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The Enhancement of Biodesulfurization Activity in a Novel Indigenous Engineered *Pseudomonas putida*

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ABSTRACT

Background: The combustion of sulfur-rich fossil fuels leads to release of sulfur oxide pollution in the environment. In biodesulfurization process, an organism is able to remove sulfur from fossil fuels without decreasing the caloric value of those substrates. The main aim of this research was to design a recombinant microorganism to remove the highest amount of sulfur compounds in fossil fuels. **Methods:** Three genes (*dszA,B,C*) from *dsz* operon are responsible for the 4S pathway (biodesulfurization pathway) in *Rhodococcus erythropolis* IGTS8 were inserted into the chromosome of a novel indigenous *Pseudomonas putida*. The reaction catalyzed by products of *dszA,B,C* genes require $FMNH_2$ supplied by dszD enzyme. Thus, *pVLT*31 vector harboring *dszD* gene was transferred into this recombinant strain. **Results:** The results demonstrated a higher biodesulfurization activity when the flavin reductase gene was transferred into recombinant *P. putida* harboring *dszA,B,C*. These results were approved by the Gibbs test and HPLC analysis. **Conclusion:** These analyses showed that this novel indigenous engineered *P. putida* could be a promising candidate for an industrial and environmental application for Biodesulfurization process. *Iran. Biomed. J. 13 (4): 207-213, 2009*

Keyword: Pseudomonas aeruginosa, *Rhodococcus erythropolis* IGTS8, *pVLT31* vector, Biodesulfurization

INTRODUCTION

The combustion of fossil fuels disseminates sulfur oxide compounds. Many researchers have been performed to develop a biocatalyst to remove organic sulfur from coal and petroleum products [1]. The conventional hydro-desulfurization technology is a high pressure and high temperature catalytic process using various transition metal catalysts. However, the biodesulfurization process using microorganisms to remove the organic sulfur from coal and petroleum products could be performed safely under mild condition. Dibenzothiophen (DBT) is generally considered as a model compound for desulfurization in fossil fuels [2].

A significant number of organisms were found to remove sulfur from DBT via the 4S pathway. In this pathway, DBT is desulfurized and converted to 2-hydroxybiphenyl (2-HBP) without degradation the carbon skeleton in this compound and thus the calorific value of the fuel is conserved [3]. The 4S pathway has been already characterized for *Rhodococcus erythropolis* IGTS8, *Mycobacterium*, *Pseudomonas*, *R. erythropolis* D-1 and *Gordilla* species [1, 4-6]. There are four genes *(dsz A,B,C,D*) which involve in the pathway and allow the sulfur to release after four-step enzymatic reaction. Three catabolic genes, *dszA,B,C*, responsible for DBT desulfurization are clustered in an operon. DszC enzyme catalyzes two consecutive monooxygenation reaction converting DBT to DBT sulfone and

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Table 1. Bacterial strains used in this study.

Species and Strains	Relevant genotype/phenotype	Reference/origin
E. coli DH5α	[F´], *end*A1, *hsd*R17(rk^-mk^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A(Nal^r), *rel*A1, △(*lac*ZYA-*arg*F)un69, (ø80*lac*Z△M15), *LacZ* recombination deficient host	[11]
E. coli HB101	*Pro*A2, *leu*B6, *thi*-1, *lac*Y1, *hsd*S20,(rB^-mB^+), *rec*A13, *rps*120(Str^r), *ara*-14, *gal*k2, *xyl*-5, *mtl*-1, *sup*E44, *mcr*B, *mrr*, General recombination deficient plasmid host	[11]
Rhodococcus erythropolis IGTS8	Biodesulfurization	NIGEB (National Institute of Genetic Engineering and Biotechnology)

subsequently dszA as a second monooxygenase enzyme converting the DBT sulfone to DBT sulfonate.

