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Oil-Biodegradation and Biosurfactant Production by Haloalkaliphilic *Archaea* isolated from Soda Lakes of the Wadi An Natrun, Egypt

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Haloalkaliphilic archaea isolated from Soda lakes of Wadi An Natrun, Egypt, were screened for biosurfactant/bioemulsifier production by using crude oil as the sole carbon source. Haloalkaliphilic isolates from water and sediment samples were screened for biosurfactant/bioemulsifier production using haemolytic activity, emulsification activity, drop collapsing test as well as oil displacement test. Twenty nine strains exhibited clear zone onto the minimal growth medium supplemented with crude oil (0.2% v/v). Sixteen strains were able to emulsify weathered crude oil into oil-broth medium during cultivation. Analyses of partial 16S rRNA gene sequences of the biosurfactant-producing strains indicated that they belonged to the family Halobacteriaceae, and were referred to *Natronococcus*, *Natronolimnobius*, *Halorubrum* and *Natronomonas* genera. Two strains exhibited the highest activity for oil displacement test and emulsification activity against Kerosene. Crude oil-in-water emulsions were stabilized over a broad range of conditions, from pH 5 to 12, with up to 35% sodium chloride in the aqueous phase. The search for biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environments that can potentially increase their stability in the harsh environments in which they are to be applied in biotechnology.

Key words: Archaea, Alkaliphiles, Oil-biodegradation, Biosurfactants, Biotechnology.

Biosurfactants are a diverse group of surface-active agents produced by many living organisms[1,2]. These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between different fluid phases. Their uses and potential commercial applications have been reported in several fields, including surfactant-assisted flooding for enhanced oil recovery in the oil industry, emulsifiers in the food industry and moisturizers in the cosmetic industry[3-6] The search for biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environments that can

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potentially increase their stability in the harsh environments in which they are to be applied in biotechnology[7,8].

There are very few reports on biosurfactant producers in hypersaline environments[9]. Halophiles, which have a unique lipid composition (phytanylglycerol), may have an important role to play as surface-active agents. The archaeal ether-linked phytanyl membrane lipid of the extremely halophilic archaea has been shown to have surfactant properties[10]. These surfactants were active over a wide range of pH (5–10) and at very high salt concentrations (up to 200 g l^{-1}).

Lately, interest in the mass cultivation of microorganisms from hypersaline environments has grown considerably, because this represents an innovative low technology approach to biotechnological exploitation[11,12]. Moreover, the importance of biodiversity in the Soda lakes of Wadi An Natrun has been recognized[13]. However, no information regarding the biosurfactant-producing haloalkaliphilic archaea has been reported. Hence, the study screened for the distribution of surface-active agent producing haloalkaliphilic archaea from Soda lakes of Wadi An Natrun, Egypt.

MATERIAL AND METHODS

Site description and sample collection

Water and sediment samples were collected from four Soda Lakes of Wadi An Natrun, Egypt, in October 2009. Lake Hamra, a less saline lake (30°23'48.28'' N, 30°19'13.39'' E), Lake Zugm (30°23.9172 N, 30°18.3832 E), Lake Beidah (30°25'57'' N, 30°14'30'' E), and Lake Fazda, a hypersaline lake situated at 30°19'43.50'' N, 30°24'29.68'' E, were sampled. All samples were immediately stored at +4° C and upon arrival at the Suez Canal University were analyzed for pH, electrical conductivity (EC), and concentration of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, HCO_3^{-}, CO_3^{-}, SO_4^{2-}, according to standard procedures[14].

