domain, and mainly from genus *Rhodococcus* (Denis-Larose et al., 1997). From eukaryotic organisms, *Cunninghamella elegans* has been reported to desulfurize DBT (Crawford et al., 1990). This fungus grows on DBT and produce DBT-5-oxide and DBT-5-dioxide but not biphenyl. The fungus *Paecylomyces* sp. desulfurizes DBT by producing 2,2'-dihydroxybiphenyl (Faison et al., 1991). Among yeasts, *Rhodosporidium toruloides* is the first known yeast that utilizes DBT. When this yeast was grown on glucose in the presence of a commercial emulsion of bitumen, 68% of the benzo and dibenzothiophenes were removed after 15 days of incubation (Baldi et al., 2003). *Rhodosporidium* DBVPG 6662 is able to utilize the organic sulfur of DBT and a large variety of thi-

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ophenic compounds (Baldi et al., 2003). In this study, *Trichosporon* sp. was isolated from soil and capable of growing at phenol and dibenzothiophene as the only sources of carbon and sulfur, respectively.

MATERIALS AND METHODS

Medium and cultivation

Basal salt medium (BSM) was used to assay *Trichosporon* for its ability to desulfurization of DBT. BSM contained (per liter) 5.57 g of $NaH_2PO_4.H_2O$, 2.44 g of $KH_2PO_4.3H_2O$, 2 g of NH_4Cl, 0.2 g of $MgCl_2.6H_2O$, 0.001 g of $CaCl_2.2H_2O$, 0.001 g of $FeCl_3.6H_2O$, and 0.004 g of $MnCl_2.4H_2O$. 5 g of glucose as the carbon source was added. DBT (0.3 mmol) dissolved in ethanol was used as sulfur source with or without glucose (Piddington et al., 1995). All chemicals were obtained from Merck and Sigma. Media were sterilized at 110°C for 20 min and DBT was added after sterilization of media.

Utilization of DBT by *Trichosporon*

Desulfurization assay were performed in liquid culture. For this assay cells were incubated 2-3 days at 30°C in Sabaura dextrose Agar (SDA) supplemented with DBT. The colonies were then suspended in BSM to give an A_{600} of 0.5, and from this suspensions 1 ml added to 50 ml of BSM containing test substrate, DBT (0.3 mmol/L in ethanol) with and without glucose in a 250 ml screw cap Erlenmeyer flask. During cultivation of yeast, measuring of growth and DBT degradation was performed (Baldi et al., 2003).
Measurement of degraded compound from DBT by yeast was performed by using Gibb's reagent for detection of biphenyl (phenolic compound that is a by-product of 4S pathway), extraction of the products from acidified culture (with hydrochloric acid) by ethyl acetate and then measurement of UV-visible spectra at λ323 and λ285 (Tanaka et al., 2002). For detection of HFBT production from DBT (by-product from kodama pathway) by yeast, supernatant of yeast culture acidified with citric acid was measured at UV-visible spectra at λ392 (Bressler and Fedorak 2001). For determination of formaldehyde that is in kodama pathway, 5 ml of broth medium were mixed with equal volumes of Hantzch reagent according to Sihn et al. (1997).

Yeast growth

Yeast growth was determined by measuring A_{600} with a UV-visible light spectrophotometer (Baldi et al., 2003).

Effect of resting cells on DBT utilization

The yeast was grown on BSM containing 5 g of D-glucose L^{-1}, and 0.3 mmole DBT L^{-1} or $MgSO_4$ replacing $MgCl_2$ in BSM and incubated at 30°C with 130 rpm shaking for 2-3 days. Then cells were harvested by centrifugation at 3000 rpm for 10 min, washed twice with phosphate buffer (pH 7.4), and resuspended in BSM with adjustment of the cell mass to an OD_{600} of 30. This cell suspension was inoculated into BSM containing DBT for determination of DBT utilization with resting cells of yeast (Tanaka et al., 2002). After 3 h and 24 h of incubation in 30°C and 130 rpm shaking degradation assay was performed.

