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Short communication

Industrial-scale culturing of the crude oil-degrading marine *Acinetobacter* sp. strain HC8-3S

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ABSTRACT

The marine bacterium *Acinetobacter* sp. strain HC8-3S, capable of degrading petroleum hydrocarbons, was previously shown to be applicable for bioremediation. Here, we evaluated the performance of the strain during industrial-scale fermentation, long-term storage, and biodegradation processes. Fermentation parameters were optimized for industrial-scale culturing using lower salinity and fewer inorganic salts in the culture medium. The shelf-life of the fermentation broth was evaluated in different storage conditions. Biodegradation efficiency of the strain was evaluated using gas chromatography. Results indicated that the optimum conditions for one-ton scale fermentation were 30 °C, pH 7.4–7.8, and rotation speed 90 rpm; the cell density reached $3.6 \pm 1.9 \times 10^{10}$ CFU ml^{-1} after 12 h. The low temperature can preserved fermentation broth for longer time. When the storage temperature was down to 4 °C from room temperature, half-life of strain HC8-3S extended from 9 days to 34 days. The biodegradation rate of the saturated hydrocarbon fraction of crude oil was 94% after treatment with the strain for 5 days. The results indicate that *Acinetobacter* sp. strain HC8-3S can be enriched efficiently on a large scale, making it a potentially useful industrial strain. This crude oil degradative capability of the strain HC8-3S provides possible application for the clean-up of crude oil-contaminated environment.

1. Introduction

Petroleum products derived from crude oil are essential for modern industry and daily life. Oil spills occur frequently during exploration, production, refining, transportation and storage of petroleum products (Pasquevich et al., 2013). Marine water, groundwater, and soil are damaged, and biological survival is challenged by disastrous oil spills, for example the Deepwater Horizon oil spill in the Gulf of Mexico (2010) and the Penglai 19-3 oil spill in Bohai Bay, China (2011). Crude oil might be the most complex organic mixture and contains more than 17,000 distinct components (Marshall and Rodgers, 2004). Thus, crude oil cannot be completely and easily removed using conventional physical and chemical methods. Transferring contaminants probably causes secondary pollution (Gan et al., 2009; Gavrilescu, 2010).

Bioremediation is an effective method to remove petroleum pollutants because of the diverse mechanisms of metabolism in various microorganisms (Brown, 2010). Microorganisms can use hydrocarbons as their carbon and energy sources via various enzyme systems. Bioremediation of crude oil can occur by natural attenuation, biostimulation, or bioaugmentation (Bento et al., 2005). Microorganisms play key roles in bioremediation processes of complex compounds (Gomez et al., 2007). In crude oil contaminated environments, environmental factors and biological characteristics, e.g., salinity, oil density, and microbial species, can also affect the crude oil degradation rate (Elias et al., 2015; Liang et al., 2012; Tong et al., 2013).

Most current studies focus on discovering highly efficient bacterial strains for bioremediation (Al-Awadhi et al., 2002; Kumar et al., 2014). In laboratory-scale study, *Rhodococcus corynebacterioides* immobilized on chitin and chitosan flakes were examined under different storage temperatures (Gentili et al., 2006). Compared to room temperature, 4 °C and −20 °C could extend the shelf life. After 15 days, immobilized inoculants removal percentage of crude oil degraded the crude oil the most successfully. Previous experiments have proven that bacteria can be used to research the degradation of oil after fermentation. However, a greater amount of

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bacteria should be produced on an industrial scale for field bioremediation study.

Bacteria from the genus *Acinetobacter* are widespread in nature and can be obtained from various oil-polluted environments (Al-Bader et al., 2012; Hassanshahian et al., 2012; Kostka et al., 2011). In our previous paper, *Acinetobacter* sp. strain HC8-3S, isolated from seabed sediment, could effectively degrade crude oil; especially, the saturated hydrocarbon fractions were degraded by 83% after 5 days of treatment (Lin et al., 2014). Furthermore, the crude oil biodegradation efficiency of strain HC8-3S immobilized on cotton fibers increased by 30% compared with that of planktonic cells. The strain could grow well at salinity ranging from 30 to 70 g L^{-1} and pH from 5.6 to 8.6, while maintaining >60% degradation efficiency (Lin et al., 2014).

Based on this previous research, an industrial-scale fermentation study of *Acinetobacter* sp. strain HC8-3S was performed here. The research involved four main processes: shaken flask culture, 5-L reactor fermentation, industrial-scale production (one ton), and crude oil degradation. Fermentation conditions of strain HC8-3S were optimized. Lower salinity of the medium and fewer inorganic salts were used in the industrial reactors. The shelf-lives of the fermentation broth were investigated at different storage temperatures. Biodegradation efficiency of strain HC8-3S was evaluated using gas chromatography (GC). The results support the *in situ* bioremediation of crude oil by *Acinetobacter* sp. strain HC8-3S.

