Effect of Various Isolated Microbial Consortiums on the Biodegradation Process of Precipitated Asphaltenes from Crude Oil

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ABSTRACT: One of the serious problems in the oil industry is precipitation and deposition of asphaltenes in the different oil production stages including formation, wellbore, production tubing, flow lines, and separation units. This phenomenon causes a dramatic increase in the cost of oil production, processing, and transferring. Thus, it seems to be very necessary to use the removing methods for precipitated asphaltenes in different crude oil production and transferring stages. In this study, the ability of microorganisms for biodegradation of precipitated asphaltenes was investigated. For this purpose, four bacterial consortiums were isolated from oil-contaminated soil, crude oil, reservoir water, and oil sludge samples of an oil field located in the southwest of Iran. Based on the results of the designed experiments, by using response surface methodology (RSM) and central composite design, the bacterial consortiums were cultured in the flasks. Three levels of temperatures, salinity, pH, and initial asphaltene concentration as the substrate were considered as the parameters of culture medium and incubated growth mediums for 60 days. The maximum asphaltene biodegradation was 46.41% caused by the crude oil consortium including Staphylococcus saprophyticus sp. and Bacillus cereus sp. at 45 °C, salinity 160 g L⁻¹, pH 6.5, and 25 g L⁻¹ initial asphaltene concentration. Also, it was observed that the negative or positive impacts of culture media conditions such as temperature and salinity on asphaltene degradation depended on the type of the available bacterial consortium. The carbon–hydrogen–nitrogen–sulfur analysis showed that carbon, hydrogen, nitrogen, and in some cases, the sulfur in biodegraded samples are less than in control samples. Moreover, Fourier transform infrared analysis indicated that the alkyne groups were less resistant to biodegradation and were eliminated thoroughly after 2 months of incubation. In addition, alkane components were partially removed in treated asphaltene fraction. The parameters of culture medium were optimized by RSM, and besides, their effects on the performance of bacteria in the asphaltene biodegradation process were discussed. The validity of some available kinetic models to describe the behavior of the studied bacteria consortium was investigated, and it was observed that Tessier, Moser, and Contois models accurately predict the values of asphaltenes and biomass concentration at 30, 45, and 60 °C, respectively.

1. INTRODUCTION

Asphaltenes are considered as a fraction of crude oil that is insoluble in normal alkanes, e.g., pentane, heptane, and decane, but can be dissolved in aromatic solvents, e.g., toluene, benzene, and xylenes, at room temperature.¹⁻⁵ X-ray diffraction analysis demonstrated the asphaltene structure as flat plates of aromatics systems that are interconnected by sulfur, ether, aliphatic chains, and/or naphthenic rings.⁶⁻⁷

Asphaltene precipitation from reservoir fluids in all stages of oil production and transportation causes a serious problem and increases the cost of production.⁸⁻¹² Because of these problems, it seems to be mandatory to find and employ asphaltene precipitation removal methods such as chemical methods, physical techniques, thermal methods, and microbial methods.¹³⁻¹⁶ Recently, biological processes as one of the affordable and environment-friendly methods have encouraged researchers to use them in removing precipitated asphaltenes. Hydrocarbon biodegradation is a very important mechanism that causes the elimination of crude oil and other pollutant hydrocarbons from ecosphere.¹⁷

Received: July 5, 2019
Accepted: October 10, 2019
Published: February 17, 2020

DOI: 10.1021/acsomega.9b02056
ACS Omega 2020, 5, 3131−3143
The limitations of microorganisms’ culture medium are one of the significant controlling factors in the biodegradation of oil pollution in the environment. The hydrophobic nature of the hydrocarbon components of the petroleum makes them connect with soil components and causes reducing the oil components elimination or degradation. The microorganisms’ effects on hydrocarbons and the possibility of hydrocarbons biodegradation are different. Resistance downtrend of hydrocarbons in the biodegradation process between linear alkanes and asphaltenes is as follows:

Asphaltenes > resin > polycyclic aromatics > cyclic alkanes > small aromatics > branched alkanes > linear alkanes.

The alteration of the crude oil structure due to biodegradation processes and the effect of microorganisms on its chemical and physical properties have been studied significantly. In comparison, there is not enough studies on the asphaltene biodegradation and the alteration of asphaltene structure in the biodegradation process. Pineda et al. inoculated microbial consortium including Corynebacterium sp., Bacillus sp., Brevibacillus sp., and Staphylococcus sp. to the medium containing mineral salts and asphaltene precipitation. The asphaltene precipitation as the sole source of energy and carbon was added to the mineral medium and asphaltenes biodegradability evaluated using the ISO 9439 method after 13 days of incubation. On the other hand, it was confirmed that just a part of asphaltenes and bitumen is affected by enzymes and microbial activity. Also, it was proven that asphaltene bio-catalytic reformation can be occurred by some hemeproteins such as chloroperoxidase, cytochrome C peroxidase, and lignin peroxidase from Caldariomyces fumago, Bacillus megaterium, and Escherichia coli and remove the nickel and vanadium from petro-porphyrins and asphaltenes. Moreover, the possibility of isolating some species from the Dorood oil field and Bangestan reservoir in Iran which can utilize asphaltene precipitation as the sole source of carbon and energy at 28 and 40 °C under shaking and static conditions was studied. Aditiawati and Kamarisima evaluated the performance of four isolated bacteria from a sludge oil sample from Balikpapan on precipitated asphaltene. Bacillus sp. and Lysinibacillus fusiformis have the best ability to degrade asphaltene, and percentage of their asphaltene biodegradation was 50 and 55%, respectively. Moreover, Asadollahi et al. identified Bacillus cereus as a bio surfactant-producing bacterium in the oil-contaminated soil and oily sludge samples around the Kermanshah Oil Refining Company which could degrade 40% of asphaltenes after 60 days at 28 °C. Iraji and Ayatollahi studied on the effect of a surfactant on the microorganism adhesion and cell surface hydrophobicity of Enterobacter cloacae, E. cloacae, and Pseudomonas aeruginosa and evaluated the performance of these bacteria in the asphaltene biodegradation at 40 °C. They observed that cell hydrophobicity alteration leads to more asphaltene biodegradability up to 49%. The asphaltene precipitation phenomenon in oil equipment is a serious challenge in the production facilities in the Iranian

