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Biodesulfurization of light crude oil using *Bacillus subtilis* Wb600

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ABSTRACT

Bacillus subtilis Wb600 is capable of desulfurizing the light crude oil from a two phase system including the aqueous microbial culture and the organic crude oil. This microorganism grows on sulfur-free basal salt medium in the presence of light crude oil with 1.5% total sulfur. *B. subtilis* Wb600 utilizes the sulfur-containing compounds of the oil as a source of sulfur without degrading the hydrocarbon content. The microorganism produces biosurfactants which transfers the organic sulfur compounds into the aqueous phase. The quantity of biosurfactants produced in the microbial culture is about two times higher than the ones produced in the control culture with inorganic sulfur source. Desulfurization capability of the microorganism was tested during 35 h exponential growth phase in a flask with an oil/water phase ratio of 0.2. A total sulfur removal of 40% from light crude oil was found.

Key words: biodesulfurization; biosurfactant; light crude oil; Bacillus subtilis Wb600

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1. INTRODUCTION

Petroleum as primary energy source has found a vast number of application from power generation in thermal power plants to synthesis of chemical derivatives. Petroleum products are mainly composed of three carbon, hydrogen, and sulfur elements. The sulfur content of crude oil ranging from 0.03 to 7.89% that is found in both inorganic and organic forms(1, 2). The high sulfur content in these energy resources and the world's high energy demand on fossil fuels would result in many health, environmental, and technical problems. To avoid these issues arising from the combustion of high sulfur content fuel reserves, legislations imposed by US Environmental Protection Agency and European Union restricted the fuel sulfur content to about 10 ppm(3, 4). Both researchers in academia and industry have developed many techniques such as electrochemical pretreatment(5), alkaline desulfurization(6), absorption and catalytic conversion(7, 8), and hydrodesulfurization(9, 10) in order to decrease the sulfur content of fossil fuels. These techniques were successful in the reduction of sulfur to a great extent. Unfortunately, these thermo-chemical techniques have high installation and O&M costs, are energy intensives, and does not work well on recalcitrant heterocyclic organo-sulfur compounds(4, 11). These organo-sulfur compounds compose a substantial fraction of the petroleum. Table 1 presents the percentage of organo-sulfur compounds in petroleum resources of different parts of the world. The sulfur constituents of these petroleum fractions can be converted to easily removable forms by the use of microorganisms. The biological approach to fossil fuel desulfurization is an attractive alternative from both environmental and energy perspective.

Table 1. Organic sulfur content of some crude oil resources(12)

Source	Organo-sulfur content (%)	Percentage of world crude oil
North Africa	0.18	7.2
West Texas	0.05-5.0	0.25
East Texas	0.26	
Middle East	1.5-3.0	50.3
California	1.0	0.17
Canada	0.44	13.2
Kuwait	2.6	6.9
Venezuela	1.7	20.0

A variety of aerobic and anaerobic microorganisms can be applied for crude oil desulfurization. These microorganisms selectively break the carbon-sulfur bonds in petroleum compounds without degrading the carbon skeleton of the fuel(13). They utilize the sulfur compound as sole sulfur source. The most common biochemical pathway for desulfurization of dibenzothiophene (DBT) and its derivatives is 4S pathway carried out by aerobic bacteria. DBT is a model organo-sulfur compound typically found in crude oils accounting a significant percentage of its total sulfur content. To exploit this pathway in aerobic bacteria for desulfurization of petroleum fractions, a large number of investigations were conducted in recent years so as to develop an efficient desulfurization process. Table 2 summarized a few of these researches. In these investigations, two objectives were considered by the researchers. The first one was to increase the rate and extent of desulfurization from real target molecules by metabolic engineering strategies. The second one was to commercialize the biological process through both innovative design of chemical operating units and the techno-economic optimization of the overall process.

