

Original Research Article

Isolation of halophiles from the Dead Sea and exploring their potential biotechnological applications

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ABSTRACT

Dead Sea is a hypersaline terminal desert lake with 34.8% salinity and is one of the extreme habitats for the sustenance of life on earth. Present ionic composition of the lake is 2M Mg²⁺, 0.5M Ca²⁺, 1.5M Na⁺, 0.2M K⁺, 6.5M Cl⁻ and 0.1 M Br⁻, which is inhibitory to most of life forms. Dead Sea harbours numerous halophilic microorganisms such as algae, bacteria, archaea and fungi; which can withstand the extreme conditions of the lake. During the years of heavy rainfall such as 1980 and 1992, upper water layer of the Dead Sea becomes diluted and leads to development of algal blooms which is followed by bloom of red halophilic Archaea. Metagenomic studies revealed the significant difference between microbial community during the algal bloom and resident community during the interbloom period. Halophilic archaea and bacteria isolated from the lake include the members of the genera *Haloferax*, *Haloarcula*, *Halobaculum*, *Halorubrum*, *Halomonas*, *Chromohalobacter* and *Salibacillus*. The halophilic microorganisms are utilized for various biotechnological applications and present study focuses on the isolation and screening of halophilic microorganisms from the Dead Sea for biotechnologically important properties such as extracellular protease production, Polyhydroxy alkanooates (PHA) production, halocin production and bio emulsifier production. Highest extracellular protease activity of 12.45 U/ml and highest PHA concentration of 27.5 % of cell dry weight was obtained. Isolates showing halocin production were also tested against the human pathogens such as *S. aureus* and *P. aeruginosa*; while this is one of the few reports, describing the bioemulsifier production from the halophiles.

Keywords

Dead Sea,
Halophiles,
Protease,
Polyhydroxy
alkanoates
(PHA),
halocin, Bio
emulsifier

Introduction

Dead Sea is a hypersaline terminal desert lake with 34.8% salinity and gains its name due to absence of any macroscopic living creatures. Part of Syrian-African Rift valley, is located between the present political boundaries of Jordan, Israel and Palestine. Its surface and shore is located 429 meter below sea level making it lowest terrestrial elevation on earth. The lake consists of deeper northern basin and shallow southern basin which is recently dried up and used for commercial mineral production (Figure 1). Jordan River is main tributary to the Dead Sea and the water level is dependent on the delicate balance between amount of fresh water inflow and evaporation (Oren, 2002). Also, several water springs contributes to fresh water inflow at Dead Sea and a complex system of underwater springs is recently discovered (Ionescu et al, 2012).

Water balance at Dead Sea is negative since from last century and water levels has been continuously decreasing; which leads to further increase in salinity and reduction in surface area (940 sq. km in 1960's to 600 sq. km present) (Khlaifat et al, 2010; Bashitialshaeer et al, 2011). Before 1979 the Dead Sea was meromictic; stratified into less saline, less dense and warmer upper layer while lower layer was anoxic, more salty, dense and cooler (Anati et al, 1987). Since then the lake is holomictic with no stratification except the rainy seasons in 1980 and 1992; characterized by NaCl supersaturation and halite deposition at the lake bottom (Oren, 2010a).

In an attempt to restore the Dead Sea levels, the Red Dead Sea conveyer project has been proposed which would convey seawater from the Red Sea (Gulf of Aqaba) to the Dead Sea. However, the environmental impact of this project is still under debate (Qdais, 2008).

The Dead Sea is an increasingly extreme environment with water activity less than 0.67, lowest ever to support the life (Oren et al, 2005). Due to continuous evaporation, Na⁺ precipitates as halite while the Mg²⁺, whose salts are more soluble, is further concentrated. Present ionic composition is 2 M Mg²⁺, 0.5 M Ca²⁺, 1.5 M Na⁺, 0.2 M K⁺, 6.5 M Cl⁻, 0.1 M Br⁻; which is inhibitory for most of the living forms (Oren and Gunde-Cimerman, 2012). Despite of its extreme hypersaline environment, the Dead Sea is habitat for numerous microorganisms; however, in low abundance.

