

## Biotransformation of Dibenzothiophene by Resting Cells of a Newly Isolated *Serratia marscens* Sp. Strain Originated from Industrial Wastewater

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### Abstract

Five isolates able to use dibenzothiophene (DBT) as a sole sulfur source with high rates were selected to investigate their potentialities as biocatalysts of biodesulfurization reactions. The desulfurizing activities of selected strains were investigated in growing and resting cell state. The biodegradation yields were considerably higher in resting cell reaction especially for two strains tentatively named S1 (98.8%) and S27 (97.5%). These results insinuated that biodegradation activity was mainly related to secondary metabolism on these strains. Their biotransformation potentialities were also evaluated under various conditions in order to evaluate their stability in both aqueous and organic media; and their sensitivity to the presence of squalene, used in this study as a representative of hydrocarbons in petroleum. The results showed that the 5 selected strains were still active in the presence of 95% of squalene but no transformation observed at 99% of squalene. The sulfur substrate selectivity was studied in presence of other organosulfur compounds such as dimethylsulfoxide DMSO and benzothiazole BTH. The presence of these substrates inhibited the DBT uptake by the bacteria and consequently decreased its degradation rate. Moreover, conventional analysis of 16S ribosomal DNA sequencing showed that the strain with highest bioconversion rates belonged to *Serratia marcescens* species. To far of our knowledge, *Serratia* sp. was rarely reported as DBT degrader strain. Thus, the rate and the extent of the biodesulfurization reaction, exhibited by the strain *Serratia marcescens* S27, suggested that it could be used in practical scale.

**Keywords:** Dibenzothiophene; Biodesulfurization; Organosulfur compounds; Xenobiotics; Biodegradation; *Serratia* sp.

### Introduction

Organic sulfur compounds including dibenzothiophene (DBT), benzothiophene (BT) and their alkylated derivatives in fossil fuels have been the major cause of worldwide environmental problems including air pollution and acid rain [1] and healthy problems such as carcinogenicity to humans [2,3]. In addition, with the increasing demands for energy and more stringent environmental policies, deep desulfurization of petroleum is becoming more and more required [4].

Biodegradation of organosulfur compounds has been previously studied using different strains like *Corynebacterium* sp. [5,6], *Paenibacillus* sp. A11-2 [7], *Pseudomonas* sp. [8-10], *Rhodococcus* sp. [11], *Mycobacterium* sp. [12,13], *Sphingomonas* sp. [14], *Bacillus subtilis* WU-S2B [15], *Gordonia* sp. [16-18], and *Brevibacterium* sp. [19].

DBT has been used as a polyaromatic sulfur compound model for the isolation and characterization of bacteria capable of bioconversion of recalcitrant organosulfur compounds found in variety of fossil fuels [20,21]. The dibenzothiophene (DBT) shows high toxicity and mutagenicity which may affect human health and ecosystems in general [2]. A small number of genera such as *Rhodococcus* and *Gordonia* are known to remove sulfur from DBT via a sulfur-specific

pathway [22,23]. *Rhodococcus* and *Gordonia* have the specificity to oxidize sulfur in DBT without cleaving the carbon skeleton into low-carbon-number hydrocarbons. This property has a great advantage when applied to industrial processes.

DBT biodesulfurization pathway, so called 4S route, is based on multi-enzymatic system involving four consecutive reactions [24]. The reaction started by DBT oxidation using two monooxygenases: DBT monooxygenase (DszC) and DBT sulfone monooxygenase (DszA) and ended by the conversion to 2-hydroxybiphenyl as the end product using the desulfinase enzyme (DszB) [25].

Microbial desulfurization of DBT have an increasing interest as it is easy to maintain, does not require hydrogen gas, offers less environmental disturbance lower operating costs comparing to hydrodesulfurisation [20]. However, biodesulfurization yield could be limited due to the bacterial enzymatic activities and substrate transport rate across the cell membrane [26-28].

Considering the relevance of the overall impact of pollution generated by DBT, we report in this study the characterization of DBT bioconversion using five strains isolated from wastewater in minimum medium with DBT as the sole sulfur source. Examination of DBT consumption by selected strains was investigated under growing and resting cell state. DBT biodegradation in biphasic medium was also examined in order to simulate a real conditions and in presence of other organosulfur compounds to examine to broad specificity of the finally selected strain. Identification of stains was achieved using 16S

rRNA identification. Detection of desulfurizing genes was also highlighted in order to characterize the metabolic pathway used by the strain with a high degradation potential.

