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Biodesulfurization of Dibenzothiophene by Two Bacterial Strains in Cooperation with Fe_3O_4, ZnO and CuO Nanoparticles

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Abstract

Fossil fuels contain a considerable amount of sulphur which after burning will have negative effects such as generating acid rain in the environment. Biodesulfurization is assumed to be a promising process for desulfurization due to no need for extreme conditions and furthermore breaking the C-S bond by microorganisms without destruction of the heterocyclic structure, through a pathway called 4S. In this study dibenzothiophene (DBT), as a model target compound was applied for sulfur removal by a cooperative system of two bacterial strains; *Rhodococcus erythropolis* IGTS8 and *Pseudomonas aeruginosa* PTSOX4, and Fe_3O_4, ZnO and CuO nanoparticles. Results of spectrophotometry and further HPLC analysis demonstrated that addition of the ZnO nanoparticles into the microbial culture, significantly resulted in the increment of desulfurization rate and conversion of DBT to 2-hydroxybiphenyl. The maximum value of almost 1.4 fold improvement in biodesulfurization activity was obtained for *P. aeroginusa* PTSOX4 in the presence of ZnO nanoparticles.

Keywords: Biodesulfurization, Dibenzothiophene, *Rhodococcus erythropolis* Igts8, *Pseudomonas aeruginosa* Ptsox4, Nanoparticles

Abbreviations: DBT: Dibenzothiophene; BDS: Biodesulfurization; 2-HBP: 2-Hydroxybiphenyl; HDS: Hydrodesulfurization; NIGEB: National Institute of Genetic Engineering and Biotechnology

Introduction

Numerous sulfur-containing compounds found in petroleum are able to cause various effects such as endanger living-things health and making acid rain after being emitted into the atmosphere and also poison catalytic converters of exhaust system. Sulphur can also decrease the value of the fuel by disabling the antioxidant features of fuel and interfere in installations [1,2].

Hydrodesulfurization (HDS) is the current method used in petrochemical plants to reduce the amount of sulfur from fossil fuels. Although this process is plenty useful for removing the element from aliphatic and some aromatic compounds, it does not have enough influence on some cyclic molecules such as dibenzothiophene and its alkylated derivatives. On the other hand, HDS requires high temperature and high pressure which cost a huge budget [3-5]. Biodesulfurization has been the promising technology to compensate the weak points of HDS. In this process microorganisms remove sulfur selectively without breaking the heterocycles. The fuel value is not reduced in this four-step (4S) pathway [5,6]. By using DBT as a substrate in 4S pathway, three intermediate and a final compounds are produced which 2-hydroxybiphenyl (2-HBP) is the last one [7]. There are many microorganism species such as Arthrobacter, Gordonia, Brevibacterium, Rhodococcus and Pseudomonas which are capable of applying 4S [8]. Among all the organisms used in this field, *Rhodococcus erythropolis* IGTS8 is the most studied bacteria for desulfurization of DBT and its alkylated derivatives. This strain contains a plasmid carrying dsz operon genes which encode 4S enzymes [9,10]. After introduction of BDS many genetic manipulations has been exerted to gain efficient microorganisms. *Pseudomonas aeroginusa* PTSOX4 is a species which is generated after transferring dsz genes from *R. erythropolis* IGTS8 to *P. aeroginusa* [11,12].

In recent decade some researches have been performed in studying the effect of Fe_3O_4 nanoparticles coated on bacteria cell wall as a magnetic factor to give a better gathering and separation to the organisms during BDS [13-17]. In some studies also the effect of ZnO and CuO nanoparticles on desulfurization in different conditions such as acidic or as coated on graphene and active carbon has been investigated in which considerable results have been demonstrated [18,19]. Another reports have suggested that nanoparticles (NPs) make the cell membranes of bacteria more permeable thus facilitating transportation of HBP out of the cells [2].

