

## Assessing the Biological Inhibitors Effect on Crude Oil Wax Appearance Temperature Reduction

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### ABSTRACT

To assess the effect of micro-organisms on the reduction of wax appearance temperature (WAT) of waxy crude oil, some appropriate strains were obtained from contaminated samples exposed to hydrocarbon compounds for a long time. By conducting some screening tests, four strains were chosen and aerated in a bioreactor; they were then grown in some hydrocarbon environments in order to produce biological inhibitors. The ability of the biological inhibitors in wax deposition prevention or reduction of WAT is assessed. The WAT is determined by means of the optical absorption spectroscopy method. The absorption plots show that biological compounds are highly effective in reducing WAT; however, different strains are not of the same efficiency. In some cases, the efficiency of biological inhibitors is more than chemical inhibitors. The optimization experiments were run with the objective of achieving the maximum WAT reduction through the Taguchi design method, and the optimum cultivation condition was identified. According to the analysis of variance, pH with a contribution of 49.63% is the most influential factor on the cultivation of the most efficient micro-organisms. The factors of temperature, the inoculation fluid, and nitrogen concentrations are ranked after pH with the contributions of 32.39, 7.92, and 1.39% respectively.

**Keywords:** Waxy Crude Oil, Biological Inhibitors, Wax Appearance Temperature

### INTRODUCTION

Wax deposition phenomenon always threatens oil industry companies by serious problems such as damaging the oil reservoir, choking the pipelines, causing accumulation of oil sludge at the bottom of oil tanks [1], increasing viscosity, necessitating to pump power increment, and forming waxy gel leading to serious situations after long shutdowns periods [2]. More frequent pipeline cleaning operations and implication of

various wax inhibitor methods impose more costs on crude oil transportation companies [3]. Since wax deposition removal operations, especially in deep-water pipelines, are technically very difficult and costly, oil companies always pursue for new methods to prevent wax deposition or its removal. There exists various methods, including pipelines thermal insulation [4], coating the pipelines inner surfaces [5], mechanical methods [6,7], and chemical methods [8-10] in order to control and

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reduce wax deposition or remediate wax deposit. Very detailed schedules should be utilized to minimize the cost and frequency of mechanical pipeline cleaning operations like pigging [6] or a combination of several chemical and mechanical methods which are implemented to increase the wax removal efficiency [11]. Normally, efficient and economic strategies are adopted with respect to determining deposition rate, the characteristics of the studied location, available methods, and previous experiences [12].

The wax appearance temperature (WAT) is one of the important stability characteristics of waxy crude oil and is referred to the highest temperature where the first wax crystals are formed during cooling condition [13]. The pipeline design depends on the WAT [14], and the efficiency of wax inhibitors can be assessed by measuring the influence of their operational factors. Several laboratory methods are suggested to measure this characteristic, but the accuracy of these methods depend on the techniques and materials applied [15]. These techniques include the ASTM D2500, light transmission measurement, viscosity and density measurement [16-18], microscopic techniques based on cross polarized light [15], differential calorimetry, Fourier transform infrared spectroscopy (FTIR) [19], and near-infrared spectrum (NIR) [20].

Biological methods are considered as the manners against heavy deposits in addition to the mechanical, thermal, and chemical methods since the early 90s [1, 21-25], and the number of academic researchers and oil companies R&D's interested in adopting these methods are rising. Although the strains identifying techniques capable of destructing hydrocarbons have improved, conditions and

methods of their development [26], diagnosis of metabolic pathways of degradation, preparation of databases of biochemical metabolic, genetic engineering tools, and many of the principals involved in this issue still remain unexplored [27]. Several methods such as applying bacteria which is able to consume the sole carbon source [28], using extracellular enzymes capable of gradual destruction of chemical bonds within or between wax deposit molecules [29], and producing metabolic compounds to increase the dissolution and availability of heavy wax deposit [21] are proposed to explain the micro-organism activities in preventing, dispersing, and remediating wax deposits. In general, micro-organisms able to degrade the organic compounds which create deposits or produce biological solvents and bio-surfactants can be used to prevent or remove deposits. Bio-surfactants with a variety of structures and chemical compositions are produced by many types of micro-organisms. According to many studies, consuming inhibitors in order to prevent wax deposit is one of the most rewarding and economical methods [30-33]. Considering the wide range of inhibitors properties and many difficulties involved in chemical inhibitor synthesis process, it is clear that biological inhibitors are a good choice to encounter wax deposition problem [25].

