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Metabolic and process engineering for biodesulfurization in Gram-negative bacteria

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Highlights

- Biodesulfurization is a real technology to reduce sulfur levels in fuel
- Metabolic and genetic engineering can overcome the major bottlenecks of the process
- Gram-negative bacteria are suitable hosts for engineering desulfurizing biocatalysts
- More efforts in process engineering are needed for the industrial application

Abstract

Microbial desulfurization or biodesulfurization (BDS) is an attractive low-cost and environmentally friendly complementary technology to the hydrotreating chemical process based on the potential of certain bacteria to specifically remove sulfur from *S*-heterocyclic compounds of crude fuels that are recalcitrant to the chemical treatments.

The 4S or Dsz sulfur specific pathway for dibenzothiophene (DBT) and alkyl-substituted DBTs, widely used as model *S*-heterocyclic compounds, has been extensively studied at the physiological, biochemical and genetic levels mainly in Gram-positive bacteria. Nevertheless, several Gram-negative bacteria have been also used in BDS because they are endowed with some properties, e.g.,, broad metabolic versatility and easy genetic and genomic manipulation, that make them suitable chassis for systems metabolic engineering strategies. A high number of recombinant bacteria, many of which are *Pseudomonas* strains, have been constructed to overcome the major bottlenecks of the desulfurization process, i.e., expression of the *dsz* operon, activity of the Dsz enzymes, retro-inhibition of the Dsz pathway, availability of reducing power, uptake-secretion of substrate and intermediates, tolerance to organic solvents and metals, and other host-specific limitations. However, to attain a BDS process with industrial applicability, it is necessary to apply all the knowledge and advances achieved at the genetic and metabolic levels to the process engineering level, i.e., kinetic modelling, scale-up of biphasic systems, enhancing mass transfer rates, biocatalyst separation, etc. The production of high-added value products derived from the organosulfur material present in oil can be regarded also as an economically viable process that has barely begun to be explored.

Keywords:
Biodesulfurization, Dibenzothiophene, Gram-negative bacteria, Metabolic engineering, Genetic engineering, Scale-up

1. Introduction

Global society is moving towards zero-sulfur fuel due to the negative impact that the combustion of sulfur containing fuels causes to the environment and to the health. The conventional hydrodesulfurization (HDS) is the most employed technology to reduce sulfur (S) content in fuels. However, HDS suffers many limitations, e.g., it

works under severe and hazardous operation conditions, it is not efficient in desulfurization of some refractory S-containing compounds, it needs high capital and operating costs, and it generates the hazardous H_2S end product, among others. Due to these facts, during the last decades several advances have been made in developing chemical, physical and biological technologies complementary to HDS to achieve ultra-low sulfur fuel (S < 15 ppm for on-road and non-road diesel) (Stanislaus et al., 2010). Biodesulfurization (BDS) is one of these emerging non-conventional technologies that can be merged with other desulfurization technologies, such as the oxidative desulfurization process, to produce S-free fuels.

The BDS process involves the use of free or immobilized microorganisms, their enzymes or cellular extracts, as catalysts to remove the S present in fuels (Soleimani et al., 2007). BDS combines a high S removal efficiency and substrate selectivity, due to the use of microbial enzymatic systems, with a low generation of undesirable by-products (Ohshiro and Izumi, 1999). The sulfur compounds targeted in BDS are mainly the aromatic compound dibenzothiophene (DBT) and its alkylated derivatives. Other sulfur-containing heterocycles have been scarcely studied (Ahmad et al., 2014).

Despite the first reports describing bacterial BDS were accomplished with Gram-negative bacteria, such as *Pseudomonas* and *Desulfovibrio* strains, the most extensively studied and used for BDS processes are Gram-positive bacteria, e.g., *Rhodococcus*, *Gordonia*, *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Paenibacillus*, or *Bacillus* strains (Kilbane, 2006; Soleimani et al., 2007: Mohebali and Ball, 2008; Kilbane and Stark, 2016). However, several Gram-negative bacteria present some characteristics, e.g., high tolerance to organic solvents and metals, broad metabolic versatility and easy genetic manipulation, that make them ideal candidates for developing recombinant biocatalysts for BDS. Some interesting reviews have been recently published on BDS, but most of them are mainly focused on the biocatalyst or bioprocess levels (Boniek et al., 2015; Kilbane and Stark, 2016; Moheballi and Ball 2016). To provide a comprehensive vision of the sequential steps needed for a correct development of a BDS process, here we review not only the studies at the genetic and metabolic levels, but also the advances in both process engineering and scale-up when using Gram-negative bacteria as target biocatalysts.

2. Biodesulfurization: microorganisms and pathways

S-hererocyclic compounds desulfurizing bacteria are widespread in different environments and geographic locations, suggesting that desulfurization is an important sulfur-scavenging strategy for these bacterial species (Mohebali and Ball, 2016). The metabolic pathways/reactions used for degradation/conversion of aromatic S-heterocyclic compounds can be classified into four different types: i) sulfur oxidation; ii) C-C cleavage; iii) C-C and C-S cleavage; iv) C-S cleavage (sulfur specific cleavage).

The sulfur oxidation of DBT and other S-containing aromatic compounds is catalyzed by some fungal laccases and bacterial ring hydroxylating dioxygenases, mostly from Gram-negative bacteria, that oxidize DBT to DBT-sulfone and other sulfur-containing hydroxylated derivatives as dead-end products (Fig. 1) (Gupta et al., 2005; Xu et al., 2006; Soleimani et al., 2007; Mohebali and Ball, 2016).

The C-C cleavage of DBT is known as the "Kodama pathway", and it consists of three main steps catalyzed by Dox enzymes responsible for the degradation of naphthalene, or other polycyclic aromatic hydrocarbons, yielding 3-hydroxy-2-formyl-benzothiophene as the S-containing end-product (Fig. 1) (Gupta et al., 2005). All the microorganisms able to carry out this pathway are Gram-negative bacteria, e.g., *Pseudomonas* strains, *Rhizobium meliloti*, *Burkholderia fungorum* DBT1, *Xhantobacter polyaromaticivorans* 127W, *Beijerinckia* sp., *Sphingomonas* sp., or some fungi such as *Cunninghamella elegans* (Ohshiro and Izumi, 1999; Gai et al., 2007; Andreolli et al., 2011).

