Study on the removal performance of modified *Enteromorpha*-immobilized bacterial agent in oil contaminated seawater

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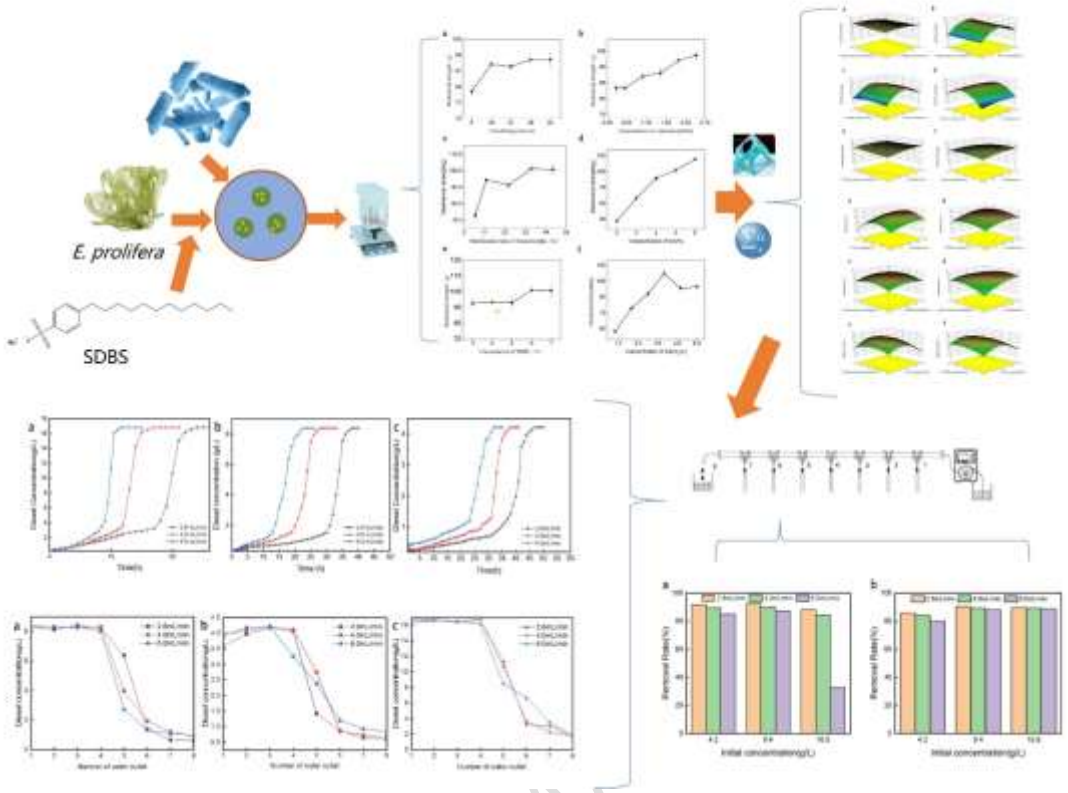
GRAPHICAL ABSTRACT

The modified *Enteromorpha*-immobilized bacterial agent was optimized to reach excellent removal efficiency of diesel (90.39%) and recyclability.



Abstract

Compared with free bacteria, immobilized bacteria have a higher oil degradation rate, making them highly promising for the bioremediation of offshore oil pollution. In our study, preparation conditions of modified *Enteromorpha*-immobilized bacterial agent were optimized by response surface method. A reactor was constructed and the effect of diesel concentration and flow rate on the removal rate were investigate. The recyclability of modified *Enteromorpha*-immobilized bacterial agent was further studied. The result showed that, under the optimal preparation conditions, the removal rate of diesel in 5 days reached 90.39% and the mechanical strength reached a maximum value of 173.67g. When the inlet flow rate was not greater than 6.0mL/min and the concentration of diesel was less than 16.8g/L, the removal rate of diesel was guaranteed within 10.5h. After three cycles of diesel breakthrough, the removal rate can still attain 82.35%. This study provides a valuable insight into the practical application of immobilization technology to remove petroleum from seawater in the future.

Keywords: Oil pollution; *Enteromorpha prolifera*; Modification; Immobilized bacterial agent

1. Introduction

Marine oil pollution was caused by the leakage of oil during the process of exploitation, transportation, refining and use, which has brought huge disasters to marine life and environment(Chen et al., 2020a; Chen et al., 2020b; Li et al., 2020; Naeem and Qazi, 2020). As oil pollutants are persistent and bioaccumulative(Yang et al., 2021), the establishment of practical and effective restoration and control measures is imminent.

To remediate areas contaminated with oil, various traditional technologies have been employed, including thermal extraction, steam stripping, adsorption, and bioremediation(Xue et al., 2019). Among them, bioremediation has been proved to be a low-cost, easy-to-operate, and pollution-free repair method, which has developed rapidly in recent years(Tang et al., 2019; Wang et al., 2019). Microbial agents has been widely employed to remediate oil-contaminated soil. However, their effectiveness may be compromised when added to the ocean, as the agents can be inactivated and their density reduced. Simultaneously, the low solubility, non-polarity and hydrophobicity of oil also limited the oil degradation(Fu et al., 2019; Pereira et al., 2019; Varjani and Upasani, 2017; Xu et al., 2018b). The immobilized microorganism technology confines microbial agents within an enclosed space, making it an effective solution for the aforementioned issues(Kumar et al., 2019). Compared with free cells, immobilized system provides a more stable living environment for microorganisms(Abu Talha et al., 2018; Qin et al., 2013; Xue et al., 2022).