Finally, the dszB as a desulfinase enzyme catalyzes the rate-limiting step in the pathway to transform DBT sulfone to 2-HBP and sulfite [7, 8]. In this pathway, the monooxygenase C and A require a free $FMNH_2$ for their activity. Therefore, it is provided as a dszD enzyme in the form of high expression recombinant plasmid in this strain [9, 10]. In this work three genes (*dszA,B,C*) were inserted stably into the chromosome of newly indigenous *Pseudomonas putida* and a broad-host-range expression vector (*pVLT31)* harboring the *dszD* gene was transferred into this recombinant strain. The Gibbs test and HPLC analysis were applied to measure the amount of 2-HBP production in this recombinant strain and compared to that of *R. erythropolis* IGTS8.

MATERIALS AND METHODS

Restriction endonucleases were purchased from Fermentas (Germany). All enzymes were used in accordance with manufactures recommendation. DBT and 2-HBP were purchased from Fisher (Germany) and Gibbs reagent, 2, 6-dicholoroquinone-4-chloromide from Sigma (USA). Ethyl acetate, aceto nitril and all other chemicals were purchased from Merck (Germany).

Bacterial strain and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Media and growth conditions. Deionized water was used to prepare all media and stock solutions. The sulfur-free medium used in this study was a modification of the standard basal salt medium (BSM) containing (per liter) 2.44 g KH_2PO_4, 5.57 g Na_2HPO_4, 2.0 g NH_4CL, 0.2 g $MgCl_2.6H_2O$, 0.001 g $CaCl_2.2H_2O$, 0.001 g $FeCl_3.6H_2O$, 0.004 g $MnCl_2.4H_2O$, DBT as a sulfur source to the final concentration of 60 ppm and glycerol (1.6 ml l^{1-}) as a carbon source to the final concentration of 100 ppm. Occasionally, citrate was added after sterilization of medium. Citrate was used to isolate Pseudomonas strain that are resistant to antibiotic. Indigenous Pseudomonas strains were grown at 40°C. *Escherichia coli* strains were grown in Luria Bertani (LB) at 37°C. Transformants were selected on 1.5% (w/v) LB agar containing ampicillin or kanamycin (50 μg $m\,l^{1-}$ each).

PCR and cloning of dszA,B,C genes in P. putida. The genomic DNA of *R. erythropolis IGTS8* was extracted by phenol/chloroform extraction method [11]. PCR was carried out for the identification and amplification of *dszA,B,C* genes of *R. erythropolis* IGTS8. The sequence of forward and reverse primers were 5´GAATTCCGCGATGACTCAAC AACGAC 3´ and 5´ AAGCTTTCAGGAGGTGAA GCCGGGAA 3´, respectively. Restriction sites for *EcoRI* and *HindIII* introduced at the 5' ends of forward and reverse primers. Fast start *taq* DNA polymerase and a high fidelity kit (Roche, Germany) were used for PCR. The annealing temperature was 65°C. The PCR product with 3.8 kb length was purified and concentrated by using a high pure PCR product purification kit (Roche, Germany). The PCR product containing *dszA,B,C* genes was digested by *EcoRI* and *HindIII* and cloned into the *pVLT31* containing *tac* promoter. *Ptac dsz* fusion together. *pVLT31* plasmid was transformed into competent *E. coli* DH5. The bacteria were harvested and

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Table 2. Plasmids used in this study that is previously been described by Gallardo *et al*. [12].

Plasmid	Genotype/phenotype
pRK600	Cm^r, oriColE1/PK 2-Mob^+, PK2-Tra^+, pESOX1 into pVLT31
pESOX3	TC^r, 3.8 kb, dsz operon subclone of pESOX1 into pVLT31
pESOX4	Km^r, Ap^r, 5.8 kb fragment subclone of pESOX3 into pBSL118
pVLT31	TC^r, Broad-host-range plasmid
pBSL118	Km^r, Ap^r Mini-*Tn5* transposon carrier