First report of presence of microscopic living forms such as bacteria, algae and ciliate protozoa emerged in 1930's and 1940's when the salinity of the lake was much lower than today (Wilkansky, 1936, Elazari-Volcani, 1940,1943a,1943b,1944). Since then the unicellular green algae of the genus *Dunaliella* and numbers of novel archaea and eubacteria have been isolated from water column and sediments that includes autotrophs as well as heterotrophs and aerobes as well as anaerobes (Table 1) (Oren, 2010a). 70 species of fungi are reported from the deep waters and near shore localities of the Dead Sea, however, their role in the ecosystem of the lake is yet to be studied (Oren and Gunde-Cimerman, 2012).

In 1964 when the lake was in meromictic stage, the unicellular green algae of the genus *Dunaliella* was primary producer in surface water of the lake (Kaplan and Friedmann, 1970); while prokaryotes, probably dominated by red halophilic Archaea, live on the expense of the organic material produced by algae (Bardavid and Oren, 2008). After the end of meromictic stage in 1979 algae is disappeared from the water column of the lake, except for the

years 1980 and 1992 when the lake witnessed massive algal blooms followed by the dense bloom of the halophilic archaea giving red colour to the lake due to its carotenoid pigments (Oren, 2005). During both of these blooms the large amount of floodwater diluted the surface water layer of the lake which is sufficient to establish a meromictic regime for a period of few months.

Metagenome analysis of the microbial communities of algal bloom period and residual community at interbloom period shows the dominance of archaea and both the communities are completely different (Bodaker et al, 2010). Dominant 16S rRNA sequence phylotype during the bloom period is different than any cultured archaea from the lake till date while the phylotypes of the residual community shows little sequence similarity with any reported sequence in GenBank (Rhodes et al, 2012). Occurrence of abundant virus like particles during the decline phase of archaeal bloom in 1992, suggests the role of phages in community dynamics (Oren et al, 1997a). Thus, the Dead Sea is a unique ecosystem harbouring the various halophilic microorganisms adapted to its extreme hypersaline environment; however, complete understanding of its diversity and ecology is yet to be achieved.

Halophilic microorganisms are known for various potential biotechnological applications such as pigment production, hydrocarbon degradation, PolyHydroxy Alkanoate (PHA) production, halocin production, exopolysaccharide production, bioemulsifier production and halotolerant enzyme production (Oren, 2010b). Present study focuses on isolation and screening of halophilic bacteria for potential biotechnological applications such as extracellular protease production, PHA

production, bioemulsifier production and halocin production.

Materials and Methods

1) Sample collection and Isolation of halophiles

Sediment sample was collected from the Northeast coast of the Dead Sea from Balqa governorate, Jordan in December 2008 (31°44'37"N; 35°35'32"E). Isolation was carried out by enrichment and membrane filtration method using eight different isolation media, designated '**A-H**', of varying salt concentration and composition (Table 2).

In enrichment method, 1gram of mud sample was inoculated in 100 ml of enrichment media and kept for incubation at 37°C for 5 days on shaking incubator at 180 rpm. Further loopful of enriched culture was streaked on respective solidified medium for 5-8 days at 37°C to obtain the isolated colonies. The colonies were purified by repeated transfer on the solidified medium (Oren et al, 1999).

In membrane filtration method, 1 gram of mud sample was dissolved in 100 ml of 10% saline and filtered through cellulose acetate membrane (0.45µm) using membrane filter assembly. Membrane was placed on solidified medium and incubated at 37°C for 5-8 days (Elvi et al, 2004). All the isolates obtained were further maintained on the medium designated as '**I**' (Table 2).

2) Morphological, physiological and biochemical characteristics of isolates

Colony characters and cell morphological features were examined after 5-8 days of incubation. Gram staining was performed according to procedure of Dussalt, 1955;

after fixation with 2% acetic acid. The optimum salt requirement for growth was determined using medium 'I' with varying NaCl concentrations and all the isolates were categorized into moderate halophiles (optimum growth at 2.9% - 14% (w/v) NaCl), borderline to extreme halophiles (optimum growth at 8.8% - 23.4% (w/v) NaCl) and extreme halophiles (optimum growth at 14.6% - 32.1% (w/v) NaCl) (Nieto et al, 1989). Sugar fermentation, amylase test, indole test and nitrate reduction tests were performed as previously described (Elvi et al, 2004).