A few microorganisms, e.g., *Brevibacterium* sp. DO and *Arthrobacter* DBTS2, are able to use sulfur organic compounds as both sulfur and carbon source via oxygenolytic attack of the C-C and C-S bonds (Fig. 1), but the genes/enzymes involved in this destructive pathway have not been yet characterized (Bressler and Fedorak, 2000).

The discovery of an aerobic sulfur-specific pathway, usually known as "4S pathway", in the Gram-positive *Rhodococcus erythropolis* IGTS8 strain (Denome et al., 1993) represented a turning point on BDS because is a non-destructive pathway which retains the full combustion capacity of DBT (Fig. 1). According to this pathway, sulfur is removed selectively whereas the carbon skeleton and the caloric value of the resulting S-free 2-hydroxybiphenyl (2HBP) end product remain intact. The 4S pathway transforms DBT into 2HBP and sulfite by four serial reactions catalyzed by DszC (DBT monooxygenase), DszA (DBT-sulfone monooxygenase) and DszB (2-hydroxybiphenyl-2-sulfinate (HBPS) desulfinase) enzymes (Fig. 1). These enzymes are codified by the

dszC, *dszA* and *dszB* genes, respectively, which are part of the *dszABC* operon located in a 120 kb plasmid in strain IGTS8 (Gray et al., 1996). A NADH:FMN oxidoreductase enzyme, encoded by the chromosomally-located *dszD* gene, provides $FMNH_2$ required for the activities of DszC and DszA enzymes (Fig. 1) (Gray et al., 1996). Since the discovery of *R. erythropolis* IGTS8, a large number of mesophilic and thermophilic microorganisms containing the 4S pathway have been isolated from soil using traditional selective screenings in the presence of DBT, and they are mostly Gram-positive Actinobacteria (Gray et al., 2003; Kilbane, 2006; Moheballi and Ball, 2016).

Despite most microorganisms containing the 4S pathway are Gram-positive bacteria, several Gram-negative bacteria of different proteobacterial groups, i.e. *Pseudomonas, Pantoea, Serratia, Stenotrophomonas, Enterobacter, Klebsiella, Shewanella, Acinetobacter* (gammaproteobacteria), *Agrobacterium, Sphingomonas, Chelatococcus* (alfaproteobacteria), *Achromobacter, Acidovorax, Ralstonia* (betaproteobacteria), *Desulfobacterium* (deltaproteobacteria), able to desulfurize DBT, have been also described and some of them have been used in BDS processes (Table 1) (Papizadeh et al., 2011; Gunam et al., 2013; Liu et al., 2015; Bordoloi et al., 2016; Mohebali and Ball, 2016; Dejaloud et al., 2017; Gunam et al., 2017; Papizadeh et al., 2017). Nevertheless, the genetic characterization of the desulfurization gene clusters in these Gram-negative bacteria is still missing. As with the Gram-positive bacteria, some of the Gram-negative desulfurizers are thermophilic strains, e.g. *Klebsiella* sp. 13T (Bhatia and Sharma, 2012), or psychrophilic strains, e.g. *Pseudomonas*, *Acinetobacter*, and *Sphingomonas* strains (Gunam et al., 2013). Another way to look for novel biodesulfurizing microorganisms is by *in silico* screening of the available genomic databases using the *dsz* genes from strain IGTS8 as query. By using this approach, at least nine novel potential DBT-desulfurizing Gram-negative bacteria have been identified (Bhatia and Sharma, 2010a).

Despite of the standard 4S pathway produces 2HBP as final product, an "extended 4S pathway" has also been reported in some *Mycobacterium* strains. In this extended pathway, 2HBP is methoxylated to 2-methoxybiphenyl (2MBP) by an *O*-methyltransferase (Fig. 1) (Xu et al., 2006). This extended pathway has been also found in some Gram-negative bacteria such as *Achromobacter* sp. (Bordoloi et al., 2014) and *Chelatococcus* sp. (Bordoloi et al., 2016). Another extended pathway that converts 2HBP to biphenyl has been described in a *Rhodococcus* strain but the genes have not been yet characterized (Akhtar et al., 2009, 2015).

Some Gram-negative sulphate-reducing bacteria, e.g. *Desulfovibrio* and *Desulfomicrobium* strains, are able to specifically remove sulfur from benzothiophene and DBT under anaerobic conditions (Kim et al., 1995)..Although anaerobic BDS avoids aeration costs and has the advantage of liberating sulfur as a gas, the low rate and extent of BDS and lack of knowledge on the biochemistry and genetics of the anaerobic microorganisms makes this anaerobic process commercially unviable (Gupta et al., 2005).

Despite of the large number of microorganisms able to perform BDS via the 4S pathway, their native activities are too low to develop a commercial process. Moreover, most of the oil samples are usually constructed from scratch with selected hydrocarbons and often over-simplified formulations, instead of using actual refinery products. In this sense, several bottlenecks have been identified at the biocatalyst level and many efforts to avoid them will be summarized in the next section (Fig. 2).

3. Genetic and metabolic engineering for BDS in Gram-negative bacteria

Since the first recombinant desulfurizer biocatalyst was successfully developed in *R. erythropolis* (Denome et al., 1993), genetic manipulation of the 4S pathway from *Rhodococcus* and other Gram-positive bacteria has been widely used to try to achieve higher desulfurization rates in many recombinant bacteria. Gram-negative bacteria of easy genetic manipulation and endowed with relevant properties of environmental and industrial interest have been commonly used as ideal hosts to express the *dsz* genes from Gram-positive desulfurizers either in multicopy plasmids or stably inserted into the host chromosome (Gallardo et al., 1997; Reichmuth et al., 2000; Watanabe et al., 2002; Noda et al., 2003.; Meesala et al., 2008; Aliebrahimi et al., 2015). The three-genes operon *bdsABC* from *Gordonia terrae* C-6 involved in the desulfurization of benzothiophene, through a pathway similar to the 4S pathway, was also successfully expressed in *Escherichia coli* and it allowed the conversion of benzothiophene into sulfite and *o*-hydroxystyrene in this Gram-negative bacterium (Wang et al., 2013). In this section, the most relevant advances for genetic engineering of the 4S pathway and for metabolic engineering of Gram-negative host cells will be presented.

