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Selective Plugging Strategy Based Microbial Enhanced Oil Recovery Using *Bacillus licheniformis* TT33

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The selective plugging strategy of Microbial Enhanced Oil Recovery (MEOR) involves the use of microbes that grow and produce exopolymeric substances, which block the high permeability zones of an oil reservoir, thus allowing the water to flow through the low permeability zones leading to increase in oil recovery. *Bacillus licheniformis* TT33, a hot water spring isolate, is facultatively anaerobic, halotolerant, and thermotolerant. It produces EPS as well as biosurfactant and has a biofilm-forming ability. The viscosity of its cell-free supernatant is 120 mPa·s at 28°C. Its purified EPS contained 26% carbohydrate and 3% protein. Its biosurfactant reduced the surface tension of water from 72 to 34 mN/m. This strain gave 27.7±3.5% oil recovery in a sand pack column. Environmental scanning electron microscopy analysis showed bacterial growth and biofilm formation in the sand pack. Biochemical tests and Amplified Ribosomal DNA Restriction Analysis confirmed that the oil recovery obtained in the sand pack column was due to *Bacillus licheniformis* TT33.

Keywords: Selective plugging, Microbial Enhanced Oil Recovery, exopolymeric substances, *Bacillus licheniformis*

Among the various sources of energy, crude oil plays an important role in providing energy supply to the world [8]. It also serves as a raw material for feed stock in the chemical industry. Oil recovery from oil wells is conducted in two phases. In the primary phase, oil is recovered by natural stored energy or pumping, which recovers about 10% to 15% of the Original Oil In Place (OOIP). The secondary phase involves water flooding, where water is forcibly flooded into the reservoirs to recover oil trapped in rock pores. Water flooding is effective in all reservoirs, usually recovering about another 15% of OOIP. In view of the worldwide oil demand and the fact that almost 65% of oil is left behind in the reservoir at the end of conventional oil recovery methods, the importance of tertiary oil recovery or Enhanced Oil Recovery (EOR) is obvious to increase the recovery of crude oil [15]. EOR is divided into two major groups, namely, thermal processes and chemical flooding processes. These processes are environmentally hazardous, costly, and leave undesirable residues difficult to dispose off without adversely affecting the environment [4].

Microbial Enhanced Oil Recovery (MEOR) is a process where microorganisms are used to retrieve unrecoverable oil from oil wells. In comparison with the existing EOR processes, MEOR is a cost-effective and ecofriendly process [3]. There are several mechanisms by which microorganisms may contribute to tertiary oil recovery. These mechanisms include gas production leading to increased pressure; acid production resulting in degradation of limestone matrices; solvent production causing decrease in viscosity; biosurfactant production leading to reduction of interfacial tension between oil/water and oil/rock; and biomass accumulation leading to selective plugging of high permeability zones [1, 3, 24].

Earlier reports on oil recovery studies have shown that the major factor limiting oil recovery is the variation of permeability. Because of this, water flows through the high permeability zones, leaving oil present in the low permeability zones unrecovered [20]. Selectively plugging the most permeable regions of the reservoir reduces permeability variation and improves oil recovery. A variety of techniques has been developed to selectively plug reservoirs, employing polymers, clays, cements, and waxes [23]. The instability and difficulty in controlling placement of some of these agents has limited their use. The use of microorganisms to resolve permeability variation has been suggested by several workers. EPS-producing microbes form a large amount of biomass, which selectively plugs the highly

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permeable zones and diverts the water flow to oil-rich zones in order to push the oil out of the reservoir [11, 19].

There are few reports related to selective plugging, but none of them have focused on oil recovery in a sand pack column by selectively plugging it with injected microbes. Jang *et al.* [10] studied bacterial migration through a nutrient-enriched sand pack column for *in situ* recovery of oil. Raiders *et al.* [20] studied the permeability profile of Berea sandstone cores using both indigenous and injected microorganisms. Torbati *et al.* [23] investigated the pore entrance size distribution of plugged cores using a mathematical model based on Poiseuille's equation. Raiders *et al.* [19] further studied the ability of indigenous populations of microorganisms in Berea sandstone to improve volumetric sweep efficiency and increase oil recovery by *in situ* growth and metabolism following injection of nutrients. They also showed that gas produced by microorganisms is an important factor leading to increased oil recovery. Yakimov *et al.* [24] used *Bacillus* spp. to demonstrate selective plugging in Berea sandstone cores. Dunsmore *et al.* [7] investigated biofilm accumulation and bacterial transport in porous matrices by using time-lapse photomicrography.

