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REVIEW

Biodesulfurization: a mini review about the immediate search for the future technology

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Abstract A major concern among the environmental agencies includes the emission of sulfurous gas into the environment. Consequently, the oil agencies are in constant search of alternative processes aiming the reduction of sulfur content in fuels. One of the technologies commonly used is the hydrodesulfurization (HDS), but this is a high-cost process that also requires high temperature and pressure. A complementary alternative to HDS is biodesulfurization (BDS) involving the use of specific microorganisms to the removal of sulfur present in the carbon chain, using the oxidation pathway "4S", in which there is cleavage of carbon–sulfur bond, and maintaining the calorific value of the organic molecule. The BDS is a low-cost technique when compared with HDS. For this process to occur, activation of specific enzymes is needed, which is controlled by *dsz*ABC genes. Therefore, strategies to optimize this process have been of great importance to the oil refineries. For decades, attempts to try to implement BDS in the industry have been made, but difficulties in obtaining satisfactory results led the researchers to seek new knowledge about this bioprocess. The need of more studies concerning implementation on an industrial scale of this process is evident, since this biotechnology is a promising alternative to refineries in the near future.

Keywords Biodesulfurization · Biotechnology · Environmental · Sulfur · *Rhodococcus erythropolis* IGTS8

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Introduction

The process of oil combustion causes the emission of certain hazardous pollutants into the atmosphere. Among these pollutants, the sulfur dioxide (SO_2), which causes environmental impacts, such as air pollution, acid rain, as well as problems for human health (Mohebali and Ball 2008; Dube et al. 2014). Aiming to reduce these emissions, the pollutant concentration is controlled during the refining process through physical chemical methods known as hydrodesulfurization (HDS). However, this process is of high cost for the industries (Gupta et al. 2005). An alternative process and also complementary to this technology is the use of microorganisms capable of metabolizing the sulfur residues contained in petroleum hydrocarbon chains by a specific metabolic pathway (Kilbane 2006). This process is called biodesulfurization (BDS), a biotechnology of great interest for many industries and researchers due to its lower costs when compared to HDS. In addition, this process occurs under conditions of atmospheric pressure and environmental temperature (20–25 °C). It also lacks formation of undesirable products or pollutants, such as hydrogen sulfide (H_2S) (EPA/USA 2011). For over a decade, several reviews on this subject have been published

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(Kropp and Fedorak 1998; Ohshiro and Izumi 1999; Monticello 2000; Babich and Moulijin 2003; Soleimani et al. 2007; Nuhu 2012). As a result, this review aims at a brief overview on the process of BDS from the biochemical, genetics, and molecular enzyme views, also discussing about the extensive research on this subject and little success from the point of view of industrial applicability.

Sulfur levels required by legistalation

The sulfur contained in oil may range from 0.03 to 7.89 % (w/w) (Kilbane and Le Borgne 2004). Sulfur atom preferably associates with components of higher molecular weight in crude oil (Amin 2011, Muzic et al. 2012). When crude oil is refined, the sulfur concentrates among fractions of high molecular weight (Swaty 2005). Many governmental agencies have recognized that gases such as H_2S and SO_2, are hazardous to human health and as a result, it is desirable to reduce sulfur emissions which are required by law (EPA/USA 2011). Increasingly stringent worldwide environmental standards have demanded the production of vehicles and fuels that meet these legislative requirements from car manufacturers and refineries. The simplest and most effective way to decrease the emission of SO_2 is to limit the amount of sulfur in the fuel (Davoodi-Dehaghani et al. 2010). In 2003, the Environmental Protection Agency (EPA) of the United States of America (USA) determined that the sulfur in diesel fuel should be reduced from 3400 to 500 ppm by the year 2007 (Song 2003). Moreover, the European Union stated that the concentration of sulfur contained in diesel should be reduced to 50 ppm by 2005 and 10 ppm by the year 2009 (Marcelis 2002). The maximum allowable sulfur content in diesel in USA is currently 15 ppm, with the compromise of decreasing this level to 10 ppm by 2014 (Song 2003).

