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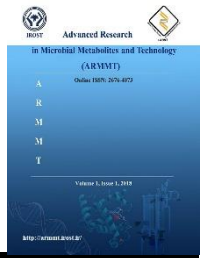

Enhancement /evolution of biodesulfurization 4S pathway by genetic engineering and bioinformatic approaches

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Abstract

Biodesulfurization could be a beneficial method in the industry for lowering the sulfur content of crude oil and could improve the efficiency of currently and costly used method, *i.e.*, hydrodesulfurization. But, to achieve this goal, it is critical to enhancing the BDS rate using genetics and other omics approaches. The reason attributed to the fact that the desulfurization activity of currently available natural biocatalysts does not meet the needs of the petroleum industry. Based on the expression of the 4S pathway genes in several heterologous hosts with no ability of desulfurization, the involved genes are ideal targets for enhancement through genetics or metabolic engineering approaches. This review provides an overview of the reported solutions concerning identification of new desulfurizing genes and their regulation, elimination of the inhibitory effects of end products, solving the problem of mass transfer, increasing the production, specific activity, and stability of the involved enzymes, and enhancing the resistance of recombinant strains to the biodesulfurization conditions. Also, the results of some bioinformatics studies which can help in the genetic engineering of desulfurizing strains were provided. Overall, genetic engineering and bioinformatics techniques can be effective in solving the problems of the biodesulfurization process in the near future as a complementary method of hydrodesulfurization.

1. Introduction

There is stringent international legislation about the sulfur content of crude oil (up to 10ppm). The reason related to the fact that the combustion of sulfur-containing fuels produces such as sulfur oxides (SO_x) adversely affect the environment and human health (Srivastava 2012).

Biodesulfurization (BDS) is a microbial, low cost, high energy savings, environmentally friendly and effective process in desulfurizing recalcitrant sulfur compounds of crude oil in contrast to the physicochemical method, *i.e.*, hydrodesulfurization (HDS) (Bhatia and Sharma 2010a; Ma 2010). Dibenzothiophene (DBT), 4-methyldibenzothiophene (4-MDBT), and 4,6-dimethyldibenzothiophene (4,6-DMDBT) are some of the examples of refractory organic sulfur compounds in crude oil (Babich and Moulijn 2003; Song 2003; Muzic and Sertic-Bionda 2013).

Various pathways including Kodama, mineralization, reductive or anaerobic, and 4S have been reported in different microorganisms to sulfur desulfurization. Among them, the

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petroleum desulfurization using the 4S pathway has been proposed because it has not the disadvantages of the anaerobic pathway, such as slow desulfurization rate and high cost of maintaining anaerobic conditions. Besides, it is a sulfur specific pathway which doesn't reduce the calorific value of the fuel in contrast to Kodama and mineralization pathways (Kodama et al. 1973; McFarland 1999; Monticello 2000; Gray et al. 2003; El-Gendy 2006; Mohebali and Ball 2016; Li and Ma 2019). Therefore, this review focused on the 4S pathway.

However, a successful commercial BDS process needs about a 500-fold increase in desulfurization activity of currently available natural biocatalysts and requires improvement in BDS operation. The reasons attributed to the fact that natural biocatalysts have been showed low desulfurization rate because they need negligible amounts of sulfur for their growth. In addition, the responsible genes expression is regulated in a sulfur-dependent manner as a result of the feedback inhibition (Li et al. 1996; Kilbane II 2006). In this regard, we review the updates of feasible solutions to overcome BDS limitations through genetic approaches. For more information about the recombinant biodesulfurization strains the previous reviews also recommended (Mohebali and Ball, 2008; Martínez et al., 2017). Besides, the authors suggested the following recent reviews for better understanding the BDS process (Pokorna and Zabranska, 2015; Stark 2016; Sadare et al., 2017; Chen et al., 2018).

2. The 4S pathway genes and related enzymes:

The 4S pathway is an aerobic desulfurization pathway (Fig. 1). The name of pathway "4s" comes from the four produced intermediate compounds in this pathway including DBT sulfoxide (DBTO), DBT sulfone ($DBTO_2$), hydroxyphenyl benzene sulfonate (HBPS) and finally sulfite (SO_3^{-2}). The reaction is energy-intensive and needs oxygen and the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin mononucleotide ($FMNH_2$).

