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Screening of Minimal Salt Media for Biosurfactant Production by *Bacillus* spp.

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***Abstract*—**Crude oil is a major source of global energy. The major problem is its widespread use and demand resulted is in increasing environmental pollution. One associated pollution problem is 'oil spills'. Oil spills can be remediated with the use of chemical dispersants, microbial biodegradation and microbial metabolites such as biosurfactants. Four different minimal salt media for biosurfactant production by *Bacillus* isolated from oil contaminated sites from Oman were screened. These minimal salt media were supplemented with either glucose or sucrose as a carbon source. Among the isolates, W16 and B30 produced the most active biosurfactants. Isolate W16 produced better biosurfactant than the rest, and reduced surface tension (ST) and interfacial tension (IFT) to 25.26mN/m and 2.29mN/m respectively within 48h which are characteristics for removal of oil in contaminated sites. Biosurfactant was produced in bulk and extracted using acid precipitation method. Thin Layer Chromatography (TLC) of acid precipitate biosurfactant revealed two concentrated bands. Further studies of W16 biosurfactant in bioremediation of oil spills are recommended.

***Keywords*—**Oil contamination, remediation, *Bacillus* spp, biosurfactant, surface tension, interfacial tension.

I. Introduction

CRUDE oil is an essential source of energy and one of the main factors for the economic developments in the world. The exploitation of oil resources in existing mature reservoirs is essential for meeting future energy demands. Petroleum oil consists of complex array of gaseous, liquid, and solid n-alkanes, branched paraffins, cyclic paraffins and substituted cycloparaffins, aromatic compounds, sulfur compounds including benzo (b) thiophene and dibenzothiophene, and many other organic compounds [1]. The biggest problem associated with the widespread use and demand of the crude-oil is the ever-increasing pollution. Amongst all, the major problem is 'Oil Spills'. The term 'oil spill' is usually applied where oil is released into the ocean or coastal waters or land. The major reasons of oil-spills are: releases of crude oil from tankers, off-shore platforms, drilling rigs and oil-wells, as well as spills of refined petroleum products and their by-products. In addition, heavier fuels used by large ships such as bunker fuel, or the spill of any oily refuse or waste oil are common. Due to the geographical position of Oman, its shore lines are vulnerable to oil pollution from normal tanker operations, ballast water, illegal discharges and accidental spills. When unloaded, the returning tankers fill sea water as ballast to be carried in the compartments previously occupied by oil. In December 19, 1972 one of the worst major oil spills in history was recorded in the Gulf of Oman. The amount of oil spill was 35.3 million gallons when the South Korean supertanker, Sea Star, collided with the Brazilian tanker, Horta Barbosa, off the coast of Oman [2]. Oil spill cleanup is difficult and depends upon many factors, including the type of oil spilled, the temperature of the water (affecting evaporation and biodegradation), and the types of shorelines. Different types of bacteria have been isolated in Oman feeding on oil and therefore, can be used in oil spills cleanup avoiding chemical and physical means which can lead to other types of environmental pollutions. Bacteria bioproducts such as biosurfactants, biosolvents, or enzymes have been used in oil bioremediation. Biosurfactants are microbial secondary metabolites, which have both 'hydrophillic' and 'hydrophobic' moieties in their molecules, which helps making 'oil-water' emulsions. There are many studies associated with the use of biosurfactants in environmental applications such as bioremediation, dispersion of oil spills, and treatment of waste [3], [4]. *Bacillus* spp., are spore forming, Gram positive, bacteria. They can survive under petroleum reservoir conditions where high temperature, high salinity and anaerobic condition. Youssef et al. [5] reported that several biosurfactants, especially lipopeptides, produced by *Bacillus* spp. which can reduce the interfacial tension between hydrocarbon and aqueous phases to mobilize the oil. Members of *Bacillus* spp. are known to inhabit soil which are easily cultivated and consequently used in commercial production of biosurfactants.