Isolation procedure and growth conditions

Aliquots of water and sediment samples were spread onto Minimal Salt Medium (MSM) [15] supplemented with Na_2CO_3 10 g l^{-1} and diesel oil as the sole carbon source (0.2%). The medium was solidified with 20 g of agar per liter; the pH was adjusted to 10 ±0.2 and incubated for 21 days at 37°C. After incubation, morphologically different colonies were selected (approximately 15 to 30 colonies per plate), purified by re-streaking and screened for biosurfactant production. Isolated colonies were inoculated into 100 ml of MSM containing 0.2% of crude oil (obtained from Sharm El-Maiya Bay) and incubated with continuous shaking (200 rpm) for two weeks at 37°C. Colonies possessing biosurfactant producing activity, as evidenced by emulsification of crude oil, were chosen. In addition, the cell suspensions of isolates, cultivated without crude oil, were tested for the presence of surfactant by using haemolytic activity, the qualitative drop collapsing test, quantitative oil displacement test and emulsification activity. The selected colonies were phenotypically characterized in accordance with proposed minimal standards[16].

Biosurfactant activity assays

Haemolytic activity

Isolated strains were screened on blood agar plates containing fresh human blood (5%, v/v) and incubated at 37°C for two weeks. Haemolytic activity was demonstrated by the occurrence of greenish zone around the colony and was correlated with the production of biosurfactant[17].

Drop collapsing test

Wells of a micro-titer plate were coated with two µl of mineral oil. The lid was equilibrated for 1 h at room temperature, and then five µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after a minute. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production[18, 19].

Oil displacement test

To test oil displacement, 15 µl of crude oil were placed on the surface of distilled water (40 µl) in a Petri dish (150 mm in diameter). Then, 10 µl of the culture supernatant were gently put on the center of the oil film. The diameter and area of clear halo, visualized under visible light, were measured and calculated after 30'' [20].

Emulsification activity assay

Emulsification activity was measured by a modification of the method of Cooper and Goldenberg[[21]. Briefly, 4 ml of Kerosene were added to 4 ml of culture supernatant, and vortexed at high

speed for 5 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage[22].

Properties of emulsions

Stabilization of emulsions from haloalkaliphilic strains was evaluated over a range of pH and of NaCl concentrations. The pH was adjusted between 5 and 12 with NaOH. The emulsifier was tested with 10, 15, 20, 25 and 35% (w/v) sodium chloride in the aqueous phase. The stability of the formed emulsions (ES, %) was measured in intervals up to 48 h [22].

Biodegradation of crude oil assay

The degradation of crude oil by selected strains (WN23 and WN26) was determined using gas chromatography (GC) as it is a precise method for the analysis of the stable mixture of hydrocarbons[23]. The used GC was (6890 series, Hewlett Packard) equipped with a flame ionization detector (FID) and coupled with a spectra physics integrator programmed to calculate automatically the signal peak areas and corresponding concentration. A splitless injection mode was used to analyze the individual of total aliphatic and aromatic hydrocarbon fractions. The separation was carried out using the conditions described by[24].

Identification of strains

Strain identification was carried out using the sequence of the gene encoding 16S rRNA according to Rochelle *et al*. [25]. The 16S rRNA gene of the archaeal isolates was amplified with a set of universal primers (Invitrogen, USA). The primers 5'-ATTCCGGTTGATCCTGCCGG-3' (positions 6–25 in *Escherichia coli* numbering) and 5'AGGAGGTGATCCAGCCGCAG-3' (positions 1540–1521) [26]. The PCR conditions: 50 µl of reaction system, reaction cycles 30 times, 95°C pre-denaturation 5 min, 94°C denaturation 1 min, 60°C annealing 1 min, 72°C extension 1 min 30 s, 72°C final extension 10 min, 4°C hold. 50 ng/µl of each PCR product was used to prepare the samples which were delivered to MacroGen Company in Korea (http://www.macrogen.com) following their specifications. The sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST/) to get a preliminary identification of the strains. The cluster analysis was performed using the MEGA 5 software package.