The observation of significantly increased HBP production in the decorated cells suggests that the magnetic nanoparticles might facilitate transport of HBP out of the cells—assuming that it is produced in the cytoplasm. A possible mechanism for the enhancement is that the nanoparticles bound to the bacteria make their membranes more permeable. In order to investigate this hypothesis, the possible effect of nanoparticles on membrane permeability In this study we examined the effect of Fe_3O_4, ZnO and CuO nanoparticles as free factors on the BDS. The bacterial strains used as the biological catalysts were *Rhodococcus erythropolis* IGTS8 and *Pseudomonas aeroginusa* PTSOX4 to have a comparison between a natural bacteria and a genetically manufactured one in desulfurization activity. DBT was also the model molecule we chose for the research which has been intensively utilized as a model material as it is easy to be manufactured [3]. To the best of our knowledge, no previous studies to determine the effect of nanoparticles

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on desulfurization ability of bacterial strains have been carried out so far.

Materials and Methods

Chemicals

Chemicals were purchased as following: DBT and 2-HBP from Fischer chemical co, Gibbs reagent (2, 6 dichlorchinon), Agar, ethyl acetate, glycerol, pure ethanol and HCl from Merck chemical Co., Muller Hinton broth from Sigma-Aldrich Co. All three types of nanoparticles (Fe_3O_4, ZnO and CuO) were purchased from US Research Nanomaterial co with the following characteristics:

Bacterial strain and cultivation

Bacterial strain *Rhodococcus erythropolis* IGTS8 and *Pseudomonas aeroginusa* PTSOX4 were obtained from NIGEB reservoir.

In this work we used Luria Broth (LB) broth for growth of the bacteria. For examination of nanoparticles toxicity, we utilized Muller Hinton Broth (MHB). The media applied for desulfurization was the standard Basal Salt Medium (BSM) which is composed with the following procedure: At first 2.44 g KH_2PO_4, 5.57 g Na_2HPO_4 and 2 g NH_4Cl and then 0.2 g $MgCl_2.6H_2O$, 1 mg $CaCl_2.2H_2O$, 1 mg $FeCl_3.6H_2O$ and 4 mg $MnCl_2.6H_2O$ were dissolved in 850 ml and 150 ml deionized water respectively and eventually mixed together. As source of carbon 105 ml pure glycerol was added into every Erlenmeyer flask containing 100 ml BSM.

Inoculum built-up

Inoculum was gained by means of a standard method. One loop of LB agar was inoculated into a 250 ml Erlenmeyer flask containing 50 ml LB broth then retained in shaker incubator to reach the mid logarithmic phase. Afterward the washing and dilution were applied to obtain the OD_{600}=0.8 – 1 (0.5 McFarland).

Nanoparticles toxicity test

The microtiter plate application was used for determination of possible toxicity of the used nanoparticles. The range of nanoparticles concentration was 1-100 ppm. After the inoculum was prepared, the first column of the microtiter plate contained no bacteria as the negative control. All cells of the second column were filled by culture which contained only bacteria as positive control. The remained cells were loaded with bacteria and target nanoparticles concentration (1-100 ppm). All the cells were sealed to avoid evaporation and then heated 24 h in incubator (30°C) and finally the growth rate was examined by Elyssa reader.

Gibbs assay

1 ml of the bacterial solutions obtained from inoculum preparation was poured into 250 ml Erlenmeyer flasks containing 100 ml BSM with 55 ppm DBT as the sulfur source. Then nanoparticles were added with the final concentration of 20 ppm. Finally the containers were placed in shaker incubator with 120 rpm in 30°C. We also provided flasks containing no bacteria to see if the nanoparticles could run the process without microorganism cooperation.

Every 24 h 5 ml aliquot was centrifuged to remove cells and NPs and then their pH was adjusted to 8 with 10% (w/v) sodium carbonate. Afterward 2 ml supernatants were tested by mixing them with 20 µl Gibbs indicator (0.1 g 2, 6 Dichloroquinone-4-chloroimide in 10 ml ethanol). After a 30 min in 30°C, if the solution was turned to blue, it evinced that DBT had been transformed to 2-HBP so sulfur removal was successfully achieved [20].