The microbes used in present study were isolated from crude-oil contaminated soil samples from various sites in Oman. The isolates belong to *Bacillus* spp. Biosurfactant production from these strains was studied in four different minimal salt media. The biosurfactant was produced in bulk and extraction using acid precipitation method, partially analyzed using thin layer chromatography (TLC).

II. Materials and Methods

Several *Bacillus* strains previously isolated from oil contaminated soil samples were tested for biosurfactant

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production. The strains were identified as *Bacillus subtilis* and *B. licheniformis*, by 16S rRNA sequencing (3130xl Genetic analyzer) at Department of Biology, College of Science, Sultan Qaboos University [6]. Six bacterial strains (W16, B25, B26, B27, B28, and B30) were selected in this study.

Luria Bertani (LB) broth was used as seed medium (50ml LB broth in 250ml Erlenmeyer flasks), which was inoculated with loop-full of previously isolated strains of on LB agar plates. Flasks were incubated aerobically at 40°C, 160 rpm for 8-12h. Four different minimal salt media were used for screening best production media with glucose or sucrose as a carbon source (Table I). The components of the basal minimal salt medium were prepared and distributed 50ml each in 250ml Erlenmeyer flasks, sugar (glucose or sucrose) was sterilized separately and added after autoclaving (121^{0}C for 15 minutes). Each different production media flasks were inoculated with 2% (v/v) of seed media. After inoculation, the flasks were incubated in shaking incubator with agitation at 40^{0}C, 160rpm. Samples were collected every 24h (till 72h) and analyzed for microbial growth (OD_{660}), pH, and biosurfactant production (ST and IFT).

TABLE I
BASAL COMPOSITION OF FOUR MINIMAL SALT MEDIA USED FOR BIOSURFACTANT PRODUCTION

Medium Components	Concentration (g/L)			
	Nazina Medium [7]	McInerney Medium [8]	Cooper's Medium [9]	Mukherjee Medium [10]
NaCl	7	50	-	0.01
K_2HPO_4	0.09	13.9	-	2.2
KH_2PO_4	0.21	2.7	4.083	0.14
NH_4Cl	0.8	-	-	-
$MgCl_2.5H_2O$	0.2	-	-	-
$CaCl_2.2H_2O$	0.05	-	-	0.04
$NaHCO_3$	2.2	-	-	-
$(NH_4)_2SO_4$	-	1.0	-	-
$MgSO_4$	-	0.25	0.197	0.6
Glucose	-	-	20	20
Sucrose	20	20	-	-
NH_4NO_3	-	-	4.002	3.3
$NaHPO_4$	-	-	7.119	-
$FeSO_4$	-	-	-	0.2
Trace mineral solution	1ml/l[a]	10ml/l[b]	1ml/l[c]	0.5ml/l[d]

[a] TMS composition (g/l): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.18; $ZnSO_4.7H_2O$, 0.022; $CuSO_4.5H_2O$, 0.08; $CoCl_2.6H_2O$, 0.06; Na $MoO_4.2H_2O$, 25.0.
[b] TMS composition (g/l): Na_2-EDTA, 1.0; $MnSO_4$, 3.0; $FeSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1; $CoCl_2.6H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01; Na_2MoO_4, 0.01.
[c] TMS composition (g/l): $CaCl_2$, 0.00077; $FeSO_4.7H_2O$, 0.0011; $MnSO_4.4H_2O$, 0.00067; Na_2-EDTA, 0.00148.
[d] TMS composition (g/l): $ZnSO_4.7H_2O$, 2.32; $MnSO_4.4H_2O_4$, 1.78; H_3BO_3, 0.56; $CuSO_4.5H_2O$, 1.0; $Na_2MoO_4.2H_2O$, 0.39; $CoCl_2.6H_2O$, 0.42; Na_2-EDTA, 1.0; $NiCl_2.6H_2O$, 0.004; KI, 0.66.