pVLT31 plasmid was extracted and digested by EcoRV restriction enzyme. *DszA,B,C* genes and *tac* promoter in a fragment were the result of EcoRV restriction enzyme. Then, *Ptac*::*dsz* fragment was ligated in *pBSL118* plasmid (*pBSL118* harboring *Ptac*::*dsz* is named *pESOX4*). *The pESOX4* Plasmid contains the RP4-mediated mobilization function and carries a mini-*Tn5* transposon that causes the stable migration of the heterologous DNA fragment from plasmid into the chromosome of *P. putida. The pESOX4* transformed into the competent *E. coli* CC118λpir bacterium. The tri-parental filter-mating method was applied by using *E. coli* CC118λpir as the donor and *E. coli* HB101 as the helper to transfer genetic information to *P. putida* as a recipient cell. Bacteria were cultivated in a M9 medium containing kanamycin antibiotic and citrate as only carbon source [12].

Southern-blot hybridization analysis. To confirm the cloning of *dszA,B,C* genes, DNA hybridization experiment was performed by Southern-blot method as described by Maniatis *et al.* [11]. DNA was labeled non-radioactively by using the random primer method with a Dig label and detection kit (Bohering Mannheim, Germany). Genomic DNA was electrophoresed on a 0.7% agarose gel then incubated in 0.25 M HCl at 20°C for 10 min, in the denaturation solution for 45 min and in the neutralization solution for 30 min. The denaturation solution contained NaOH (50 ml of 10 M solution) and NaCl (87.66 g) made up to 1 L with distilled water. The neutralization solution contained Tris (121.14 g), NaCl (87.66 g) and Tri-sodium citrate (88.2 g) in distilled water (L). Separated DNA transferred from gel to nitrocellulose membrane. The hybridization between the Dig-labeled probe and immobilized DNA on the membrane was carried out by using the procedure as described by Maniatis *et al.* [11].

Cloning of dszD gene into recombinant P. putida. Plasmid *pTZ57RT* containing *dszD* gene of *R. erythropolis* IGTS8 was digested by *EcoRI* and *HindIII* restriction enzymes. *dszD* gene was purified and ligated in plasmid *pVLT31* comprising tetracycline resistance site and *tac* promoter. Then, it was cloned into the recombinant *P.* putida containing *dszA,B,C* in its chromosome by electroporation method [11]. After cloning, the bacteria were cultivated in M9 medium containing tetracycline antibiotic and citrate as a carbon source. In order to identify bacteria containing *dszD* gene, PCR technique was employed. The nucleic acid sequences of the PCR primer were designed using conserved nucleic acid sequence of the flavin reductase enzyme of *R. erythropolis* IGTS8. The primers were as follow: 5′ -GAA TTC ATG TCT GAC AAG CCG AAT GCC-3′ (forward) and 5′-TCT AGA CTA TTG ACC TAA CGG AGT CGG-3′ (reverse). Annealing temperature was 55ºC for 1 min (30 cycles)**.**

Quantitative Gibbs assay. Recombinant *P. putida* containing *dszA,B,C* in the chromosome and *dszD* on *pVLT31* vector and *R. erythropolis* IGTS8 were grown in LB medium at 30ºC. Bacteria were cultured to reached to an optical density of 2.0 at 600 nm, harvested by centrifugation and resuspended in BSM medium(400 ml) to reach to an optical density of 0.05 at 600 nm.. Then supplemented by DBT in acetone (250 µl of 40 mM solution) as a sulfur source until the final concentration of DBT reached 60 ppm and glycerol (200 µl) as a carbon source until the final concentration of glycerol reached 100 ppm and shacked 200 rpm at 30ºC. A volume of 40 ml of this solution was removed every 4 h and its pH was adjusted to 2 by using concentrate HCl. For Gibbs assay, the pH of solution was adjusted to 8 with 10% (w/v) sodium carbonate. Gibbs reagent (2, 6 dichloroquinon -4-chloroamide, 10 µl of a10-mM solution in acetone) was then added to the solution and kept at 30°C for 30 minute. The solution was then centrifuged (8000 ×g) for 5 minute to remove cells. Absorbance of the supernatant was determined at 610 nm [11]. Biodesulfurization activity of recombinant *P. putida* and *R. erythropolis* IGTS8 was compared in 6, 12, 18, 24, 30 and 36 h. Standard Gibbs curve [8] was designed and 2-HBP production of other samples was measured based on comparing with 2-HBP concentration and OD of standard peak.

