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Enhanced Desulfurization Activity in Protoplast Transformed *Rhodococcus erythropolis*

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Abstract: Petrochemically-derived fuels may contain a range of organosulfur compounds that produce sulfur dioxide when combusted, culminating in atmospheric and soil pollution. Sulfur constituents include thiophenes, such as Benzothiophene and Dibenzothiophene (DBT), with the latter often used as a model for the isolation of bacteria capable of selective removal of sulfur for potential use in the desulfurization of fuel oils. A bacterium capable of desulfurizing DBT was isolated from a gasoline-contaminated soil in Isfahan, Iran. Desulfurization employs the 4S metabolic pathway that leads to the production of 2-hydroxybiphenyl (HBP) as an end product. Biochemical analyses and 16S rRNA gene sequencing identified the organism as *Rhodococcus erythropolis* (strain R1) with a rapid capability to desulfurize DBT when using it as its sole sulfur source. Plasmids from *R. erythropolis* strain R1 (HBP-positive) were used to produce a stable desulfurizing mutant (mut23) of a HBP-negative strain by polyethylene glycol (PEG)-mediated protoplast transformation. *R. erythropolis* strain R1 and transformed *Rhodococcus* strain (mut23) were able to degrade 100% of the original DBT after 72 h. However, mut23 desulfurized DBT and produced HBP more efficiently than wild type R1 strain.

Key words: *Rhodococcus erythropolis* . Dibenzothiophene . Desulfurization . Transformation

INTRODUCTION

Members of the genus *Rhodococcus* are gram-positive, aerobic and non-spore forming, partially acid fast *Nocardiaceae*. *Rhodococcus* exhibit a wide range of metabolic activities such as degradation of alkanes and aromatic hydrocarbons compounds [1]. Between sulfur aromatic hydrocarbons, dibenzothiophene (DBT) is a representative or model organo-sulfur compound present in fossil fuels [2-6]. Although a significant number of organisms have been found to remove sulfur from DBT via a hydrocarbon degradative pathway, such a means of sulfur removal involves the destruction of carbon-carbon bonds and thus results in an unacceptable reduction of fuel value [2, 7]. However, *Rhodococcus* [8-13], *Panibacillus* [14] *Bacillus subtilis* WU-S2B [15], *Corynebacterium* and *Arthrobacter* species have been shown to remove sulfur from DBT via 4S sulfur-specific pathway. These bacteria selectively cleaving sulfur from DBT without ring destruction and therefore maintaining the fuel content [16].

2-Hydroxybiphenyl (HBP) has been found as a metabolite of bacterial desulfurization of DBT in 4S metabolic pathway [2, 5].

The genes *dsz*ABC, coding for the enzymes of the 4S metabolic pathway in *R. erythropolis* IGTS8, have been found in large plasmids [17]. Loss of the DBT-desulfurizing phenotype has been clearly related to the loss of these plasmids [18]. These genes are grouped in a 3.7 kb operon called the *dsz* operon. This system is repressed in the presence of easily metabolizable sources of sulfur, such as sulfate or sulfur containing aminoacids [19]. The first and key reaction for biodesulfurization of DBT is enzyme DszC, thus this enzyme catalyzes two consecutive monooxygenation reactions that convert DBT to DBT-sulfune [20]. Two other enzymes consist of DszA and DszB catalyze reactions tend to produce HBP and sulfate [15].

The objective of this study is to isolate microorganisms capable to degrade heterocyclic compounds, such as *Rhodococcus erythropolis* to use as novel biocatalysts in the processes of petroleum refining (desulfurization)

In this study, *Rhodococcus erythropolis* strain named (R1) that produces HBP from DBT was isolated from gasoline-soil and the native plasmids were extracted from the (R1) isolated strain and transformed into HBP-negative *Rhodococcus* by polyethylene glycol (PEG)-assisted transformation of *Rhodococcus*

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protoplasts in order to obtain a strain with higher ability to biodesulfurize DBT.