Microbial growth was determined by using spectrophotometer (Helios ε, Thermo Spectronic USA) to measure the optical density at 660 nm (OD_{660}). Both ST and IFT were measured by using Drop Shape Analyzer (Pendant drop Tensiometer, Kruss, DSA100, Germany)

For extracting biosurfactant in bulk quantity, W16 strain were grown in 1500ml (30 flasks each containing 50ml) Mukherjee medium with sucrose as a carbon source and incubated for 48h. This strain and medium was chosen because it showed better reduction than other strains. After 48h broth was centrifuged at 10000 x g for 15 min to get cell free broth. Biosurfactant was precipitated by adjusting the pH of the cell free broth to 2.0 using 6 N HCL and keeping it overnight at 4^{0}C [11]. The precipitate was collected by centrifugation (12000 x g, 20 min) and dissolved in 100ml of distilled water. Its pH was adjusted to pH 8.0 with 1 N NaOH. Thus, 15 times concentrated biosurfactants (from 1500ml to 100ml) was collected.

The concentrated biosurfactant, acid precipitated sample, untreated broth and control uninoculated medium were spotted on thin layer chromatography (TLC) plates. The TLC plates were saturated using the solvent system – chloroform: methanol: water (65:25:4) [12]. After separation, the plate was developed by the iodine vapor.

III. RESULTS AND DISCUSSION

The bacterial strains used for this study were isolated from different oil-contaminated sites in Oman. *Bacillus* isolates were screened for their biosurfactant production capabilities in four different minimal salt media. The biosurfactant production was analyzed using Pendant Drop Tensiometer (Fig. 1) [11]. Where, the change in the shape of the drop (Fig. 2) was measured for all samples.

The production of surface active compounds (biosurfactants) by microorganism has been a subject of increasing interest in recent years, especially due to their potential applications in enhancing oil recovery and environmental pollution. This study attempted to find the most reliable four minimal salt media reported [7]-[10] supplemented with C-source (glucose or sucrose) to optimize biosurfactant production.

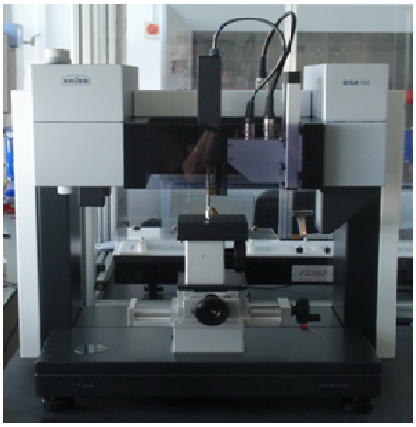


Fig. 1 Pendant drop DSA100 tensiometer (Kruss) was used to measure the IFT and ST

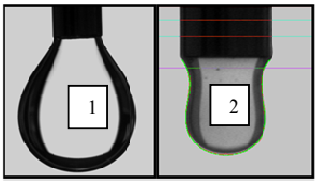
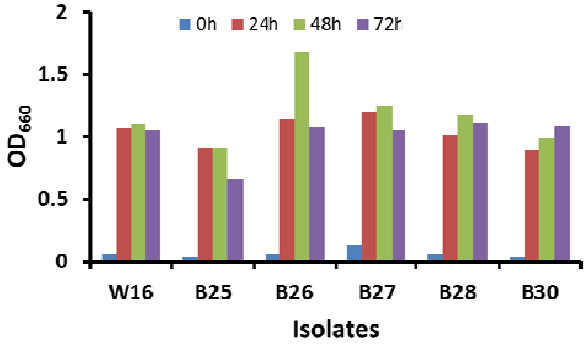


Fig. 2 Reduction in ST/IFT (1) Control: IFT 26.08 mN/m, (2) Isolate W16: IFT 2.06 mN/m in Mukherjee medium (IFT against hexadecane)

Among the four media, Nazina did not supported biosurfactant production of the isolates during the 72h period and was not used in this study. McInerney medium supported better microbial growth than Nazina medium (Fig. 3 (a)). There were minor fluctuations in pH between the six strains during the three-day incubation period (Fig. 3 (b)). Moreover, better activity of biosurfactants was observed in this medium. The lowest values were 48.38mN/m and 16.43mN/m for ST and IFT respectively (Figs. 3 (c), (d)).



