

Screening of extracellular polymeric substance producing bacteria isolated from environmental sources and testing their ability to seal fractures zones of oil reservoir rocks

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Abstract. In this study, 21 environmental bacterial isolates isolated from different environmental sources in Basra Governorate identified phenotypically and genetically based on 16 rRNA gene sequence analysis were screened to reveal their ability to produce biopolymer based on the viscosity scale formed in the cultural media and use of Sudan Black stain. Six isolates showed their ability to produce biopolymer, and the two isolates of *Bacillus licheniformis* strain CC91, *Bacillus thuringiensis* strain LU3 recorded the best results. The isolate of the *Bacillus licheniformis* strain CC91 was tested in the process of sealing fractures and openings with high permeability for samples of oil reservoir rocks.

Keywords: biopolymer, byproducts, MEOR, petroleum microbiology.

Introduction

Biopolymers are polymers produced derived from natural renewable resources synthesized by either microorganisms under different environmental conditions or chemically synthesized from a biological material [1][2]. Biopolymers they are composed by monomeric units covalently linked to form larger structures of Biopolymers [3]. Often the production of polysaccharides biopolymer by bacteria for the purpose of protecting from desiccation conditions or the risk of predation by other microbes as well as to assist in adhesion to surfaces [4][5]. Ates ,2015 [6] mentioned that in 1861 Pasteur was the first noticed the production of dextran polymer in wine by bacteria, the first person to identify this bacterium *Leuconostoc mesenteriodes* was Van Tieghem in the year 1878. Bio-polymers have now gained much attention due to their importance in industrial sectors all over the world [7][8]. Many bacteria can produce different types of biopolymers such as *Bacillus* sp. produce Levan [9], *Xanthomonas* sp. produce Xanthan gum [10], *Sclerotium* sp. Produce Scleroglucan ([11] and many others [4]. The proposed processes of biopolymers in microbial enhanced oil recovery (MEOR) are application in indirectly reducing water mobility towards undesirable zones due to the ability of some microorganism to produce viscous biopolymers (Nwidee *et al.*, 2016 [12] ; Song *et al.*,2020[13]). Because these zones take the majority of the injected aqueous flow, it is expected that they will be successfully stopped, forcing water floods into the less permeable regions [4] [14] [15]. The current study aimed at screening a number of

bacterial isolates for their ability of biopolymer production and selecting the most efficient one in production to test the ability of the product to close the high permeability openings of the oil reservoir rocks.

Material and methods

Screening of biopolymer production

In this study, 21 diagnosed bacterial isolates were used [16] which were isolated from various oil sources. For screening of biopolymer production activity 2 ml of overnight broth culture of each isolate were added to 250 ml Erlenmeyer flask containing 100 ml of sterilized biopolymer producing media modification of [17] including (per liter distilled water) 40 g sucrose as a carbon source, 0.5 g NaNO₃, 1.0 g yeast extract, 0.5 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, pH 7.0. 1 ml stock solution was composed of (g/L): FeCl₃·6H₂O 0.08, CuSO₄·5H₂O 0.075, H₃BO₃ 0.15 solid media were prepared by the addition of 15 g·L⁻¹ agar. Wet and viscous colonies were selected and identified as the bacteria that produce biopolymers.

The Erlenmeyer flask was infected with 2% 12 hour old inoculum and incubated for 72 hours at 30 °C on a shaker at 180 rpm. The culture was centrifuged for 30 minutes at 12,000 rpm, and the exopolysaccharide-containing cell-free supernatant was used to assess viscosity.

Viscosity measurement

Biopolymers production was detected by measuring the viscosity of supernatant solution using a glass capillary viscometer at 25°C in a refrigerated bath and circulator. The sample broth was first cooled in a refrigerated bath for 2 minutes before viscosity was measured [18].

Sudan black stain

The biopolymer production by the microbes can be confirmed by staining with Sudan black stain in two methods:-

- Slide Method

After heat fixing the smear, the slide was submerged in 5 percent Sudan Black (w/v in 70% ethanol) staining for 5 minutes and allowed to dry in air, the excess stain was de-stained using xylene several times and blot dried with absorbent paper, safranin was added for 30 seconds and washed with tap water and dried. Under an oil immersion microscope, the stained cells were examined. The biopolymer granules appeared as blue black droplets and cytoplasm part of microorganisms appeared as pink color [19].