MATERIALS AND METHODS

Bacterial isolation and culture: To isolate DBT-desulfurizing microorganisms, approximately 20 samples of soil, gasoline-soil, oil-soil, crude oil from refinery of Isfahan and gasoline were collected as sources of microorganisms. Ten gram of each sample were suspended in 90 ml distilled water and 5 ml of this suspension was inoculated into a 250 ml flask containing 45 ml basal salt medium (BSM) supplemented with glucose (5 g l^{-1}) and 5 mM per liter DBT. BSM containing: 4 g KH_2PO_4, 4 g Na_2HPO_4, 2 g NH_4Cl, 0.2 g $MgCl_2$, 0.001 g $CaCl_2$ and 0.001 g $FeCl_3$ in 1000 ml twice distilled water [10]. Cultivation was performed at 30°C in rotary shaker for 5 d. After five sub cultivations, the culture broth medium was spread onto BSM agar supplemented with glucose, glycerol and DBT. After cultivation at 30°C for 3-5 d, colonies formed on the plates were again inoculated into liquid BSM with DBT. One strain named (R1) that showing a Gibbs assay positive in BSM supplemented with DBT was selected.

Identification of *Rhodococcu erythropolis* isolate strains: Identification of *Rhodococcu erythropolis* isolate was based on colony morphology, microscopic observation of the cell cycle, Gram stain, acid-fast stain, catalase test, oxidase test, oxygen requirement, motility and casein hydrolyze. The ability to grow on different carbon, carbon and nitrogen sources and in the presence of some inhibitors were also tested for further identification according to *Bergey's* [21]. Another *Rhodococcus erythropolis* strain 68 was used as HBP-negative (Gibbs-negative) strain in this study.

For molecular identification, genomic DNA was isolated from *Rhodococcus* strain according to Duarte *et al*. [22], then PCR amplification was performed using two primers from 16S rDNA gene of *E. coli* [23] as shown in Table 1. The PCR amplification reaction was carried out in a DNA thermocycler (Eppendorf, Hamburg, Germany).

PCR reaction was carried out in final volume of 25 µl with 2.5 µl 10X PCR buffer containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 1 µl of template DNA, 0.1 mM of each deoxynucleoside triphosphate, 10 pM (each) primer and 2.5 U of *Taq* DNA polymerase (Promega).

PCR conditions consisted of an initial denaturation at 94°C for 4 min, followed by 30cycle of 92°C for 1 min, annealing to primers at 60°C for 2 min and extension at 72°C for 2 min with a final extension step at 72°C for 10 min. PCR amplified product was separated using agarose gel electrophoresis in 1.4% TAE buffer and stained with 0.5 µg/ml ethidium bromide according to Sambrook and Russell [24]. Amplified band was excised from agarose gel in TAE buffer, according to the manufacturer directions from the Fermentas DNA extraction kit (Fermentas Diagnostics). Then extracted PCR product was sent for sequencing and the first 523 bases of the 16S rRNA gene were sequenced by Macrogen Company (South Korea). Sequences were compared to known other sequences in GenBank by using the BLASTn search tools with NCBI software.

Plasmid isolation: Plasmids were isolated from *Rhodococcus* sp. using alkaline lysis method according to Denis-Larose *et al*. [17]. Electrophoresis was performed overnight using 0.55% agarose gel and 50 V overnight [17].

PCR amplification of the dszC gene: Plasmid DNA isolated from *Rhodococcus* sp. was used as a template in PCR analysis. The PCR primers for the amplification of the desulfurization *dsz*C gene were designed from *Rhodococcus* strain IGTS8 (GenBank accession number U08850) as shown in Table 1.

