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Original Article

Biodeodorization of Barrels Containing Natural Gas Odorants by *Bacillus cereus*

Daryush Arabian [1*], **Peyvand Amiri** [1]

[1] University Institute of Applied Science, Malek Ashtar University of Technology, Isfahan, Iran

Corresponding Author: Daryush Arabian, PhD, Assistant Professor, University Institute of Applied Science, Malek Ashtar University of Technology, Isfahan, Iran. Tel: +98-3145914515, E-mail: darabian@mut.ac.ir

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Abstract

Introduction: All sulfur organic odorants used in the Iranian gas industry enter the country in 200-liter barrels. There are ways to clean up the empty barrels contaminated with these materials. In the Gas Company, the currently used method is chemical oxidation (using sodium hypochlorite and caustic). In this study, the biological desulfurization and degradation method of mercaptan was studied.

Materials and Methods: Desulfurizing bacteria in the university microbial collection, together with bacteria isolated from gas odorant barrels, were examined, among which one of the species had the highest and fastest decomposition rate. This bacterium belongs to the *Bacillus cereus* family. The most important factors affecting biological desulfurization including initial bacterial concentration, the concentration of odorant, and the Oil Fraction Phase (OFP) were optimized.

Results: These three factors were studied using an experimental design. Initial bacterial concentrations were evaluated at five levels from 10 to 50 ml with an optimum concentration of 30 ml. The OFP was also evaluated at five levels from 10 to 90%, with 50% being optimized. Concentrations of odorant were also evaluated from 2500 to12500 ppm, with an optimum concentration of 7500 ppm.

Conclusions: Operational testing was carried out in one of the barrels in the optimized conditions for 48 h. The results showed 79.8% efficiency in removing odorant.

Keywords: *Bacillus Cereus*, Bio-degradation, Bio-desulfurization, Mercaptan, Optimization

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Introduction

Biotechnology has introduced new applications in the oil and gas industry. These applications include the removal of undesirable substances such as sulfur, nitrogen, heavy metals and aromatic compounds.[1] Sulfur atoms make up 0.5 to 1 percent of the bacterial cell's weight. Microorganisms need sulfur for their growth and biological activity. Sulfur is usually found in the structure of some cofactor enzymes (such as cofactor A, thiamine and biotin) and amino acids and proteins. Microorganisms can supply the required sulfur from a variety of sources depending on their enzymes and metabolic pathway.[2-5] In the process of biodesulfurization, the sulfur atom of organic compounds is separated by bacteria without breaking the carbon structure, the process being carried out in the presence of water and oxygen and under mild operating conditions (room pressure and temperature) and without the need for hydrogen. Numerous studies have been done to select suitable microorganisms that have the ability to desulfurize organic compounds.[6-9] The development of bi-sulfurization method requires access to active microorganisms that can use sulfur organic compounds as a source of sulfur. Much research has been done to determine the factors affecting the bio-desulfurization process. It has been shown that the activity of desulfurization is strongly dependent on the number of alkyl carbons and the location of these groups on the benzene rings also has a great influence on the activity of the microorganisms. Also, the overall kinetics of conversion of these compounds depend on the concentration and distribution of different types of attached groups and the number and length of the alkyl groups. The most important influencing variables are: nutrient culture medium for the bacterium during the process, solvent toxicity for different bacterial species, the effect of organic to aqueous phase ratio on bacterial growth and germination rate, bacterial concentration and initial sulfur content.[10] Numerous studies have been conducted on the composition of aqueous medium. In a study conducted to optimize the culture medium of the bacterium *Rhodococcus erythopolis*, glycerol and ammonium chloride have been proposed as carbon and nitrogen sources, respectively.[11] Despite the results published on optimizing the bacterial culture medium, it is difficult to use the information provided. Because of the