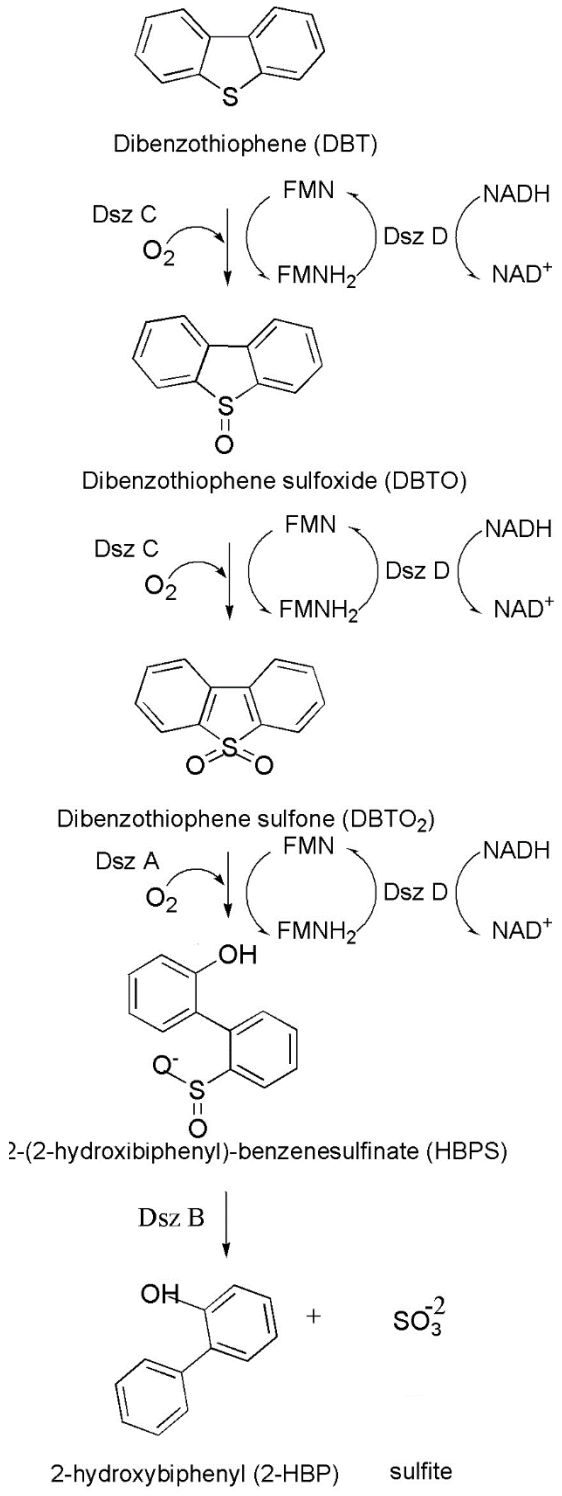

Fig. 1 4S pathway for DBT desulfurization

Sulfite and 2-hydroxybiphenyl (2-HBP) are end products (Monticello 2000; Mohebali et al. 2008; Li and Ma 2019). The genes of 4S Pathway were initially called as *sox* (sulfur oxidation) but later they were called *dsz* that came from desulfurization (*dszA*, *dszB*, *dszC*, *dszD*) (Denome et al. 1994). The properties and the reaction catalyzed with each desulfurizing enzyme in *Rhodococcus erythropolis* IGTS8, one of the first microorganisms to be identified at the molecular level, are summarized in Table 1. The *dszABC* operon in this strain has about 4 kb and is located on a 150 kb megaplasmid named pSOX (Denis-Larose et al. 1997; Denis-Larose et al. 1998). Transcription of the *dszABC* operon is controlled by a single promoter (Piddington et al. 1995; Li et al. 1996).

