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Detection of DBT biodesulfurization strains via PCR and conservation analysis among different bacterial strains

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Abstract

Eight bacterial strains, *Brevibacillus brevis* HN1, *Staphylococcus gallinarum* NK1, *Corynebacterium variabilis* Sh42, *Paenibacillus* sp. SW11, *Rhodococcus erythropolis* HN2, *Bacillus sphaericus* HN, *Micrococcus luteus* RM1 and *Pseudomonas aeruginosa* Asph2, capable to grow on sulfurous-hydrocarbons as a sole sulfur source, were investigated to use as biocatalysts in fuel desulfurization process. The biodesulfurization strains were determined by PCR using primers targeting the conserved regions of *dsz*A gene. PCR results showed HN1, NK1, Sh42 and HN2 strains are carrying the *dsz* genes, consequently these strains have the ability to desulfurize dibenzothiophene DBT selectively. In order to analyze the conservation, the bands (≈ 370 bp) amplified by PCR were sequenced and analyzed. The nucleotide sequences exhibit identity up to 99% to those of other known *dsz*A genes. A comparison of 15 *dsz*A sequences, 5 sequences obtained via PCR and 10 sequences obtained from GenBank databases, revealed a large degree of conservation between the four detected strains although they are taxonomically different.

Key words: Biodesulfurization, Dibenzothiophene, *dsz*A gene, PCR, conservation analysis

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1. Introduction

Emission of sulfur dioxide (SO_2) during combustion of fossil fuel causes many adverse effects on human health and environments, and it is considered as the principal cause of acid rain formation [1]. Concerns regarding the emission of SO_2 to atmosphere have forced many governments and regulatory agencies throughout the world to reduce sulfur emissions by establishing stringent regulations on sulfur level of all fuel products [2]. Up to 70% of total sulfur in various crude oil fractions are organic sulfur, as Benzothiophene (BT), Dibenzothiophene (DBT) and their alkylated derivatives [3], therefore DBT has generally accepted as a model sulfur compound present in fossil fuels [4]. These compounds are highly recalcitrant in removal by the well-known hydrodesulfurization (HDS) method, used in refineries process [5]. Hence, biodesulfurization (BDS) or microbial desulfurization was considered as an alternative technology to remove the organic sulfur compounds found in oil fractions in order to achieve the low sulfur-concentrations that is required in fuel [6].

Microbial desulfurization was first investigated in the 1970s and many microorganisms have been isolated to remove sulfur from organic sulfur compounds. These microbes are classified into four different categories according to their modes of action: Oxidative C–C cleavage, Kodama pathway [7,8]; Oxidative C-S cleavage, ring-destructive pathway [9]; Reductive C–S cleavage, anaerobic pathway [10]; and Oxidative C–S cleavage, sulfur-specific pathway and also known as "4S pathway" [11].

Over the past two decades various studies have been focused on microbes using oxidative sulfur-specific pathway, due to its specificity for sulfur atoms remove without affecting the carbon skeleton and the removal operation occurs under aerobic conditions. During this pathway DBT molecules are converted to 2-hydroxybiphenyl (2-HBP) and sulfite (SO_3^{2-}) via four enzymatic steps, so it is named a 4S pathway [12]. This catabolic pathway was first characterized in *Rhodococcus erythropolis* strain IGTS8 (formerly *Rhodococcus rhodochrous* IGTS8, ATCC 53968) by Denome et al. [13].

The genes involved in 4S pathway called *dsz*A, -B, and -C genes (also called in some studies *bds*, *tds*, *mds* and *sox* instead of *dsz*), they are clustered in an operon located on large plasmid [14]. The operon codes two monoxygenases (dszC and dszA) and one desulfinase (dszB) controlled by single promoter and transcribed in a one direction [15,16]. dszC enzyme catalyze the conversion of DBT to DBT sulfone, dszA convert the DBT sulfone to HBP sulfinate, and the dszB catalyzes the release of sulfur atom from HBP sulfinate producing 2-HBP and sulfite [13,17].

Kilbane and Robbins [18] identified conserved and unique regions in *dsz* genes and designed universal PCR primers targeting these regions that could be useful to use as a diagnoses for detection of the biodesulfurization strain. In the present study, the two primers which targeting conserved regions ARTLERG and RYDRADEFL in *dsz*A gene was used to determine the desulfurization activity of eight locally isolated strains via PCR to be used as biocatalysts in fuel desulfurization process.

2. Material and Methods

2.1. Bacterial strains

Characteristics and references of the bacterial strains that used in this study are listed in Table (1). *R. erythropolis* IGTS8 (ATCC 53968), a well characterized strain has *dsz* genes, was used as a positive control. Strains HN1, NK1, Sh42, SW11, HN2, HN, RM1 and Asph2, aerobic and mesophilic bacteria, were isolated from different geographical locations. The eight strains were previously isolated by Petroleum Biotechnology Laboratory, Egyptian Petroleum Research Institute, Cairo, Egypt.

2.2. Chemicals

Dibenzothiophene (DBT) (99%) was purchased from Merck, Germany. Potassium phosphate monobasic (99%), Glycerol (99%), Sodium chloride (99%), Ferric chloride (97%), Ammonium chloride, Magnesium sulfate and Ethanol (99.8%) were purchased from Honeywell, Germany. Tryptone, Agar-Agar bacteriological and Yeast extracts were obtained from Oxoid, United Kingdom. Diethyl ether (99.8%) and Ethyl acetate (99.8%) were purchased from Sigma-Aldrich, Spain. Lysozyme and RNAse are products of Bio Basic inc, Canada. Taq DNA polymerase, dNTPs and DNA Ladder are products of Thermo scientific, Lithuania and the Agarose type II was purchased from Applichem, Germany. The PCR Purification Kit was purchased from Qiagen, Germany. All other chemicals were of analytical grade, commercially available and used without further purification.