3.1. Engineering the 4S pathway

Although there is no indication that the P_{dsz} promoter driving expression of the *dszABC* operon from *R. erythropolis* IGTS8 is inducible, it is strongly repressed by

sulfate and sulfur-containing amino acids, which constitutes an important bottleneck when designing efficient DBT desulfurizer biocatalysts. To alleviate this constraint, the *dszABC* operon was engineered under control of heterologous broad-host-range regulatory signals ($lacI^q/P_{trc}$), and stably inserted into the chromosomes of different *Pseudomonas* strains that were able to efficiently desulfurize DBT even in culture media containing sulfur (Gallardo et al., 1997).

The inhibition caused by the intermediates of the 4S pathway is also an important bottleneck in BDS, and HBPS and 2HBP are responsible of the major inhibitory effects on the Dsz enzymes and cell growth (Abin-Fuentes et al., 2013). Engineering a biocatalyst that can convert 2HBP into a less toxic compound may alleviate the toxicity and the inhibition caused by this metabolite. The extended 4S pathway able to convert 2HBP into 2-methoxybiphenyl in some Gram-negative bacteria, e.g., *Achromobacter* and *Chelatococcus* strains (Bordoloi et al., 2014, 2016), provides a possible solution to overcome the feed-back inhibitory effect of 2HBP on the Dsz enzymes (Xu et al., 2006; Akhtar et al., 2009; Abin-Fuentes et al., 2013). A different strategy to overcome the toxicity of 2HBP on the initial DszC monooxygenase was carried out by synthetic biology approaches. Since the 4S pathway consists of three serial and irreversible modules, it was possible to develop a collection of synthetic *dsz* cassettes formed by different combinations of the *dsz* genes that were functional both alone and by combination in different *P. putida* biocatalysts (Martínez et al., 2016a). Compartmentalization of the functional units of the 4S pathway into different hosts allows for their individual optimization and demonstrates that through division of labor synthetic consortia can overcome process limitations, e.g. 2HBP feedback inhibitions, difficult to achieve when using monocultures. Thus, the generation of an artificial 4S pathway by combining the *dszC1-D1* and the *dszB1A1-D1* cassettes in an optimized synthetic bacterial consortium, and the *dszB1* cassette as a cell-free extract, is a promising alternative to the use of naturally existing or recombinant *dsz* pathways for enhanced conversion of DBT into 2HBP (Martínez et al., 2016a). On the other hand, the combination of the *dszC1-D1* and the *dszA1-D1* cassettes in the absence of the DszB activity allows the accumulation of the valuable product HBPS, an intermediate used for the synthesis of surfactants (Martínez et al., 2016a).

The concentration of DszB desulfinase in the cytoplasm is several-fold lower than those of DszA and DszC monooxygenases, becoming the rate-limiting step in the whole process (Gray et al., 1996). The overexpression of the *dszB* gene and the

optimization of its ribosome binding site in *E. coli* can overcome the rate-limiting step caused by the low DszB activity (Reichmuth et al., 2004). The broad substrate range of the Dsz enzymes is one of the driving forces for the development of BDS as a commercial process. Gain-of-function mutations and protein engineering of the Dsz enzymes from *R. erythropolis* have been shown to be an efficient way to improve biodesulfurization (Gray et al., 2003), and these modified enzymes can be then expressed in Gram-negative bacteria to engineer more efficient biocatalysts.

The design of recombinant biocatalysts harboring such *dszABC* operon from *R. erythropolis* IGTS8 requires also the synthesis of precise amounts of the DszD flavin-reductase to achieve an efficient desulfurization activity. Due to the ability of some flavin-reductases to efficiently couple with the DBT monooxygenases, heterologous reductases from non-DBT-desulfurizing organisms were used to enhance the activity of DszA/DszC enzymes. Thus, a recombinant *P. putida* KTH2 strain was constructed containing the *hpaC* gene, that encodes the flavin-reductase component of the 4-hydroxyphenylacetate monooxygenase from *E. coli* W, stably inserted into the chromosome, and the *dszABC* genes from strain IGTS8 cloned into a broad-host-range pESOX3 plasmid. The resulting *P. putida* KTH2 (pESOX3) strain, named as *P. putida* CECT5279, showed higher DBT desulfurization activity than the same strain harboring the pESOX3 plasmid but lacking the *hpaC* gene (Galán et al., 2000). In another example, a flavin-reductase from *Vibrio harveyi* was overexpressed in *E. coli* cells harboring the *dszABC* genes and an increase in the rate of DBT removal was achieved, although a reduction in the rate of 2HBP production was observed (Reichmuth et al., 2000).

Due to the fact that the petroleum refining and HDS require high temperatures, the use of thermophilic bacterial strains and enzymes that may avoid the use of cooling steps is a very interesting approach in BDS. Although different thermophilic Gram-positive bacteria have been shown to desulfurize DBT, *Klebsiella* sp. 13T is the only Gram-negative bacterium so far described able to carry out the thermophilic desulfurization of DBT (Bhatia and Sharma, 2012). Nevertheless, genetic engineering approaches allowed the cloning and expression of the *dsz* genes from Gram-positive mesophilic bacteria in well-characterized Gram-negative thermophilic hosts, such as *T. thermophilus*. Thus, the *dszC* gene from *R. erythropolis* IGTS8 was successfully expressed in *T. thermophilus*, although the thermolability of the other Dsz enzymes prevented the functional expression of the full desulfurization pathway in this host

(Kilbane, 2006). Nevertheless, since *T. thermophilus* is genetically amenable and grows at temperatures ranging from 55 to 85 ºC, the *dsz*-containing recombinant strains become nice tools to use directed evolution and selective pressure to gradually evolve desulfurization enzymes able of functioning at higher temperatures (Kilbane, 2006).

3.2. Engineering the host cell

The contribution of the host cell harboring the 4S pathway is another major key factor to take into account for developing an efficient BDS. Thus, the expression of the *dsz* genes in more advanced heterologous hosts could be a successful strategy to increase the BDS activity in comparison with natural DBT-desulfurizing strains. In this respect, Gram-negative bacteria offer some advantages/alternatives to the native Gram-positive hosts.