Table 1. Some features of desulfurization genes and enzymes belonging to *Rhodococcus* sp. (strain IGTS8)

Gene name	Gene length (Kb)	Gene location	Protein name	Protein length (No. of residue)	Protein weight (KDa)	Reaction
sox A (*dsz* A)	1.45	plasmid	DBT desulfurization enzyme A (DszA); DBT sulfone monooxygenase ($DBTO_2$-MO)	453	49.6	Converts DBT sulfone to HPBS using $FMNH_2$
sox B (*dsz* B)	1	plasmid	DBT desulfurization enzyme B (DszB); hydroxyphenyl benzene sulfonate (HPBS) desulfinase	365	39	Converts HPBS to 2-HBP (rate determining step)
sox C (*dsz* C)	1.25	plasmid	DBT desulfurization enzyme C (DszC); DBT monooxygenase (DBT-MO)	417	45	Converts DBT to DBTO and then $DBTO_2$ using $FMNH_2$
dsz D	0.579	chromosome	DBT desulfurization enzyme D (DszD); NADH-dependent FMN oxydoreductase; NADH: FMN oxidoreductase; flavin reductase	192	20.5	Reduces FMN to $FMNH_2$ using NADH

3. Application of Genetic Engineering to Biodesulfurization

Identification of the responsible genes in desulfurizing bacterial strains was the first genetic engineering researches concerning BDS. As mentioned before, the genes involved in BDS were characterized probably first in *R. erythropolis* IGTS8. For this purpose, a gene cluster that returned the activity of a desulfurization-negative IGTS8 mutant was cloned and sequenced (Denome et al. 1993). The product of the genes, their organization in the operon, direction of their transcription and the promoter and associated regulatory regions were also determined (Denome et al. 1993; Denome and Young 1995; Li et al. 1996). Also, it was reported recently that a TetR family protein could act as an activator of *the dszABC* operon for *R. erythropolis* IGTS8 and so enhance the levels of DSZ enzymes and BDS activity (Murarka et al. 2019). Identification of the *dsz* genes was also reported in other strains of *R. erythropolis* (Hirasawa et al. 2001; Santos et al, 2007; Etemadifar et al. 2014). *Rhodococcus* strains with the capacity of DBT

desulfurization have often conserved *dsz* genes. However, it was reported that a desulfurizing strain named *Rhodococcus* sp. FUM94 encoded a truncated *dsz* operon (Khosravinia et al. 2018a). Also, BDS genes have been reported in other bacterial strains such as *Gordonia terrae* C-6 (BT biodesulfurization or *bds* genes), *Paenibacillus* sp. A11-2 (thermophilic DBT-desulfurizing or *tds* genes) and *Bacillus subtilis* WU-S2B (*bds* genes) (Ishii et al. 2000; Kirimura et al. 2004; Kilbane and Robbins, 2007; Wang et al. 2013; Su et al. 2018). Interestingly, some differences were reported about the gene location and their promoter sequences. For example, the *dsz* genes in *Gordonia alkanivorans* RIPI90A located on chromosome unlike the *dsz* operon of *R. erythropolis*, which located on the plasmid. In addition, the promoter sequences of these two strains were different (Shavandi et al. 2010). The metagenomics approaches helped the researchers to identify new DBT desulfurization genes in nature from nonculturable bacteria. In this regard, Abbasian et al. (2016) identified three new DBT desulfurization genes using a metagenomics study and analyzed their activity using expression cloning in *Escherichia coli* DH5α cells (Abbasian et al. 2016). Martin-Cabelo et al. (2020) found a complete operon of *dszEABC* by functional metagenomics. This operon included an FMN-oxidoreductase (DszE), and other Dsz enzymes (DszA, DszB, DszC), which caused desulfurization ability in *Escherichia coli* for using DBT as sole sulfur source.