RESULTS

Physico-chemical analysis of sediments and water samples

Characteristics of water and sediment

Table 1. Physico-chemical characteristics of water and sediment samples collected from different Soda Lakes of Wadi An Natrun, Egypt

Physicochemical properties	sample sites							
	Water samples				Sediment samples[a]			
	Hamra	Zugm	Beidah	Fazda	Hamra	Zugm	Beidah	Fazda
pH	9.03	8.89	8.7	8.8	8.58	8.98	8.64	8.56
EC (dS m^{-1})	151.3	188.3	137.4	197.9	100.8	195.1	141.5	119.2
Cations (gl^{-1})								
Na^{+}	24.244	31.427	21.956	30.228	16.005	31.867	23.166	18.458
K^{+}	0.114	0.057	0.076	0.038	0.1045	0.057	0.066	0.038
Ca^{2+}	0.1	0.2	0.3	0.5	0.2	0.3	0.1	0.2
Mg^{2+}	0.48	0.72	0.42	1.5	0.12	0.42	0.18	0.6
Anions (gl^{-1})								
CO_3^{2-}	10.8	21.3	9.33	8.22	5.1	5.1	3.3	0.3
HCO_3^{2-}	7.75	13.95	6.603	7.75	5.27	3.1	1.457	0.837
Cl^{-}	26.18	28.951	24.65	36.482	18.751	42.67	32.3	26.52
SO_4^{2-}	6	5.48	5.04	15.2	2.28	4.4	3.72	8.12

[a] The properties of sediment samples is relate to ratio (1:1)

Table 2. Phenotypic characteristics of haloalkaliphilic biosurfactant-producing isolates from Soda Lakes of Wadi An Natrun, Egypt

	Strain										
	WN11	WN13	WN14	WN16	WN17	WN18	WN23	WN24	WN25	WN26	WN27
Colony size (mm)	2	2	1	1	2	1	1	3	1	3	2
Colony elevation	flat	raised	flat	flat	flat	flat	raised	raised	flat	raised	flat
Colony edge	entire	entire	entire	entire	entire	entire	entire	entire	entire	entire	entire
Pigmentation	bright-pink	red	pink	orange-red	faint-pink	pink	orange-red	orange-red	pink	faint-pink	red
Cell shape	coccoid	coccobacilli	pleomorphic	pleomorphic	coccoid	pleomorphic	pleomorphic	pleomorphic	coccoid	coccoid	rod
Motile	-	+	+	-	+	-	+	+	+		+
NaCl range for growth (%, w/v)	15-30	15-30	15-30	15-35	15-30	15-30	15-35	15-35	15-30	10-25	15-30
pH range for growth	8.5-11	8.5-11	8.5-11	8.5-11	8.5-11	8.5-11	8.5-12	8.5-11	8.5-11	7-10	8.5-11
Temperature range for growth (^{o}C)	22-50	30-55	30-55	30-55	22-50	30-55	30-55	30-55	22-50	22-55	30-50
Carbohydrates used											
Glucose	+	+	+	+	+	+	+	+	+	+	-
Fructose	-	-	-	+	-	+	-	-	-	+	-
Sucrose	+	+	-	+	+	+	+	-	+	+	-
Mannitol	-	-	-	+	-	-	-	-	-	+	-
Acetate	-	+	+	+	-	+	+	+	-	+	-
Gelatin hydrolysis	+	-	-	+	+	+	-	+	+	+	+
Amylase activity	-	-	+	+	+	-	-	++	+	+++	-
Protease activity	-	+	-	-	-	+	++	-	-	-	-
Tween 80 hydrolysis	++	++	-	-	-	++	++	-	-	+	-
Formation of indole	+	+	+	-	+	+	+	+	+	+	+
Reduction of nitrate	-	+	+++	+	-	++	+	+	+++	+++	+

samples are reported in Table 1. The studied lakes are highly alkaline and hypersaline, the pH were in the range from 8.7 to 9.03 for water sample of Lake Beidah and Lake Hamra, respectively, and EC were from 137.4 to 197.9 dS m^{-1} for sample of Lake Beidah and Lake Fazda, respectively.

Characterization of the isolates

All colonies that developed on solid media after three weeks of incubation at 37°C were about 1-5 mm in diameter, circular, entire and pigmented in different shades of red. Twenty nine morphologically distinct colonies were selected for phenotypic characteristics. Some colonies consisted of pleomorphic cells with different sizes. All isolates, except strain WN26, required at least 15% (w/v) NaCl and pH 8.5 for growth. Further information on the phenotypic characteristics of the biosurfactant-producer isolates is given in Table 2.