- Plate Method

In this method grown colonies on agar plates were flooded with 3% of Sudan Black (w/v in 70% ethanol) solution for 20 min. Later, the ethanol solution was added as a dye remover solution of Sudan black stain. Finally the solution was drained off and observed for screening of biopolymer producers as black mucous colonies [20].

Extraction of Crude biopolymer:

Centrifugation of broth culture yielded cell-free supernatant, which was then treated with half volume of distilled water before being placed in a shaking incubator at 30 °C for one hour. To precipitate the extracellular protein fractions, 7% trichloroacetic acid (TCA) was added to the cell-free supernatant. The protein fractions were then collected by centrifugation at 9,000 rpm for 30 minutes at 4°C. To the clear supernatant, an equal volume of 100%

ethanol was added. Precipitation was gathered around the glass rod. Four to five times the dissolving and precipitation process was repeated until uniform white precipitates were obtained. Vacuum desiccators were used to dry the sample. The crude biopolymer was dialyzed and lyophilized against distilled water [21].

Characterization of polymer using Fourier Transform Infra-Red (FTIR)

To determine the basic functional groups of the two partial purified polymer, Fourier Transform Infra-Red (FT-IR) spectroscopy analysis in the Chemistry department /College of Sciences/ University of Basra was used. 1 mg of dried biopolymer was milled with 100 mg of KBr to form a very fine powder to be analyzed by FT-IR spectra at the wavelength of 400–4000 cm⁻¹ [22].

Production of extracellular polymeric substance to seal fracture zones in tested cores

Three oily saturated cores having high permeability zone and deep cracks were selected in this experiment, the core plugs were dried in a hot air oven at 65°C for 24 h and mass of each dry core was measured using a sensitive balance then saturated with crude oil using vacuum pump. Each core was placed separately to 500 ml Erlenmeyer flask contain 250 mL of sterile polymer production medium which mentioned previously. Each flask was inoculated with 2% of the bacterial suspension of broth culture of *Bacillus licheniformis* strain CC91 and incubated at 30 ° C for 7 days. One of cores treated as control without bacteria.

Results and discussion

Screening of biopolymer producing bacteria

Twenty-one of bacterial isolates were tested for the biopolymer production. Ten isolates were observed in sucrose containing media. Many researchers showed using sucrose as carbon source in biopolymer production media [23] [24] [25]. Each colony was grown clearly on the nutrient and give apposite result in screening test. The best result showed with two gram positive strains *Bacillus licheniformis* strain CC91 and *Bacillus thuringiensis* strain LU3. Biopolymer was reported to be created by a huge number of gramme positive bacteria in prior studies. [26],[27],[28].

3.7.2.1 Viscosity measurement

After an incubation period of 3-5 days, a viscosity was observed in the medium containing the polymer-forming bacteria that could be distinguished during shaking of the bottle, as well as the presence in **Figure 1**. Supernatant viscosity test for all isolates indicated that there are 6 isolates with the highest values ranged between (3.7643-2.4543 c.st) **Table 1**. The two highest values recorded (3.7643 c.st) and (3.6942 c.st) for *Bacillus licheniformis* strain CC91, *Bacillus thuringiensis* strain LU3 respectively, as shown in **Figure 2**. The results of the viscosity measurement in our study were identical to the results reported by Ilias *et al.* [29] in the viscosity measurements of several biopolymers production strains of the genus *Bacillus* spp. used in their study. According to Lee & Tripathi, (2005) [30], the viscosity measuring approach is very relevant in many chemical, biological, and medicinal applications where sample numbers are limited..