## Materials and Methods

### Chemicals

Dibenzothiophene (DBT) (Wako Pure Chemicals Co., Japan), Benzothiazole (BTH, Wako Pure Chemicals Co., Japan) and Dimethylsulfoxide (DMSO; Wako Pure Chemicals Co., Japan) were of liquid chromatography grade and other chemicals were of analytical grade, commercially available. All other reagents were of the highest purity commercially available and were used without further purification. All media and solutions were prepared with deionized water.

### Enrichment and isolation

Wastewater was obtained from different industrial facilities discharge (tannery, agro-food, plastic industry) in Tunisia. Several strains growing with DBT as a sole source of sulfur were isolated from sampled wastewater. Thus, 10 mL of wastewater were suspended in 100 mL of BHMS medium (composition: 1 g/L  $\text{KH}_2\text{PO}_4$ ; 0.2 g/L  $\text{K}_2\text{HPO}_4$ ; 0.2 g/L  $\text{MgSO}_4\cdot\text{H}_2\text{O}$ ; 0.02 g/L  $\text{CaCl}_2$ ; 1 g/L  $\text{NH}_4\text{NO}_3$ ; and 2 drops of  $\text{FeCl}_3$  60%), inoculated with 0.25 mM of DBT as a unique sulfur source and 5 g/L glucose as a carbon source. The resulted suspension was incubated on a rotary shaker (200 rpm) at 30°C until turbidity for 24 h. Then, the mixture was centrifuged at 14000 rpm for 3 min and the culture was then transferred into fresh medium which was inoculated with 5% v/v inocula followed by a sub-culturing with 2% v/v inocula five times [29]. Bacterial isolation was made by streaking a single-colony onto the same medium containing 1.5% agar.

### Culture conditions

The five selected strains cultivated in 50 mL of culture medium in 250 mL flasks were inoculated with 25 mg/L of DBT and incubated for 120 hours at 30°C under shaking conditions. During cultivation time, aliquots of the culture were sampled for measurements of DBT concentrations by HPLC and cell growth by turbidimetric assay at an Optical Density of 660 nm (O.D.).

The culture medium was prepared as mentioned by Izumi et al. [30]. The standard minimal sulfate-free medium (SMM) contained: 5 g carbon source (glucose or glycerol), 0.5 g of  $\text{KH}_2\text{PO}_4$ , 4 mg of  $\text{K}_2\text{HPO}_4$ , 1 g of  $\text{NH}_4\text{Cl}$ , 0.2 g  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ , 0.02 g of  $\text{CaCl}_2$ , 0.01 g of  $\text{NaCl}$ , 10 mL of metal solution in 1000 mL of deionized water (pH 7.7). The metal solution contained 0.5 g of  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ , 0.5 g of  $\text{ZnCl}_2$ , 0.5 g of  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ , 0.1 g of  $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ , 0.05 g of  $\text{CuCl}_2$ , 0.05 g of  $\text{Na}_2\text{WO}_4\cdot 2\text{H}_2\text{O}$  and 120 mmol of HCl in 1000 ml of deionized water. The sole sulfur source in this experiment was DBT (25  $\text{mg}\cdot\text{L}^{-1}$  added in solution in ethyl alcohol).

### DBT bioconversion by resting cells

Cells were cultivated in 50 mL of SMM in 250 mL flasks. They were harvested at the end of the growth phase by centrifugation (20000 g for 15 min), washed twice with sterilized deionized water and once with 0.1 M phosphate buffer (pH 7.0) and suspended in an appropriate volume of the phosphate buffer to adjust the cell concentration to an optical density at 660 nm of 10. The cell suspension was heated at

121°C for 5 min in case of heat inactivated cells to examine the possibility of substrates adsorption to the resting cells. The reaction was started by adding DBT used here as a substrate. DBT was added at a finale concentration of 1 g/L. The bioconversion experiments were carried out in 250 mL flasks at 30°C and 200 rpm. Samples were collected every 2 hours of intervals for assay of DBT by HPLC. Resting cells activity was determined by the rate of DBT consumption during 24 h of the experiment.