The *dsz* genes are ideal targets for enhancement through genetics or metabolic engineering approaches because the expression of the 4S pathway genes in heterologous hosts with no capacity of DBT desulfurization has resulted in more effective desulfurizing strains (Martínez et al. 2016). For example, the plasmid from *R. erythropolis* strain R1 (HBP-positive) was transferred to an HBP non-producing strain using polyethylene glycol (PEG)-mediated protoplast transformation. The mutant degraded 100% of DBT and produced HBP more efficiently (Etemadifar et al. 2008). Also, *R. erythropolis* KA2-5-1 *dsz* gene cluster was transferred into a non-desulfurizing strain of *R. erythropolis i.e.*, MC1109 which resulted in a recombinant strain with the ability to remove about twice as much sulfur concentration that of the parent strain (Watanabe et al. 2003). Also, it was reported that a BT-desulfurizing bacterium (*Rhodococcus* sp. T09) which has been received the DBT-desulfurizing genes of *R. erythropolis* KA2-5-1 could desulfurize both alkylated BTs as well as various alkylated DBTs. This result reminds that the inability of DBT desulfurization in BT-desulfurizing wild type strain is due to enzyme specificity (Matsui et al. 2001).

One of the main problems in the BDS process is the adverse effect of sulfate on the expression of the 4S pathway enzymes. Various strategies have been adopted to overcome this problem. Using a sulfate non-repressible promoter is one of the strategies (Noda et al. 2002; Gupta et al. 2005; Shavandi et al. 2009). For example, Khosravinia et al. used pCom8 as an alkane responsive promoter for expression of *Rhodococcus* sp. FUM94 *dsz* genes and made the recombinant strain insensitive to inorganic sulfate compounds in the culture medium (Khosravinia et al. 2018b). Deletion of the *dszB* gene from the operon is another strategy to eliminate sulfate and 2-HBP inhibition effect (Monticello 2000). Pan et al. (2013) selected another solution for this problem and inserted a synthetic gene encoding a peptide named Sulpeptide1 (S1) into the *dsz* operon of *R. erythropolis* IGTS8 aimed to make it hard for the microbial cell to eliminate or inactivate the S1 gene without loss of BDS activity. Also, its insertion in the operon caused to deplete intracellular deposits of preferred sulfur sources, such as methionine, cysteine, and sulfate. Therefore, S1 prevented the suppression of the operon by preferential sulfur sources and led to an increase in the expression of desulfurization genes. Also, a signal sequence was inserted into the S1 gene to secrete the S1 protein extracellularly.

Therefore, the cell demand for sulfur increased, and as a result, the expression of the desulfurization genes was improved. The desulfurization activity of the resulted recombinant cell (*dszAS1BC*) showed more than a twentyfold increase (Pan et al. 2013). In addition, mutagenesis of sulfur metabolism-related genes such as cystathionine β-synthase (Cbs) was also an effective strategy in increasing the expression of 4S pathway enzymes even in the presence of sulfate (Tanaka et al. 2002).

2-HBP is another end product of BDS reaction, which can be toxic for biocatalyst and inhibiting cell growth and their metabolism. 2-HBP can inhibit desulfurization enzymes at concentrations lower significantly than that of it accumulated during the BDS process. Among the four involved enzymes in the 4S pathway, the first one, *i.e.,* DszC is affected more by 2-HBP feedback inhibition. For this reason, Li et al. (2019) generated a mutant of DszC, which showed substantially reduced susceptibility to the feedback inhibition (Li et al. 2019). However, DszB (the last enzyme in the 4S pathway that converts HPBS to 2-HBP) has the highest tolerance to 2-HBP (Kilbane 2016). As mentioned earlier, disruption of the *dszB* gene could decrease cell toxicity and the desulfurizing enzymes inhibitory effect. It also resulted in the accumulation of HBPS instead of 2-HBP with the benefit that HBPS could be used for biosynthesis of surfactants (Monticello 2000).

