Turkish Journal of Chemistry

Volume 39 | Number 2 Article 5

1-1-2015

# Revisiting the biodesulfurization capability of hyperthermophilic archaeon Sulfolobus solfataricus P2 revealed DBT consumption by the organism in an oil/water two-phase liquid system at high temperatures

GÖKHAN GÜN

YUDA YÜRÜM

GİZEM DİNLER DOĞANAY

TÜBİTAK

Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2015) 39: 255 – 266

doi:10.3906/kim-1407-52

# Revisiting the biodesulfurization capability of hyperthermophilic archaeon *Sulfolobus solfataricus* P2 revealed DBT consumption by the organism in an oil/water two-phase liquid system at high temperatures

**Gökhan GÜN[1], Yuda YÜRÜM[2], Gizem DİNLER DOĞANAY[1,*]**

[1]Department of Molecular Biology and Genetics, İstanbul Technical University, Maslak, İstanbul, Turkey
[2]Faculty of Engineering and Natural Sciences, Materials Science and Engineering Program, Sabancı University, Orhanlı, Tuzla, İstanbul, Turkey

**Received:** 23.07.2014 • **Accepted/Published Online:** 05.11.2014 • **Printed:** 30.04.2015

**Abstract:** The ability of the hyperthermophilic archaeon *Sulfolobus solfataricus* P2 to grow on organic and inorganic sulfur sources was investigated. A sulfur-free mineral medium was employed with different sources of carbon. The results showed that inorganic sulfur sources display growth curve patterns significantly different from the curves obtained with organic sulfur sources. *Solfataricus* has the ability to utilize DBT and its derivatives, but it lacks BT utilization. *Solfataricus* utilizes DBT at a rate of 1.23 $\mu$mol 2-HBP $h^{-1}$ g $DCW^{-1}$ even at 78 °C, at which DBT is known to be unstable. After enabling DBT stabilization using a two-phase culture system, stable microbial growth was achieved showing a desulfurization rate of 0.34 $\mu$M DBT g $DCW^{-1}$ $h^{-1}$. *Solfataricus* offers beneficial properties compared to the other desulfurizing mesophilic/moderate thermophilic bacteria due to its capacity to utilize DBT and its derivatives under hyperthermophilic conditions.

**Key words:** Biodesulfurization, dibenzothiophene, gas chromatography, *Sulfolobus solfataricus* P2, sulfur compounds

## 1. Introduction

Combustion of fossil fuels leads to the atmospheric emission of sulfur oxides that contribute to acid rain and air pollution.[1] Strict government regulations throughout the world have been implemented to reduce these emissions.[2] Nowadays, the current technology used to reduce the sulfur composition in fuels is hydrodesulfurization (HDS), which is the conventional method carried out with chemical catalysis at high temperature (290–450 °C) and pressure (1–20 mPa).[1] Heterocyclic organosulfur compounds (dibenzothiophene (DBT) and substituted DBTs) represent significant sulfur (up to 70%) quantities in petroleum and are recalcitrant to HDS.[3] Therefore, biological desulfurization (BDS) using microorganisms and/or enzymes is an attractive alternative or complementary method to HDS due to its low cost, mild reaction conditions, and greater reaction specificity.[4]

DBT is a widely used model compound in desulfurization studies.[5] Sulfur-specific cleavage of DBT (4S pathway) is a preferable pathway in biodesulfurization, in which DBT is selectively removed without carbon skeleton rupture. This pathway includes four reactions through the conversion of DBT into a free sulfur product, 2-hydroxybiphenyl (HBP), and sulfite/sulfate.[6]

Various DBT desulfurizing microorganisms have been reported to date; for instance, mesophilic bacteria

*Correspondence: gddoganay@itu.edu.tr

such as *Rhodococcus* sp. IGTS8,[7] *Rhodococcus erythropolis* H-2,[8] *Corynebacterium* sp.,[9] *Bacillus subtilis* WU-S2B,[10] and a moderately thermophilic *Mycobacterium pheli* WU-F1[11] are known to use the 4S pathway in DBT desulfurization. Since these bacteria exhibit high DBT-desulfurization ability at around 30 °C and 50 °C for mesophilic and moderately thermophilic bacteria, respectively, their use in fossil fuel desulfurization as an alternative or complementary to hydrodesulfurization requires an additional cooling process of the fuel to ambient temperature following HDS. This additional cooling process causes an economical burden when used in large scale fossil fuel desulfurization. Thus, hyperthermophilic microbial desulfurization is desirable and makes the crude oil biodesulfurization process more feasible due to the low viscosity of the crude at high temperature.[3]

There have been various attempts to use hyperthermophiles in biodesulfurization to date.[12–15] Most of these studies were able to clearly delineate the pyritic sulfur desulfurization, but failed to show reliable sufficient amounts of organic sulfur removal efficiency. A study that examined the usage of hyperthermophilic *Sulfolobus acidocaldarius* in DBT utilization revealed the oxidation of sulfur present in DBT to sulfate at 70 °C.[13] Unfortunately, that study did not include DBT degradation at high temperatures in the absence of microorganism;[13] therefore, the obtained rate of desulfurization does not represent the real biodesulfurization rate. Another attempt to study heterocyclic organosulfur desulfurization using a thermophile, *Sulfolobus solfataricus* DSM 1616,[15] at 68 °C showed DBT self-degradation in the absence of microorganism at high temperatures; thus no substantial DBT utilization could be observed. That study clearly showed the difficulty of using a DBT model compound at high temperatures in biodesulfurization by *S. solfataricus.*[15] Nonetheless, the same study showed the oxidation of thiophene-2-carboxylate by *S. solfataricus*;[15] therefore, the organic sulfur desulfurization molecular mechanism was shown to be present in this hyperthermophile, and further investigations are necessary to optimize the conditions for better organic sulfur removal with possibly a different *Sulfolobus* strain, which might lead to better efficiency for desulfurization.

