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Research Article

Construction and Performance Evaluation of Dual-Metabolite Oil-Producing Engineering Bacteria Suitable for Low-Permeability Reservoir

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Microbial oil recovery (MEOR) technology has a wide application prospect in the field of enhanced oil recovery in low-permeability reservoirs, and genetic engineering plays an important role. At present, oil-production engineering bacteria constructed by genetic engineering are mainly used to obtain high metabolites and strong environmental adaptability; there are few reports on genetically engineered bacteria with different functional metabolites. However, the bacteria which could efficiently produce one metabolite have a poor ability to produce other metabolites. Therefore, in order to reduce the cost of nutrients and improve the effect of microbial oil recovery, dual-metabolite bacteria have been constructed for low-permeability reservoir. In this paper, four rhamnolipid expression plasmids are extracted from *E. coli*-produced biosurfactant. Then, the rhamnolipid expression plasmids are transferred into the bacteria-produced biopolymer by electrotransformation, and the metabolite performance of the constructed bacteria is evaluated. Finally, the profile control and displacement performance of the constructed bacteria are studied through low-permeability core experiments. The experimental results show that three bacteria, WS1, WS2, and WS3, are successfully constructed that have preferable ability, among which the bacteria WS2 has the best capacity for producing biopolymer and biosurfactant at the same time. After culturing 96 h, the viscosity of the bacteria WS2 fermentation broth could increase to 42.1 mPa·s, and the surface tension and interfacial tension of the fermentation broth could decrease to 24.3 mN/m and 0.035 mN/m, respectively. This time-varying biological viscosity ensures that the microbial system can enter the low-permeability reservoir at a low injection pressure, and the oil recovery of the low-permeability core could be increased by 10.18% after injecting 0.5 PV WS2 microbial system. The findings of this study can help for better understanding of gene construction and technical support for further popularization and application of MEOR in low-permeability reservoirs.

1. Introduction

Chemical flooding is widely used in conventional reservoirs, but the application of chemical flooding in unconventional reservoirs such as low permeability and ultra-low permeability is limited due to high viscosity of the polymer system, difficult injection, and large reservoir damage [1–3]. At present, microbial oil recovery technology has a good application prospect in the field of enhanced oil recovery in low-permeability reservoirs, which is in line with the concept of low cost green development reservoirs. Microbial enhanced oil recovery (MEOR) technology has been effectively applied to increase crude oil production by microorganisms or products of their metabolism such as the biosurfactant and biopolymer [4–6]. Biosurfactants can reduce the interfacial tension between oil and water, accelerate the emulsification in order to improve the microcosmic displacement efficiency. Biopolymer can improve oil-water flow ratio and plug large pores or small cracks in order to extend the macroscopic swept volume [7, 8]. At present, the bacteria which could efficiently produce the biosurfactant have a poor ability to produce biopolymer, and the bacteria which could efficiently produce biopolymer also have a poor ability to produce biosurfactant. Therefore, it is common to use compound bacteria

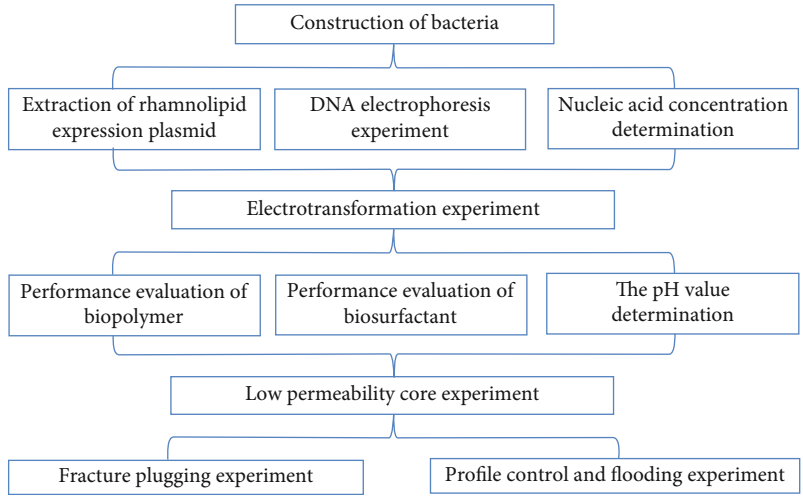


Figure 1: The general sketch of the study.

for microbial oil production, but there are some problems such as whether the compound strains can coexist well and whether they can adapt to the physicochemical properties of the low reservoir at the same time.