2.3. Culture media and growth conditions

Tryptone glucose yeast extract (TGY) medium [26] was used for maintenance and cultivation of all the utilized bacterial strains. The well grown bacterial cells inoculated in TGY broth medium and incubated for 24 h at 30°C in shaking incubator (150 rpm) were harvested and washed by sterile saline (0.85% NaCl), then resuspended into sterile saline to be used as inoculum. These inocula were used to inoculate Basal salt medium (BSM) supplemented with 250 ppm DBT, prepared according to Nassar et al. [27]. Medium contained (per liter): Na_2HPO_4, 5.57 g; KH_2PO_4, 2.44 g; NH_4Cl, 2.0 g; $MgCl_2.6H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.001 g; $FeCl_3.6H_2O$, 0.001 g; $MnCl_2.4H_2O$, 0.004 g; yeast extract, 0.1 g; 6.4 ml of glycerol. The cultures were incubated at 30°C for 72 h in a shaking incubator (150 rpm).

2.4. DNA extraction

Total DNA was extracted using lysozyme-sodium dodecyl sulfate method of Godson and Vapnek [28] as described in Sambrook and Russell [29]. DNA concentrations were determined spectrophotometrically by measuring the absorbance at λ_{260nm} using UV-VIS Spectrophotometer, Model: UV-240 (Shimadzu, Japan).

2.5. PCR amplification

DNA amplification was performed using primers targeting conserved regions ARTLERG and RYDRADEFL that was identified by Kilbane and Robbins [19], (F primer) 5′-GCS CGK ACH CTS GAG CGS GGC-3′ and (R primer) 5′-AAY TCR TCR GCV CGG TCR TAB CG-3′, respectively. PCR amplification were done in thermal cycler, MJ Research PTC-100 (MJ Research Inc, MA, USA) using extracted DNA as a template. Amplification mixtures was prepared using Dream-Taq DNA polymerase (Thermo scientific) with 40 ng DNA of each strains, 0.2 mM of dNTPs and 20 pmol of each oligonucleotide primers. The PCR thermal profile was as follows: 5 min at 94°C (pre-denaturation); 35 cycles of 40 sec at 94°C (denaturation), 40 sec at 56°C (annealing), and 1 min at 72°C (extension); followed by a final 10 min at 72°C (final extension). After the completion of PCR program, a portion of amplification products was analyzed by electrophoresis on 2.0% (w/v) agarose gels in 40mM Tris-acetate, 1mM EDTA buffer.

2.6. DNA sequencing and analysis

Amplified fragments were purified by QIA-quick PCR Purification Kit (Qiagen, Germany), and sequenced on ABI 3730XL (Applied Bio-systems, USA), service was provided by Macrogen Inc (South Korea). Sequences data were aligned to database sequences of NCBI using BLASTn search tools [30]. Multiple sequence alignments were performed using ClustalW2 provided by EMBL-EBI [31]; then, Jalview 2.8 software was used to shad result of alignments [32] and MEGA6 software was applied to construct the phylogenetic tree [33].

2.7. Accession numbers

The selected nucleotide sequences of the desulfurization genes amplified in this study were deposited in the GenBank database as a *dsz*A partial sequence of strains, *Brevibacillus brevis* HN1, *Staphylococcus gallinarum* NK1, *Corynebacterium variabilis* Sh42 and *Rhodococcus erythropolis* HN2, under accession numbers, KJ002079, KJ002080, KJ002081 and KJ002082, respectively.

3. Results and Discussion

3.1. Detection of strains carrying dsz genes

There is significant interest in isolation of microbial strains capable of selectively remove sulfur from recalcitrant organosulfur compounds without degrading the carbon backbone, i.e., following 4S metabolic pathway of DBT, to be suitable for commercial fuel desulfurization process. Many results have been reported on isolation of active strains when grow on the organosulfur compounds, but most of them affect the C-C structure [34]. Duarte et al. [35] reported the isolation of bacterial strains following 4S pathway are not easily obtained from everywhere, desulfurization strains exist only in environments where sulfurous-hydrocarbons are the pollutant and inorganic sulfate are limited. Since inorganic sulfur are relatively abundant in many environments, the distribution of desulfurization-strains in nature are restricted [36]. The present work focused on detection of the biodesulfurization ability of eight bacterial strains isolated previously from different geographical locations contaminated with hydrocarbon and identified as a DBT-metabolizing strains (Table 1).

The strains were screened by PCR using primer specific for detection of *dsz* genes targeting ARTLERG and RYDRADEFL conserved regions in *dsz*A gene. In a first step, strain IGTS8 (+ve control), and locally isolated strains *Brevibacillus brevis* HN1, *Staphylococcus gallinarum* NK1, *Corynebacterium variabilis* Sh42, *Paenibacillus* sp. SW11, *Rhodococcus erythropolis* HN2, *Bacillus sphaericus* HN, *Micrococcus luteus* RM1 and *Pseudomonas aeruginosa* Asph2, were cultivated on BSM containing 250 ppm DBT as a sole sulfur source. This step was applied to assure their potency to grow on the organosulfur compounds instead of the readily available inorganic sulfate which are essential element for the growth of all microorganisms and also to activate the desulfurization genomic material because the most of desulfurization strains that have been reported previously are not competent cultures [36]. In a second step, total DNA were extracted by lysozyme-sodium dodecyl sulfate method because in many cases the desulfurization operon locates on large plasmid [13,14,37] and the most efficient way to get the genetic material of the desulfurization from any DBT-desulfurizing strain is to isolate the total genomic DNA which will include the large plasmids and the main chromosome (M). Finally, PCR screening was done using the extracted DNA, resulting in four strains, HN1, NK1, Sh42 and HN2 (Figure 1), were demonstrated positive amplification as same as standerd biodesulfurization strain (IGTS8).