Although considerable attention has been focused on developing immobilization carriers with several specific properties (including strength, chemical and thermal stability, durability and surface properties), the activity of immobilized bacteria is still limited due to the lack of nutrients. Chen (2012) applied polyurethane-polyurea copolymer as an immobilization carrier and determined that the degradation efficiency

is only 47.25% without providing sufficient nutrients(Dehnavi and Ebrahimipour, 2022). Alginates are a heterogeneous group of polymers, with a wide range of functional properties, their success as immobilization matrices will rely on an appropriate choice of materials and methodology for each application(Mardiana et al., 2019; Smidsrod and Skjakbraek, 1990). The alginate could improve the degradation rate of diesel. Common methods for microbial immobilizing include adsorption, embedding, cross-linking, and covalent bonding(Zhang et al., 2010). The embedding immobilization technique is widely used due to its simple operation, mild reaction conditions, low risk of microorganism leakage, excellent stability and reusability, high microbial activity and cell capacity, and high immobilization efficiency (>70%) compared to other methods like adsorption, cross-linking, and covalent bonding(Kuyukina et al., 2009; Wu et al., 2009).

The *E. prolifera* formed large-scale blooms (the so-called "green tide") from 2007 in the Yellow Sea, China. Currently, the majority of *E. prolifera* collected from the sea is considered as solid waste. Therefore, it is urgent to develop *E. prolifera* utilization technique and reduce the pressure of marine environmental pollution. At present, the most common utilization of *E. prolifera* is fertilizer and feed. *E. prolifera* was used to prepare a tunable amphiphilic phyllox modified graphene gascoagulant for oil-water separation(Ji et al., 2021). Hydrogel calcium alginate microparticles derived from *E. prolifera* were utilized as a biomaterial to remove heavy metals from water(Duc et al., 2021). *E. prolifera* is composed of a single layer of cells, surrounded by a tubular or adhesive ribbon, this unique physical structure provides considerable surface for adsorption. In addition, it would be more porous after modification by sodium dodecylbenzene sulfonate (SDBS), and its adsorption capacity was further improved(Xu et al., 2018a; Yang et al., 2021). Meanwhile, it can provide nutrients for growth of bacteria such as N and P(Shi et al., 2020). Therefore modified *E. prolifera* is a suitable carrier for microbial immobilization, and this provides a new idea for the resource utilization of *E. prolifera*.

In this paper, the *E. prolifera* was modified and combined with oil-degrading bacteria to remove oil. The single factor experiment, response surface and the

breakthrough curve were used for this study. Therefore, to the aim of this study is: (1) determine optimal preparation conditions of the modified *Enteromorpha*-immobilized bacterial agent; (2) study the removal performance of the agent under the optimal conditions; (3) evaluate the recyclability of the agent.

2. Material and methods

2.1 Materials and strain

All the chemicals used in this study are of analytical grade. *E. prolifera* was collected from 35°57′33.22″N, 120°14′55.36″E in Golden Beach, Qingdao, July 2020. The highly efficient oil-degrading strain Sp8 (*Shewanella algae*) was isolated from seawater samples from 35°56′27.92″N, 120°12′52.59″E in Tangdao Bay, Qingdao. Diesel (0#) was purchased from Petro China (Qingdao Center, Huangdao District), and filtered with 0.22 μm membrane before using.

2.2 Single factor experiment

Table 1. Single factor experimental design scheme

Single factor	Fixed condition
SDBS concentration （3%、4%、5%、6%、7%，w/v）	Digging time 24 h SA concentration 4% Cross-linking time 27 h $CaCl_2$ concentration 3% *E. prolifera* concentration 1.5%
Digging time （10 h、20 h、30 h、40 h、50 h，w/v）	SDBS concentration 5% SA concentration 4% Cross-linking time 27 h $CaCl_2$ concentration 3% *E. prolifera* concentration 1.5%
SA concentration （2%、3%、4%、5%、6%，w/v）	SDBS concentration 5% Digging time 24 h Cross-linking time 27 h $CaCl_2$ concentration 3% *E. prolifera* concentration 1.5%
E. prolifera concentration （0.25%、0.5%、1%、1.5%、2%、2.5%，w/v）	SDBS concentration 5% Digging time 24 h SA concentration 4% Cross-linking time 27 h $CaCl_2$ concentration 3%

$CaCl_2$ concentration （1%、2%、3%、4%、5%，w/v）	SDBS concentration 5% Digging time 24 h SA concentration 4% Cross-linking time 27 h *E. prolifera* concentration 1.5%
Cross-linking time （9 h、18 h、27 h、36 h、45 h）	SDBS concentration 5% Digging time 24 h SA concentration 4% $CaCl_2$ concentration 3% *E. prolifera* concentration 1.5%

The basic process of modified *Enteromorpha*-immobilized bacterial agent preparation was described by Yang et al(Yang et al., 2021). *E. prolifera* was dipped in a SDBS solution, then dried and ground for later usage. After enrichment, bacteria and modified *E. prolifera* were mixed into SA solution and transferred into $CaCl_2$ with a dropper to form beads. At last, the beads were stored at 4 °C containing $CaCl_2$ to cross-linking. The modified *Enteromorpha*-immobilized bacterial agent was stored in distilled water before use. Concentration of SA, SDBS, *E. prolifera*, and time of dipping were used as influencing factors. A single factor experiment was designed to optimize the conditions and parameters of the immobilization process. Meanwhile the mechanical strength of modified *Enteromorpha*-immobilized bacterial agent was target. The design of single factor experimental was shown in Table 1. The mechanical strength was performed as described by Xue et al (Xue et al., 2019).