Implementing the information available from earlier reports, a sand pack column was designed so as to imitate an actual oil reservoir [22]. In this study, a selective plugging strategy of MEOR using microbes that grow and produce exopolymeric substance (EPS) under reservoir conditions was used. Vegetative cells of *Bacillus licheniformis* TT33 were injected into the sand pack column, and the oil recovery obtained by biomass accumulation in highly permeable zones was estimated. The sand pack column, used in the present study, is easy to construct and simulates the oil reservoir suitably, replacing the live Berea sandstone cores used in earlier studies. This work demonstrates the selective plugging mechanism of oil recovery *in situ* in simply operated sand pack columns. Some of the advantages of this study include the following: a battery of columns can be run simultaneously; the selective plugging mechanism can be studied microscopically; and the process can be scaled up to a higher possible level.

MATERIALS AND METHODS

Microorganism and Cultivation Condition

A biopolymer-producing isolate, TT33, used for the present investigation, was isolated from Tuva-Timba hot water spring, Gujarat, India. It was identified as *Bacillus licheniformis* based on its phenotypic characteristics and 16S ribosomal RNA gene sequence analysis (GenBank Accession No. FJ392828). *Bacillus licheniformis* TT33 is facultatively anaerobic, halotolerant, and thermotolerant. The medium used for cultivation of this strain was K. Jenny's medium [12] containing glucose 10 g/l, $NaNO_3$ 2.8 g/l, KCl 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, H_3PO_4 2 ml of 84%/l, $CaCl_2 \cdot 2H_2O$ 0.03 g/l, EDTA 0.2 g/l, $ZnSO_4 \cdot 7H_2O$ 0.8 mg/l, $MnSO_4 \cdot H_2O$ 0.2 mg/l, H_3BO_3 60 µg/l, $Na_2MoO_4 \cdot 2H_2O$ 20 µg/l, $CuSO_4 \cdot 5H_2O$ 0.1 mg/l, $CoCl_2 \cdot 6H_2O$ 0.1 mg/l, and $FeSO_4 \cdot 7H_2O$ 0.3 mg/l. 50 ml of sterile medium in a 250-ml Erlenmeyer flask was inoculated with 2% 12 h old inoculum and incubated at 37°C on a shaker at 180 rpm for 72 h. The culture was centrifuged at 12,000 rpm for 30 min and the EPS-containing cell free supernatant (CFS) was used for viscosity and surface tension (ST) measurements.

Viscosity and Surface Tension Measurements

The viscosity of the CFS was measured using an Ostwald glass viscometer based on the flow rate to check for biopolymer production. The ST of the CFS was measured using a Du-Nuoy's tensiometer (Win-Son & Co., Kolkata, India) based on the ring detachment technique.

Biofilm Analysis

A standard microscopic slide (75×25 mm) was dipped in a 250-ml Erlenmeyer flask containing 50 ml of K. Jenny's medium. This flask was then sterilized and inoculated with the strain under study. After 72 h of incubation at 37°C under static condition, the slide was removed from the flask and washed with distilled water. The biofilm formed on the slide was stained by LIVE/DEAD Baclight (Invitrogen) according to the manufacturer's instructions. The images of the biofilm formed on the slide were collected by a LSM 510 META confocal microscope (Carl Zeiss, Germany). The biofilm structure was quantified from the confocal stacks by using the image analysis software package COMSTAT [9]. In the present study, maximum biofilm thickness, average biofilm thickness, roughness coefficient, substratum coverage, and biomass were estimated to characterize the biofilm structure formed by *Bacillus licheniformis* TT33.

Effects of Different Parameters on Growth and Biopolymer Production

The effects of different parameters on the growth of *Bacillus licheniformis* TT33 and its EPS production were studied by inoculating 50 ml of sterile K. Jenny's medium in a 250-ml Erlenmeyer flask with 2% 12 h old inoculum and incubating at 37°C on a shaker at 180 rpm for 72 h. To study the effect of salinity, the medium was supplemented with 5%, 10%, and 15 % NaCl at 37°C; for temperature, the flasks were incubated at 30°C, 37°C, and 50°C in the presence of 10% NaCl; for pH, the pH of the medium was adjusted to 5, 7, and 9 and incubated at 50°C in the presence of 10% NaCl; and for different environmental conditions, the flasks were incubated in aerobic, static, and anaerobic conditions at 50°C in the presence of 10% NaCl. The culture was then centrifuged at 12,000 rpm for 30 min. The bacterial cell pellet obtained after centrifugation was washed twice with distilled water and used to measure the growth of *Bacillus licheniformis* TT33 in terms of dry weight (g/l). The CFS was used to measure the specific viscosity of biopolymer produced by *Bacillus licheniformis* TT33.