The first pilot project of microbial treatment was run successfully in the 1980's [34]. In a study, some strains isolated from several hydrocarbon contaminated samples were used to biodegrade paraffin deposits successfully [29]. Some emulsifier compounds and commercial products were employed in enhancing the heavy crude oil flow in Venezuela, and the heavy oil viscosity was reduced from 105 to 70 mPa.s, which allowed the transport

of heavy oil for 600 km from the reservoir. In a similar attempt, crude oil viscosity was reduced from an initial value of 20000 to 1 mPa.s by applying a combination of bio-surfactants leading to an easy transportation in a long pipeline [35]. In another study, the viscosity, surface tension, and pour point of a crude oil sample were reduced by 34, 31, and 31% respectively [36]. One of the most successful results was obtained in Kuwait oil industry; the researchers used two tons of microbial solution containing bio-surfactants for remediating the formed deposits in the bottom of a large storage tank and retrieved a significant portion of the accumulated petroleum hydrocarbons worth 150 thousand dollars per tank [37]. The results of a study on the effect of three groups of bacteria on the removal of paraffin wax deposits in four wells in China indicated that microbial coalition contributed to reducing these deposits, which led to an increase in the production perfectly [38]. A similar study showed the high ability of microorganisms in the degradation of paraffin wax and deposits removal within the wells and pipelines for the easier transportation of waxy crude oil [39]. According to the results obtained in another work [40], some biological surfactants could reduce the adhesion of wax crystals on steel pipeline wall and cause the deposits removal. The thermophilic *Bacillus subtilis* could reduce the crude oil viscosity efficiently [41]. In addition, the high efficiency of Z25 strain in the removal of wax deposits, the degradation of alkanes, pour point reduction, and crude oil flow properties improvement is verified elsewhere [23]. Adding microbial strain to two crude oil types in China led to a destruction rate of 64% and the deposits removal of 55% [42]. The advantage of this method compared to chemical

treatment methods is less harmful to human and the environment, thereby attracting the attention of the involved in this field [24].

In order to compare WAT reduction performance of some biological inhibitors extracted from various strains, Iranian waxy crude oil sample and the optical spectroscopy technique were used and implemented. The best microorganisms' cultivation state, which leads to the highest reduction of wax appearance temperature, is identified by adopting the Taguchi experimental design method. The objective here is to emphasize the importance of properly adjusting the cultivation conditions in order to achieve the most effective strains and to provide the practical comparison between the biological and chemical inhibitor efficiencies.

## EXPERIMENTAL PROCEDURES

### Material and Method

Dehloran (SW, Iran) crude oil specimen is used for the experiments in this study. The subject sample is dead oil. The kerosene with low sulfur content, glycerol, methanol, a chemical surfactant Tween 80, agar, nutrient broth and nutrient agar were purchased from Merck Company (Germany) and the solid paraffin was provided by Sepahan Oil Company (Isfahan, Iran). The related physical properties of Dehloran crude oil and solid paraffin are tabulated in Tables 1 and 2 respectively.

**Table 1: Physical properties of Dehloran crude oil.**

Properties	Values
Density at 15°C (g/m <sup>3</sup> )	0.8659
Viscosity at 45 °C (mm <sup>2</sup> /s)	0.5123
Wax content (wt.%)	12.66
Asphaltene content (wt.%)	0.86

**Table 2: Physical properties of solid wax.**

Property	Density at 15 °C (kg/m <sup>3</sup> )	Oil content (wt.%)	Flash point (°C)	Melting point (°C)
Method	ASTM D 4052/96	ASTM D 721	ASTM D 92	ASTM D 127
Value	850-884	22-25	245-262	54-62

## Resources and Mediums for Strain Cultivation

In order to isolate the strains which are able to digest the petroleum hydrocarbons and produce suitable bio-surfactants, the soil samples contaminated with crude oil and some sludge samples were collected from six national wide refineries. Since the most successful method for the isolation of desired strains is a mineral salt reinforced medium containing the hydrocarbon source, three types of the following mineral media are consumed:

Mineral medium (g/L): K<sub>2</sub>HPO<sub>4</sub> (0.775), KH<sub>2</sub>PO<sub>4</sub> (0.35), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.1), FeSO<sub>4</sub>.7H<sub>2</sub>O (0.001), and CaCl<sub>2</sub> (0.04).

Bushnell-Haas medium (g/L): K<sub>2</sub>HPO<sub>4</sub> (1), KH<sub>2</sub>PO<sub>4</sub> (1), NH<sub>4</sub>NO<sub>3</sub> (1), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.2), FeCl<sub>3</sub>.6H<sub>2</sub>O (0.05), and CaCl<sub>2</sub> (0.02).

Oil Medium (g/L): K<sub>2</sub>HPO<sub>4</sub> (1), KH<sub>2</sub>PO<sub>4</sub> (1), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.04), and FeCl<sub>3</sub>.6H<sub>2</sub>O (0.004).

The effect of iron as a multi-electrovalence metal on the production of biological inhibitors is assessed by adding FeCl<sub>3</sub>.6H<sub>2</sub>O to the Bushnell-Haas medium. It should be noted that FeCl<sub>3</sub>.6H<sub>2</sub>O is not added to the media when the crude oil or oil sludge are used as the carbon source in the Bushnell-Haas medium.

## Apparatuses

Jasco UV-VIS-NIR V570 spectrometer device (Japan) equipped with quartz cuvette and 1 cm-light

pathlength was utilized to obtain the absorption plots of the waxy samples. A laboratory bioreactor was made with the working volume of 5.1 L and a height to diameter ratio of 0.6. The sterile air is blown into the medium by two spargers after passing through some 0.45-micron filters.

