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The Study of Biodesulfurization Activity in Recombinant *E. coli* Strain by Cloning the *dsz* Genes Involve in 4S Pathway

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Abstract

Several research groups have attempted to make recombinant bacteria capable of efficient desulfurization of oil fraction. The main aim of this study was to design a recombinant strain in order to desulfurize dibenzothiophen (DBT) and its components found in petroleum. In this study the pVLT31 vector harboring *dszABC* genes was transferred into recombinant *E. coli* BL21 which contained the *dszD* gene previously. We found that the recombinant *E. coli* BL21 containing the pVLT31 plasmid harboring *dszABC* genes and the pET21a plasmid harboring *dszD* gene had the highest capability for desulfurization of dibenzothiophen, when these four genes co-expressed. The data obtained in this study were confirmed by using Gibbs test, High-performance liquid chromatography (HPLC) and measuring of dry Cell weight. These analyses showed that *dszABC* and *dszD* genes co-express under the heterologous promoter in the strains which are stable in oil and petroleum compounds. In the next step the pUC18 plasmid harboring *dszAB* genes of *Rhodococcous* FMF was transferred into *E. coli* DH5α in order to evaluate the Effect of elimination of *dszC* gene in the rate of biodesulfurization process. This analysis demonstrated that 2-hydroxybiphenyl (2-HBP) production of recombinant *E. coli* DH5α using broken 4s pathway was less than that of *Rhodococcous* FMF in comparison. In this report we showed that the existence of the third gene (*dszC*) is necessary for enhancement of desulfurization activity.

Keywords: Desulfurization; Dibenzothiophen; 2-Hydroxybiphenyl

Introduction

Fossil fuel Combustion releases Sulfur oxide into the atmosphere, which is the main cause of acidic rain and air pollution [1, 2]. Hydrodesulfurization treatment is a catalytic process converting organic sulfur to hydrogen

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sulfide gas by reacting crude oil fractions with high hydrogen pressures and high temperatures. However, a number of cyclic organic sulfur compounds are recalcitrant against the hydrodesulfurization (HDS) method [3-5]. Biodesulfurization with mild operational conditions and lower capital could be an alternative technology to remove sulfur from the recalcitrant organic compounds. In this process, the organism is able to remove sulfur from fossil fuel without decreasing the caloric value of those substrates [6-9]. Most of reported strains such as *Rhodococcus erythropolis* IGTS8, *Mycobacterium* G3, *Pseudomonas*, *Gordona* and *Rhodococcus* Sp. SY1 can remove sulfur from DBT and its derivatives in a sulfur-specific manner without affecting the carbon skeleton by following the 4S pathway [9-13]. The biodesulfurization via the 4S pathway, is a process in which the DszC enzyme catalyzes the oxidation of DBT to DBT sulfone (DBTO2) and subsequent oxygenation occurs to yield 2-(2-Hydroxyphenyl) benzensulfinat (HPBS) by DszA enzyme. HPBS is converted to 2-hydroxybiphenyl (2-HBP) and sulfite/sulfate by desulfinase DszB enzyme [14, 15, 16]. DszD enzyme is a flavin oxidoreductase and absolutely required in the reaction catalyzed by DszC and DszA [17]. The biodesulfurization activity of naturally-occurring bacterial cultures is not commercial. Therefore gene manipulation can be as a promising strategy for enhancing the rate and efficiency of biodesulfurization process. In this study the *dszABC* genes have been cloned into the pVLT31 vector and the plasmid harboring *dszABC* genes was transferred into *E. coli* BL21 which already contained the *dszD* gene**.** In addition the biodesulfurization activity of recombinant *E. Coli* BL21 containing *dszABC* gene on the pVLT31 plasmid and *dszD* on the pET21a plasmid was measured by methods such as quantitative Gibbs test, High-performance Liquid chromatography (HPLC) and measuring the dry Cell weight. In the next step the 4s pathway was broken by separating *dszC* gene and *dszAB* genes were transferred into *E. coli* by using pUC18 plasmid. After confirmation of cloning, the biodesulfurization activity of recombinant *E. coli* which containing short 4s pathway was compared with that of *Rhodococcous* FMF as the native bacterium by quantitative Gibbs test.