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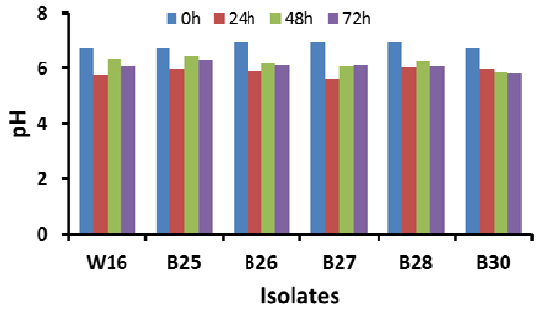

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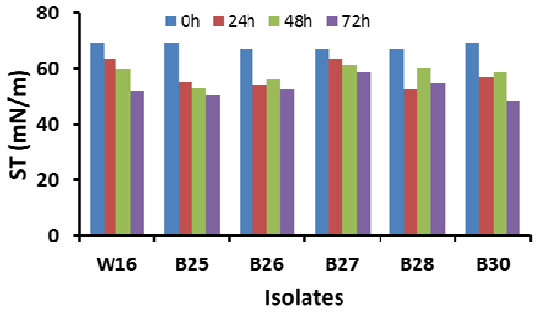

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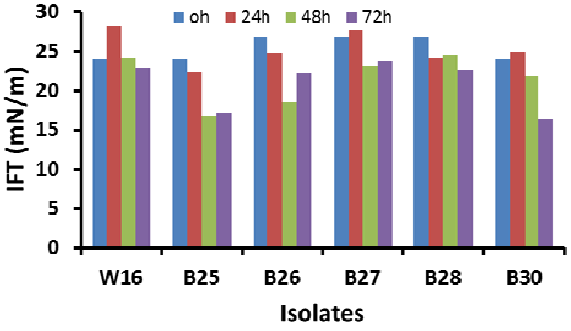
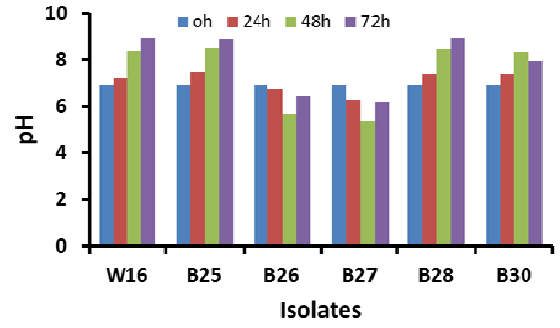


(d)

Fig. 3 Production profile for all six bacterial isolates in McInerney medium: (A) pH, (B) growth (OD660), and biosurfactant production – ST (C), IFT (D)

Significant difference in production profile was observed in Cooper's medium compared to the McInerney medium. The pH was closely stable throughout the fermentation (Fig. 4 (a)), because of strong buffering capacity in the medium. Except for isolate B26, more or less similar patterns of growth were observed for the rest of the isolates (Fig. 4 (b)). Isolates B30 and W16 were the best strains with the lowest ST - <35mN/m (Fig. 4 (c)) and IFT <11mN/m (Fig. 4 (d)). Haghighat et al. [13] reported in their experiment that nitrogen plays an important role in biosurfactants production. They used peptone, ammonium nitrate, yeast extracts and sodium nitrate as a nitrogen source in their medium and concluded that sodium nitrate showed the lowest ST (30mN/m).



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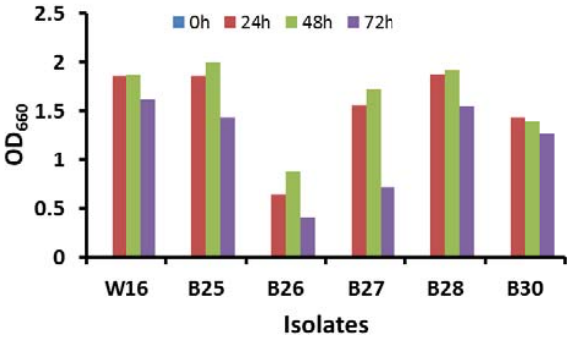