complexity of the effects of nutrients and permeability and the composition of different substances in the medium, it is impossible to express a simple relationship between the composition of the material and the rate of germination.[10] Another factor that should be taken into account is the effect of the organic phase on bacterial activity. The importance of this is in isolating and restoring bacteria. In a study, the effects of two toxic solvents of octanol and toluene were evaluated on seven species of *Rhodococcus*. The species were in contact with the solvent and the desulfurization activity, growth and oxygen consumption were evaluated. A relationship has been observed between the solvent inhibition of oxygen consumption and desulfurization activity but no relationship has been seen between bacterial growth and desulfurization activity. As a result, oxygen consumption can be investigated as a sign of the inhibitory effect of the solvent.[12] The effect of bacteria on the formation and breakdown of aqueous-organic emulsions should also be investigated. The importance of this agent in biodesulfurization of low boiling point feeders, which are the most toxic environment for bacteria is higher.[13-15] In a study on *Rhodococcus erythopolis*, a reduction in sulfur content was observed with increasing bacterial concentration. In this system, when the concentration of bacteria exceeds a certain level, limiting mass transfer issues become more important. This is due to the inability of some cells to contact the organic phase. In this case, the problem of mass transfer increases because the rate of removal of sulfur within the cell is greater than the rate of transfer from the organic phase to the cell. This limitation is also probably due to the difficulty of transferring external mass from the organic phase into the cell, which is more difficult than mass transfer from the cell membrane. As a result, the transfer from the membrane is an issue that is of greater importance in large and multi-branched derivatives.[16] Biodesulfurization is known as an aqueous-organic two-phase process due to the need of bacteria to water. Numerous studies have been conducted on this factor.[17] In a study on *Rhodococcus erythopolis*, the rate of removal of sulfur compounds increased with increasing organic phase volume.[18] Bacterial concentration is an important factor in the design and performance of the process. The more activity of the bacteria, the less biocatalyst needed. However, high bacterial concentration even in the case of highly active biocatalysts can reduce process costs. As a result, it is important to find the maximum bacterial count. In many studies, an optimal value for bacterial concentration has been reported.[10] Another factor that should be evaluated in this process is the concentration of sulfur in the feed. In a study on *Pseudomonas putida* and *Rhodococcus erythopolis* bacteria, the effect of initial concentrations of sulfur was investigated. It is observed that the two bacteria have the same function. *Pseudomonas putida* had more bio-desulfurization activity with increasing sulfur content. This is due to the increased penetration of matter into the aqueous phase. The increase in desulfurization activity of *Rhodococcus erythropolis* is also due to its ability to bind to organic solvents and to react at aqueous-organic levels.[18] In the field of biodegrading of mercaptan, in a study by Badr et al., in 2014, the removal of methanothiol from gas and water was investigated biologically by *Thiobacillus thioparos*. This study was carried out inside a semi-batch bioreactor and the removal efficiency and optimum conditions were obtained. In this study, the effect of operating parameters such as initial methanothiol (MT) pH, temperature and dissolved oxygen and initial bacterial concentration along with reaction time were evaluated, with 94% efficiency, which was achieved in 300 minutes.[19] Badr et al. investigated the mathematical model of the biological elimination of methyl mercaptan in batch bioreactor. In this study, they proved that the main product of decomposition of mercaptan is low oxygen or high concentration of methyl mercaptan sulfur and is the predominant product at high oxygen concentration. This actually showed that the model can easily predict the behavior of the system. At low oxygen concentrations, only 14% of the sulfide is converted to sulfate, and at high oxygen concentrations (4.5 ppm) it reaches 60%.[20] Another study by Angelis in 2012 examined the removal of mercaptan and H_2S from natural gas.[21] Methane is a major element in natural gas, but there are other substances such as ethane, propane, butane and pentane, along with nitrogen water, carbon dioxide and sulfur compounds. Among the sulfur compounds, H_2S and mercaptan with a small amount of carbonyl sulfide are present. In this research, optimization of the biological degradation and desulfurization of gas odorants (mecaptan and disulfides) process was studied inside the reactor. The most important factors affecting the processes including initial bacterial concentration, concentration of odorant and solvent concentration were selected and optimized.

Materials and Methods

Bacterial Characteristics

In this study, the performance of 17 bacteria isolated from oil-contaminated soil was first investigated. Then the appropriate bacterium with the highest degradation and desulfurization rate was selected.