The sulfur content in diesel fuel allowed in Japan was drastically reduced from 2,000 ppm in 1992 to 500 ppm in 1997, and 10 times less in 2004, and by 2007 it was reduced to 10 ppm. Moreover, in this eastern country, both fuels like gasoline and diesel have a lower quantity than the 10 ppm sulfur. As per Europe, in 2003, legislation was put in place to reduce the sulfur content from 350 to 50 ppm in 2005 and a subsequent decrease to 10 ppm for later in 2007 (Quian 2008).

Since the year 2012, two distinct qualities of diesel have been available in Brazil: the rural diesel sold in rural areas, and the metropolitan diesel, sold within a ratio of 40 km from big city centers (CONAMA 2012). By the year 2008, diesel sold in rural areas has a concentration of 0.2 % of sulfur, whereas the metropolitan diesel reaches a concentration of 0.05 % of this element (Monticello 1998). Since the aforementioned year, a Brazilian multinational launched a new diesel on the market known as S-50, with a limit of 50 mg kg^{-1} of sulfur. Thus, this new product may be considered as less polluting, also reducing up to 80 % of particulate material emission. According to automotive diesel legislation, S-50 fuel must contain 5 % of biodiesel.

Hydrodesulfurization (HDS) as a physical–chemical

Hydrodesulfurization is a technology used in refineries which reduces the sulfur content (< 15 ppm) in distilled or fossil fuel (Monticello 1998). Removal of organic sulfur from fossil fuels is difficult due to the fact that it may only be separated from organic molecules when the chemical bonds are broken (Soleimani et al. 2007). However, high temperatures and pressures are needed for this chemical rupture to occur, with the conditions being directly linked to the type of hydrocarbon (Erdogan et al. 2014). Depending on the hydrocarborn and the efficiency of the chosen desulfurization process, HDS may occur at 200–425 °C and 150–250 psi (Izumi et al. 1994).

Despite the large amount of simple inorganic and organic sulfur that can be removed by HDS technology, around 70 % sulfur belonging to oil as thiophenes, such as dibenzothiophene and its alkylated derivatives are particularly recalcitrant to HDS (Monticello 2000; Le Borgne and Quintero 2003; Chen et al. 2009).

However, the HDS technology has some disadvantages that limit its use. Among them we can mention that H_2S generated by physical–chemical process is damaging and reduces the useful life of the equipment. Another disadvantage is the need for a large capital investment due to a higher cost of operation (Egorova 2003). Moreover, exposure of crude oil fractions under severe conditions of temperature and pressure, above 360 °C decreases the value of the fuel coming from the treated oil (Monticello 1996). Finally, the HDS atmospheric hydrogen results in a hydrogenation reaction of olefinic compounds, leading to a reduction of the calorific value of the fuel. To make up for loss of calorific value it is necessary that these compounds treated by HDS undergo to a new catalytic fluid, resulting in an increased cost (Hernández-Maldonado and Yang 2004). Considering the negative points of HDS, BDS technology, which has great potential for desulfurize refractory compounds, alike DBT, has a lower cost and is not harmful to the environment and presents more advantages (Bhatia and Sharma 2012).

Microbial activity in BDS

Biodesulfurization is an alternate technology based on biological activity and processes. It is a complementary method used with HDS and highly advantageous for removal of