Effect of supernatant (extra cellular enzymes) on DBT utilization

Culture supernatant of previous assays was used for determination of the effect of supernatant on DBT utilization. For this, supernatant added to BSM containing DBT, and after 3 and 24 h, assay was performed (Lei et al., 1996).

Effect of NaCl on DBT utilization

Different concentrations of NaCl in BSM medium were used with DBT. For blank medium no NaCl added to BSM. Reduction of DBT was measured after inoculation of yeast to these medium. All spectrophotometric assays were performed with a UV160A Shimatzu spectrophotometer.

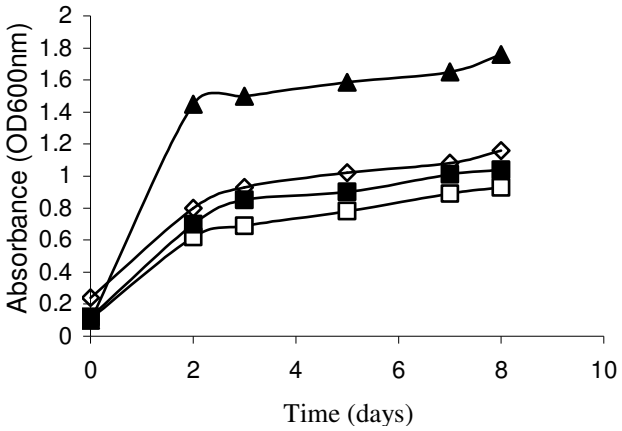

Figure 1. The growth rate of yeast grown on basal salt medium containing 0.3 mM DBT dissolved in ethanol (□), glucose (▲), glucose with 0.3 mM DBT (■), and ethanol with inorganic sulfur source (◊), incubated at 30°C, pH 7 for 7days.

RESULTS

Isolation

In phenol-rich medium, a yeast was isolated that can use 0.3 g/L phenol in 24 h. The reduction of phenol was assayed by Gibb's reagent. This strain identified as *Trichosporon sp.*, and did not show any growth on octane, isooctane, toluene, ethyl acetate, dimethylformamide, acetone, ether, and tetrachlorocarbon (TCC). However it seems this strain can utilize dibenzothiophene (DBT) as the only source of sulfur in present of another carbon sources. Production of biphenyl was not seen during utilization of DBT. The growth rate of this isolate on different media showed that the yeast have the same growth on ethanol with or without DBT (Figure 1)

λmax of DBT in 285 and 323 nm reduced when yeast was grown on basal salt medium (BSM) with DBT dissolved in ethanol (0.3 mM). The results are shown the reduction of λmax in 285 nm was almost 60% and the reduction of λmax in 323 nm was more than 70% in 9 days, and after 14 days λmax in 323 was zero. The result

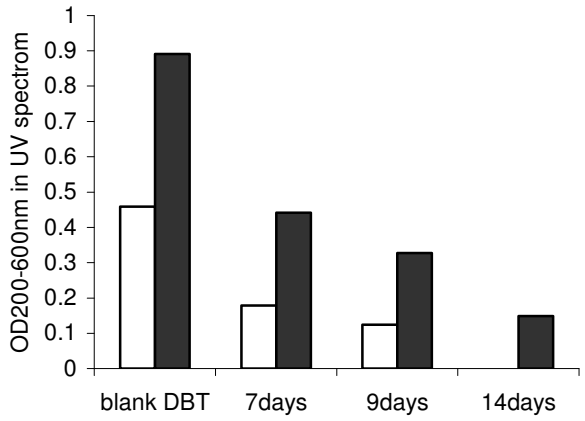

Figure 2. Reduction of λmax in 323 nm (□), and λmax in 285 nm (■), when isolated yeast was inoculated into 0.3 mM DBT in ethanol (Incubation at 30°C, pH 7, T=14 days and shaking=180 rpm).