The specific viscosity of the biopolymer was calculated using the following formula:

$$\eta_{sp}=t-t_0/t_0$$

where η_{sp}=specific viscosity, t=flow time of the unknown liquid, and t_0=flow time of the reference liquid. In the present study, uninoculated sterile medium was taken as a reference liquid.

Purification of Biopolymer

The CFS was precipitated with three volumes of isopropanol, followed by air drying. The air-dried material was dissolved in distilled water,

dialyzed (12,000 Dalton, cut-off analysis tubing; Sigma), and lyophilized. The product obtained was dissolved in distilled water and mixed thoroughly. Then, a few drops of 5% cetyltrimethylammoniun bromide (CTAB) were added into it until white precipitates appeared. The precipitates were collected by centrifugation and dissolved in 2 M NaCl. This solution was reprecipitated with three volumes of isopropanol, followed by air drying. The aqueous solution of the air-dried material was dialyzed and lyophilized [17].

Effect of Shear Rate on the Viscosity of Biopolymer

Purified biopolymer (0.1% and 0.2%) dissolved in distilled water and in saline (10% NaCl solution) was subjected to different shear rates (20, 40, 60, 80, and 100 s^{-1}) using a Brookfield's viscometer. Effects of these shear rates on the stability of biopolymer were compared with respect to its viscosity in distilled water and in saline.

Characterization of Purified Biopolymer

The purified biopolymer was hydrolyzed with an equal volume of 6 M HCl at 100°C and neutralized with 6 M NaOH. Thin layer chromatography of hydrolyzed and unhydrolyzed biopolymers was carried out to detect the protein moiety, using a solvent system comprising chloroform–methanol–acetic acid–water (25:15:4:2) as mobile phase and 2% ninhydrin in acetone as a developing agent [5]. The IR spectrum of the purified biopolymer was recorded in potassium bromide pellets using FT–IR spectroscopy (Perkin Elmer Rx 1). The UV absorption spectrum of 1.0% (dry w/v) solution of purified biopolymer was recorded using a spectrophotometer. The carbohydrate and protein contents of purified biopolymer were measured colorimetrically by the method of Dubois *et al.* [6] and Lowry *et al.* [14] respectively.

Sand Pack Column Experiments

The sand pack column was designed to simulate an oil reservoir and used to check the selective plugging mechanism based oil recovery efficiency of *Bacillus licheniformis* TT33. The construction and operation of the sand pack column have been described in our earlier work [22]. The column was first saturated with brine. The brine was then replaced by oil saturation. The sand pack was again flooded with brine until there was no oil coming in the effluent. Thereafter, 0.6 pore volume (PV) of *Bacillus licheniformis* TT33 (OD_{600}=0.2) in K. Jenny's medium was injected into the column and incubated for 20 days at 50°C [24]. After incubation, the column was flooded with brine (10% NaCl) and the additional oil recovered (AOR) over residual oil saturation was measured. At the end of the experiment, the packed sand was analyzed using environmental scanning electron microscopy (ESEM). Effluent collected from the outlet of the column was used for viable count. The isolate obtained from the effluent was identified using biochemical tests and antibiotic sensitivity tests [21].

Oil Recovery Measurements

To determine the selective plugging based oil recovery efficiency of *Bacillus licheniformis* TT33, the additionally mobilized amount of oil was calculated using the formulae given below.

Pore Volume (PV) (ml)=Volume of brine required to saturate the column

Original Oil in Place (OOIP) (ml)=Volume of brine displaced by oil saturation

S_{orwf} (ml)=Residual oil saturation after water flooding

S_{orbp} (ml)=Oil collected over residual oil saturation after biomass plugging

$$\text{Initial water saturation } (S_{wi})\ (\%)=\frac{X}{PV}\times 100$$

where X=Pore volume−Volume of brine replaced by oil

$$\text{Initial oil saturation } (S_{oi})\ (\%)=\frac{OOIP}{PV}\times 100$$

$$\text{Residual oil saturation } (S_{or})\ (\%)=\frac{X_i}{OOIP}\times 100$$

where X_i=OOIP−Volume of oil collected after initial water flooding

$$\text{Additional Oil Recovery over OOIP (AOR) (\%)}=\frac{\text{Oil recovered using biomass plugging}}{\text{Oil in column after water flooding}}\times 100$$

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Genomic DNA was isolated from the bacterium present in the effluent of the column; strain *Bacillus licheniformis* TT33 and standard strains of *Bacillus mojavensis, Bacillus subtilis,* and *Bacillus thuringiensis.* The genomic DNAs were used as templates for eubacterial-specific 16S rDNA primer-based PCR. Approximately 1 µg of the amplified 16S rDNA product of isolates and reference strains, in five separate tubes, was cleaved with 5 units of restriction enzyme, *HhaI.* 2 µl of corresponding enzyme buffer was added to the assay mixture and the final volume was adjusted to 20 µl with distilled water. The reactions were carried out at 37°C for 3 h. The reaction product was analyzed by agarose gel electrophoresis (2.5% w/v) in TBE buffer containing 50 ng/ml ethidium bromide. The gels were compared visually.