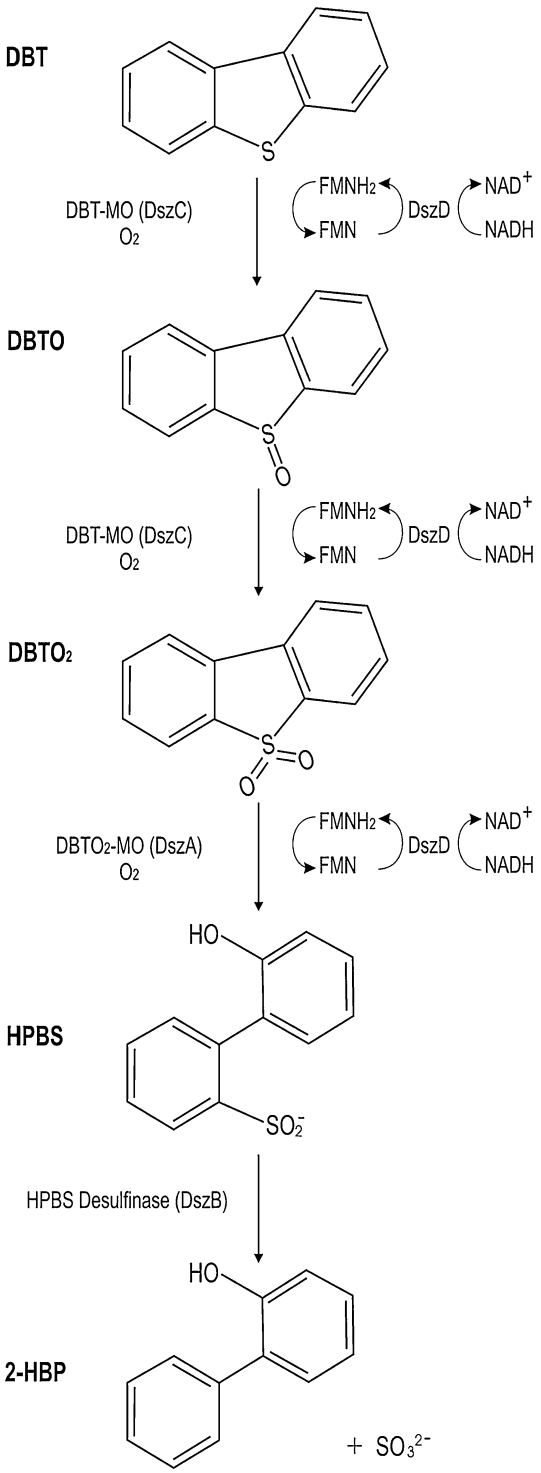

Fig. 1 The "4S" pathway for the biodesulfurization of DBT. *DBT* dibenzothiophene, *DBTO* dibenzothiophene sulfoxide, *DBTO*$_2$ dibenzothiophene sulfone, *HPBS* hydroxyphenylbenzene sulfonate, *2-HBP* 2-hydroxybiphenyl. The enzymes in the "4S" pathway are dibenzothiophene monooxygenase (DBT–MO or DszC), encoded by the *dsz*C gene, dibenzothiophene sulfone monooxygenase (DBTO$_2$–MO or DszA), encoded by the *dsz*A gene, HPBS desulfinase, encoded by the *dsz*B gene, and the NADH/FMN oxidoreductase encoded by the *dsz*D gene

sulfur in fossil fuel (Caro et al. 2007; Muzic et al. 2012). This process allows the removal of the sulfur atom at moderate temperatures and pressures (Monticello 2000). In addition, it may also be considered more advantageous than the HDS, since it retains more energy (Gray et al. 2003).

Biodesulfurization microorganisms require sulfur as a growth factor and as important element for their physiological activities. This component is present in bacterial cells, representing about 0.5–1 % of their dry weight (Guobin et al. 2006). The microorganisms that depend on specific enzymes and metabolic pathways may have the ability to acquire the necessary sulfur in different forms, consuming it and consequently decreasing the fuel acidity (Gupta et al. 2005). Due to the involvement of biocatalystic enzymes in these biological activities, the BDS process may be classified as highly selective and specific, corroborating with the data obtained by (Denome et al. 1994).

Metabolic pathways

Kilbane (1989) proposed oxidative desulfurization, where the sulfur from dibenzothiophene (DBT) could be removed in a specific manner. Through this pathway the carbon skeleton of DBT is released intact resulting in no calorific value loss of the fuel (McFarland 1999; Mohebali and Ball 2008). The pathway was called "4S" in reference to the four intermediates formed (DBT sulfoxide, DBT sulfone, hydroxyphenyl benzene sulfonate, sulfite), and this pathway occurs through successive oxidations of DBT (Kertesz 1999) that is metabolized to 2-HBP (2-hydroxybiphenyl).