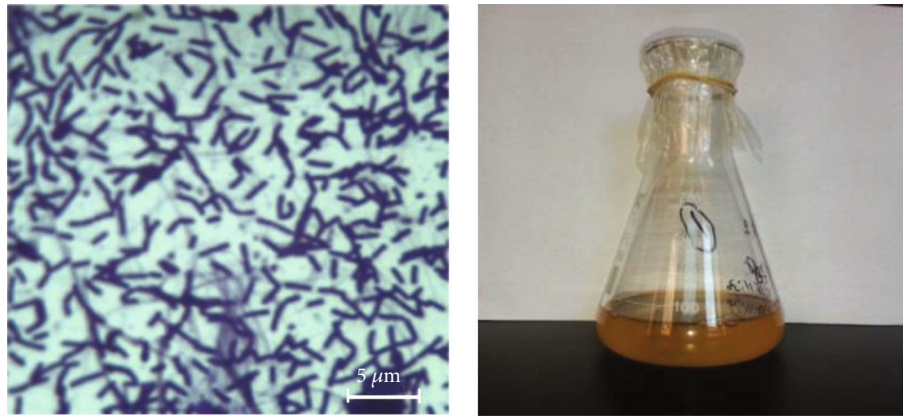
Genetic engineering technology can construct new bacteria by multiple bacteria with different genetic characters, so that they can have the ability to efficiently metabolize the desired products [9, 10]. The genetic engineering technologies used in MEOR mainly include recombination, protoplast fusion, and mutagenesis. At present, oil production engineering bacteria constructed by genetic engineering are mainly used to obtain high metabolites and strong environmental adaptability. Zhao constructed *Pseudomonas aeruginosa* PoprAB by protoplast fusion [11]. The rhamnolipid production of the bacteria under anaerobic conditions could reach 1094.6 mg/l, which was 60.2% higher than that before gene construction. Song cloned the gene *slad* A into plasmid pSTE33 to obtain plasmid pSTalk [12]. The plasmid pSTalk was transformed into thermophilic Geobacillus thermodenitrificans ZJ-3, and a genetically engineered bacteria SL-21 was constructed. SL-21 could degrade 75.08% of the hydrocarbon after 14 d at 70°C, and it has both functions of thermophilic living and hydrocarbon degrading. Sun et al. constructed an engineered bacterium ZR3 by protoplast fusion of *Enterobacter cloacae* and thermophilic geobacter, which produced extracellular polysaccharides [13]. The temperature-adapted growth value of ZR3 was increased from 30°C to 45°C. Although genetic engineering has been applied in the field of microbial oil recovery, there are few reports on genetically engineered bacteria with different functional metabolites.

Combined with the relevant knowledge in the fields of bioengineering and oil production engineering, this paper provides a new method for the construction of oil-producing engineering bacteria suitable for low-permeability reservoir. The bacteria could metabolize both biopolymer and biosurfactant efficiently. First, the construction of dual-metabolite oil-producing engineering bacteria is described. Then, the performance of the bacteria metabolites is evaluated. In the end, the core experiment of EOR is carried out. The microbial system could enter low-permeability reservoirs with a low injection pressure due to the low initial viscosity; when the biopolymer and biosurfactant are increased, the microbial system can improve oil recovery by expanding the swept volume and improving the oil washing efficiency. Therefore, dual-metabolite oil-producing engineering bacteria could be effectively applied to profile control and flooding in low-permeability reservoirs with microfractures. The general sketch of the study is shown in Figure 1.

2. Materials and Methods

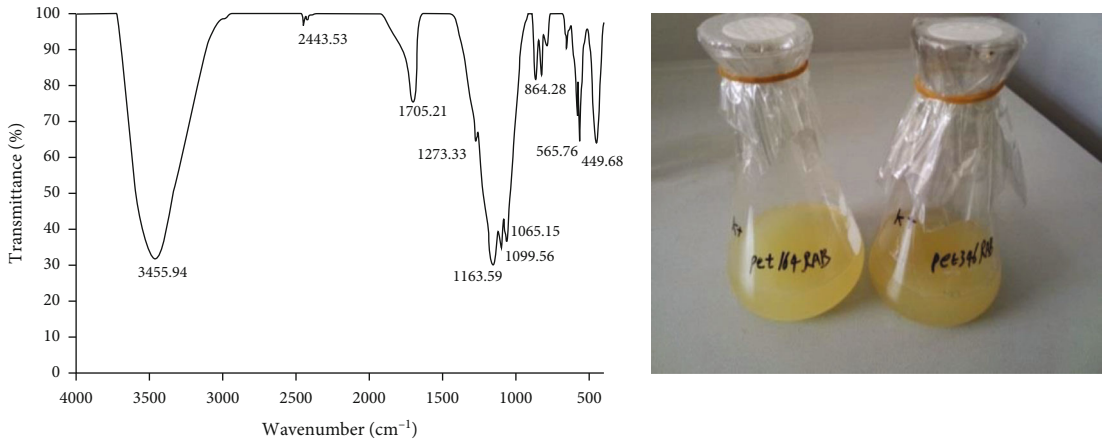
2.1. Bacteria. Pseudomonas SP1 is an original bacteria strain screened in the laboratory from the low-permeability reservoir of Daqing Oilfield, which can produce biopolymer efficiently [14]. After 72 hours of fermentation (cornstarch 20 g/l, yeast powder 10 g/l, Na_2HPO_4 1.5 g/l, KH_2PO_4 1.5 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.05 g/l, NH_4NO_3 3 g/l, NaCl 2 g/l, 40°C, 120 r/min shake culture), the viscosity of fermented liquid can reach 45 mPa·s. According to the analysis of the biopolymer produced by the metabolism of the bacteria, it is identified that it is a biological polysaccharide. The components of the biopolymer include D-galactose and D-glucose, belonging to succinic acid type dextran. The microscopic image of *Pseudomonas* SP1 and the amplified culture medium are shown in Figure 2.