HPLC analysis

To quantification of the obtained results, 4 ml samples from the 24, 48, 72 and 96 h of the process were subjected to HPLC analysis. For this, extraction of the produced 2-HBP in each sample was performed by adding 4 ml of ethyl acetate as organic phase. The upper phase (organic) was extracted and the process was repeated twice. After purification, samples were dissolved in acetonitrile and analyzed by HPLC. The mobile phase was a mixture of 40% water and 60% acetonitrile with the flow rate of 1.5 ml/min. The column was C-18 nucleusil-100, 60 × 4.6 mm. The UV detector utilized for this research worked in 245 nm.

Results and Discussion

Growth rate

The bacteria were grown on BSM to obtain growth curve. As it is shown in Figure 1, both bacteria spent a short time about 4 hours to pass the lag phase which is the result of repetitive culturing on the media. The logarithmic phase for Rhodococcus started a little bit slowly till the 16th hour and after that growth rate became faster and the curve changed to be sharper while the stage for Pseudomonas began initially steeply and eventually after about 24 h PTSOX4 and after 37 h IGTS8 reached the stationary stage which remained for 2-3 h. Afterward growth rate turned to a descending level or in other words bacteria death rate was more than the generating rate. As BDS is an application which is performed in logarithmic phase, it is important to have this stage shorter because it shows a faster metabolism and more metabolites (enzymes) released.

Nanoparticles toxicity

Minimum inhibitory concentration (MIC) was examined in Muller Hinton broth in the range 1-100 ppm. The growth rate was recorded by Elyssa reader system for every cell showing values near zero as compared to the negative controls (the cells which contained no bacteria) which is a proof for no growth. In other words, none of the three NPs showed any toxicity on any of the two strains. As the examination was performed three times and every time in triple and as it was carried out based on CLSI standard procedures, it can be concluded that the test has been dependable.

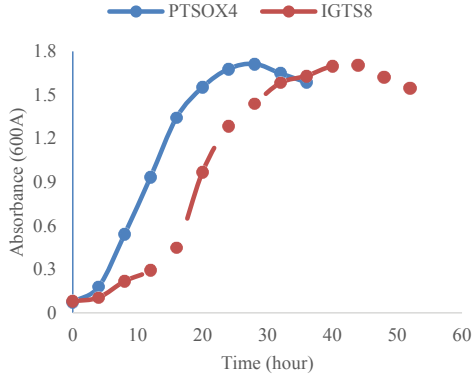

Figure 1: Growth curve for *Pseudomonas aeroginusa* PTSOX4 and *Rhodococcus erythropolis* IGTS8 at 30°C over a 50 h incubation. Each point is a mean of three experiments.

Gibbs assay

Interaction between Gibbs indicator and 2-HBP and therefore appearance of the blue color demonstrated the elimination of sulfur from DBT. Figure 2 shows how the process proceeds as the time continued till 96 h.

Investigation on the cooperation effect of NPs and bacterial strains on the production of HBP

Biodesulfurization of DBT was performed in cooperative systems containing two bacterial strains and Fe_3O_4, ZnO and CuO nanoparticles with different particle sizes. The results were analyzed with HPLC after 48, 72 and 96h of each reaction as described above. As can be seen from Figure 3, cooperation between ZnO nanoparticles and both bacteria shows considerable higher desulfurization of 94% and 58% in case of using *P. aeroginusa* PTSOX4 and *R. erythropolis* IGTS8 respectively. These results show significant improvement in biodesulfurization process of both bacteria in the absence of NPs, in which 68% and 48% were obtained for PTSOX4 and IGTS8, respectively. The observation of significantly increased HBP production in the presence of ZnO nanoparticles may be attributed to permeabilization of cell membranes which facilitates the mass transferring of DBT and HBP into or out of bacterial cells. It is also well documented that the first and rate-limiting step in the oxidative biodesulfurization of DBT is the transferring of DBT from the oil into the cell [21]. The system of using Fe_3O_4 nanoparticles alongside bacteria stands as the second by 70% for PTSOX4 and 49% for IGTS8. The obtained results show that Fe_3O_4 NPs have almost no effect on biodesulfurization yield compared to the results obtained from the process in the absence of NPs. This is in accordance with the results previously reported by Shan et al., in which Fe_3O_4 nanoparticles were utilized as immobilization matrix for bacterial cells [17]. Their investigation showed that magnetic nanoparticles had no direct effect on the BDS process. The obtained results may also be attributed to the effect of nanoparticles to alter microbial metabolism, which leads