### Table 1. Experimental Results of Asphaltene Biodegradation and Dry Weight of Soil and Reservoir Water Consortiums

| run no. | temperature (°C) | salinity (g L⁻¹) | pH | asphaltene concentration (g L⁻¹) | response Soil consortium biodegradation (%) | dry weight (g L⁻¹) | response Reservoir water consortium biodegradation (%) | dry weight (g L⁻¹) |
|---------|-----------------|-----------------|----|-------------------------------|--------------------------------------------|-------------------|----------------------------------------------------------|-------------------|
| 1       | 30.00           | 0.00            | 5.50 | 15.00                         | 17.56                                      | 13.33             | 14.28                                                    | 1.16              |
| 2       | 30.00           | 0.00            | 7.50 | 15.00                         | 9.19                                       | 3.63              | 40.35                                                    | 12.55             |
| 3       | 30.00           | 160.00          | 5.50 | 15.00                         | 11.56                                      | 2.55              | 4.10                                                     | 4.30              |
| 4       | 30.00           | 160.00          | 7.50 | 15.00                         | 1.18                                       | 8.8               | 8.12                                                     | 3.66              |
| 5       | 30.00           | 80.00           | 6.50 | 25.00                         | 3.87                                       | 4.8               | 21.07                                                    | 5.53              |
| 6       | 30.00           | 0.00            | 5.50 | 35.00                         | 14.65                                      | 1.2               | 8.30                                                     | 1.6               |
| 7       | 30.00           | 0.00            | 7.50 | 35.00                         | 6.48                                       | 1.6               | 28.03                                                    | 1.92              |
| 8       | 30.00           | 160.00          | 5.50 | 35.00                         | 15.02                                      | 5.94              | 12.67                                                    | 1.14              |
| 9       | 30.00           | 160.00          | 7.50 | 35.00                         | 4.85                                       | 2.27              | 21.39                                                    | 6.72              |
| 10      | 45.00           | 80.00           | 6.50 | 15.00                         | 5.06                                       | 1.21              | 6.07                                                     | 2.00              |
| 11      | 45.00           | 0.00            | 6.50 | 25.00                         | 5.76                                       | 7.55              | 12.5                                                     | 11.66             |
| 12      | 45.00           | 80.00           | 5.50 | 25.00                         | 22.0                                       | 1.27              | 8.54                                                     | 3.83              |
| 13      | 45.00           | 80.00           | 6.50 | 25.00                         | 6.9                                        | 4.76              | 5.54                                                     | 11.8              |
| 14      | 45.00           | 80.00           | 6.50 | 25.00                         | 7.1                                        | 3.8               | 5.85                                                     | 12.4              |
| 15      | 45.00           | 80.00           | 6.50 | 25.00                         | 6.88                                       | 5.46              | 6.76                                                     | 13.55             |
| 16      | 45.00           | 80.00           | 6.50 | 25.00                         | 6.95                                       | 4.22              | 6.37                                                     | 12.88             |
| 17      | 45.00           | 80.00           | 6.50 | 25.00                         | 6.85                                       | 3.77              | 5.07                                                     | 10.75             |
| 18      | 45.00           | 80.00           | 6.50 | 25.00                         | 7.15                                       | 4.66              | 5.79                                                     | 11.33             |
| 19      | 45.00           | 80.00           | 7.50 | 25.00                         | 12.28                                      | 7.36              | 6.87                                                     | 2.8               |
| 20      | 45.00           | 160.00          | 6.50 | 25.00                         | 4.13                                       | 9.43              | 7.82                                                     | 6.00              |
| 21      | 45.00           | 80.00           | 6.50 | 35.00                         | 4.83                                       | 2.60              | 13.08                                                    | 3.81              |
| 22      | 60.00           | 0.00            | 5.50 | 15.00                         | 18.56                                      | 3.41              | 13.04                                                    | 2.00              |
| 23      | 60.00           | 0.00            | 7.50 | 15.00                         | 9.31                                       | 1.26              | 8.30                                                     | 0.6               |
| 24      | 60.00           | 160.00          | 5.50 | 15.00                         | 16.93                                      | 6.20              | 11.46                                                    | 2.27              |
| 25      | 60.00           | 160.00          | 7.50 | 15.00                         | 3.68                                       | 4.28              | 4.48                                                     | 0.76              |
| 26      | 60.00           | 80.00           | 6.50 | 25.00                         | 6.019                                      | 5.94              | 12.67                                                    | 2.45              |
| 27      | 60.00           | 0.00            | 5.50 | 35.00                         | 14.44                                      | 3.33              | 27.25                                                    | 5.5               |
| 28      | 60.00           | 0.00            | 7.50 | 35.00                         | 5.39                                       | 4.43              | 4.10                                                     | 0.88              |
| 29      | 60.00           | 160.00          | 5.50 | 35.00                         | 19.19                                      | 1.22              | 7.23                                                     | 1.52              |
| 30      | 60.00           | 160.00          | 7.50 | 35.00                         | 8.13                                       | 5.25              | 15.37                                                    | 2.33              |
asphaltenes and microorganisms available kinetic models to predict the concentration of biodegradation. Besides, the validation of some molecules during biodegradation was investigated using spectroscopy and elemental analysis. The alteration of asphaltene bacterial consortium were optimized through the response surface methodology (RSM). The main goal of this study was an investigation on the precipitated asphaltene biodegradation process by isolated bacterial consortia, from oil contaminated soil, crude oil, reservoir water, and oil sludge samples in the Bangestan reservoir of an Iranian southwest oil field. Furthermore, the effects of cultural medium factors including temperature, salinity, pH, and substrate concentration on the performance of identified microorganisms were evaluated. The values of these parameters have been attempted to be close to operating conditions.