### Bioconversion reactions in biphasic medium

The capability of selected strains to convert DBT in two-phase system was also investigated. Thus, we used a biphasic medium with squalene as a representative of hydrocarbons. The two-phase desulfurization systems contains resting cells suspended in 25 mL phosphate buffer (0.1 M; pH 7) inoculated with 3 mM DBT and the appropriate concentration of squalene: 0, 50, 90, 95, 99 vol.%; with respect to the total volume. The reaction was stopped by centrifugation and sampling of the organic phase. Substrate concentrations were determined in the organic phase by HPLC analysis. Residual DBT degradation activity was calculated as the percentage of the activity in presence of squalene relative to the activity measured in absence of squalene (100% activity=activity in aqueous phase).

### Degradation of other organosulfur compounds

To investigate the range of organic sulfur compounds that can be assimilated by selected strains, two compounds were used: benzothiazole BTH and dimethylsulfoxide DMSO. The organic sulfur compounds; in addition to DBT were added as the sole sulfur source in growth experiments at a final concentration of 30 mg/L. The cultivation was extended for 7 days at 30°C. By the end of the experiment, HPLC analysis was performed using aqueous phase. Cell growth was estimated by measuring the optical density at 660 nm.

### HPLC analysis

All compounds concentrations were measured by High Performance Liquid Chromatography (HPLC) (Type LC-10A, Shimadzu Co., Kyoto, Japan), equipped with a diode-array detector and an automatic injector.

To analyze by HPLC 1.5 mL of sample was centrifuged at 13000 rpm for 5 min, into Eppendorf tubes (aqueous samples with acetonitrile, in ½ dilutions). When both phases were separated, the compounds in oil once dissolved was measured with a column for reverse-phase analysis (Type VP-ODS Shim-pack, 150 mm 4.6 mm, Shimadzu Co., Kyoto, Japan). In this case, isocratic elution was performed with a 55:45 (v/v) acetonitrile: water mobile phase at 1 mL  $\text{min}^{-1}$ . DBT was detected at a wave length value of 280 nm.

### 16S rRNA identification

The Genomic DNA were prepared using ISOPLANT (Nippon-gene Co., Tokyo, Japan) and used for PCR. The 16S rRNA gene locus was amplified by PCR as described previously [31]. The blast program (<http://ncbi.nlm.nih.gov/BLAST/NCBI>, MD, USA) was used for the gene homology search with the standard program default. The phylogenetic tree based on the 16S rRNA gene sequence was constructed by the neighbor-joining method [32], with the Kimura two-parameter model as a distance corrector [33] after alignment of

sequences with the CLUSTAL X multiple sequence alignment program [34].

### Detection of *dszA*, *dszB*, and *dszC* genes based on PCR

The isolated strain was screened by PCR for the presence of the desulfurizing genes; *dszC*, *dszA* and *dszB* genes. They are:

- *dszA* forward: 5'-TCGATCAGTTGTCAGGGG-3', *dszA* reverse: 5'-GGATGGACCGACTGTTGAG-3'
- *dszB* forward: 5'-ATCGAACTCGACGTCCTCAG-3', *dszB* reverse: 5'-GGAACATCGACACCAGGACT-3'
- *dszC* forward: 5'-CTGTTTCGGATACCACCTCAC-3', *dszC* reverse: 5'-ACGTTGTGGAAGTCCGTG-3'. All PCR amplifications were performed under conditions according to Duarte et al. [29] protocol.

## Results

### Isolation and selection of efficient strains for DBT biotransformation

Five pure strains able to grow with DBT (Figure 1) as a sole sulfur source were isolated from industrial wastewaters facilities. Strains were subjected to the analysis of DBT consumption evaluated as DBT biodegradation. The bacterial growth was continued for 120 h, in the parallel way with the depletion of DBT. As shown in Figure 2, the strains tentatively named S1, S27 and S7 grew well on DBT as a sole sulfur source with a specific growth rate respectively equal to 0.055 h<sup>-1</sup>, 0.056 h<sup>-1</sup> and 0.052 h<sup>-1</sup>. However, S19 and S26 had a slower growth under the same conditions with  $\mu_{max}$  of 0.036 h<sup>-1</sup> and 0.049 h<sup>-1</sup> respectively. The growth profile by S27 showed a short lag phase of growth early in the first 10 hours, followed by the exponential growth phase from 30 hours. The maximum growth was observed after 80 hours of incubation followed by a small stationary phase maintained for 120 hours. In contrast, for the strain S26 and S1, there was a slight inhibition in the period 0-8 hours of growth and the maximum growth rate was observed respectively at 96 h and 108 h. The selected strains belong probably to different species due to their different growth curve.