The desulfurization efficiency can boost by increasing of DszB and DszC proteins, and in the ratio of A:B:C equal to 1:4:2, approximately 100% biodesulfurization rate of DBT to 2-HBP is accomplishing in vitro (Li et al. 2020). Overproduction of Dsz enzymes, especially DszB enzyme, can enhance the BDS efficiency. The reason attributed to the fact that despite the *dszABC* operon is controlled by a single promoter, the production of DszB is lower as compared with other enzymes. It is worth to remind that the level of transcription or translation of the involved genes in an operon usually decreases as the distance of the gene from the promoter increases. Also, there is a gene overlap between the *dszA* and *dszB* genes (Kilbane 2017). This genetic overlapping exists likely to decrease the *dszB* expression and thus reduce the adverse effects of its product, *i.e.,* 2-HBP. Several strategies were adopted to enhance DszB production in previous studies. For example, Reichmuth *et al.* increased the copy number of *dszB* as well as changed the ribosome binding site of the related gene (Reichmuth et al. 2004). Li *et al.* rearranged the *dsz* operon as *dszBCA*, instead of *dszABC*, in *R. erythropolis* DRA. Their results revealed that the *dszB* and *dszC* expression levels had been enhanced insofar as the desulfurization activity increased a more than twelvefold concerning the resting cells with the natural operon (Li et al. 2008). Removing the overlap between the two genes, *i.e., dszA* and *dszB,* is another strategy for this purpose (Li et al. 2007). Low levels of the flavin reductase activity can influence the BDS yield. Therefore, some previous works focused on this issue. Overexpression of a heterologous flavin reductase in the host cell is one of the solutions of this problem (Galán and García 2000; Reichmuth et al. 2000; Raheb et al, 2010).

The identification and mutagenesis of the residues of Dsz enzymes which affect their specific activity or stability could lead to improving the desulfurization process. For example, Kamali *et al*. used site-directed mutagenesis to substitute DszD Thr62 residue with Asn (T62N) and Ala (T62A). In this work, both variants, *i.e.*, T62N and T62A, showed an increase in *R. erythropolis* desulfurizing activity as much as five- and sevenfold, respectively (Kamali et al. 2010). Also, Ohshiro *et al*. found that two amino acid substitutions (Tyr63 and Gln65) could enhance the DszB catalytic activity and thermostability (Ohshiro et al. 2007).

BDS strains use oxygen not only for the monooxygenase activity but also for their metabolism. Because of relatively high Michaelis constant (*Km*) for oxygen in the oxygenase enzymes, it is necessary to maintain

O_2 pressure properly so that the oxygenases can be allowed to compete for O_2 with the cellular respiratory enzymes. However, an increase in air intake can lead to the volatilization of diesel or gasoline and, as a result, may lead to an explosion hazard. So, it would be promising to find and develop a biocatalyst which shows high desulfurization activity under hypoxic conditions. For this purpose, Xiong et al. (2007) increased desulfurizing activity and also the biomass production of *R. erythropolis* LSSE8-1 by transferring *Vitreoscilla* hemoglobin gene (*vgb* gene) into the strain (Xiong et al. 2007).

In other studies, the reason for the construction of recombinant strain was to have higher solvent resistance strain so the problem of the biocatalyst low viability in petroleum can be solved. Introduction of *dsz* genes from desulfurizing strains to solvent-tolerant bacteria such as strains of *Pseudomonas putida* is one of the strategies reported by previous studies. The recombinant strains showed the ability of desulfurizing DBT in the 4S-pathway, had the better growth rate than that of the parent cell at the same concentration of DBT, and had the same substrate range as desulfurizing strains (Tao et al. 2006; Meesala et al. 2008; Aliebrahimi et al. 2015). In addition, organic solvent-responsive expression vectors increased the BDS activity of *Pseudomonas putida* DS23 in the presence of various solvents (Tao et al. 2011).

The transfer of desulfurizing genes to surfactant-producing *Pseudomonas* strain has been adopted as a strategy to increase the uptake of DBT from the oil phase (Gallardo et al. 1997). Also, it was reported that the DBT uptake and the desulfurization activity of a recombinant strain, which received a hydrophobic compounds specific transport system named *hcu*ABC gene cluster, has been increased (Noda et al. 2003; Wang et al. 2011). The other reported strategy was displaying a BDS enzyme on the bacterial host strain surface to overcome the mass transfer limitation (Rangra et al. 2018).