Screening and identification of biosurfactant-producing haloalkaliphiles

Twenty nine isolates were cultivated in liquid minimal growth medium supplemented with 0.2% petroleum oil and biosurfactant production was confirmed. Different reactions were noted for biodegradation of weathered crude oil (Figure 1). Only sixteen strains (named as shown in table 3) exhibiting haemolytic activity and emulsifying activity showed the positive result with drop collapsing test, emulsifying activity and oil displacement test (Table 3). From the results, WN23 and WN26 strains exhibited the highest activity for both oil displacement test against weathered crude oil (1.6 and 1.4 cm respectively) and emulsifying activity against Kerosene (55 and 45% respectively).

Properties of emulsions

WN23 and WN26 strains were selected

Table 3. Oil displacement and emulsification activity (expressed as mean value ± standard deviation), and drop collapsing test of cell-free supernatants of haloalkaliphilic strains isolated from Soda Lakes of Wadi An Natrun, Egypt

Strain	Testing methods		
	Oil displacement (cm)	Emulsification activity (%)	Drop collapsing
WN11	1.1±0.01	40±0.2	+
WN13	1.2±0.01	40±0.1	+
WN14	0.7±0.03	30±0.5	+
WN15	0.6±0.01	25±0.6	+
WN16	0.6±0.03	25±0.3	+
WN17	0.5±0.02	25±0.1	+
WN18	0.3±0.03	20±0.2	+
WN19	1.0±0.01	40±0.3	+
WN21	0.8±0.02	30±0.7	+
WN22	1.1±0.01	40±0.2	+
WN23	1.6±0.01	55±0.4	+
WN24	0.3±0.03	20±0.2	+
WN25	0.4±0.05	25±0.1	+
WN26	1.4±0.02	45±0.2	+
WN27	1.1±0.03	50±0.2	+
WN29	1.1±0.01	40±0.1	+

Fig. 1. Biodegradation extent of the crude oil after two weeks of incubation by strain WN23 (a), strain WN27 (b) and strain WN26 (c) in comparison with the negative control (d)

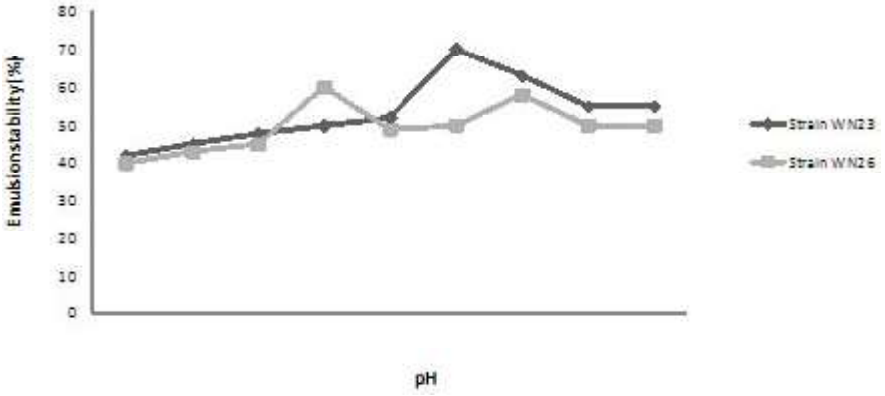

Fig. 2. Influence of pH on the stability of the emulsion formed by the strains WN23 and WN26. The emulsions were measured after 1 h of rest at room temperature