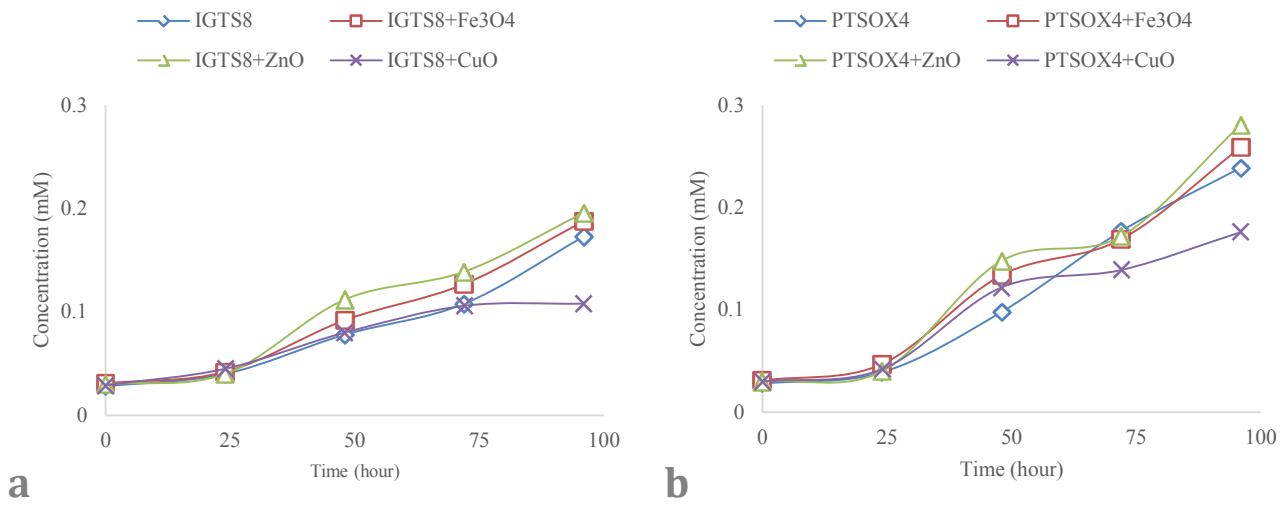

Figure 2: Gibbs assay for *Rodococcus erythropolis* IGTS8 (a) and *Pseudomonas aeroginusa* PTSOX4 (b) in presence and absence of Fe_3O_4, ZnO and CuO NPs (each point is the mean of three experiments).