Hyperthermophiles are isolated mainly from water-containing volcanic areas such as solfataric fields and hot springs in which they are unable to grow below 60 °C. *Sulfolobus solfataricus* P2, belonging to the archaebacteria, grows optimally at temperatures between 75 and 85 °C and at low pHs between 2 and 4, utilizing a wide range of carbon and energy sources.

This paper describes the potential of a hyperthermophilic archaeon, *S. solfataricus* P2, to utilize several inorganic and organic sources of sulfur for growth in various conditions, and shows *S. solfataricus* P2's ability to remove sulfur from DBT via the sulfur-selective pathway even under high temperatures with the elimination of DBT self-degradation. To the best of our knowledge, this is the first report showing the DBT desulfurization kinetics analysis of *S. solfataricus* P2.

## 2. Results and discussion

### 2.1. Influence of carbon source on the growth of *S. solfataricus* P2

The ability of *S. solfataricus* P2 to use several sources of carbon was investigated. Four types of carbon sources were applied to the SFM medium: D-glucose, D-arabinose, D-mannitol (Figure 1), and ethanol. All these experiments were carried out employing 2 g $L^{-1}$ as the initial concentration of carbon source. Figure 2 shows the effects of different sources of carbon on archaeal growth. The highest growth rate, 0.0164 $h^{-1}$ (60.9 h), and the maximum biomass density, 0.149 g dry weight $L^{-1}$, were observed when D-glucose was employed as a carbon source (Figure 2). On the other hand, D-arabinose, D-mannitol, and ethanol (at a concentration of 2 g $L^{-1}$) did not support growth (Figure 2). Our data in Figure 2 clearly show that glucose is a better carbon source for the growth of *S. solfataricus* P2 compared to the other carbon sources tested. *S. solfataricus* harbors

a semiphosporylative Entner–Doudoroff (ED) pathway for sugar metabolism.[16,17] Since D-glucose is the first metabolite necessary to initiate glycolysis, better D-glucose utilization than the other sugars is expected. For both D- and L-arabinose a well-defined pentose mechanism exists in *S. solfataricus*.[16] Both pentose mechanisms may include intermediates that are not heat stable; thus these products may become degraded while enough ATP is accumulated to allow cells to survive. As presented in a recent study, unstable intermediate metabolites exist for the semiphosporylative ED pathway in glucose metabolism for hyperthermophiles that grow at extreme temperatures.[17] Therefore, a similar type of unstable intermediate production in the pentose mechanism may prevent the growth of *S. solfataricus* cells under scarce sugar supplies.

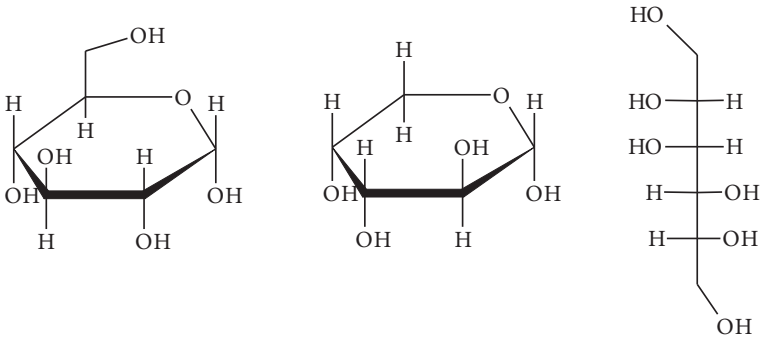


**Figure 1.** Some of the carbon sources used in the study. Molecular structures of D-glucose, D-arabinose, and D-mannitol are shown.

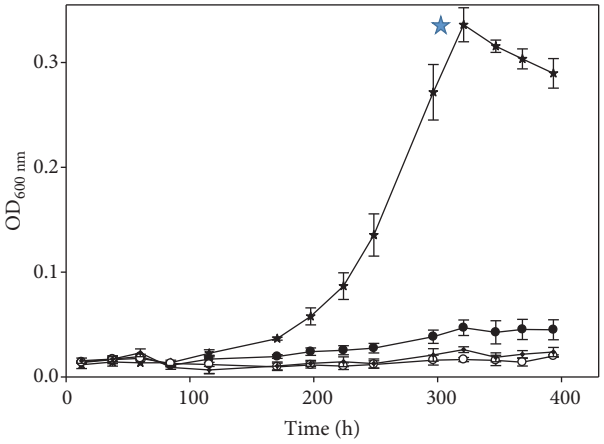


**Figure 2.** Effects of different carbon compounds (concentrations of 2 g $L^{-1}$) on the growth of *S. solfataricus* P2 in SFM medium. (○) D-mannitol, (●) D-arabinose, (+) ethanol, (⋆) D-glucose. The white star represents the highest growth rate observed for D-glucose.

To further determine the optimum growth condition of *S. solfataricus* P2 in SFM medium when glucose is the source of carbon, various concentrations of glucose ranging from 2 g $L^{-1}$ to 20 g $L^{-1}$ on SFM culture were employed. The results revealed that the highest growth rate (0.0339 $h^{-1}$ (29.5 h)) and biomass concentration (0.157 g $L^{-1}$) were obtained when 20 g $L^{-1}$ glucose was used (Figure 3). It can be affirmed that the higher the glucose concentration is, the higher the growth rate is (Table 1). Figure 3 also indicates that with increasing concentrations of glucose, an enhanced growth rate was observed, and the time required to reach the maximum biomass value was decreased; however, the maximum cell densities obtained with increasing concentrations of

glucose were similar for all of the concentrations (ranging from 0.14 to 0.157 g DCW $L^{-1}$). At the same time, the lag time decreased with the highest concentration of glucose application, and cells reached the stationary phase faster as the concentration of glucose was increased. The observed increased rate for growth with higher glucose concentration might due to allowing cells to steadily obtain all the necessary intermediate metabolites; even if some of them get degraded under high temperatures,[17] excess amounts for productive glycolytic cycles would still be enough for cells to proliferate. Although an acceptable growth profile was observed when glucose was employed as the carbon source, overall, in SFM medium, the presence of glucose was not sufficient to obtain optimal growth; additional micronutrients were necessary to optimize the growth conditions.

