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Biodegrdation of Crude Oil by Using Bacterial Floc Consortium in Wastewater

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Abstract: Flocculation is the dynamic processes have special advantages such as safety, strong effect, biodegradability and harmlessness to humans and the environment. Fifteen bacterial isolates obtained from waste water contaminated with oil were screened for floc formation ability, hydrophobicity, biodegradation of crude oil ability and EPS production. The results showed flocculation index by bacterial isolates namely 6E, 7E, A6 and C21 were able to produce high percentage of floc formation 31.9%, 26%, 25% and 24.9%, respectively. The hydrophobicity of the cells express as ability of adherence to crude oil was ranged from 96.5% to 91.7%. All the isolates were able to produce high amount of EPS varied from 1.1g/L to 7.7g/L. Isolate A6 showed the highest degradation of crude oil at 78.3%,whereas isolate A4, 6E and 12C only at 60.1, 57.2% and 55.3%, respectively. The isolates are combination as five groups tends to for high flocculation by exopolysaccharides and remove significant amount of crude oil by biodegradation. Approximately isolates (6E, A6 and A4) results revealed that there degradation percentage and flocculation index more than single isolates were 78.9% and 57.2 % respectively. On the other hand, the bacterial cells of culture consortium (CC) attached closely after seven days than 0 days this can possibly be due to ionic and hydrophobic interaction. The aim of this study is to screen bacterial isolates with the ability of producing biopolymer exopolysaccharides (EPS) to forming floc.
Keyword: Bioremediation; Bacterial floc formation; Crude oil, Exopolysaccharides, Culture consortium.

Introduction

Microorganisms have tendency to adhere together and attachment under different condition to form aggregation as a primary step towards flocculation [1]. The bio floccules process is known to be triggered by environmental factors such as physical or chemical stress. Flocculating agents are widely used in industrial processes and drinking water purification including wastewater treatment. Flocculants are usually used to accelerate or improve the settling of suspended solids in various types of wastewater [2]. The hydrophobic cell surface which is one of the important factors in the formation of bio floccules and provides the interaction of cells with hydrocarbon droplets effectively and further aggregate into larger clumps [3]. Floc-forming bacteria mean glued together by biopolymers, which is termed as EPS exopolysaccharides [4]. [5] Showed the bio flocculants are environment friendly and EPS produced by microorganisms play a definite role in flocculation. EPS can form chemical bonds with the surface and intermediate / promote the chemical reactions and the presence of protein outside layer lead to hydrophobic surface [6].

Cell surface hydrophobicity is believed to be important to flocculation; higher hydrophobicity produces the higher degree of adhesion to the flocs [7]. The formation of bioflocculation usually accompanies with the biodegradation crude oil but both biodegradation and biosorption occur during bioremediation process. [8]Noted polysaccharides in the cell surface this makes cells contact to oil droplets more easily. Biopolymer

EPS contain more than two types of monosaccharide units these extracellular materials (polysaccharides, lipids, glycoprotein and lipopolysaccharides) can be used as stabilizers, gelling adhesives, thickening agents, emulsifying agents, flocculants and flushing agents [9]. In this study some isolates have been abilities to degrade crude oil by mixed bacterial floc consortium, oil pollutants could be effectively degraded [10].

In recent years, researchers are working hard to find an effective and efficient way to remove the oil contaminants from the environment [11, 12]. This method provided a new strategy of bioremediation and made an interesting contribution to the biological treatment of hydrocarbons, Therefore, we try to find out the reasons why bacteria to form biofloccules. Systematic this study has been conducted to identify the flocculation among 15 isolates. The degradation crude oil, hydrophobicity and EPS production have been demonstrated. The objective of this study was to screen bacteria isolates which have the ability to produce biopolymer exopolysaccharides (EPS) to floc formation, while the main emphasis of the degradation hydrocarbons and effects on bioremediation.

Materials and Methods

Source of bacteria

Fifteen bacterial isolates that have been isolated from soil or water contaminated with oil were used in this study. The isolates were previously studies identified using biochemical tests and sequencing of the 16s rDNA (Table 1).