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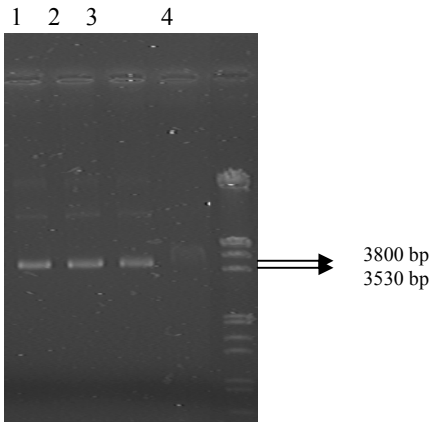


Fig. 1. Amplification of *dszA,B,C* gene from *Rhodococcus ertyropolis* IGTS8. Lane 1-3, PCR of *dszA,B,C* gene (3800 bp) and lane 4, molecular weight III (Roche, Germany).

HPLC analysis. To recognize standard peak, a certain value (0.1 mM) of pure 2-HBP was inserted in BSM medium and injected (standard peak). After sample preparation according to the Maniatis *et al.* [11], each sample was injected and the the production of 2-HBP was monitored and the peak area on HPLC was compared with standard 2-HBP peak. The time course for DBT desulfurization for all the strains were carried out by evaluation of the producing of 2-HBP. The 2-HBP production of recombinant *P. putida* and *R. erythropolis* IGTS8 was measured after 12, 24 and 36 h. The properties of HPLC analysis were model 1100 Cecil (England), C18 column, elution phase performed with 50:50 (v/v) acetonitrile/water mobile phase at 1 ml min^{-1}. Detection was realized with an UV detector at 280 nm, flow rate 0.9 ml/min [11, 13].

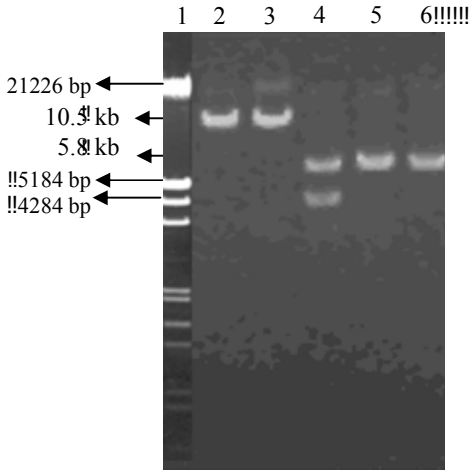


Fig. 2. Confirmation of the accuracy of cloning by digestion. pESOX4 plasmid was digested by EcoRV restriction enzyme. Lane 1, molecular weight III (Roche); lanes 2 and 3, plasmid containing insert fragment (10.5 kb); lane 4, digestion of pESOX4 by EcoRV enzyme and two bands of *dszA,B,C* gene under *tac* promoter (5.8 kb) and plasmid (4.7 kb) reveal; Lane 5 and 6, plasmid without insert.

RESULTS

Cloning of dszA, B, C genes into the chromosome of P. putida. *dszA,B,C* genes (3.8 kb) of *R. erythropolis* IGTS8 was amplified using PCR method (Fig. 1) and ligated into the plasmid *pVLT31* under control of *tac* promoter to make the construct