2.3 Optimization of preparation conditions

Table 2. Experimental design and experimental results of condition optimization

Run	A: Digging time(h)	B:SA concentration(%)	C:SDBS concentration(%)	D: Enteromorpha concentration (g)	Mechanical strength (g)	Removal rate(%)
1	24	5	7	2.0	135.333	60.459
2	48	4	6	2.0	88.333	67.488
3	36	5	6	2.0	164.750	85.214
4	36	5	5	1.5	137.000	49.016
5	24	5	5	2.0	148.000	57.216
6	36	4	6	1.5	94.333	52.530
7	36	6	6	1.5	118.667	47.394
8	48	5	6	1.5	187.333	95.241

9	48	6	6	2.0	107.000	49.106
10	48	5	5	2.0	173.667	79.472
11	36	5	6	2.0	174.000	78.543
12	48	5	6	2.5	167.000	73.255
13	24	5	6	2.5	149.667	63.343
14	36	5	6	2.0	154.000	80.421
15	36	5	5	2.5	155.667	66.406
16	24	5	6	1.5	148.333	55.233
17	36	4	6	2.5	175.000	76.776
18	24	6	6	2.0	115.667	36.221
19	36	5	7	2.5	165.000	86.590
20	36	6	5	2.0	124.333	54.422
21	24	4	6	2.0	84.000	60.550
22	36	5	6	2.0	164.750	87.672
23	36	6	7	2.0	119.000	49.286
24	36	4	7	2.0	81.000	65.596
25	36	6	6	2.5	135.667	66.406
26	36	5	7	1.5	157.000	64.514
27	48	5	7	2.0	137.000	88.753
28	36	4	5	2.0	89.000	62.262
29	36	5	6	2.0	156.000	87.554

Statistical Product Service Solutions (SPSS 15.0) was used for data analysis, according to the mechanical strength, the range of the four factors were: the dipping time of *E. prolifera* of 24-48(h), SA concentration of 4-6%(w/v), SDBS concentration of 5-7%(w/v), *E. prolifera* concentration of 1.5-2.5%(w/v) (Table 2).

Using the central combination design principle of Box-Benhnken, the factors of SA, SDBS, *E. prolifera* concentrations and the dipping time were analyzed by response surface(Calera et al., 2020; Maleki and Karimi-Jashni, 2020), and the influences of agent prepared under different combinations of four factors on removal rate of diesel (%) in 5 days and mechanical strength (g) were discussed. The data of response surface method was analyzed using analysis of variance. The regression results were fitted to the experimental results using Design-Expert 8.0 software to gain quadratic multiple regression models, 3D surface plots with contour plots were established to obtain the optimal preparation conditions of modified *Enteromorpha*-immobilized microbial agent.

The mechanical strength of the modified *Enteromorpha*-immobilized bacterial agent and its removal rate of diesel were taken as response values. The optimal preparation conditions of the agent were obtained. The removal rate of diesel was obtained according to the following formula:

$$D = (1 - C_1/C_0) \times 100\% \quad (1)$$

Where C_0 (mL/L) is the initial concentration of diesel, C_1 (mL/L) is the residual concentration of diesel, D (%) is the removal rate of diesel.

The predicted value was compared with the experimental value, and the reason was analyzed.

2.4 Research of removal rate

The diesel (control group) and remaining diesel by modified *Enteromorpha*-immobilized bacterial agent at 7 days were detected via gas chromatography-mass spectrometry (GC-MS) (Agilent 19091S-433, USA). The method of the pre-treatment GC-MS was performed as previously described by Shi et al. (Shi et al., 2019).

2.5 Research on removal performance

A reactor was designed to study removal performance of modified *Enteromorpha*-immobilized bacterial agent. The reactor was composed of four parts: peristaltic pump, sample tube, reaction column and outlet pipe (Figure 1). The reaction column is divided into 8 sections, each length of 10 cm, and a water outlet and a final water outlet are set between each two sections, numbered from outlets 1 to 8, to detect the concentration of water (Figure 1). The modified *Enteromorpha*-immobilized bacterial agent was filled into the reaction column.

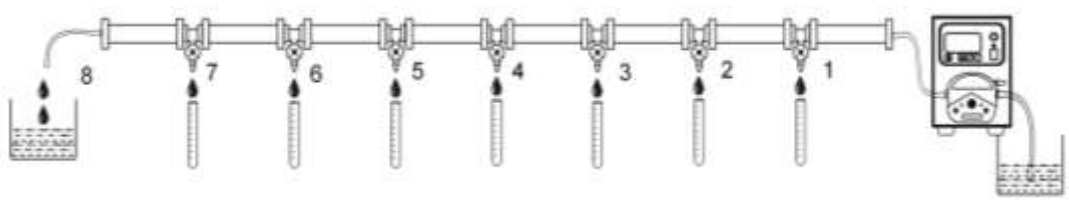


Figure 1 Reactor

To evaluated the impact of flow rates and diesel concentrations on the degradation rate and removal effectiveness, the inlet flow rates were set as 2.6 mL/min, 4.0 mL/min, 6.0 mL/min, and inlet diesel concentrations were set as 4.2 g/L, 8.4 g/L, 16.8 g/L, respectively. Degradation rates of outlets before breakthrough point at different inlet flow rates and diesel concentration was measured, the breakthrough time (T) ,1/2T and 10h for different diesel concentrations and flow rates was determined.