Table1. Diameters of colonies on SDA media after 27h for 15 isolates after 7 days grow in MSM medium with 1% crude oil at 37°C for 150 rpm

Sample code	Name of bacteria	Diameters of colonies
6E	*Bacillus cereus*	0.5±0.1
7E	*Bacillus flexus*	0.4±0.1
A6	*Pseudomonas aeruginosa* HNYM41	0.5±0.1
C21	*Delftia tsuruhatensis*	0.3±0.1
R60	*Alcaligenes faecalis*	0.3±0.1
S15	*Enterobacter sp.*	10±0.1
B4	*Acinetobacter sp.*	0.4±0.05
A4	*Pseudomonas aeruginosa 28*	0.7±0.1
CT	*Streptomonas maltophylia*	0.3±0.05
12C	*Acinetobacter baumanii*	0.5±0.05
13C	*Acinetobacter jejuni*	0.4±0.1
C15	*Enterobacter sp.*	10±0.2
B18	*Enterobacter sp.*	10±0.15
M6	*Pseudomonas aeruginosa*	0.3±0.1
M4	*Bacillus subtillus*	0.4±0.057

Screening of bacterial isolates producing exopolysaccharides (EPS)

a) Bacterial growth

Isolates were streak on sabourauds dextrose agar (SDA) and incubated at 25°C for 48-72 h. The growth with mucous colonies (which displayed viscous or sticky growth) showed the production of EPS [5]. Positive isolates will be selected for further test.

b) Determining the index of flocculation

Single bacterial isolate was grown in 100 mL nutrient broth (NB). After an overnight incubation at 37°C with shaking at 150 rpm. The culture was centrifuged at 4000 rpm for 15 min; the pellet was suspended in

10 mL normal saline. Standardized inoculate was prepared as described by [13]. Ten percent of standard inoculums was inoculated into 25mL nutrient broth (NB) and incubated at 37°C in 150 rpm. After 24 h samples were simply allowed to stand and were measured after 5, 10, 30 and 60 min this indicated as A_t, samples were immediately centrifuged at 650g for 2 min and the OD 550 nm of the supernatant (upper layer) was measured indicate as A_s. Index of flocculation (%IF) was calculated as follows (14, 15] in Equation (1). All tests were done in triplicates:

$$100*(A_t-A_s)/A_t \quad (1)$$

c) Math assay (microbial adhesion to hydrocarbon)

Single bacterial isolate was grown in 100 mL of (NB) and incubated in an orbital shaker at 37°C, 150 rpm for 24 h. The cells from standard inoculums (2 mL) was added to 100 µl crude oil and vortexes for 3 min in test tubes and the aqueous phase were allowed to separate for 1 h. The OD was read from the aqueous phase at 550 nm [16]. Hydrophobicity is expressed as the percentage of cell adherence to crude oil calculated as follows Equation (2):

100 *(1-OD of the aqueous phase / OD of the initial cell suspension) (2)

Degradation of crude oil

A total of 15 isolates to quantify for the percentage of oil degradation. Degradation of hydrocarbon was measured by gravimetric method. Ten percent of standardized inoculums were inoculated into 100 mL of mineral salt medium (MSM) with 1% (v/v) of crude oil (pH 7.0) and incubated at 37°C, 150 rpm for 7 days. The medium without the inoculation of bacteria was used as a control. The residual hydrocarbon was extracted from the culture medium with 100 mL of chloroform in 500 mL separator funnel. The solvent was then removed by evaporation using a rotary evaporator at 50°C and the total hydrocarbon obtained was weighed [17].

Determination EPS

Bacterial cultures were grown in MSM added with 1% crude oil and incubated at 37°C, 150 rpm for 7 days. The bacterial cultures were centrifuged at 10000 g for 15 min at 4°C and 10 mL of supernatant were precipitated with 30 mL ethanol by incubation at -20°C for 24 h. The liquid was centrifuged at 10000 g for 15 min and the supernatant was removed. Bound EPS was extracted from the cell pellet using EDTA method. The precipitate from free and bound EPS was dried at 105°C for 24 h and weighed [18].

Preparation of consortium culture

Single bacterial 4 isolates was grown in 100 mL (NB) and incubated in an orbital shaker at37°C, 150 rpm for 24 h. The cells were separated by centrifugation (4000 rpm, 15 min), the pellet was suspended in 10 mL in NaCl (0.85). Standardized inoculate was prepared as described by [13]. The bacterial culture was inoculated in MSM. Subsequently, mixed culture were conducted using of standard inoculums of each bacterium by appropriate volume, 10% (v/v) were transferred into 100 mL with 1% (v/v) tapes crude oil as sole carbon and incubated for 7 days at 37°C in orbital shaker at 150 rpm for study degradation percentage.