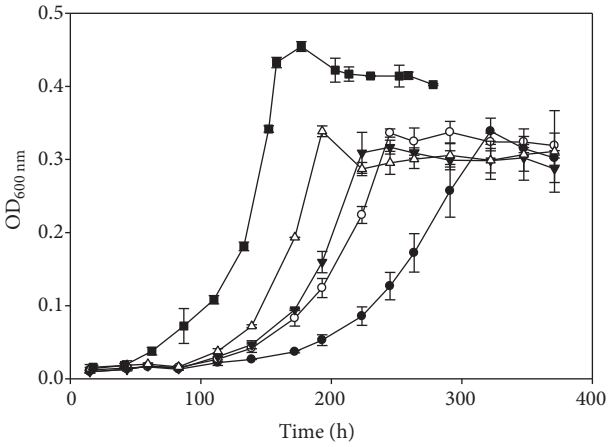


**Figure 3.** Glucose gradients from 2 g $L^{-1}$ to 20 g $L^{-1}$ were performed in SFM medium. (●) 2, (○) 5, (▼) 10, (△) 15, and (■) 20 g $L^{-1}$ glucose.

**Table 1.** Calculated growth rates and maximum cell densities corresponding to experimental growth data of *S. solfataricus* P2 cells when treated with increasing glucose concentrations as the sole source of carbon.

| | Growth rate ($h^{-1}$) | Maximum cell density (g $L^{-1}$) |
|---|---|---|
| 2 g $L^{-1}$ glucose | 0.0164 ± 0.0006 | 0.149 ± 0.008 |
| 5 g $L^{-1}$ glucose | 0.0192 ± 0.0004 | 0.148 ± 0.003 |
| 10 g $L^{-1}$ glucose | 0.0217 ± 0.0006 | 0.139 ± 0.002 |
| 15 g $L^{-1}$ glucose | 0.0276 ± 0.0014 | 0.149 ± 0.005 |
| 20 g $L^{-1}$ glucose | 0.0345 ± 0.0011 | 0.199 ± 0.003 |

### 2.2. Utilization of organic sulfur compounds

The ability of *S. solfataricus* P2 to utilize organic sulfur compounds was evaluated toward 4,6-DMDBT, DBT sulfone, DBT, and BT. Each acted as the sole source of sulfur for growth with an initial concentration of 0.3 mM in SFM culture except for the presence of trace amounts of sulfur originating from the culture stocks. ICP-OES analysis revealed the presence of 0.00168 ± 0.0008 g $L^{-1}$ sulfur in the 100-mL control flasks. Unless otherwise noted, all the cultivation experiments were done in the same manner, and their initial sulfur contents were estimated to be similar to the initially determined value. Moreover, for all of the growth, 20 g $L^{-1}$ glucose was employed as a carbon source in SFM medium. The effects of the organic sulfur compounds on growth are shown in Figure 4. When the cultures were incubated initially with DBT, DBT-sulfone, 4,6-DMDBT, and BT, there was no archaeal growth (data not shown). Instead of employing organic compounds at the beginning of growth, each organic sulfur compound was separately added to SFM medium after a moderate optical density

(OD between 0.35 and 0.4, around the midst of log phase during *S. solfataricus* P2 growth) was attained. Thus, supplementation of organic compounds in this way enabled *S. solfataricus* P2 cells to grow well on media containing DBT-sulfone and 4,6-DMDBT as the sole sources of sulfur; however, addition of BT resulted in abrupt interruption of cell growth and subsequently led to cell death (Figure 4). DBT addition, on the other hand, progressively ceased the growth of the cells (Figure 4). Maximum biomass densities and specific growth rates are given in Table 2. Maximum cell density was achieved with 4,6-DMDBT, yielding 2.5 times higher cell density compared to that of the control. DBT-sulfone presence enabled cells to achieve 1.4 times higher cell density with respect to the control. These results revealed that *S. solfataricus* P2 can utilize organic sulfur compounds containing DBT and its derivatives; however, even among them, it has certain preferences for some types of organic molecules over others. The results indicated that *S. solfataricus* P2 cannot utilize BT. Since DBT and BT desulfurization pathways were shown to be different for various desulfurizing bacteria,[18,19] it can be concluded that *S. solfataricus* P2 has a metabolic pathway specific for DBT and its derivatives.

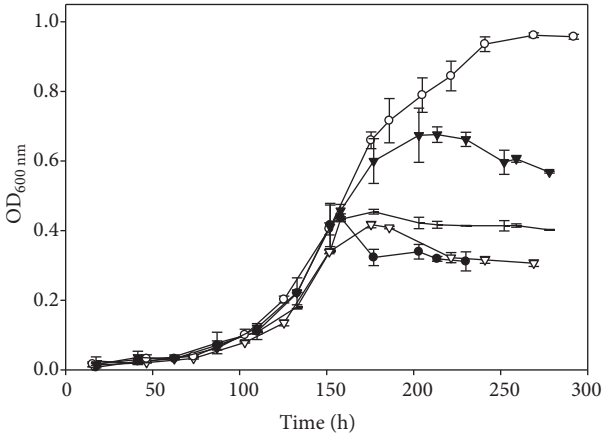


**Figure 4.** Growth of *S. solfataricus* P2 in the presence of 0.3 mM organic sulfur sources in SFM medium supplemented with 20 g $L^{-1}$ glucose. (●) BT, (○) 4-6 dimethyldibenzothiophene, (▼) DBT sulfone, (▽) DBT, and (–) SFM-only medium. Sulfur sources were supplemented to the growing cultures at $OD_{600}$ near 0.4.