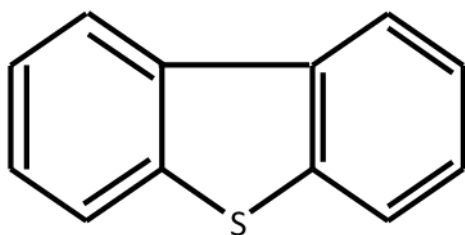


Figure 1: Chemical structure of Dibenzothiophene DBT.

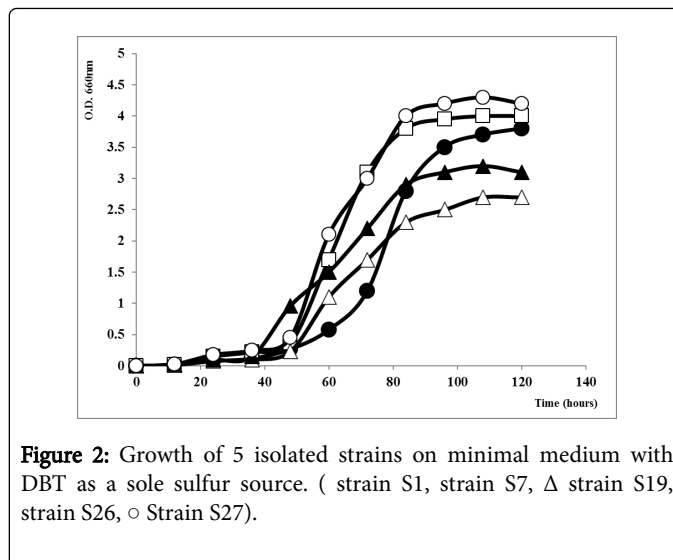


Figure 2: Growth of 5 isolated strains on minimal medium with DBT as a sole sulfur source. ( strain S1, strain S7,  $\Delta$  strain S19, strain S26,  $\circ$  Strain S27).

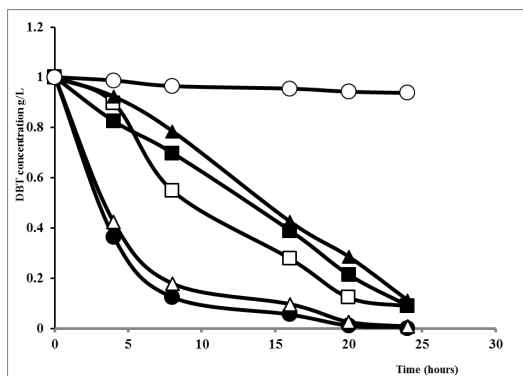
Table 1 showed the DBT degradation using bacterial growing cells supplemented with 0.25 mM DBT as the sole sulfur source and 5 g/L of glucose as carbon source. The degradation proceeded with the cell growth, and most of DBT was depleted at 120 h of reaction time. Under growing conditions the highest DBT bioconversion rate was observed for S27 and S1 which were respectively 93% and 90.7% within 120 hours of reaction time.

	Cell concentration (OD 660)	Residual DBT (mM)	Degradation (%)
S1	3.8	0.023	90.7
S7	4	0.076	69.48
S19	2.7	0.088	64.64
S26	3.1	0.073	70.62
S27	4.2	0.016	93.5
no cells	--	0.25	0

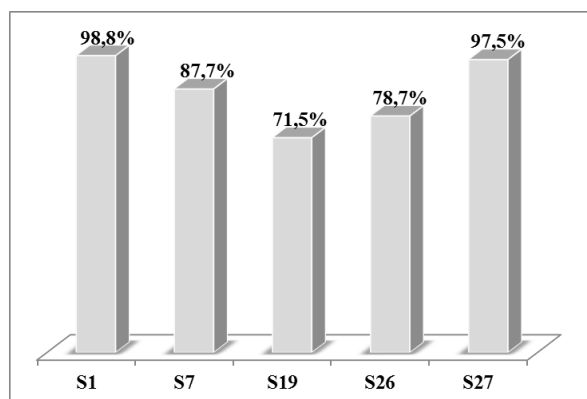
Table 1: DBT degradation by growing cells of selected bacteria.