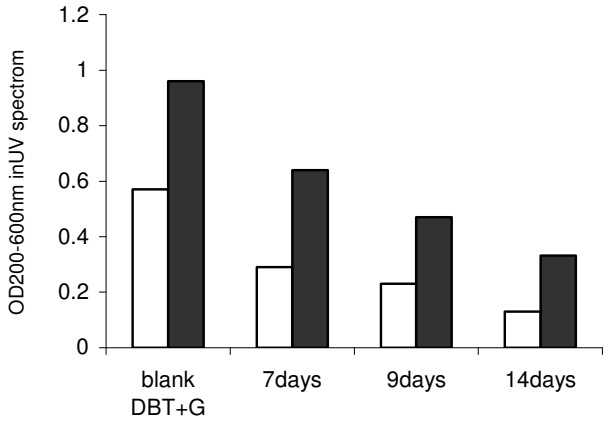

Figure 3. Reduction of λmax in 285nm (■) and λmax in 323nm (□), when isolated yeast was inoculated to 0.3 mM DBT in present of glucose (incubation at 30°C, pH 7, T=14 days and shaking=180 rpm).

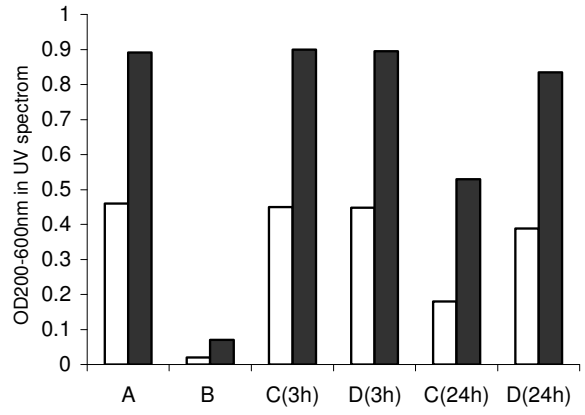

Figure 4. Effect of yeast supernatant grown on glucose with sulfate (S) or DBT as the sole sulfur source on reduction of λmax in 285 nm (■) and λmax in 323 nm (□) of DBT dissolved in ethanol after 3 and 24 h. A = 0.3 mMDBT blank, B = blank of DBT supernatant, C = supernatant of inorganic S, and D= supernatant of DBT medium.

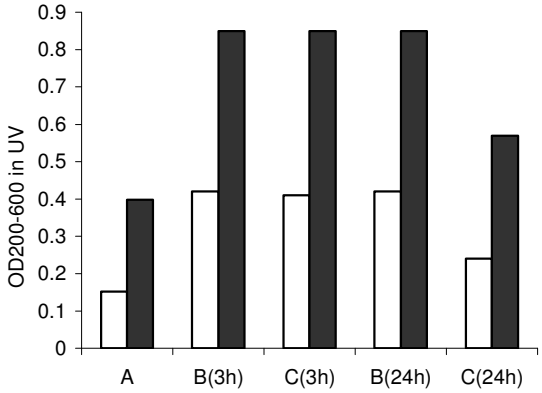

Figure 5. Effect of resting cells of yeast grown on glucose with DBT or sulfate (S) as the sole sulfur source on reduction of λmax in 285 nm (■) and λmax in 323 nm (□) of DBT dissolved in ethanol in BSM medium. A = blank of resting cells in DBT, B = resting cells of medium with inorganic S, C =r esting cells of medium with DBT.

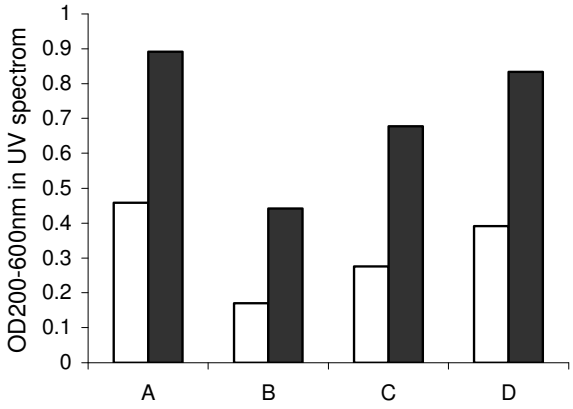

Figure 6. Effect of NaCl on reduction of λmax in 285 nm (■) and λmax in 323 nm (□) of DBT dissolved in ethanol in BSM medium. A=blank DBT, B=DBT without NaCl, C=1%NaCl, D=5%NaCl, incubated at 30°C with 140 rpm shaking.

showed that ethanol produced biomass and DBT was utilized during ethanol utilization (Figure 2). The effect of glucose is also shown in Figure 3. Glucose does not increase reduction of DBT.