The bacterial uptake of DBT from the oil phase to the cytoplasm is an important bottleneck in BDS. The use of transport systems that facilitate the uptake of low-solubility substrates from the oil to the biocatalyst could enhance BDS. Thus, a transport system for hydrophobic compounds of *P. aeruginosa* NCIMB9571, encoded by the *hcuABC* cluster, was cloned in a recombinant *P. putida* IFO13696 strain carrying the *dszABCD* genes. The resulting strain was able to desulfurize DBT dissolved in *n*-tetradecane, more likely due to the increased uptake of DBT from the oil phase (Noda et al., 2003). Recently, it was shown that HBPS, the substrate of DszB, can be secreted out of the cell to the medium and is unable to enter again into the cell. This observation reinforces the critical role of DszB in BDS and stresses the need of searching for HBPS transporters that may facilitate the uptake of the secreted intermediate and, thus, enhance the BDS efficiency (Martínez et al., 2016a). A proteomic study with *Chelatococcus* cells grown in DBT revealed the up-regulation of enolase and MFS transporters, that may play an important role in transporting DBT and its metabolites across the cell membrane, as well as the ATP synthase enzyme that provides the required energy supply for the transporters. Several stress proteins and chaperones were also up-regulated, confirming their role in DBT desulfurization and adaptation of bacteria to S-containing aromatic compounds (Bordoloi et al., 2016). The beneficial role of biosurfactants in the acquisition of hydrophobic compounds from the crude oil has been also utilized to engineer rhamnolipid-producing recombinant *Pseudomonas* strains that express the *dsz* genes (Gallardo et al., 1997; Raheb and Hajipour, 2011). The combination of a biocatalytic oxidation of organosulfides and thiophenes with

hemoproteins, e.g., fungal peroxidases, to form more soluble and bioavailable sulfoxides and sulfones, has been also proposed as an alternative treatment to enhance BDS (Ayala et al., 1998). Nanobiocatalytic approaches offer another alternative to improve the transfer rate of sulfur compounds from diesel oil to the microbial cells. Al_2O_3 nanoparticles were assembled onto *Acidovorax delafieldii* (former *P. delafieldii*) R-8 biodesulfurizing cells to selectively adsorb DBT from the organic phase resulting in an increase in BDS rate (Guobin et al., 2005).

Solvent-tolerant bacteria, mainly *Pseudomonas* strains, can be promising hosts for BDS and several examples have been reported in the literature. A solvent-tolerant DBT-desulfurizing strain, *P. putida* A4, was generated by cloning the *dszABC* operon from *R. erythropolis* XP into the solvent-resistant *P. putida* Idaho strain (Tao et al., 2006). The combination of a solvent resistant bacterial host, e.g., the *P. putida* S12 strain, with an organic solvent-responsive expression system that drives the transcription of the *dsz* genes, resulted in a highly efficient DBT removal in a biphasic system with *n*-hexane (Tao et al., 2011). On the other hand, *P. azelaica* HBP1 and related strains, which can use 2HBP as carbon and energy source, express a MexAB-OprM efflux system that confers tolerance to high concentrations of 2HBP, which makes these strains promising hosts for heterologous expression of *dsz* genes and the development of biocatalysts more resistant to the toxic effect of 2HBP (Czechowska et al., 2013; García et al., 2014; El-Said Mohamed et al., 2015).

In silico studies using genome-scale metabolic models show that the cellular metabolism of the sulfite released in the 4S pathway is another critical factor that may affect BDS efficiency (Aggarwal et al., 2011; Aggarwal et al., 2013). *Chelatococcus* cells grown in the presence of DBT revealed the up-regulation of the cysteine synthase enzyme, suggesting that the synthesis of the sulfur-containing amino acid cysteine is a predominant mechanism of sulfur assimilation in this bacterium. Moreover, the up-regulation of the thioredoxin enzyme also suggests the involvement of this protein in sulfur assimilation and as a target for improving desulfurization (Bordoloi et al., 2016). The use of high quality genome scale flux-based metabolic models in recombinant biodesulfurizers, e.g. *P. putida* derived strains (Nogales et al., 2008), may reveal unnoticed bottlenecks that should be addressed in the future to further improve the BDS process.

4. Process engineering for BDS with Gram-negative bacteria

We have presented before the main strategies to design more efficient biocatalysts; in this section, the main advances in process engineering for the development of an industrial BDS process are reviewed.

4.1. Process development

A comparison of different BDS efficiencies using Gram-negative bacteria as biocatalysts is given in Table 1. It becomes clear that although the use of growing cells can be an easy way to check the ability of different microorganisms to carry out BDS, the use of resting cells yields better BDS efficiencies and a higher DBT concentration can be desulfurized in comparison with growing cells conditions.

The optimizations of the medium composition and the operational conditions in aqueous phase are preliminary studies prior to approach desulfurization in biphasic systems, which is the final aim of the BDS process (del Olmo et al., 2005a and b; Deriase et al., 2012; Derikvand et al., 2015). Using Response Surface Design (RSD) to optimize pH, temperature and rotary shaker speed, *Stenotrophomonas maltophilia* sp. Kho1 growing cells led a BDS yield of 6.3% after 96 h (Table 1) (Ardakani et al., 2010).

After optimization of the medium composition for recombinant *P. putida* CECT5279 cells (Martín et al., 2004), the study of the operational conditions revealed a different trend in growth rate and desulfurization activity. Thus, increasing temperature and the oxygen transfer rate favored growth but decreased the desulfurization capacity of the cells (Martín et al., 2005). Remarkably, it was shown that the activity of Dsz enzymes depends significantly on the physiological state of the cells. Thus, whereas both DBT monooxygenases (DszC and DszA) present maximal activity in cells collected at around 23h of growth time, the highest activity of the DszB desulfinase was found in cells collected at only 5h of growth time; longer incubation times led to a significant decrease in DszB activity (Calzada et al., 2009a). As a consequence, mixtures of cells of *P. putida* CECT5279 collected at different growth ages, corresponding to those at which the DszA/DszC monooxygenases and DszB desulfinase reached the maximum activity, were shown to be 2.5-fold more efficient than cells collected at a single growth age (Calzada et al., 2009b; Calzada et al., 2011). In addition, the use of some short-chain organic acids, e.g., acetate, as co-substrate in the desulfurization medium may provide higher intracellular concentration of reducing

equivalents, hence leading to a further increase in the yield of the BDS process (Martínez et al., 2015).