Methods for Isolating Bacteria

Nutrient broth culture medium was prepared and 100 ml of it was added to 250 ml Erlenmeyers. Different percentages of the odorant sample include 1%, 2%, 3%, 4%, 5% were then added to the culture medium and incubated for one week at 35 and 45 °C. After this stage, 0.1 ml of each culture medium was transferred to Nutrient agar medium to obtain pure bacteria.

Table 1. Parameters and Levels set for Use in the RSM Method and Conditions of Experiments Suggested

Factor name	Unit	Low	High	levels				
Bacterial Con.	ml	10	50	10	20	30	40	50
OFP	%	10	90	10	30	50	70	90
Odorant Con.	ppm	2500	12500	2500	5000	7500	10000	12500

Run	Factor 1 A:Bacterial Con.	Factor 2 B:Solvent Con. (Isopropanol)	Factor 3 C:Odorant Con.
	ml	%	ppm
1	30	117	7500
2	10	10	12500
3	10	90	12500
4	50	90	12500
5	10	10	2500
6	30	40	7500
7	30	40	7500
8	30	40	7500
9	30	40	7500
10	50	30	7500
11	10	50	7500
12	30	40	7500
13	30	40	7500
14	10	90	2500
15	30	50	2500
16	50	10	2500
17	30	50	15909
18	50	10	12500
19	50	90	2500
20	30	20	7500

Investigation of Variables Affecting Biodegradation

The most important variables affecting biodegradation are: bacterial concentration, OFP (isopropanol used as solvent in this study), and sulfur content.[16] The effect of each of these variables on bacterial performance was investigated.

RSM Optimization

The RSM method was used to study the kinetics of the process and to obtain optimal conditions. In this method, three parameters of bacterial concentration, solvent concentration and odorant concentration are considered and for each of these variables, five levels are set as shown in Table 1. Using the Design Expert 10 software and using the level shown, 20 experiments are proposed. The test instructions are shown in Table 1.

Sample Analysis

Samples were analyzed by gas chromatography-mass spectrometry, consisting of two parts GC (Model 780 A) and mass spectrometer (MS Model 5975C, manufactured by US AGLIENT). The injector was heated to a constant temperature of 250 °C and a split ratio of 100.1. The oven was initially at 40 °C for one minute and kept at this temperature. The incremental temperature was then heated to 10 °C per minute, then to a final temperature of 232 °C. The increase of 12 °C per minute was maintained at this temperature for a minute. The helium flow 1.3 ml/min was used as a carrier gas.

Results

Select the Appropriate Microorganism and Identify Its Features

The performance of 17 strains of bacteria isolated from oil-contaminated soil was evaluated along with two bacteria isolated from mercaptan barrel samples. The experiments were carried out at mercaptan concentration of 500 mg/L. Bacteria were grown in a volume of 2 ml of the broth (pre-culture) and added to the organic phase mixture and the specified culture medium (100 ml). Among the isolated bacteria from the oil medium, 12 strains did not change the amount of mercaptan. The degradation rate of the other five bacteria is shown in Table 2, while among the two bacteria isolated from mercaptan barrel samples, one sample was able to reduce mercaptan levels. Accordingly, the two species, C and D, along with the specimen isolated from the barrel, had the most activity. The effect of time on this process was investigated to select the appropriate bacteria. The total duration was four days and analysis was performed at 24-h intervals. The results are shown in Figure 1. The degradation activity of the bacterium C is noticeable only for about 48 h and then it becomes slow. Therefore, bacterium D was selected as a suitable microorganism for the next stages of research. After selecting the appropriate microorganism, its cell growth was evaluated. The cell growth chart at 600 nm over time is shown in Figure 2. The 16S rRNA detection test was also performed with the target kettle (Type D). This strain was previously identified as *Bacillus cereus* (GenBank databases accession number is JF705198).[22]