2.6 Recyclability of modified *Enteromorpha*-immobilized bacterial agent

Continuous removal and elution were used as reusability experiments. The removal rate of diesel, surface adsorption and internal intake of each cycle was measured by ultraviolet spectrophotometry. After the removal rate of diesel, surface adsorption and internal intake was measured in each cycle, the modified *Enteromorpha*-immobilized bacterial agent was filtered out and washed with physiological saline for 2–3 times, and then the agent was added to the new degradation medium to start a new cycle of experiments. Take five days as an experimental cycle, a total of three cycles of cyclic experiments were carried out. In order to reduce experimental errors, each group of experiments had three parallel samples.

3. Results and discussion

3.1 Influence of different factors on mechanical strength

The influence of four factors on the mechanical strength of the modified *Enteromorpha*-immobilized bacterial agent was illustrated in Figure 2. The mechanical strength increased as the concentration of SA and *E. prolifera* increased, with a tendency to further increase as the SDBS concentration rose. The most significant change was observed when the SDBS concentration increased from 5% to 6%. In addition, With the extension of the modification time of the *E. prolifera*, the mechanical strength tended to increase significantly, and the change was not obvious after 36 h.

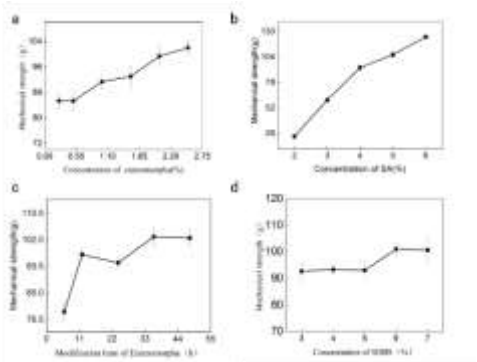


Figure 2 Mechanical strength of immobilized petroleum degrading bacteria prepared under different (a) Concentration of *E. prolifera*; (b) Concentration of SA; (c) Modification time of *E. prolifera*; (d) Concentration of SDBS.

The concentrations of SA, SDBS, and *E. prolifera*, as well as the modification time, were included in the multi-factor experimental design (Table 3), which is consistent with the findings of Fu et al. (2022) (Fu et al., 2022). The multivariate ANOVA results of mechanical strength and removal rate of the modified *Enteromorpha*-immobilized bacterial agent was significant (Table 3, $P < 0.01$).

Table 3 Experimental settings of four factors and three levels

Factor	Level		
	-1	0	1
A: Dipping time	24	36	48
B:SA concentration	4	5	6
C: SDBS concentration	5	6	7
D: *E. prolifera*concentration	1.5	2	2.5

3.2 Optimization of preparation conditions of modified *Enteromorpha*-immobilized microbial agent

Table 4. ANOVA response surface quadratic model

Source	Sum of Squares		df	Mean Square		F-Value		P-Value		
	Mechanical strength	Removal rate		Mechanical strength	Removal rate	Mechanical strength	Removal rate	Mechanical strength	Removal rate	
Model	24975	6129.06	14	1783.93	437.79	13.88	13.88	< 0.0001	0.0018	significant
A	524.48	1205.87	1	524.48	1205.87	4.08	14.56	0.0629	0.0019	
B	2914.11	259.27	1	2914.11	259.27	22.68	3.13	0.0003	0.0986	
C	92.6	179.44	1	92.60	179.44	0.72	2.17	0.4103	0.1631	
D	60.76	148.82	1	60.76	148.82	0.47	1.80	0.5029	0.2014	

AB	42.25	8.84	1	42.25	8.84	0.33	0.11	0.5755	0.7487	
AC	144	9.11	1	144.00	9.11	1.12	0.11	0.3077	0.7450	
AD	117.36	226.44	1	117.36	226.44	0.91	2.73	0.3555	0.1204	
BC	1.78	17.94	1	1.78	17.94	0.014	0.22	0.9080	0.6488	
BD	53.77	114.01	1	53.77	114.01	0.42	1.38	0.5282	0.2602	
CD	28.45	5.49	1	28.45	5.49	0.22	0.066	0.6453	0.8005	
A^2	316.58	423.82	1	316.58	423.82	2.46	5.12	0.1388	0.0401	
B^2	19940.00	3641.68	1	19940.00	3641.68	155.16	43.98	< 0.0001	< 0.0001	
C^2	573.49	455.12	1	573.49	455.12	4.46	5.50	0.0531	0.0343	
D^2	23.14	663.13	1	23.14	663.13	0.18	8.01	0.6777	0.0134	
Residual	1799.20	1159.22	14	128.51	82.80					
Lack of Fit	1799.20	1159.22	10	179.92	115.92					not significant