The bacterial consortium’s performance in asphaltenes biodegradation process at 30, 45, and 60 °C with salinity 0, 80, and 160 g·L⁻¹, pH 5.5, 6.5, and 7.5; and initial asphaltene concentration as substrate 15, 25, and 35 g·L⁻¹ for two months was assessed. In addition, the culture conditions of each available bacterial consortium were optimized through the response surface methodology (RSM). The alteration of asphaltene molecules during biodegradation was investigated using spectroscopy and elemental analysis. Besides, the validation of some available kinetic models to predict the concentration of asphaltenes and microorganisms’ dry weight at pH 6.5, salinity 80 g·L⁻¹, initial asphaltene concentration 25 g·L⁻¹, and temperature 30, 45, and 60 °C have been studied.

2. RESULTS AND DISCUSSION

2.1. Identification of Microorganisms. Based on the results of 16S rRNA sequence analysis and morphological and biochemical tests which are listed in Table 2, isolated species from the same oil polluted environment was categorized in a consortium. The Soil consortium which was obtained from a polluted soil sample in the burning pit includes *Bacillus cereus* YSH-1 and *Microbacterium paraoxydans* YSH-2. Three strains including *E. cloacae* YSH-3, *Bacillus cereus* YSH-4, and *Stenotrophomonas maltophilia* YSH-5 were extracted from the reservoir water sample and placed in Reservoir water consortium. *Staphylococcus saprophyticus* YSH-6 and *Bacillus cereus* YSH-8, and *Pseudomonas* YSH-9 were categorized as the Crude oil consortium. Moreover, Sludge consortium includes *E. cloacae* YSH-8, and *Bacillus cereus* YSH-9 strains as isolated strains from the crude oil sample could degrade asphaltene precipitation and use it as a sole source of carbon and energy.

Table 2. Experimental Results of Asphaltene Biodegradation and Dry Weight of Crude Oil and Sludge Consortium

| run no. | temperature (°C) | salinity (g·L⁻¹) | pH | asphaltene concentration (g·L⁻¹) | response Crude oil consortium | response Sludge consortium |
|---------|------------------|------------------|----|----------------------------------|-------------------------------|-----------------------------|
|         |                  |                  |    | biodegradation (%)               | dry weight (g·L⁻¹)            | biodegradation (%)           | dry weight (g·L⁻¹)         |
| 1       | 30.00            | 0.00             | 5.50 | 15.00                            | 4.93                          | 2.6                          | 8.55                        | 1.88                        |
| 2       | 30.00            | 0.00             | 7.50 | 15.00                            | 8.98                          | 2.2                          | 3.05                        | 1.13                        |
| 3       | 30.00            | 160.00           | 5.50 | 15.00                            | 16.5                          | 4.6                          | 39.37                       | 3.2                         |
| 4       | 30.00            | 160.00           | 7.50 | 15.00                            | 24.02                         | 5.30                         | 6.01                        | 2.23                        |
| 5       | 30.00            | 80.00            | 6.50 | 25.00                            | 15.48                         | 5.2                          | 19                          | 1.6                         |
| 6       | 30.00            | 0.00             | 5.50 | 35.00                            | 4.88                          | 1.16                         | 4.92                        | 3.05                        |
| 7       | 30.00            | 0.00             | 7.50 | 35.00                            | 1.7                           | 1.4                          | 11.65                       | 1.37                        |
| 8       | 30.00            | 160.00           | 5.50 | 35.00                            | 15.92                         | 3.54                         | 33.07                       | 3.94                        |
| 9       | 30.00            | 160.00           | 7.50 | 35.00                            | 12.88                         | 1.5                          | 12.36                       | 4.18                        |
| 10      | 45.00            | 80.00            | 6.50 | 15.00                            | 10.02                         | 3.16                         | 1.6                         | 1.4                         |
| 11      | 45.00            | 0.00             | 6.50 | 25.00                            | 21.55                         | 5.55                         | 8.75                        | 3.38                        |
| 12      | 45.00            | 80.00            | 5.50 | 25.00                            | 4.54                          | 1.33                         | 15.42                       | 4.4                         |
| 13      | 45.00            | 80.00            | 6.50 | 25.00                            | 13.38                         | 1.88                         | 11.34                       | 5.32                        |
| 14      | 45.00            | 80.00            | 6.50 | 25.00                            | 13.45                         | 2.24                         | 10.25                       | 5.45                        |
| 15      | 45.00            | 80.00            | 6.50 | 25.00                            | 14.01                         | 1.11                         | 11.11                       | 4.61                        |
| 16      | 45.00            | 80.00            | 6.50 | 25.00                            | 13.98                         | 1.55                         | 11.55                       | 4.32                        |
| 17      | 45.00            | 80.00            | 6.50 | 25.00                            | 13.88                         | 1.22                         | 10.44                       | 5.23                        |
| 18      | 45.00            | 80.00            | 6.50 | 25.00                            | 13.88                         | 3.66                         | 11.34                       | 1.23                        |
| 19      | 45.00            | 80.00            | 7.50 | 25.00                            | 4.72                          | 5.83                         | 10.42                       | 2.77                        |
| 20      | 45.00            | 160.00           | 6.50 | 25.00                            | 46.71                         | 8.2                          | 15.67                       | 4.8                         |
| 21      | 45.00            | 80.00            | 6.50 | 35.00                            | 7.35                          | 1.81                         | 8.03                        | 1.25                        |
| 22      | 60.00            | 0.00             | 5.50 | 15.00                            | 4.062                         | 2.64                         | 5.79                        | 1.38                        |
| 23      | 60.00            | 0.00             | 7.50 | 15.00                            | 15.51                         | 8.16                         | 16.56                       | 1.45                        |
| 24      | 60.00            | 160.00           | 5.50 | 15.00                            | 5.22                          | 4.76                         | 18.93                       | 2.25                        |
| 25      | 60.00            | 160.00           | 7.50 | 15.00                            | 20.82                         | 1.15                         | 3.56                        | 0.625                       |
| 26      | 60.00            | 80.00            | 6.50 | 25.00                            | 14.78                         | 8.54                         | 39.72                       | 9.45                        |
| 27      | 60.00            | 0.00             | 5.50 | 35.00                            | 9.12                          | 4.77                         | 12.26                       | 4.25                        |
| 28      | 60.00            | 0.00             | 7.50 | 35.00                            | 11.41                         | 2.12                         | 35.58                       | 5.22                        |
| 29      | 60.00            | 160.00           | 5.50 | 35.00                            | 7.16                          | 0.78                         | 23.075                      | 4.33                        |
| 30      | 60.00            | 160.00           | 7.50 | 35.00                            | 12.37                         | 4.5                          | 19.015                      | 2.66                        |
comparison of pure bacteria culture, the consortium of them can provide the maximum asphaltene biodegradation.\textsuperscript{25,28,29}