Table 2. Biological desulfurization from different sulfur-containing compounds

Bacterium	Sulfur substrate	Total sulfur removal (%)	References
Paenibacillus sp. A11-2	Methyl, ethyl, dimethyl, trimethyl, and propyl dibenzothiophene	> 25	**(14)**
Rhoddococcus erythropolis KA2-5-1	Alkylated dibenzothiophene	Not available	**(15)**
Rhoddococcus erythropolis XP	4-methyl dibenzothiophene, 4,6-dimethyl dibenzothiophene, benzothiophene	> 50	**(16)**
Mycobacterium sp. G3	4,6-dibutyl dibenzothiophene, 4,6-diphenyl dibenzothiophene, 4,6 –dimethyl dibenzothiophene, 4,6 diethyl dibenzothiophene,	Not available	**(17)**
Pseudomonas delafieldii R-8	Hydrodesulfurized treated diesel oil	47	**(18)**
Pseudomonas putida CECT 5279	Dibenzothiophene	83.6	**(19)**
Gordonia alkanivorans RIPI90A	Diesel	50	**(20)**

This study was conducted on desulfurization of light crude oil by using *Bacillus subtilis* Wb600. This bacterial species is a fast growing bacterium that specifically degrades the sulfur content of fossil fuel with no loss of the calorific value of the fuel. The high specific growth rate and inexpensive culture media are two advantages which can make feasible the commercialization of this bacterial desulfurization process through careful consideration. The metabolic activity of these bacterial species was examined in this work.

2. MATERIALS AND METHODS

2.1. Chemicals

Light crude oil was obtained from Analytical Laboratory of Exporting Petroleum (Kharg Island, Iran). Physical properties of the oil presented in Table 3. The oil was used for biological desulfurization studies without any pretreatment. The oil was autoclaved just before use to remove any microbial flora. All the other chemicals for the preparation of bacterial culture were purchased from Merck (Darmstadt, Germany) and used as received without any further purification.

Table 3. Physical properties of light crude oil

Total Sulfur content (%)	Density ($g.cm^{-3}$)	°API	Salt content (ppm)	Water and sediment content (%)	Viscosity at 10°C (cSt)	Pour point (°C)
1.5	0.8602	33	15	0.1	7	-33

2.2. Microorganism and culture composition

B. subtilis Wb600 was obtained from Persian Type Culture Collection (Tehran, Iran). The *B. subtilis* Wb600 is cultivated in one liter of basal salt medium composed of 5 g glycerol, 0.5 g KH_2PO_4, 4 g K_2HPO_4, 1 g NH_4Cl, 2 ml of 1% $CaCl_2$, 2 ml of 10% $MgCl_2$, 200 µl of 1 % $FeCl_3$, 200 µl of 5 % NaCl, and 0.2 g Na_2SO_4. It should be mentioned that sodium sulfate was removed from the medium in two phase cultures. The pH of the medium was adjusted to 7.0 by the use of concentrated HCl and NaOH solution prior to autoclaving.

2.3. Adaptation of B. subtilis Wb600 to two phase culture

A typical culture of the bacterium uses the sodium sulfate as the sole sulfur source. To desulfurize the light crude oil, the bacterial species were adapted to two phase culture composed of an aqueous basal salt medium free of any sulfur source and the organic phase with various organic and inorganic sulfur compounds. To this end, the microorganisms were cultivated at 308 K on a shaker incubator operating at 170 rpm. A 250 ml Erlenmeyer flask with a working volume of 100 ml was used. The cultivation was continued in the typical basal salt medium for 48 h. After this time period, the cells were separated by centrifugation at 9000 rpm and recultivated in the two phase system at 308 K on a shaker incubator operating at 150 rpm. A 250 ml Erlenmeyer flask with a working volume 180 ml including 150 ml aqueous sulfur-free medium and 30 ml of light crude oil was used.

2.4. Batch growth and desulfurization of bacterial culture

B. subtilis Wb600 was cultivated in the basal salt medium free of any sulfur source. The batch cultivation of the microorganisms was performed in a 500 ml Erlenmeyer flask with a working volume of 180 ml composed of 150 ml aqueous microbial culture and 30 ml of light crude oil. The flasks were incubated at 308 K on a shaker incubator operating at 170 rpm for a period of 50 h. The bacterial growth was determined by measuring the optical density of the culture at 650 nm using UV/visible T80 spectrophotometer (PG Instruments, UK). The organic phase was analyzed for total sulfur concentration at definite time intervals during the exponential growth phase (35 h). Total sulfur content was measured by X-Ray Fluorescence spectrometer available in Research Institute of Petroleum Industry (Tehran, Iran). All the measurements were done in triplicates.