## Microorganism Isolation

The microorganisms' isolation is performed in two direct and indirect procedures. In the direct procedure, some cultivation plates containing Bushnell-Haas agar and mineral salts medium agar were prepared, and the oil contaminated soil and oil sludge were placed on each solid medium by loop. In the indirect approach, a 100-mL Erlenmeyer flask containing mineral salts, Bushnell-Haas [43], and nutrient broth was prepared. In order to isolate the appropriate strains from the contaminated soil samples, 0.5 grams of soil was screened on each of the media, and to isolate the possible strains available in the sludge, 1 mL of the same sludge was added to the minerals and Bushnell-Haas mediums. After 72 hours, 2 mL of each Erlenmeyer flasks were inoculated to the mediums containing sterile hydrocarbons.

## Strain Production and Purification

After one week, the strains produced by the direct procedure were purified. For this purpose, three Erlenmeyer flasks with different carbon sources at a concentration of 1% were prepared [44], and the contents of each plate were inoculated to each of the Erlenmeyer flasks. The strains grew during 4 to 10 days of incubation at 30 °C while stirring at 120 rpm. The growth of the strains was confirmed after the mediums turbidity and microscopic observation. In order to re-purify the strains, they

were cultivated on a solid medium with a similar composition. It should be noted that due to the very low solubility of hydrocarbons in this medium, Kiyohara standard method [45] was used to prepare the plates containing hydrocarbons [46]. The purification process in the indirect procedure was performed in the same manner. Thus, after the growth of strains, the proper plates were inoculated by the loop .

### Screening Experiments

After successive stages of separation and purification through the direct and indirect procedures, a total of 44 types of bacteria capable of consuming hydrocarbons, as the sole carbon source, are isolated from the collected samples. In order to select the most appropriate strains, several screening experiments such as the determination of growth potential of strain in different hydrocarbon sources, the emulsification index, the ability to disperse or remove solid deposits, the growth rate of microorganisms, and the amount of bioactive

compounds production were run. Provided that adding an organic solvent to an aqueous solution leads to emulsion or the appearance of two phases of foam and liquid, it could be concluded that there exist bioactive compounds in the solution [47]. By definition, mediums containing bioactive compounds form flat drops, while a medium without bioactive compounds forms round drops [48, 49], indicating the lack of the two phases emergence. The distribution of medium on the oil surface is an indication of the presence of bioactive compounds [50]. The emulsification index is a quantitative measure of the strain ability to form the emulsion of hydrocarbon, while surface tension is the best criterion to confirm the presence of bioactive compounds in the culture medium. Running these tests according to the procedures mentioned in [51], the four strains named  $S_0$ ,  $S_2$ ,  $S_4$ , and  $S_8$  are selected for further experiments; these belong to a gram-positive strain group and their microscopic images are shown with a magnification of 1000x in Figure 1.

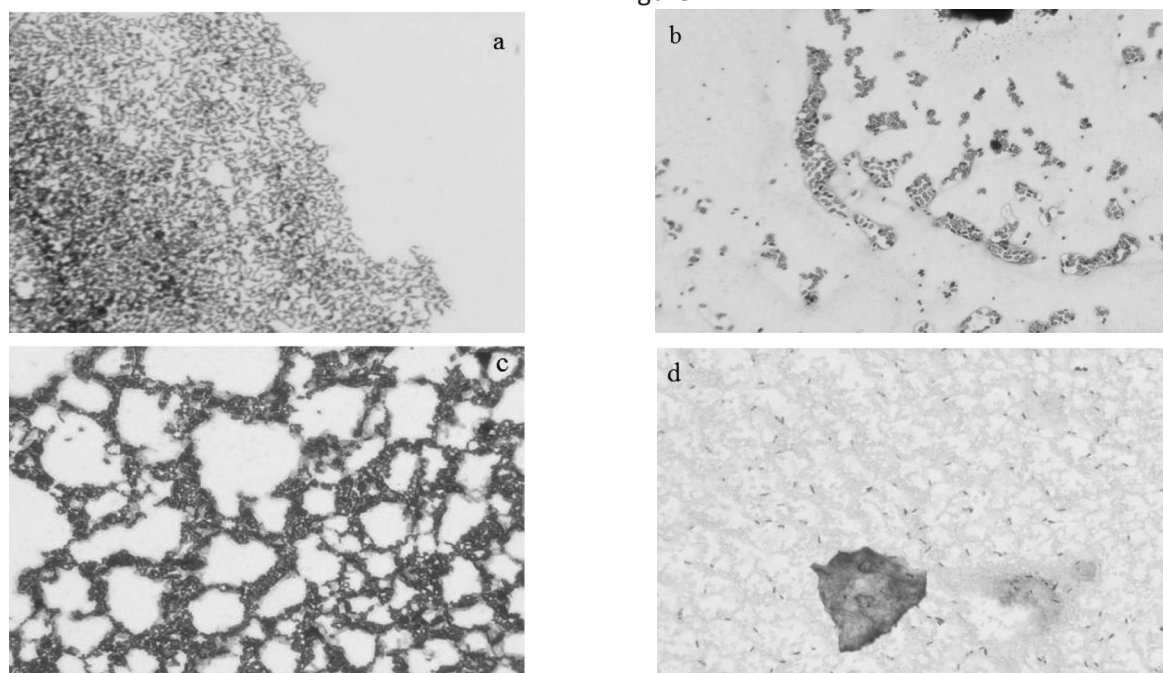


Figure 1: Microscopic images of the strains; a)  $S_0$ ; b)  $S_2$ ; c)  $S_4$ ; and d)  $S_8$ .