**Table 2.** Utilization of various organic sulfur compounds by *S. solfataricus* P2 in SFM medium.

| | Growth rate ($h^{-1}$) | Maximum cell density (g $L^{-1}$) |
|---|---|---|
| 4.6 DMDBT | 0.0172 ± 0.0011 | 0.423 ± 0.031 |
| DBT-sulfone | 0.0179 ± 0.0056 | 0.281 ± 0.011 |
| BT | - | 0.192 ± 0.009 |
| DBT | - | 0.183 ± 0.004 |

### 2.3. Utilization of inorganic sulfur compounds

To compare the effects of organic and inorganic sulfur sources on growth, 0.3 mM inorganic sulfur sources as sole sulfur sources (elemental sulfur, sodium sulfite, sodium sulfate, potassium persulfate, and potassium disulfite) were employed in the SFM medium at $OD_{600}$ around 0.32. Growth curve patterns of cultures containing inorganic sulfur sources were similar except for the elemental sulfur case (Figure 5). All the growth curves reveal a short stationary period after supplementation of the inorganic sulfur compounds, suggesting a certain adaptation time for the cells to the new nutrient environment. This adaptation period may correlate to the immediate uptake of inorganic sulfur molecules by the cells. A logarithmic enhancement in the growth followed

by this short stationary period shows that *S. solfataricus* P2 utilizes the supplied inorganic sulfur sources. Similar growth rates were observed for the sulfate and sulfite cases (Table 3). Elemental sulfur supplemented growth revealed a longer adaptation period and showed a slower growth rate compared to that of the sulfate and sulfite supplemented growths (Table 3). The growth curves showed maximum cell densities with the sulfate compounds; a very similar maximum cell density (0.651 g DCW $L^{-1}$) with minor errors was obtained (Table 3). Inorganic sulfur sources led to rapid cell death after a maximum biomass cell density was obtained except for in the elemental sulfur case, which showed a sustained stationary phase (Figure 5) after a maximum cell density, 0.586 $\pm$ 0.016 g DCW $L^{-1}$, was reached (Table 3). Rapid cell death after sulfate and sulfite utilization could be explained by the excess uptake of these anions by the cells, leading to a demand for counter ion balance, which can be maintained by excess accumulation of cations to cells, causing an osmotic imbalance. The observation of a prolonged stationary phase in the elemental sulfur case was similar to that of the control growth, where even after 150 h of growth in the stationary phase still a certain cell density can be measured but the estimated cell density for the control was almost 4 times less than that of the elemental sulfur supplemented trial (Figure 5; Tables 1 and 3). In SFM medium, when inorganic sulfur sources were used as the sole sulfur source instead of organic sulfur compounds, faster growth rates and larger biomass concentrations were observed for *S. solfataricus* P2. It is thought that not all glucose was used after cells reached a cell density of 0.157 g DCW $L^{-1}$. At this point, sulfur became the growth limiting factor and supplementation of inorganic sulfur sources led to faster growth and higher biomass density.

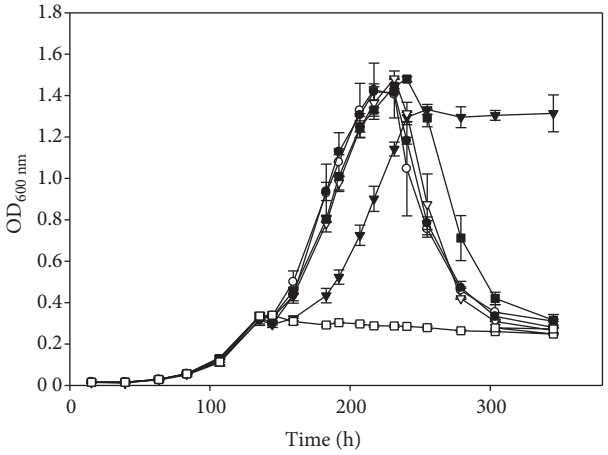


**Figure 5.** Growth of *S. solfataricus* P2 in the presence of 0.3 mM inorganic sulfur sources in SFM supplemented with 20 g $L^{-1}$ glucose. (▼) Elemental sulfur, (○) sodium sulfite, (■) sodium sulfate, (▽) potassium persulfate, (●) potassium disulfite, and (□) SFM-only medium. Sulfur sources were supplemented to the growing cultures at $OD_{600}$ near 0.4.

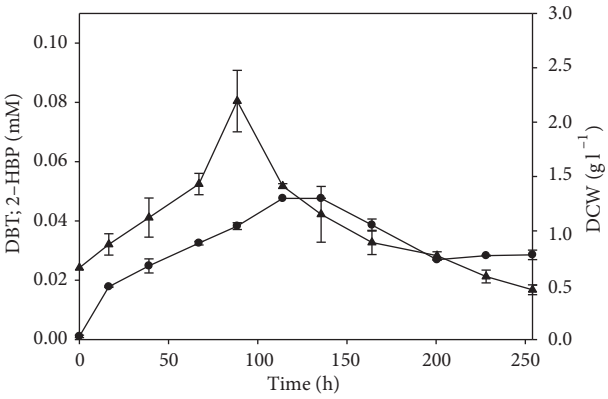


**Figure 6.** Formation of 2-HBP by the growing cells of *Sulfolobus solfataricus* P2. DBT was supplemented to growing cultures in minimal medium at 0.66 g dry cell $L^{-1}$. (▲) DCW, (●) 2-HBP.