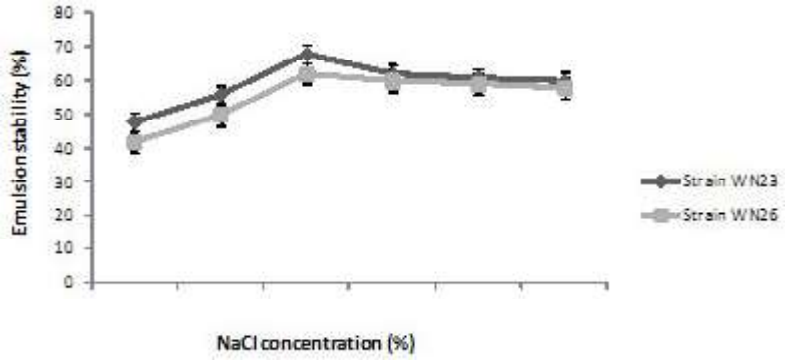

Fig. 3. Effect of different concentrations of salt (NaCl) on the stability of the emulsion formed by strains WN23 and WN26. The emulsions were measured after 48 h of rest at room temperature

to study the stability of the emulsions formed under various conditions. The emulsion stabilizing capacity of the two archaeal strains WN23 and WN26 is kept constant with a value of 55.2 and 45.8%, respectively. Figure 2 shows that the pH of the aqueous phase had little effect on the amount of diesel oil phase emulsified between pH 5 and 12. In the basic environments, it is clear that the strain WN23 has an emulsion stabilizing capacity that is more important than those in the neutral and acidic environment, with an optimum at pH 9. After 48 h at room temperature, the relative emulsion stability is equal to 100%. The strains WN26 shows the same result except that its highest emulsion-