The complete removal of sulfur from DBT requires four enzymes (Ohshiro and Izumi 1999). Two of these, DBT monooxygenase (DBT-MO or DszC, encoded by the *dsz*C) and DBT-sulfone monooxygenase (DBTO$_2$-MO or DszA, encoded by the *dsz*A), are flavin-dependents. These both require a third enzyme (the flavin reductase or DszD, encoded by the *dsz*D) for activity. The fourth enzyme, HPBS desulfinase (DszB, encoded by the *dsz*B), completes the reaction sequence, which results in a phenolic product, the 2-hydroxybiphenyl (2-HBP) and SO_3^{2-} (Gray et al. 2003) (Fig. 1). The 2-HBP produced is very soluble in oil and in practice finds its way back to the petroleum fraction, thus conserving the fuel calorific value (Monticello 2000), and sulfite formed can be oxidized to sulfate, and that the sulfur can suffer assimilation by microbial cell or be deposited in the form of aqueous waste (Gupta et al. 2005).

The existing studies have shown that there is no natural specificity concerning the oxidation of the carbon–sulfur bonds. As a result, in 1990 the Institute of Gas Technology (IGT), located in the city of Chicago, USA, has developed new strains through selection of mutagens. The IGT isolated indigenous microorganisms from soil samples

contaminated with hydrocarbons sulfur. The soil samples were placed in a continuous flow bioreactor where it was exposed to mutagenic agents (NTG -1 methyl-3-nitro-nitrosoguanidine (Kilbane and Bielaga 1990).

After the mutagenic action provided by the bioreactor exposure, the remaining species showed a specific capacity of breaking the carbon–sulfur bond and were able to remove 90 % of the organic sulfur contained in that sample (Kilbane and Jackowsky 1992b). This new group of microorganisms was called IGTS7. The naming was given due to the fact that seven different types of colonies were obtained from this process. Among these colonies, two were capable of desulfurizing. They also presented a small population and slow growth. The species were *Rhodococcus rhodochrous* and *Bacillus sphaericus*. Thus, these two new mutant strains were named *R. rhodochrous* IGTS8 and *B. sphaericus* IGTS9 (Ohshiro et al. 1994). In 1997, an analysis of the nucleotide sequence of the 16S rRNA operon revealed that the strain previously named *R. rhodochrous* IGTS8 presented high similarity with *R. erythropolis* IGTS8. Since then, researches on the molecular biology of this strain, as well as other strains involved in BDS processes are being developed with the objective of having a better understanding on the enzymatic machinery and genetics involving specific sulfur removal (Monticello 2000; Denome et al. 1993; Abbad-Andaloussi et al. 2003; Kilbane and Robins 2007).

Activity of the genetic process in BDS

The desulfurization genes and encoded enzymes were initially called as *sox* (sulfur oxidation). Later, the abbreviation *sox* was replaced by *dsz* (desulfurization) (Denome et al. 1994). The *dsz* genes are located in a 4 kb fragment present in the megaplasmid pSOX of 150 kb in *R. erythropolis* IGTS8 (Denis-Larose et al. 1997). This fragment contains three genes organized in a single operon called *dsz*ABC (Alves et al. 2006). Furthermore, this operon undergoes a negative regulation in the presence of sulfur-containing compounds such as sulfate, methionine and cysteine (Alves et al. 2008).

The *dsz* genes and their proteins were compared with other enzymes and genes with sequences deposited in databases such as the GenBank and the Swiss-Prot. The researches showed no significant homology, suggesting that the desulfurization genes *dsz*C, *dsz*A, and *dsz*B are encoded by specific enzymes (Piddington et al. 1995).

Multienzymatic complex DszABC

Studies about this pathway revealed a multienzyme system with three distinct activities. The first three enzymes, DszC, DszA, and DszB are included among the monooxygenases type, capable of catalyzing the transformation of DBT through successive oxidations to DBT-sulfone and then to 2-hydroxybiphenyl 2-sulfonic acid (Gray et al. 1996). The desulfatase (DszB) is responsible for the formation of HPB from HPB-sulfate. The multienzymatic nature of pathway "4S" and the requirements of cofactors NADH and $FMNH_2$ prevents oxidation reactions from occurring when any of the enzymes needed in the process of BDS are missing (Monticello 1998). Another protein involved in the process is DszD which is a flavin mononucleotide (FMN) dependent on reduced nicotinamide adenine dinucleotide (NADH) and is encoded by gene *dsz*D located in chromosomal DNA (Santos et al. 2006). Note that the enzymes involved in this pathway (DszC, DszA and DszB) are encoded by the operon *dsz* (Van Hamme et al. 2003) which contains the three genes (*dsz*C, *dsz*A and *dsz*B) sufficient for conversion of DBT to 2-HBP (Ohshiro and Izumi 1999).