From this result the HN1, NK1, Sh42 and HN2 strains were identified as biodesulfurization strains and have the desulfurization activity toward organic sulfur compounds. These strains follow the 4S pathway when grow on DBT because they are carrying the *dsz* genes. On the other hand, SW11, HN, RM1 and Asph2 strains showing negative amplification at the corresponding size, i.e., have no specific desulfurization activity, they may follow a destructive pathway when grow on DBT due to lack of desulfurization genes.

3.2. Comparative analysis for sequences of amplified dszA with other known sequences

PCR-based screening for presence of the *dsz* genes resulted in amplifying a portion from *dsz*A gene of strains IGTS8 (+ve control), HN1, NK1, Sh42 and HN2. These amplicons were sequenced in both directions using the same primers used in PCR amplification. Obtained sequences data were compared with other known desulfurization genes found in GenBank database of NCBI using BLASTn search tool. BLASTn result showed the *dsz*A sequences of HN1, NK1, Sh42 and HN2 strains exhibit significant homology with all desulfurization genes, *dsz*A *sox*A, *bds*A, *tds*A, in the GenBank database (Table 2). As determined by BLASTn DNA sequences analysis, strains HN1, NK1, Sh42 and HN2 have up to 99% identity to those of canonical *dsz*A sequences that described previously and available in the GenBank database. Moreover, the most of *dsz*A sequences were found highly similar and internally conserved.

3.3. Phylogenetic analysis for sequences of amplified dszA and related sequences

From BLASTn result, ten *dsz*A sequences were selected as a reference in the phylogenetic analysis, representing all genera and/or species that demonstrate a significant homology in BLASTn result. The reference sequences were aligned with the five sequences that obtained in this study, *dsz*A sequences of IGTS8 (+ve control) and HN1, NK1, Sh42 and HN2 strains (Figure 2). Multiple sequence alignments were performed using ClustalW2 program, online tool provided by EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default settings, and shaded by Jalview 2.8 software. After multiple alignments, the result was underwent a phylogenetic analysis. Neighbor joining phylogenetic tree has been constructed using MEGA6 software (Figure 3), based on the Tamura-Nei method and gamma distribution (parameter = 2.2), with 1000 bootstrap replicates [33, 38-40].

In Figure 3, the 15 *dsz*A sequences were grouped into two clusters. Group I, was clustered at 98 % similarity, and group II was ≥ 70 % similar to group I. The first group was consisted of 10 sequences including the 5 *dsz*A sequences of IGTS8 (+ve control), HN1, NK1, Sh42 and HN2 strains, while group II was consisted of the sequences that derived from GenBank database directly. The phylogenetic analysis revealed a great similarity of all detected sequences to the reference *dsz*A sequences of *Agrobacterium tumefaciens* FD-3 (GenBank AY960127.1), *Brevibacillus brevis* (GenBank DQ062161.1), *Gordonia nitida* (GenBank AY714057.1), *Nocardia globerula* (GenBank AY714059.1) and *Rhodococcus* sp. strain IGTS8 (GenBank L37363.1). On the phylogenetic tree, although the HN1, NK1, Sh42 and HN2 strains are taxonomically different and isolated from different sites, the *dsz* genes of these strains were found highly conserved. These finding strongly support the hypothesis of the *dsz* genes that discussed by Kilbane and Le Borgne [36] and Pylro et al. [41], the *dsz* genes are commonly subjected to horizontal transfer by conjugative plasmid-encoded the *dsz* genes and rarely acquired by vertical evolution.

Table 1: List of bacterial strains

No.	Strain	Characteristics	Reference
1	*Rhodococcus erythropolis* IGTS8 (ATCC 53968)	Standard DBT-desulfurization bacterium, formerly named *R. rhodochrous* IGTS8	Kayser et al. [19]
2	*Brevibacillus brevis* HN1	Isolated from Egyptian El-Nasr coke, capable to metabolize DBT to 2-HBP.	unpublished
3	*Staphylococcus gallinarum* NK1	Isolated from hydrocarbon polluted water, for its ability to metabolize different aromatic compounds such as DBT.	El-Gendy and Abo-State [20]
4	*Corynebacterium variabilis* Sh42	Isolated from petrogenic polluted water of El-Lessan area in Egypt, capable to metabolize different Poly aromatic compounds (PACs).	El-Gendy et al. [21]
5	*Paenibacillus* sp. SW11	Isolated from petroleum hydrocarbons polluted sea water for its ability to metabolize DBT.	unpublished
6	*Rhodococcus erythropolis* HN2	Isolated from Egyptian El-Nasr coke, for its ability to grow on DBT as a sulfur source and metabolize it to 2-HBP.	El-Gendy et al. [22]
7	*Bacillus sphaericus* HN	Isolated from oil polluted soil for its ability to metabolize DBT using 4S and Kodama pathways.	Deriase et al. [23]
8	*Micrococcus luteus* RM1	Isolated from oily sludge contaminated soil to metabolize different poly aromatic hydrocarbon.	Soliman et al. [24]
9	*Pseudomonas aeruginosa* Asph2	Isolated from oil-polluted sea water, halotolerant, utilize different Poly aromatic compounds (PACs) as asphaltene and DBT.	Ali et al. [25]