Results of the RSM Method

The proposed experiments of the RSM method were performed (Table 3). After performing the experiments and recording the data on degradation rate, the results were analyzed using the Design Expert software. The first step in

analyzing the results is selecting the right model for the system that can predict the results accurately. To this end, the software proposes a quadratic model. The model proposed for the system consists of three terms of single component effects (A, B, C), three terms of dual effects (BC, AC, AB) and three terms of curvature effects (C^2, B^2, A^2). However, not all of these parameters have a significant effect on the model, so eliminating ineffective parameters can make the model simpler. The p-value scale is used to eliminate ineffective parameters. The specified value for each parameter in the software indicates the effect of the parameter on the system response so that if the scale value for each factor is less than 0.05, it indicates the significant effect of this factor on the system response. Factors with p-value of greater than 0.1 are removed from the model so that eventually factors with a p-value less than 0.1 remain in the model. Although the p-value of the factor impact factor is less than 0.05, factors with p-value less than 0.1 remain in the model because these factors may also influence the response. The p-values for the factors affecting the amount of degradation for the initial model are presented in Table 3.

Table 2. Deodorization Rate of five Bacterial Strains

Species	Remained Mercaptan (ppm)	Biodegradation (%)
A	361.16	27.7
C	271.1	45.8
D	247.7	50.4
F	350.2	29.9
G	410.4	17.9
Barrel Bacteria	290.6	41.8

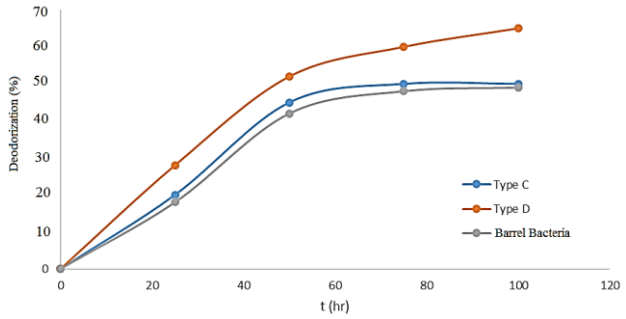

Figure 1. Changes in the Activity of the Isolates Over the Time.

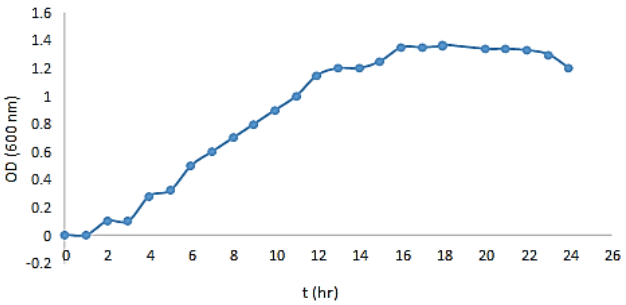

Figure 2. Cell Growth Over the Time.

Regarding the remaining parameters in the system, finally a model is presented to predict the amount of degradation with five parameters affecting the system, which has a regression constant of 0.9845. Therefore, it can be said that this model predicts the rate of gas odorant biodegrading very well. As it can be seen in Table 4, the p-value for the model is less than 0.0001, which indicates the significance of the model. Adequate precision (comparison between the predicted range using the model and the average prediction error of more than 4 is desirable) is equal to 23.496 and more than 4, which is also a desirable factor for the model. Adjusted-R^2 and predicted-R^2 values are 0.9706 and 0.9555, respectively. In addition, the p-value for lack of fit is 0.9687 which indicates that the test is unimportant and that is desirable for the model. The model presented for the update rate according to the coded factors is given below. The model presented for the degradation rate according to the coded factors is also given below. This model includes single and curvature effects. The following equation is the final equation after eliminating ineffective factors in the model.

$$\text{Bio-Degradation} = +87.02 + 8.10^{*} A - 5.50^{*} B + 5.30^{*} C + 4.00^{*} AC - 0.75^{*} BC - 12.17^{*} A^2 - 13.23^{*} B^2 - 7.97^{*} C^2$$