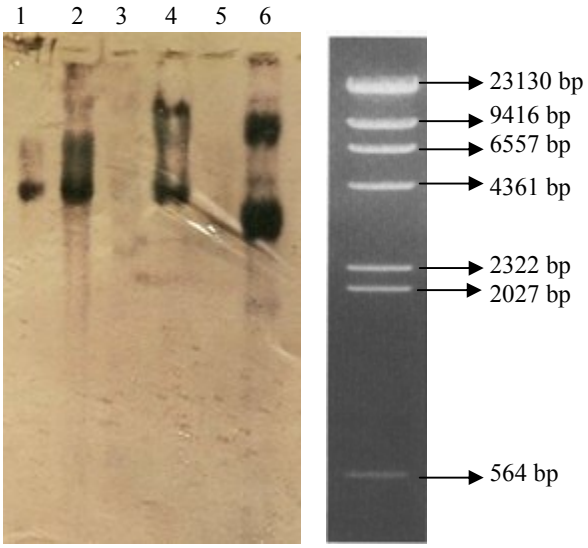


Fig. 3. Southern-blot of *dszA,B,C* gene in chromosome of recombinant *P. putida*. Lane 1, blot of *dszA,B,C* gene as probe; lane 2, blot of chromosomal DNA of SOX4 demonstrated the accuracy of insertion of *dszA,B,C* gene into chromosome of *P. putida*; lanes 4 and lane 6 are blots of $pTSOX_3$ and $pTSOX_4$, respectively as positive control. Lanes 3 and 5 are native *P. Putida* as negative control.

pTSOX3. Then, a 5.8-kb fragment containing *dsz* operon and *tac* promoter of *pTSOX3* plasmid was digested with EcoRV restriction enzyme and ligated into EcoRV site of a suicide vector (pBSL118) which carries the *mini-Tn5* transposon genes to make the construct *pTSOX4*. Afterwards, the construct, *pTSOX4* was transferred into the *E. coli* CC118λpir cells. To confirm the accuracy of cloning, the *pESOX4* plasmid was digested by EcoRV restriction enzyme. According to Figure 2, two bands of *dszA,B,C* gene under heterologous *tac* promoter (5.8 kb) and plasmid (4.7 kb) were seen. Then, desulfurization phenotype of recombinant *P. putida* was approved by Gibbs assay.

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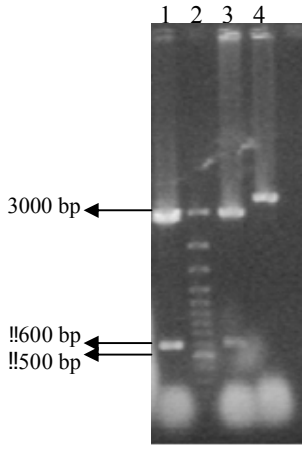


Fig. 4. Digestion of *pTZ57RT* containing *dszD* gene. Lane 2, marker (100 bp) (Roche,, Germany), lanes 1 and 3, digestion of *PTZ57R* containing oxidoreductase gene by *EcoRI* and *HindIII* showing two bands of *pTZ57R* (2800 bp) and oxidoreductase gene (586 bp); lane 4, band of non-cut *pTZ57R* plasmid.

Southern-blot. To confirm the presence of *dszA,B,C* gene into the chromosome of recombinant *P. putida* (*pTSOX4*), Southern-blot was performed. Southern-blot of chromosomal DNA demonstrated location of *Ptac::dsz* cassette in recombinant *P. putida*. According to Figure 3, Lane 2 is blot of chromosomal DNA of SOX4 which demonstrated the accuracy of insertion of *dszA,B,C* gene into chromosome of *P. putida*. The data confirmed that the *dsz* cluster was functional when stably inserted in a single-copy into the chromosome of *P. putida*.

Cloning of dszD gene into recombinant P. putida. Plasmid *pTZ57RT* containing *dszD* gene was digested by *EcoRI* and *HindIII* (Fig. 4). In Figure 4, lanes 2 and 3 reveal bands of *dszD* gene (586 bp) and *pTZ57RT* plasmid. The *dszD* gene was ligated in *pVLT31* plasmid and transferred into recombinant *P. putida*. *Oxidoreductase* gene is necessary for enhancing DBT desulfurization activity. To screen *P. putida* containing *dszD* gene, PCR technique was employed. In Figure 5, lanes 5-7 reveal PCR band of oxidoreductase gene (586 bp).