### Extraction of the Biological Inhibitors

The extraction of bioactive compounds was performed through centrifugation after the cells growth in the aqueous phase (inorganic medium). The centrifugation took place at a constant temperature of 4 °C since a higher temperature may inactivate the compounds. The pH of cell-free cultivation medium was adjusted at 2 by adding the concentrated hydrochloric acid, and the medium was kept at 4 °C overnight. The solution containing the sediment was centrifuged for 10 min at 8000 rpm at 4 °C. The obtained sediment was isolated by a lower volume of distilled water, and the pH was adjusted at 7 by adding required amount of soda [44, 51-54]. In order to dry the compounds, they were poured on the pre-weighted plates and put inside a desiccator. After 4 to 6 hours, the plates were dried in the oven for 4 hours at 35 °C.

### WAT Determination through Spectroscopy Technique

The near-infrared spectroscopy (NIR) uses the NIR spectra in the range of 780 to 2500 nm [20]. The WAT determination through this method is based on the strong dependency of waxy crude oil absorption on temperature in a wide range of NIR spectrum. One appropriate wave length must be selected to perform the further NIR tests. Since the appropriate wave length depends on the oil sample, at first the absorption curve of the waxy crude oil is determined within a reasonable NIR range. The appropriate wave length is the wavelength at which the minimum absorption occurs. Next, the sample absorption value can be measured at different temperatures, at appropriate wavelength through which the absorption plot is obtained. The temperature at which the slope of the plot changes

in a sudden manner is reported as the WAT of the waxy crude oil [55].

### Taguchi Design Method

The experimental design methods, including Taguchi design is a statistical method that distinguishes the important factors affecting the response from the unimportant factors and determines the conditions to achieve the optimal performance of system. This method reduces the number of required experiments in achieving the desired results [56]. The first and most important step in this approach is selecting the factors and their levels. To assess the effect of various factors on the strain cultivation condition in order to achieve the maximum efficiency in WAT reduction, the four factors of temperature, inoculums concentration, pH, and concentration of nitrogen source are of concern. The factors and their levels are tabulated in Table 3.

Table 3: Factors and their levels.

Factor	Level 1	Level 2	Level 3
Temperature (°C)	30	40	45
Coalition liquid (%)	25	50	75
pH	5	6	7
Nitrogen source concentration (g/L)	0.8	1	1.2

The coalition liquid is made of four strains  $S_0$ ,  $S_2$ ,  $S_4$ , and  $S_8$ . According to the results obtained from the screening tests, the concentration of  $S_2$  is found to be more than the rest of the strains, and in the Taguchi table, inoculation concentration is indicative of the concentration of  $S_2$  in the coalition liquid. Finally, by applying the  $L_9$  Taguchi table (Table 4) the experiments are conducted.

Table 4: Design of experiments: an  $L_9$  Taguchi table.

Run	Temperature (°C)	Coalition liquid (%)	pH	Nitrogen source concentration (g/L)
1	30	25	5	0.8
2	30	50	6	1
3	30	75	7	1.2
4	40	25	6	1.2
5	40	50	7	0.8
6	40	75	5	1
7	45	25	7	1
8	45	50	5	1.2
9	45	75	6	0.8

## RESULTS AND DISCUSSION

### WAT Determination

The minimum wavelength at which the oil medium has the minimum absorption must be determined because at this wavelength oil absorption is very low, and it helps us to detect the solid phase as soon as it appears. When the medium is one liquid phase, the absorption is minimum, but once the first solid crystal appears, it absorbs the spectra, and the absorption value rises; hence, we can detect the solid crystal presence, and as a result WAT will be determined exactly.

The absorption plot of Dehloran crude oil sample in the range of 1200 to 1900 nm is shown in Figure 2, where the minimum absorption is observed in the vicinity of 1608 nm. After determining the appropriate wavelength (1608 nm), three grams of solid paraffin is added to 60 mL of the sample oil to determine WAT.

The results of measuring the absorption at different temperatures and at the wavelength of 1608 nm are expressed in Figure 3. Each of the 9 samples had the same thermal history.

The results indicate that a reduction in temperature will increase absorption. The significant absorption of wax crystals leads to a change in the slope of the absorption plot, which is very different in the single phase system (right side) compared to the two-phase system (left side). Since the technique applied in this study is of high accuracy, it is accepted that the measured temperature is a good estimation of the actual WAT. Based on the mentioned approach and using Figure 3, the WAT of Dehloran crude oil is 36.6 °C.