Antibiotic sensitivity was tested by disc diffusion method (Oren et al, 1997b); using discs containing Ampicillin (100 µg/ml), Azithromycin (50 µg/ml), Chloramphenicol (50 µg/ml), Ciprofloxacin (50 µg/ml), Gentamicin (50 µg/ml), Linezolid (30 µg/ml), Rifampicin (30 µg/ml), Streptomycin (100 µg/ml). Sensitivity to Simvastatin (20 µg/ml), inhibitor of archaeal enzyme hydroxy methyl glutaryl coenzyme A reductase, was tested by disc diffusion assay as described by Dyal-Smith, 2008. Sensitivity to bile salt was tested by inoculating the isolates on the solidified medium 'I' containing taurocholic acid at concentration of 0.25g/L (Kamekura et al, 1988).

3) Screening of isolates for potential biotechnological applications

a. Protease production:

Enzymes from halophilic origin are highly stable at saturated salt concentrations and could have novel applications. (Oren, 2010b). Isolates were screened for extracellular protease production by spot inoculation on medium 'I' supplemented with 10% milk, incubated at 37°C and occurrence of zone of proteolysis was considered as positive activity. Isolates

having positive proteolytic activity were further grown in modified medium 'I', in which 1% yeast extract was replaced by 1% soybean meal; incubated for 120 hours at 37°C on rotary shaker at 180 rpm. The culture was centrifuged at 10,000 rpm for 20 minutes at 4°C and supernatant was used as source of protease (Akolkar et al, 2009). The caesinolytic activity at 24, 72 and 120 hours was estimated using modified Arnon's method as described by Joo et al, 2002. One unit of enzyme was defined as the amount of enzyme that liberated 1µg tyrosine⁻¹ from casein under assay conditions.

b. PHA production:

Polyhydroxy alkanates (PHA) is class of polymeric compounds which serves as energy reserve in bacteria and polyhydroxy butyrate (PHB) is most common type of polymer found; while many different polymers and copolymers of this class are produced by variety of organisms (Suriyamongkol et al, 2007). The cultures were grown for 10 days in PHA enhancing medium as described by Lillo and Rodriguez-Valera, 1990. Accumulation of PHA granules was detected by Sudan black B staining and later confirmed by Nile blue fluorescent staining using fluorescence microscope with episcopic fluorescence attachment (Lab-Phot, Olympus) at 460nm excitation wavelength (Ostle and Holt, 1982). PHA was further extracted and quantified on dry cell weight basis as described by Kulkarni et al, 2010.

c. Halocin production:

Halocins are proteinaceous antimicrobial compounds produced by halophilic archaea and is considered as their universal feature (Torreblanca et al, 1994). Halocins vary in their spectrum of activity and known to act across domain barrier (Atanasova et al, 2013). Halocin production by isolates was

detected by disc diffusion method (Shand, 2006). Briefly, supernatant of freshly grown culture of each isolate was obtained by centrifugation and filtered through 0.2 μm syringe filters and tested against other isolates. Seed agar of test isolates was prepared by spreading 100 μl of freshly grown culture, adjusted to 0.1 OD at 600nm. Filter paper discs soaked in culture supernatant were placed on seed agar and zone of inhibition was observed. Culture supernatant of Halocin producing strains was also tested against the human pathogenic bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* by disc diffusion method with uninoculated media as control.

d. Bioemulsifier production:

Bioemulsifiers are surface active compounds which reduce the interfacial tension between immiscible liquids and leads to formation of stable emulsion. Bioemulsifiers are produced by various microorganisms, including halophiles and have potential applications in environmental bioremediation and oil recovery (Satpute et al, 2010). Primary screening of isolates for bioemulsifier production was performed by modified drop collapse method (Jain et al, 1991) as described below. Cultures were separately inoculated in 'T' media containing 1% of either parafin oil, coconut oil, sunflower oil or olive oil. Further, cultures were incubated on rotary shaker at 37°C for 5 days. Cultures were centrifuged and the supernatant was filtered through 0.2 μm membrane filter. In a petri dish containing water, a 20 μl drop of oil was taken and 10 μl aliquot of the above supernatant was added on the drop of oil. Uninoculated broth was used as a negative control and 1% SDS solution was used as a positive control. Bioemulsification activity of positive isolates was further assessed as described by Radhakrishnan et al, 2011 and one unit of

emulsifying activity defined as an increase in absorbance of 0.1 with respect to the quantity of bioemulsifier added.