2.2. Asphaltene Biodegradation Results. The effect of four bacterial consortiums and one mixed culture of all available strains on the asphaltene degradation in the different culture mediums was evaluated. The experimental results of asphaltenes degradation percent (% w/w) and biomass dry weight after two months of incubation are presented in Tables 1–3. The uncertainty in the samples’ weight was ±0.0001 g. It can be seen that in the different growth medium, a specific bacterial consortium had various performances and in similar conditions, the ability of consortiums to degrade asphaltenes was dissimilar. After 60 days, the highest asphaltene biodegradation by the Crude oil consortium was 46.71% at 45 °C, 160 g·L\(^{-1}\) salinity, pH equal to 6.5 and 25 g·L\(^{-1}\) as initial asphaltene concentration. In addition, 39.72, 18.90, 40.35, and 26.03% were the maximum asphaltene biodegradation using sludge, soil, reservoir water, and all-isolated strain consortiums, respectively. Also, it was determined that biodegradation ability of a specific bacterial consortium can be higher or lower than the all-strain consortium. Soil and Crude oil consortiums had the maximum performance at the lowest temperature, salinity, pH and asphaltene concentration, respectively. Furthermore, at the maximum temperature, salinity, pH and asphaltene concentration, the best and worst efficiencies were observed for Sludge and Soil consortiums, respectively.

In a previous work, it was observed that \textit{Bacillus cereus} YSH-9 which was isolated from oil sludge had maximum efficiency in the biodegradation of asphaltene precipitation. Maximum biodegradation was achieved using YSH-1, YSH-2, YSH-4, YSH-5, YSH-6, and YSH-8 strains as 41.5, 38.1, 41.51, 30.64, 30.65, and 33.33%, respectively.\textsuperscript{10} A comparison between the performance of pure isolates and consortia showed that microorganisms in this consortium did not have synergy activity in all growth mediums and in some cases, biodegradation potential of the bacterial consortium can be higher than pure bacteria.

It was reported that compared with pure bacteria, a mixed culture of \textit{Pseudomonas} sp., \textit{Bacillus licheniformis} sp., \textit{Bacillus lentus} sp., \textit{Bacillus cereus} sp., and \textit{Bacillus firmus} sp. had the highest reported degradation result, 48%, and obtained at 28 °C.\textsuperscript{28} In addition, the best consortiums’ performance was achieved by an isolated bacterial consortium from a soil sample from the Shiraz refinery including \textit{P. aeruginosa} and \textit{Pseudomonas fluorescens} at 40 °C and 35 g·L\(^{-1}\) of asphaltenes concentration under shaking and static conditions. The highest observed precipitated asphaltene degradation was 51.5 and 32% under shaking and static conditions, respectively.\textsuperscript{29}

The contribution of the assimilation or dissimilation metabolisms in asphaltene biodegradation would be evaluated by measuring the biomass dry weight. In other words, the values of biomass dry weight explained which one of them is more sensible. In assimilation, carbon was absorbed and causes an increase in microorganisms’ dry weight by increasing the substrate utilization. In the dissimilation metabolism, carbon may be released as CO\(_2\) and lead to lack of microorganisms’ dry weight.
weight with increases in substrate consumption. For example, investigation on asphaltene biodegradation and biomass dry weight results of Soil consortium performance in three different growth mediums (run number 1, 20, and 25) shows that despite the rise in asphaltene consumption in growth medium 25 compared with 20, the biomass dry weight decreases. It seems that in medium 25, dissimilation metabolism is more effective than medium 20. On the other hand, such as asphaltene biodegradation percent, the amount of dry weight of biomass in medium 1 is higher than medium 20. It can be concluded that in medium 1, assimilation metabolism is more operative and compared with medium 20, and more carbon is absorbed by bacterial members of the Soil consortium.