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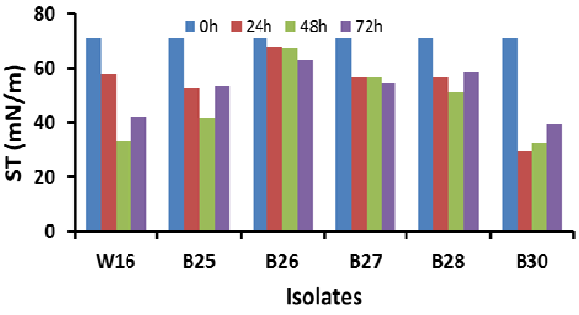

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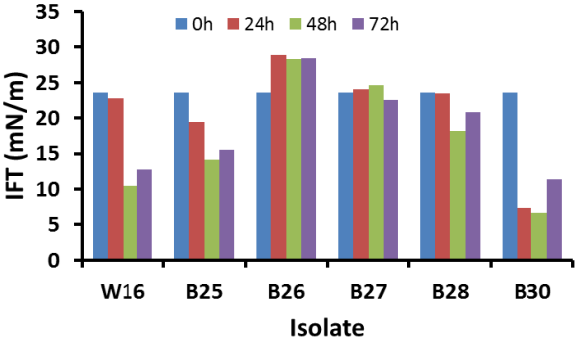
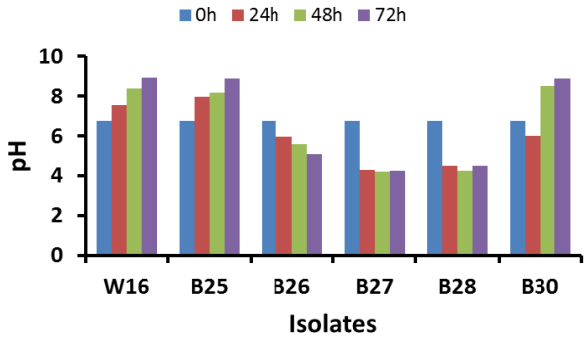


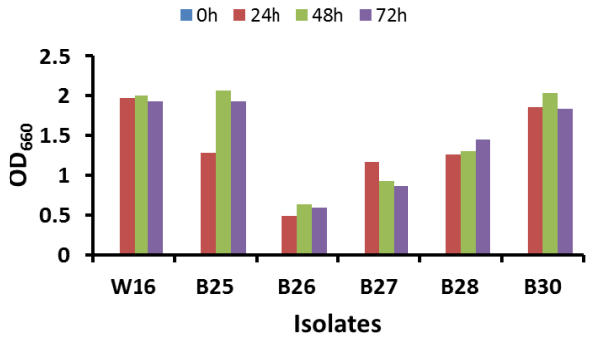
(d)

Fig. 4 Production profile for all six bacterial isolates in Cooper's medium: (A) pH, (B) growth (OD660), and biosurfactant production – ST (C), IFT (D)

Like Copper's medium, Mukhrejee medium also supported better microbial growth and biosurfactant production. The pH was almost stable throughout the fermentation, except for isolates B27 and B28 where it became acidic (Fig. 5 (a)). In this medium isolate B26 also showed less growth as compared to the other 5 isolates (Fig. 5 (b)). Isolates B30 and W16 were the most suitable strains that showed the lowest ST - <30mN/m (Fig. 5 (c)) and IFT <7mN/m (Fig. 5 (d)). Isolate W16 showed much better reduction in ST (25.26mN/m) and IFT (2.29mN/m) within 48h of incubation as compared to Cooper's medium (ST - 33.01mN/m and IFT - 10.43mN/m). These results are equivalent to other investigations for *Bacilli* strains [13], [14].