The PCR mixtures were prepared with 2 µl of target plasmid DNA, 5 µl of 10X PCR buffer (10 mM Tris-HCl [pH 8.3], 10 mM KCl), 200 µM of each desoxyribonucleoside triphosphate, 3.75 mM $MgCl_2$, 20 pmol of each of appropriate primers, 1% (v/v) formamide and 5 U Taq polymerase in a 50 µl final volume. Negative control (PCR mixture without added target DNA) was included in PCR. PCR conditions were as follow: one cycle of 4 min at 94°C; 35 cycles consisting of 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C; and a final cycle of 10 min at 72°C [22].

Table 1: Characteristics of designed primers specific to 16S rDNA gene and *dsz*C gene

Primers	Sequences of direct (f) and reverse (r) primers (5´...3´)		Start	Product size (bp)
16S rDNA gene	AGAGTTTGATCTTGGCTCAG	F	8	528
	GTATTACCGCGGCTGCTGGCAC	R	536	
*dsz*C gene	CTGTTCGGATACCACCTCAC	F	277-296	392
	ACGTTGTGGAAGTCCGTG	R	651-668	

Rhodococcus protoplast preparation and PEG-mediated transformation [25]: Strain 68 was grown at OD_{600} 2-3 in LBSG medium containing 1.0 g tryptone, 0.5 g yeast extract, 0.5 g sodium chloride, 10.3 g sucrose and 3.0 g glycine and stored for up to 1 month at 4°C. A 50 µl of LBSG medium cultured cells was used for transformation. These cells were rinsed twice in autoclaved B buffer (0.3 M sucrose, 0.01 M $MgCl_2$ and 0.025 M Tris pH (7.2) and resuspended in the original volume of B buffer containing 5 mg/ml lysozyme. After 90 min incubation at 37°C, cells were rinsed gently in freshly made P buffer (B buffer with 2% 1M $CaCl_2$ and 1% 0.03M KH_2PO_4), gently resuspended in the original volume of P buffer and 20 µl plasmid DNA was added. After 5 min, an equal volume of PPEG (P buffer with 50% PEG 6000) was gently mixed in and left for 5 min. The cells were spread onto plates (as LBSG, with glycine omitted and 1.5% agar and 0.4% $MgCl_2$ were added. After autoclaving and immediately before pouring the plates 2% 1M $CaCl_2$, 1% 0.03 M KH_2PO_4 and 1.7% 0.25M Tris pH 7.2 were added. After 12 h recovery at 30°C, incubation continued until distinct colonies appeared. The colonies were screened for biphenyl producing strains by replica plating to BSM containing 0.5% glucose and 0.3 mM DBT. Gibbs assay was performed according to Yu *et al*. [5] on colonies to select Gibbs-positive colony or induced 4S-pathway bacteria.

DBT desulfurization in wild and transformed strains: BSM (a sulfur free medium) containing 5 g l^{-1} glucose was used and DBT-ethanol solution was added to the sterilized medium to a final concentration of 0.3 mM. Cultivation was performed at 30°C with rotation at 140 rpm for 72-100 h. The analytical methods include following:

Cell growth was measured turbidimetrically at 600nm [11, 26]. The concentration of DBT was determined by spectrophotometric analysis in a Specord S 10 UV spectrophotometer (Carl Zeiss Technology), using absorption maximum at 323.8 nm after acidifying the samples to pH 2.0 and extraction with ethyl acetate. On the basis of standard curve of DBT the concentration of remained DBT was estimated [26]. HBP that is by-product of DBT desulfurization in 4S pathway was detected by the Gibbs assay as following: an aliquot (2 ml) of culture broth were put into Eppendorf tube and centrifuged (12000 rpm, 2 min) to remove cells. 1 ml of supernatant was transferred to Eppendorf tube and then 200 µL of sodium bicarbonate (pH 8) and 20 µL of 1 µg μl^{-1} Gibbs reagent solution in ethanol were added. The reaction mixture was agitated at 30°C for 30 min to allow the formation of a blue complex by the Gibbs reagent and aromatic hydroxyl groups such as HBP. Then the absorbance was measured at 610nm [5]. The standard curve of HBP concentrations was prepared and the content of produced HBP was estimated by mmole per liter (mM).