Given the coefficients of the factors in the above equation, the importance of each factor on the rate of gas odorant degradation can be understood. From the equation above and Figure 3, it can be concluded that the individual effects of all parameters except the solvent concentration (OFP) on the rate of bio-degradation are positive. This means, by increasing all the parameters except the OFP, the rate of degradation increases. Regarding the coefficients, the trend of the most important factors on the process are: bacterial concentration, OFP, odorant concentration. In addition, according to the obtained equation, all three factors have a curvature effect on the process. Figure 1 shows a diagram of the changes of the factors affecting the system at one point in the test space (concentration of odorant 7500 ppm, bacterial concentration 30 ml equal to 18×10^7 and solvent concentration equal to 50%). Once the model is properly verified, the software allows process optimization. The goal is to maximize the odorants degradation. The limitations that apply to optimization are that all three parameters fall within the initial defined range. Among the modes the software proposes, conditions are chosen that have a higher solvent concentration, as one of the problems with the industrialization of the biological degradation process is the large-volume aqueous phase needed. Optimal points obtained from RSM experiments are bacterial concentration 18×10^7 (cell/ml), solvent concentration 50 (%) and odorant concentration 7500 ppm with a maximum of 92% biodegradation efficiency.

Table 3. Results of RSM Test and ANOVA Analysis

	Factor 1	Factor 2	Factor 3	Response 1
Run	A:Bacterial Con.	B:Isopropanol	C:Odorant Con.	biodegradation
	ml (1ml=0.6 *10^7 cell/ml)	%	ppm	%
1	30	117	7500	40
2	10	10	12500	60
3	10	90	12500	35
4	50	90	12500	60
5	10	10	2500	44
6	30	40	7500	92
7	30	40	7500	89
8	30	40	7500	93
9	30	40	7500	86
10	50	30	7500	80
11	10	50	7500	66
12	30	40	7500	85
13	30	40	7500	82
14	10	90	2500	44
15	30	50	2500	75
16	50	10	2500	50
17	30	50	15909	72
18	50	10	12500	84
19	50	90	2500	55
20	30	20	7500	83

Factor	p-value
A-Bacterial Con.	< 0.0001
B-Solvent Concentration	0.0004
C-Odorant Con.	0.0004
AB	0.4592
AC	0.0058
BC	0.0002
A^2	< 0.0001
B^2	< 0.0001
C^2	< 0.0001

Effect of Bacterial Concentration

As shown in Figure 4, it is observed that by increasing the bacterial concentration from 10 to about 30 ml, the degradation efficiency increases. At higher concentrations, the rate remained constant and no significant difference was observed in the 40 ml concentration compared to the optimal concentration (30%) and the highest rate (90%) was obtained at 30 ml concentration.

The Effect of Solvent Concentration (OFP)

According to Figure 5, increasing the solvent concentration by about 45 to 50% leads to a slight increase in the efficiency, but the higher percentage decreases the bacterial activity sharply. The difference in efficiency at the highest concentration (90%) is reduced by 30% compared to the optimal concentration (50%).

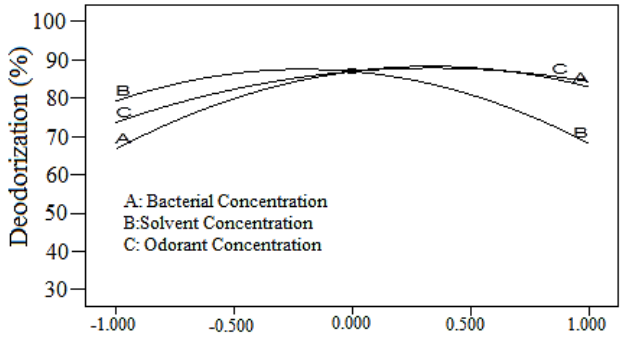

Figure 3. Changes in the Factors Affecting the System at One Point in the Experiment.

Effect of Concentration of Odorant Substances

As it can be seen in Figure 6, increasing the concentration of odorant has a less effect than the bacterial concentration on the process yield, and the rate increases with a slower slope and its optimum value is about 8000 ppm, after which the yield remains constant. However, according to the results of analysis of variance, these three parameters have significant interaction which are shown as surface diagrams. In fact, surface-level diagrams are three-dimensional diagrams that are plotted as a function of two different independent variables in the range of experiments, while other variables are on a fixed surface.