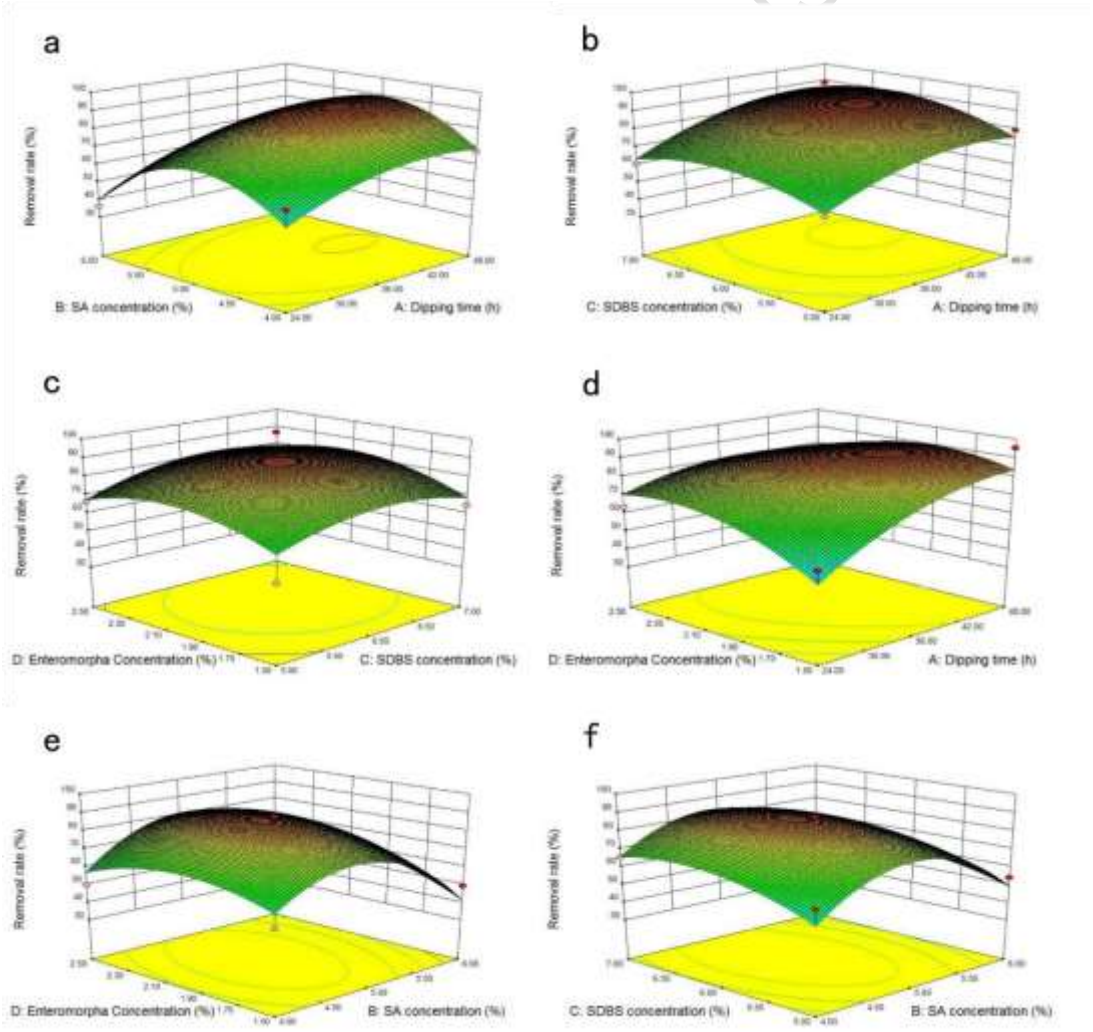


Figure 3 Response surface graph (response value: diesel removal rate. (A: Dipping time; B: SA concentration; C:

SDBS concentration; D: *E. prolifera* Concentration)

The coded independent variables (SA, SDBS, *E. prolifera* concentrations, and dipping time) and experimental values (removal rate of diesel and mechanical strength) used in the RSM design are summarized in Table 4. The removal rate of diesel ranged from 36.22% to 95.24%, while the mechanical strength varied between 81.00 and 187.33 g throughout the experiments. And the sum of squares (SS) should also be regarded(Maleki and Karimi-Jashni, 2020). As the SS value increases, the importance of the variable increases as well. The influences of SA, SDBS, *E. prolifera* concentrations and the dipping time on removal rate of diesel and mechanical strength are revealed in Table 2. The results distinctly revealed that the model fit was statistically significant ($P < 0.002$). The SA concentration presented a significant influence on mechanical strength ($P < 0.0005$, F=22.68, SS=2914.11). For removal rate of diesel, the influence of the digging time was significant ($P < 0.002$, F=14.56, SS=1205.87).

From Figure 3 and 4, the maximum of removal rate of diesel and mechanical strength under optimum condition were obtained. The maximum predicted values of the removal rate of diesel and mechanical strength of the modified *Enteromorpha*-immobilized bacterial agent was 90.71% and 166.56 g, respectively. The observed removal rates of diesel using the modified *Enteromorpha*-immobilized immobilized bacterial agent are higher than those reported for bacteria immobilized on cinnamon shell(Fu et al., 2019), maize straw(Xue et al., 2019), and wood chips(Xue et al., 2017). Under optimal conditions, the modified *Enteromorpha*-immobilized bacterial agent was prepared and tested for removal rate of diesel and mechanical strength. Results showed a removal rate of 90.39% and mechanical strength of 173.67 g.