The effect of yeast supernatant on DBT is shown in Figure 4, indicating that the enzymes for DBT oxidation are extracellular, and for induction of these enzymes DBT must used as the only source of sulfur. The results showed that when the isolated yeast grown on sulfate the supernatant cannot reduce λmax of DBT in 24 h. Also the resting cells grown on glucose with sulfate or DBT as the only sources of sulfur can reduce the λmax of DBT without any carbon sources (Figure 5). So by immobilization of this yeast, it is possible to reduce DBT in oil without any changes of hydrocarbon. The yeast biomass can be easily removed from the oil bulk.

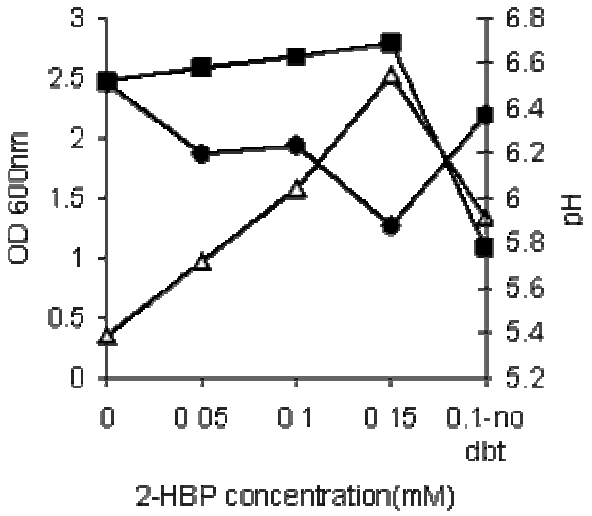

Figure 7. Effect of different concentration of 2-HBP on maximum growth (●), pH variation (■) and Gibbs' test (Δ) in DBT medium supplemented with glucose (1 gL^{-1}) inoculated by the yeast, incubated at 30°C with 140 rpm shaking.

The effect of NaCl on utilization of DBT is shown in Figure 6. In the presence of 1% NaCl, 40% of λmax of DBT in 323 nm was reduced, while in control medium 62% reduction occurred. In medium with 5% NaCl, reduction of DBT was low (approximately 13%). The effect of 2-HBP on growth rate of yeast was is shown in Figure 7. The maximum growth is in the 7th day is using 0 mM of 2-HBP that includes 0.2 mM DBT and also in 0.1 mM 2-HBP without DBT. When the yeast cultured in Sabaura broth and 2-HBP added to it after 24 h, the culture showed a change in UV absorbance curve that is shown in Figure 8.

Assessment of effect of DBT concentration on growth rate (data are not shown) was carried out. Results of this part of study showed that the isolated *Trichosporon* showed greatest growth at DBT concentrations of 0.1- 4 mmole, but the yeast were unable to grow when the concentration is higher than 5 mM, possibly due to toxicity of DBT.