Biodesulfurization currently

Nowadays, a race among researchers aiming the development of industrial-scale BDS processes has been observed due to the economic advantages that this type of biotechnology offers. Over the past 10 years, researches on improving the efficiency of desulfurization processes have increased considerably.

The application of large scale desulfurization technology is still only at the pilot project level study. One of these projects was executed by the U.S. Company Energy Bio-Systems Corporation, which has developed a pilot project where sulfur can be removed from up to 5 barrels of oil per day. In this process, the biocatalyst and the fossil fuel are mixed in a bioreactor where the biological desulfurization occurs. After that, petroleum product already desulfurized is sent to a container for disposal of sewage, through a series of filters. Finally, it is added to the aqueous basic solution to neutralize. The sodium sulfate resulting from chemical reaction is easily removed in a wastewater treatment station (Alves et al. 1999).

According to Monticello (2000), the pilot project depicted (Fig. 2), shows the beginning of the industrial process of BDS. This research also emphasizes the need to use three bioreactors in order to reach low concentrations of sulfur, required by new U.S. legislations, since the degradation rates from the metabolism of microorganisms studied to this day are very low. In addition, microbial biomass would have to be renewed during the manufacturing process, enabling commercially viable bioprocess desulfurization.

The search for new desulfurizing psychrophilic strains from the Antarctic continent revealed the presence of

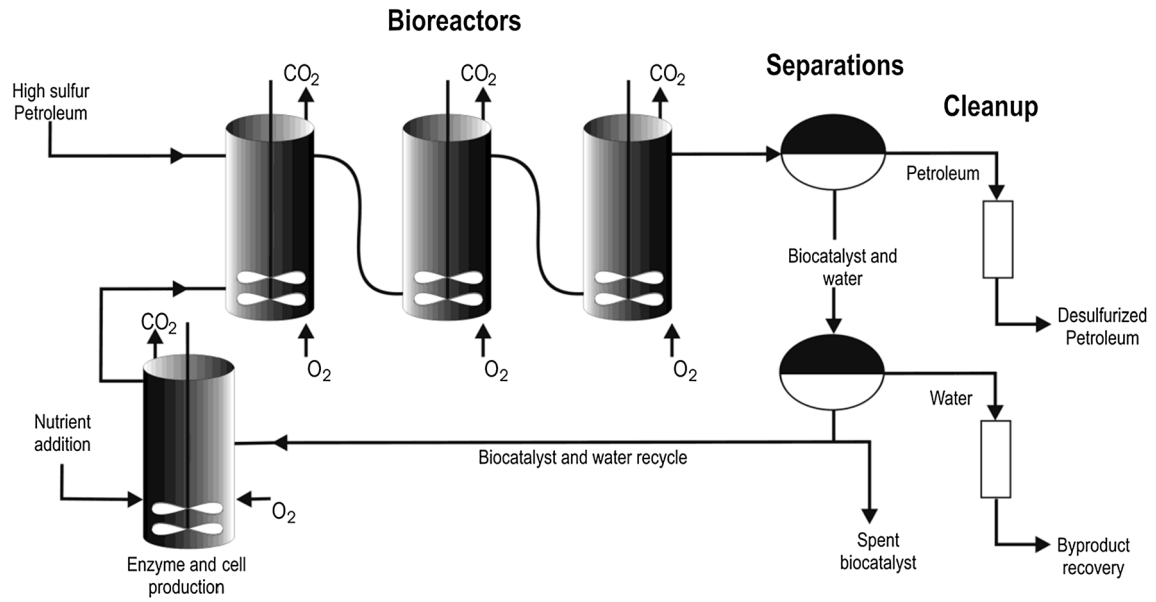

Fig. 2 Schematic drawing of the conceptual process of desulfurization. (Adapted from Monticello 2000). In this diagram the three reactors that are required to achieve very low sulfur concentrations. The microbial biomass grown and reactivated in the bioprocess, a necessary step to obtain the long biocatalyst residence times needed for a commercial bioprocess