### 2.4. DBT consumption kinetics by *S. solfataricus* P2

Our results revealed that *S. solfataricus* P2 can utilize 4,6-DMDBT and DBT sulfone efficiently, but DBT utilization was not as effective as that of the former compounds in SFM culture medium. Since DBT has been used as the model molecule of the thiophenic compounds present in fossil fuels, we aimed to optimize DBT

**Table 3.** Utilization of various inorganic sulfur compounds by *S. solfataricus* P2 in SFM medium.

| | Growth rate ($h^{-1}$) | Maximum cell density (g $L^{-1}$) |
|---|---|---|
| Elemental S | 0.0165 ± 0.0012 | 0.586 ± 0.016 |
| Sodium sulfite | 0.0226 ± 0.0006 | 0.628 ± 0.053 |
| Potassium disulfite | 0.0254 ± 0.0005 | 0.623 ± 0.008 |
| Sodium sulfate | 0.0220 ± 0.0008 | 0.651 ± 0.005 |
| Potassium persulfate | 0.0222 ± 0.0003 | 0.651 ± 0.001 |

utilization levels of *S. solfataricus* P2 by changing the growth medium conditions. Addition of yeast extract to the minimal medium significantly enhanced the utilization levels of DBT by *S. solfataricus* P2. The effect of different concentrations of DBT was tested in the growth of *S. solfataricus* P2 (Table 4); with 0.1 mM DBT supplementation, cell density was enhanced significantly compared to the control, where no DBT was added to the minimal medium, and to the increasing DBT concentrations. Higher amounts of DBT use showed significantly lower maximum cell density; therefore, 0.1 mM of DBT was used in our DBT desulfurization kinetics studies (Table 4). Continuous growth was observed until 89 h with simultaneous production of 2-HBP, which was determined by both Gibbs assay and GC (Figure 6). It was observed that DBT concentration decreased sharply under abiotic conditions (data not shown). Earlier work also revealed DBT to be unstable at higher temperatures in an aqueous environment.[15] However, even under these conditions, desulfurization activity was observed in growing cultures, and is estimated to be 1.23 $\mu$mol 2-HBP $h^{-1}$ g $DCW^{-1}$. The specific production rate of 2-HBP was decreased sharply after 16.5 h, as can be seen in Figure 7. A similar abrupt decrease in the production rate of 2-HBP was observed previously in most of the BDS studies,[20–23] and was explained by the production of HBP in the medium causing substrate inhibition type of enzyme kinetics.[24] Although 93% of DBT depletion was observed within 39 h, 2-HBP production continued to increase up until 114 h to a concentration of 47.6 $\mu$M. Growth of *S. solfataricus* P2 stopped near where the maximum levels of 2-HBP were produced (Figure 6). Similar growth inhibition behavior with 2-HBP production was also observed in previous BDS studies.[25,26] It was reported that 2-HBP above 200 $\mu$mol/L was toxic to the bacterial cells and inhibitory to biodesulfurization.[8] Even though the maximum levels of 2-HBP concentration produced in our studies were not close to the toxic level, a decrease in 2-HBP production rate was observed with cell death. Another explanation may be other products that developed in the biodesulfurization pathway becoming toxic to cells.

Since DBT was not stable at 78 °C in the aqueous environment (90% DBT depletion was observed within 16.5 h (data not shown)), an oil phase was used to prevent the effects of temperature and aqueous medium on DBT stabilization. DBT was preserved under abiotic conditions when the xylene was used as the second phase. Although addition of xylene containing DBT ceased the growth at the mid-log phase, 22% DBT utilization was observed within 72 h (Figure 8). The specific rate of DBT degradation in the first 23 h was 0.34 $\mu$M DBT g $DCW^{-1}$ $h^{-1}$. After 24 h of xylene addition, *S. solfataricus* P2 secreted a biosurfactant into the culture medium. Emulsification was observed only in growing cultures, not in the control. It was suggested in a previous study that formation of biosurfactant may play a role in the DBT desulfurization process by increasing the contact surface of cells with the oil phase.[27] A two-phase system has been tested in many BDS studies in which hexane, heptane, and xylene were mainly used as the oil phase.[27,28] Since the growing temperature necessary for *S. solfataricus* P2 growth was higher than that in other BDS studies using the two-phase systems,[27–30] an oil having a high boiling temperature, xylene (bp 134–139 °C), was selected as the oil phase. Although DBT