Rhamnolipid is an anionic biosurfactant composed of homologous multistructures; it can decrease the interfacial tension between the mobile phases effectively and has excellent detergency, emulsification, and flocculation ability; in addition, it also has the advantages of nontoxicity and biodegradability [15]. *E. coli* pET-164RAB and *E. coli* pET-346RAB used in this paper are two types of *E. coli* containing the rhamnolipid expression plasmid, and the average yield of rhamnolipid reached 71 mg/l and 56 mg/l, respectively. These two bacteria are very desirable plasmids for extracting strains because they contain the rhamnosyl transferase gene promoter as well as the Kana resistance. The amplified culture medium of the two bacteria and the infrared spectrum of the metabolite rhamnolipid are shown in Figure 3.



(a) (b)

Figure 2: Microscopic image and amplified culture medium of *Pseudomonas* SP1: (a) microscopic image of *Pseudomonas* SP1; (b) amplified culture medium of *Pseudomonas* SP1.



(a) (b)

Figure 3: Infrared spectrum of metabolites and amplified culture medium of *E. coli* pET-164RAB and *E. coli* pET-346RAB: (a) infrared spectrum of rhamnolipid; (b) amplified culture medium.

2.2. Extraction of Rhamnolipid Expression Plasmid. The SDS alkaline lysis method is used in the paper. The principle is to use DNA membrane to selectively adsorb DNA to achieve the purpose of rapid purification of plasmid DNA [16]. The extraction volumes of high-purity DNA plasmids are more than 20 micrograms from 1 to 4 ml bacterial culture. These kits are used for sequencing, in vitro transcription and translation, restriction enzyme digestion, bacterial transformation, and other molecular biology experiments. The main experimental steps are as follow.

(1) 2 ml of the bacterial solution grown in LB (Luria-Bertani) medium is centrifuged at 12,000 rpm/min for 1 min in a centrifuge, and the supernatant is discarded

(2) 250 μl of Buffer S1 suspension bacterial precipitation is added; we cannot leave small bricks through the suspension which is suspended evenly (RNase A should be added in Buffer S1)

(3) 250 μl of Buffer S2 is added, gently and fully, up and down 4~6 times, so that the solution mixed evenly. The bacteria fully cracks until the translucent solution is formed; this step should not be more than 5 min

(4) 350 μl of Buffer S3 is added, gently and fully, up and down 6~8 times, then centrifuge for 10 min at 12,000 rpm/min. And the centrifugal method is also used to continue the experiment

(5) The centrifugal supernatant from step 4 is sprayed with a micropipettor and transferred to the preparation tube (placed in a 2 ml centrifuge tube) and centrifuged for 1 min in a centrifuge at 12,000 rpm/min; then, the filtrate is discarded

(6) The preparation tube is placed into the centrifuge tube; 500 μl of Buffer W1 is added and centrifuged

for 1 min in a centrifuge at 12,000 rpm/min; then, the filtrate is discarded

(7) The prepared tube is returned to the centrifuge tube, 700 μl of Buffer W2 is added and centrifuged at 12,000 rpm/min for 1 min, and the filtrate is discarded. The filtrate is washed once again with 700 μl of Buffer W2 in the same manner and discarded (it should be confirmed that absolute ethanol had been added in Buffer W2 as the volume of the reagent bottle)

(8) The preparation tube is returned to a 2 ml centrifuge tube and centrifuged at 12,000 rpm/min for 1 min in a centrifuge

(9) The preparation tube is transferred to a new 1.5 ml centrifuge tube. 60~80 μl of deionized water (ultrapure water) is added to the center of the preparation membrane and allowed to stand at room temperature for 1 min and centrifuged at 12,000 rpm/min for 1 min (heating deionized to 65°C for elution effectiveness)

2.3. DNA Electrophoresis Experiment. The main experimental steps are as follows.

(1) Weighted the appropriate amount of agarose and placed it in a triangle bottle, add a certain amount of 1× TBE (0.7% concentration), and melt the agarose gradually by heating

(2) When the gel temperature is dropped below about 55°C, add 2~3 μl of ethidium bromide (EB) at a concentration of 0.5 mg/l

(3) Place the gluing plate into the chamber, then place the comb vertically above the gluing plate, and finally pour the melted agarose into the plastic sheet

(4) When the gel is completely cooled and solidified to become milky white opaque solids, gently pull the comb out vertically from the gel

(5) Place the gel into the electrophoresis bath of the 1× TBE electrophoresis buffer and continue to add the electrophoretic buffer to allow the electrophoresis buffer immersed in the gum surface to be higher than the gel

(6) Drop 1~2 μl sample buffer on the sealing film, mix with a certain amount of DNA sample, and then add it to the gel hole. The sample should be deposited at the bottom of the hole. In addition, the appropriate DNA relative molecular weight standard should be added

(7) Cover the electrophoresis bath cover and select the appropriate electrophoretic voltage (120 V) and the direction of the electrophoresis (point to the negative end). Turn on the power switch and start the electrophoresis. The DNA sample would move from the negative to the positive [17].