Microtitre plate for physiological assay in wild and mutant bacteria: The strains were grown in BSM medium supplemented with Glucose (0.5%) and DBT (0.3 mM dissolved in ethanol) for 48 h. The colonies were washed once in BSM and diluted to optical density of 0.4 at 600 nm with same medium. Then 50 µl of diluted inoculums used to inoculate individual wells of a 96-well microtitre plate containing 200 µl sterile BSM supplemented with different carbon sources and different concentrations of DBT separately. The microtitre plates were incubated for 1 to 4 days at 30°C. All treatments were done in triplicate with blank wells. After each day of incubation absorbance was measured with a microplate autoreader or ELISA reader (Stat Fax-2100) at 630 nm [27, 28]. 200 µl of each concentration at triplicate in wells used as blank wells. The absorbance of each blank well was subtracted from well by inoculation with same concentration of DBT to obtain the true absorbance of growth. Gibbs assay performed in microtitre plate as follow: after 3-4 d incubation, 40 µl of sodium bicarbonate (pH 8) and then 26 µl of Gibbs reagent were added to wells. After 30 min at room temperature the absorbance at 630 nm was read by ELISA reader. The absorbance of plates was read before addition of reagent was subtracted from subsequent reading (after 30 min) to obtain the value of Gibbs test.

Chemicals: Dibenzothiophene (DBT, C12H8S) was purchased in Iran that was made by Merck-Schuchardt Hohenbrunn, Germany. 2-Hydroxybiphenyl (2-HBP, $C_{12}H_{10}O$) was purchased in Iran that made by Fluka (EC No. 2019935). Gibb's reagent (2,6-Dichloroquinone-4-chloroimide, $C_6H_2C_{13}NO$) made by Merck in Germany. All other chemicals were of analytical grade and used without further purification.

All experiments were done in triplicate with proper blanks.

RESULTS AND DISCUSSION

There is significant interest in the development of desulfurizing bacteria for commercial fuel desulfurization. Specific interest in desulfurizing bacteria is a consequence of the inability of these strains to degrade the carbon backbone of the original organosulfur compound, following desulfurization. This obtains in 4S metabolic pathway for desulfurization of DBT [16]. In this study we isolated one partially

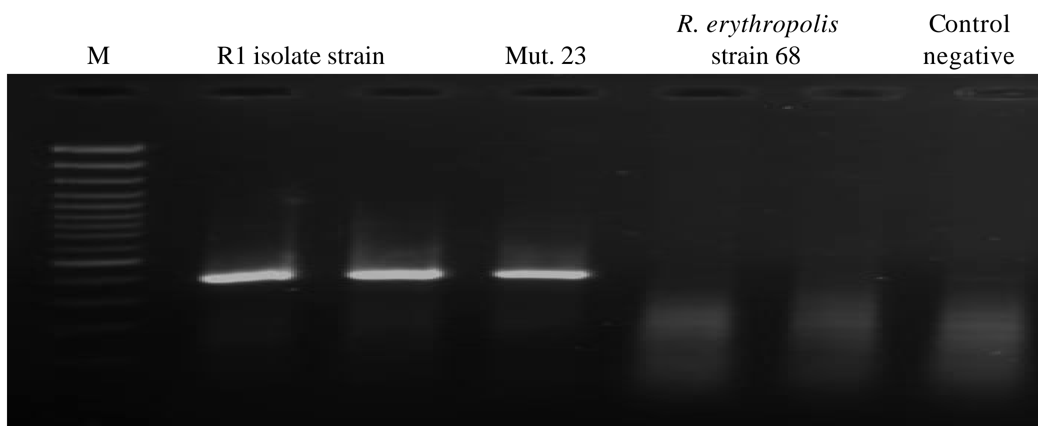

Fig. 1: PCR amplification of *dsz*C gene in *Rodococcus* strains. M= DNA ladder with molecular size from 100 to 3000 bp