Degradation of crude oil as consortium

Fifteen bacteria isolates were used in this study. These isolates included the genus *Bacillus cereus*, *Acinetobacter baumanii, Pseudomonas aeruginosa 28*, *Pseudomonas aeruginosa* HNYM41 were selected to test degradation as consortium as 3 bacteria. The mixed consortium for the study based on the efficiency of tapis crude oil degradation. All bacteria consortium grow in mineral salts medium as describe above.

Results and Discussion

Screening of EPS-producing bacterial isolates:

The ability of the 15 bacterial isolates to produce EPS was determined by plating on SDA media. Fig. 1

shows the colonies when grown on SDA media have produced mucous on the growth medium. Diameters of these colonies after 3 days ranged between 0.2 to 10 mm (Table 1). The possible reason for mucous colonies in SDA medium may be contain higher amount from dextrose as carbon sources enhancement to produced EPS. All these 15 isolates were screened to possess the potential for EPS production. The ability to produce EPS is a distinct advantage to the bacteria because it improves contact between cell surface and hydrocarbon, thus allowing formation of micelles (oil droplets) [19]. Some isolates were capable of producing mucous colony from EPS in medium and each of them could form an EPS more than 0.5 mm until 10 mm. In general EPS production slim or capsule by different isolates to protect them against unfavorable condition [5].

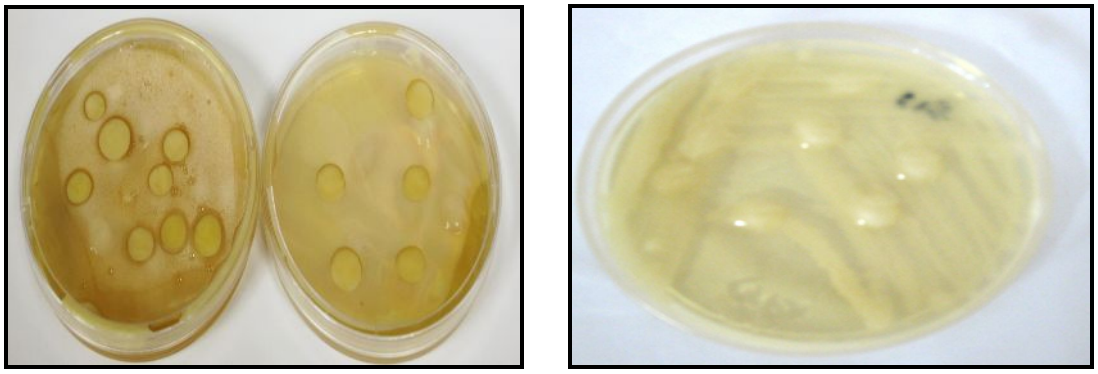

Fig.1. Potential isolates showed formation mucous colonies on (SDA) media after 3days incubated in 25 °C due to production of large quantities of EPS

Growth of microorganism

The bacterial growth was expressed as colony forming units (CFU) and absorbance reading as optical density (OD). Correlating the reading to the numbers of colonies on nutrient agar. All isolates grow in MSM media with1% crude oil as carbon sources range OD between 1.2 to 1.7 absorbance. Growth 4 isolates choice as consortium at (1.65, 1.6, 1.59 and 1.57) for (6E, A4, A6 and 12C) respectively. Among this 4 isolates were found to be more dominant with count exceeding the mean (2.9×10^{11}). The overall population means of isolates a range between (3.16×10^{10} to 3.09×10^{8} CFU/mL for isolates (A4, A6, 12C and 6E) (Fig. 2).

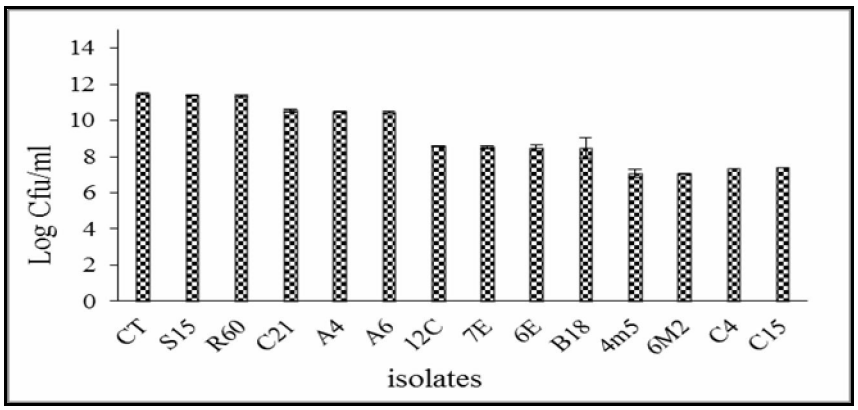

Fig 2. Growth of 15 isolates in MSM with 1% crude oil incubated at 37°C for 150 rpm after 7 days