2.5. Measurement of surface tension

To monitor biosurfactant production during cultivation, *B. subtilis* Wb600 was cultivated in the basal salt medium free of any sulfur source. The batch cultivation of the microorganisms was performed in a 250 ml Erlenmeyer flask with a working volume of 180 ml composed of 150 ml aqueous microbial culture and 30 ml of light crude oil. The flasks were incubated at 308 K on a shaker incubator operating at 150 rpm for a period of 90 h. The microbial culture was sampled at definite time intervals and then centrifuged at 9000 rpm to reach a microorganism free supernatant. The surface tension of the supernatant was then measured by ring method using a Sigma703D Tensiometer (Biolin Scientific, Sweden) equipped with a De Nouy platinum ring at 300 K. All the measurements were repeated three times to be sure of the reproducibility of the results.

2.6. Online monitoring of B. subtilis Wb600 metabolic activity

For in depth study of the cellular growth and for exploration of the possible diauxic growth, activity of the bacterial species was continuously monitored by a milibioreactor equipped with oxygen and carbon dioxide sensor. This instrument is capable of online measurement of oxygen and carbon dioxide transfer rate, and respiratory quotient. The bacterial cells were cultivated at 308 K with a rotation speed of 170 rpm in the flasks of the equipment. Each flask has a working volume of 25 ml. Three flasks were inoculated: (1) a control culture with the typical basal salt medium; (2) a two phase culture composed of 23 ml aqueous sulfur free medium and 2 ml of crude oil; (3) a two phase culture composed of 21 ml of sulfur free medium and 4 ml of crude oil.

3. RESULTS AND DISCUSSION

The desulfurization activity of *B. subtilis* Wb600 in the two phase system was studied by online monitoring of the cellular respiration. Fig 1 shows the oxygen transfer rate of three flasks inoculated with the species during 48 h incubation at 308 K and a rotation speed of 170 rpm. Flask No. 4 belongs to one phase culture (control sample) composed of the typical basal salt medium. Flask No. 1 belongs to a two phase culture with an organic to aqueous phase ratio of 0.1. Flask No. 3 is for a two phase system with an organic to aqueous phase ratio of 0.2. The working volume of all these flasks is 25 ml. The cells in the flaks with an organic source of sulfur grow as the one with a typical basal salt medium. This shows the desulfurization

capability of the bacterial species. The specific growth rate of the species in the two phase system is also higher than that of the single phase culture so that the cells go to stationary phase 15 h after incubation and experience death phase 5 h thereafter.

The growth limiting substrate in all these systems is carbon source (*i. e.* glycerol). The cells in flask No. 4 go to stationary phase after 20 h incubation. The cells in flasks No. 1 and No. 3 exhibits no diauxic growth during cultivation in two phase systems providing a reason to lack of any degradation of the carbon skeleton of the light crude oil. Some sharp peaks in the growth phase of the bacterial species can be related to specific metabolic activities or some errors in the measurement by the instrument; however, they are not the issues to be investigated in this study.