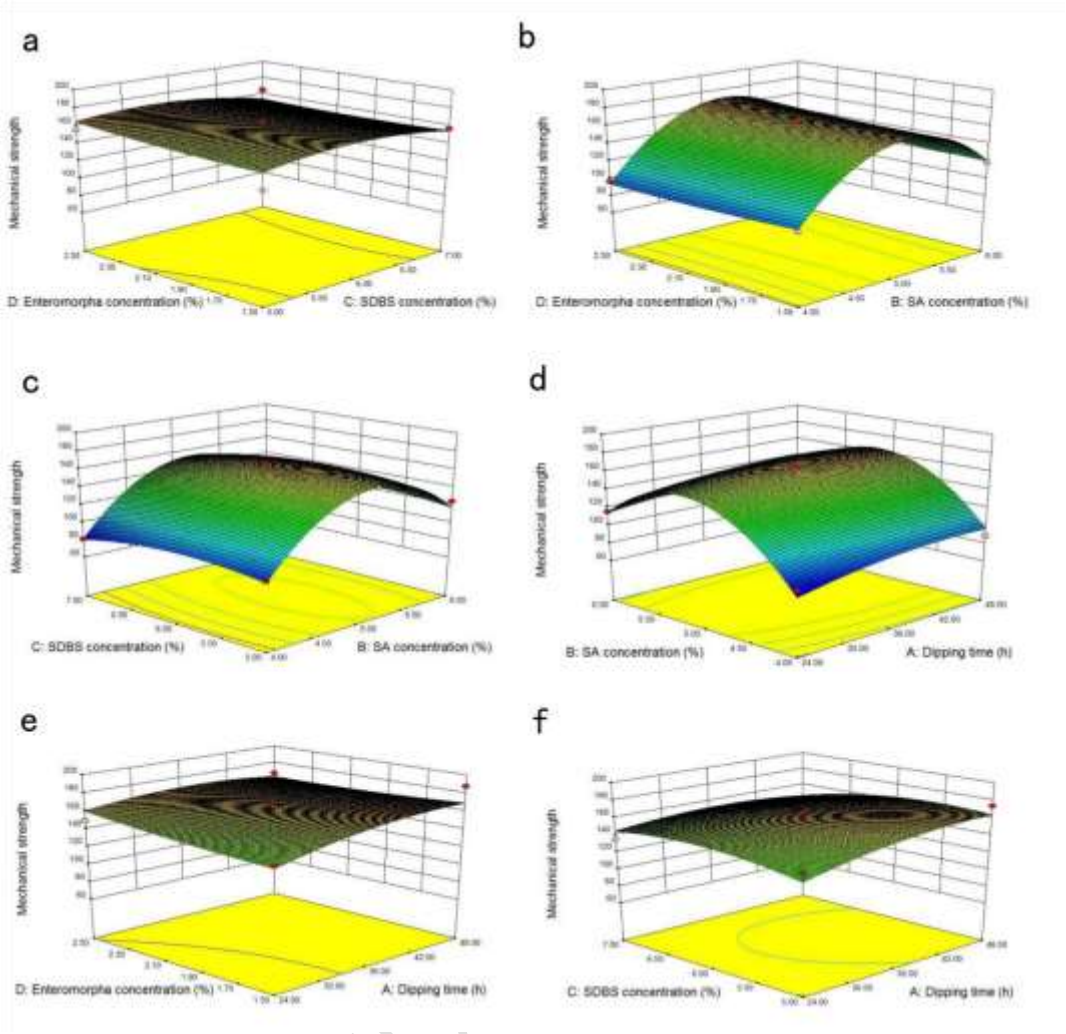


Figure 4 Response surface graph (response value: mechanical strength. A: Dipping time; B: SA concentration; C: SDBS concentration; D: *Enteromorpha* Concentration.)

3.3 Removal rate of diesel by modified *Enteromorpha*-immobilized bacterial agent

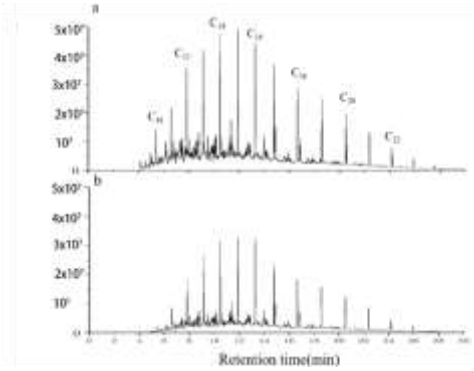


Figure 5 GC-MS analysis of inlet diesel (a) and outlet diesel (b)

The chromatographic peak in Figure 5 indicated a decrease in all organic content, with C_{17} demonstrating the most significant reduction and a removal rate of 73%. The

removal rate for C10 was also high at 64%, while removal rates for other hydrocarbons were approximately 60%. Therefore, immobilized cells are particularly effective when pollutants are predominantly composed of C_{17}.