Results and Discussion

1) Isolation, Morphological, Physiological and Biochemical characterization of halophiles:

Total 22 isolates were obtained (Table 2), with colonies appearing after 5-8 days of incubation. All the isolates were Gram negative, motile and extremely pleomorphic rod shaped cells occurring as single cells or in pairs or in clusters or in chains. Gas vacuoles were observed in isolates H1 and H3. Based on optimum NaCl concentration for growth isolates were categorized as moderate halophiles (11), borderline to extreme halophiles (8) and extreme halophiles (3) (Table 3). Results of Sugar fermentation, amylase test, indole test and nitrate reduction test are summarized in Table 4. Most of the isolates were sensitive to Ampicillin, Azithromycin, Ciprofloxacin, Rifampicin, Streptomycin and Sodium taurocholate (Table 5). All the isolates were highly sensitive to Linezolid and only one isolate was susceptible to Simvastatin. However, the biochemical characterization and sensitivity to antimicrobial compounds were not sufficient for identification. Sequencing of 16S rRNA gene will be required for the identification of isolates.

2) Screening of isolates for potential biotechnological applications

a. Protease production:

13 isolates showed the zone of proteolysis after 72 hours (Table 6) and protease production by these isolates was determined by enzyme activity assay (Figure 2). Most of the isolates showed maximum enzyme activity after 24 hours of inoculation.

Table.1 Novel microorganisms isolated from the Dead Sea (Oren, 2010a)

Archaea		Bacteria
Aerobes	Anaerobes	
<i>Haoarcula marismortui</i>	<i>Halobacreroides halobius</i>	<i>Halomonas halmophila</i>
<i>Haloferax volcanii</i>	<i>Sporohalobacter lortetii</i>	<i>Chromohalobacter marismortui</i>
<i>Halorubrum sodomense</i>	<i>Orenia marismortui</i>	<i>Chromohalobacter israelensis</i>
<i>Halobaculum gomorreense</i>	<i>Selenihalanaerobacter shrifitii</i>	<i>Salibacillus marismortui</i>
<i>Haloplanus natans</i>		

Isolates A6, A7, E1, G2 and G3 showed enzyme activity more than 10 Units/ml at 24 hours with maximum enzyme activity of 12.45 Units/ml by isolate A7. Isolates C1, F1 and H3 showed increase in enzyme activity with time and isolate H1 showed maximum enzyme activity (7.15 Units/ml) at 72 hours.

The protease from the *Halobacterium halobium*, previously isolated from the Dead Sea, was active at NaCl concentration of 2M or above and maximum enzyme activity was observed at 4.3M NaCl (Izotova et al, 1983). Similarly, the optimum NaCl concentrations for the enzyme activity of isolates of this study need to be characterized. Also, the production of protease could be enhanced by media optimization.

b. PHA production:

9 isolates showed the presence of PHA granules with Sudan black B staining and later confirmed by Nile blue staining (Table 6). Cells were fluoresced as orange under the fluorescence microscope at excitation wavelength of 460nm (Figure 3). Isolates A1, B1, E3 showed highest PHA production of 27.5% while isolate D1 showed the PHA production of 25% on dry cell weight basis (Figure 4). The highest PHA production of 27.5 % of cell dry weight basis in this study is comparable with previous reports of PHA production by halophiles (Poli et al, 2011)

and could be enhanced using media optimization.

c. Halocin production

Four isolates B1, E1, F2, G1 were detected as producer of halocin, which was active against the four isolates F2, H3, B2, A7 respectively (Table 7). The culture supernatant of the producer isolate E1 was active against the *Staphylococcus aureus*; however, none of the producer isolates inhibited the growth of *Pseudomonas aeruginosa*. Also, the culture supernatant of the isolates B1, E1, F2 showed the zone of pigment enhancement in *S. aureus* around the disc (Table 8). Previous reports have mentioned that pigment production in *S. aureus* has been associated with enhanced bacterial survival in harsh environment. (Samuel Katzlf, et al; 2005).

