Bioresource Technology 102 (2011) 6153–6158

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Exopolysaccharide production by a genetically engineered *Enterobacter cloacae* strain for microbial enhanced oil recovery

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ARTICLE INFO

Article history:
Received 10 November 2010
Received in revised form 1 March 2011
Accepted 2 March 2011
Available online 9 March 2011

Keywords:
Microbial enhanced oil recovery (MEOR)
Water-insoluble exopolysaccharide
Electrotransformation
Genomic DNA
Core flooding

ABSTRACT

Microbial enhanced oil recovery (MEOR) is a petroleum biotechnology for manipulating function and/or structure of microbial environments existing in oil reservoirs for prolonged exploitation of the largest source of energy. In this study, an *Enterobacter cloacae* which is capable of producing water-insoluble biopolymers at 37 °C and a thermophilic *Geobacillus* strain were used to construct an engineered strain for exopolysaccharide production at higher temperature. The resultant transformants, GW3-3.0, could produce exopolysaccharide up to 8.83 g l^{-1} in molasses medium at 54 °C. This elevated temperature was within the same temperature range as that for many oil reservoirs. The transformants had stable genetic phenotype which was genetically fingerprinted by RAPD analysis. Core flooding experiments were carried out to ensure effective controlled profile for the simulation of oil recovery. The results have demonstrated that this approach has a promising application potential in MEOR.

1. Introduction

Biotechnology has found an increasing application in petroleum engineering. Microbial enhanced oil recovery (MEOR) is one of such biological efforts which may impact the petroleum industry substantially in current energy shortage period. For the crude oil production, it was concluded that the recovery percentage by both primary and secondary methods is typically 30–50% (Brown, 2010; Tzimas et al., 2005). Current tertiary recovery, or enhanced oil recovery (EOR), allows another 5–15% of the reservoir's residual oil to be recovered (Green and Willhite, 1998). Therefore, it is important to develop novel approaches to improve the efficiency of EOR of oil entrapped in porous media to increase economic profits.

MEOR is a petroleum biotechnology for manipulating function and/or structure of microbial environments existing in oil reservoirs for prolonged exploitation of the largest source of energy. MEOR possesses several advantages over other enhanced oil recoveries, which include low costs, broad applications, stable bacterial activity, environmentally friendly, and so on (Lazar et al., 2007). The microorganisms used in MEOR may produce a variety of bioproducts, such as biosurfactants, biopolymers, biomass, acids, solvents, gases and enzymes which could be utilized to extend the life of the oil reservoirs (Sen, 2008).

One of the main challenges in the oil production by water flooding is the variation of reservoir permeability. This can be circumvented by selective plugging of high permeability areas (Nemati et al., 2005). The role of microbial biopolymer in enhanced oil recovery is to improve the volumetric sweeping efficiency of waterflood by selective plugging of high permeability zones or water-invaded zones (Lazar et al., 2007; Yakimov et al., 1997). Several bacteria have been found to be capable of secreting biopolymers. For instance, xanthan gum can be produced by *Xanthomonas campestris* (Becker et al., 1998), glucan is found to be secreted by *Lactobacillus suebicus*, *Pediococcus parvulus* or *P. parvulus* (Garai-Ibabe et al., 2010), dextran is produced by *Leuconostoc mesenteroides* (Kim and Fogler, 1999), lewan is produced by *Halomonas* species (Poli et al., 2009) or *Bacillus licheniformis* (Garai-Ibabe et al., 2010; Liu et al., 2010; Ramsay et al., 1989) and pullulan is one of the metabolic products by *Aureobasidium pullulans* (Singh et al., 2009). *Enterobacter cloacae* is a gram-negative bacteria which is capable of producing insoluble polymer. This type of microbe can be grown at 4–60 °C and pH 5–7 (Prasertsan et al., 2006). It is reported that the yield of exopolysaccharide from *E. cloacae* can be up to 7.28 g l^{-1} at optimum environmental conditions and its specific growth rate is 3 times higher than that of pullulan production from *A. pullulan* (Prasertsan et al., 2008).