Results

Bacillus licheniformis TT33, a hot water spring isolate, produces biopolymer as well as biosurfactant. The viscosity of its CSF is 120 mPa·s at 28°C. About 4.51 g/l dry weight of the crude biopolymer was obtained after ethanol precipitation of its CSF. It reduced the ST of water from 72 to 34 mN/m.

Biofilm Analysis

Biofilm parameters quantified by COMSTAT are shown in Table 1. The values obtained for maximum biofilm thickness (46.72±0.19 µm) and average biofilm thickness (33.23±7.73 µm) indicated the formation of a dense biofilm on the substratum. The roughness coefficient (0.06±0.02) represented a homogenous biofilm. The substratum coverage (0.12±0.07) reflected that the strain *Bacillus licheniformis* TT33 colonized the substratum efficiently. The biomass (11.10±5.61 $\mu m^3/\mu m^2$) represented a high volume of biofilm cells present in a given confocal image stack [13]. Hence, COMSTAT analysis of the biofilm structure confirmed that a dense, homogeneous, and confluent biofilm was formed by *Bacillus licheniformis* TT33 (Fig. 1).

Table 1. Different parameters of the biofilm formed by *Bacillus licheniformis* TT33.

Microorganism	Thickness (μm)		Roughness coefficient	Substratum coverage	Biomass ($\mu m^3/\mu m^2$)
	Maximum	Average			
B. licheniformis TT33	46.72±0.19	33.23±7.73	0.06±0.02	0.12±0.07	11.10±5.61

Effects of Different Parameters on Growth and Biopolymer Production

Bacillus licheniformis TT33 could grow and produce biopolymer at a wide range of salinity, temperature, and pH, and under static, aerobic, and anaerobic conditions. Biopolymer production was measured in terms of specific viscosity. It was highest in the presence of 10% NaCl (Fig. 2A); hence, the effect of temperature was checked in the presence of 10% NaCl. *Bacillus licheniformis* TT33 produced maximum biopolymer at 50°C (Fig. 2B); hence, the effects of pH and different environmental conditions were checked at 50°C in the presence of 10% NaCl. pH 7 and aerobic conditions were found to be most suitable for biopolymer production (Figs. 2C and 2D).

Effect of Shear Rate on the Viscosity of Biopolymer

The rates of decrease in viscosity of the biopolymer dissolved in distilled water and in saline (10% NaCl) at shear rates of 0–100 s^{-1} were compared. As the shear rate increased, the viscosity of solutions decreased, which indicated the pseudoplastic and non-Newtonian nature of the biopolymer. Aqueous solutions of biopolymer showed higher viscosity at each shear rate than its saline solutions, but the viscosity decreased more rapidly in aqueous solutions as compared to saline solutions (Fig. 3).

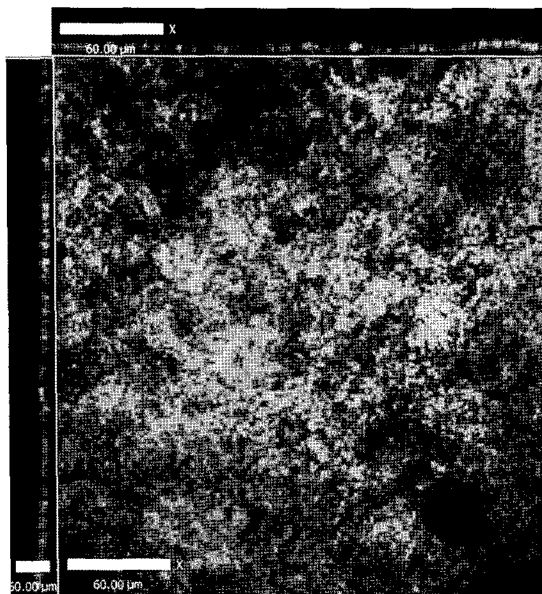

Fig. 1. Biofilm image of *Bacillus licheniformis* TT33 obtained by confocal microscopy through LIVE/DEAD Baclight staining.