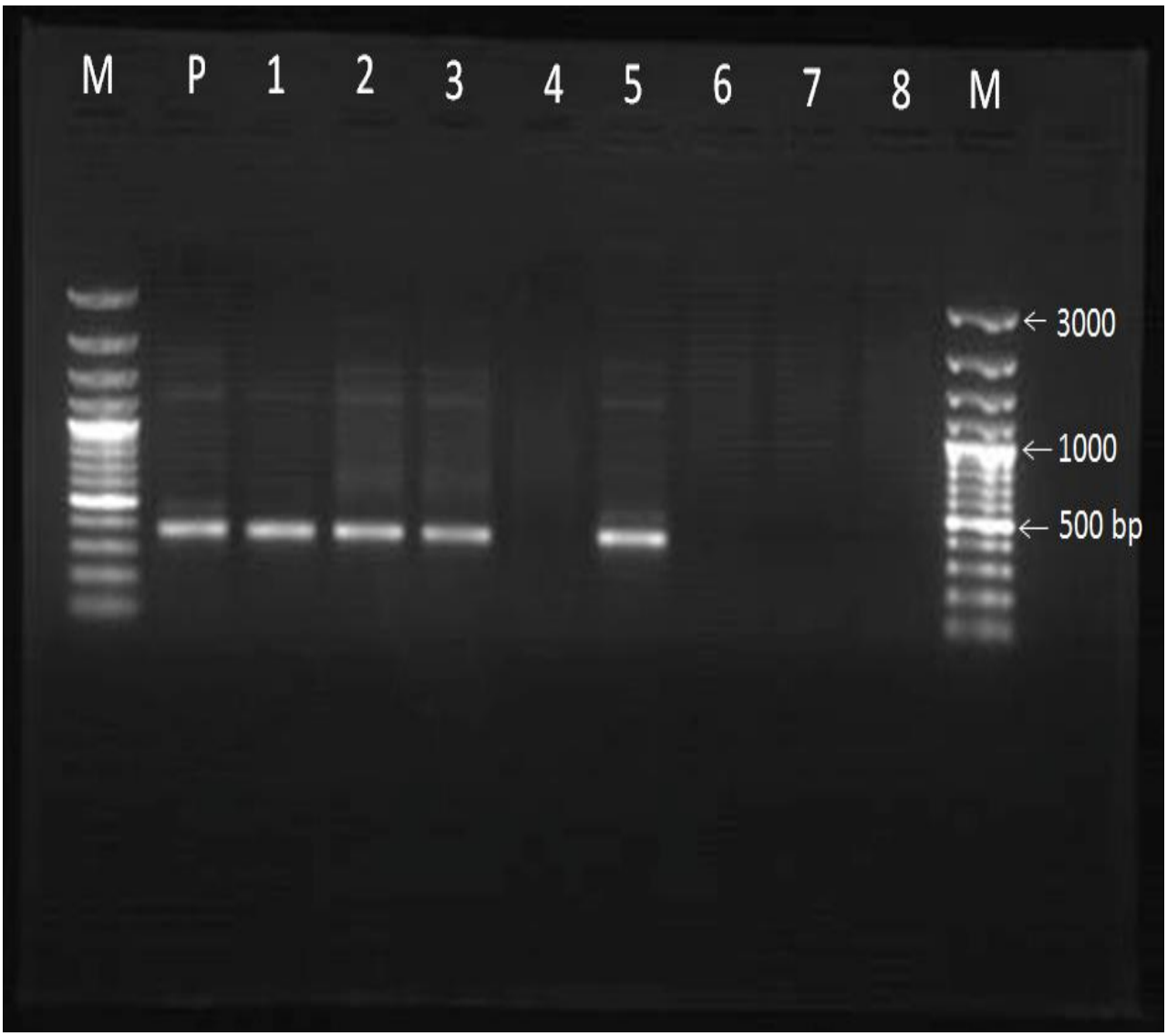

Figure 1. Agarose gel electrophoresis for PCR-based detection of *dsz* genes. Lane: M, DNA size marker (GeneRuler™ 100 bp Plus DNA Ladder, Thermo Scientific #SM0323); P, positive control (*Rhodococcus erythropolis* IGTS8, ATCC 53968); 1, *Brevibacillus brevis* HN1; 2, *Staphylococcus gallinarum* NK1; 3, *Corynebacterium variabilis* Sh42; 4, *Paenibacillus* sp. SW11; 5, *Rhodococcus erythropolis* HN2; 6, *Bacillus sphaericus* HN; 7, *Micrococcus luteus* RM1; 8, *Pseudomonas aeruginosa* Asph2.

Table 2: Sequences homology of detected *dsz*A to related sequences.