To better understand the biodegradation of dibenzothiophene using selected strains, the experiment was carried out with bacterial resting cells. Figure 3 showed the transformation of DBT by the resting cells of the 5 selected strains. Nearly, all the strains degrade the DBT after 25 hours of experiment time. Obviously, DBT conversion rate with resting cells was considerably higher for all strains comparing to the growing cell conditions in a shorter time. The resting cells of S1 and S27 displayed the highest degradation in a shorter time rate evaluated respectively as 98.8% and 97.5% as illustrated in Figure 4.

No transformation was observed with the heat inactivated cells, indicating that transformation was proceeded by a biochemical reaction; neither by absorption of substrate by the cells nor by spontaneous transformation.



**Figure 3:** Time course of DBT degradation by resting cells of the selected strains. (○ Heat inactivated cell; strain S1; strain S7; strain S19; strain S26; Δ strain S27).



**Figure 4:** Biodegradation rates (%) of DBT by resting cells of selected strains named S1, S7, S19, S26 and S27.

### DBT bioconversion in biphasic medium

The effect of the presence of an oil fraction is essential in this kind of processes as it can affect both the biocatalyst growth and the 4S-pathway development. Mass transfer and toxic effects are expected [3]. Table 2 reported the desulfurization assay in aqueous and two-phase systems by resting cells of the 5 isolated strains. The effect of the presence of an organic medium on desulfurization activities of the strains was studied using squalene which is supposed to be a good model of diesel oil in terms of hydrophobicity. Various concentrations of squalene were used, ranging from 50% to 99% of the total reaction volume. Activities in squalene compared to activities in aqueous phase are shown in Table 2 with the consideration that: 100% activity=activity in aqueous phase.

Squalene concentration (%)	Residual DBT-consuming activity (%)				
	S1	S7	S19	S26	S27
50	64	58	50	62	74
90	40	33	26	39	58

95	30	20	11	18	28
99	0	0	0	0	0

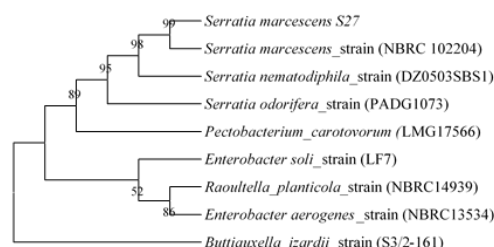
**Table 2:** Effect of squalene concentration on DBT-consuming activities in biphasic medium. Squalene concentration (vol.%) is calculated with respect to the total reaction volume. Residual activity is given as the percentage of the activity in presence of squalene relative to the activity measured in absence of squalene (100% activity=activity in aqueous phase).

According to the Table 2, DBT consuming activities of all strains decreased with the addition of squalene. As expected, no biotransformation activity was detected in micro-aqueous media with 99% squalene. However, all strains were still biologically active in 95% squalene with distinctive rates. DBT consumption yield was still high in the presence of 90% squalene for the strains S1 and S27; 40 and 58% respectively. However, the higher sensitivity of selected strains due to the presence of squalene was observed from 90% of squalene. The strain S27 was the strain displaying the highest activities activities in 95% squalene.

In fact, the effect of the presence of squalene on DBT-consuming activities depended upon strain [35]. DBT-degrading activity was less stable in the presence of the squalene. Nevertheless, the 5 selected strains were still active in the presence of 95% of squalene (more than 5% of the activity in aqueous medium). However, no activity was detected in microaqueous (99% squalene) media. Our results confirmed that an extended range of solvent can be used for a biodesulfurization process. Moreover, the sensitivity of the strains to the presence of solvent is variable. For instance, S1 was the strain displaying the highest DBT-degrading activity when the reaction medium contained 95% squalene whereas S27 was the best one in the presence of 50% solvent.

### Characterization of S27 strain

The isolate S27 was found to be aerobic, motile and Gram negative bacteria. 16S rRNA sequencing of isolate S27 showed a similarity of 99% with that of *Serratia marcescens* (Gene bank accession No. NBRC\_102204) and hence categorized as *Serratia marcescens* S27 strain (phylogenetic tree shown in Figure 5). The 16S rDNA sequence of isolate S27 has been submitted to the Gene bank (accession No. KY780304).