Due to the organic nature of the fuels, an industrial BDS process must be carried out in the presence of an organic phase. Simulated oils are usually employed in order to simplify the BDS studies, using long chain alkanes, such as *n*-tetradecane or *n*-hexadecane, which are representative of hydrocarbons contained in fuels. In these biphasic systems the mass transfer rate of compounds from organic to aqueous phase and *vice versa* becomes a new limiting step of the BDS process (Boltes et al., 2013). Some approaches to increase mass transfer rates in BDS have been accomplished. Thus, the use of surfactants reduces the viscosity of the oil and increases the mass transfer of sulfur compounds from the fuel to the aqueous phase. In this sense, the use of Tween 20 or Triton X-100 was found to be essential for the sulfur removal in bunker oil (Li and Jiang, 2013). Other surfactants, such as Tween 80, have also been satisfactorily used in BDS (Feng et al., 2006). Similar effects can be achieved using β-cyclodextrins (Caro et al., 2007a).

Desulfurizing bacterial cells in stirred oil-water systems are distributed in different populations: single free cells in aqueous phase, aggregate cells in the aqueous phase, cells adhering to the oil droplet, cells trapped in the water droplets within the oil phase, and cells in the emulsion phase. The fraction of each of these bacterial populations and the mechanistic steps of their involvement in the BDS process, as well as the kinetics of mass transfer of the substrate and product across the different phases, present a big challenge in order to optimize the operation conditions of the BDS process. Some examples of BDS in biphasic oil-water media are shown in Table 1, and it can be noticed the variability of the results presented. In desulfurizing *P. putida* CECT5279 resting cells the BDS capacity increased with the increase of cell concentration but it decreased when oil/water ratio increased (Caro et al., 2007b; Boltes et al., 2013). These studies also highlight the major influence of the biocatalyst nature when BDS takes place in biphasic media. Thus, unlike the hydrophobic *R. erythropolis* cells, *Pseudomonas* cells cannot uptake DBT directly from the organic solvent without using co-solvents (Caro et al., 2007b). Growing cells of *Pantoea agglomerans* D23W3 were able to remove from 26.38 to 71.42 % of sulfur present in different petroleum oils in 5 days, showing a strong influence of the fuel source in the final sulfur removal rate (Bhatia and Sharma 2010b). The use of lyophilized cells was studied in *A. delafieldii* R-8 in a model oil (*n*-dodecane containing DBT) (Luo et al., 2003). Desulfurization was

strongly dependent on the oil-to-water ratio, cell concentration and DBT concentration, which support the mass-transfer limitation reported in other works (Caro et al., 2007b).

The separation of the mixture microorganisms-oil-water is also another bottleneck since an economical satisfactory separation procedure has not been implemented yet. Immobilization of *Sphingomonas subarctica* T7b by entrapment with polyvinyl alcohol (PVA) increased the desulfurization activity and cells were reused for more than 8 cycles without losing activity (Gunam et al., 2013). The separation for further reuse of the resting cells can be simplified using *in situ* magnetic separation of cells immobilized in superparamagnetic Fe_3O_4 nanoparticles (Li et al., 2009).

4.2. Kinetic modelling of the BDS process

When the BDS is carried out by growing cells, cell growth is directly associated with 2HBP/sulfate production, and therefore, classic models of substrate consumption or product generation can be used to describe the evolution of the process (Liu et al., 2015). A kinetic model able to describe the evolution of bacterial growth and desulfurizing capacity under several conditions, such as different medium compositions, temperature and pH values, was developed for *P. putida* CECT5279 (Martín et al., 2005). The desulfurization during growth of *Enterobacter* sp. D4 was modeled taking into account the 2HBP inhibition and a dynamic model was used to describe DBT consumption and 2HBP formation (Liu et al., 2015).

Regarding the kinetics of the 4S pathway, most of the studies have described only the evolution of some of the intermediate compounds with time, or the kinetic equation of some of the reactions of the 4S pathway, mainly the first one. Often, Michaelis-Menten kinetic is proposed to describe the first reaction of the 4S pathway (oxidation of DBT and its derivatives) (Kobayashi et al., 2001; Luo et al., 2003; Alcón et al., 2005; Zhang et al., 2013). When a mixture of sulfur compounds is used as substrate, a decrease in the DBT removal rate is observed. Some studies suggested that this could be due to a competitive inhibition (Kobayashi et al., 2001; Zhang et al., 2013) or to a product inhibition (Kobayashi et al., 2001). However, a complete kinetic model should contain all differential equations that describe the rate of appearance and disappearance of all the compounds involved in the 4S pathway, not only DBT removal (Gray et al., 1996; Santos et al., 2007). A complete kinetic model, including all the reactions involved in the 4S pathway, has been developed over years for *P. putida* CECT5279. Initially, the kinetic of the three first reactions was described by simple

Michaelis-Menten equations, while the last reaction took into consideration the inhibition by the final product (2HBP) (Alcón et al., 2008). This model is useful for short reaction times or for description of a fed-batch operation, because as reaction time increases, all the reaction rates decrease. To solve this problem, it is possible to use an empirical enzymatic deactivation (Calzada et al., 2012) or to reformulate the model, including cofactor concentrations, where both batch and fed-batch operations were adequately described (Martínez et al., 2017) .

4.3. Scale-up of the process and reactor engineering

To scale-up BDS it is necessary to apply all the knowledge achieved by flask-scale work to the bioreactor scale. Although the reaction rates must be independent of the reactor size and geometry, and even of the operation type, the overall process rate can be strongly influenced by transport phenomena. Most of the studies carried out at bioreactor-scale did not take into account the selection of the operational conditions (Kobayashi et al., 2001; Guobin et al., 2006; Yang et al., 2007; Lin et al., 2012).

The effect of oxygen mass transfer is recognized as a key aspect to develop the BDS process (Martín et al., 2005; García-Ochoa and Gómez, 2009; García-Ochoa et al., 2010). The influence of the oxygen mass transfer in the BDS capacity of *P. putida* CECT5279 showed that while a high oxygen concentration increased the biomass growth rate, the BDS capability decreased (Gómez et al., 2006). The use of a ceramic micro-sparging aeration system (adapted from animal cell culture processes) can enhance gas-liquid mass transfer and increase 2-fold the activity of the biocatalyst when comparing to a traditional bubble aeration process (Lin et al., 2012). In this way, stirred tank bioreactors (Matínez et al., 2016b) and airlifts bioreactors (Lee et al., 2005; Boltes et al., 2008; Zhang et al., 2013) are the two configurations most studied. The use of scale-up criteria, such as maintaining oxygen transfer rate at a constant value, has also been demonstrated as a correct strategy (Boltes et al., 2013; Martínez et al., 2016b). Other bioreactor configurations have been used satisfactorily in BDS. A derivative of traditional Microbial Fuel Cell, named Microbial Electroremediating Cell (MERC), was used to enhance more than 3-fold the removal of DBT by native electrogenic microbes (Rodrigo et al., 2014), although the 2HBP production is not described. Continuous processes present some advantages over batch processes, including the reduction of costs and a better process control. Trying to approach industrial scale conditions, some work has been carried out using continuous processes with Gram-positive biocatalysts

(Yang et al., 2007), but no work with Gram-negative bacteria has been reported until now.