Flocculation index and hydrophobicity of crude oil

In the first part of our study the flocculation index and hydrophobicity of 15 isolates these are summarized in (Table 2). About 40% of the bacterial isolates were good floc formation (flocculation index 20% or more) while 60 % the flocculating bacterial less than 20%. Isolates 6E, 7E, A6 and C21shared similar properties relatively high flocculation index range between 31.9% to 24.9% with hydrophobicity higher than 90%. A large flocculation index value means relatively higher floc formation and good settling characteristics of the culture [3]. The percentages of adhesion to crude oil of the 15 isolates showed the diversity of their surface hydrophobicity the most hydrophobic 96.5% to isolate 6E.

Table 2. Index flocculation and hydrophobicity of crude oil and n-octane for 15 isolates.

No of Bacteria	IF%	Math assay Crude oil %	Math assay n-octane
6E	31± 0.14	96.5± 0.98	83.7± 1.2
7E	26.4± 1.9	94.3± 0.70	91.3± 2.1
A6	25.4± 0.43	92.7± 0.70	96.6± 1.9
C21	24.9± 1.3	89.6± 0.70	69.5± 3.8
R60	23.7± 0.98	91.7± 2.5	98.3± 2.1
S15	21.9± 1.6	76.7± 2.5	88.7± 8.06
B4	21± 1.1	84.1±0.14	90.6± 1.6
A4	20.4± 1.7	92.6± 0.84	98.9± 1.2
CT	18± 0.28	63.7± 0.14	72.2± 5.09
12C	15± 0.14	90.7± 0.14	80.1± 6.3
13C	14.8± 0.67	86.6± 1.1	98.2± 0.56
C15	12.1± 1.1	85.9± 0.42	86.7± 6.3
B18	12± 0.24	80± 0.28	85.8± 1.9
M6	10.05± 1.2	88.7± 0.14	83.2± 4.8
M4	4.5± 0.74	65.1± 1.2	89.8± 1.6

Present results display that the more hydrophobic cells have higher flocculation index, while this isolate S15 has lower hydrophobicity to hydrocarbons this contradiction results that the relationship between flocculation and hydrophobicity may depend on the bacterial species. Study [20] proven cell surface properties of bacterial isolates depend on their surface structure. Another possible reason can explain by this bacterium has hydrophobicity to another chemical such as heavy metals than hydrocarbon. [21] Showed hydrophobicity is an important prerequisite for flocculation to occur but some other factor is also involved in the regulation of flocculation behavior this related with bacteria behavior. On the other hand isolates 12C have high hydrophobicity than the flocculation index. The most hydrophobic isolate 6E with 96% adhesion to crude oil and isolate M4 was the most hydrophilic with 63% adhesion.

Biodegradation of crude oil

Five potential isolates (A6, A4, 6E, 7E and 12C) in floc formation ability and hydrophobicity showed the highest percentage of degradation were (78.3%, 60.1%, 57.2%, 55.5%, and 55.3%) respectively (Fig 3). These bacterium possess adheres strongly to hydrocarbons. Good flocculation properties with high values of relative hydrophobicity demonstrate that hydrophobic interaction is important in the flocculation by high percentage of hydrophobic surfaces (R-correlation coefficient at 0.76) between index flocculation and degradation percentages. This result agree with [20] showed correlation the hydrophobicity with flocculation.