3.4 Study of immobilizing agents for the removal performance of diesel

The breakthrough time ranged from 10.5 h to 47 h, with the highest value observed at a diesel concentration of 4.2 g/L and an inlet flow rate of 2.6 mL/min, and the lowest value observed at a diesel concentration of 16.8 g/L and an inlet flow rate of 6.0 mL/min (Figure 6). The breakthrough time increased as the inlet concentration decreased, which may be attributed to the increased viscosity of oil at higher initial concentrations, making it easier for the filler to become saturated and adsorbed[32]. In addition, contaminated water diffuses rapidly through the adsorption saturation section and partial saturation section, reaching the non-adsorption section faster and reducing the overall adsorption time. The results of our study demonstrate that the breakthrough point for diesel spills occurs earlier at higher concentrations.

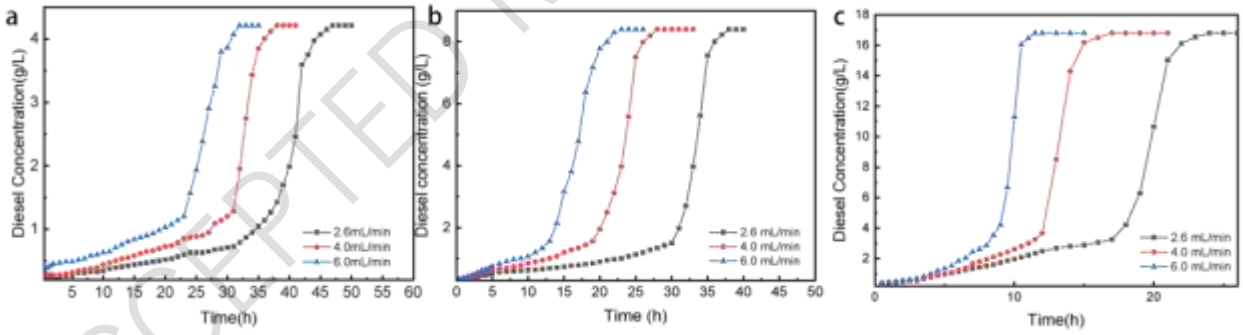


Figure 6 Breakthrough curve at different inlet flow rates and different inlet diesel concentration. (a:4.2g/L; b:8.4g/L; c:16.8g/L.)

At 1 / 2 T, the diesel concentrations of outlet 1 ~ 4 were closed to that of inlet diesel, and as the distance from the outlet decreased, the concentration of diesel oil at the outlet approached 0. This is consistent with the premise that contaminated water is adsorbed and saturated in the reactor progressively (Figure 7). When the initial concentration of diesel was 4.2 g/L, the effluent concentration of outlets 1 ~ 4 was lower than the inlet concentration of diesel, indicating that when the concentration is low, the

modified *E. prolifera* fixed fossil oil degrading bacteria filled in the reactor have begun internal absorption or biodegradation after adsorption saturation.

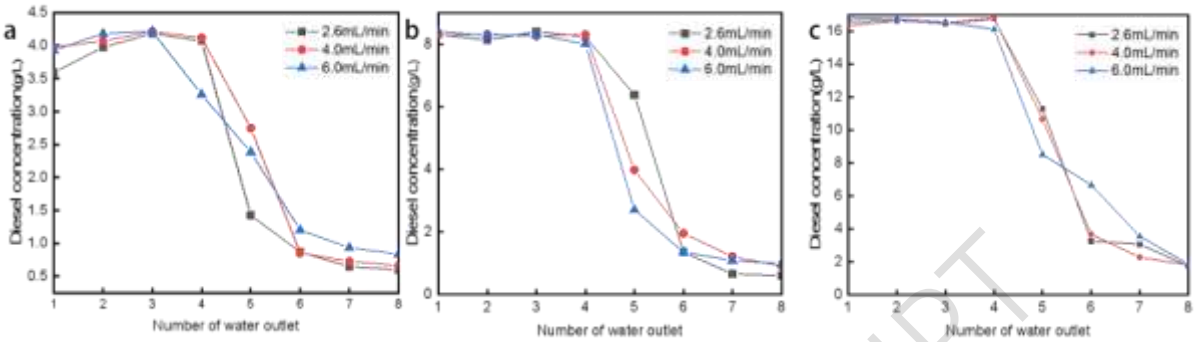


Figure 7 Diesel concentration at each outlet at 1/2 T under different initial concentrations and flow rates. (a:4.2g/L; b:8.4g/L; c:16.8g/L.)

When the inlet flow rate was 6.0mL/min and the concentration of diesel was 16.8g/L, the removal rate was decreased obviously and approached the breakthrough point before 10.5h (Figure 8a). However, when the initial concentration was kept constant, higher diesel removal rates were achieved at slower flow rates. There was little difference($P > 0.05$) in diesel removal rate under different initial concentration of rate and flow rate (Figure 8). This showed that the flow rate and concentration of diesel oil will not have much influence on the removal rate when the diesel oil flow is not completely penetrated. When the inlet flow rate was not greater than 6.0mL/min and the concentration of diesel was less than 16.8g/L, the removal rate of diesel was guaranteed within 10.5h.