(8) When the bromophenol blue pigment is close to the gel near the positive side (generally, the bromophenol blue band is about 1/2 or 2/3 of the gel length), turn off the power to stop the electrophoresis (120 V, 30 min), and open the bath cover

(9) Take out the sample and observe and photograph the sample under the ultraviolet lamp. The position of DNA presents fluorescence, and if the time is longer than 4~6 h, the fluorescence effect decreased

2.4. Determination Experiment of Nucleic Acid Concentration. The main experimental steps are as follows.

(1) Preheat nucleic acid protein analyzer 10~20 min

(2) Put 10 μl of plasmid DNA sample into 990 μl of ddH_2O and mix well. Then, place in the quartz cuvette of the nucleic acid protein analyzer

(3) Read the number under the A260 condition using ddH_2O as a blank comparison

(4) Calculate the content of DNA using the following formula. The content of DNA = $A_{260} \times 50 \times 100$ (μg/mL) [18].

2.5. Electrotransformation Experiment. The main experimental steps are as follows.

(1) The recipient cells are mixed with plasmids (foreign DNA fragments) and placed into the electric shock cup together

(2) After the voltage is added to the electric shock cup, the cell membrane component is polarized, and some instantaneous holes are formed on the cell surface

(3) The plasmids (foreign DNA fragments) enter the cell through the holes formed above

(4) When the external electric field is withdrawn, the cell surface pores are closed, preventing the external molecules from entering the cell. The flow chart is shown in Figure 4, and the operational parameters set in the electrotransformation experiment are shown in Table 1

2.6. Performance Evaluation Experiment of Biopolymer. The viscosities of the transformed bacteria fermentation broth and the medium are monitored by a Brookfield viscometer (LVDV-II+P, USA) [19].

2.7. Performance Evaluation Experiment of Biosurfactant. The surface tension of the transformed bacteria fermentation broth and the medium are monitored by the surface tensiometer (A201, USA), and the interfacial tension of WS1, WS2, WS3, and culture are monitored by the interfacial tensiometer (TX500C, USA) [20].

2.8. pH Value Determination of Bacteria Fermentation Broth. The pH values of WS2 and medium are monitored by the pH meter (Orion Star A211, USA) [21].

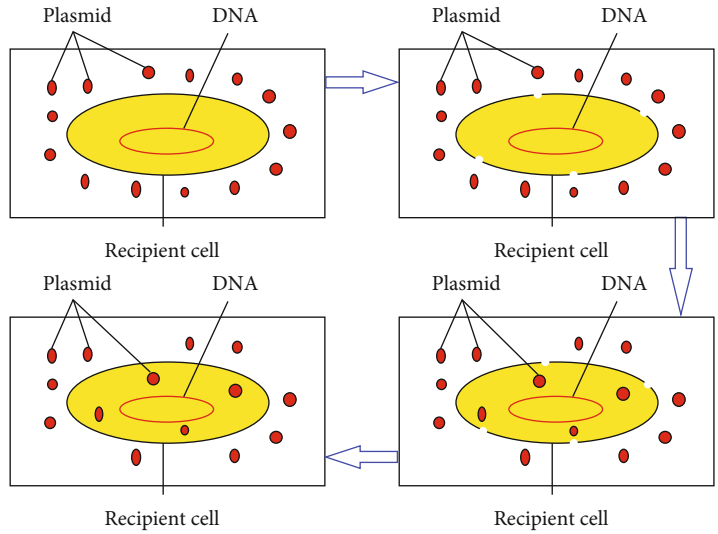


Figure 4: The flow chart of electrotransformation experiment.

Table 1: The operational parameters set in the electrotransformation experiment.

Parameter	Capacitance (μF)	Voltage (kV)	Resistance (Ω)	Time (ms)
Value	25	2.0	200	5.2

2.9. Fracture Plugging Experiment of Low-Permeability Core. Daqing natural sandstone cores with permeability of about 200×10^{-3} μm^2 are selected to simulate microfractures in low-permeability reservoirs. After 0.5 PV WS2 microbial system is injected, the well is shut down for 4 days [22–24]. The experimental temperature is 40°C, the salinity of formation water is 4057.32 mg/l, the viscosity of simulated crude oil is 9.8 mPa·s, the injection velocity is 0.1 ml/min at constant velocity mode, and the back pressure at the outlet is 11.5 MPa.

2.10. Profile Control and Flooding Experiment of Low-Permeability Core. The heterogeneous cores are selected for the experiment, in which the permeability of the middle layer is 200×10^{-3} μm^2 to simulate the microfractures in the low-permeability reservoir, and the upper and lower layers are 20×10^{-3} μm^2 to simulate the matrix. The thickness of the single layer is 1.5 cm. After injecting 0.5 PV three microbial systems (*Pseudomonas* SP1, *E.coli* pET-346RAB, WS2), respectively, the injection system is shut down for 4 days. The experimental conditions are the same as those in Section 2.9 except that the injection velocity is 0.3 ml/min at constant velocity mode. The flow diagram of the experiment is shown in Figure 5, and the artificial core parameter data is shown in Table 2.