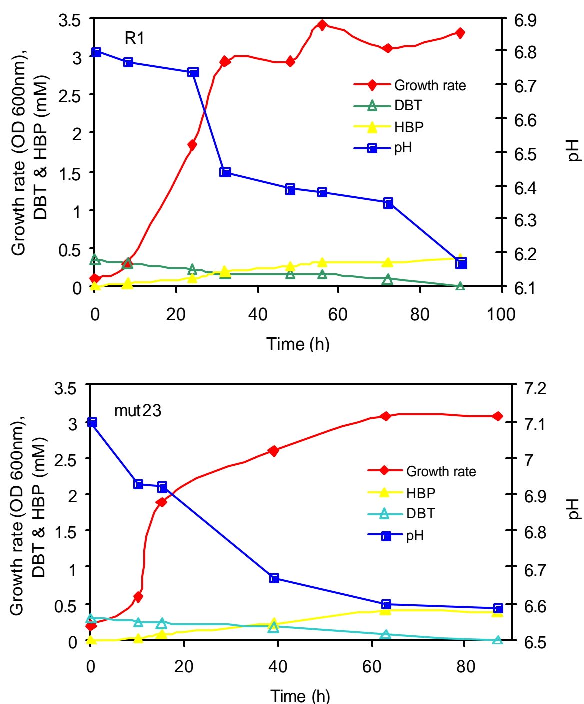

Fig. 2: Growth and desulfurization of DBT in R1 isolated wild strain and transformant *Rhodococcus* (mut 23)

acid-fast bacterium in coccuid form, able to grow using DBT as sole source of sulfur from gasoline soil contaminated. This strain was named R1 and identified as *Rhodococcus* sp. by biochemical tests. Partial sequences of 16S rRNA gene showed 97% homology with *Rhodococcus erythropolis* GenBank accession numbers: EF690428.1, EF204438.1, etc. Thus isolated strain has been assigned *Rhodococcus erythropolis* strain R1 dsz (sox) plasmid was found in the soil-isolated *R. erythropolis* strain R1 with plasmid curing and lost of plasmid in consecutive sub culturing. Denome *et al*. [10], showed the location of desulfurization genes on an endogenous 120 kb linear plasmid in *Rhodococcus erythropolis* strain IGTS8. Denis-Larose *et al*. [29], also showed a large plasmid of 150 kb in IGTS8 and 100 kb in the other strains of *Rhodococcus*. Despite this difference in location the *dsz* operon is apparently high conserved across different strains since PCR amplification of *dsz* genes yielded DNA of the same size, as that of strain IGTS8 [17]. In this study isolated *Rhodococcus erythropolis* strain R1 also showed the PCR product of *dsz*C as the same size of IGTS8.

The extracted plasmids from strain R1 were used to transform protoplasts of *R. erythropolis* strain 68, a related organism that does not desulfurize DBT normally. All of the *R. erythropolis* 68 transformants could convert DBT to HBP, indicating that the genetic information required for DBT desulfurization resides on this plasmid. The protoplast regeneration efficiency was 45% and we obtained transformants of *R. erythropolis* strain 68 with an efficiency of about 4×10^3 transformants per µg plasmid DNA. One of the transformed mutants of the strain 68 named mut23. Denome *et al*. [18], showed that the ability to desulfurize DBT to HBP could be transferred to a desulfurization-negative mutant of IGTS8 and also to a separate species, *R. fascians* by plasmid transformation. The positive transformation confirmed by Gibbs test for production of HBP and PCR assay for *dsz*C gene (the first and key gene for desulfurization of DBT). Result of PCR amplification of *dsz*C gene is shown in Fig. 1.