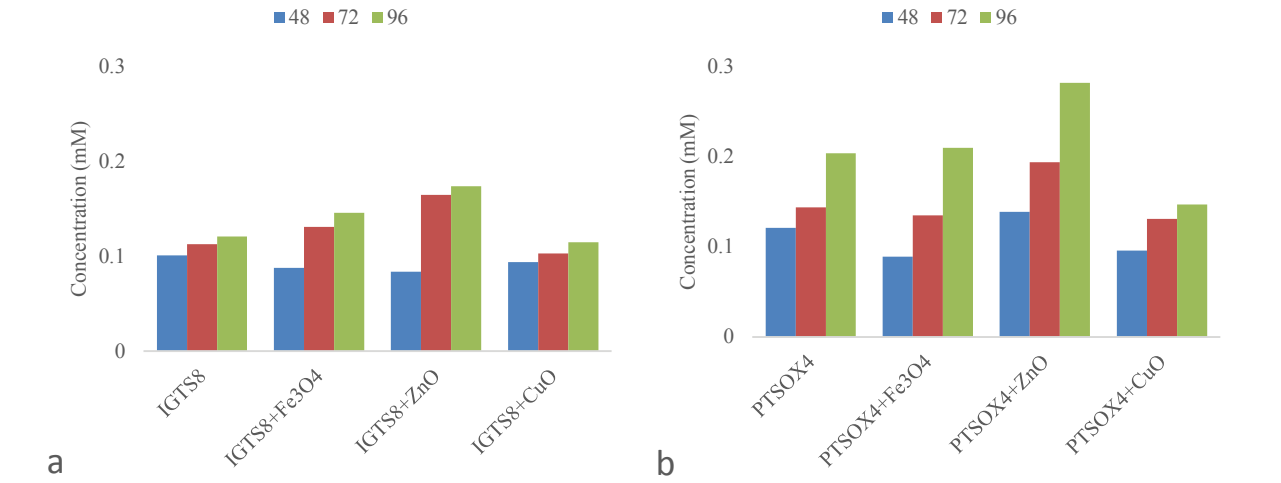

Figure 3: Comparison of 2-HBP production capability by *R. erythropolis* IGTS8 (a) and *P.aeroginusa* PTSOX4 (b) in presence and absence of Fe_3O_4, ZnO and CuO NPs.

