J Ind Microbiol Biotechnol (2010) 37:425–429
DOI 10.1007/s10295-010-0697-6

SHORT COMMUNICATION

Mining of genomic databases to identify novel biodesulfurizing microorganisms

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Received: 22 October 2009 / Accepted: 6 February 2010 / Published online: 20 February 2010

Abstract The commercialization of the biocatalytic desulfurization process does not seem to be realistic in the near future because of the low desulfurization rate of the known microorganisms. Hence, the future development will depend on either genetically modifying the currently available bacteria or identifying novel biodesulfurizers. In this study an in silico method to identify new biodesulfurizing microorganisms was adopted. By screening the available genomic databases, 13 novel desulfurizing microorganisms belonging to 12 genera were identified. Several of these could be of immense utility as they have both environment pollutant and industrial waste degrading capability.

Keywords Biodesulfurization · Genomic databases · Fuel · Thermophile · Mesophile

Upgrading the quality of fossil fuels to deal with sulfur-related environmental problems has been a major thrust in the recent past. This is mainly because of the stringent government regulations in several countries for lower levels of sulfur in transportation and non-road fuels [1]. Moreover petroleum refineries are also facing the problem of unavailability of low sulfur crudes, since the feedstock for refining processes are becoming heavier day by day with high sulfur contents [2, 3]. In the refineries hydrodesulfurization (HDS) is the technology presently being used for the pre-combustion desulfurization of fuels. HDS involves the use of chemical catalysts containing metals at high pressures and temperatures. However, organic sulfur compounds such as dibenzothiophene (DBT) and its alkylated derivatives are found to be highly recalcitrant to HDS [4, 5]. Moreover, increasing generation of used up chemical catalysts as hazardous wastes is leading to the problem of their disposal. The past decade has seen the development of biocatalytic desulfurization (BDS) as an alternative or compliment to HDS for the removal of organic sulfur from fuels. BDS has the potential benefits of lower operating costs, production of fewer greenhouse gases and degradation of HDS recalcitrant organic sulfur compounds. These features would lead to high energy savings in the refinery in addition to being environmentally more benign [1].

The impetus for BDS was the discovery of the 4S pathway in *Rhodococcus erythropolis* IGTS8 [6]. Since then, various microorganisms have been characterized as possessing biodesulfurization ability [1]. The pathway deals with the removal of sulfur without affecting the carbon content and thus preserving the fuel value [7–9]. It involves four key enzymes: dibenzothiophene monooxygenase (DszC), dibenzothiophene sulfone monooxygenase (DszA), hydroxyphenyl benzene sulfonate (HPBS) desulfinase (DszB) and NADH:FMN oxidoreductase (DszD). Despite considerable progress in the field of BDS, microbial desulfurization up to now has not been that productive for many reasons, and therefore its commercialization is unlikely to take place in the near future [3]. These reasons include, but are not restricted to, (1) sulfur specificity, (2) activity, (3) broad spectrum, (4) mesophilic nature of the organisms, etc. Moreover, the known biodesulfurizers have an approximately 500-fold lower desulfurization rate than what is required in industrial processes [10]. Hence, there exists a quest to identify and characterize new organisms that may have superior characteristics

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to the known ones. However, conventional methods of isolating novel microorganisms demand high inputs of time, labor and cost.

With the advancement in sequencing technology, the past few years have seen mushrooming of the genomes of various microorganisms. Exploration of this genomic information has accelerated progress in a few fields, for example, the area of natural products and biosynthetic pathways [11]. However, their use has never been exploited in BDS research. Therefore, in this study an in silico approach was used to identify novel biodesulfurizers. To the best of our knowledge, this is the first report of exploring genomic databases for identifying novel sulfur degrading microorganisms. Amino acid sequences of the Dsz proteins of *R. erythropolis* IGTS8 [6] were used as a query to perform protein-protein BLAST (BLASTp, http://www.ncbi.nlm.nih.gov/blast/index.shtml) in order to search for the homolog protein(s) in the entire finished, non-redundant and unfinished microbial genome database. All the database hits having an *E* (expect) value <0.001 were taken as a putative homolog(s) of the query sequence [12]. Further, microbes reported in the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database were also screened for the identification of putative homologs.