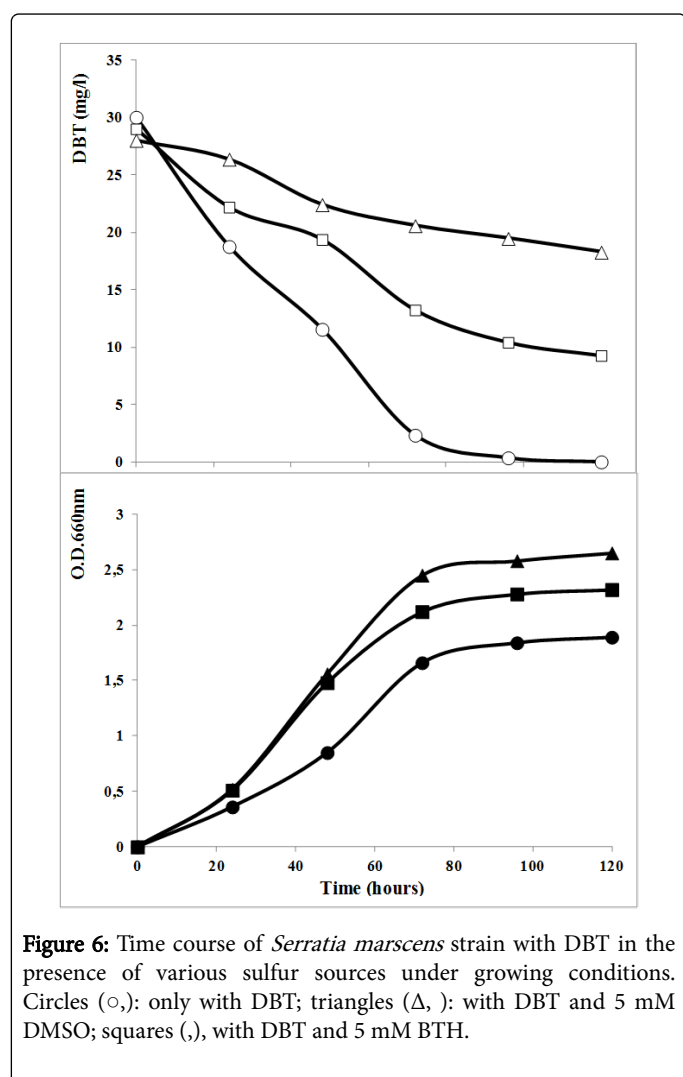


**Figure 5:** Phylogenetic analysis of *Serratia marcescens* S27 based on the 16S rRNA sequence. Bootstrap probabilities are indicated at the branch points. The accession numbers are shown in parentheses.

DBT desulfurization property of *R. erythropolis* IGTS8, first bacterium to be reported for possessing the ability of removing sulfur from DBT [22] has been shown to be due to the presence of the desulfurization genes i.e., *dsz* ABC operon [36]. Therefore, PCR was carried out for the molecular evidence for the presence of *dsz* genes in the isolate S27. Analysis of the sequences of the PCR products revealed 100% identity with the corresponding *dsz* genes (*dsz* A, *dsz*B, *dsz*C) from *R. erythropolis* IGTS8. This is in agreement with the conserved nature of the *dsz* genotype of desulfurizing bacteria. Genes involved in DBT metabolism have been found to be present in almost all of the DBT degrading bacteria and have been shown to have almost 70% of the sequence homology [1].

### DBT bioconversion in the presence of various sulfur sources

Many types of organo-sulfur compounds apart from DBT such as BT and alkylated DBT's derivatives have been shown to be present in diesel, crude oil and industrial wastewater [6]. To assess the broad specificity of the isolated stain; *Serratia marcescens* S27 sp., the strain was subjected to a growth test in presence of DBT, Benzothiazole (BTH) as an aromatic organosulfur compound and Dimethylsulfoxide (DMSO) as an aliphatic organosulfur compound.



**Figure 6:** Time course of *Serratia marcescens* strain with DBT in the presence of various sulfur sources under growing conditions. Circles (○): only with DBT; triangles (Δ): with DBT and 5 mM DMSO; squares (◻), with DBT and 5 mM BTH.