5. On the way to an industrial BDS process and to alternative applications

In spite of all the advances obtained in the knowledge of the 4S pathway, it is clear that a more detailed information, including all the stages of the bioprocess development, is required to develop an industrial BDS process. Modern biotechnology provides new tools that can be applied to the BDS process. Genome sequencing, high-throughput omic techniques and systems biology and synthetic biology approaches are some of the technologies that will pave the way for the rational design of optimized metabolic pathways and bacterial hosts. Some authors suggest that the currently available biocatalysts require an activity increase in their BDS rate of about 500-fold for developing a commercially viable process (Soleimani et al., 2007; Xu et al., 2009). Protein engineering to design Dsz enzymes with higher catalytic efficiency, broader substrate range, and not susceptible to the inhibition caused by 4S intermediates, is a future challenge. Genome mining and metagenomics will facilitate the search for novel pathways/genes for biodegradation/biotransformation of S-containing compounds. From the host cell perspective, the design of engineered cells with higher thermal and solvent tolerance, increased substrate uptake rates, and longer duration life in bioreactors are also some key points to be addressed. Moreover, a major limiting factor that should be alleviated in biphasic processes is the mass transfer rate (Boltes et al., 2013; García-Ochoa and Gómez, 2009). From the point of view of the process development, current processes require two separation steps including biocatalyst separation and oil/water emulsion separation, which extremely increase the operational costs. A straightforward approach is the use of membranes as physical barriers between oil and aqueous phases, through which DBT could be transported from the oil and 2HBP could be returned from the aqueous phase. This system has been successfully used for biotransformations of hydrophobic compounds but its use in BDS is still unexplored. The use of immobilized cells directly in the organic phase, and the formation of biofilms of catalytic bacteria, are alternative strategies under development (Mohebali and Ball, 2016). So far, biocatalysts used are planktonic cells, usually very sensitive to the stressful conditions prevalent in bioreactors that are dispersed in large volumes of reaction medium. However, biofilms of catalytic bacteria may be advantageous over suspended cells not only in that the physical proximity limits the unwanted diffusion of intermediates but

also in that they exhibit a superior tolerance to physicochemical insults and harsh reaction conditions and they allow the operation of catalysts in packed column reactors. Recently, it was shown that the physical form and the morphology of the active biomass can be genetically programmed *in vivo* by implanting a synthetic device on an endogenous regulatory network that controls the planktonic versus biofilm lifestyle in *P. putida*, thus boosting biodegradation of the target substrate (Benedetti et al., 2016).

Energy BioSystems Corporation was the only company that implemented the BDS process at industrial scale but the project ended more than twenty years ago (Kilbane , 2006). Since then, no serious attempts have been done in this direction. In the scientific literature, as previously commented, only a few works go in depth in the scale-up of the process, and definitely, more efforts are needed in this direction.

Even though there are currently few to none industrially established desulfurization biocatalysts, the production of higher value products derived from the organosulfur material present in a cheap feedstock such as petroleum could be an economically viable alternative bioprocess. As a consequence of the broad substrate range of the 4S pathway enzymes, they can be used to convert sulfides, disulfides, mercaptans, sulfoxides, sulfones, sulfinates, sulfonates, thiophenes, and benzothiophenes present in oil to wide range of chemicals (Kilbane and Stark, 2016; Kilbane, 2017). Recombinant biocatalysts that express the DszC and DszA monooxygenases but lack the DszB desulfinase have been constructed to efficiently convert DBT into sulfonate compounds that can be useful for the production of surfactants (Martínez et al., 2016a). On the other hand, efficient biocatalysts containing the complete 4S desulfurization pathway convert DBT into 2HBP that can be used as precursor of other phenolic compounds for the synthesis of polymers, or as fungicides, disinfectants, preservatives, dyes or antioxidants, and that can be reacted to add nitrate, halogen, alkyl or acyl groups to create a wide range of chemical derivatives (Kilbane and Stark, 2016). Similarly, the phenyl styrene compounds derived from benzothiophene-like precursors could be polymerized to yield plastic material (Kilbane and Stark, 2016; Kilbane, 2017). Desulfurization enzymes could potentially also be used to modify organosulfur compounds to make novel monomers that can be polymerized to produce polythioesters employed as thermoplastics and biodegradable polymer films (Khairy et al, 2015). Some DBT monooxygenases are also able to oxidize indole to the blue pigment indigo in the presence of a heterologous flavin reductase, and this feature can be used as a selective indication that may be widely applicable for the

screening of novel genes encoding either a flavin reductase or a flavin-dependent monooxygenase from two-component monooxygenases (Furuya et al., 2004). Flavin reductases may be beneficial in many biotechnological applications as enzymes coupling to monooxygenases or, in some cases, as nitroreductases (Takahashi et al., 2009).

The development of desulfurization-competent hosts, such as those adapted to express a sulfur-rich polypeptide (Pan et al., 2013; Wang et al., 2017), represents a new tool for studying the expression, protein folding, disulfide bond formation, and protein secretion of high-sulfur-content proteins. Polypeptides that contain a high concentration of cysteine/methionine include some of the most potent venoms and some therapeutic compounds such as bacteriocins, defensins, and various synthetic peptides with anti-microbial and anti-tumoral properties (Kilbane and Stark, 2016). Several antibiotics, e.g., penicillin, bacitracin, cephalothin, cephalexin and sulfanilamide, contain carbon-sulfur bonds, thus biodesulfurization enzymes could be helpful to design new molecules useful to treat bacterial pathogens resistant to current antibiotics (Kilbane and Stark, 2016; Kilbane, 2017). Biodesulfurization can be also applied to detoxification of some chemical warfare agents, e.g., mustard gas, by cleaving their carbon-sulfur bonds (Kilbane and Jackowski, 1996).