(a)



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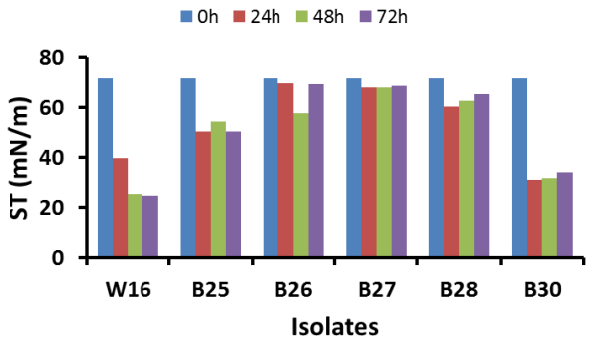

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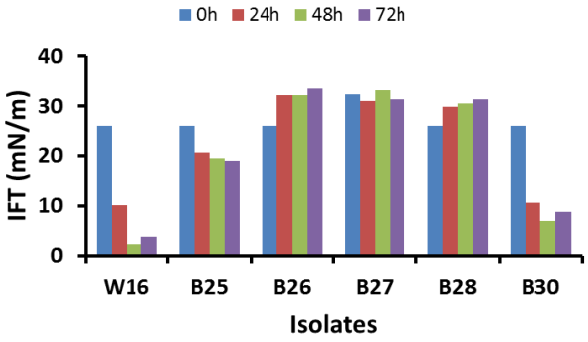

(d)

Fig. 5 Production profile for all six bacterial isolates in Mukherjee medium: (A) pH, (B) growth (OD660), and biosurfactant production – ST (C), IFT (D)

As isolate W16 produced more potent biosurfactant, it was selected for further experiments. W16 biosurfactant was extracted and concentrated using acid precipitation method [11]. Fig. 6 shows the 15 times concentrated biosurfactant (100ml from 1.5l initial broth). The ST and IFT of concentrated biosurfactant were 29.67mN/m and 4.08mN/m respectively. The ST and IFT of acid precipitated sample were 50.29mN/m and 17.55mN/m respectively compared to the control uninoculated medium (ST - 71.71mN/m and IFT - 26.08mN/m). This indicates that some percentage of biosurfactant was not extracted after acid precipitation.

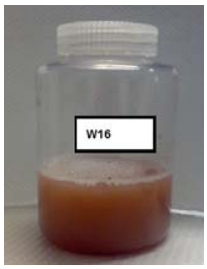

Fig. 6 Concentrated biosurfactant of isolate W16 using acid precipitation method

Biosurfactant was partially analyzed by TLC, showed two bands of the concentrated biosurfactant with high intensity compared with biosurfactants in the untreated broth (Fig. 7). Iodine vapor bound to lipid and protein portions of *B. subtilis* and *B. licheniformis* lipopeptide-biosurfactants. Therefore, it was used to develop and visualize the biosurfactants on TLC plates [12]. Two bands appeared in the concentrated biosurfactants sample. Moreover, higher intensity was in the concentrated biosurfactants than the untreated broth which indicated that the acid precipitation method used in this study, concentrated the biosurfactant. Further investigations are needed to identify and characterize the type of biosurfactant produced by W16 and its applications in oil bioremediation.

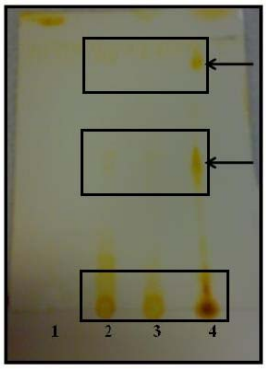

Fig. 7 TLC results (1) control, (2) untreated broth, (3) acid precipitated sample, and (4) concentrated biosurfactant

IV. Conclusion

Biosurfactants have a potential application in bioremediation and in treatments of oil spills. In this study, out of six isolates of *Bacillus* strains, W16 and B30 were found to be the best biosurfactant producers. The best minimal media supporting biosurfactant production of the two strains were Cooper's and Mukherjee media. However, isolate W16 gave better results in sucrose containing Mukherjee medium reducing ST and IFT to 25.26mN/m and 2.29mN/m from 71.71mN/m and 26.08mN/m respectively. Further studies are needed to identify and characterize the type of biosurfactant produced by isolate W16, and its application in bioremediation of oil spills and oil pollutions.

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