4. Application of In Silico analysis to Biodesulfurization

Identifying novel biodesulfurizers through in silico methods have not reported much for biodesulfurization studies. Isolation of novel biodesulfurizing microorganisms using conventional methods takes a lot of time, labor, and cost. However, mining genomic databases solved the mentioned problems. For this purpose, BLASTp and Kyoto Encyclopedia of Genes and Genomes database can be used to find putative homolog protein(s). The mining of genomic databases using bioinformatics tools resulted in the identification of 13 novel desulfurizing strains (Bhatia and Sharma, 2010b). Furthermore, the Dsz phylogenies can provide valuable information to understand the origin of *dsz* gens, as well as the species adaptation during the evolutionary period (Wee et al., 2019).

The results of multiple sequence alignment analyses can be useful in recognizing the conserved residues of Dsz proteins which are most probably responsible for their activity. Interestingly, it had shown that a large number of *Rhodococcus* strains with the capacity of DBT desulfurization have conserved *dsz* genes (Khosravinia et al. 2018a). For example, it was found that the DszA enzymes variation among 14 different microorganisms was related only to the residues ranged from 434 to 474 concerning the reference sequence (AAP80182) through Jotun Hein, Clustal V, and Clustal W algorithms (Karnwal and Goindi, 2012). In addition, the analysis of Dsz protein structure in recognizing the key residues in enzyme activity was also valuable. Zhang et al., (2014) concluded that the C-terminus of DszC of *R. erythropolis* (residues 410–417) is critical to the stabilization of the enzyme substrate-binding pocket. Enzymatic assay and analytical ultracentrifugation analysis have also confirmed these results (Zhang et al., 2014). In addition, Liu et al., (2014) suggested that H391 residue of DszC from *Rhodococcus* sp. XP may have a critical

role in the enzyme activity based on the results of the molecular docking and site-directed mutagenesis (Liu et al., 2014).

In contrast to the conserved residues, the non-conserved residues are responsible for variation in the physicochemical properties. Therefore, changing the non-conserved residues can affect the function and other desirable properties of proteins.

The in-silico analysis is often performed before the genetic engineering methods to design appropriate mutations in a much easier, faster, and more economical way. Here we summarized the results of some previous studies concerning this issue. Fallahzadeh et al., (2019) were identified that A79I and A79N mutants have the lower binding free energies toward FMN among the key residues on the active site regions of the DszD enzyme of *Rhodococcus erythropolis* IGTS8 using the molecular docking and molecular dynamics simulation. These results were confirmed through experimental work and the biodesulfurization activity of A79I and A79N mutants increased as much as 1.9- and 2.3-fold, respectively (Fallahzadeh et al., 2019). In silico docking analysis of DszB wild type and mutant proteins of *Streptomyces* sp. VUR PPR 101 showed that Y63A mutant had the highest affinity towards its substrate and therefore the highest activity (Reddy and Vanga, 2017). The best point mutations for increasing *R. erythropolis* DszC hydrophobicity and stability were predicted previously. It was reported that changing the surface residues of DszC enzyme to hydrophobic residues increased the enzyme affinity to oil (Torktaz et al. 2012).

5. Conclusion

Using ultra-deep HDS processes is a costly method to reduce or eliminate the refractory organosulfur compounds present in the crude oil. Therefore, BDS can be helpful as a complementary method for petroleum desulfurization. However, controlling the microbial cells and their desulfurizing enzymes in the harsh conditions encountered in the industry are faced with numerous problems including low production, activity and thermal stability of the desulfurizing enzyme, the inhibitory effect of the final products, the susceptibility of microbial cells and enzymes to the solvents, the supply of cofactors for the enzymes, and the problem of mass transferring to the biocatalyst, and the narrow substrate spectrum for desulfurization. Genetic engineering methods helped researchers not only to identify the genetic basis of BDS pathway in various strains to a better understanding of microbial processes but also to overcome these BDS process problems. Further studies are still needed. Engineered biocatalyst which can degrade or transform 2-HBP into other less toxic metabolites, investigation of thermophilic desulfurizing enzyme resistant to end-product inhibition using metagenomics approaches, increasing the substrate spectrum of biocatalyst using site-directed mutagenesis are some of the recommendations for future researches.

Conflict of interest: The authors declare no conflict of interest.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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