2.3. Influence of Independent Variables on the Asphaltene Biodegradation. The effect of temperature, salinity, pH, and asphaltene concentration on the asphaltene biodegradation as independent variables can be illustrated by perturbed figures. These figures are derived from RSM and include some curves that each presents the influence of one of the factors (in this study: growth medium condition) on the response (in this study: precipitated asphaltene biodegradation) qualitatively and can be observed in Figures 1–5.

Figure 1. Effect of culture medium variables [(A) temperature, (B) salinity, (C) pH, and (D) initial asphaltene concentration] on asphaltene biodegradation with the Soil consortium.

Figure 2. Effect of culture medium variables [(A) temperature, (B) salinity, (C) pH, and (D) initial asphaltene concentration] on asphaltene biodegradation with the Reservoir water consortium.

Figure 3. Effect of culture medium variables [(A) temperature, (B) salinity, (C) pH, and (D) initial asphaltene concentration] on asphaltene biodegradation with the Crude oil consortium.

Figure 4. Effect of culture medium variables [(A) temperature, (B) salinity, (C) pH, and (D) initial asphaltene concentration] on asphaltene biodegradation with the Sludge consortium.

Figure 5. Effect of culture medium variables on [(A) temperature, (B) salinity, (C) pH, and (D) initial asphaltene concentration] asphaltene biodegradation with the All-strain consortium.

The negative and positive slopes of curves point to that the factors have negative and positive effects on consortium performance, respectively. The zero slope means that increasing or decreasing the values of these parameters does not affect the bacterial consortium activity. As can be observed the influence of temperature, salinity, pH, and substrate concentration on...
asphaltene degradation depended on the type of the available bacterial consortium. For instance, before the intermediate (center coded) quantities, pH and initial asphaltene concentration had a positive and after that had a negative effect on Crude oil consortium performance. Moreover, in comparison with pH and initial asphaltene concentration, salinity had a reverse effect and temperature did not significantly effect on the biodegradation process by this mixed culture. As can be observed in each perturbed figure, the effect of two independent variables on asphaltene biodegradation are more significant. For example, salinity and pH influences on the Crude oil consortium performance and temperature and initial asphaltene concentration effect on Sludge consortium activity are more remarkable. The concurrent effect of two variables which had the most significant influence on bacteria consortium performance have been illustrated by 3D surface images in Figures 6–10.

In these figures, the influences of temperature and pH for the Soil consortium, temperature and salinity for the Reservoir water consortium, salinity and pH for the Crude oil consortium, temperature and initial asphaltene concentration for the Sludge consortium, and pH and initial asphaltene concentration for the all-strain consortium have been studied, respectively. Two other factors for each bacterial consortium have been kept constant in their mid-range.

Figure 6 shows that in a salinity of 80 g\cdot\text{L}^{-1} and asphaltene concentration of 25 g\cdot\text{L}^{-1} when increasing the temperature and pH simultaneously, asphaltene degradation by the Soil consortium was initially reduced and then increased. Also, at the highest pH value, increase in the temperature had a positive influence on the Soil consortium activity and more precipitated asphaltene degraded. At the maximum temperature, pH growth before 6.5 led to degradation decrease and after that increase. In addition, asphaltene biodegradation was more sensitive to pH and the effect of the pH alteration on it is more remarkable. Also, maximum biodegradation was obtained in the lowest and highest pH and temperature, respectively.

Figure 7. Response surface of the asphaltene biodegradation percentage using the Reservoir water consortium on salinity and temperature in pH = 6.5 and an initial asphaltene concentration of 25 g\cdot\text{L}^{-1}.

Figure 8. Response surface of the asphaltene biodegradation percentage using the Crude oil consortium on pH and temperature at a temperature of 45 °C and an initial asphaltene concentration of 25 g\cdot\text{L}^{-1}.

Figure 9. Response surface of the asphaltene biodegradation percentage using the Sludge consortium on initial asphaltene concentration and temperature in a salinity of 80 g\cdot\text{L}^{-1} and pH = 6.5.
have been demonstrated in Figure 7. It can be observed that the growth values of salinity and temperature before intermediate values caused in the performance of the Reservoir water consortium drop and then increase. It is notable that biodegradation in the lowest values of salinity and temperature rather their maximum values occurs more.

The 3D surface figure of the asphaltene biodegradation percent using the Crude oil consortium (Figure 8) demonstrates that at 45 °C and an initial substrate concentration of 25 g L\(^{-1}\), simultaneous increase in salinity and \(pH\) before their mid-range led to degradation reduce and after that increase. Furthermore, maximum biodegradation occurred in the highest value of salinity and the mean value of \(pH\).

Figure 9 shows that in salinity 80 g L\(^{-1}\) and \(pH\) 6.5, at the same time, the increase in the initial asphaltene concentration and temperature before and after mean values caused to decrease and increase asphaltene biodegradation by the Sludge consortium, respectively. Also, it is detected that maximum biodegradation has been obtained at maximum temperature and mid-range of asphaltene concentration.

In Figure 10, it is revealed that at 45 °C and a salinity of 80 g L\(^{-1}\), simultaneous increase in asphaltene concentration and \(pH\) led to improve the All-strain consortium performance. However, the graph illustrates that the rate of asphaltene biodegradation growth in the higher values of asphaltene concentration and \(pH\) was lower than the smaller amounts of their value.