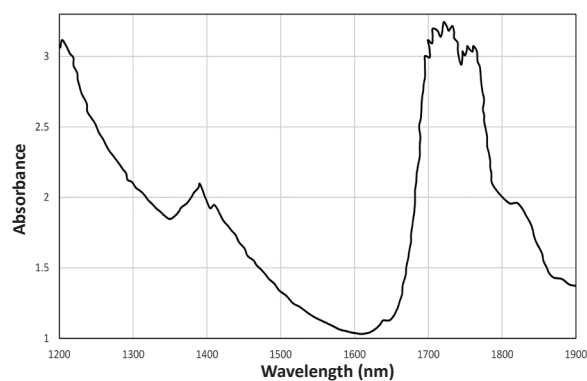
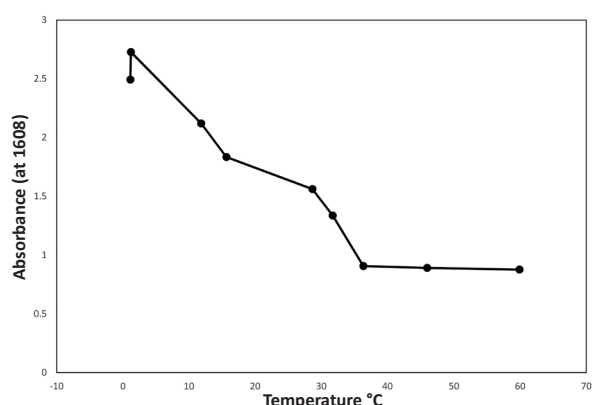


Figure 2: Absorbance data of Dehloran crude oil.



**Figure 3: Absorbance-temperature plot of Dehloran crude oil.**

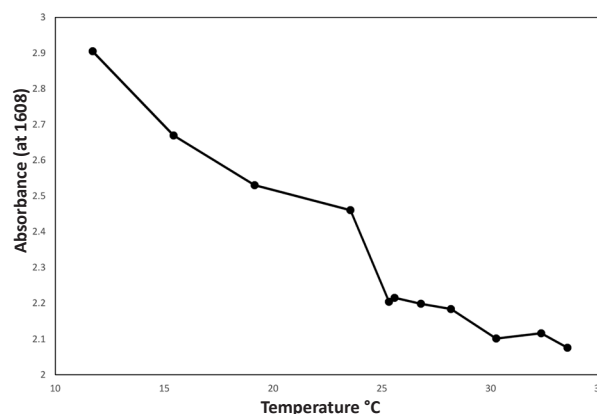
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### Effect of Chemicals Inhibitor on WAT

In order to provide a standard for comparing the performance of biological inhibitors in WAT reduction, the effect of a mixture of some well-known chemical inhibitors is studied. For this purpose, 350 mL of xylene and methyl-ethyl-ketone mixture (at a ratio of 6 to 1) is added to 20 mL of the oil sample. The absorption values of this treated oil are measured at various temperatures (Figure 4). The trend of absorption-temperature plot reveals that as the temperature decreases to

25.3 °C, the absorption rate increases suddenly, indicating first detectable wax crystals formation; hence, this temperature is reported as the WAT of crude oil containing chemical inhibitor. The chemical inhibitor can reduce the WAT from 36.6 to 25.3 °C.



**Figure 4: Absorbance-temperature plot of Dehloran crude oil containing methyl-ethyl-ketone at 1608 nm.**

### Effect of Biological Inhibitors on WAT

In order to produce the significant quantities of biological inhibitors in a short period of time, microbial strains are cultivated as pure and as coalition in the bioreactor. Four strains of  $S_0$ ,  $S_2$ ,  $S_4$ , and  $S_8$  are selected as biological inhibitors by screening the obtained strains.

Each of the strains  $S_0$ ,  $S_2$ , and  $S_4$  is added to the sample at the concentration of 3.0 g/L. The corresponding absorption-temperature plots are shown in Figure 5. Based on these plots, the WAT of the oil samples containing  $S_0$ ,  $S_2$ , and  $S_4$  are 27.8, 22.5, and 26.8 °C respectively. In other words, the bio-inhibitors of strains  $S_0$ ,  $S_2$ , and  $S_4$  cause a drop of 24, 38.5, and 26.77% in WAT values respectively. Usually, the use of a coalition of bacteria has some advantages in a medium with some growth restrictions. In order to evaluate this claim, a coalition of strains  $S_0$ ,  $S_2$ ,  $S_4$ , and  $S_8$  are cultivated



together in the bioreactor. This coalition consists of 25% of  $S_0$ , 50% of  $S_2$ , 12.5% of  $S_4$ , 12.5% of  $S_8$ . According to Figure 5-d, the WAT of the oil sample containing microbial coalition is 19.5 °C, indicating a decrease of about 46.72% in WAT.