3. Results and Discussion

3.1. Construction of Dual-Metabolite Oil-Producing Engineering Bacteria. The electrotransformation method is used in this paper. The cost of this method is relatively low, there are almost no biological and chemical side effects, and it has a wide range of application [25]. The efficiency of gene transformation is much higher than that of chemical methods [26].

In order to increase the success rate of bacteria construction, plasmids are extracted twice from *E. coli* pET-164RAB and *E. coli* pET-346RAB separately. Plasmids pET-164RAB (1) and pET-164RAB (2) are obtained by extraction from *E. coli* pET-164RAB; plasmids pET-346RAB (1) and pET-346RAB (2) are obtained by extraction from *E. coli* pET-346RAB. Plasmids pET-164RAB and pET-346RAB are all medium copy number plasmids with different promoter strengths, and the copy number of genes is the main factor affecting gene heterologous expression. The extracted plasmids are kept in the -40°C temperature environment, left to be used in subsequent experiments.

The DNA electrophoresis experiments are carried out with the BIO-RAD protein electrophoresis apparatus for rhamnolipid expressing plasmids pET-164RAB (1), pET-164RAB (2), pET-346RAB (1), pET-346RAB (2), and pET-346RAB (W), and the experimental results are shown in Figure 6.

From the DNA electrophoresis map, we could see that the four kinds of rhamnolipid expression plasmids are displayed on the electrophoresis map, which showed that the plasmid is successfully extracted.

The determination experiments are carried out with the BIO-RAD protein electrophoresis apparatus for rhamnolipid expressing plasmids pET-164RAB (1), pET-164RAB (2), pET-346RAB (1), and pET-346RAB (2), and the experimental results are shown in Table 3.

The results show that the nucleic acid concentration of rhamnolipid expression plasmids are 69 mg/l, 76 mg/l, 56 mg/l, and 45 mg/l. These concentrations can meet the requirement and can be used in subsequent experiments.

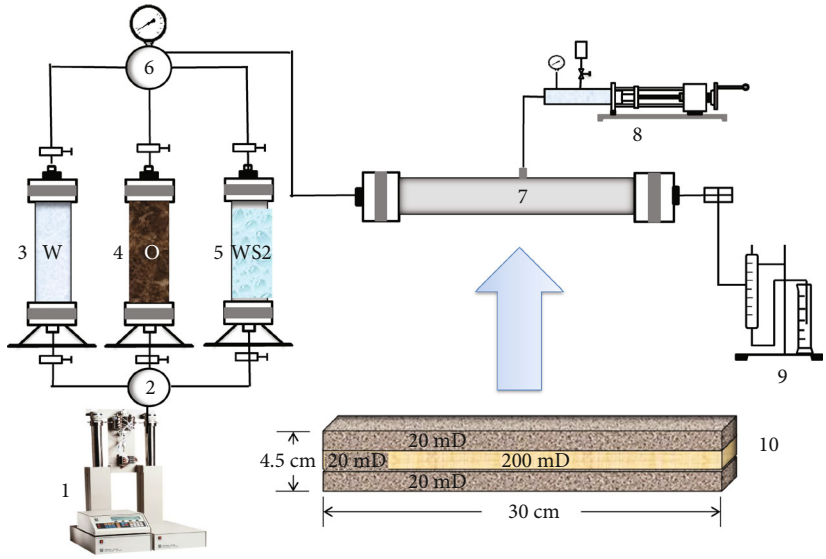


Figure 5: Flow diagram of experiment. 1: ISCO pump; 2: six-port valve; 3: stratum water container; 4: oil water container; 5: WS2 microbial system container; 6: pressure gauge; 7: core gripper; 8: hand pump; 9: metering device, 10: low-permeability microfracture core model.

Table 2: The artificial core parameter data.

Core number	Core type	Length (m)	Cross-section area (m^2)	Microfracture length (m)	Microfracture cross-section area (m^2)
RZ1	Artificial core	0.304	0.002	0.304	0.0006
RZ2		0.306	0.002	0.306	0.0006
RZ3		0.298	0.002	0.298	0.0006
RZ4		0.301	0.002	0.301	0.0006

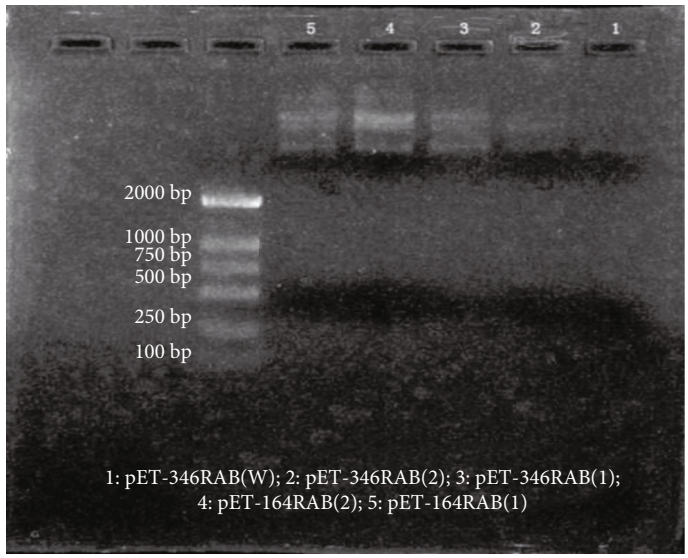


Figure 6: Electrophoresis map of rhamnolipid-expressing plasmid DNA.