Figure 6 showed the DBT degradation by strain S27 in the presence of various sulfur sources under growing condition. DMSO is an aliphatic organosulfur compound. DMSO is more realistic sulfur source than DBT for the production of industrial amounts of biocatalyst because of its cost and availability. The strain grew considerably better with dimethyl sulfoxide and DBT than with DBT as the sole sulfur source. However, DBT consuming rate reached 33.9% in the presence of DMSO comparing to 98% with DBT only. The DBT degradation seemed to be inhibited by the presence of DMSO. Similar result was obtained in the presence of BTH. The bacterial growth was enhanced by the presence of the aromatic organosulfur compound BTH (Figure 6). However, DBT consumption was about 68% in the presence of BTH.

### Discussion

Five strains named S1, S7, S19, S26 and S27; selected from the initial 35 isolates able to grow on DBT as the sole sulfur source were isolated from industrial wastewater samples. First steps of selection included high DBT biotransformation activity of growing and resting cells. The five selected strains had different DBT degradation rates. Further investigation of their properties confirmed that they had different behavior towards DBT. The desulfurizing activity of growing cells was considerably high for the two strains S1 and S27 (90.7 and 93.5% respectively). The DBT consuming rate was considerably relevant in resting cell state for all strains comparing to growing state. This behavior could be explained by the fact that degradation ability is probably due to a second metabolism on these strains. Knowing the suitable cell state for DBT biotransformation could have an impact for practical application.

Moreover, keeping high bioconversion activities in an organic medium is a prerequisite for an industrial biodesulfurization process. Thus, the effect of the presence of squalene was measured on the DBT consuming activity. The DBT degradation activity for all strains was reduced comparing to their activity in aqueous medium. Nevertheless, the five strains were still active in the presence of 95% squalene. This result is mainly due to the byproduct accumulation in the oil phase. However, almost no activity was detected in microaqueous (99% squalene) media for all strains; the organic phase prevents the substrate to reach the bacterial cell. In fact, bioconversion of DBT seems to be a too complex process involving many steps to be active in such a medium.

The strain S27 having the highest DBT bioconversion rate was identified as *Serratia marcescens* strain. The detection of the desulfurization genes i.e., *dsz* ABC operon lead us to conclude that 4S pathway is mainly involved in DBT degradation. Based on previous research, the 4S pathway is a specific pathway for biodesulfurization of DBT and its conversion into 2-Hydroxybiphenyl. In this case the carbon skeleton of DBT is released intact [2]. Furthermore, Bioconversion pathways of DBT by bacteria, has been widely studied, as: *Arthrobacter* [37], *Brevibacterium* [38], *Mycobacterium* and *Rhodococcus rhodochrous* IGTS8 [39]. These strains were only able to remove sulfur of DBT converting to compound hydroxybiphenyl 2(2-HBP) [40]. These microorganisms have the specificity to selectively remove organic sulfur without degrading the carbon atoms. To the best of our knowledge, very few reports have demonstrated the desulfurization potentials of genus *Serratia* sp. reported in this study.

Even if DBT is generally taken as the model compound of heterocyclic organosulfur OSC, other OSC represent a high proportion

of these molecules, known to be recalcitrant to hydrodesulfurization. In this study two OSCs were taken into account: DMSO (aliphatic OSC) and BTH (aromatic OSC). The presence of BTH and DMSO has obviously enhanced the bacterial growth related to the abundance of sulfur element. The uptake of DMSO and BTH by the strain *Serratia marcescens* S27 was considerably higher than DBT uptake. Consequently, DBT bioconversion considerably decreased. This suggested that the degradation activity of DBT on this strain seemed to be inhibited by these compounds and the strain predominantly utilized these compounds as sulfur source rather than DBT. This behavior could be a limitation when applied in practical scale containing a mixture of organosulfur compounds.

## Conclusion

Five isolates able to desulfurize DBT in growing and resting state were isolated from industrial wastewater. The biotransformation rate was considerably higher in resting cell state than in growing cell state which meant that DBT biodegradation rate belonged to the secondary metabolism on these strains. The strain with high degradation rate was identified as *Serratia marcescens* strain. However, the bioconversion of DBT using this strain is highly influenced by the organosulfur compounds existing in the medium. Along with the fact that the strain expressed desulfurizing activity in a growing and resting cell state, as shown previously, this bacterium could contribute to the DBT containing wastewater treatment. Although it appears that the *S. marcescens* is capable of desulfurizing DBT, the bioconversion of this compound still required a lot of research to discover the real mechanism of the metabolic pathway of desulfurization of DBT.

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