Environmental factors such as temperature, \(pH\), salinity, and alkene groups, respectively. It was detected that the fact that consortiums have broken asphaltene molecules, consumed the elemental carbon and released hydrogen.

Table 4. Elemental Analysis of Untrained Asphaltene Precipitation (Control Sample) and Biodegraded Asphaltene after 60 days Incubation with Bacterial Consortiums of Bacterial Isolates at 45 °C, 80 g L\(^{-1}\) Salinity, \(pH\) Equal to 6.5 and 25 g L\(^{-1}\) Initial Asphaltene

| consortium               | % carbon | % hydrogen | % nitrogen | % sulfur | carbon/hydrogen | nitrogen/carbon | sulfur/carbon |
|--------------------------|----------|------------|------------|----------|-----------------|-----------------|---------------|
| Control sample           | 80.65    | 9.68       | 2.65       | 5.24     | 8.33            | 0.032           | 0.064         |
| Soil consortium          | 66.78    | 1.09       | 0.05       | 25.40    | 61.26           | 0.00075         | 0.3803        |
| Reservoir water consortium| 70.52    | N/A        | 0.43       | 9.96     | N/A             | 0.0060          | 0.141         |
| Crude oil consortium     | 55.39    | 0.40       | 0.17       | 1.56     | 138.47          | 0.0030          | 0.0281        |
| Sludge consortium        | 58.31    | 0.01       | 0.14       | 5.38     | 5831            | 0.0024          | 0.0922        |
| All-strain consortium    | 79.20    | 1.90       | 1.54       | 3.49     | 41.68           | 0.0194          | 0.0440        |
components such as n-fatty acids and linear aliphatic alcohol which bonded to the asphaltene core by ester and hydrogen bonds were affected. On the other hand, because of the strong covalent bonds such as C–C and ether bonds, the linear alkyl group which was attached by them was degraded at heavy—severe stages. FT-IR analysis of other samples gave similar results.

2.6. Optimization of Asphaltene Biodegradation. To optimize the variables of the asphaltene biodegradation process, independent factors including temperature (Tem), salinity (Sal), pH (pH), and initial asphaltenes concentration (Asp) have been applied as input data in quadric models as below

\[
Y(\%) = \alpha_0 + \alpha_1 \times \text{Tem} + \alpha_2 \times \text{Sal} + \alpha_3 \times \text{pH} + \alpha_4 \\
\times \text{Asp} + \alpha_5 \times \text{Tem} \times \text{Sal} + \alpha_6 \times \text{Tem} \times \text{pH} + \alpha_7 \\
\times \text{Tem} \times \text{Asp} + \alpha_8 \times \text{Sal} \times \text{pH} + \alpha_9 \times \text{Sal} \times \text{Asp} + \alpha_{10} \times \text{pH} \times \text{Asp} + \alpha_{11} \times \text{Tem}^2 + \alpha_{12} \times \text{Sal}^2 + \alpha_{13} \\
\times \text{pH}^2 + \alpha_{14} \times \text{Asp}^2
\]

(1)

where \(Y\), \(\alpha_0\), and \(\alpha_i\) (\(i = 1−14\)) are biodegraded asphaltenes, intercept and coefficients of the model, respectively, and have been listed in Table 5. The high value of \(R^2\) as the coefficient of determination pointed to the adequate fit of this equation for each system over the given experimental domain. The analysis of variance (ANOVA) of the selected model has been presented in Table 6. It can be seen that the proposed quadric model has been significant for each sample. For example, the model \(F\)-value of 11485.87 and values of \(\text{Prob } F\) less than 0.0001 for the Soil consortium indicates that the model is significant. The optimum value of factors predicted the percentage of biodegradation and desirability and is given in Table 7.

2.7. Kinetic Studies on Asphaltene Biodegradation. The ability of Monod, Contois, and Tessier models as kinetic models to predict the concentration of substrate and biomass dry weight of the Crude oil consortium has been evaluated. The asphaltene concentration and microorganism dry weight were measured every five days during 60 days of incubation. In general, the mass balance equations for asphaltenes and biomass are as follows

\[
\frac{dx}{dt} = \mu x
\]

(2)

\[
\frac{dS}{dt} = -\frac{\mu x}{Y_S/S}
\]

(3)

where \(x\), \(\mu\), \(S\), \(Y_S/S\), and \(t\) are the biomass concentration in liquid medium (g·L\(^{-1}\)), the specific growth rate (day\(^{-1}\)), the substrate concentrations in the liquid medium (g·L\(^{-1}\)), the Biomass yield on asphaltenes (g/g) and time, respectively.

The various equations have been proposed for the specific growth rate as a key role in kinetic models. Some of them are as follows

Monod model: \(\mu = \frac{\mu_m S}{K_S + S}\)

(4)

Contois model: \(\mu = \frac{\mu_m S}{K_S x + S}\)

(5)

Tessier model: \(\mu = \mu_m \left(1 - \exp\left(-\frac{S}{K_S}\right)\right)\)

(6)

where \(\mu_m\) and \(K_S\) are the maximum specific growth rate (day\(^{-1}\)) and kinetic constant (g·L\(^{-1}\)), respectively. The kinetic
It was revealed that in some growth medium conditions, the Tessier model was the best model, and unlike some other models such as Monod, Moser, and logistic models, it had the highest $R^2$ for the description of asphaltene biodegradation using bacterial consortiums and Bacillus lentus. 28,29