An *E. cloacae* strain has been identified which could produce water-insoluble extracellular polysaccharide at 37 °C when glucose was supplied. The *E. cloacae* was named JD and will be used in this study. The exopolysaccharide produced by JD could be used for

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doi:10.1016/j.biortech.2011.03.005

sealing high permeability areas in the mature oil reservoirs. The aim of this paper was to construct an engineered *E. cloacae* strain from JD and a thermophilic *Geobacillus* strain by electrotransformation. The transformants could produce exopolysaccharide at elevated temperature (up to 50 °C). The engineered *E. cloacae* strain was characterized in liquid culture with growth parameters such as pH, temperature and salt tolerance in order to seek for optimal conditions for cell growth and exopolysaccharide production, Random amplification of polymorphic DNA (RAPD) PCR was used to confirm the success of DNA transformation. Core flooding experiments were conducted to evaluate the effects of simulated MEOR.

2. Methods

2.1. Microorganisms

The *E. cloacae* strain, JD, was originally isolated from the wastewater in Jilin Oil Field of PetroChina Company Ltd., Jilin province, China. This bacterium was, ampicillin resistant and exopolysaccharide-producing. However, when the growth temperature was higher than 37 °C, JD would cease to produce exopolysaccharide.

The *Geobacillus* sp. bacteria, GW3 and GW4, were isolated from the wastewater in Daqing Oil Field of PetroChina Company Ltd., Heilongjiang province, China. The two donor strains used in this work, GW3 and GW4, were gram-positive bacteria, ampicillin sensitive, and their most suitable growth temperature was in the range of 60–70 °C. GW3 and GW4 were not capable of producing biopolymer under any condition.

2.2. Preparation of electrocompetent cells

Competent JD cells for electrotransformation were prepared with the protocol as follows (Dorella et al., 2006; Olubajo and Bacon, 2008): 5 ml of Luria–Bertrani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter) in a test tube was inoculated with a single colony of JD and incubated at 37 °C with shaking at 200 rpm overnight. Then the overnight culture was diluted 10-fold with 45 ml LB broth and incubated with shaking at 250 rpm at 37 °C to an optical density of $OD_{600\ nm}$ = 0.6–1.0. The culture was chilled on ice for 30 min; 1 ml of JD cell culture broth was sampled and then harvested by centrifugation at 13000 rpm at 4 °C for 30 s. The supernatant was removed, and the cell pellets were resuspended with 1 ml of ice-cold 10% glycerol in water (v/v), and centrifuged at 13000 rpm for 30 s at 4 °C. This step was repeated three times. Following the last centrifugation, the cells were resuspended in 400 μl of 10% ice-cold glycerol and stored at −70 °C.

2.3. Extraction of GW genomic DNA

GW genomic DNA was extracted according to a cetryltrimethylammonium bromide (CTAB)-NaCl protocol (Conn and Franco, 2004), with some modifications. Briefly, 1 ml of GW3/GW4 cell culture broth was sampled and harvested by centrifugation at 13000 rpm at 4 °C for 30 s. The supernatant was removed, and resuspended with 567 μl TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and 3 μl 20 mg ml^{-1} proteinase K (Takara, Shiga, Japan). This suspension was lysed with 30 μl 10% sodium dodecyl sulfate (SDS) at 37 °C for 1 h. Subsequently, 100 μl of 5 M NaCl and 80 μl of CTAB-NaCl (700 mM NaCl, 275 mM CTAB) were added, and the mixture was incubated at 65 °C for 10 min. The cell lysates were extracted first with 780 μl of chloroform-isoamyl alcohol (24:1, v/v) at room temperature for 1 min with slow shaking. After centrifugation (10,000 rpm, 5 min), the supernatant was mixed with one volume of phenol–chloroform-isoamyl alcohol (25:24:1, v/v/v) and centrifuged at 14,000 rpm for 5 min. DNA was precipitated from supernatant by 0.6 volume of isopropanol, washed with 1 ml 70% ice-cold ethanol, dissolved in 50 μl sterile H_2O containing 20 μg RNase (Takara) per milliliter, and stored at −20 °C. The DNA was semiquantified in 0.8% agarose gel in 1 × Tris–Acetic–EDTA and visualized by staining with ethidium bromide (EB). The purity and concentration of DNA were estimated by spectrophotometry (Zhang et al., 2003).