Interaction between Bacterial Concentration and Odorant Concentration

According to the curve in Figure 7, by increasing the bacterial concentration and with an increase in the concentration of odorant, the activity of bacterial increases. Thus, according to the curve in Figure 7, the two parameters in the range of 30 ml < bacterial concentrations < 40 ml and 8500 ppm < odorant concentration < 10000 ppm were in their optimum range. This interaction increases the optimum concentration of odorant relative to the single-factor mode.

Interaction between Odorant and Solvent Concentrations

According to the curve in Figure 8, with increasing solvent concentration, and concurrently with increasing concentration of odorant ,biodegradation grows. Thus, as it is evident in the curve in Figure 8, these two parameters were in their

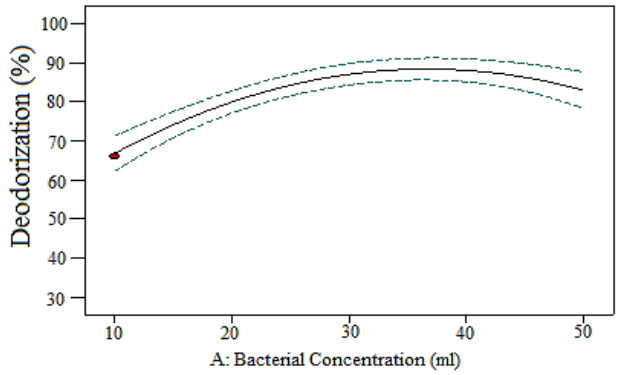

Figure 4. Effect of Bacterial Concentration on the Process Rate.

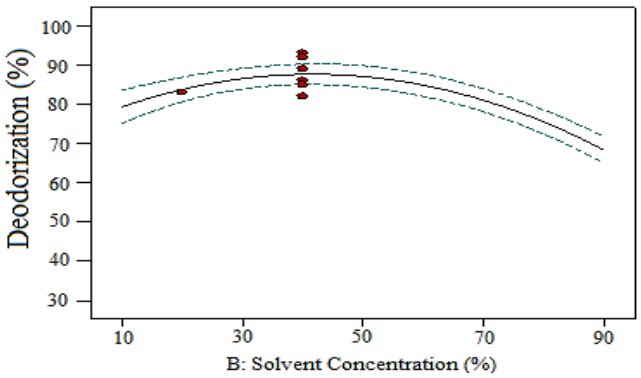

Figure 5. Effect of Solvent Concentration on the Process Rate.

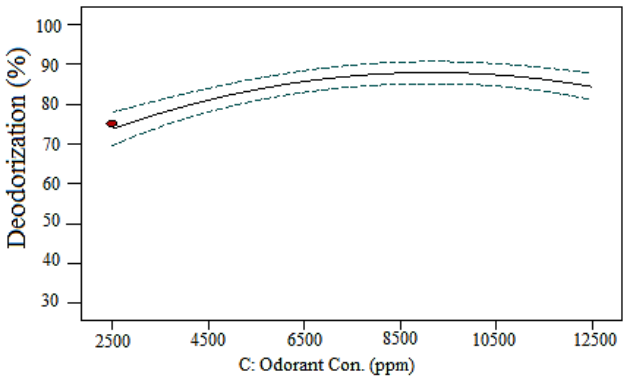

Figure 6. Influence of Odorant Concentration on the Process Rate.

optimum range in the range of 50 < solvent concentration < 60 and 8500 < odorant concentration < 10000. This interaction increases the solvent concentration required for single-factor brewing.