Nucleotide Source	Significant Alignments			
	Sequences title	**Query cover**	**Identity**	**Accession no.**
Brevibacillus brevis HN1	*Rhodococcus* sp. DS-3 , *dsz*A	100 %	98 %	DQ444325.1
	Rhodococcus sp. SDUZAWQ, *dsz*A	100 %	98 %	AY789136.1
	Nocardia globerula, *dsz*A	100 %	98 %	AY714059.1
	Rhodococcus erythropolis, *dsz*A	100 %	98 %	AY714058.1
	Rhodococcus sp. XP, *dsz*A	100 %	98 %	AY278323.1
	Brevibacillus brevis, *dsz* operon	100 %	98 %	DQ062161.1
	Agrobacterium tumefaciens, *dsz*A	100 %	98 %	AY960127.1
	Rhodococcus sp., *sox*A	100 %	98 %	U08850.1
	Rhodococcus sp., *dsz*A	100 %	98 %	L37363.1
	Gordonia nitida, *dsz*A	100 %	98 %	AY714057.1
	Gordonia sp. CYKS2, soxA	100 %	93 %	AY396519.1
	Gordonia alkanivorans 1B, *dsz*A	100 %	91 %	AY678116.1
	Gordonia amicalis, *dsz*A	100 %	83 %	EF026089.1
	Mycobacterium goodii X7B, *dsz* operon	100 %	76 %	JF740062.1
	Mycobacterium sp. G3, *dsz*A	96 %	76 %	AB070603.1
	Bacillus subtilis, *bds*A	100 %	75 %	AB076745.1
	Paenibacillus sp. A11-2, *tds*A	85 %	71 %	AB033997.2
Staphylococcus gallinarum NK1	*Rhodococcus* sp. DS-3, *dsz*A	100 %	99 %	DQ444325.1
	Rhodococcus sp. SDUZAWQ, *dsz*A	100 %	99 %	AY789136.1
	Rhodococcus erythropolis, *dsz*A	100 %	99 %	AY714058.1
	Rhodococcus sp. XP, *dsz*A	100 %	99 %	AY278323.1
	Brevibacillus brevis, *dsz* operon	100 %	99 %	DQ062161.1
	Agrobacterium tumefaciens, *dsz*A	100 %	99 %	AY960127.1
	Rhodococcus sp., *sox*A	100 %	99 %	U08850.1
	Rhodococcus sp., *dsz*A	100 %	99 %	L37363.1
	Nocardia globerula, *dsz*A	100 %	99 %	AY714059.1
	Gordonia nitida, *dsz*A	100 %	99 %	AY714057.1
	Gordonia sp. CYKS2, *sox*A	100 %	95 %	AY396519.1
	Gordonia alkanivorans 1B, *dsz*A	100 %	93 %	AY678116.1
	Gordonia amicalis, *dsz*A	100 %	85 %	EF026089.1
	Mycobacterium goodii X7B, *dsz* operon	100 %	77 %	JF740062.1
	Mycobacterium sp. G3, *dsz*A	96 %	76 %	AB070603.1
	Bacillus subtilis, *bds*A	100 %	76 %	AB076745.1
	Paenibacillus sp. A11-2, *tds*A	100 %	70 %	AB033997.2
Corynebacterium variabilis Sh42	*Rhodococcus* sp. DS-3, *dsz*A	100 %	99 %	DQ444325.1
	Rhodococcus sp. SDUZAWQ, *dsz*A	100 %	99 %	AY789136.1
	Rhodococcus erythropolis, *dsz*A	100 %	99 %	AY714058.1
	Rhodococcus sp. XP, *dsz*A	100 %	99 %	AY278323.1
	Brevibacillus brevis, *dsz* operon	100 %	99 %	DQ062161.1
	Agrobacterium tumefaciens, *dsz*A	100 %	99 %	AY960127.1
	Rhodococcus sp., *sox*A	100 %	99 %	U08850.1
	Rhodococcus sp., *dsz*A	100 %	99 %	L37363.1
	Nocardia globerula, *dsz*A	100 %	99 %	AY714059.1
	Gordonia nitida, *dsz*A	100 %	99 %	AY714057.1
	Gordonia sp. CYKS2, *sox*A	100 %	94 %	AY396519.1
	Gordonia alkanivorans 1B, *dsz*A	100 %	92 %	AY678116.1
	Gordonia amicalis, *dsz*A	100 %	84 %	EF026089.1
	Mycobacterium goodii X7B, *dsz* operon	100 %	76 %	JF740062.1
	Mycobacterium sp. G3, *dsz*A	96 %	76 %	AB070603.1
	Bacillus subtilis, *bds*A	100 %	75 %	AB076745.1
	Paenibacillus sp. A11-2, *tds*A	100 %	70 %	AB033997.2
Rhodococcus erythropolis HN2	Rhodococcus sp. DS-3, dszA	100 %	99 %	DQ444325.1
	Rhodococcus sp. SDUZAWQ, *dsz*A	100 %	99 %	AY789136.1
	Nocardia globerula, *dsz*A	100 %	99 %	AY714059.1
	Rhodococcus erythropolis, *dsz*A	100 %	99 %	AY714058.1
	Rhodococcus sp. XP, *dsz*A	100 %	99 %	AY278323.1
	Brevibacillus brevis, *dsz* operon	100 %	99 %	DQ062161.1
	Agrobacterium tumefaciens, *dsz*A	100 %	99 %	AY960127.1
	Rhodococcus sp., *sox*A	100 %	99 %	U08850.1
	Rhodococcus sp., *dsz*A	100 %	99 %	L37363.1
	Gordonia nitida, *dsz*A	100 %	99 %	AY714057.1
	Gordonia sp. CYKS2, *sox*A	100 %	94 %	AY396519.1
	Gordonia alkanivorans 1B, *dsz*A	100 %	92 %	AY678116.1
	Gordonia amicalis, *dsz*A	100 %	85 %	EF026089.1
	Mycobacterium goodii X7B, *dsz* operon	100 %	77 %	JF740062.1
	Mycobacterium sp. G3, *dsz*A	96 %	77 %	AB070603.1
	Bacillus subtilis, *bds*A	100 %	76 %	AB076745.1
	Paenibacillus sp. A11-2, *tds*A	100 %	70 %	AB033997.2