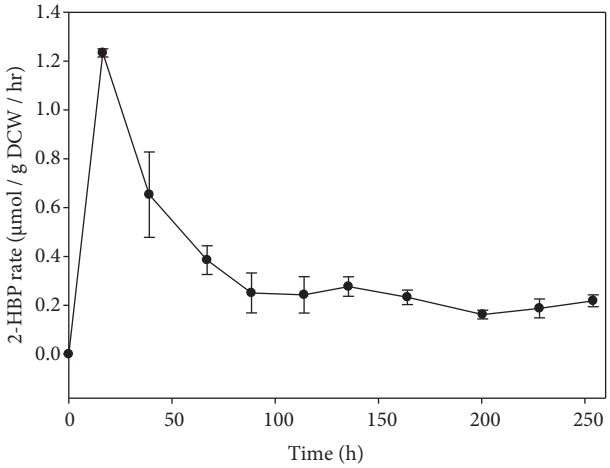


**Figure 7.** The time course of specific production rate of 2-HBP from 0.1 mM DBT by *Sulfolobus solfataricus* P2.

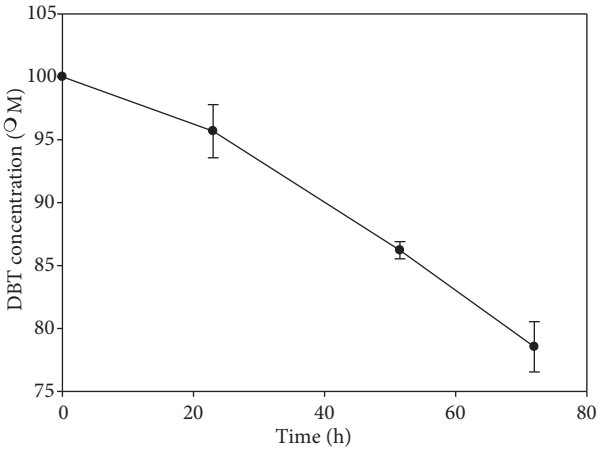


**Figure 8.** Consumption of DBT. Experiments were performed in minimal medium containing 40% (v/v) xylene.

**Table 4.** Utilization of increasing DBT concentrations by *S. solfataricus* P2.

| | Growth rate ($h^{-1}$) | Maximum cell density (g $L^{-1}$) |
|---|---|---|
| 0.1 mM DBT | 0.0122 ± 0.0014 | 2.19 ± 0.28 |
| 0.2 mM DBT | 0.0061 ± 0.0011 | 2.13 ± 0.11 |
| 0.3 mM DBT | 0.0020 ± 0.0002 | 0.87 ± 0.01 |
| 0.4 mM DBT | - | 0.73 ± 0.01 |
| Yeast medium (control) | 0.0149 ± 0.0010 | 1.57 ± 0.05 |

containing a xylene phase ceased the growth of the microorganism when it was applied in the two-phase system at 40% (v/v), equilibrium between xylene concentration, amount of DBT in the oil phase, and initial cell concentration can be optimized for effective DBT biodesulfurization when applied in industrial usage.

A two-oil-phase system has been used for enhancing the poor solubility of many organic compounds in aqueous cultures.[29,30] Since the solubility of DBT is 0.005 mM in water,[30] an aqueous/apolar culture system is advantageous for the biodesulfurization of DBT and its derivatives.

In conclusion, since biodesulfurization performed under high temperatures has potential for an alternative/complementary method to lower the sulfur content of fossil fuels, hyperthermophilic *S. solfataricus* P2 with its potential DBT-desulfurization ability can serve as a model system for the efficient biodesulfurization of fossil fuels. Further molecular biology studies for the characterization of the genes responsible for DBT desulfurization, undertaken already by our group, will enable us to delineate the exact BDS mechanism of *S. solfataricus* P2.

## 3. Experimental

### 3.1. Chemicals

*S. solfataricus* was obtained as a powder from the American Type Culture Collection (ATCC 35091). DBT (99%) was obtained from Acros Organics, DBT-sulfone (97%) was from Sigma Aldrich, 4,6-Dimethyldibenzothiophene (97%) and elemental sulfur (99%) were from ABCR, and DMF was from Riedel-de Haën. All other reagents were of the highest grade commercially available.

### 3.2. Culture media and growth conditions

Sulfur-free mineral (SFM) medium was prepared by dissolving 70 mg of $CaCl_2.2H_2O$, 1.3 g of $NH_4Cl$, 0.25 g of $MgCl_2.6H_2O$, 0.28 g of $KH_2PO_4$, and 0.5 mL of trace elements solution in 1 L of Milli-Q water, and this mixture was adjusted to pH 3 with HCl. Trace elements solution[18] was prepared with 25 g $L^{-1}$ EDTA, 2.14 g $L^{-1}$ $ZnCl_2$, 2.5 g $L^{-1}$ $MnCl_2.4H_2O$, 0.3 g $L^{-1}$ $CoCl_2.6H_2O$, 0.2 g $L^{-1}$ $CuCl_2.2H_2O$, 0.4 g $L^{-1}$ $NaMoO_4.2H_2O$, 4.5 g $L^{-1}$ $CaCl_2.2H_2O$, 2.9 g $L^{-1}$ $FeCl_3.6H_2O$, 1.0 g $L^{-1}$ $H_3BO_3$, and 0.1 g $L^{-1}$ KI. Minimal medium[31] was adjusted to pH 3 and supplemented with yeast extract (0.15% w/v) and glucose (20 g $L^{-1}$). Initial stocks of *S. solfataricus* culture were initially made by using minimal medium and were kept at –80 °C as 10% glycerol stocks of 1-mL aliquots. Cell cultivation was carried out at 78 °C in 250-mL flasks containing 100 mL of medium with 160 rpm shaking.

### 3.3. Carbon utilization

SFM culture medium was employed as the base medium and was supplemented with D-arabinose, ethanol, D-glucose, and D-mannitol as different sources of carbon to a final concentration of 2 g $L^{-1}$. To find out the optimum sulfur-free growth conditions, various concentrations of the most effective carbon source, glucose, was added to SFM culture medium at concentrations of 2, 5, 10, 15, and 20 g $L^{-1}$. The data are represented as the means of triplicate cultures ± standard error.

### 3.4. Sulfur utilization

The ability of *Sulfolobus solfataricus* P2 to utilize organic and inorganic sulfur sources was investigated. Several organic and inorganic sulfur compounds including DBT, BT, DBT-sulfone, 4,6-dimethyldibenzothiophene (4,6-DMDBT), elemental sulfur, sodium sulfide, sodium sulfate, potassium persulfate, and potassium disulfite were added at an initial concentration of 0.3 mM to SFM culture as the sole source of sulfur. However, there was a trace amount of sulfur contamination from the stocks of the culture, which were first prepared using minimal medium. Sulfur content originating from the stocks of *S. solfataricus* in SFM was measured using inductively coupled plasma-optical emission spectrometry (ICP-OES, PerkinElmer, USA) as described in a previous study.[32] In all of these media, 20 g $L^{-1}$ glucose was used as the sole source of carbon. SFM culture containing only the carbon source (20 g $L^{-1}$ of glucose) was used as a control. Stock solutions of organic sulfur compounds, DBT, BT, 4,6-DMDBT, and DBT-sulfone were dissolved in N,N-dimethylformamide (100 mM). In all of these experiments, organic sulfur compounds were added to the growth culture after a certain exponential growth was achieved, corresponding to an $OD_{600}$ (optical density at 600 nm) value between 0.35 and 0.4. Data are represented as the means of triplicate cultures ± standard error.