Characterization of Biopolymer

FT–IR of the purified biopolymer showed a broad absorption band near 3,400 cm^{-1}, indicative of O-H content. The characteristic absorption of the N-H group was in the region of about 3,500–3,300 cm^{-1}; N-H bending of primary amines showed absorption between 1,650 and 1,580 cm^{-1}; the absorption range of the aldehyde group was 1,725–1,685 cm^{-1}; and bands at 2,376 cm^{-1} and 2,928 cm^{-1} indicated C-H stretching. A strong band at 1,630 cm^{-1} indicated the presence of C=O stretching. A band at 1,200 cm^{-1} indicated the presence of an ether group (C-O-C) and absorption at 1,460 cm^{-1} indicated the presence of a C-N bond (Fig. 4). TLC of the hydrolyzed biopolymer showed a purple-colored spot upon spraying ninhydrin, which indicates the presence of protein in the biopolymer. The UV absorption spectrum of 1 mg/ml solution of purified biopolymer showed a strong absorption peak at 200 nm, a wavelength specific for carbohydrate. Quantitative analysis using colorimetric methods indicated that the purified biopolymer contained 26% carbohydrate and 3% protein.

Sand Pack Column Studies

To check the selective plugging based oil recovery efficiency of *Bacillus licheniformis* TT33, sand pack column experiments were carried out in triplicates. PV of the sand pack column was in the range of 54–65 ml. The volume of oil required to saturate the column was in the range of 45–51 ml, which formed about 81.1±2.9% of the PV. Thereafter, brine flooding was able to recover about 53.1±2.6% of OOIP, leaving about 46.9±2.7% of oil unrecovered in the column (Table 2). To recover this unrecovered oil, *Bacillus licheniformis* TT33 with nutrient medium was injected into the column and incubated for 20 days. Brine flooding of the sand pack after the incubation period recovered about 27.7±3.5% of the unrecoverable oil (Table 2). ESEM analysis of the packed sand showed that *Bacillus licheniformis* TT33 grew and produced enough amount of biopolymer forming a biofilm in the sand pack (Fig. 5). Comparison of the biochemical characters (Table 3), antibiotic sensitivity (Table 4), and ARDRA profiles (Fig. 6) of the bacteria isolated from the effluent of the column and *Bacillus licheniformis* TT33 showed identical results, indicating that the strain injected into the sand pack column is responsible for oil recovery through a selective plugging mechanism. The results indicate that a large amount of unrecoverable oil was recovered by selective plugging of the highly permeable zones of sand pack

Fig. 2. Effects of (**A**) salt, (**B**) temperature, (**C**) pH, and (**D**) growth conditions on growth of and biopolymer production by *Bacillus licheniformis* TT33.

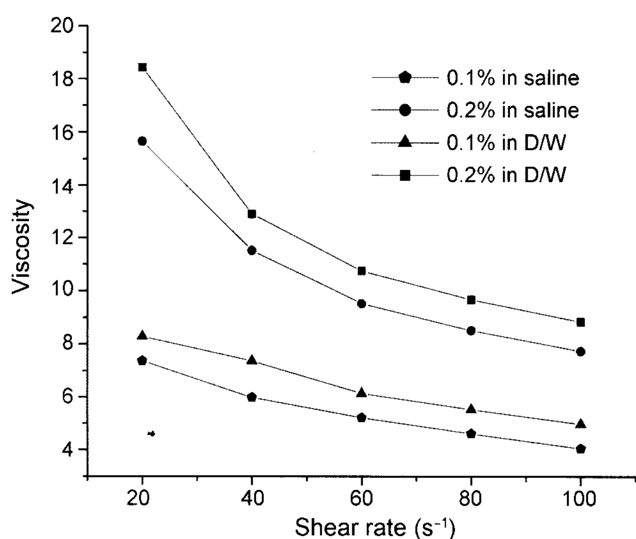

Fig. 3. Effect of shear rate on the viscosity of biopolymer produced by *Bacillus licheniformis* TT33.

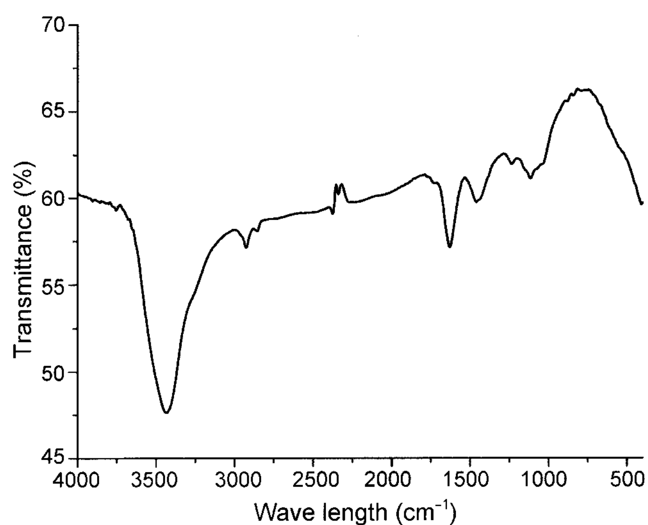

Fig. 4. FT–IR spectrum of biopolymer produced by *Bacillus licheniformis* TT33.