Table 3: Nucleic acid concentration.

Plasmid	pET-164RAB (1)	pET-164RAB (2)	pET-346RAB (1)	pET-346RAB (2)
Concentration (mg/l)	69	76	56	45

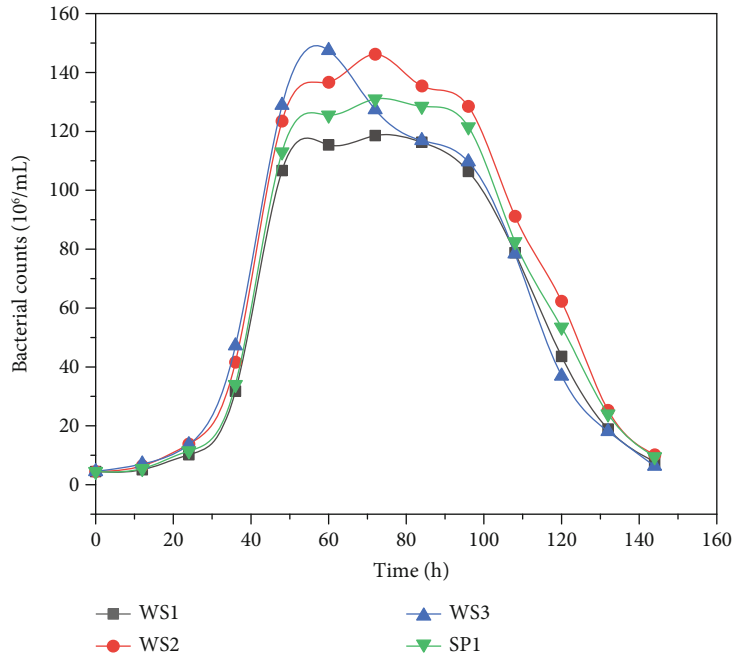


Figure 7: Growth curves of transformed bacteria and the original bacteria.

Four groups of electrotransformation experiments are carried out using pET-164RAB (1), pET-164RAB (2), pET-346RAB (1), and pET-346RAB (2) in the above-mentioned methods. They are cultured in LB medium containing antibiotics (Ampicillin 100 μg/ml). It is found that the bacterial colonies grown on the plates transformed by pET-164RAB (1), pET-346RAB (1), and pET-346RAB (2) plasmids showed colony growth. Each of the plasmids is picked up as a single colony and cultured and labeled WS1, WS2, and WS3, respectively, for subsequent performance evaluation. There is no bacterial colony grown in the plate transformed by pET-164RAB (2).

3.2. Performance Evaluation of New Engineering Bacteria Metabolites

3.2.1. Growth Regularity of Bacteria. The bacterial counts of new constructed bacteria and original bacteria are determined by the plate count method. The results of bacterial count data at different times are shown in Figure 7. The growth curves of new constructed bacteria and original bacteria are similar at different growth stages, and the maximum counts of bacteria can reach 1.46×10^8/ml in the stable growth stage. The results showed that the three plasmids had little effect on the growth of the recipient bacteria *Pseudomonas* SP1.

3.2.2. Performance Evaluation Experiment of Biopolymer. The results of viscosity data at different times are shown in Figure 8. From the viscosity change curves, it can be seen that the viscosity of the medium has always been around 10 mPa·s due to the presence of corn starch in the medium, and the viscosities of three transformed bacteria fermentation broths are decreased firstly and then increased. The microorganisms firstly reduce the transformed bacteria liquid viscosity by consuming the corn starch in the culture medium and then increase the viscosity by producing the biopolymer. The viscosities of bacteria WS2 and WS3 are all higher than that of bacteria WS1; they are higher than 40 mPa·s after culturing for 96 h. It is indicated that the bacteria WS2 and WS3 had better performance in producing biopolymer. This time-varying biological viscosity ensures that the microbial system can enter the low-permeability reservoir at a low injection pressure. At the same time, the system could artificially and controllably produce biopolymers in microfractures by adjusting the concentration of the nutrient solution.

3.2.3. Performance Evaluation Experiment of Biosurfactant. The results of surface tension data and interfacial tension at different times are shown in Figures 9 and 10, respectively. It can be seen from the surface tension and interfacial tension curves that the surface tension and interfacial tension of the medium are 51-54 mN/m and 4.7-4.9 mN/m, respectively, while the values of the corresponding three transformed bacteria fermentation broths are lower. Among them, WS2 has the strongest ability to reduce interfacial tension; the interfacial tension could reach 0.034 mN/m after culturing for 120 h. The experiment results show that the bacteria WS2 have the best capacity of producing biopolymer and biosurfactant at the same time. This may be because the plasmid pET-346RAB has a stronger promoter than pET-164RAB, and more of plasmid pET-346RAB are fused in WS2 during the fusion process. Therefore, the bacteria WS2 should be used as the optimum oil-producing engineering bacteria for further experiments.