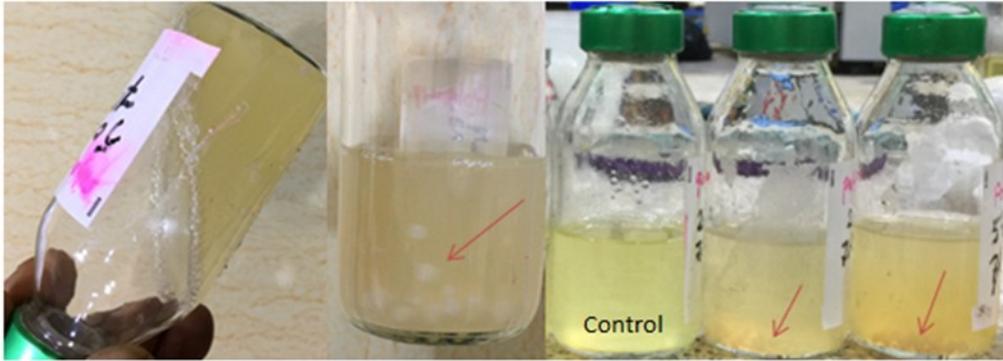


Fig. 1. Viscosity of biopolymer producing

Table 1: Viscosity measurements of isolates

Strain		Viscosity centistokes (c.st)	Sudan black stain
1	Control	0.8334	-
2	<i>Bacillus cereus</i> strain ASWISA1	1.5432	-
3	<i>Lysinibacillus boronitolerans</i> strain SWTPB36	0.9987	-
4	<i>Bacillus subtilis</i> strain HDZK-BYSB7	1.0876	-
5	<i>Bacillus thuringiensis</i> strain ASWISA2	2.2655	+
6	<i>Enterobacter aerogenes</i> strain B19	1.6543	-
7	<i>Bacillus sonorensis</i> strain ASWISA3	1.8766	-
8	<i>Bacillus licheniformis</i> strain CC91	3.7643	+
9	<i>Bacillus cereus</i> strain ASWISA4	1.2333	-
10	<i>Bacillus subtilis</i> ASWISA5	1.4432	-
11	<i>Bacillus subtilis</i> ASWISA7	1.4236	-
12	<i>Pseudomonas stutzeri</i> strain ASWISA6	1.6675	+
13	<i>Bacillus cereus</i> strain A	1.8776	-
14	<i>Bacillus cereus</i> ASWISA8	0.9877	-
15	<i>Bacillus cereus</i> Strain US04	0.8776	-
16	<i>Bacillus thuringiensis</i> strain LU3	3.6942	-

1 7	<i>Bacillus paramycooides</i> strain ASWISA9	2.4543	+
1 8	<i>Bacillus paramycooides</i> strain ASWISA10	2.5655	+
1 9	<i>Brevibacillus brevis</i> strain ASWISA11	1.0987	+
2 0	<i>Enterobacter cloacae</i> strain ASWISA12	0.8766	-
2 1	<i>Enterobacter cloacae</i> strain FC1375	1.0432	-
2 2	<i>Bacillus cereus</i> strain LDPE1	0.9232	-