Table 2. Oil recovery obtained in sand pack columns using *Bacillus licheniformis* TT33.

B. licheniformis TT33	Column1	Column2	Column3
PV (ml)	54	62	65
OOIP (ml)	45.5	50	51
X (ml)	8.5	12	14
S_{orwf} (ml)	25.5	25.9	26.2
Sorbp (ml)	8.1	6.5	7
S_{wi} (%)	15.74	19.35	21.53
S_{oi} (%)	84.25	80.64	78.46
S_{or} (%)	43.95	48.2	48.62
AOR (%)	31.76	25.09	26.72

column by *Bacillus licheniformis* TT33. Therefore, it can be a potential candidate for selective plugging strategy mediated MEOR in oil reservoirs.

DISCUSSION

Selective plugging strategy based MEOR requires organisms that can grow and produce a high amount of biomass under the extreme conditions generally prevalent in oil reservoirs. *Bacillus licheniformis* TT33 is a facultatively anaerobic, halotolerant, thermotolerant, and biofilm-forming microorganism. It was found to produce biopolymer as well as biosurfactant. Its biosurfactant acts as a cleaning agent and helps in adherence of the biofilm to a solid surface [2]. Its biopolymer increases the thickness of the biofilm. Thus, it could form a thick and dense biofilm on a solid surface. Therefore, *Bacillus licheniformis* TT33 was selected for selective plugging strategy based MEOR.

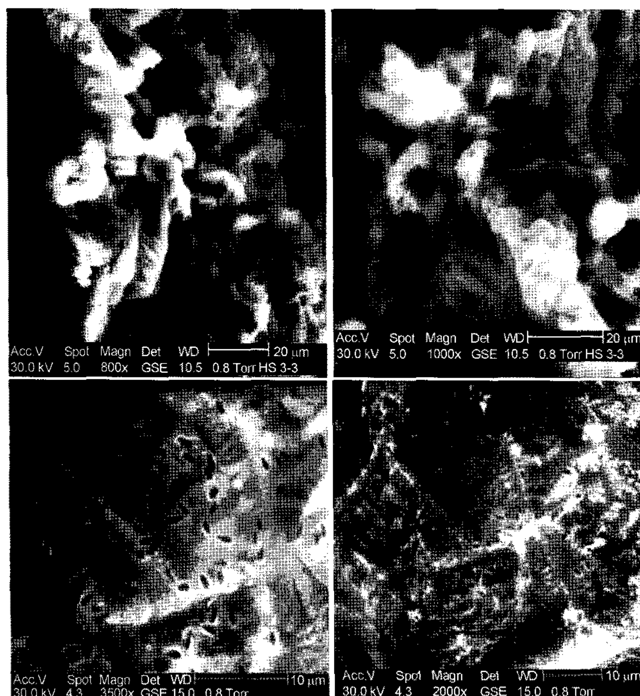

Fig. 5. ESEM analysis of sand packed in column showing biofilm and bacterial cells.

Table 3. Biochemical tests for *Bacillus licheniformis* TT33 and isolates obtained from effluents of the sand pack columns (C1, C2, and C3).

Test	*B. licheniformis* TT33	C1	C2	C3
Mannitol	+	+	+	+
Catalase	+	+	+	+
Citrate	-	-	-	-
Nitrate	+	+	+	+
Starch	+	+	+	+
VP	-	-	-	-
Glucose	+	+	+	+
Gram staining	+	+	+	+
Endospore staining	+	+	+	+
Anaerobic growth	+	+	+	+
Growth at 50°C	+	+	+	+
Growth in 10% NaCl	+	+	+	+

+=Positive result; −=Negative result

Oil occupies minute pores in the rocks of an oil reservoir. About 60% to 90% of the PV of an oil reservoir is filled with oil and the rest with water [23]. Similarly, in an oil-saturated sand pack column, about 81.1±2.9% of the PV was filled with oil and the rest with brine. This oil can be recovered by displacing it with brine. Generally, water is flooded into a reservoir to achieve an adequate displacement of oil [19]. The flooded water passes through highly permeable zones and displaces the oil present in these areas. It eventually establishes a path in the reservoir and flows through the same path (highly permeable zones). As a consequence, the low permeability areas are surpassed by the water. The oil remains trapped (unrecoverable) in these areas [15, 16, 19]. Likewise, in a sand pack column about 46.9±2.7% of oil remained unrecovered after brine flooding.