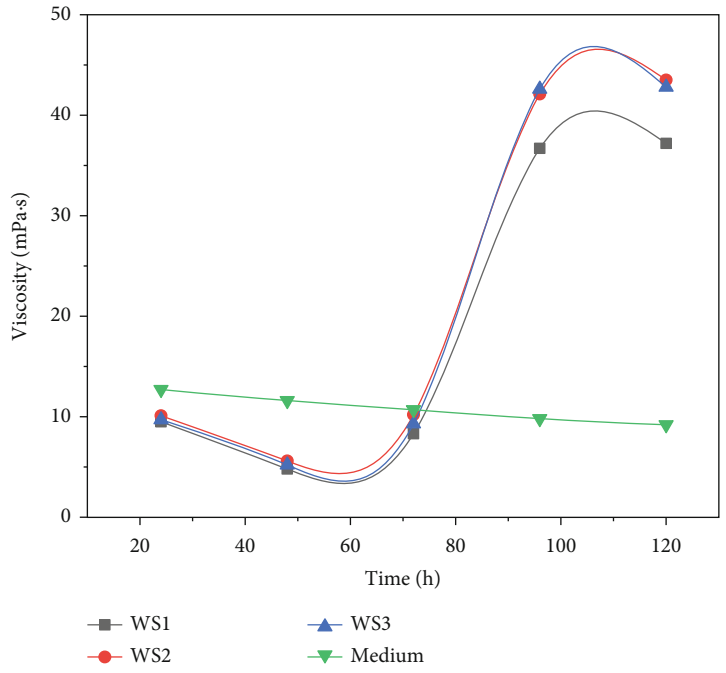


Figure 8: Viscosity curves of transformed bacteria fermentation broths (40°C).

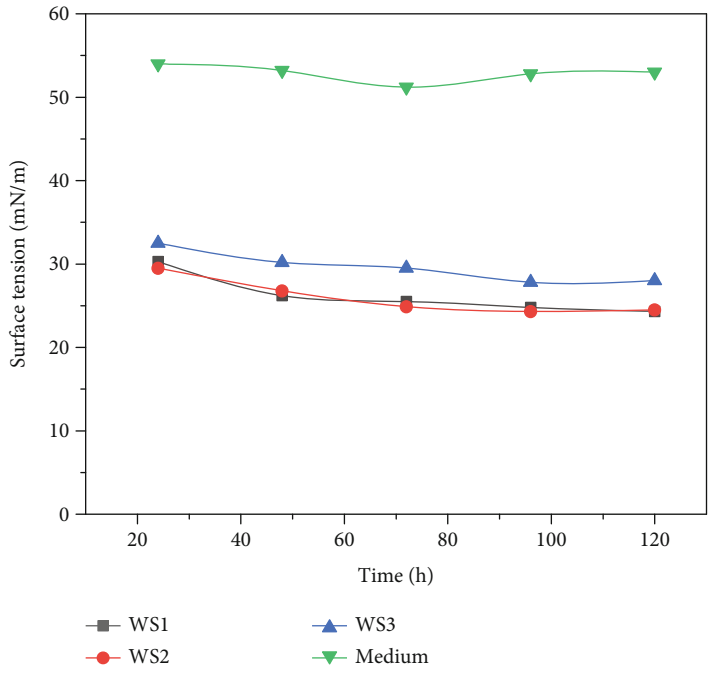


Figure 9: Surface tension curve of transformed bacteria fermentation broths (40°C).

3.2.4. pH Value Determination of Bacteria Fermentation Broth. The results of pH value data at different times are shown in Figure 11. It can be seen that the pH value of the medium is 6.7~7.0, and the pH value of bacteria WS2 fermentation broth is 7.0~7.5. If the pH value of the WS2 fermentation broth is higher than 7.5 or lower than 6.5, the pH value needs to be adjusted to the neutral environment because the neutral environment fermentation broth is conducive to the sustainable growth and metabolism of microorganisms.

3.3. Profile Control and Flooding Effect of Microbial System

3.3.1. Fracture Plugging Experiment of Low-Permeability Core. The core permeability change data before and after injection are shown in Table 4. The experimental results

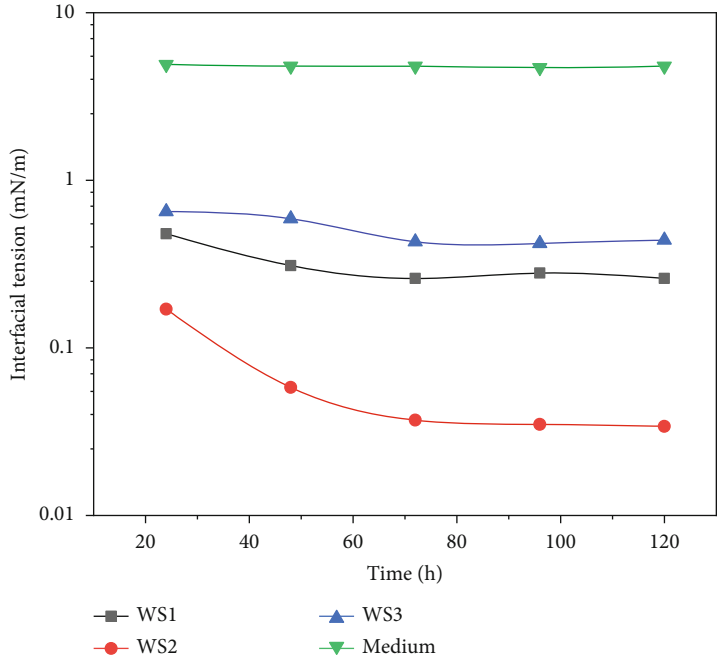


Figure 10: Interfacial tension curve of transformed bacteria fermentation broths (40°C).