All these new applications of the desulfurization biocatalysts for oil valorization have barely begun to be explored and they should be subject of future research and process development.

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Figures

Fig. 1. Main pathways involved in the bacterial metabolism of DBT.
Desulfurizing pathways are those that release sulfur (in red) from the DBT molecule via: i) aerobic cleavage of the C-S bond by the 4S pathway (blue arrows) and two extended 4S pathways, E1 and E2 (purple arrows); ii) aerobic cleavage of the C-C and C-S bonds by a destructive pathway (grey arrows); and iii) anaerobic cleavage of the C-S bond (green arrows). Non-desulfurizing pathways, which metabolize the molecule of DBT but do not release the sulfur, include: i) DBT oxidation to DBT-sulfone (orange arrows), and ii) oxidative cleavage of C-C bonds by the Kodama pathway (red arrows). The metabolites of the Kodama pathway are: I, cis-1,2-dihydroxy-1,2-dihydro DBT; II, 1,2-dihydroxyDBT; III; cis-4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; IV, 3-hydroxy-2-formyl-benzothiophene.

Fig. 2. Main bottlenecks for DBT biodesulfurization in native biocatalysts and genetic engineering approaches proposed to overcome them in an optimized bacterial chassis. The bottlenecks are indicated with red stars, and the corresponding solutions with blue stars. Bottlenecks are: A, expression of the *dsz* operon; B, activity of the Dsz enzymes; C, retro-inhibition of the Dsz pathway; D, availability of reducing power; E, up-take/secretion of 4S pathway substrate and intermediates; F, other host-specific limitations. Solutions proposed are: A, use of heterologous promoters driving the expression of *dsz* genes, synthetic *dsz* operons with optimized transcription and translation signals, increased gene dosage by expression in multicopy plasmids. B, protein engineering to design enzymes with higher catalytic efficiency, broader susbstrate range, thermo-stability, etc. C, further metabolism of the final products 2HBP and sulfite, engineering synthetic microbial consortia that express individualized Dsz enzymes, disruption of *dszB* to avoid 2HBP formation. D, optimizing metabolic fluxes by using genome-scale metabolic reconstructions and synthetic biology approaches. E, membrane permeabilization, engineering hydrophobic compounds transport systems. F, use of bacterial hosts endowed with properties of biotechnological interest, i.e., solvent resistant, metal resistant, biosurfactants producers, thermophiles, etc.

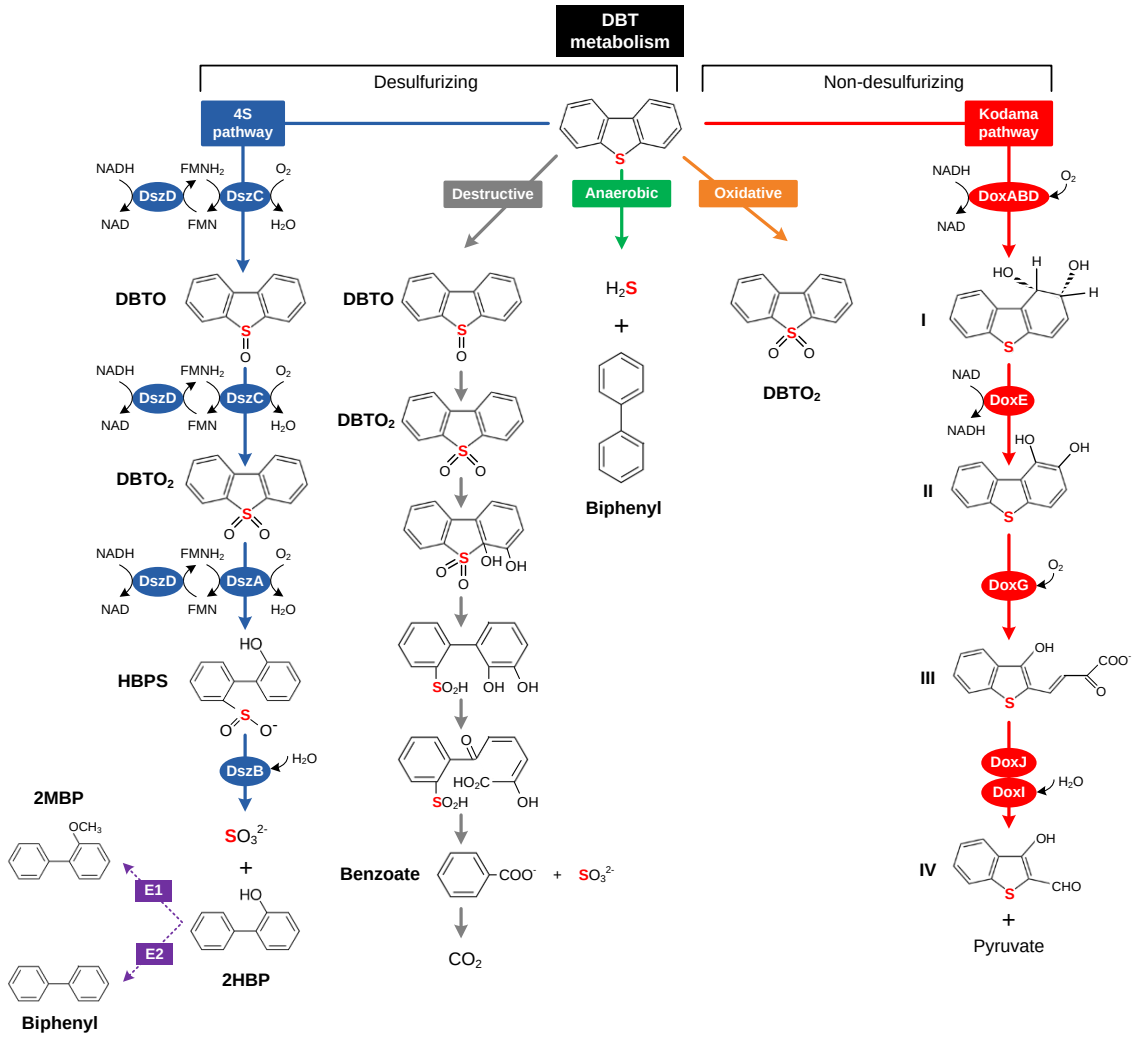

Fig. 1.