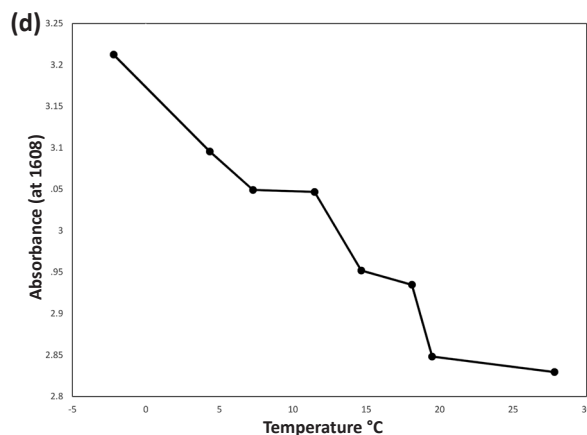
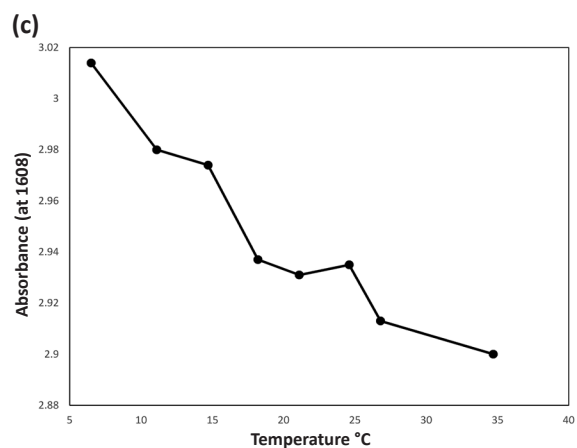
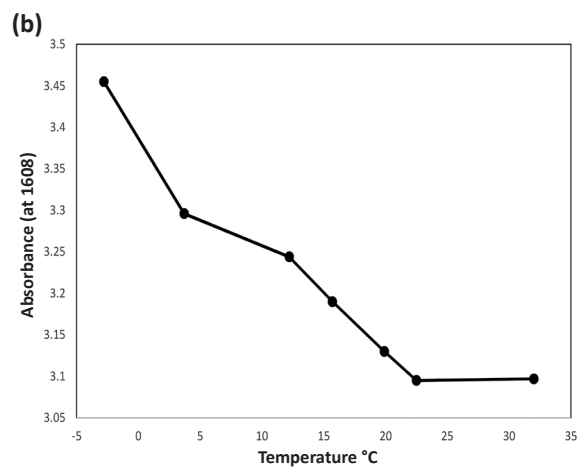
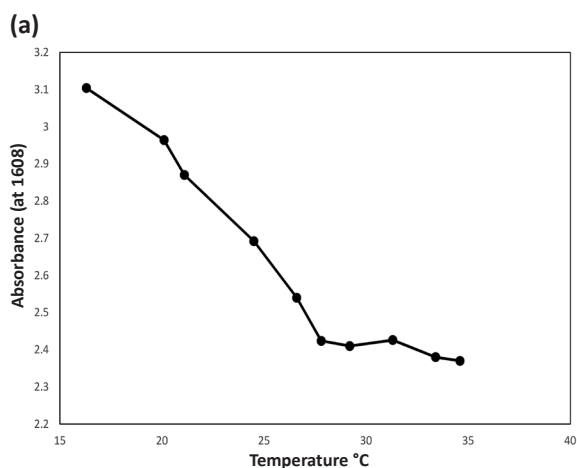


Figure 5: Absorbance data of Dehloran crude oil containing a)  $S_0$ , b)  $S_2$ , c)  $S_4$ , and d) microbial coalition.

### Comparison of Performance of Different Strains in WAT Reduction

The treatment of the crude oil by the chemical inhibitor and biological inhibitors of  $S_0$ ,  $S_2$ ,  $S_4$ , and microbial coalition respectively leads to an 11.3, 8.8, 14.1, 9.8, and 17.1 °C drop in the WAT of untreated oil sample. Therefore, biological inhibitors of microbial coalition is the most influential WAT reducer, followed by bio-inhibitor of  $S_2$ , xylene, and methyl-ethyl-ketone chemical mixture, bio-inhibitors of strain  $S_4$ , and  $S_0$  respectively.

As observed, the slope of the absorption plot of  $S_4$  (Figure 5-b) is more than that of the  $S_0$  plot slope (Figure 5-a), indicating that in the presence of  $S_4$  the wax crystal formation decreases significantly. In addition to the fact that  $S_4$  is a better WAT reducer than  $S_0$ , it can reduce the wax crystals concentration below the WAT in a significant manner.

Although the use of microbial coalition leads to such a desired result in this study, the detailed analysis of this mechanism is complicated. In a hypothetical sense, the dual nature compounds (hydrophilic-hydrophobic) can be the justification of these observation and findings. The result of a test where  $FeCl_3 \cdot 6H_2O$  is added to coalition strains medium

indicates that the use of this agent only causes an improvement of about 0.6 °C in the WAT reduction. It is found that this agent has no significant effect on reducing the wax crystals production rate below WAT. Therefore, due to the difficulties in experimental measurement, consuming  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  is not reasonable under normal conditions.

### Parameters Optimization

After the cultivation of the strains under the condition adjusted in accordance with the experiments design table (Table 4), the obtained biological compounds are consumed to reduce the

WAT of the crude oil samples. Each test of table  $L_9$  Taguchi is repeated once and the findings are tabulated in Table 5.