Desulfurization of DBT was compared in strains R1 and mut23. The results are given in Fig. 2. As it is shown *R. erythropolis* strain R1 and transformed *Rhodococcus* strain (mut23) were able to degrade

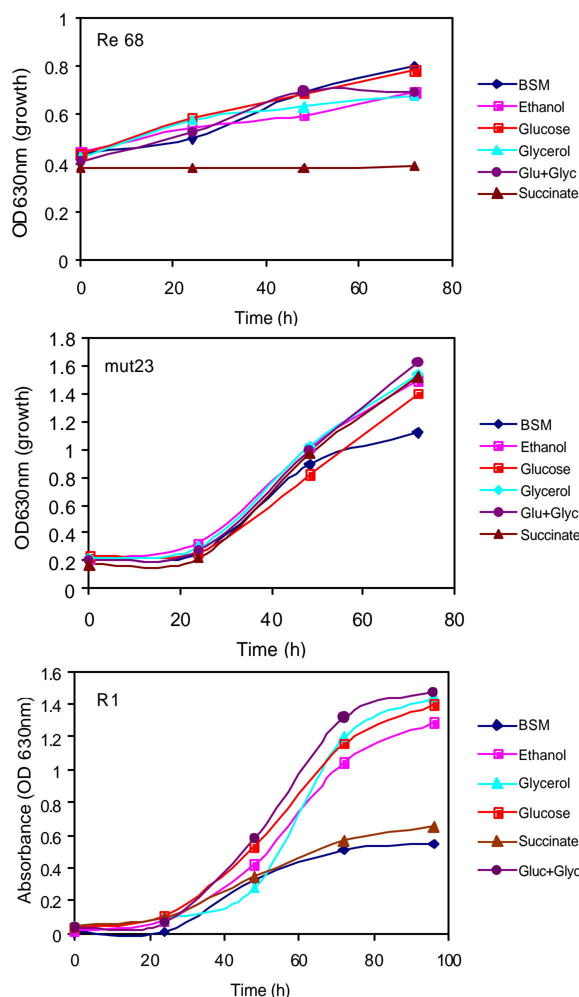

Fig. 3: Typical growth rate of *Rhodococcus* (strains: R.e 68, mut23 and R.1) grown on different carbon sources in presence of DBT as sole sulfur source in microtitre plate incubated at 30°C for 4 d

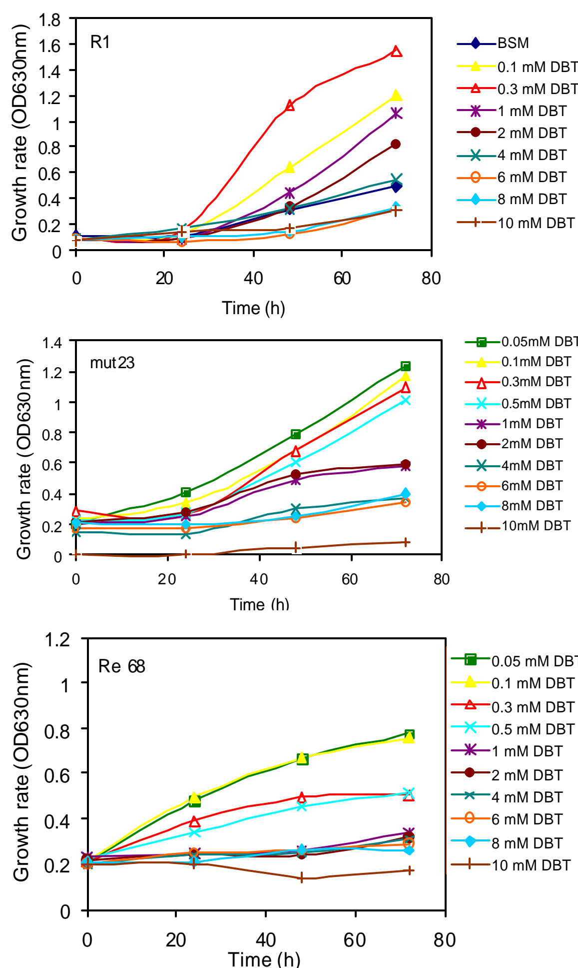

Fig. 4: Growth of wild *R. erythropolis* strain 68, its mutant (mut23) and *R. erythropolis* strain R1 on different concentrations of DBT in microtitre plate incubated at 30°C for 3 d

