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Metabolic and process engineering for biodesulfurization in Gram-negative bacteria

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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.

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,

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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; Mohebali 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; Mohebali 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*-