For the desulfurization kinetics assay, minimal medium supplemented with 0.1 mM DBT, 0.15% w/v yeast extract, and glucose (20 g $L^{-1}$) was used in the presence and absence of 40% (v/v) xylene. Cells grown at the mid-log phase ($OD_{600}$ being 1.5) were supplemented with DBT or DBT dissolved in xylene in a two-state oil phase.

### 3.5. Analytical methods

Cell densities were measured at 600 nm wavelength using a Shimadzu UV visible spectrophotometer (model UV-1601). The correlation between $OD_{600}$ and dry cell weight (DCW) was established to determine the concentration of cells. One unit of optical density corresponded to 0.44 g DCW $L^{-1}$.

### 3.6. Analysis of organic sulfur compounds and metabolites

For gas chromatography (GC) experiments, aliquots of the culture during the course of bacterial growth were acidified below pH 2.0 with 1 N HCl; then the culture was extracted with equal volumes of ethyl acetate during a 5 min vortex and 10 min centrifugation at 2000 rpm. For the two-phase system, xylene fractions were directly used for DBT quantification. Next, 2 $\mu$L of the organic fraction was used for the detection of DBT and 2-HBP by using a GC (HP-Agilent Technologies 6890N GC Systems, USA) equipped with a flame ionization detector. An Agilent JW Scientific DB-5 capillary 30.0 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m column was used for the measurements. Temperature was set to 50 °C for 5 min followed by a 10 °C min$^{-1}$ rise up to 280 °C and was kept at this temperature for 5 min. Injector and detector temperatures were both maintained at 280 °C. Quantification of DBT and 2-HBP was performed using standard curves with a series of dilutions of the pure DBT and 2-HBP compounds as reference. All the reaction mixtures were prepared in triplicate.

### 3.7. Gibbs assay/Desulfurization assay

The Gibbs assay was used in conjunction with GC analyses to detect and quantify the conversion of DBT to 2-HBP produced by *Sulfolobus solfataricus* P2 in the culture media lacking xylene. The assay was carried out as follows: 1 mL of culture was adjusted to pH 8.0 with 10% (w/v) $Na_2CO_3$; then 20 $\mu$L of freshly prepared Gibbs reagent (2,6-dicholoroquinone-4-chloroimide, 5 mM in ethanol) was added. The reaction mixtures were allowed to incubate for 60 min at 30 °C for color development. The mixtures were then centrifuged at 5000 rpm for 10 min to remove cells, and absorbance of the supernatant was determined at 610 nm (UV 1601, Shimadzu, Japan). Concentration of 2-HBP produced from the Gibbs assay results was determined from the standard curve obtained by different concentrations of pure 2-HBP. Results correspond to the means of three different experiments with the standard errors included.

### Acknowledgments

This work was supported in part by a grant, 110M001, awarded by the Scientific and Technological Research Council of Turkey (TÜBİTAK), and İstanbul Technical University internal funds.

## References

1. Monticello, D. J. Biodesulfurization and the upgrading of petroleum distillates. *Curr. Opin. Biotechnol.* **2000**, *11*, 540–546.
2. EPA. Heavy-duty Engine and Vehicle Standards and Highway Diesel Fuel Sulfur Control Requirements. EPA420-F-400-057, 2000.
3. Le Borgne, S.; Quintero, R. Biotechnological processes for the refining of petroleum. *Fuel Process Technol.* **2003**, *81*, 155–169.
4. Chen, H.; Zhang, W. J.; Chen, J. M.; Cai, Y. B.; Li, W. Desulfurization of various organic sulfur compounds and the mixture of DBT + 4,6-DMDBT by *Mycobacterium* sp. ZD-19. *Bioresour. Technol.* **2008**, *99*, 3630–3634.
5. Matsui, T.; Onaka, T.; Maruhashi, K.; Kurane, R. Benzo[b]thiophene desulfurization by *Gordonia rubropertinctus* strain T08. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 212–215.
6. Gray, K. A.; Pogrebinsky, O. S.; Mrachko, G. T.; Xi, L.; Monticello, D. J.; Squires, C. H. Molecular mechanisms of biocatalytic desulfurization of fossil fuels. *Nat. Biotechnol.* **1996**, *14*, 1705–1709.
7. Patel, S. B.; Kilbane, J. J.; Webster, D. A. Biodesulphurisation of dibenzothiophene in hydrophobic media by *Rhodococcus* sp. strain IGTS8. *J. Chem. Technol. Biotechnol.* **1997**, *69*, 100–106.