Materials and Methods

All restriction enzymes and InsT/A clone PCR product cloning kit were provided from fermentase (Germany). The molecular weight marker, DNA detection kit, high pure PCR product purification kit and agarose gel DNA extraction kit were bought from Roche (Germany). All other chemical were purchased from Merck (Germany).

Bacteria and Plasmids

Strains and plasmids used in this study are listed in Tables 1 and 2.

Media and Growth Conditions

The sulfur-free medium used in this study was a modification of the standard Basal salt medium (BSM), deionized water was used to prepare all media and stock solution. BSM medium contained (per liter) 2.44g of KH_2PO_4, 5.57g of Na_2HPO_4, 2.0g of NH_4CL, 0.2g of $MgCl_2 .6H_2O$, 0.001g of $CaCl_2. 2H_2O$, 0.001g of $FeCl_3. 6H_2O$, 0.004g of $MnCl_2. 4H_2O$, DBT as sulfur source and Glycerol as carbon source were added to BSM medium, until the final concentration reached 60 ppm and 100ppm respectively. *Escherichia coli* strains were grown in Luria-Bertani (LB) at 37°C. Transformants were selected on Luria Broth (LB) (10 g/*l* Bacto-trypytone, 5 g/*l* yeast extract and 5 g/*l* NaCl, PH 7.2) and on luria Agar (LBA) (10 g/*l* Bacto-tryptone, 5 g/*l* yeast extract, 5 g/*l* NaCl and 15 g/*l* Agar). Plates were incubated at 37ºC LB agar supplements with 1.5% (w/v) agar and containing tetracycline (10 µg m l^{-1}) and/or ampicillin (50 µg m l^{-1}).

Amplifying of dszABC Genes of R. Erythropolis IGTS8

PCR reaction was used for isolation and amplification of *dszABC* genes in *Rhodococcus erythropolis* IGTS8. The primers were designed as follow: forward Primer ABC1 and the reverse Primer ABC2; Primer ABC1: 5′ GAATTCCGCGATGACTC AACAACGAC 3′, and the reverse primer ABC2: 5′ AAGCTTTCAGGAGGTGAAGCCGGGAA 3′. The restriction sites for *Eco*RI and *Hin*dIII introduced at the 5′ ends of forward and reverse primers. Fast-start *Taq* DNA polymerase and a high fidelity kit were used for PCR. The gene amplification kit was provided by Corbett Research.

Transformation of dszABC genes into E. coli BL21 bacteria

The PCR product containing *dszABC* genes was cloned into the pVLT31 plasmid. This plasmid has a *bla* marker for tetracycline resistance and can carry out α-complementation. The pVLT31 plasmid harboring *dszABC* gene was transferred into and *E. coli* BL21

bacteria using transformation method [18]. Transformed bacteria (100 µl) were spread on an LB agar plate containing tetracycline (10 mg/ml), IPTG (1 mM) and X-Gal and were incubated at 37°C. The presence of the *dszABC* genes were shown in the white colonies by culturing and plasmid extraction.

Transformation

dszAB genes of *Rhodococcus* FMF which ligated in pTZ57RT vector previously, was digested by *Eco*R1 and *Hin*dIII enzymes and then purified using High pure product purification kit (Roche). After purification, *dszAB* genes were ligated into pUC18 plasmid and transferred into *E. coli* DH5 (this reaction containing 2µl *oxidoreductase* gene, 6 µl pVLT31 plasmid vector, 1 µl T4 ligase, 1 µl T4 buffer maintained 24h, in 22 °C). These methods were used according to sambrook and Russel, 2001.