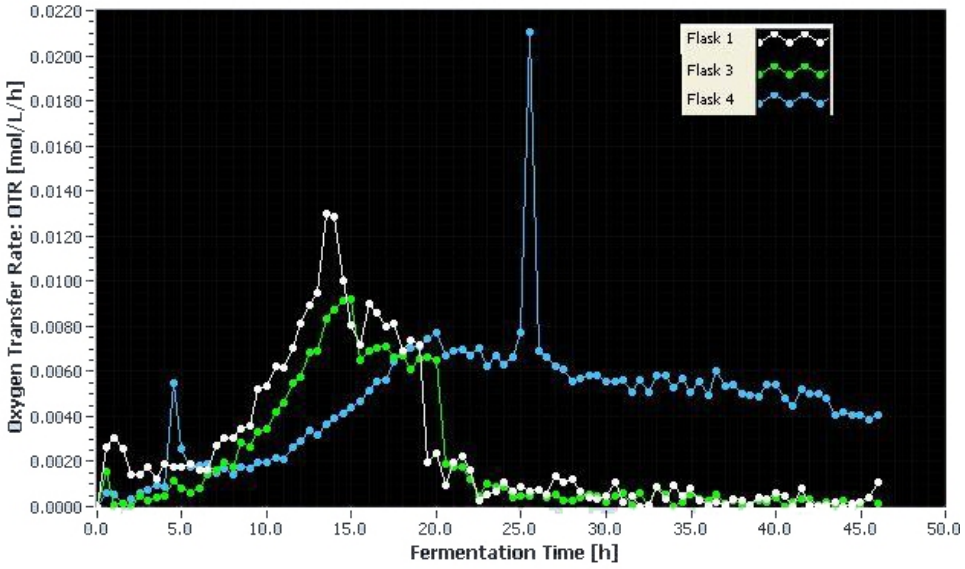

Figure 1. Growth curve of *B. subtilis* Wb600. (blue curve) The bacterial species cultivated in a one phase culture composed of a typical basal salt medium; (green curve) the bacterial species cultivated in two phase culture with oil/water phase ratio of 0.1; (white curve) the bacterial species cultivated in two phase culture with an oil/water phase ratio of 0.2

The oxygen and carbon dioxide transfer rate and respiratory quotient for cells cultivated in flask No. 3 are shown in Fig. 2. The trend of carbon dioxide transfer rate is similar to that of oxygen transfer rate. This provides an explanation that the oxygen transfer rate shown in Fig. 1 is related to cellular respiration and not supposed to be an unexpected error or physicochemical reactions in the system.

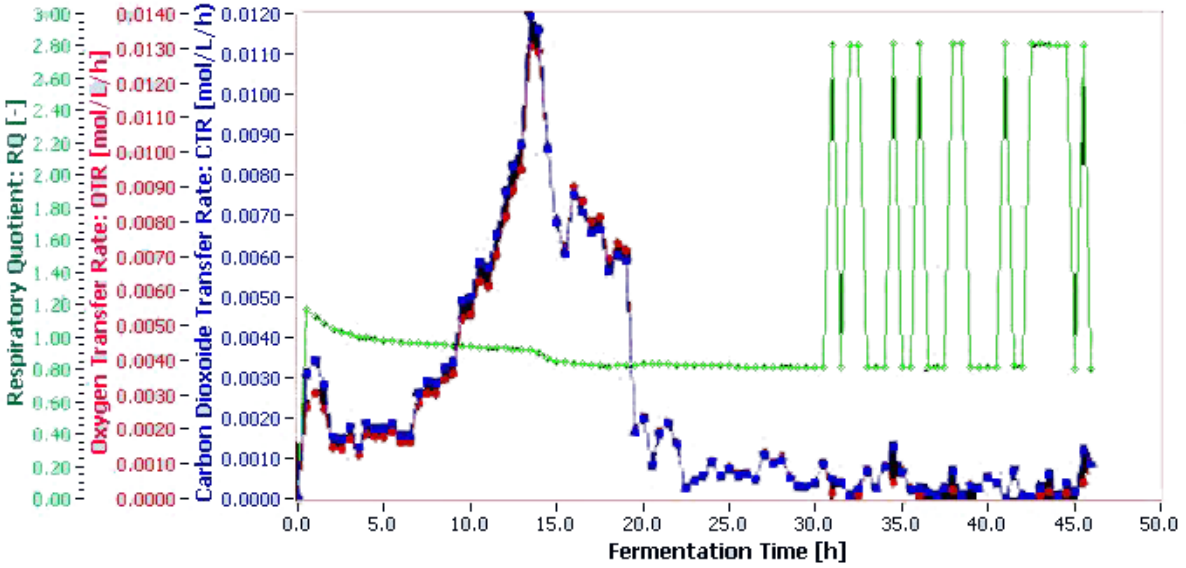

Figure 2. Oxygen and carbon dioxide transfer rate and respiratory quotient in shaking flasks of milibioreactor operating at 170 rpm. The bacterial species were cultivated at 308 K in two phase system with an oil/water phase ratio of 0.2