Field Test Result

At the optimum point obtained from the previous stage, the pilot test was performed. For this purpose, the residual amount of odorant in the barrel was obtained by measuring the weight of the barrel, which was about 500 ml. The remaining 500 ml of the solvent was added to the residual solution and was placed on the stirrer as shown in Figure 9 for 1 h to wash the barrel. Then, 1 liter of cultured bacteria was added to the contents of the barrel and was placed on the stirrer for 48 h. Sampling was done within 48 h to measure the amount of material removed and analyzed by gas chromatography. According to Figure 10, after 48 h the amount of biodegradation reached 79.8% near the laboratory value. Since the obtained efficiency after one time purification was 79.8%, the samples were once again poured into the barrel for higher efficiency, and by adding a new bacterial sample, the biodegradation process of the barrel's content was evaluated for another 48 h. Samples were taken at 24 h and 48 h intervals and chromatographic analysis was performed. During the first 24 h of re-testing, the process rate was high and the residual odorant content was less than 9% but a slight decrease was observed in the odorant content in the second 24 h and the remaining amount was about 6.5%. Therefore, it is concluded that in order to achieve maximum efficiency, at least three barrels should be triangulated together and the barrel output to be rotated within 72 h to obtain maximum biodegradation inside the barrels.

Discussion

In examining the effect of bacterial concentration, it was observed that by increasing the bacterial concentration from 10 to about 30 ml, the degradation efficiency increases and at higher concentrations the rate remained constant. The reason is, as the bacterial concentration increased, some cells were unable to contact the organic phase. In fact, in this case, the rate of conversion of the odorant material into the cell is higher than the rate of transfer from the organic phase to the cell, and the transfer of sulfur from the organic phase to the cell is the controlling mass transfer resistance. On the other hand, with nutrient deficiencies and increased carbon dioxide in the environment, resulting in more acidification, cell death increases. Consequently, the odorant degradation rate decreases even after overcoming the mass transfer resistance and transferring the odorant material to the aqueous phase due to the lack of active biocatalyst. In this regard, an optimal value for the initial bacterial concentration has been reported in literature.[16,23] Achieving a better desulfurization rate with higher biomass concentrations is also reported by other studies.[18,24,25]

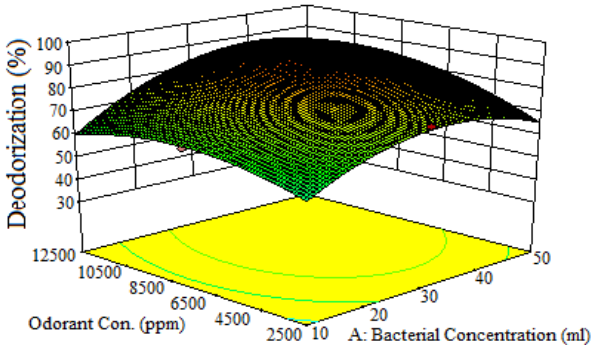

Figure 7. The Interaction between Bacterial and Odorant Concentrations on the Process Rate.

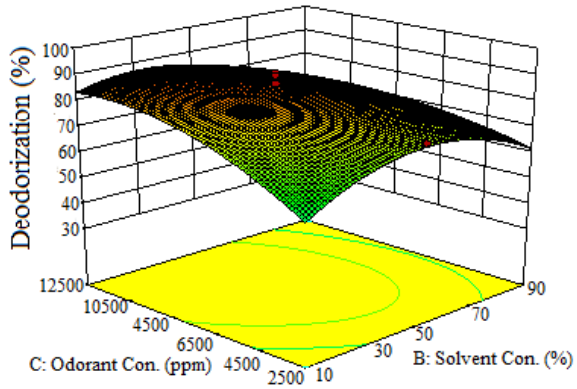

Figure 8. The Interaction between Solvent and Odorant Concentrations on the Process Rate.

Figure 9. How to Close the Barrel to the Stirrer.

It should be noted that since the present study is the first study on bio-deodorizing of barrels containing odorants (mercaptan and disulfides), the results are compared and discussed with studies on the desulfurization of various organic materials.