PCR Detection

PCR was used for the identification of *dszAB* genes. The proper primers were designed: forward Primer AB1 and the reverse Primer AB2 as follows; Primer AB1: 5′ GAATTCCGCGATGACTCAACAACGAC 3′, and the reverse primer AB2: 5′ AAGCTTCTATCGGTGGC GATTGAGGC 3′ and restriction sites for *Eco*RI and *Hin*dIII introduced at the 5' ends of forward and reverse primers. Fast-start *Taq* DNA polymerase and a high fidelity kit were used for PCR. The gene amplification kit was provided by Corbett Research and used an annealing temperature of 65°C. The PCR product in the 2.48 kb region was purified and concentrated by using a high pure PCR product purification kit.

Gibbs Assay

E. coli BL21 was grown at 37 °C in LB medium. When the cultures reached an optical density at 600nm (OD_{500}) of about 2.0, it was allotted to an OD_{500} of about 0.05 in to BSM medium. Then DBT in acetone (250 µl of 40 mM solution) as sulfur source was added to culture, until the final concentration of DBT reached 60ppm and glycerol (200 µl) as carbon source was added to culture, until the final concentration of glycerol reached 100ppm and shake 200 rpm at 37 °C. 40 ml of this solution was removed each 4hours 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. Then Gibbs reagent (2, 6 dichloroquinon - 4-chloroamide) (10 µl of a10 mM solution in acetone) was 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 and the absorbance of the supernatant was determined at 610 nm [19].

HPLC

For HPLC analysis of culture medium, the medium was acidified to pH 2.0 with 1 N HCl and then 2-HBP was extracted with equal volumes of ethyl acetate. In two-phase systems the concentration of 2-HBP was also measured after separation of the hydrocarbon phase from the aqueous phase containing cells by centrifugation (5,000 g) for 20 min at 25°C and 10-fold dilution. The concentration of 2-HBP production was measured with a Cecil 1100 England, C18 column. The mobile phase was a 50:50 (v/v) mix of acetonitrile: water at 1 ml min^{-1}, Detection was realized with an UV detector at 280 nm and flow rate: 0.9 ml/min. HPLC quantification 2-HBP was performed by reference to standard curves with a series of dilutions of pure 2-HBP.

Results

Transformation of dszABC Genes of R. Erythropolis IGTS8 into Recombinant E. coli BL21

Due to the commercial application of recombinant *E. coli* BL21, we tried to introduce the pVLT31 plasmid harboring *dszABC* genes (under *tac* promoter) into *E. coli* BL21 in order to increase the biodesulfurization activity. Flavin reductase (DszD) catalysed the reduction of flavins, such as flavin mono nucleotid, FAD and riboflavin by NADH and/or NADPH to form the reduced flavin. The pVLT31 plasmid containing *dszABC genes* of *R. erythropolis* IGTS8 (Fig. 1) was transferred into recombinant *E. coli* BL21 (*E. Coli* BL21 containing *dszD*). The presence of the *dszABC* genes was approved by digestion (Fig. 2) and Gibbs test.

Analysis of Gibbs Test, Cell Concentration (OD_{660}) and pH Alteration

Quantitative Gibbs test was performed to determine the 2-HBP production. In these experiments, the Gibb's assay calorimetrically quantities the amounts of 2-HBP produced from DBT. The quantitative Gibbs, pH alteration and cell concentration were performed at the presence and absence of the isopropyl-β-d-thiogalacto-side (IPTG) inductor. According to Figure 3, Gibbs test showed that the highest amount of 2-HBP production of

recombinant *E. coli* BL21 was achieved after 99 hours, (OD_{610} =4.274 nm (+ IPTG induction) and OD_{610}=2.342 nm (- IPTG induction)). Cell concentration was estimated by measuring the optical densities at 660 nm (OD_{660}). According to the measuring of cell concentration there was a linear relationship between Times (hour) and culture OD. The data obtained here demonstrated that the highest concentration of this bacterium was achieved after 124 hours. The rate of pH alteration was around 7.17—6.75 by IPTG induction and 7.16—6.08 without IPTG induction (Fig. 3).