Dead Sea water and mud is known to possess medicinal properties from ancient times and is currently used in various cosmetic and skin care products (Harari, 2012). Dissolved minerals in the Dead Sea water and mud are known to confer its medicinal properties; however, role of biotic component is not studied (Portugal-Cohen et al, 2015). Therefore it is possible that the halocins or other compounds of halophilic origin might be responsible for medicinal activity of Dead Sea water and mud related to skin diseases.

Table.2 Enrichment media used: A (Ventosa et al, 1982); B (Nieto et al, 1989); C (Dyall-Smith, 2008); D (Arahal et al, 1999); E (Rodriguez-Valera et al, 1980); F (Modified medium of Ventosa et al, 1982); G (Oren et al, 1995); H (New media composition devised according to relative proportions of major salts present in the Dead Sea water); I (Elvi et al, 2004)

Components (g/L)	A	B	C	D	E	F	G	H	I
NaCl	178	89	240	103	156	178	125	60.8	150
KCl	2	1	7	0.2	4	2	-	8.8	2
MgSO ₄ .7H ₂ O	1	0.5	35	18	20	1	-	-	20
MgCl ₂	-	-	30	-	13	-	160	70.4	-
CaCl ₂	0.36	0.18	0.5	0.25	1	0.36	0.1	28.8	-
NaBr	0.23	0.15	0.8	-	0.5	0.23	-	-	-
NH ₄ Cl	-	-	-	-	2	-	-	-	-
NaHCO ₃	0.06	0.03	0.2	-	0.2	0.06	-	-	-
FeCl ₃	0.06	0.03	-	-	0.005	0.06	-	-	-
KH ₂ PO ₄	-	-	-	-	0.5	-	-	-	-
K ₂ SO ₄	-	-	-	-	-	-	5	-	-
Casamino acids	5	2.5	-	0.5	-	5	1	-	-
Tri-sodium citrate	-	-	-	-	-	-	-	-	3
Peptone	10	5	10	-	1	10	-	5	-
Yeast extract	1	-	1	-	10	-	1	10	10
Glucose	10	-	-	-	-	-	-	1	-
Starch	-	-	-	-	-	-	2	-	-
Glycerol	-	-	-	-	-	10	-	-	-
Sea water	-	-	-	-	-	-	-	100	-
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Number of isolates obtained	7	2	1	1	3	2	3	3	NA

Table.3 Classification of isolates on the basis of optimum NaCl concentration for growth

Category	Isolates	Total
Moderate halophiles (optimum growth at 2.9% - 14% (w/v) NaCl)	A5, C1, D1, E1, E2, E3, F1, F2, H1, H2, G3	11
Borderline to extreme halophiles (optimum growth at 8.8% - 23.4% (w/v) NaCl)	A1, A2, A4, A6, A7, B1, B2, G1	8
Extreme halophiles (optimum growth at 14.6% - 32.1% (w/v) NaCl)	A3, G2, H3	3

Figure.1 Dead Sea satellite view (Image Source: Google Earth)



Table.4 Results of sugar fermentation, Amylase test, Indole test, Nitrate reduction test

Isolate	Dextrose	Fructose	Lactose	Maltose	Mannitol	Sucrose	Amylase test	Indole test	Nitrate reduction test
A1	-	+	-	-	-	-	-	+	-
A2	-	-	-	-	-	-	+	-	-
A3	-	-	-	-	-	-	+	+	+
A4	+	+	-	-	+	+	-	+	-
A5	+	-	+	-	+	-	+	-	-
A6	+	-	-	-	-	-	-	+	-
A7	+	-	-	-	-	-	+	-	-
B1	+	+	+	-	+	+	+	+	-
B2	+	+	-	+	+	+	+	+	-
C1	-	+	-	-	-	-	+	-	-
D1	-	+	-	-	-	-	-	-	-
E1	-	-	-	-	-	-	-	+	-
E2	+	+	-	-	-	-	+	+	+
E3	+	+	-	-	-	+	-	-	-
F1	-	-	-	-	-	-	-	-	-
F2	+	+	-	-	-	-	-	-	-
G1	+	-	-	-	-	-	-	-	-
G2	+	+	-	-	-	-	-	-	-
G3	+	-	-	-	+	-	+	-	-
H1	-	-	-	-	-	-	-	-	-
H2	+	-	+	-	-	-	-	-	-
H3	-	-	-	+	-	-	-	-	-