### 3. CONCLUSIONS

The performances of four bacterial consortiums which identified in the oil-contaminated soil, crude oil, reservoir water, and oil sludge on precipitated asphaltene biodegradation at three levels of temperatures, pH, salinity, and initial substrate concentrations during two months’ incubation were studied. The results showed that after the incubation period, the Crude oil consortium degraded the highest amount of asphaltene precipitation, 46.71%, at 45 °C, 160 g·L⁻¹ salinity, 6.50 pH, and 25 g·L⁻¹ initial asphaltene concentration. The share of assimilation and dissimilation metabolism was not same in all culture mediums and a more powerful effect of assimilation makes increase in the biomass dry weight concurrent with increasing the substrate utilization. The elemental analysis presented that the bacterial consortium broke asphaltene molecules into smaller segments and consumed the carbon, hydrogen, nitrogen, and in some cases, sulfur. The spectroscopy results indicated that alkyne groups and aldehyde groups in asphaltenes have the least and most resistances to biodegradation, respectively. Using the RSM, the statistical optimization was done and the optimum values of culture media parameters were obtained. The results of the kinetic study showed that at different temperatures, Tessier, Moser, and Contois models have the different precision to predict the concentration of precipitated asphaltene and microorganisms’ dry weight of the Crude oil consortium as a function of time and Monod and Tessier models could better correlate the experimental data.

### 4. MATERIALS AND METHODS

#### 4.1. Culture Medium Preparation

The content of broth culture medium was mineral salts as the nutrient, precipitated asphaltenes as the substrate and inoculated bacterial consortium. The composition of mineral salts medium used in this study was similar to the previous work, and asphaltene was extracted by the ASTM D3279-90 standard procedure. 42 The members of consortiums were isolated from oil-contaminated soil in the burning pit, reservoir water, crude oil, and oil sludge samples from an Iranian southwest oil field and 16S rRNA gene sequencing analysis, and some biochemical tests were carried out to identify them. 10,43,44 The utilized general bacterial primer sets were (5′-CCGAATTTCGTCCGACACAGAGTTT-GATCCTGGCTCAG-3′) as a forward primer and (5′-
as a reverse primer. The polymerase chain reaction (PCR) was performed on a thermal cycler by using 25 μL reaction mixture. The reaction mixture contains 1, 12.5, 9.5, and 1 μL of each forward and reverse primers, master mix 2×, Milli-Q water and genome, respectively. The reaction mix was exposed to the denaturation process at 94 °C for 5 min; next, the amplification was performed with 25 cycles of 1 min at 94 °C, 40 s at 55 °C, 2 min at 72 °C chased by 1 cycle extension of 10 min at 72 °C. The analysis of PCR products was carried out by using 1% agarose gel. As mentioned, the precipitated asphaltene was added to culture medium as sole substrate. Therefore, the isolated strains were able to utilize asphaltene precipitation as a sole source of carbon and energy.

4.2. Asphaltene Biodegradation and Biomass Quantification. The experiments of asphaltene biodegradation were carried out in the flasks, containing 5 mL of inoculum, 50 mL of the broth growth medium in three pH levels 5.5, 6.5, and 7.5, and three levels of salinity 0, 80, and 160 g·L⁻¹, with considering three initial concentrations of asphaltenes, 15, 25, and 35 g·L⁻¹. The flasks were incubated at 30, 45, and 60 °C for two months. A pure colony of each bacterium on the latest solid culture was removed, added to 10 mL of Muller-Hinton broth, were incubated at 30 °C for 24 h and utilized as pure inoculum. Then, based on the oil-contaminated source of the bacterial consortiums, 2 mL of pure members of each consortium was inoculated to same fresh Muller-Hinton broth, and the culture

Table 6. Analysis of variance (ANOVA)

| response                      | sum of squares | DOF | mean squares | F-value | P-value | Prob > F |
|-------------------------------|----------------|-----|--------------|---------|---------|----------|
| % biodegradation Soil consortium | model          | 845.45 | 14 | 60.39 | 11485.87 | <0.0001 |
|                               | residual       | 0.079 | 15 | 0.0058 |          |          |
| % biodegradation Reservoir water consortium | model          | 1458.81 | 14 | 104.20 | 2.65 | 0.0357 |
|                               | residual       | 590.25 | 15 | 39.35 |          |          |
| % biodegradation Crude oil consortium | model          | 1885.40 | 14 | 134.67 | 10.43 | <0.0001 |
|                               | residual       | 193.76 | 15 | 12.92 |          |          |
| % biodegradation Sludge consortium | model          | 2871.22 | 14 | 205.09 | 16.30 | <0.0001 |
|                               | residual       | 188.74 | 15 | 18.73 |          |          |
| % biodegradation All-strain consortium | model          | 532.19 | 14 | 38.01 | 17.45 | <0.0001 |
|                               | residual       | 32.67 | 15 |          |          |          |

Table 7. Optimum Conditions of Asphaltene Biodegradation Predict Degradation and Desirability

| optimum value                  | temperature (°C) | salinity (g·L⁻¹) | pH  | asphaltene concentration (g·L⁻¹) | predict biodegradation (%) | desirability |
|--------------------------------|------------------|------------------|-----|---------------------------------|---------------------------|--------------|
| Soil consortium                | 49.49            | 76.26            | 5.5 | 26.72                           | 22.0724                   | 1.00         |
| Reservoir water consortium     | 30.00            | 0.00             | 7.42| 15.00                           | 31.7818                   | 0.764        |
| Crude oil consortium           | 30.15            | 160.00           | 6.56| 22.97                           | 38.896                    | 0.826        |
| Sludge consortium              | 30.01            | 146.73           | 5.55| 28.55                           | 39.7995                   | 1.00         |
| All-strain consortium          | 30.21            | 0.67             | 6.39| 34.94                           | 26.4188                   | 1.00         |