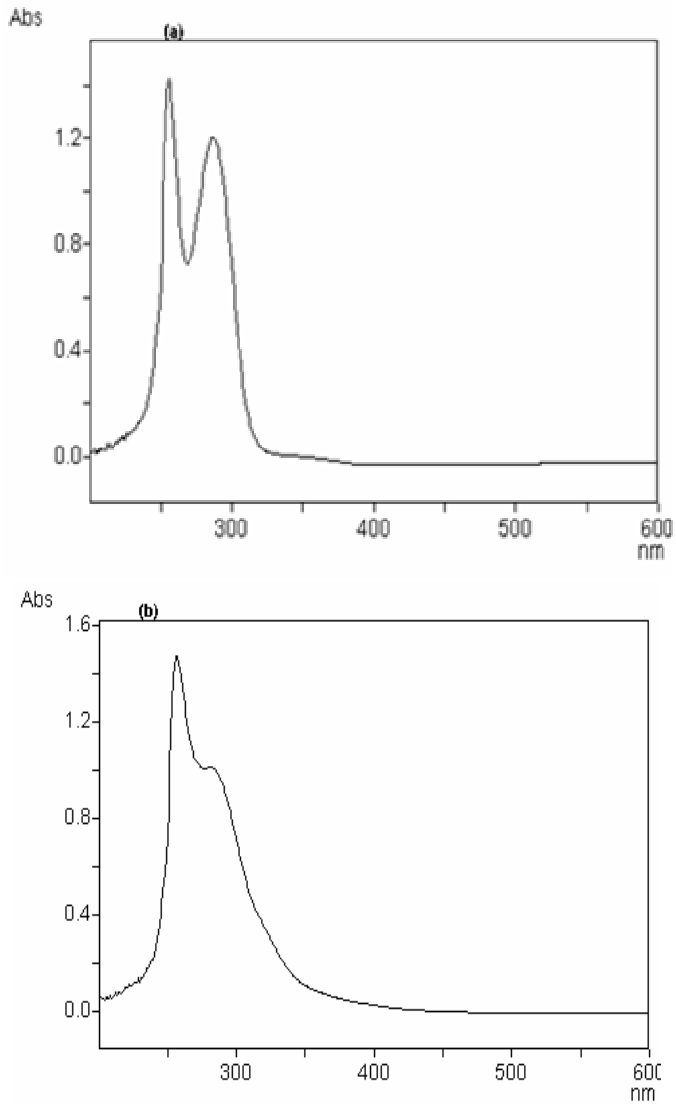

Figure 8. Absorbance curve of 2-HBP in UV spectrum ($OD_{200-600nm}$), (a): blank of 2-HBP 0.2 mM in saboura broth (SB), and (b): 7-day culture of the yeast in SB with 2-HBP 0.2 mM, incubated at 30°C with 140 rpm shaking.

DISCUSSION

The yeast, *Trichosporon* sp. was isolated from phenol-rich industrial wastes and was the only microorganism capable of growing at high concentration of phenol. This strain can use sulfate, thiosulfate, organic sulfur and DBT as the only source of sulfur. The utilization of the organic sulfur is not dependent on the presence of glucose in the culture medium when enough cells exist in the media. However Baldi et al. (2003) indicated that the utilization of the organic sulfur compounds by *Rhodosporidium toruloides* is strictly dependent on the presence of in the culture medium. Here ethanol was used as DBT solvent and the utilization of DBT was observed when DBT was dissolved in ethanol without glucose. Even the cell supernatant or resting cell could remove DBT.

The utilization of DBT cannot be through the kodama pathway since there was no growth on DBT as the only source of carbon and sulfur. There was no sign of production of formaldehyde or HFBT intra- or extra- cellular and determination of HFBT (increasing the absorbance in λ392 in UV spectrum) or formaldehyde assays were negative. The growth of this microorganism in BSM medium containing glucose and 2-Hydroxybiphenyl with or without DBT was also assessed. Results showed that in 1% 2-HBP, the best growth occured. Spectrometric assay and Gibb's test also showed that the quantity of 2- HBP

was partially decreased and pH also was reduced to 5.8 on the 7th day of culture.

The absence of detectable sulfate or 2-HBP may indicate that the metabolites were produced in quantities too small for detection by the analytic techniques used in this study or it is possible that *Trichosporon* changes DBT rapidly to produce 2-HBP to other metabolites. According to Sietmann et al. (2001) the yeast *Trichosporon mucoides* was able to transform biphenyl into a variety of mono-, di-, and trihydroxylated derivatives hydroxylated on one or both aromatic rings.

ACKNOWLEDGEMENT

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