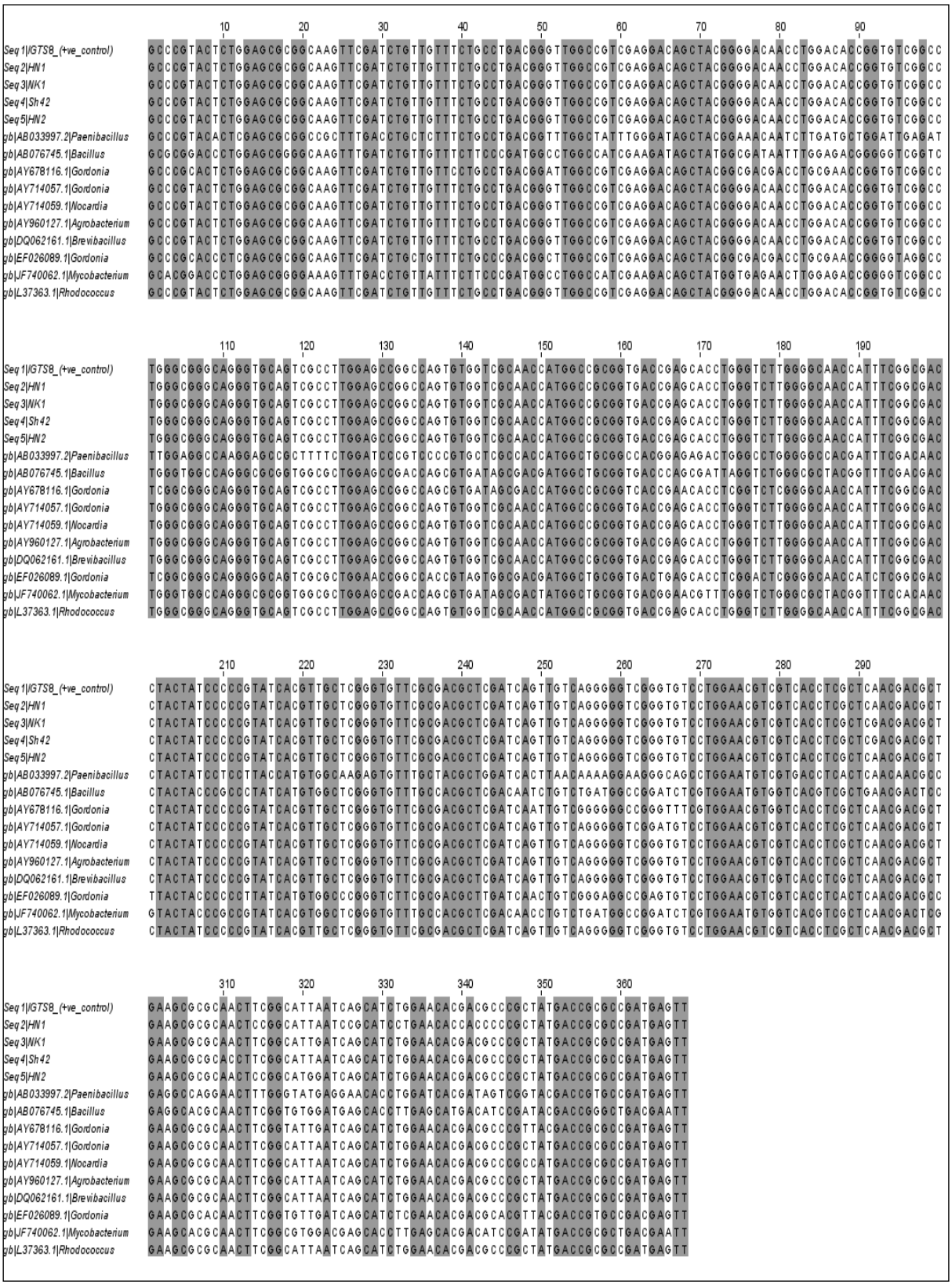

Figure 2: Multiple sequence alignments for 368-bp of *dsz*A gene from various desulfurization species. The 5 sequences obtained (Seq1: IGTS8, +ve control; Seq2: strain HN1, Seq3: strain NK1, Seq4: strain Sh42 and Seq5: strain HN2) were aligned with 10 *dsz*A reference sequences (dbj: AB033997.2, *Paenibacillus* sp. strain A11-2; dbj: AB076745.1, *Bacillus subtilis*; gb: AY678116.1, *Gordonia alkanivorans* 1B; gb: AY714057.1, *Gordonia nitida*; gb: AY714059.1, *Nocardia globerula*; gb: AY960127.1, *Agrobacterium tumefaciens* FD-3; gb: DQ062161.1, *Brevibacillus brevis*; gb: EF026089.1, *Gordonia amicalis* F.5.25.8; gb: JF740062.1, *Mycobacterium goodii* X7B and gb: L37363.1, *Rhodococcus* sp. strain IGTS8). Shaded columns are identity nucleotides conserved in all sequences.