The microorganism cultivation in two phase systems was performed in two steps. At first, all species were grown in one phase culture of basal salt medium and collected by centrifugation at the end of their exponential phase. Adaptation of the species was then done by inoculating the centrifuged species into a two phase culture composed of an aqueous sulfur free medium and a light crude oil. The microorganisms were stayed 2 days in the lag phase and went to the exponential phase thereafter. These adapted species produced biosurfactants so as to bring the organic phase into the aqueous phase and use the available sulfur compounds of the oil. These species were centrifuged at the end of their exponential phase and used for inoculation of the Flasks No. 1 and 3. The cells adaptation to the two phase system and their capability of exploiting the sulfur compounds of the organic phase was also tested by measuring the surface tension of the aqueous phase. The microorganisms produce biosuractants so as to transfer sulfur-containing oil into aqueous phase. Consequently, the cells would be able to exploit the organo-sulfur compounds in their metabolism. The biosurfactant production was measured at definite time intervals during cultivation of the adapted species. Fig. 3 shows reduction in surface tension of a microbial culture during cultivation in two phase systems (oil/water ratio = 0.2). The cultivation was done in 250 ml Erlenmeyer flasks with a working volume of 180 ml. The flasks were incubated at 308 K on a shaker incubator operating at 150 rpm for a period of 90 h. The biomass growth was investigated during cultivation through turbidity measurement as well.

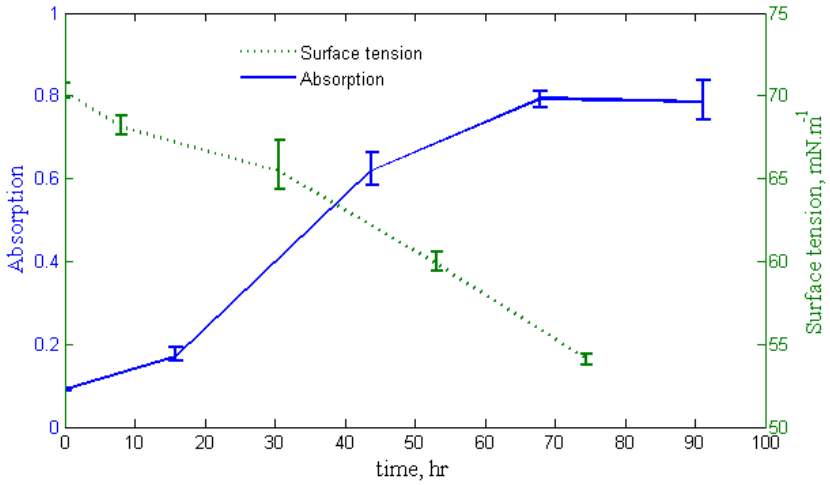

Figure 3. Changes in surface tension and light absorption of the *B. subtilis* Wb600 culture during 90 h incubation on a shaker incubator operating at 150 rpm

The bacterial species are commonly exploited for biosurfactant production in the academic and industrial settings(20, 21) To be sure that the reduction in surface tension is due to production of surfactants with the aim of consuming the organo-sulfur compounds, the reduction in surface tension of two cultures were measured during cultivation. Two cultures are a one phase aqueous media with the typical basal salt medium and a two phase culture composed of a sulfur free aqueous phase and a sulfur containing organic phase (oil/water ratio = 0.2). Fig. 4 shows the trend of surface tension in these two systems. The steeper reduction in surface tension of two phase system can provide a well explanation to capability of the microorganisms in transferring the sulfur containing organic phase into the aqueous one for possible sulfur consumption.