Since the purpose of determining the optimum conditions is achieving the maximum reduction of WAT, the “small is better” option in the Taguchi method is chosen to analyze the results through the Qualitek-4 software. The results of the analysis of variance are tabulated in Table 6, where the pH with an influence of 49.63% is the most influential factor. The temperature, inoculum concentration, and the concentration of nitrogen source are ranked next by 32.39, 7.92, and 1.39% respectively.

**Table 5: Results of wax appearance determination.**

Temperature (°C)	Coalition liquid (%)	pH	Nitrogen source concentration (g/L)	Wax appearance temperature (°C)		Average (°C)
				First run	Second run	
30	25	5	0.8	25	26	25.5
30	50	6	1	16	17	16.5
30	75	7	1.2	23	20	21.5
40	25	6	1.2	15	16	15.5
40	50	7	0.8	18	20	19
40	75	5	1	21	21	21
45	25	7	1	26	27	26.5
45	50	5	1.2	23	25	24
45	75	6	0.8	21	20	20.5

**Table 6: ANOVA table.**

Factor	DF	Sum of Square	Variance	F-ratio	Influence Percentage
Temperature	2	80.11	40.05	32.77	32.39
Coalition liquid	2	21.44	10.72	8.77	7.92
pH	2	121.44	60.72	49.68	49.63
Nitrogen Source Concentration	2	5.77	2.88	2.36	1.39
Error	9	10.99	1.22	-	8.667

## Effect of Temperature

The performance of biological inhibitors produced by the strains grown at 40 °C (medium level) is more than the others at other temperatures (Figure 6). Temperature affects the solubility of the hydrocarbons in aqueous phase same as the physiological activity of micro-organisms. The micro-organisms produce the bioactive compounds in order to increase the availability of carbon source, and an increase in temperature increases the solubility and availability of hydrocarbons; therefore, the micro-organisms have no tendency to produce bioactive compounds at high temperatures. Also, the biological inhibitors are ineffective at low temperatures, because these bacteria are isolated in high temperature mediums and cannot grow at temperatures lower than 40 °C, which may have a negative effect on their bio-inhibitor production capacity.

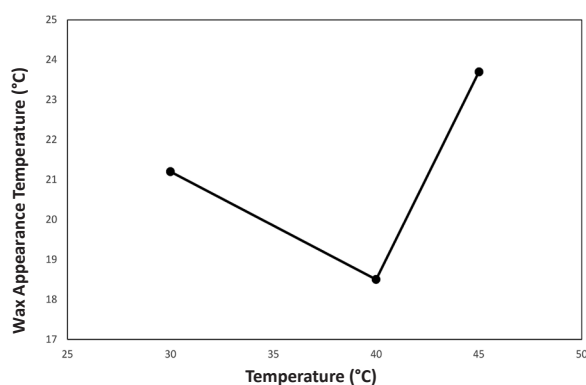


Figure 6: Effect of temperature on WAT.

## Inoculums Concentration

The effect of the inoculum concentration on the performance of the strains on WAT reduction is shown in Figure 7. According to the previous results,  $S_2$  strain outperforms the other strains; however, inoculation strain is better than  $S_2$  in WAT reduction, which might be a result of an increase in desirable co-metabolic effects. There exists

an optimum concentration at which the strain performance is at the highest level. By increasing the amount of inoculum concentration to more than 50%, WAT is increased as well, because here  $S_2$  is predominant. The inoculums concentrations of other strains are neither low so that their effects are subtle nor high so that they would diminish the  $S_2$  effect.

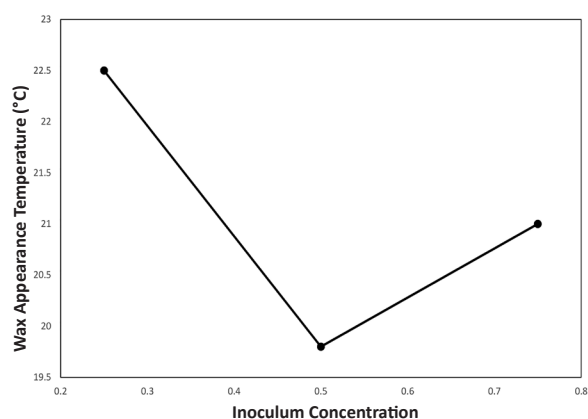


Figure 7: Effect of inoculums concentration on WAT

## Effect of pH

The assessment of pH effect on the ability of strains in WAT reduction is conducted at three levels. pH is one of the factors influencing the growth and activity of micro-organisms. The strains' growth is very difficult and restricted in basic mediums. Provided that pH is adjusted at 6 or is slightly acidic, the strain performance will be appropriate which would lead to a higher WAT reduction (Figure 8).