This approach leads to the identification of protein(s) in microorganisms, which have already been shown experimentally to possess *dsz* genes and their respective proteins. These microorganisms were left aside, and microbes that have not been shown to possess the property of DBT degradation to date were selected. These included 13 novel putative DBT degraders belonging to 12 genera. The list of these 13 microbes along with their unique properties and the presence/absence of Dsz proteins (based on the present search) has been shown in Table 1. All the identified microorganisms had DszA, DszC and DszD proteins except *Methylobacillus flagellatus*, which does not seems to have DszC and DszD proteins. The rate-limiting step in the "4S

Table 1 Potential dibenzothiophene (DBT) degrading microorganisms discovered on the basis of in silico search and their unique properties

Organisms	DszA	DszB	DszC	DszD	Unique properties
Burkholderia fungorum[a]	+	+	+	+	Good bio-degraders of polychlorinated biphenyls (PCBs), commercially important for bioremediation, pollutant degrading ability, role in global C-cycle
Burkholderia cepacia	+	+	+	+	Capacity to fix nitrogen, biodegradation of pollutants, biocontrol of root diseases, model for comparative genomic studies
Bradyrhizobium japonicum	+	+	+	+	Nitrogen-fixing ability, excellent model organism for studying respiratory enzymes
Methylobacillus flagellatus	+	+	−	−	Obligate methylotrophic bacterium, ideal producer for biotechnology industry, used for the overproduction of amino acids and vitamins
Magnetospirillum magnetotactium	+	+	+	+	Exhibit magnetotaxis, model for biomineralization, potential geobiological tracer, commercially can be used for magnetic targeting of pharmaceuticals, cell separation, and applications in magnetic resonance imaging
Thermobifida fusca[b]	+	−	+	+	Degrade all major plant cell wall polymers except lignin and pectin, its spores cause the condition farmer's lung
Azotobacter vinelandii	+	+	+	+	Nitrogen-fixing ability, highly amenable to genetic manipulation, produces 5-alkylresorcinols, alginates and poly-b-hydroxybutyric acid, which are of importance in medicine, food and biodegradable plastics industries, respectively
Mesorhizobium loti	+	−	+	+	Nitrogen-fixing ability
Oceanobacillus iheyensis	+	−	+	+	Extremely halotolerant and facultative alkaliphilic capability
Novoshingobium aromaticivorans[a]	+	−	+	+	Glycosphingolipid as a cell wall component, degrades a wide variety of aromatic hydrocarbons including tolune, xylene, naphthalene, fluorine, etc.
Brevibacterium linens	+	−	+	+	Produce volatile sulfur compounds, bacteriocin, self-processing extracellular proteases. Can metabolize heterocyclic and polycyclic ring structures
Rubrobacter xylanophilus[b]	+	−	+	+	Gamma radiation resistant, degrades hemicellulose and xylan
Ralstonia metallidurans	+	−	+	+	Resistant towards various heavy metals like Zn, Cd, Co, Pb, Cu, Hg, Ni and Cr

[a] Unfinished genomes

[b] Thermophiles

The amino acid sequences of the enzymes viz. DszA (accession no. AAA99482), DszB (accession no. AAA99483), DszC (accession no. AAA99484) and DszD (accession no. AAC38226) involved in the 4S pathway of DBT desulfurization found in *Rhodococcus erythropolis* IGTS8 was used [6, 10] to BLAST the available genomic databases

pathway" is the final step that leads to the accumulation of 2-hydroxybiphenyl (2-HBP) and sulfate, and requires DszB enzyme [7]. Hence, one of the ways to improve the rate of sulfur removal could be to develop/identify the DszB$^-$ biocatalyst [7, 10]. Further absence of *dsz*B gene would allow the accumulation of hydroxybiphenyl benzene sulfinate (HPBSi), a more valuable product than the sulfate. HPBSi can be recovered from the aqueous phase and used as surfactant [13]. Seven of the identified microbes in the present study do not have DszB protein (DszB$^-$ microorganisms; Table 1). Of these, *T. fusca* and *R. xylanophilus* are thermophilic in nature. These microbes could be advantageous as if a BDS reaction could be carried out at higher temperature, then there would be no need to cool the HDS-treated oil to ambient temperature, which is required in case of mesophiles [9]. Moreover, at higher temperature, BDS would afford a more practical approach to a large-scale industrial process and could result in higher rates and low processing costs [14]. In addition higher temperature decreases oil viscosity and contamination by undesirable bacteria, which affects the BDS process [15, 16].