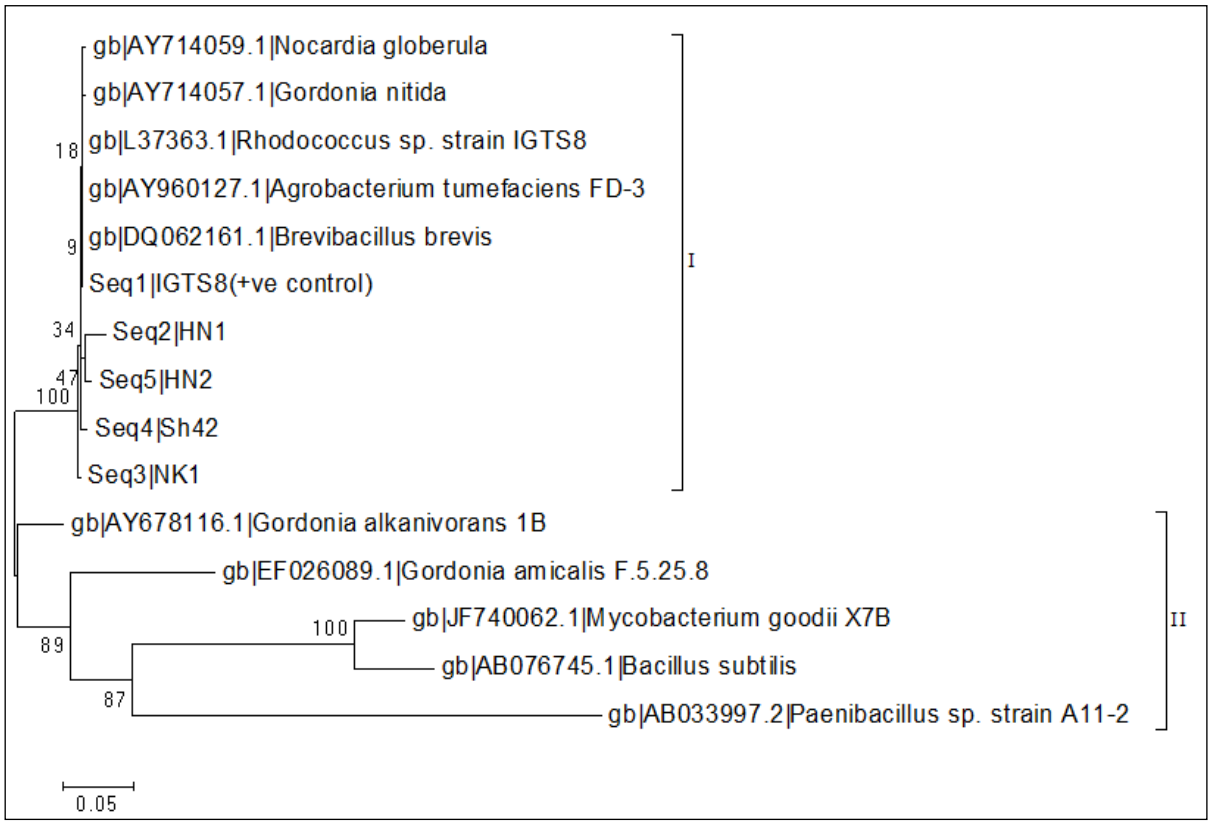

Figure 3. Neighbor-Joining phylogenetic tree for *dsz*A nucleotide sequences of 15 desulfurization strains. Sequences of strain IGTS8 (+ve control) and detected local-strains (HN1, NK1, Sh42 and HN2) are highlighted with gray color. The bar below the tree indicates the branch length corresponding to 0.05 nucleotide substitutions per site. Numbers at nodes represent the percentage of bootstrap replicates.

Therefore, the *dsz* genes of HN1, NK1, Sh42 and HN2 strains might have acquired by horizontal transmission not by vertical evolution, due to the *dsz* sequences of these strains are highly similar and conserved although they have no taxonomic relation.

In conclusion, the results presented here, based on PCR amplification and conservation analysis, clearly indicating that the HN1, NK1, Sh42 and HN2 are biodesulfurization strains since they have the *dsz* genes of the 4S pathway. In contrast, SW11, HN, RM1 and Asph2 are not biodesulfurization strains due to the lack of *dsz* genes, they might follow the Kodama pathway or complete degradation pathway when grow on the organosulfur compounds. In this study, four novel biodesulfurization strains have been determined and in order to reach a commercial desulfurization process using one of these strains, further researches are required.

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References

[1] W.P. Cunningham, M.A. Cunningham, (2012). Environmental Science-A Global Concern, 12th edition, McGraw-Hill, USA.

[2] G. Mohebali, A.S. Ball, (2008). Biocatalytic desulfurization (BDS) of petrodiesel fuels, Microbiology, 154: 2169-2183.

[3] K.G. Kropp, P.M. Fedorak, (1998). A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum, Candian Journal of Microbiology, l44: 605-622.

[4] D.J. Monticello, (2000). Biodesulfurization and the upgrading of petroleum distillates. Current Opinion Biotechnology, 11: 540-546.

[5] D.J. Monticello, W.R. Finerty, (1985). Microbial desulfurization of fossil fuels, Annual Review Microbiology, 39: 371-389.

[6] M. Soleimani, A. Bassi, A. Margaritis, (2007). Biodesulfurization of refractory organic sulphur compounds in fossil fuels, Biotechnology Advances, 25: 570-596.

[7] K. Kodama, S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, K. Yamada, (1973). Identification of microbial products from dibenzothiophene and its proposed oxidation pathway, Agricultural and Biological Chemistry, 37: 45-50.

[8] K. Kodama, S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, K. Yamada, (1970). Microbial conversion of petro-sulfur compounds. III. Isolation and identification of products from dibenzothiophene, Agricultural and Biological Chemistry, 34: 1320-1324.

[9] M. Van Afferden, D. Trappe, M. Beyer, H.G. Tüpper, J. Klein, (1993). Biochemical mechanisms

for the desulfurization of coal-relevant organic sulfur compounds, Fuel, 72(12): 1635-1643.

[10] B.Y. Kim, H.Y. Kim, T.S. Kim, D.H. Park, (1995). Selectivity of desulfurization activity of *Desulfovibrio desulfuricans* M6 on different petroleum products. Fuel Processing Technology, 43: 87-94.

[11] J.R. Gallagher, E.S. Olson, D.C. Stanley, (1993). Microbial desulphurisation of dibenzothiophene: a sulfur-specific pathway. FEMS Microbiology Letters, 107: 31-36.

[12] N. Gupta, P.K. Roychoudhury, J.K. Deb, (2005). Biotechnology of desulfurization of diesel: prospects and challenges, Appl Microbiol Biotechnol, 66: 356-366.

[13] S.A. Denome, C. Oldfield, L.J. Nash, K.D. Young, K.D., (1994). Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8, Journal Bacteriology, 176: 6707-6716.

[14] C. Denis-Larose, D. Labbe, H. Nergron, A.M. Jones, C.W. Greer, J. Al-Hawari, M.J. Grossman, B.M. Sankey, P.C.K. Lau, (1997). Conservation of plasmid-encoded dibenzothiophene desulfurization genes in several *Rhodococci*, Applied Environmental Microbiology, 1997**;** 63: 2915-2919.