2. Materials and methods

2.1. Culture and media

Acinetobacter sp. strain HC8-3S used in this study was previously isolated from crude oil contaminated sea sediment from Bohai Bay, China (Lin et al., 2014). The strain was isolated using Zobell 2216E medium. In order to adapt the conditions for industrial fermentation, the 2216E medium was simplified by reducing the salinity and removing trace inorganic salts. Per liter of distilled water, the fermentation medium contained: tryptone 8 g, yeast extract 4 g, $CaCl_2$ 0.02 g, $MgSO_4{\cdot}7H_2O$ 0.41 g, K_2HPO_4 1 g, KH_2PO_4 1 g, $FeSO_4{\cdot}7H_2O$ 0.13 g, and NaCl 10 g; pH 7.6 ± 0.2. All media used in this study were sterilized by autoclaving at 121 °C for 30 min. The fermentation medium was used in the containers, including flask, 5-L fermenter, and 1 ton fermenter.

2.2. Effect of sodium chloride concentration on bacterial growth

The influence of NaCl concentration on growth of the strain HC8-3S was investigated by using different NaCl concentrations set at 10, 20, 30 g L^{-1} in the fermentation medium, respectively. One milliliter of bacterial suspension (approximately 2.8×10^{10} colony-forming units (CFU) ml^{-1}) was added into 250 ml of medium in 1000-ml shaken-flasks. After 2 days' inoculation at 30 °C and 180 rpm, the growth conditions of bacteria was measured. The volume of sample for the analysis was 4 ml, taken at regular intervals; the absorbance at 600 nm (OD_{600}) was measured using typical bacterial growth curve method. All data are expressed as the mean of triplicate experiments. The generation time (G) was calculated using the formula:

$$G = \frac{(t_b - t_a)}{3.3 log\, b/a}$$

where t_a and t_b were cultivation times and a and b were the corresponding OD_{600} values.

2.3. Experimental set-up

Strain HC8-3S can degrade crude oil (Lin et al., 2014). First, fermentation conditions were optimized in the laboratory. Then, the fermentation was enlarged to a bench-scale 5-L fermenter. The fermentation parameters were adjusted to meet the requirements of larger scale fermentation. Subsequently, industrial-scale fermentation was performed in a manufactory. The process flow and the fermenters are shown in Fig. 1.

2.4. Laboratory-scale fermentation

A single colony of *Acinetobacter* sp. strain HC8-3S was inoculated into 100 ml of fermentation medium in 250-ml shaken flasks. The bacterial strain was cultured at 30 °C, 180 rpm for 12 h, before fermentation in a 5-L fermenter with a working volume of 3.5 L of medium. The seed liquid was inoculated into fermentation medium at a concentration of 10% (v/v). The fermentation was operated at 30 °C, 120 rpm. The pH was adjusted to 7.6 ± 0.2 throughout the process by addition of 1 M NaOH or 1 M HCl.

2.5. Industrial-scale fermentation

Industrial-scale study was conducted at a manufacturing site of Diyuan Biological Technology Co., Ltd., Yantai, China. The system mainly involves seed fermenters (100 L) and fermentation tanks (one ton). In mesophilic conditions (30 °C), bench-scale experiments were conducted in shaken flasks and a 5-L fermenter in the laboratory. Then, broth was fed into a 100-L seed fermenter. Bacterial seed was then transported to a one-ton tank through a pipeline. The bacterial community was evaluated by Gram-staining for fermentation processing control. The bacterium liquid was observed with the microscope, and culture using the dilution-plate method, to determine whether there are other bacterial contamination during the experiment. The quality control points were as follows: a, microscopic examination before inoculation; b, examination before expansion culture; c, inspection after seed fermenter sterilization; d, sample examination after seed fermentation; e, inspection of product from one-ton fermenter. The fermentation medium was supplemented with 0.3% polyether modified polysiloxane defoamer. Fermentation was carried out at 30 °C and 90 rpm in the simplified medium described above.

2.6. Microflora activity assay

Stability (bacterial survival) tests were conducted at two temperatures during long-term storage of fermentation broth in 2 L Plastic Flexible Package (4 °C and ambient temperature (25 °C–30 °C)), starting with the same cell density (3.6×10^{10} CFU ml^{-1}). The broth was sampled every 30 d. Dilution plate and spread plate methods were used. One milliliter of broth was added into 9 ml 0.9 g L^{-1} physiological saline for gradient dilution. Then, fermentation medium plates were coated with 0.1 ml cell suspension from 1×10^6, 1×10^7 and 1×10^8 fold dilutions, respectively. The plates were incubated at 30 °C for 18 h. Colonies on the plates, which ranged in number from 30 to 300, were counted. The concentration of bacterial cells was calculated according to the sampling volume (Mellefont and Ross, 2007). The data were analyzed by software of SPSS (version 10.0).

2.7. Crude oil biodegradation

The crude oil used in this study was from Shengli oil field, Dongying, China. One-hundred milliliters of fermentation broth ($3.6 \times 10^{10} \pm$ CFU ml^{-1}) were mixed with crude oil (0.5 g) in 250-ml