Table 4. Antibiotic sensitivity tests for *Bacillus licheniformis* TT33 and isolates obtained from effluents of the sand pack columns (C1, C2, and C3).

Antibiotic	*B. licheniformis* TT33	C1	C2	C3
Chloramphenicol	S	S	S	S
Ampicillin	R	R	R	R
Tetracycline	S	S	S	S
Gentamycin	S	S	S	S
Kanamycin	S	S	S	S
Co-Trimoxazole	S	S	S	S
Amikacin	S	S	S	S
Streptomycin	S	S	S	S

S=Sensitive; R=Resistant

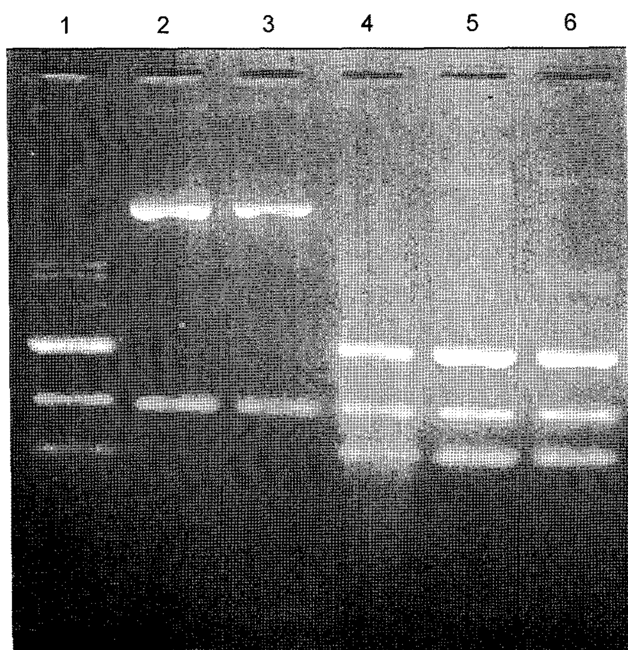

Fig. 6. ARDRA profile of (1) *Bacillus mojavensis*, (2) *Bacillus thuringiensis*, (3) *Bacillus subtilis*, (4) *Bacillus licheniformis*, (5) *Bacillus licheniformis* TT33, and (6) isolate from the sand pack column effluent.

To recover this unrecoverable oil, the flow of brine needs to be diverted from the already flooded areas (high permeability zones) to the unflooded areas (low permeability zones) by blocking the already established path of brine and forcing it to enter the low permeability zones, thus displacing oil from these areas. This could be done by selective plugging of the highly permeable zones (already flooded areas). Earlier studies have shown that *in situ* microbial growth substantially reduces the permeability of sandstone cores; microbial growth occurs preferentially in the high permeability regions, and plugging of highly permeable regions diverts the fluid flow to less permeable regions [18, 20]. In this study, *Bacillus licheniformis* TT33 was injected into the sand pack saturated with residual oil. *Bacillus licheniformis* TT33 grew and produced EPS, thus forming a biofilm in the sand pack, as observed by ESEM [24]. During the incubation period, biofilm formed by *Bacillus licheniformis* TT33 narrowed and finally blocked the pore throats [7]. Hence, the brine flooded after biomass plugging could not pass through the same path. The biofilm protected the biomass from getting washed away during brine flooding and developed a pressure to divert the flow of brine to low permeability zones. Thus, brine passed through the low permeability zones (previously unflooded areas) and released oil from these areas leading to additional oil recovery.

The sand pack column is similar to an oil reservoir in every aspect. The selective plugging mechanism can be studied effectively in a sand pack column. It provides a suitable approach to study the ability of injected microorganisms to recover oil by selectively plugging the highly permeable zones of the sand pack column. In the present study, the selective plugging ability of *Bacillus licheniformis* TT33 was analyzed using sand pack columns. The amount of unrecoverable oil (AOR=27.7±3.5%) recovered by *Bacillus licheniformis* TT33 proved its potential for MEOR. Therefore, it can be an ideal strain to recover the unrecovered oil left behind in an oil reservoir by using the selective plugging strategy.