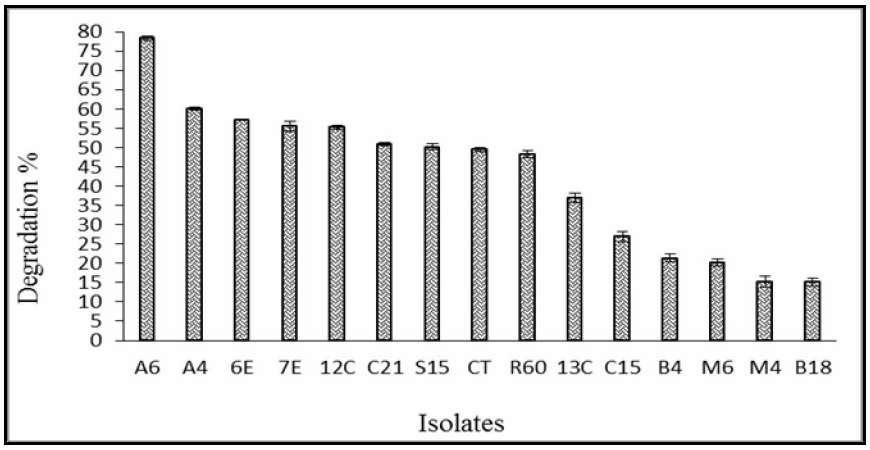

Fig 3. Degradation of crude oil (%) by 15 potential isolates grown in MSM. All values are mean of three replicates ± standard deviation

The adhesion of a microbe to the oil–water interface enhances the rate transfer of Hydrocarbon to the cell by reducing the effective distance between a microorganism and its substrate [22]. These results strongly indicate that hydrophobic interaction is an important driving force. Hydrophobicity is a common strategy can increase biodegradation and growth bacteria on hydrocarbons these mechanism only to enable rapid uptake of hydrocarbons but in biodegradation process not necessary cell adhesion to the hydrocarbon phase [23]. This reason can explain isolates S15 and CT have less hydrophobicity at 76.7and 63% respectively with high percentage in degradation were 50.2 and 49.5%.

EPS production

Most of the exopolysaccharides are produced by microorganisms during the growth phase. Showed different isolates produced EPS in the same cultural condition. In many cases the types of EPS produced specific to species. Isolate 6E produced the higher EPS 7.7 g/L but lowest amount at 0.5 g/L produced by isolates CT and 4M. The capacity of isolates to produce different concentration of EPS demonstrated different metabolic activity [9]. EPS produced by bacterial cells to protect them against unfavorable environmental conditions. This variation in EPS concentration depended on characteristic of EPS production by different isolates (Fig 4) [5].

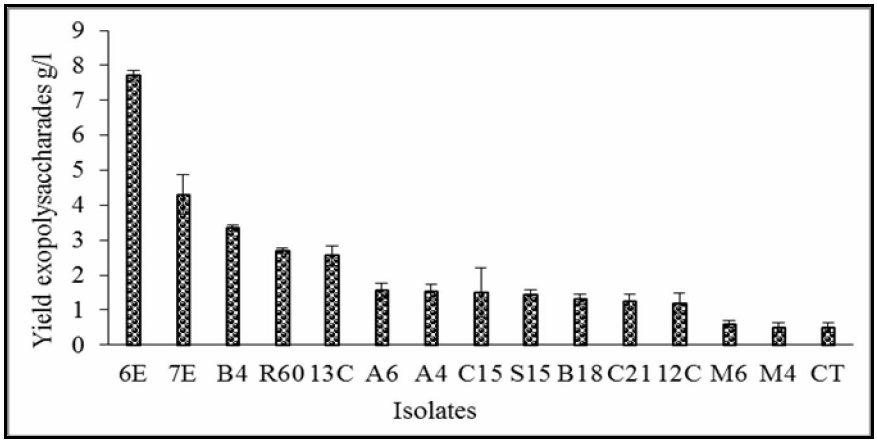

Fig 4. EPS (g/L) production by the 15 potential isolates at the end 7 days at initial pH 7.0. All values are mean of three replicates ± standard deviation

Adhesions also influenced by chemical species on the cell surface that determine hydrophobicity with the length and conformation of attached biopolymers [24]. While showed the relationship between the concentration of biopolymer EPS and flocculation index [25, 2]. The production biopolymer EPS demonstrated occurs more extensively during the specific adhesion stage [26] and reported EPS compounds apply on the environment in degradation process of organic substances.