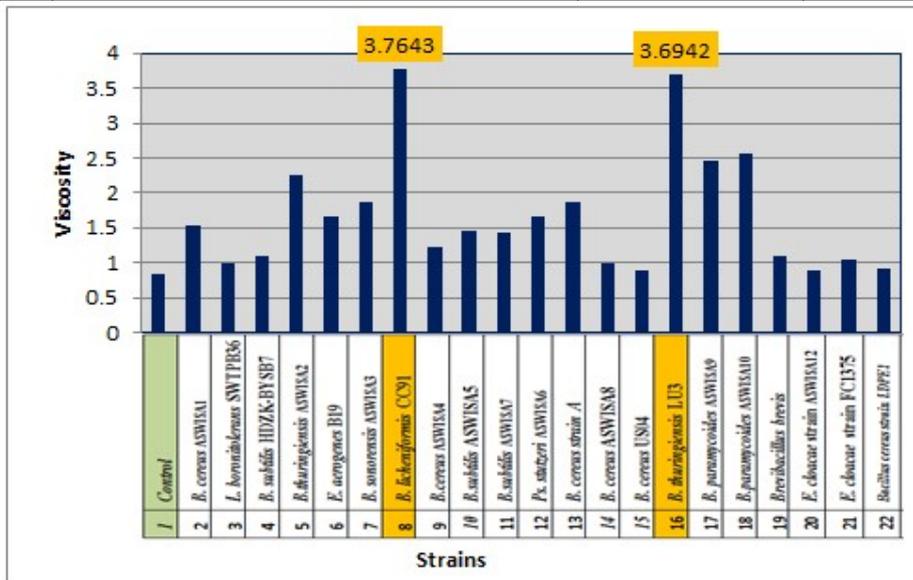


Fig. 2. Viscosity values for supernatant of isolates

Sudan black stain

Use of Sudan black stain in slides and plate method gave positive results similar to all isolates that gave positive results of viscosity measurements testing. **Figure 3** shows the black color of sticky colonies growing on the plates for both isolates for *Bacillus licheniformis* strain CC91, *Bacillus thuringiensis* strain LU3. As well as spores stained in black color while the cells stained in red color in slide stain method **Figure 4**. Yasin & Al-Mayaly,2020 [31] had the same finding, showing granules loaded with black staining, as biopolymer granules can be seen as dark spots under the light microscope.

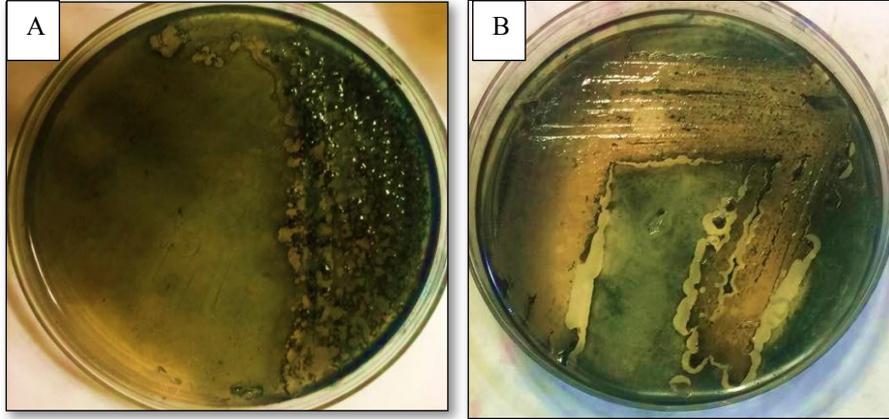


Fig. 3. Sudan black stain plate methods (A) *Bacillus licheniformis* strain CC91(B) of *Bacillus thuringiensis* strain LU3

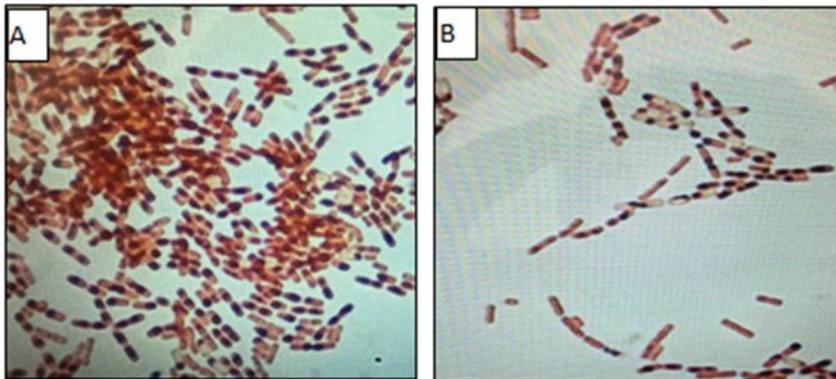


Fig. 4. Sudan black stain slide methods (A) *Bacillus licheniformis* strain CC91(B) *Bacillus thuringiensis* strain LU3

Extraction of Crude biopolymer

The results of the extraction of biopolymer using solvents are shown to separate the top layer containing the polymer for each of the three isolates: *Bacillus licheniformis* strain CC91(8) , *Bacillus thuringiensis* strain LU3as shown in the **Figure 5**.