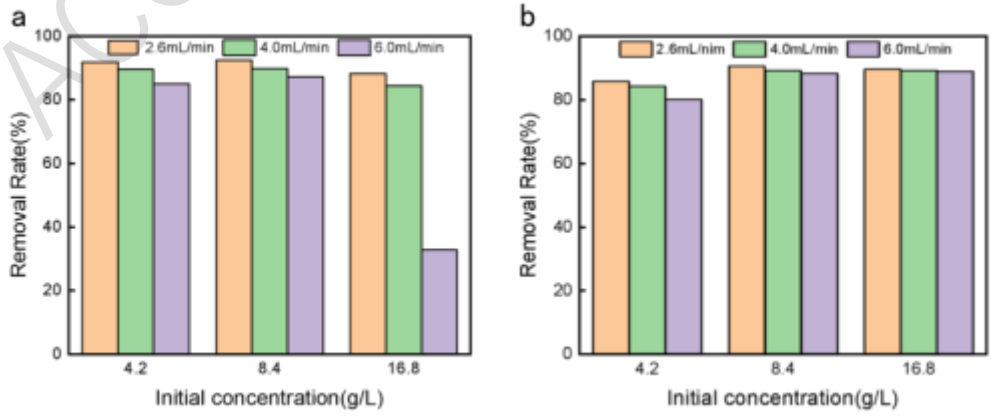


Figure 8 Diesel removal rate at 10 h(a) and 1/2 T(b) under different initial concentrations and flow rates

3.5 Recyclability of immobilization on the microbial community

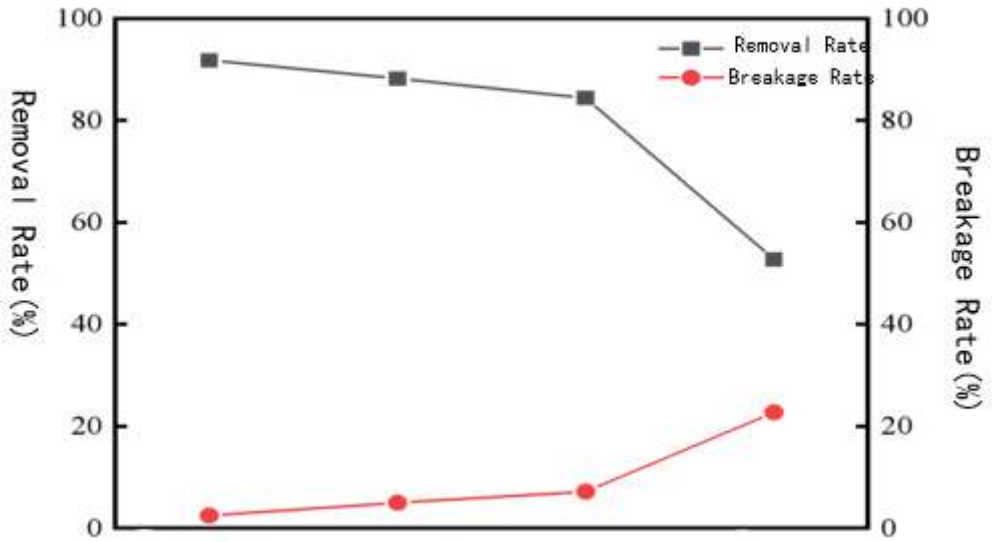


Figure 9. Changes in the diesel removal rate and fragmentation rate of modified *Enteromorpha*-immobilized bacterial agent under different utilization times

During multiple recycling processes, the removal rate of diesel oil decreased gradually with the increase of reuse times of immobilized bacteria, and the fragmentation rate of immobilized fossil oil degrading bacteria of modified *E. prolifera* increased gradually (Figure 9, Table S4). For the first three utilization cycles, no significant changes in diesel removal and fragmentation rates were observed ($P > 0.05$), while after the fourth cycle, the diesel removal rate decreased significantly and the crushing rate increased significantly ($P < 0.05$). Therefore, the optimum recycling times of filler in the reactor with modified *Enteromorpha*-immobilized bacterial agent as filling material was 3 times.

In the recyclability experiments, the main reasons for the increase of fragmentation rate of immobilized bacteria and the decrease of diesel removal rate might be: after a long time of immersion and scouring, the embedded materials fall off and the mechanical strength decreases continuously. In addition, washing of the normal saline may cause the falling off of embedded materials and the loss of internal oil degrading

bacteria(Ye et al., 2021).

4. Conclusion

In this study, a new type of immobilized cells, free cells combined with sodium alginate and modified *E. prolifera* that could degrade diesel, was studied. The optimal conditions for the preparation of modified *Enteromorpha*-immobilized bacterial agent were obtained by designing response surface experiment and the highest removal degraded rate reached 90.39% in 5 days When the inlet flow rate was not greater than 6.0mL/min and the concentration of diesel was less than 16.8g/L, the removal rate of diesel was guaranteed within 10.5h. The modified *Enteromorpha*-immobilized bacterial agent exhibited optimal recycling potential, with a recommended reuse limit of three times in the reactor. These findings demonstrate the feasibility of using immobilized cells for the treatment of oil pollution.

Declarations

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Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

Jinxiao Wei: Investigation, formal analysis, writing-original draft, visualization. Yuping Yang: Funding acquisition, supervision, conceptualization, methodology, resources, writing—review and editing. Yanlu Qiao: Formal analysis, writing-review. Wenhui Xu: Analysis, visualization. All the authors contributed to critically reading of the paper and approved the final manuscript.

Data Availability

All data and code generated or used during the study appear in the submitted article.

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