References

1. Almeida, P. F., R. S. Moreira, R. C. Almeida, A. K. Guimaraes, A. S. Carvalho, C. Quintella, M. C. Esperidia, and C. A. Taft. 2004. Selection and application of microorganisms to improve oil recovery. *Eng. Life Sci.* **4:** 319–325.
2. Bais, H. P., R. Fall, and J. M. Vivanco. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol.* **134:** 307–319
3. Banat, I. M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. *Bioresource Technol.* **51:** 1–12.
4. Bryant, R. S., A. K. Stepp, K. M. Bertus, T. E. Burchfield, and M. Dennis. 1993. Microbial enhanced waterflooding field pilots. *Bioresource Technol. Dev. Petr. Sci.* **39:** 289–306.
5. Cooper, D. G. and B. G. Goldenberg. 1987. Surface active agents from two *Bacillus* species. *Appl. Environ. Microbiol.* **51:** 224–229.
6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.
7. Dunsmore, B. C., C. J. Bass, and H. M. Lappin-Scott. 2004. A novel approach to investigate biofilm accumulation and bacterial transport in porous matrices. *Environ. Microbiol.* **6:** 183–187.
8. Edwards, J. D. 1997. Crude oil and alternate energy production forecasts for the twenty-first century: the end of the hydrocarbon era. *AAPG Bulletin.* **81.**
9. Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146:** 2395–2407.
10. Jang, L. K., P. W. Chang, J. E. Findley, and T. F. Yen. 1983. Selection of bacteria with favorable transport properties through porous rock for the application of microbial enhanced oil recovery. *Appl. Environ. Microbiol.* **46:** 1066–1072.
11. Jenneman, G. E., M. J. McInerney, and R. M. Knapp. 1985. Microbial penetration through nutrient-saturated berea sandstone. *Appl. Environ. Microbiol.* **50:** 383–391.
12. Jenny, K., O. Kappeli, and A. Fiechter. 1991. Biosurfactants from *Bacillus licheniformis*: Structural analysis and characterization. *Appl. Microbiol. Biotechnol.* **36:** 5–13.
13. Kierek, K. and P. I. Watnick. 2003. The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca^{2+}-dependent biofilm development in sea water. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 14357–14362.

14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
15. McInerney, M. J., R. M. Knapp, J. L. Chisholm, V. K. Bhupathiraju, and J. D. Coates. 1999. Use of indigenous or injected microorganisms for enhanced oil recovery microbial ecology of oil fields. *In* C. R. Bell, M. Brylinsky, and P. Johnson-Green (eds.). *Microbial Biosystems: New Frontiers*, Proceedings of the 8th International Symposium on Microbial Ecology. Atlantic Canada Society for Microbial Ecology, Halifax, Canada.
16. McInerney, M. J., M. Javaheri, and D. P. Nagle. 1990. Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. *J. Ind. Microbiol.* **5:** 95–101.
17. Okutani, K. 1984. Antitumour and immunostimulant activities of polysaccharides produced by a marine bacterium of the genus *Vibrio*. *Bull. Jap. Soc. Sci. Fish.* **50:** 1035–1037.
18. Portwood, J. T. 1995. A commercial microbial enhanced oil recovery process: Statistical evaluation of a multi-project database, pp. 51–76. *In* R. S. Bryant and L. K. Sublette (eds.). *The Fifth International Conference on Microbial Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems.*
19. Raiders, R. A., R. M. Knapp, and M. J. McInerney. 1989. Microbial selective plugging and enhanced oil recovery. *J. Ind. Microbiol.* **4:** 215–230.
20. Raiders, R. A., M. J. McInerney, D. E. Revus, H. M. Torbati, R. M. Knapp, and G. E. Jenneman. 1986. Selectivity and depth of microbial plugging in Berea sandstone cores. *J. Ind. Microbiol.* **1:** 195–203.
21. Sneath, P. H. A. 1986. Endospore-forming Gram-positive rods and cocci, pp. 1104–1207. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (eds.). *Bergey's Manual of Systematic Bacteriology*, 9th Ed. The Williams & Wilkins Co., Baltimore.
22. Suthar, H., K. Hingurao, A. Desai, and A. Nerurkar. 2008. Evaluation of bioemulsifier mediated microbial enhanced oil recovery using sand pack column. *J. Microbiol. Methods* **75:** 225–230.
23. Torbati, H. M., R. A. Raiders, E. C. Donaldson, M. J. McInerney, G. E. Jenneman, and R. M. Knapp. 1986. Effect of microbial growth on pore entrance size distribution in sandstone cores. *J. Ind. Microbiol.* **1:** 227–234.
24. Yakimov, M. M., M. M. Amro, M. Bock, K. Boseker, H. L. Fredrickson, D. G. Kessel, and K. N. Timmis. 1997. The potential of *Bacillus licheniformis* strains for enhanced oil recovery. *J. Petr. Sci. Eng.* **18:** 147–160.