Fig.5. -Extracted of biopolymer with solvents

Characterization of polymer using Fourier Transform Infra-Red (FTIR)

Figure 6 illustrates FTIR studies of extracted biopolymer produce by *Bacillus licheniformis* strain CC91 Which was chosen as the best isolate for the production of biopolymers. The OH (Hydroxyl) group is represented by band 3471, while the C=O (Carbonyl) and COO (ester) groups are represented by band 1743. The band at 1458 represents CH, which has asymmetrical stretching and bending vibrations in the CH₃ group, while the band at 1377 represents the COH bond. C-O bonding was seen in bands stretching from 1095 to 1283. The investigated results confirmed that the recovered biopolymer component was Polyhydroxybutyrates, as reported by Arun et al. (2009) [32]. (PHB). Alarfaj et al. (2015) [28] also came to the same conclusion.

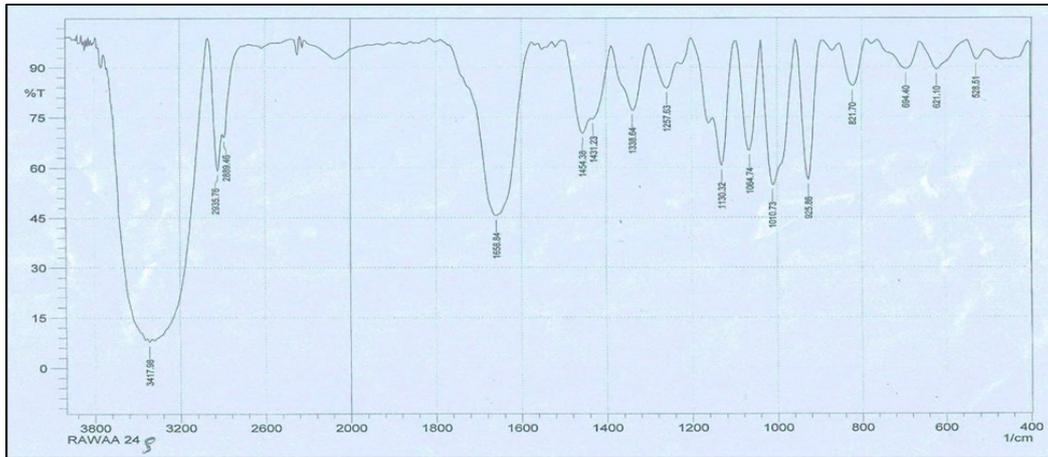


Fig.6. FTIR of extracted biopolymer produce by *Bacillus licheniformis* strain CC91

Production of extracellular polymeric substance to seal fracture zones in tested cores

The results of the inoculated of *Bacillus licheniformis* strain CC91 bacteria in biopolymer production culture medium with the presence of the cores selected from the oil fields that contain cracks and fractures showed a sticky layer of extracellular polymer and the biomass of bacteria growing in all the cracks and fractures to form a sticky layer to close these openings as in the **Figure 7** that shows the nature of the cores before (A) and after culturing (C) comparing with the core treated without bacteria (B). Several studies have indicated the use of various bacterial strains in the process of reducing high permeability areas of oil reservoir that follow the mechanism of water injection in oil recovery processes to increase oil recovery. *Bacillus licheniformis* strain BNP29 was able to produce considerable volumes of biomass, polymer with selective plugging, and boosted oil recovery from 9.3 to 22.1 percent, according to Yakimov et al. (1997)[33]. Suthar et al. (2009) [34] utilised *Bacillus licheniformis* TT33 in the sand pack to exhibit bacterial growth and biofilm formation, resulting in a 27.7±3.5 oil recovery.



Fig. 7. A- The cores before culturing B- without bacteria C- The cores after culturing

Conclusion

In the current study, biopolymer production was investigated for 22 environmental bacterial isolates, six of which showed the possibility of biopolymer production related to the viscosity measurement of the medium, as well as the use of Sudan black stain for the growth bacterial colonies or fixed cells. The isolate *Bacillus licheniformis* strain CC91 was chosen from one of twenty environmental isolates genetically diagnosed as the best isolates that have the ability to produce biopolymers.

FTIR studies of extracted biopolymer produce by *Bacillus licheniformis* strain CC91 confirming that the extracted biopolymer compound was Polyhydroxybutyrate (PHB).

The isolate *Bacillus licheniformis* strain CC91 showed ability to produced extracellular polymeric substance to seal fracture zones in tested core samples taken from an oil reservoir that may subsidize to the processes of microbial oil recovery (MEOR).

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