8. Ohshiro, T.; Hirata, T.; Hashimoto, I.; Izumi, Y. Characterization of dibenzothiophene desulfurization reaction by whole cells of *Rhodococcus erythropolis* H-2 in the presence of hydrocarbon. *J. Ferment. Bioeng.* **1996**, *82*, 610–612.
9. Wang, M. D.; Li, W.; Wang, D. H.; Shi, Y. Desulfurization of dibenzothiophene by a newly isolated *Corynebacterium* sp ZD-1 in aqueous phase. *J. Environ. Sci. (China)* **2004**, *16*, 1011–1015.
10. Kirimura, K.; Furuya, T.; Nishii, Y.; Ishii, Y.; Kino, K.; Usami, S. Biodesulfurization of dibenzothiophene and its derivatives through the selective cleavage of carbon-sulfur bonds by a moderately thermophilic bacterium *Bacillus subtilis* WU-S2B. *J. Biosci. Bioeng.* **2001**, *91*, 262–266.
11. Furuya, T.; Ishii, Y.; Nada, K.; Kino, K.; Kirimura, K. Thermophilic biodesulphurization of hydrodesulfurized light gas oils by *Mycobacterium phlei* WU-F1. *FEMS Microbiol. Lett.* **2003**, *221*, 137–142.
12. Kargi, F.; Robinson, J. M. Microbial desulfurization of coal by thermophilic microorganism *Sulfolobus acidocaldarius*. *Biotechnol. Bioeng.* **1982**, *24*, 2115–2121.
13. Kargi, F.; Robinson, J. M. Microbial oxidation of dibenzothiophene by the thermophilic organism *Sulfolobus acidocaldarius*. *Biotechnol. Bioeng.* **1984**, *26*, 687–690.
14. Kargi, F.; Robinson, J. M. Biological removal of pyritic sulfur from coal by the thermophilic organism *Sulfolobus acidocaldarius*. *Biotechnol. Bioeng.* **1985**, *27*, 41–49.
15. Constanti, M.; Giralt, J.; Bordons, A.; Norris, P. R. Interactions of thiophenes and acidophilic, thermophilic bacteria. *Appl. Biochem. Biotechnol.* **1992**, *34*, 767–776.
16. Sato, T.; Atomi, H. Novel metabolic pathways in archea. *Curr. Opin. Microbiol.* **2011**, *14*, 307–314.
17. Kouril, T.; Esser, D.; Kort, J.; Westerhoff, H. V.; Siebers, B.; Snoep, J. L. Intermediate instability at high temperature leads to low pathway efficiency for an in vitro reconstituted system of gluconeogenesis in *Sulfolobus solfataricus*. *FEBS J.* **2013**, *280*, 4666–4680.
18. Alves, L.; Salgueiro, R.; Rodrigues, C.; Mesquita, E.; Matos, J.; Girio, F. M. Desulfurization of dibenzothiophene, benzothiophene and other thiophene analogs by a newly isolated bacterium, *Gordonia alkanivorans* strain 1B. *Appl. Biochem. Biotechnol.* **2005**, *120*, 199–208.
19. Oldfield, C.; Wood, N. T.; Gilbert, S. C.; Murray, F. D.; Faure, F. R. Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus *Rhodococcus*, and related taxa. *Antonie van Leeuwenhoek* **1998**, *74*, 119–132.
20. Luo, M. F.; Xing, J. M.; Gou, Z. X.; Li, S.; Liu, H. Z.; Chen, J. Y. Desulfurization of dibenzothiophene by lyophilized cells of *Pseudomonas delafieldii* R-8 in the presence of dodecane. *Biochem. Eng. J.* **2003**, *13*, 1–6.
21. Konishi, J.; Onaka, T.; Ishii, Y.; Susuki, M. Demonstration of the carbon-sulfur bond targeted desulfurization of benzothiophene by thermophilic *Paenibacillus* sp. strain A11-2 capable of desulfurizing dibenzothiophene. *FEMS Microbiol. Lett.* **2000**, *187*, 151–154.
22. Ma, T.; Li, G.; Li, J.; Liang, F.; Liu, R. Desulfurization of dibenzothiophene by *Bacillus subtilis* recombinants carrying dszABC and dszD genes. *Biotechnol. Lett.* **2006**, *28*, 1095–1100.
23. Caro, A.; Boltes, K.; Leton, P.; Garcia-Calvo, E. Dibenzothiophene biodesulfurization in resting cell conditions by aerobic bacteria. *Biochem. Eng. J.* **2007**, *35*, 191–197.
24. Honda, H.; Sugiyama, H.; Saito, I.; Kobayashi, T. High cell density culture of *Rhodococcus rhodochrous* by pH-stat feeding and dibenzothiophene degradation. *J. Ferment. Bioeng.* **1998**, *85*, 334–338.
25. Marzona, M.; Pessione, E.; Di Martin, S.; Giunta, C. Benzothiophene and dibenzothiophene as the sole sulfur source in *Acinetobacter*: growth kinetics and oxidation products. *Fuel Process. Technol.* **1997**, *52*, 199–205.
26. Li, F. L.; Zhang, Z. Z.; Feng, J. H.; Cai, X. F.; Xu, P. Biodesulfurization of DBT in tetradecane and crude oil by a facultative thermophilic bacterium *Mycobacterium goodii* X7B. *J. Biotechnol.* **2007**, *127*, 222–228.
27. Ma, C. Q.; Feng, J. H.; Zeng, Y. Y.; Cai, X. F.; Sun, B. P.; Zhang, Z. B.; Blankespoor, H. D.; Xu, P. Methods for the preparation of a biodesulfurization biocatalyst using *Rhodococcus* sp. *Chemosphere* **2006**, *65*, 165–169.
28. Aono, R.; Aibe, K.; Inoue, A.; Horikoshi, K. Preparation of organic solvent-tolerant mutants from *Escherichia coli* K-12. *Agric. Biol. Chem.* **1991**, *55*, 1935–1938.

29. León, R.; Fernandes, P.; Pinheiro, H. M.; Cabral, J. M. S. Whole-cell biocatalysis in organic media. *Enzyme Microb. Technol.* **1998**, *23*, 483–500.

30. Maghsoudi, S.; Vossoughi, M.; Kheirolomoom, A.; Tanaka, E.; Katoh, S. Biodesulfurization of hydrocarbons and diesel fuels by *Rhodococcus* sp. strain P32C1. *Biochem. Eng. J.* **2001**, *8*, 151–156.

31. Brock, T. D.; Brock, K. M.; Belly, R. T.; Weiss, R. L. Sulfolobus - new genus of sulfur-oxidizing bacteria living at low pH and high-temperature. *Arch. Microbiol.* **1972**, *84*, 54–56.

32. Evans, P.; Wolff-Briche, C.; Fairman, B. High accuracy analysis of low level sulfur in diesel fuel by isotope dilution high resolution ICP-MS, using silicon for mass bias correction of natural isotope ratios. *J. Anal. Atom. Spectrom.* **2001**, *16*, 964–969.