important microorganisms isolated from soil and rhizosphere. These microorganisms show a higher capacity of desulfurization than previously described in the literature (Boniek et al. 2010). In earlier studies, one Gram-negative species known as *Sphingomonas subarctic* was isolated in Japan, and it was proved that it possesses an efficient ability to desulfurizate DBT considering the industrial perspectives (Gunam et al. 2006, 2013).

The interest on isolating thermophilic strains is also quite evident, since these microorganisms are considered most appropriate in the process of BDS followed by HDS (Bhatia and Sharma 2010), due to the fact that the petroleum refining process requires high temperatures favoring the development of thermophilic organisms (Konishi et al. 2000).

Figueiredo (2009) isolated new bacterial strains from soil samples contaminated with petroleum, in a *landfarming* system in Brazil. Environments contaminated with high concentrations of hydrocarbons provide new conditions which may favor the establishment of microorganisms adapted to the habitat and capable of using the nutrients available (Boniek et al. 2010). Studies of this nature envision the possibility of obtaining new isolates for BDS processes.

Nanobiotechnology has also been used in studies involving BDS. For this, strains of *Rhodococcus erythropolis* IGTS8 were decorated with Fe_3O_4 magnetic nanoparticles synthesized by a chemical method. The results on hydrocarbon absorption using this nanotechnology showed 56 % more desulfurization of DBT compared to other cells not submitted to this process (Ansari et al. 2009). After the reaction, the bacterial cells may be separated from their products through exposure of a magnetic field.

Polyphasic characterizations of microorganisms have been increasing in the past years. This technique considers phenotypic, genotypic and molecular characteristics of microorganisms. Santos et al. (2007) started this polyphasic approach with two isolates of *R. erythropolis*. The authors showed that both isolates involved in the "4S" pathway, reached desulfurization rate of 81.5 %, indicating that the isolates meet the necessary requirements for biorefining fuels. Other studies aiming at improving the efficiency of the BDS showed that diesel containing a hydrocarbon compound and submitted to the metabolic processes of *Mycobacterium* sp. ZD-19 in an airlift reactor provided lower BDS rates when compared to the DBT system. This phenomenon may be explained by a possible competition between substrates considering the Michaelis–Menten model (Zhang et al. 2012).

From the enzymatic perspective, studies about punctual mutations on enzymes involved in the "4S" tend to increase the percentage of BDS (Torktaz et al. 2012). An example that may be cited is the species *R. erythropolis*, which is the most studied bacteria concerning the identification of genes and enzymes involved in this type of biotechnology (Alves et al. 2006). The use of a synthetic gene encoding high proportions of sulfur was constructed as part of the *dsz*ABC enzymatic complex of the *Rhodococcus* genus. Increases in the desulfurization activity were

Table 1 Advantages and disadvantages of biodesulfurization technology (BDS)

BDS Advantages	BDS Disadvantages
Produce less acid rain gases (Izumi et al. 1994)	The logistics of sanitary handling, storage and use of living microbial cells within the refinery environment (McFarland, 1999)
High specificity enzymatic for the DBT (Konishi et al. 2000)	Inhibition of BDS process for the production and toxicity of 2-HBP (Alves and Paixão 2011)
Lower capital and operating costs (Guobin et al. 2006)	Metabolic pathway in common with other microorganisms degrading DBT (Aggarwal et al. 2012)
Produce ultra low sulfur fuels (Soleimani et al. 2007)	Cost of culture media used to grow the microorganisms involved in the bioprocess (Silva et al. 2013)
Remove the recalcitrant molecules under mild pressures and temperatures (Caro et al. 2007)	Separation process of oil/microbial biomass not determined (Li et al. 2009)
High valuable by-products (Alves and Paixão 2014b)	Multienzymatic nature making it difficult to ex situ performance (Alves and Paixão 2014a)

DBT dibenzothiophene, *2-HBP* 2-hydroxybiphenyl

achieved in this study, leading thus to the development of improved desulfurization biocatalysts (Pan et al. 2013).