A decrease in the amount of bio-desulfurization with increasing organic phase concentration has also been reported in other studies on bio-desulfurization.[26-28] This is due to the high hydrophobicity of sulfur compounds. It is necessary for the odorant to reach the common level of the aqueous-organic phase in order to perform the reaction with bacteria. On the other hand, by increasing the volume of the organic phase, the amount of odorant at the joint surface and consequently in the aqueous phase decreases. For this reason, a decrease in desulfurization and biodegradation is observed.[18,25,29]

At higher odorant concentrations, they do not work to improve degradation yields, but the process yields do not decrease with increasing concentration. This behavior has been previously reported for bio-desulfurization of different substances like DBT, coal, diesel etc. by researchers.[18,30-32] In the present study we achieved 79.8% desulphurization. This amount of desulphurization has already been obtained in other studies for materials such as diesel, DBT and coal but in laboratory test levels with much lower concentrations of sulfur.[29,30,33] In this study, in the operational test with much higher concentrations of sulfur, this level of efficiency was achieved.

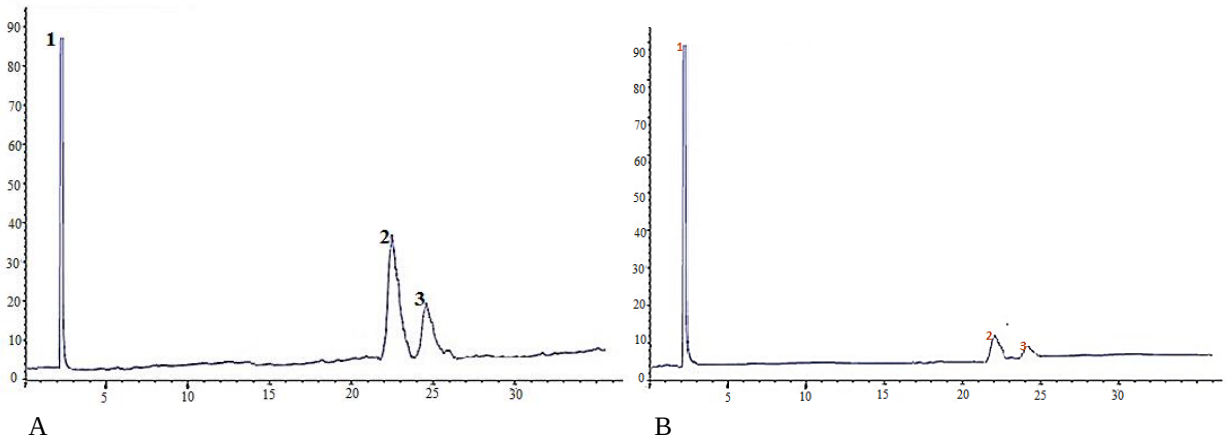

Figure 10. Gas Chromatography Analysis. (A) The Control Sample; (B) After 48 Hour's Incubation. 1) solvent (Isopropanol); 2) T-Buthylmercaptan; 3) Methyl Ethyl Sulfide.

Conclusion

Overall, two bacteria from the collection specimens and one from the barrel specimens had good performance by examining the bacteria present in the university collection as well as bacteria isolated from mercaptan-containing barrels. A temporal comparison of these three bacterial species showed that one of them performed better and faster, which was selected for further studies. The performance of this species is comparable to that of suitable chemical methods, which can be improved by optimizing its growth and activity. Summing up the optimization results show that decreasing the solvent concentration and increasing the concentration of odorant increases the degradation rate while there is an optimum value for

bacterial concentration. By using the statistical modeling with the RSM method, we obtained the equation of sum of three effective parameters. Examination of this equation shows that three parameters: bacterial concentration, solvent concentration, and odorant concentration are interacting, and bacterial and solvent concentrations have a curvature effect while the effect of odorant concentration is almost linear. In the optimum case obtained by the RSM method, the solvent concentration is 50%. This reduces the amount of aqueous phase needed, which is very desirable. The temperature effect study showed that the bacterial incubation rate at 40 °C was more than 30 °C. This remarkable property of the microorganism will significantly reduce the cost of cooling if biofuels are used after chemical biofuels.

Authors' Contributions

All authors contributed equally to this research.

Conflict of Interest Disclosures

The authors declare that they have no conflict interests.

Acknowledgment

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