Key: + Indicates positive test; - Indicates negative test

Table.5 Sensitivity pattern of isolates to various antimicrobial compounds (Key: ‘R’ – resistant; ‘S’ – susceptible)

Isolate	Ampicillin	Chloramphenicol	Gentamicin	Rifampicin	Streptomycin	Linezolid	Ciprofloxacin	Azithromycin	Simvastatin	Sodium taurocholate
A1	R	R	R	R	R	S	S	R	R	S
A2	S	R	R	S	S	S	S	R	R	S
A3	S	R	R	S	R	S	S	S	R	S
A4	S	R	R	R	R	S	S	R	R	R
A5	S	S	S	S	S	S	S	S	R	S
A6	S	S	S	S	S	R	R	R	S	S
A7	R	R	R	S	S	S	S	S	R	R
B1	S	R	S	S	S	S	S	S	R	S
B2	R	R	S	R	S	S	S	S	R	R
C1	S	R	R	S	S	S	R	R	R	S
D1	S	S	R	S	S	S	S	S	R	R
E1	R	R	R	S	S	S	S	S	R	R
E2	R	R	R	R	R	S	S	S	R	R
E3	S	R	S	S	S	S	S	S	R	R
F1	S	S	S	S	S	S	S	S	R	R
F2	S	S	R	S	S	S	S	S	R	S
G1	S	S	R	S	S	S	R	S	R	R
G2	S	S	R	S	S	S	S	S	R	R
G3	S	S	R	S	R	S	R	R	R	R
H1	S	S	R	S	S	S	S	S	R	R
H2	S	S	S	S	S	S	S	S	R	R
H3	S	R	R	S	S	S	S	S	R	R

Table.6 Results of primary screening for protease production, PHA production, Halocin production and Bioemulsifier production

Isolates	Protease production	PHA production	Halocin production	Bioemulsifier production
A1	-	+	-	-
A2	-	-	-	-
A3	-	+	-	+
A4	-	-	-	-
A5	-	-	-	-
A6	+	-	-	-
A7	+	+	-	-
B1	+	+	+	+
B2	-	-	-	+
C1	+	-	-	-
D1	+	+	-	-
E1	+	-	-	-
E2	-	-	-	-
E3	-	+	+	-
F1	+	-	-	-
F2	+	+	+	-
G1	-	-	+	-
G2	+	+	-	-
G3	+	-	-	-
H1	+	-	-	-
H2	+	+	-	-
H3	+	-	-	-

Table.7 Antagonistic interactions among isolated stains due to halocin production

Test	Producer			
	B1	E1	F2	G1
A7	-	-	-	+
B2	-	-	+	-
F2	+	-	-	-
H3	-	+	-	-

Table.8 Effect of culture supernatant of Halocin producer strains on *S. aureus*

Producer	Diameter of zone of growth inhibition (mm)	Diameter of zone of pigment enhancement (mm)
B1	Zone not observed	21.5
E1	10	20
F2	Zone not observed	14
G1	Zone not observed	Zone not observed

Figure.2 Enzyme activity at 24 hours (Blue), 72 hours (Red) and 120 hours (Green) after inoculation