To support the in silico observation, the presence of the *dsz*B and *dsz*C genes in the genomes of the two identified microbes viz. *B. fungorum* LB400 and *T. fusca* was analyzed. *B. fungorum* LB400 was initially grown at 30°C in minimal medium as described earlier [17], and slowly the organism was acclimatized on sulfur-free media (SFM) supplemented with DBT as a sulfur source [18]. Genomic DNA of *B. fungorum* LB400 and *T. fusca* was assessed by PCR for the amplification of *dsz*B *and dsz*C genes as described earlier [19]. As expected, *dsz*C was observed in both of the bacteria, whereas *dsz*B was found to be present only in *B. fungorum* LB400 (data not shown). Desulfurization activity of *B. fungorum* LB400 was further assessed by HPLC. For this 20 ml of SFM in 100-ml Erlenmeyer flasks supplemented with 100 ppm of DBT (a model compound) dissolved in n-hexane was inoculated by fresh overnight-grown culture of LB400 (initial OD_{660} of 0.02). After 120 h of incubation at 37°C in a shaking incubator, the culture broth was acidified to pH 2.0 with 6 N HCl and extracted with an equal volume of ethyl acetate. The extract was filtered and analyzed by reverse phase

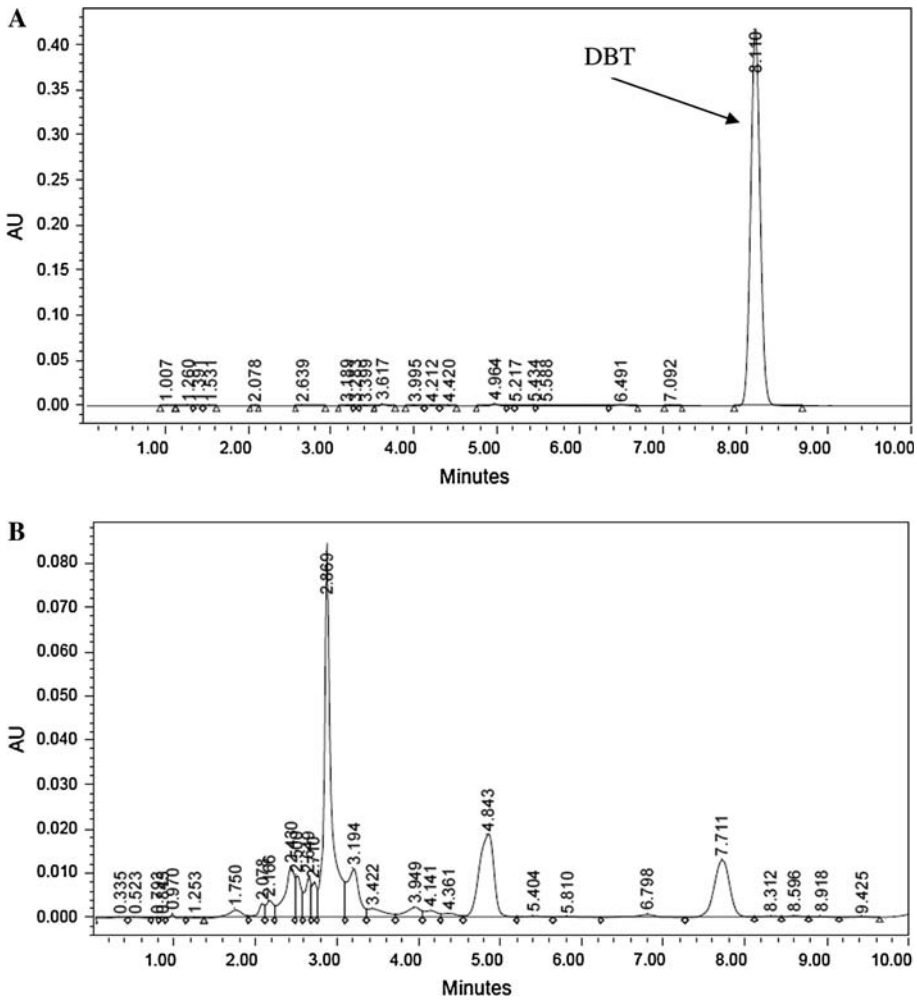

Fig. 1 DBT degradation by *Burkholderia* sp. LB400. **a** HPLC spectrum of the DBT in control sample (retention time –8.1); **b** HPLC spectrum of the DBT desulfurization products of LB400 (DBT peak has disappeared)

chromatography using a C-18 column (Waters, Germany). Elution was performed with 80/20 (v/v) acetonitrile/water as mobile phase at the flow rate of 1 ml/min, and peaks of DBT and its metabolites were detected at 280 nm. HPLC analysis showed the complete disappearance of the DBT peak (Fig. 1b) when compared to the chromatograph of the control sample (SFM supplemented with DBT but without the organism, Fig. 1a). This suggested the complete degradation of DBT by LB400. Some new peaks were observed in the HPLC chromatograph of the LB400-treated sample (Fig. 1b), but none of them was of 2-HBP, as confirmed by spiking of the culture supernatant by 100 ppm of 2-HBP. This could be because LB400 has been reported to be a polychlorinated biphenyl-degrading bacteria [20].