HPLC Analysis

The time course for DBT desulfurization of the recombinant strain was carried out by evaluation the DBT degradation using the high-performance liquid chromatography (HPLC) method. Pure DBT was injected and the concentration of DBT from other samples were measured based on comparison with the concentration of standard peak. HPLC analysis was performed for recombinant *E. coli* BL21 (in the presence and absence of IPTG inductor) and *Rhodococcus erythropolis* IGTS8. According to HPLC analysis, the highest amount of DBT degradation by recombinant *E. coli* BL21 was achieved after 99 hours. Approximately 90% and 65% of DBT concentration were consumed in the presence and absence of IPTG respectively. *Rhodococcus erythropolis* IGTS8 degraded approximately 65% of DBT concentration after 99 hours (Fig. 4).

Dry Cell Weight

The Cell concentration in the cultures was estimated by measuring the optical density at 660 nm (660_{nm}). To determine a linear relationship between dry cell weight and culture, the optical density of recombinant *E. coli* BL21 was obtained in the range of 0.464 — 0.96; g dry cells (g/l) = 0.0815 OD — 0.4548 and for the *R. erythropolis* IGTS8 were obtained in two ranges as 0.665— 1.255; g dry cells (g/l) = 0.0592OD — 0.3304 and 1.556— 2 ; g dry cells (g/l) = 0.1445OD — 0.8751 (Data not shown).

PCR Detection and Transformation of dszAB Genes into E. coli DH5α

PCR detection was used to confirm the presence of *dszAB* genes in the pTZ57R plasmid. The Sequences of *dszAB* genes from *Rhodococcus erythropolis* IGTS8 were employed to design forward and reverse primers and restriction sites for *Eco*RI and *Hin*dIII introduced at

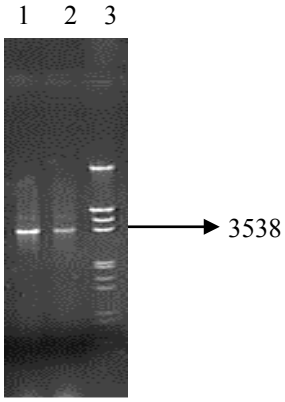

Figure 1. Amplification of *dszABC* genes of *R. erythropolis* IGTS8. lanes 1 and 2, bands of *dszABC* genes (3800 bp); lane 3, molecular weight III Roche.

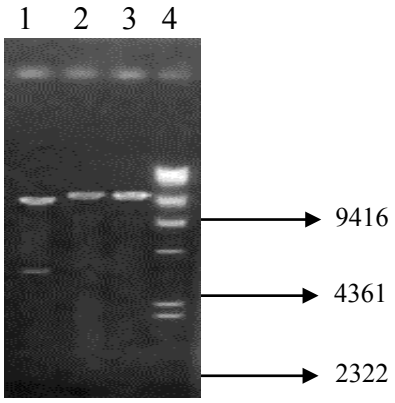

Figure 2. Confirmation of the accuracy of cloning by extraction and digestion of pVLT31 plasmid. lane 1, digestion of pVLT31 plasmid by *Eco*RI and *Hin*dIII restriction enzymes; lanes 2 and 3, pVLT31 plasmid containing *dszABC* genes (13.8 kb); lane 4, molecular weight II Roche.

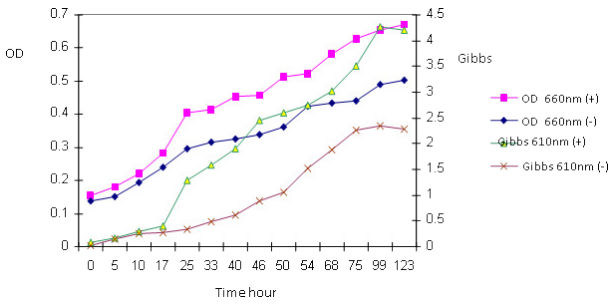

Figure 3. Comparison of optical density $_{(OD660\ nm)}$ and Gibbs $_{(OD610)}$ nm from recombinant *E. coli* BL21 SOX3 with and without IPTG induction. Square, diamond, triangle and multiple are OD660 nm (+ IPTG), OD660 nm (-IPTG), Gibbs OD610 nm (+ IPTG) and Gibbs OD610 nm (-IPTG) respectively.