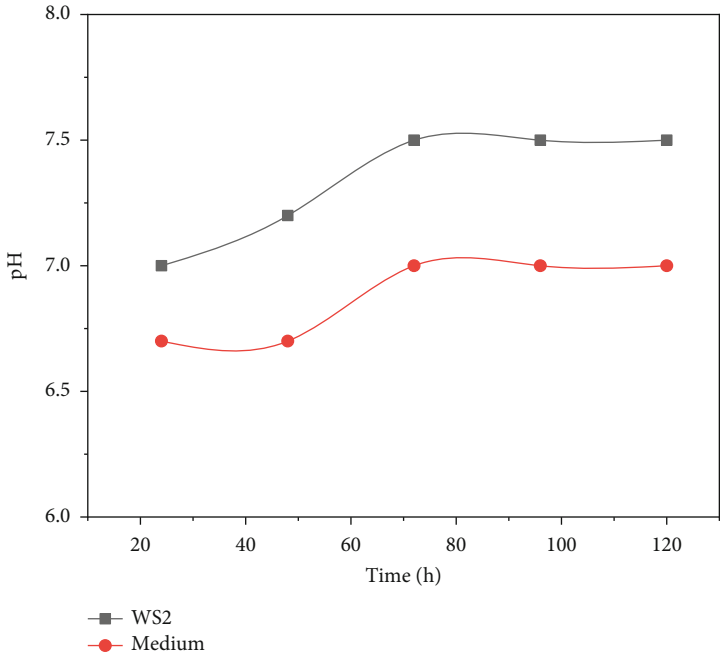


Figure 11: pH value curve of transformed bacteria.

Table 4: Permeability change data of natural cores.

Core number	Length (m)	Cross-section area (m^2)	Porosity (%)	Permeability before injection (10^{-3} μm^2)	Permeability after injection (10^{-3} μm^2)	Plugging rate (%)
TR1	0.084	0.00049	24.37	163	16.8	89.69
TR2	0.089	0.00049	24.61	185	17.1	90.76
TR3	0.087	0.00049	24.56	176	14.2	91.95

Table 5: Profile control and flooding effect of different systems.

Core number	System	Water flooding recovery rate (%)	Injection system recovery rate (%)	Post water flooding recovery rate (%)	Total recovery rate (%)
RZ1	Blank controls	26.21	—	—	—
RZ2	SP1	26.17	2.57	4.19	32.93
RZ3	E. coli pET-346RAB	26.43	2.01	1.02	29.46
RZ4	WS2	26.29	3.26	6.92	36.47

show that the microfractures in low-permeability reservoirs can be effectively plugged by the synergistic effect of microorganism growth and metabolic biopolymer after WS2 microbial system injection, and the average plugging rate is 90.82%.

3.3.2. Profile Control and Flooding Experiment of Low-Permeability Core. The oil displacement effect is shown in Table 5. Comparing the profile control and flooding effects of different microbial systems, it can be seen that the WS2 microbial system has the dual effects of expanding the vertical sweep volume and improving the oil displacement efficiency after injecting the microfracture low-permeability reservoir. After the measure, the oil recovery can be increased by 10.18%, which is better than the SP1 system and the *E. coli* pET-346RAB system at the same injection volume.

In the process of practical application, the successful construction of new oil production engineering bacteria not only reduces the amount of microbial systems injection but also effectively solves the growth competition between biopolymer-producing and biosurfactant-producing bacteria. However, this research also has some disadvantages and limitations, such as whether the genetic performance of the strain can continue to be stable and the matching relationship between the strain and different physical reservoirs. At the same time, the mechanism of enhanced oil recovery is not clear; these are also the focus of the next research work.

4. Summary and Conclusions

In this paper, three new types of oil production engineering bacteria are constructed by electrotransformation, and their properties are evaluated. The following conclusions are obtained.

(1) The allogeneic expression of the plasmid could be completed by the SDS alkaline lysis method and electrotransformation experiment, so as to realize the purpose of construction of dual-metabolite oil-producing engineering bacteria

(2) Transformed bacteria WS2 is the best constructed oil-producing engineering bacteria because that bacteria had the best capacity for producing biopolymer and biosurfactant at the same time. After culturing for 96 h, the viscosity of fermentation broths could increase to 42.1 mPa·s, the surface tension could decrease to 24.3 mN/m, and the interfacial tension could decrease to 0.035 mN/m

(3) Core experiment results show that the WS2 microbial system can effectively plug microfractures in low-permeability reservoirs, expand the vertical sweep volume, and improve the oil displacement efficiency. After injection of the 0.5 PV WS2 microbial system, the oil recovery can be increased by 10.18%.

Data Availability

The raw data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Conflicts of Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with this work.

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