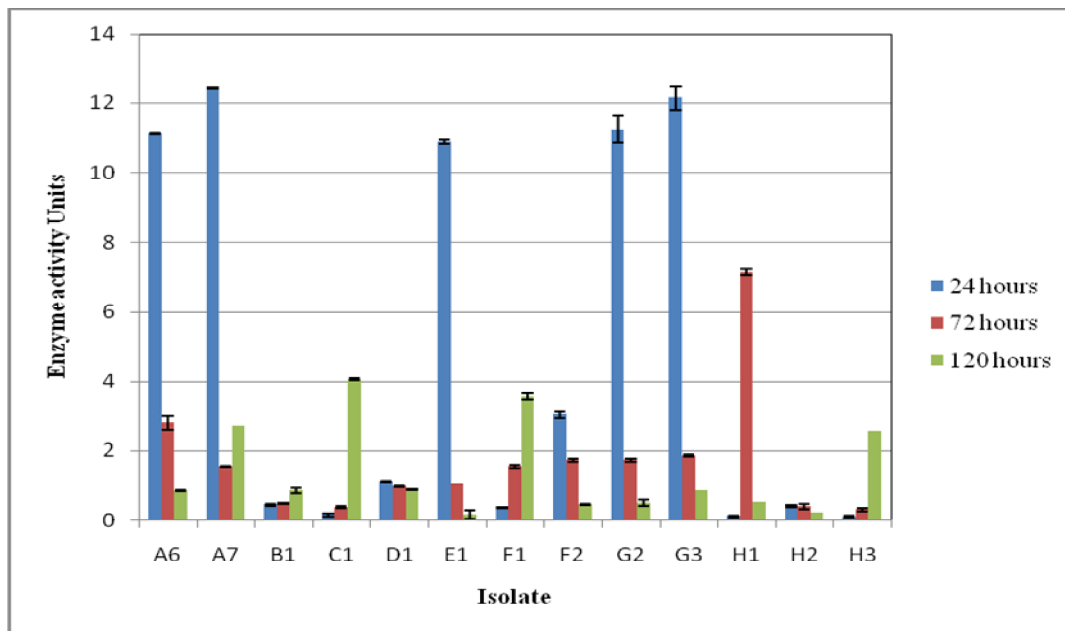


Figure.3 Nile blue A staining of PHA producing isolates; Left panel: Phase contrast microscopy;
Right panel: Fluorescence microscopy