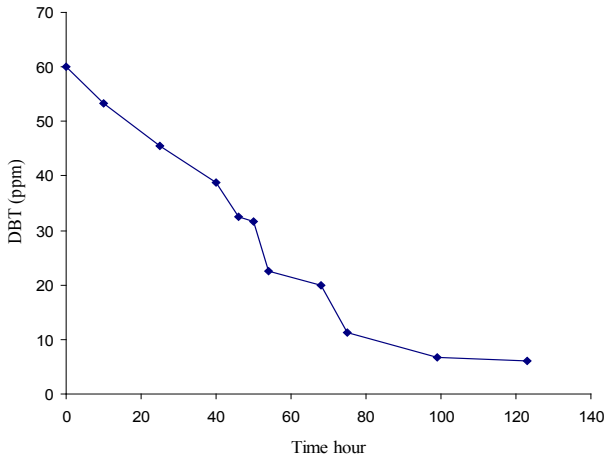

Figure 4. Analysis of biodesulfurization activity in recombinant *E. coli* BL21 via measuring the amount of dibenzothiophen reduction using HPLC.

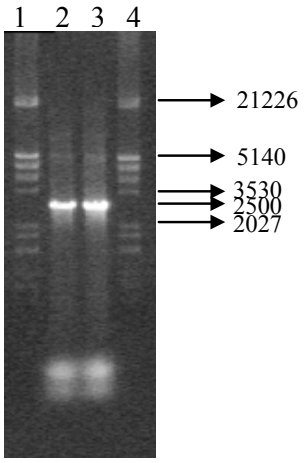

Figure 5. PCR detection of *dszAB* genes. lanes 2 and 3, *dszAB* genes (2.5 kb) which confirm the presence of *dszAB* genes in pTZ57R plasmid; lane 1 and 4, molecular weight III Roche.

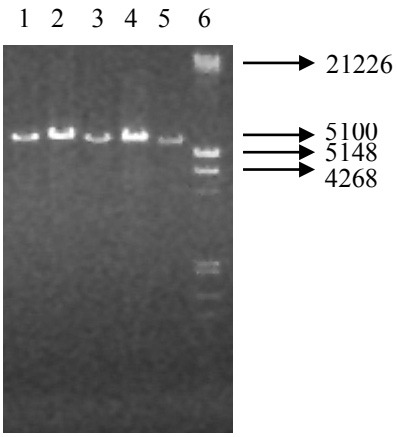

Figure 6. Extraction of pUC18AB57 plasmid. lane 1-5, extraction of pUC18AB plasmid which confirm the accuracy of cloning; lane 6, molecular weight III (Roche).

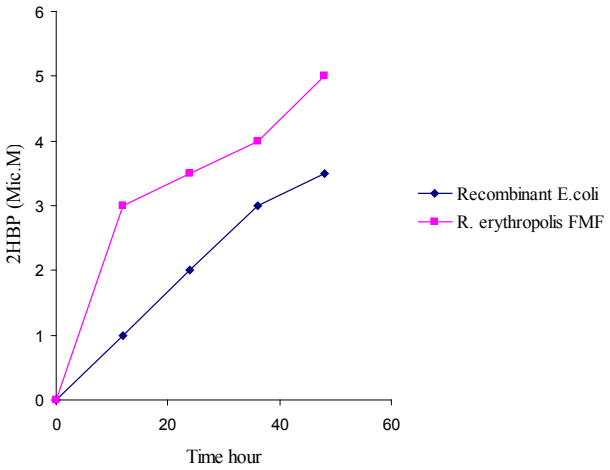

Figure 7. Comparison of 2-HBP production of recombinant *E. coli* and *Rhodococcus* FMF in quantitative Gibbs test.

the 5' ends of forward and reverse primers. According to Figure 5, lane 2 and 3 showed the presence of *dszAB* genes (2.5 kb) in the pTZ57R plasmid. In the next step pTZAB57R plasmid containing *dszAB* genes was digested by *Eco*RI and *Hin*dIII restriction enzymes (Data not shown). thereupon *dszAB* genes were ligated into pUC18 plasmid and transferred into *E. coli* DH5α. The accuracy of cloning was confirmed by extraction of the PUC18 plasmid. According to Figure 6, lane 4-10, showed the pUC18 plasmid contains the *dszAB* genes (5.1 kb).