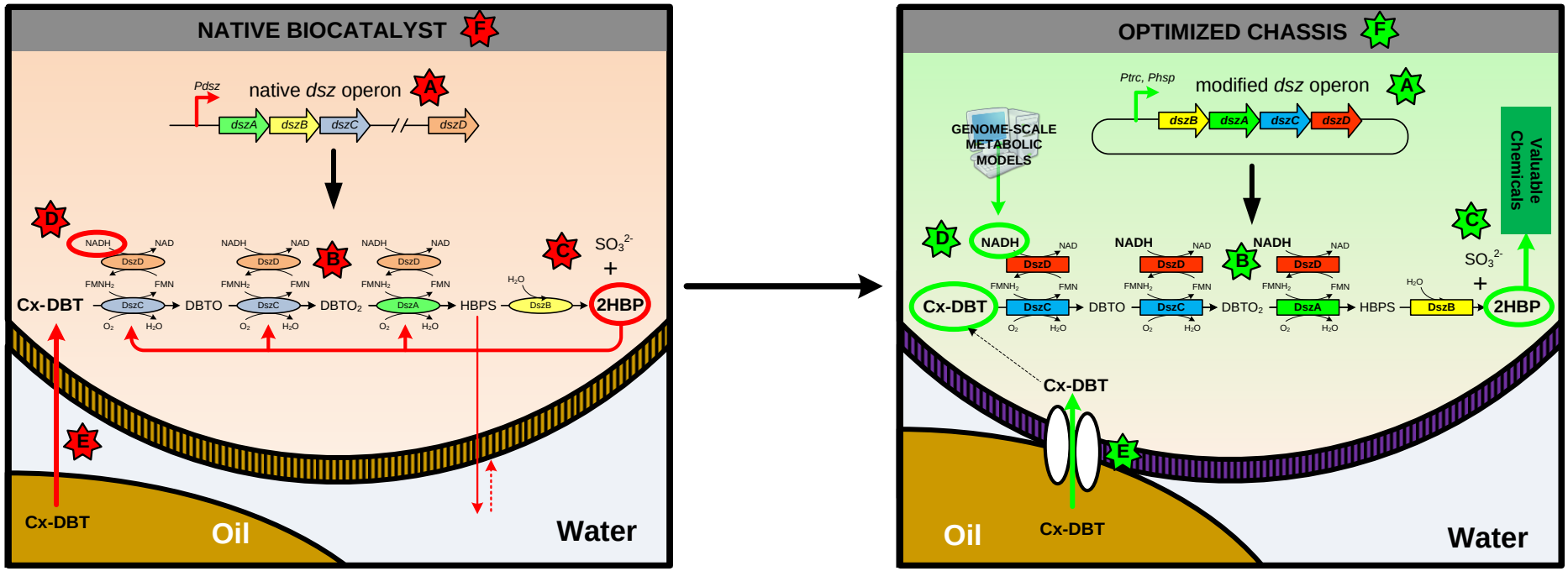

Fig. 2.

Table 1. Some Gram-negative bacteria used in biodesulfurization and reaction conditions.

-, means that it was not possible to determine the value.

* GMO, means genetically modified organism

** Percentage of DBT removal, $X_{BDS}^{DBT} = \left(\frac{C_{DBT}^{0} - C_{DBT}^{final}}{C_{DBT}^{0}} \right) \times 100$, and percentage of BDS, $X_{BDS}^{2HBP} = \left(\frac{C_{2HBP}^{final}}{C_{DBT}^{0}} \right) \times 100$

*** Specific conversion rate, $E = \frac{C_{2HBP}^{final}}{t_{BDS} \cdot C_X}$, was only determined for resting cells reactions.

Strain	**GMO***	**Biocatalyst state**	**Medium state**	C_{DBT}^{0} **(mM)**	X_{BDS}^{DBT} **(%)**		X_{BDS}^{2HBP} **	**Reaction time (h)**	E_{BDS}*** **(mmol$_{2HBP}$ /h·g$_{DCW}$)**	**Reference**
Escherichia coli DH10B (pDSR2, pDSR3)	Yes	Resting cells	Aqueous	0.4	100	|	50	45	0.005	Reichmuth et al., 2000
		Growing cells	Aqueous	0.15	87	|	33	45	-	
Acidovorax delafieldii R-8	No	Resting cells	Biphasic	1	100	|	100	10	0.005	Luo et al., 2003
Pseudomonas putida MTCC1194 (pSAD225-32)	Yes	Growing cells	Aqueous	1.2	71	|	7.5	67	-	Meesala et al., 2008
Pseudomonas putida CECT5279	Yes	Resting cells	Aqueous	0.025	100	|	100	0.5	0.012	Calzada et al., 2009b
			Biphasic	0.27	50	|	40	8	0.001	Boltes et al., 2013
Pantoea agglomerans D23W3	No	Growing cells	Biphasic	0.55	100	|	-	24	-	Bhatia and Sharma 2010b
Stenotrophomonas maltophilia Kho1	No	Growing cells	Aqueous	1	-	|	6.3	96	-	Ardakani et al., 2010
Stenotrophomonas sp. NISOC-04	No	Growing cells	Aqueous	0.8	100	|	-	240	-	Papizadeh et al., 2011
Klebsiella sp. 13T	No	Growing cells	Aqueous	0.55	97	|	-	120	-	Bhatia and Sharma 2012

Sphingomonas subarctica 17b	No	Resting cells	Biphasic	1	100	|	100	10	0.005	Gunam et al., 2013
Enterobacter sp. D4	No	Growing cells	Aqueous	0.1	63	|	47	72	-	Liu et al., 2015
Pseudomonas putida KT2440 (pIZdszB1A1C1-D1)	Yes	Resting cells	Aqueous	0.025	100	|	100	1	0.021	Martinez et al., 2016a
Enterobacter sp. NISOC-03	No	Growing cells	Biphasic	0.8	64	|	34	96	-	Papizadeh et al., 2017
Agrobacterium tumefaciens LSU20	No	Growing cells	Biphasic	1.1	77	|	-	96	-	Gunam et al., 2017
Ralstonia eutropha PTCC165	No	Resting cells	Aqueous	0.05	-	|	90	3	0.017	Dejaloud et al., 2017
		Growing cells	Aqueous	0.05	-	|	90	24	-	