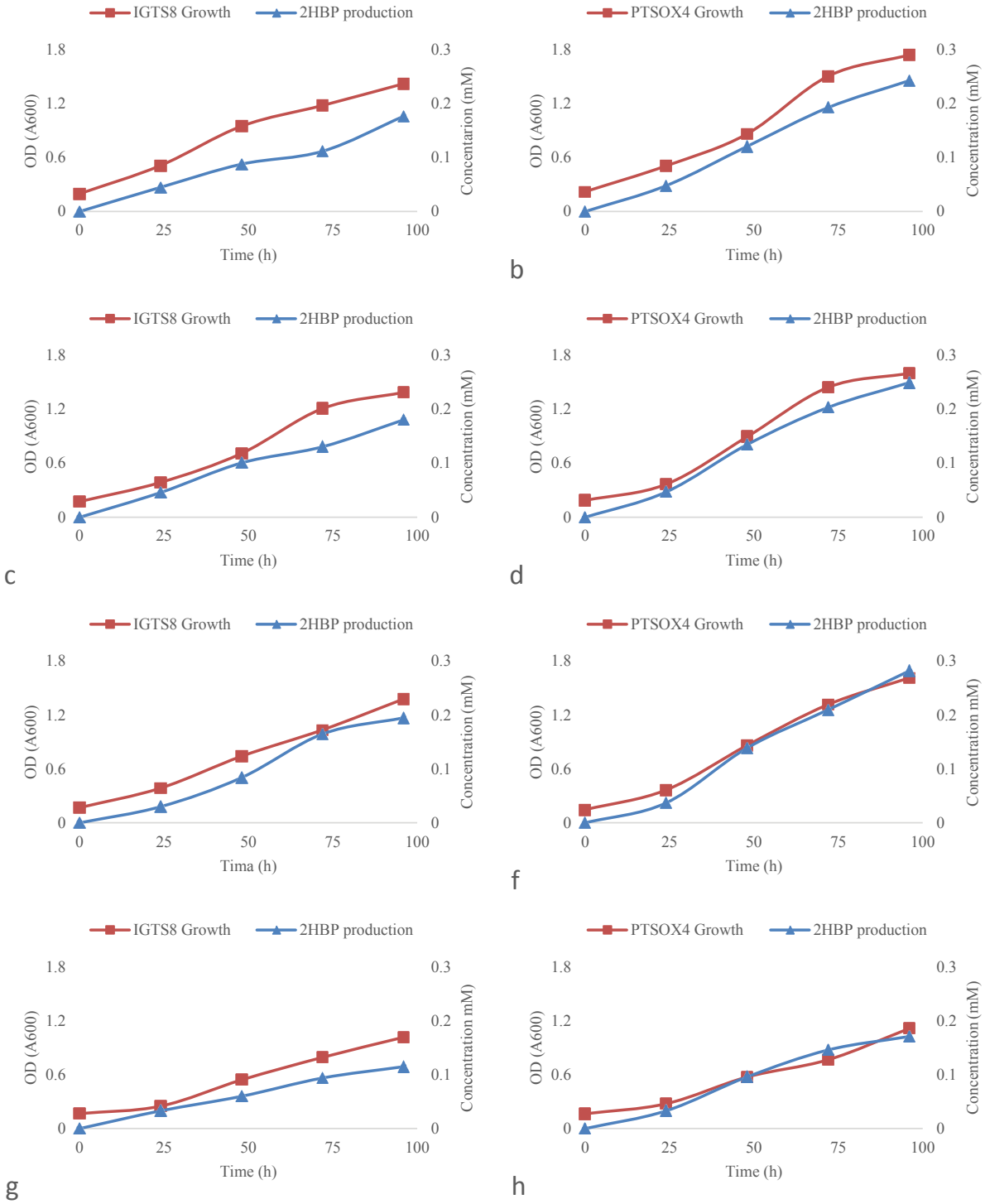

Figure 4: Relationship between bacterial growth of *R. erythropolis* and *P. aeroginusa* and BDS. (a-b) in absence of nanoparticles. (c-d) in presence of Fe_3O_4 NPs. (e-f) in presence of ZnO NPs. (g-h) in presence of CuO NPs.

to significant changes in the released substances in the surrounding environment.

PTSOX4 and IGTS8 also show decreased biodesulfurization activity of DBT in the presence of CuO NPs in comparison with other nanoparticles. Furthermore by a look at the obtained results we observe that utilization of *Pseudomonas aeruginosa* PTSOX4 as the biocatalyst causes more sulfur removal rather than *Rhodococcus erythropolis* IGTS8. Diminished biodesulfurization activities of PTSOX4 and IGTS8 in the presence of CuO may be attributed to its greater particle size of as well as the possible toxicity of these nanoparticles (Table 1).

Nanoparticle	Fe_3O_4	ZnO	CuO
Purity	+99.5%	+99%	99%
Average particle size	35-45 nm	10-30 nm	40 nm
Specific surface area	8-14 m^2/g	20-60 m^2/g	~20 m^2/g
Morphology	Spherical	Nearly spherical	Nearly spherical
True density	7.90 g/cm^3	5.606 g/cm^3	6.4 g/cm^3

Table 1: NPs characteristics.

Connection between growth and BDS

Simultaneously with BDS analysis, growth rate was measured in order to investigate the possible relation between desulfurization and bacterial growth. As it is shown in Figure 4, by increasing in bacterial density, biodesulfurization rate of PTSOX4 and IGTS8 is enhanced. However as Figures 4g and 4h show less improvement in both factors in comparison with the other six conditions can be observed for CuO nanoparticles. Regarding the bacterial growth and sulfur removal, it can be concluded that CuO nanoparticles have toxic effect on bacterial cell in long time and in the poor media, BSM. The difference between the results of toxicity examination and desulfurization is the consequence of utilization of two greatly different cultures. Muller Hinton broth is nutritious while BSM contains only a little amount of glycerol as carbon source. Therefore in MHB bacteria grow faster and as a result CuO NPs showed no reducing effect.

Conclusion

Investigation on the ability of the bacterial strains in biodesulfurization process showed that *Pseudomonas aeroginusa* PTSOX4 is more preferred than *Rodococcus erythropolis* IGTS8 as a biocatalyst. This is perhaps due to its faster metabolism and as a result more enzymes release and more sulfur removal capability. To improve desulfurization activity of these bacteria, three different nanoparticles were used in cooperative systems. According to the results, it can be deduced that utilization of ZnO NPs in the presence of the microorganisms has the potential to increase the rate of sulphur removal from cyclic sulphur containing molecules such as DBT. Higher biodesulfurization capacity of ZnO can be explained by its les toxicity and small particle size compared to CuO and Fe_3O_4 nanoparticles. On the other hand the toxic effect of CuO caused to decrease the bacterial growth of PTSOX4 and IGTS8, thereby lowering the biodesulfurization yield.

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