Table 8. Kinetic Parameters and rmsd of Applied Models for the Crude Oil Consortium in Asphaltenes Degradation at pH = 6.5, Salinity = 80 g·L⁻¹, Initial Asphaltene Concentration = 25 g·L⁻¹ and Temperatures = 30, 45, and 60 °C

| model          | temperature (°C) | μmax (day⁻¹) | Ks (g·L⁻¹) | Yx/S | rmsd |
|----------------|------------------|--------------|------------|------|------|
| Monod          | 30               | 21.785       | 2513.421   | 2.740| 0.898|
|                | 45               | 8.563        | 1198.620   | 0.334| 0.699|
|                | 60               | 161.871      | 19721.34   | 0.414| 0.959|
| Contois        | 30               | 5.231        | 1629.539   | 2.440| 0.288|
|                | 45               | 1.939        | 1578.588   | 0.439| 0.146|
|                | 60               | 0.377        | 67.844     | 0.444| 0.265|
| Tessier        | 30               | 40.34        | 5436.43    | 0.325| 1.68 |
|                | 45               | 33.491       | 4777.128   | 0.331| 0.698|
|                | 60               | 42.101       | 5123.776   | 0.416| 0.959|

Figure 13. Correlation results of the Tessier model with experimental asphaltene biodegradation data using Crude oil consortium at 45 °C, pH = 6.5, salinity = 80 g·L⁻¹ and initial asphaltene concentration = 25 g·L⁻¹.
mediums were incubated at 30 °C for 24 h. Sampling and analysis were performed at 5 days' intervals during the experiments. Two samples were provided for each test, and the average result of two experiments was reported. To separate the trained asphaltene and quantify the percentage of biodegradation, the IP 143 method was applied. The free asphaltene medium was centrifuged at 5000 rpm for 10 min, the sediment was dried at 70 °C, and the constant weight was measured and reports as the dry weight of the biomass.

4.3. Analytical Procedures. FT-IR spectroscopy (Spectrum GX model, US) was performed to determine the alteration of the asphaltene chemical structure through the biodegradation procedure. For this purpose, the samples were run as KBr pellets (Merck, Germany). The spectra were normally acquired at 4 cm⁻¹ resolutions over the range of 400–4000 cm⁻¹. Also, carbon–hydrogen–nitrogen–sulfur (CHNS) analysis was performed using the CHNSO analyser (ECS 4010 model, Italy) to evaluate the ability of bacterial consortiums in removing these elements from asphaltene precipitation at 45 °C, 80 g L⁻¹ salinity, pH equal to 6.5, and 25 g L⁻¹ of initial asphaltene concentration.

4.4. Asphaltene Biodegradation Optimization. Design of experiments and process variables optimization were done using RSM and central composition design by software design expert 7 (DX7). The process variables including temperature, salinity, pH, and initial asphaltene concentration were identified, and three levels were considered for each of them. For statistical evaluation, the lowest, middle, and highest of available independent factors were coded as −1, 0 and +1, respectively. Based on the number of desired levels and factors, 30 experiments, including six replicates at the center point to find the experimental error, were designed to determine the effect of various parameters on the precipitated asphaltene biodegradation process.

A quadratic polynomial empirical model was employed to predict the response as below

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i h_i + \sum_{i=1}^{k} \beta_i^2 h_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} h_i h_j + \epsilon
\]

where, \(Y\), \(k\), \(\beta_0\), \(\beta_i\), \(\beta_i^2\), \(\beta_{ij}\), and \(\epsilon\) represent response (asphaltene degradation percent), number of variables, intercept, coefficients of the linear parameters, variables, coefficients of the quadratic parameters, coefficients of the interaction parameters and residual associated to the experiments, respectively.

4.5. Kinetic Studies. The kinetic study provides a theoretical framework for optimal design in biotechnologies based on the employment of outdoor activity of natural microbial populations in various aspects such as fermentation, enzyme catalysis, wastewater treatment, and soil bioremediation. In this study, the ability of some kinetic models such as Monod, Contois, and Tessier models to determine the kinetic coefficients of the Crude oil consortium at three temperatures 30, 45, and 60 °C, with a salinity of 80 g L⁻¹, pH equal to 6.5, and an initial asphaltene concentration of 25 g L⁻¹ was evaluated. The cell growth and asphaltene concentration was measured every 5 days for 60 days of the incubation period, and then, the experimental data were used as the input of these models.

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
Authors thank the National Iranian South Oil Company (NISOC) for supporting this research study.

NOMENCLATURE
ANOVA analysis of variance
Asp initial asphaltene concentration (g·L⁻¹)
DOF degree of freedom
k number of variables
Kₚ kinetic constant (g·L⁻¹)
± pH
R² coefficient of determination
Tem temperature (°C)
Sal salinity (g·L⁻¹)
S substrate concentrations (g·L⁻¹)
t time
Y response (biodegraded asphaltene)
Y₀/S biomass yield on asphaltene (g/g)
b variable
x biomass concentration (g·L⁻¹)

Greek letter
α₀ intercept
αᵢ coefficients of the linear parameters
αᵢ₂ coefficients of the quadratic parameters
εᵢ coefficients of the interaction parameters
εᵢᵢ residual associated to the experiments
λ parameter of the Moser model
µ the specific growth rate (day⁻¹)
µₒ max. maximum specific growth rate (day⁻¹)

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