A new alternative for reducing the sulfur content via BDS is the use of surfactants in a type of heavy oil, commonly used in marine expeditions (Li and Jiang 2013). This type of oil is scarcely used due to its high concentrations of sulfur and consequent emission of sulfur oxides into the atmosphere favoring the incidence of acid rain (Bhatia and Sharma 2010, Mehrara et al. 2014). The use of surfactants to form an emulsion is one of the most effective methods for reducing the viscosity of heavy oil (Li and Jiang 2013) and thus optimizing the BDS process (Bandyopadhyay et al. 2013).

Perspectives on BDS

The microorganisms which are capable of metabolizing polycyclic aromatic hidrocarbons (PAH's) molecules on oil treatment processes have been considered promising for the past four decades. However, until today, the search for stable biocatalysts has not succeeded in meeting worldwide industrial standards. This is a reflection, of the lack of studies concerning the development of a viable commercial bioprocesses aiming at the removal of organic sulfur from fossil fuels. Some advantages of the BDS technology and limitations of implementing this bioprocess are listed in Table 1 and will be discussed below.

One of the main reasons for not implementing the BDS to the present day is that many of the biochemical pathways used by microorganisms for the removal of sulfur (Aggarwal et al. 2012) are also degradative pathways of hydrocarbons, which leads to unacceptable reduction in energy content of fuels (Lee et al. 1995).

The participation of four enzymes and two cofactors in BDS technology makes it difficult the use of free enzymes (Alves and Paixão 2014b). However, recent studies have shown that the use of enzymes immobilized on nanoparticles has achieved good levels of efficiency of BDS (Derikvand et al. 2014).

The use of viable cells appears to be more practical because it allows regeneration of cofactors in situ (Klein et al. 1999). However, the logistics of aseptic handling, loading, storage and use of viable microbial cells in petroleum refining industry environment, make it a barrier for commercial acceptance of BDS (McFarland 1999).

Furthermore, the process of separating oil, microbial biomass and aqueous phase are not yet well established, which generates an uncertainty in the quality and quantity of recovered fuel (Li et al. 2009). Other limitations of this biotechnology that should be taken into consideration are about the cost of culture medium used to grow the microorganisms involved in the bioprocess (Alves and Paixão 2014a), stimulating, thereby, the researchers to seek new alternatives to remedy this obstacle.

The effective commercialization of BDS will depend on the solution of numerous problems, including the issue of transfer of biomass during the BDS and the enzymatic activity of microorganisms involved (Monticello 2000). Because of these issues, studies on enzyme thermostability should be considered (McFarland 1999), besides adapting biocatalysts to high tolerances to organic solvents (Zhang Zhang et al. 2007b).

Other factors appear to limit the BDS process, when considering the formation of 2-HBP as the final product of the "4S" metabolic pathway. Alves and Paixão (2011) detected growth inhibition of *Rhodococcus erythropolis* strains due to the toxicity posed by 2-HBP. This toxicity had a direct negative effect on the entire desulfurization bioassay (Abin-Fuentes et al. 2013). Other studies have also indicated that the inhibitory effect of HBP may be observed at concentrations higher than 0.1 mmol L^{-1} (Chen et al. 2008b).

Although there are several obstacles related with the actual viability of the BDS process, the search for new microbial strains able to metabolize PAH's and thereby eliminate the sulfur in fossil fuels continue to be of utmost importance on biotechnological studies. Moreover, the emergence of new research groups worldwide is also relevant to the contribution of knowledge in this area.

Conclusions

The complete desulfurization of fossil fuel by microbial approach is not expected to occur in early future (Soleimani et al. 2007). Thus, the multidisciplinary study (such as biotechnology, engineering, and microbial biochemistry) focusing on this process makes it more possible for the BDS to change from an alternative and complementary technology to become a promising biotechnology capable of diminishing environmental impacts, health problems and even change the direction of the oil industry. Thus, this process may change from a future promise to a present useful and innovative technology.

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