Quantitative Gibbs assay. Quantitative Gibbs assay was carried out based on the color change of the solution. The time course for DBT desulfurization of Recombinant *P. putida* (containing *dszA,B,C* in its chromosome and *dszD* on *pVLT31* plasmid) and *R. erythropolis* IGTS8 was carried out by measuring the release of 2-HBP using quantitative Gibbs assay. 2-HBP production of samples were measured based on comparing with concentration and OD of standard Gibbs curve. 2-HBP production by recombinant *P. putida* was compared with *R. erythropolis* IGTS8 (Fig. 6A). Biodesulfurization activity of these samples was compared in 6, 12, 18, 24, 30 and 36 h. According to Gibbs assay, 2-HBP production of *P. putida* was 0.166 mM and 0.228 mM after 18 and 36 h, respectively. The lag phase of *P. aeruginosa* was low (3-4 h) and the highest value of 2-HBP production (0.229 mM) by recombinant *P. putida* was achieved between 16-20 h. the 2-HBP production of *R. erythropolis* IGTS8 were 0.039 mM and 0.239 mM after 18 and 36 h, respectively and the highest value of 2-HBP production (0.239 mM) by *R. erythropolis* IGTS8 was achieved after 36 h and this increasing was continued (Fig. 6A).

HPLC assay. HPLC analysis was carried out by evaluating the production of 2-HBP. The 2-HBP production of recombinant *P. putida* and *R. erythropolis* IGTS8 was measured after 12, 24 and 36 h using the high-performance liquid chromatography method. The 2-HBP production in this comparison was monitored by comparing the peak area of each sample on HPLC. The data obtained from HPLC analysis showed that 2-HBP production of recombinant *P. putida* (0.192 mM) was higher than that of *Rhodococcus erythropolis* IGTS8 (0.047 mM) in 21 h. Biodesulfurization activity of *R. erythropolis* IGTS8 was low in primary 21 h, and increased after 22 h and 2-HBP production of recombinant *P. putida* and *R. erythropolis* IGTS8 was 0.229 mM and 0.196 mM, respectively after 36 h (Fig. 6B).

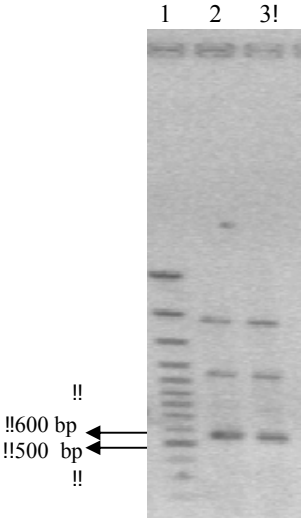


Fig. 5. PCR detection of oxidoreductase gene in the recombinant *P. putida*. Lane 1, marker (100 bp); lanes 2 and 3, PCR band of *oxidoreductase* gene (586 bp). This reaction confirmed the accuracy of cloning.

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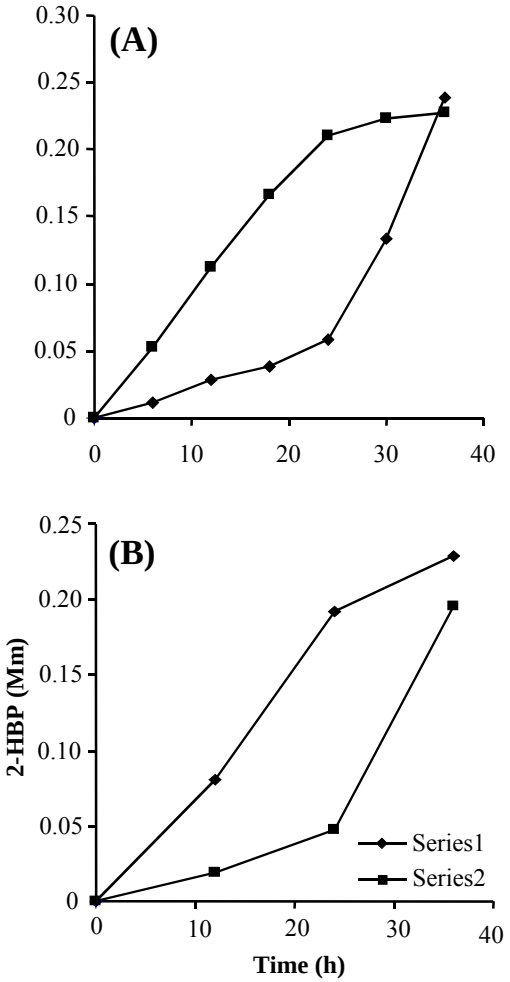