2.4. Electrotransformation

Eighty microliter of competent JD cells from −70 °C refrigerator was thawed in ice-water mixture, mixed with 1 μl GW3 or GW4 genomic DNA (1 μg μl^{-1}) and incubated on ice for 5 min. Then the mixture was transferred to a chilled 2 mm electroporation cuvette (Bio-Rad, California, USA). The samples were electroporated in the MicroPulser™ Electroporation (Bio-Rad) at voltages of 2.8 kV, 2.9 kV and 3.0 kV, respectively. After the electric pulse, 400 μl of SOC medium (2% tryptone [m/v], 0.5% yeast extract [m/v], 10 mM NaCl, 2.5 mM KCl, 10 Mm $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added into the cuvette immediately. The cell suspension was transferred to a 1.5 ml Eppendorf tube and incubated at 37 °C for 1 h with shaking at 110 rpm. Finally, all of cell culture was plated on 100 μg ml^{-1} ampicillin selective complete medium (10 g of tryptone, 5 g of yeast extract, 10 g of glucose, 5 g of beef extract and 5 g of sodium chloride per liter) and incubated at 50 °C overnight to select transformants. Positive clones were further verified with liquid ampicillin selective complete medium in static culture at 50 °C.

2.5. RAPD-PCR assay

Random amplification of polymorphic DNA (RAPD) is one of PCR-based DNA fingerprinting methods (Dubey et al., 2006). It uses short primers of arbitrary nucleotide sequence to reproducibly amplify segments of genomic DNA to detect bases changes (Williams et al., 1990).

The genomic DNA of transformants and their parents was extracted as mentioned above. Twentyfive microliter volume of PCR reaction mixtures contained 2.5 μl of a 10-fold PCR buffer without $MgCl_2$, 1.5 μl of 25 mM of $MgCl_2$, 2 μl of 2.5 mM dNTP (Promega, Wisconsin, USA), 1 μl of 5 μM of primer (Sangon, Shanghai, China; Table 1), 100 ng of template DNA, 1.5 μl of 5 u μl^{-1} DNA polymerase (Promega), and purified water to the final volume. The amplifications were carried out in a DNA thermal cycler Techgene (Techne, Cambridge, UK) using the PCR protocol including an initial denaturation at 94 °C for 4 min, 45 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 5 min. The RAPD-PCR products were separated in 0.5 × Tris–Borate–EDTA gel containing 2% agarose and stained with ethidium bromide. The gels were photographed with a gel imaging system (Vilber Lourmat, Marne la Vallée, France).

Table 1
DNA sequence of random primers used for RAPD-PCR.

Primer	DNA sequence (5′–3′)	Primer	DNA sequence(5′–3′)
S12	CCTTGACGCA	S1293	CTGACTTCCC
S86	GTGCCTAACC	S1343	TTTCCGGGAG
S208	AACGGCGACA	S1366	CCTTCGGAGG
S265	GGCGGATAAG	S1467	GTGTCAGTGG
S366	CACCTTTCCC	S1480	TTGACCCCAG
S381	GGCATGACCT	S2006	GGACGACCGT
S501	TGCGGGTCCT	S2007	GGGTCGCATC
S511	GTAGCCGTCT	S2008	CCACAGCCGA
S1142	AATCCGCTGG	S2154	ACCGTGGGTG
S1149	CCAGATGGGG	S2160	CACCGACATC