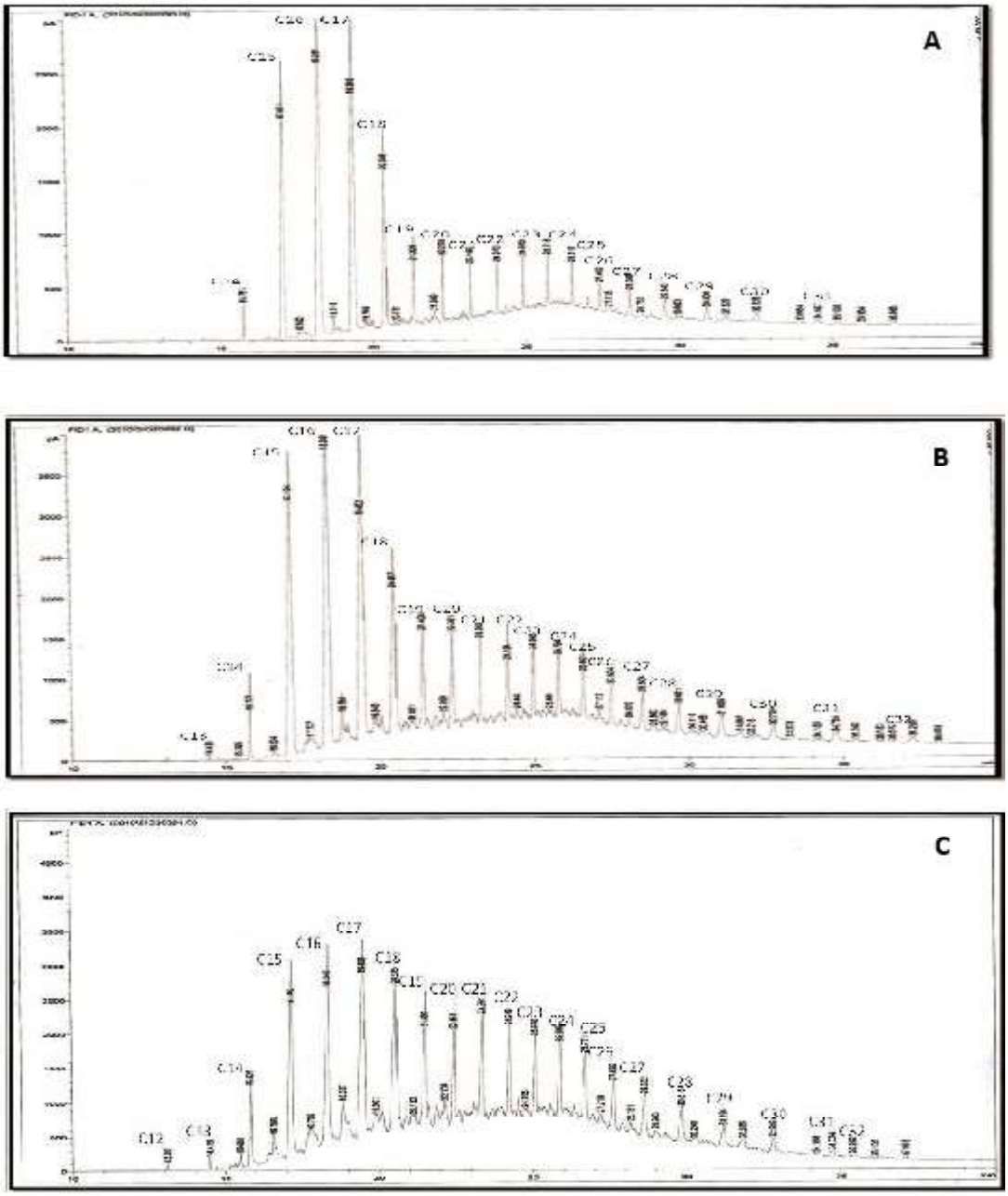

Fig. 4. Representative GC chromatograms of hydrocarbons extracted from liquid minimal growth medium supplemented with 0.2% crude oil and inoculated with strain WN23 (a) and strain WN26 (b) Negative control (c)

stabilizing capacity appears with neutral pH 7. The effect of the change in pH does not appear to affect the formed emulsions. As shown in Figure 3, in the presence of 10–35% (w/v) sodium chloride in the aqueous phase, stable and strong emulsions were formed by both strains WN23 and WN26.

Biodegradation of crude oil assay

As shown in Figure 4a, the saturated hydrocarbons are standing at equal distance above the baseline. The saturated hydrocarbons are homologous distributed covering the range of n-C12 to n-C32. GC-spectra of crude oil degraded by two strains WN23 and WN26 (Figures 4a and 4b, respectively) showed different pattern of crude oil, whereas C12 and C13 were completely disappeared by the action of strain WN23 (Figure 4a). GC-spectra of crude oil degraded by strain WN26 indicated the disappearance of C12 while distance of the range of C13 and C14 were considerably decreased than the reference chromatogram. Furthermore, there is a decrease in peak height for most aliphatic hydrocarbons particularly by the action of strain WN26.

Sequencing of 16S rRNA genes

Sixteen strains were selected for molecular identification. Isolates yielded PCR products of amplified 16S rRNA-gene-derived sequences with archaea-specific primers. The phylogenetic analysis performed by using the neighbour-joining method showed that twelve isolates were grouped with known species of the genera *Natronococcus*, *Natronolimnobius*, *Halorubrum* and with undescribed halophilic Archaea, with high similarities (e”98%) as shown in Figure 5.

Nucleotide sequence accession numbers

The 16S rRNA gene data of the twelve archaeal isolates WN11, WN13, WN14, WN16, WN17, WN18, WN23, WN24, WN25, WN26, WN27, and WN29 reported in this article have been deposited in the NCBI and GenBank nucleotide sequence databases under the accession numbers from HQ658992 to HQ659003, respectively.