[15] C.S. Piddington, B.R. Kovacevich, J. Rambosek, (1995). Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of Rhodococcus sp. strain IGTS8. Applied Environmental Microbiology, l61: 468-475.

[16] M.Z. Li, C.H. Squires, D.J. Monticello, J.D. Childs, (1996). Genetic analysis of the dsz promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. Journal Bacteriology, 178 6409-6418.

[17] B. Lei, S. Tu, (1996). Gene expression, purification and identification of a desulfurization enzyme from *Rhodococcus* sp. strain IGTS8 as a sulfide/sulfoxide monooxygenase, Journal Bacteriology, 178: 5699-5705.

[18] J.J. Kilbane, J. Robbins, (2007). Characterization of the dszABC genes of *Gordonia amicalis* F.5.25.8 and identification of conserved protein and DNA sequences, Applied Microbiology Biotechnology, 75: 843-851.

[19] K.J. Kayser, B.A. Bielaga-Jones, K. Jackowski, O. Odusan, J.J. Kilbane, (1993). Utilization of organosulfur compounds by axenic and mixed cell cultures of *Rhodococcus rhodochrous* IGTS8. Journal Genetics Microbiology 1993; 139: 3123-3129.

[20] N.Sh. El-Gendy, A.A. Abo-State, M.A., (2008). Isolation, characterization and evaluation of *staphylococcus gallinarum* NK1 as a degrader for dibenzothiophene, phenantherene and naphthalene, Egyptian Journal of Petroleum, 17(2): 75–91.

[21] N.Sh. El-Gendy, Y.M. Moustafa, S.A. Habib, Sh. Ali, (2010). Evaluation of *Corynebacterium variabilis* as a degrader for different poly aromatic compounds, Journal of American Science, 6(11): 343-356.

[22] N.Sh. El-Gendy, H.N. Nassar, S.S. Abu Amr (2014). Factorial design and response surface optimization for enhancing a biodesulfurization process, Petroleum Science and Technology, 32(14): 1669-1679.

[23] S.F. Deriase, N.Sh. El-Gendy, H.N. Nassara, (2012). Enhancing Biodegradation of Dibenzothiophene by *Bacillus sphaericus* HN1 Using Factorial Design and Response Surface Optimization of Medium Components. Energy Source Part A, 34: 2073-2083.

[24] R.M. Soliman, N.Sh. El-Gendy, S.F. Deriase, L.A. Farahat, A.S. Mohamed, (2011) Comparative assessment of enrichment media for isolation of pyrene-degrading bacteria, Revue de Microbiologie Industrielle Sanitaire et Environnementale, 5(2): 54-70

[25] H.R. Ali, N.Sh. El-Gendy, Y.M. Moustafa, M.I. Roushdy, A.I. Hashem, (2012). Degradation of aphaltenic fraction by locally isolated halotolerant bacterial strains, ISRN Soil Science; Article ID 435485 (doi:10.5402/2012/435485).

[26] H.J. Benson, (1994). Microbiological applications, 6th ed., Wm. C. Brown Publisher, pp. 447.

[27] H.N. Nassar, N.Sh. El-Gendy, M.A. Abo-State, Y.M. Moustafa, H.M. Mahdy, S.A. El-Temtamy, (2013). Desulfurization of Dibenzothiophene by a Novel Strain *Brevibacillus invocatus* C19 Isolated from Egyptian Coke, Bioscience Biotechnology Research Asia, 10(1): 29-46.

[28] G.N. Godson, D. Vapnek, (1973). A simple method of preparing large amounts of ϕX174 RFI supercoiled DNA, Biochimica et Biophysica Acta, 299: 516-520.

[29] J. Sambrook, D.W.Russell, (2001). Molecular Cloning, a laboratory manual, 3rd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor; New York.

[30] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25: 3389-3402.

[31] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23: 2947-2948.

[32] A.M. Waterhouse, J.B. Procter, D.M.A. Martin, M. Clamp, G.J. Barton, (2009). Jalview version 2: a multiple sequence alignment and analysis workbench. Bioinformatics, 25 (9): 1189-1191.

[33] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

[34] L. Alves, R. Salgueiro, C. Rodrigues, E. Mesquita, J. Matos, F.M. Girio, (2005). Desulfurization of dibenzothiophene, benzothiophene, and other thiophene analogs by a newly isolated bacterium,

Gordonia alkanivorans strain 1B, Applied Biochemistry and Biotechnology, l120: 199-208.

[35] G.F. Duarte, A.S. Rosado, L. Seldin, W. De Araujo, J.D. Van Elsas, (2001). Analysis of bacterial community structure in sulfurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (*dsz*) genes. Applied Environmental Microbiology, 67: 1052–1062.

[36] J.J. Kilbane, S. Le Borgne, (2004). Petroleum biorefining: the selective removal of sulfur, nitrogen, and metals. In: Vazquez-Duhalt R, Quintero-Ramirez R editors. Petroleum Biotechnology, Developments and Perspectives, Elsevier; Chapter 2, pp. 29–65.

[37] L. Serbolisca, F. de Ferra, I. Margarit, (1999). Manipulation of the DNA coding for the desulphurizing activity in isolate of *Arthrobacter* sp., Applied Microbiology and Biotechnology 52:122-126.

[38] J. Felsenstein, (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791.

[39] N. Saitou, M. Nei, (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

[40] K. Tamura, M. Nei, (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution,10: 512-526.

[41] V.S. Pylro, L.S. Vespoli, G.F. Duarte, K.S.C. Yotoko, (2012). Detection of horizontal gene transfers from phylogenetic comparisons. Intentional Journal of Evolutionary Biology, ID 813015.