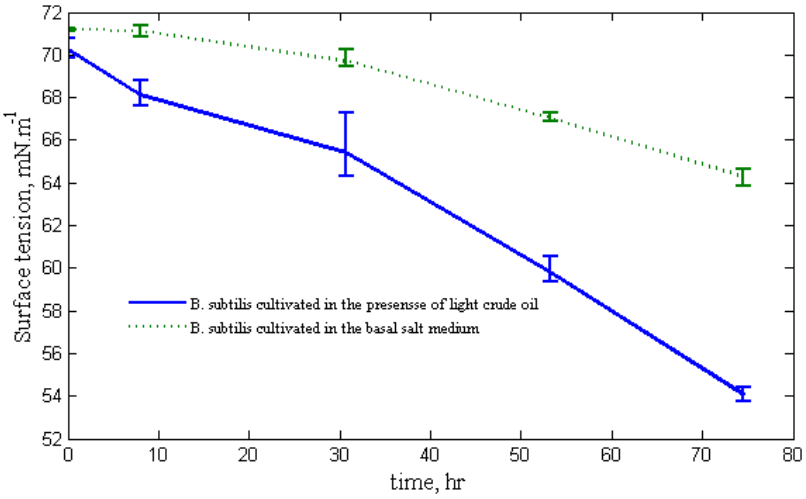

Figure 4. Changes in surface tension of the *B. subtilis* Wb600 culture during 90 h incubation in one phase (dotted line) and two phase (solid line) system on a shaker incubator operating at 150 rpm

Biodesulfurization of light crude oil was further investigated by measurement of total sulfur content of the organic phase. To this end, the batch cultivation of the microorganisms were performed in a 500 ml Erlenmeyer flask with a working volume of 180 ml composed of 150 ml aqueous microbial culture and 30 ml of light crude oil. The flasks were incubated at 308 K on a shaker incubator operating at 170 rpm for a period of 50 h. Samples from the organic fraction was taken at definite time intervals and analyzed for total sulfur content. Fig. 5 shows the continuous reduction in total sulfur content with bacterial growth. The reduction continues till the cells went to the stationary phase. However, microbial desulfurization rate higher than the one obtained in this study was reported in literature(19, 22). These studies are usually worked on desulfurization with organosulfur compounds in an organic solvent or on other bacterial species; where this work is on a light crude oil (a mixture of hydrocarbons).

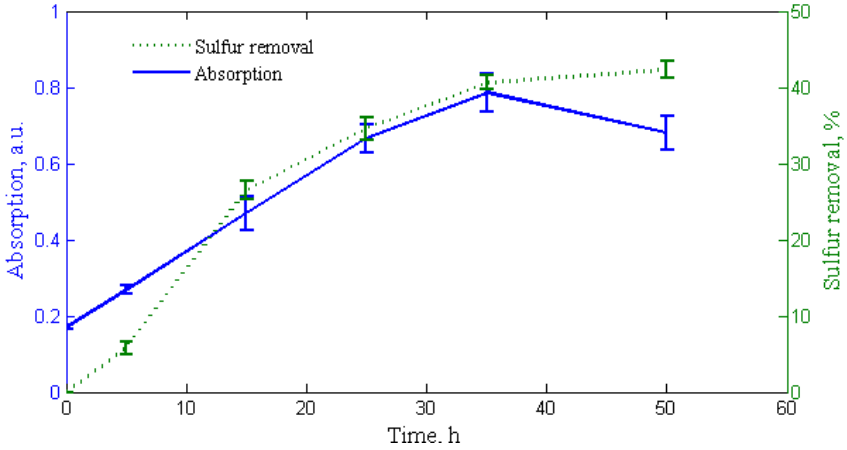

Figure 5. Sulfur removal of light crude oil and light absorption of *B. subtilis* Wb600 culture during cultivation of the species in a two phase system

4. CONCLUSION

Desulfurization capability of *B. subtilis* Wb600 from light crude oil was found in this study. This bacterial species grows on sulfur-free basal salt medium in the presence of light crude oil with a total sulfur content of 1.5%. *B. subtilis* Wb600 utilizes the sulfur-containing compounds of the oil as a source of sulfur without degrading the hydrocarbon skeleton. This bacterial species decreases the total sulfur content of the light crude oil to about 40 % during 35 h exponential growth phase. The simple culture medium of the bacterial species and high specific growth rate of 0.05 h^{-1} are two advantages of this bacterial catalyst regarding fossil fuel desulfurization. Optimization of the incubation conditions and innovative design of process equipments can lead to much higher rate of desulfurization.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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