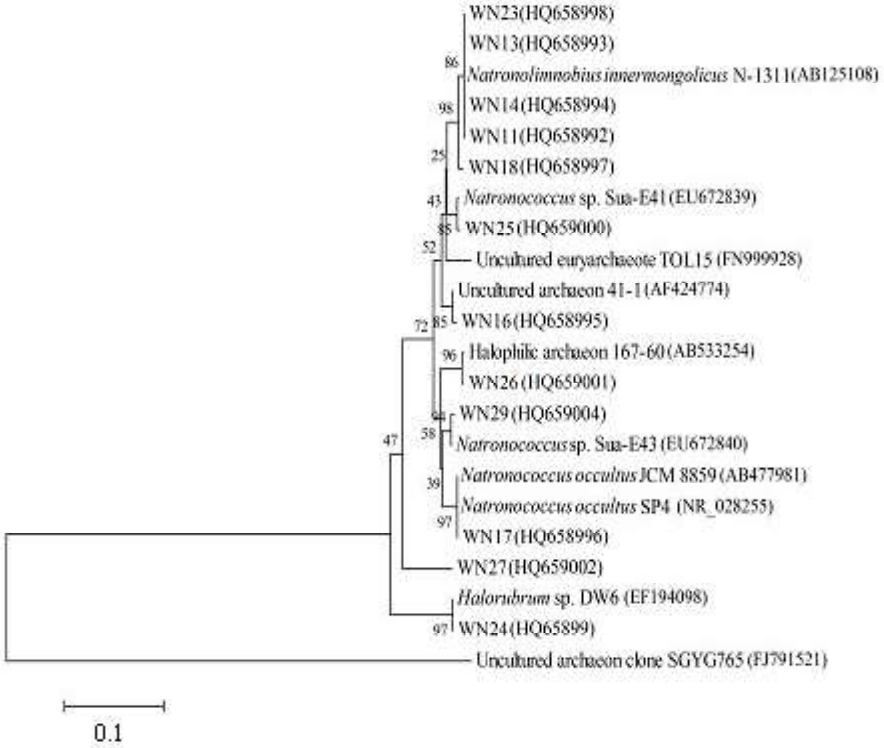

Fig. 5. Neighbor-joining tree (partial sequences ~950 bp) showing the phylogenetic relationships of archaeal 16S rRNA gene sequences of twelve isolated strains to closely related (S e” 98%) sequences from the GenBank database

DISCUSSION

The interest in biosurfactants has been increasing in recent years because of their diversity, environmentally friendly nature, prospect of large-scale production, and performance under extreme conditions. In this study, the screening for biosurfactant production under alkaline and hypersaline conditions is described for haloalkaliphilic archaeal strains. Twenty nine haloalkaliphilic isolates were used throughout this study, Nevertheless, only 16 strains exhibiting haemolytic activity and showed the positive result with drop collapsing test, emulsifying activity and oil displacement test. These strains were selected for identification by phenotypic characteristics and by partial 16S rRNA gene sequencing. From the results, only two strains (WN23 and WN26) are recognized as better biosurfactant producers using the qualitative drop-collapse test, oil displacement test, and the emulsification activity assay. The emulsion stabilizing capacity of these two archaeal strains WN23 and WN26 is kept constant with a value of 55.2 and 45.8%, respectively. The results indicated that the strains with higher emulsifying activity toward crude oil showed the greater oil displacement activity and emulsification activity, as reported for various marine bacteria[22].

Biosurfactant crude extract produced by strains WN23 and WN26 was stable in a broad pH range (5-12), and up to 35% NaCl (v/v). The effect of the change in pH did not appear to affect the emulsion stability. These results are in agreement with Kebbouche-Gana *et al.* [9]. However, strain WN23, *Natronolimnobius innermongolicus*, has higher emulsion stabilizing capacity in alkaline conditions. Furthermore, GC-spectra of crude oil degraded by strains WN23 and WN26 showed different and significant pattern, whereas, the ranges C12, C13 and C14 were completely disappeared or considerably decreased by the action of these two strains. Moreover, most of aliphatic hydrocarbons were degraded and this was confirmed by GC-analysis.

CONCLUSION

In this study, sixteen biosurfactant-producing haloalkaliphilic archaea isolated from different Soda Lakes of Wadi An Natrun, Egypt, were able to emulsify crude oil. The mode of hydrocarbon uptake is realized by the production of a biosurfactant which enhances the solubility of hydrocarbons and renders them more accessible for biodegradation. This mode has been demonstrated until now for oilfield bacteria[27] and for *Archaea* isolated from hypersaline environments[9]. The high stability of the emulsions formed under alkaline and hypersaline conditions may be valuable in various industries. Therefore, there is an increasing interest in the possible use of these biosurfactants in mobilizing heavy crude oil, oil pollution control, cleaning oil sludge from oil storage facilities, oil/sand bioremediation and enhancing microbial oil recovery not only in saline but also in saline and alkaline conditions.

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