Burkholderia sp. has been reported to have denitrogenation ability and has been shown to degrade carbazole in gas oil/water biphasic media [21, 22]. Also *B. japonicum* possesses nitrogen (compounds) degradation enzymes (KEGG database). Therefore, organisms such as *B. fungorum*, *B. cepacia* and *B. japonicum* have greater significance as they have the potential to remove not only sulfur (as revealed by the present search), but also nitrogen from the crude oil. Further, *B. fungorum* and *N. aromaticivoram* have been reported to have hydrogen (a clean fuel) production capability [12]. Thus, *B. fungorum* could be of immense importance because of its multiple functions, i.e., removal of sulfur and nitrogen from fossil fuels, waste stabilization and hydrogen production. For the development of an efficient BDS process, there is a need to develop biocatalysts that remove nitrogen (denitrogenation), metals (demetallation) as well as sulfur simultaneously, resulting in the overall up-gradation of crude oil [3]. This will help in the development of the crude oil biorefining to be applied in place of or after the treatment of oils by the HDS process. To achieve ultra-deep desulfurization, the BDS unit downstream of an HDS unit as a complementary technology has been suggested [1]. Though BDS is an energetically expensive process [1], the energy invested is gainfully utilized because the sulfur compounds obtained at the BDS step have a value-added use to produce surfactants, etc., which may be exploited for several uses, including enhanced oil recovery or in pharmaceuticals.

In conclusion, this study suggested potential novel microorganisms, especially those devoid of inhibitory steps in the 4S pathway, which were hitherto unknown to possess biodesulfurization capability. These microorganisms have the ability to degrade environmental pollutants, a range of industrial waste and aromatic compounds, and are known to play a role in the production of various valuable industrial products. Hence, this raises the intriguing possibility that they could be exploited as biodesulfurizers, waste degraders and producers for industry. The bioinformatics approach to discovering different microorganisms for the biorefining of fossil fuels would be a cost-effective technique leading to savings of time, labor and effort.

Acknowledgments The authors would like to thank the Petroleum Conservation Research Association, Ministry of Petroleum and Natural Gas, Government of India, for the financial grant to carry out this research work. *Rhodococcous erythropolis* IGTS8, *Thermobifida fusca* YX (culture and genomic DNA) and *Burkholderoia fungorum* LB400 were gifts from Drs. John Kilbane (Gas Technology Institute, Illinois, Des Plaines, IL), David Wilson (Cornell University, New York, NY) and Mary Lidstorm (University of Washington, Seattle, WA), respectively.

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