Gibbs Assay

Qualitative Gibbs assay is biochemical method which was based on the color change of solution. In this method, blue color was the sign of presence of 2-HBP. In this study, qualitative Gibbs approved the biodesulfurization activity of this recombinant strain which contains short 4s pathway. The quantitative Gibbs assay was used to measuring the 2-HBP production of recombinant *E. coli* and *Rhodococcus* FMF. The data obtained of quantitative Gibbs assay demonstrated that the biodesulfurization activity of

recombinant *E. coli* was lower than that of *Rhodococcus* FMF in all times (Fig. 7).

Discussion

The desulfurization activity of naturally-occurring bacterial cultures is low in comparison with the requirements of a commercial process. Economic analysis indicates that a successful commercial process would require a biocatalyst with high desulfurization activity and high stability in oil and petroleum [20]. Increasing the bacterial desulfurization rate requires manipulating the system through genetic engineering techniques [21]. To develop an efficient biocatalyst many investigators have constructed recombinant biocatalysts**.** Previously Denome et al. reported that *dszABC* genes are act an operon and transcribed in the same direction [10]. In this study *dszABC* genes of *Rhodococcus erythropolis* IGTS8 were amplified and ligated into pVLT31 plasmid (under *tac* promoter) and transferred into the *E. coli* DH5α. The pVLT31 plasmid containing *tac* promoter since sulfate inhibits the promoter of *dsz* operon [4]**.** Flavin reductase (DszD) catalysed the reduction of flavins, such as flavin mono nucleotid, FAD and riboflavin by NADH and/or NADPH to form reduced flavin [17]. The first two Enzymes in this pathway (4S) belong to the nonflavin containing "Two-Componenet monooxygenase" family which requires free $FMNH_2$ to enhance the bidesulfurization activity. In preliminary studies Matsubara *et al.* amplified the *flavin reductase* (*dszD*) gene with primers designed by using *dszD* gene of *R. erythropolis* IGTS8; cloned and overexpressed in *Escherichia coli* BL21, and showed that overexpressing of *flavin reductase (dszD)* gene was a the major cause of increasing of biodesulfurization activity in this recombinant bacterium [22]. In this study we have constructed a laboratory genetically engineered microorganism which harboring three genes of *dsz* operon. This operon is transferred into the *Escherichia coli* BL21 cells which containing *dszD* gene previously. In this construct the native promoter was replaced with a hybrid (*tac*) promoter, since the addition of sulfate in culture media inhibits the *dszABC* genes expression as a negative feedback via the connection to the native *dsz* promoter**.** Data obtained from HPLC analysis and Gibbs assay showed that introduction of pVLT31 plasmid harboring *dszABC* genes and pET21a plasmid harboring *dszD* gene into the *E. coli BL21* enhanced the rate and efficiency of dibenzothiophen removal multifold when these four genes co-expressed. In the next experiment, *dszAB* genes of *Rhodococcous* FMF were amplified and transferred into *E. coli* DH5α to evaluate the affection of *dszC* gene in biodesulfurization activity. The biochemical analyses showed that the presence of *dszC* gene is necessary to enhance the biodesulfurization activity. We do not know the reasons for low biodesulfurization activity of recombinant *E. coli* strain. The following reasons could be the possible (I) Absence of Oxidoreductase enzyme (DszD) in recombinant *E. coli* DH5α because of the activity of DszA monooxygenase requires the $FMNH_2$ which supplied by DszD oxidoredutase. (II) The efficiency of lac promoter is not as high as the efficiency of original promoter of *dsz* operon in spite of the fact that sulphate does not inhibit this promoter. This study is a significant step in the exploration of the biotechnological potential of novel biocatalysts for developing an efficient biodesulfurization process.

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