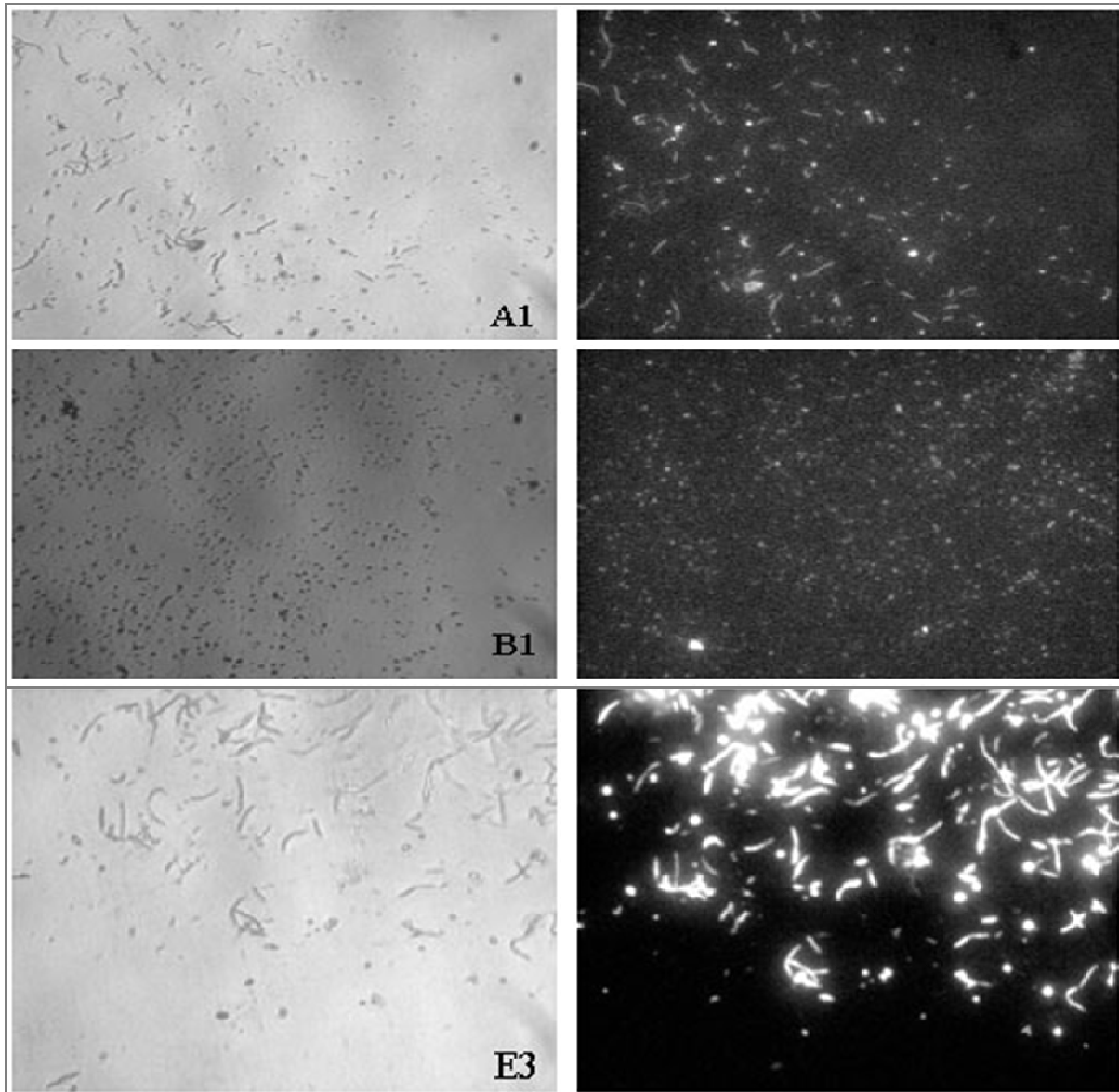
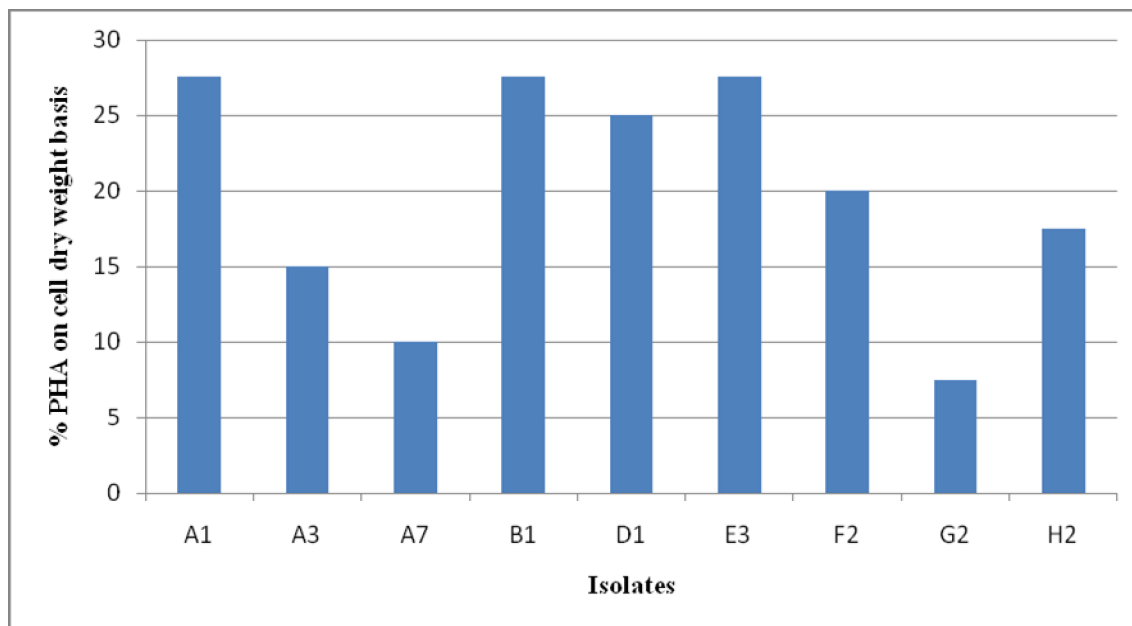


Figure.4 % PHA on dry cell weight basis



d. Bioemulsifier production:

All the 22 isolates were found positive for drop collapse method. Isolates A3, B1 and B2 were found have better ability with paraffin oil as compared to other isolates. The bioemulsifier activity of isolates A3, B1 and B2 was quantified and found to be 0.7, 4.9 and 5.1 Units/ml respectively. There are very few reports on production of bioemulsifier by halophiles and marine microorganisms (Mnif and Ghribi, 2015) and this is a novel report describing the bioemulsifier activity from the halophiles isolated from the Dead Sea.

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