completely the original DBT (0.3 mM) after 72 h. Wild *R. erythropolis* strain 68 does not desulfurize DBT even after 15 d incubation (data are not shown). Growth rate of mut23 in different carbon sources is more compare to *R. erythropolis* 68 and strain R1 (Fig. 3). As it is shown mut23 has good growth on succinate in presence of DBT as sole sulfur source, however wild strain 68 does not have any growth on succinate and DBT.

In various concentration of DBT that is shown in Fig. 4, 0.3 mM DBT reduced the growth rate of *R. erythropolis* strain 68 by 50%, but has not any significant effect on mut23. However DBT tolerance is higher in R1 strain. Yashikawa *et al.* [30], showed high desulfurizing activity in *Rhodococcus erythropolis* when DBT added at 0.1 mM and bacterial cell growth was inhibited above 1 mM DBT. The results of this study shown that the strains R1 and mut23 exhibited more tolerance to DBT, growing in higher concentrations of DBT (up to 4 mM) indicating that these strains could be more effective in the desulfurization of DBT.

HBP production is more in strain mut23 compare to R1 strain (Fig. 5) and maximum HBP is produced from 0.5 mM DBT. These results showed that conversion of DBT is more in mut23 compare to R1 strain. Kobayashi *et al.* [31], have noted that *R. erythropolis* I-19, a genetically engineered bacterium, have contributed to a 67% decrease in DBT levels. Other studies have noted different levels of DBT utilization in closed cultures. For instance, isolated *R. erythropolis* XP could reduce 94.5% of DBT in hydrodesulfurized diesel oil [5]. The isolated *R. erythropolis* strain R1 and transformed *Rhodococcus* strain (mut23) were able to utilize 100% of the original DBT levels after 72 h.

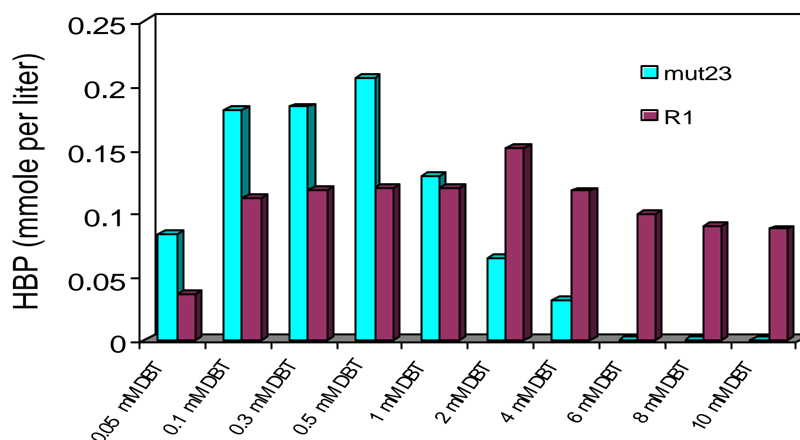

Fig. 5: The effect of DBT concentration on production of HBP in transformant strain or mut23 and wild strain R1 in microtitre plate incubated at 30°C for 3 days

The biodesulfurization rate was the highest between 20 and 60 h.

In this study we isolated bacterial biocatalysts for use in petroleum desulfurization by polyethylene glycol (PEG)-assisted protoplast transformation and produce recombinant *Rhodococcus* with higher ability to biodesulfurize DBT.

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