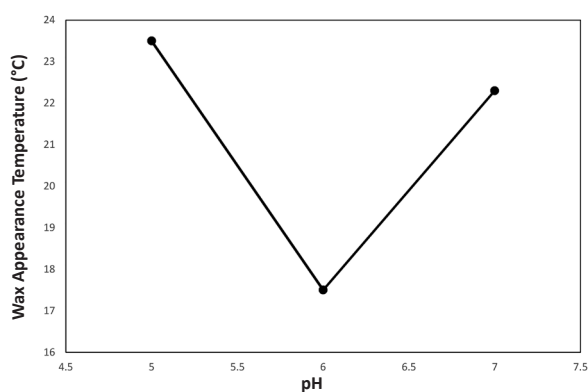


Figure 8: Effect of pH on WAT.

## The Effect of the Concentration of Nitrogen Source

The results of published studies indicate that the concentration of nitrogen source is one of the important factors influencing the type and amount of produced bioactive compounds. According to Figure 9, an increase in the concentration of nitrogen source leads to an increase in the performance of the produced bioactive compounds. However, this parameter with an influence percentage of 1.39% and an F-ratio of 2.36 has no statistical significance effect on the response of the system (WAT) in the confidence level of 95%.

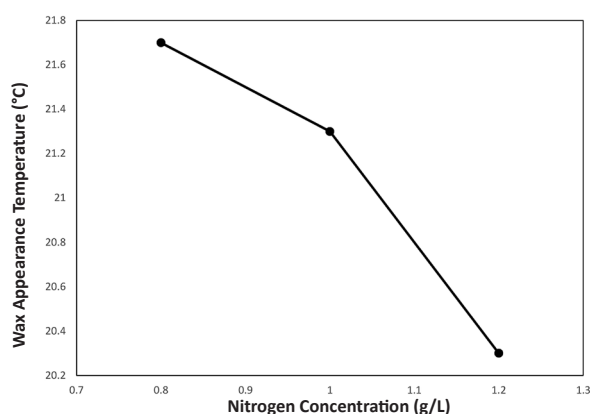


Figure 9: Effect of concentration of nitrogen source on WAT.

## Optimum Condition

The optimum condition is determined by the Qualitek-4 software (Table 7). The best result or the highest WAT reduction is obtained when the temperature, inoculum concentration of  $S_2$ , pH, and the concentration of nitrogen source are set on 40 °C, 50 vol.%, 6, and 1.2 g/L respectively. The absorption plot of Dehloran crude oil in the presence of strain coalition which has grown under optimum conditions is shown in Figure 10, and the corresponding WAT value is 15 °C. It means that when the cultivation medium condition of the

micro-organisms is adjusted under the optimum condition, WAT reduction is 21.6 °C or 60%, which is a good achievement in this context.

Table 7: Optimum conditions.

Factor	Level	Value
Temperature	2	40
Concentration of $S_2$ in coalition liquid (%v.)	2	50
pH	2	6
Nitrogen source concentration	3	1.2

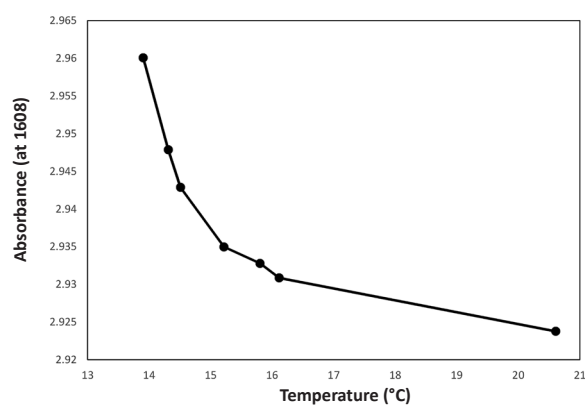


Figure 10: Absorbance data of Dehloran crude oil treated in the optimum condition.

## CONCLUSIONS

In this study, the effects of biological inhibitors on the WAT of waxy crude oil are studied. Some strains are cultivated in a medium containing waxy crude oil (as the sole carbon source), isolated, and then screened in order to achieve the most effective strains for the WAT reduction of an Iranian waxy crude oil sample. The WAT determination is performed by means of the near-Infrared spectroscopy (NIR) technique, which obtains very accurate results. The biological inhibitors produced by the strains lead to a considerable decrease in WAT value. The analysis of variance method is implemented to assess the factors influencing the

strains cultivation condition and the determination of the desirable condition suitable for the growth of most the powerful strains. The Taguchi design results indicate that the influence percentage of pH and temperature, as the most influential factors among the others, are equivalent to 49.63 and 32.39% respectively. The most powerful inhibitors in the WAT reduction are obtained when the temperature, the inoculum concentration of  $S_2$ , pH, and the concentration of nitrogen source are set on 40 °C, 50 vol.%, 6, and 1.2 g/L respectively. As a result, the WAT of Dehloran waxy crude oil reduces from 36.6 to 15 °C when the producer strains grow in this optimum condition. These results are highly desirable since, even in practical applications, the control of these parameters is very easy. Furthermore, an empirical comparison between the performance of chemical and biological inhibitors shows that the biological inhibitors are more successful than their counter parts. Despite the high efficiency of biological inhibitors, many comprehensive studies are necessary in order to improve the strain cultivation methods and medium condition optimization.

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