Fig. 6. Comparison of 2-HBP production by *Rhodococcus erythropolis* IGTS8 and recombinant *P. putida* by **(A)** Gibbs assay **(B)** HPLC analysis. (A) series 1 represents *R. erythropolis* IGTS8 and series 2 represents recombinant *P. aeruginosa*. (B) series 1 represents recombinant *P. putida* and series 2 represents *R. erythropolis* IGTS8.

DISCUSSION

Economical analysis indicate that designing a recombinant industrial strain depends on the enhancing of desulfurization rate and significant improvements in the stable biocatalysts adapted to the extreme conditions encountered in petroleum refining [14]. *R. erythropolis* IGTS8 is the most studied bacterium having the genes and enzymes responsible for DBT degradation. The rate of biodesulfurization activity and the stability of this bacterium in oil and petroleum are low.

In addition, sulfur compounds inhibit desulfurizing promoter in this organism [3, 15]. The strategy in the metabolic engineering is to change the host strain for the *dsz* genes entirely and promoter replacement for the expression of the *dsz* genes in a heterologous host [16, 17]. Gallardo *et al.* [12] previously reported that the *dszA,B,C* gene from *R. erythropolis* IGTS8 could be engineered as a DNA cassette under the control of heterologous regulatory signals to increase the ability of *P. putida* K2442 and *Pseudomonas aeruginosa* PG201 to efficiently desulfurize DBT.

Moreover, Matsubara *et al.* [3] cloned *dsz* genes of *R. erythropolis* IGTS8 into *E. coli* and showed that biodesulfurization activity of recombinant *E. coli* increased in an acceptable level.

In this study, *dszA,B,C* genes were inserted stably into the chromosome of newly indigenous engineered *P. putida* isolated from Iranian soil. The *flavin reductase* gene also transferred into recombinant *P. putida* by *pVLT31* vector. This new indigenous bacterium is an ideal biocatalyst for desulfurizing enzyme system due to the solvent-tolerant characteristic and optimum growth temperature at 40°C which is suitable for industrial biodesulfurization process. In addition, this strain produces *Rhamonolipid biosurfactant* which accelerates two-phase separation step in the biodesulfurization process through increasing emulsification. Moreover, it has a high growth rate which cause to remove sulfur compounds faster than *R. erythropolis* IGTS8 and has the highest biodesulfurization activity in shortest time. The comparison of the biodesulfurization activity of recombinant indigenous *P. putida* and *R. erythropolis* IGTS8 was performed by meaasuring 2-HBP production by HPLC analysis and Gibbs test. The data obtained from HPLC analysis and Gibbs assay showed that 2-HBP production of recombinant *P. putida* was more than that of *R. erythropolis* IGTS8 in primary 1-20 h. 2-HBP production of *R. erythropolis* IGTS8 increased after long time of cultivation (approximately 22 h). Therefore, engineered *P. putida* could be a promising candidate for industrial and environmental application in biodesulfurization due to removal of higher sulfur amounts from oil in the shortest time. In addition to a higher optimal growth temperature, ability in production of r*hamnolipid biosurfactant* and solvent toleration was the other privileges of this recombinant strain which is applicable in biodesulfurization processes.

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