Biodegradation of crude oil as consortium

Mixed bacterial consortium would be in the state of increased biodegradation of crude oil more than single isolate. In this study choice 4 isolates (6E, A6, A4 and 12C) to test as groups to degrade crude oil (Table 3). The degradation results showed that nearly 79.8% in group B after 7 days by mixed bacterial consortium high efficiency in oil removal compare than other groups. The residual concentration of crude oil was very low compared to original content by mixed bacterial consortium. Crude oil did not inhibit the degradation efficiency of mixed bacterial consortium by group A, C and D degradation percentage was (61.3%, 65.6% and 68.8%) respectively. [27] Showed the degradation of organic compounds can be more efficient by bacterial consortia than bacteria alone. Mixed cultures showed an enhanced hexadecane removal, particularly with the whole consortium (79 ± 3%) [28]. Biodegradation in aromatic structure decomposition in a short period reported by [29] after mixed bacterial consortium. The entire three mixed bacterial consortium (group B) showed maximum crude oil degradation at 37°C, 150 rpm. Table 3 illustrates the results for EPS production the highest production was shown by group B (6.5 and 0.4) g/L in bound and free EPS respectively than other groups and flocculation index 57.2%. Using scanning electron microscope SEM consortium culture was shown clearly attached closely together in the surface, this close depend on physical interaction between different isolates or hydrophobic interaction, this results agreed with [30]. The surface is coated by exopolymer exopolysaccharides EPS that can

contribute to hydrophobic interaction by functional groups such as carboxyl, phosphoryl and phosphodiesterase (Fig.5).

Table 3. Biodegradation of crude oil for bacterial consortium and EPS production for different combination after 7 days.

Groups	Biodegradation of crude oil% ± sd	Bound EPS g/L ± sd	Free EPS Flocculation	g/L ± sd index % ± sd
A(6E, A6, A 4, 12C**)**	61.3± 0.43	6± 0.56	0.33± 0.057	52.3 ± 0.98
B(6E, A6, A4)	79.8 ± 0.83	6.5± 0.6	0.4± 0.057	57.2 ± 0.28
C(6E, A6, 12C)	65.6 ± 0.56	5± 0.07	0.2± 0.07	53.8 ± 0.56
D(A6,A4, 12C**)**	68.8 ± 0.42	5.5± 0.14	0.16± 0.07	57.1 ± 0.14
E(A4,12C, 6E)	72.4 ± 0.84	4.8± 0.42	0.26± 0.11	57.± 0.77

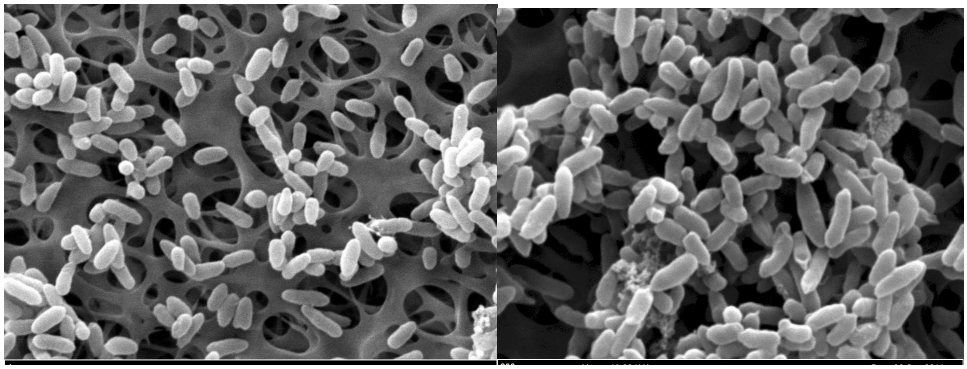

Fig 5. SEM showing floc formation by 3 isolates 6E, A6 and A4 after incubated in MSM medium + 1% (v/v) crude oil at 37 °C, 150 rpm for day-0 (A) and days -7 (B) magnifications 10000X.

Conclusions

The purpose of the present study was to investigate possible methods to enhance the rate of biodegradation of crude oil thus reducing the time usually required for bioremediation. Mixed bacterial consortium was selected among 15 isolates were successfully screened that were able to produce large quantities of EPS that facilitates crude oil degradation. The present study revealed that three isolates *Bacillus cereus, Pseudomonas aeruginosa 28* and *Pseudomonas aeruginosa* HNYM41 as consortium showed highest percentage of degradation was 78.9% thoroughly the 7 days. Environmental applications of the EPS compounds have been focused so far on the degradation process of organic substances. Results showed degradation and flocculation index as CC more than single isolates. Mixed bacterial consortium exhibited better performance in crude oil degradation.

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