Egypt. J. Microbiol. **45**, pp. 1- 13 (2010)

Biosurfactant Producing Bacteria from Oil Contaminated Egyptian Soil

E. M. Ramadan, Zeinb M. Kheiralla[*], M. A. Foaad[**], T. S. EL-Tayeb and Eman Z. Goma[**]

Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima; [*]Department of Microbiology, Faculty of Woman for Arts, Science & Education, Ain Shams University, Heliopolis and [**]Department of Biological Sciences & Geology, Faculty of Education, Ain Shams University, Heliopolis, Cairo, Egypt.

TEN PURE bacterial isolates with biosurfactant activity were obtained from autochthonous microflora of oil contaminated soil. Soil samples were selected from bus garage stations in Cairo and from different plots around oil wells of western desert of Egypt. Biosurfactant production was investigated using three parameters (hydrolyses of blood, surface tension reduction and emulsification index techniques). The biosurfactant accumulation was coincided with growth. By means of standard bacterial identification procedures, the isolates were identified as different strains of *Bacillus licheniformis*. The phylogenetic relationships between these strains were determined using the RAPD-PCR technique. The similarity between the strains was ranging from 78 to 100 %.

Keywords: Biosurfactant, Emulsification, *Bacillus licheniformis*, RAPD-PCR.

Pollution of sewage with oil spills from car stations and from oil used as fuels in factories has been a big problem in recent years. Microorganisms synthesize a wide variety of high and low-molecular-mass surfactants (Rosenberg & Ron, 1997; Richter *et al*., 1998 and Rahman *et al*., 2002). Microbial surfactants are a diverse group of surface-active molecules with potential commercial applications in the detergent industry (Rosenberg & Ron, 1998, 1999), in formulations of herbicides and pesticides (Rubinovitz *et al.,* 1982 and Patel & Gopinathan, 1986), in the petroleum, pharmaceutical and food processing industries. Potentially they can be manufactured from renewable and cheaper substrates (Maier & Soberon-Chavez, 2000; La Duc *et al.,* 2004 and Cameotra & Makkar, 2004).

Biosurfactants have unique amphipathic properties derived from their complex structures, which have both hydrophilic and hydrophobic domains. The lipopeptides and glycoproteins are interesting classes of microbial surfactants (Cameotra & Makkar, 1998 and Walzer *et al*., 2006) because of their manifold attractive properties. Biosurfactants produced by hydrocarbon-degrading

Contact authors e-mails*: tarekeltayeb@yahoo.com, mhamedfoaad@yahoo.com*

microorganisms can emulsify hydrocarbon-water mixtures, which enables them to grow on the oil droplets. These emulsification properties have also been demonstrated to enhance hydrocarbon degradation in the environment, making them potential tools for oil spill pollution-control (Banat, 1995). Biosurfactants are used for soil washing or flushing due to their ability to mobilize contaminants (Lin, 1996). They can be potentially as effective as highly used synthetic surfactants and they have some distinct advantages, including high specificity, biodegradability and biocompatibility (Kanga *et al*., 1997).

Genomic fingerprinting assays using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) had been detected as excellent methodologies and are fast and sensitive means for identifying small differences between similar complex genomes. Molecular genetic markers have been developed into powerful tools to analyze genetic relationships and genetic diversity. Main advantages of the RAPD-PCR technology include (i) Suitability for work on anonymous genomes, (ii) Applicability to problems where only limited quantities of DNA are available, (iii) Efficiency and low expense (Hadrys *et al*., 1992).

The present work isolated local bacterial biosurfactant producing strains from oil contaminated soil, examined their production of biosurfactant and assessed the similarity between them depending on the genomic differences.

Materials and Methods

Isolation of biosurfactant-producing bacteria

The bacterial strains were isolated from microflora present in oil slurry-contaminated soil from five different bus garage stations in Cairo and from different plots around oil wells of western desert of Egypt according to the procedure of Van Elsas & Smalla (1997). Five grams of each soil sample were placed into 250 ml Erlenmeyer flasks containing 50 ml of sterilized tap water and incubated at 30°C ± 2°C on a shaker at 150 rpm for 21 days. On days 3, 7, 14 and 21, a sample from each soil slurry was serially diluted, plated on PYG agar (Rocha *et al.*, 1992) and incubated for 3 days. After incubation, different slimy, yellowish and white individual colonies were repeatedly sub-cultured on PYG agar medium to obtain single isolates. The developed pure colonies on the plates were then maintained on slants of the same medium for biosurfactant production screening. Purified culture slants were kept at 4^{o}C and transferred at regular intervals.

Growth conditions for biosurfactant production

For biosurfactant production, bacterial isolates were grown aerobically on minimal salt medium (Deziel *et al.*, 1996) with the following composition (g/l): $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; KH_2PO_4, 1; K_2HPO_4, 1; $(NH_4)_2SO_4$, 1; $FeCl_3$, 0.05; pH, 7.0. Crude oil (the sole carbon source) was added to the sterilized medium at 0.5% v/v. Crude oil was obtained from Ennpi Petroleum Company, Nasr City, Cairo, Egypt. Twenty ml of the standard inoculum ($5x10^7$ cell / ml) were introduced into triplicate sets of 2l Erlenmeyer flasks containing 800 ml of medium with shaking at 200 rpm in a shaking incubator at 30°C±2°C.

Screening of biosurfactant-producing bacteria

Thirty different isolates were tested for their ability to produce biosurfactant according to the conditions described before. The preliminary investigation of biosurfactant production was detected after 12 days of incubation by using three parameters: the formation of a clear zone on blood agar plates (Morikawa *et al*., 1993), surface tension reduction (Verma *et al.,* 2006) and by the emulsification index technique (Patel & Desai, 1997).

Identification of bacteria

The bacterial cultures which gave the highest biosurfactant activity were identified by the API 50CH kit system (BioMérieux, Nürtingen, Germany) and analyzed by applying APILAB PLUS 3.3.3 software (Waldeck *et al.*, 2006).

Time course of biomass production and biosurfactant accumulation

The most efficient biosurfactant-producing strains were grown under the conditions described before. Dry biomass weight was used for monitoring the growth of bacteria according to the method described by Sifour *et al*. (2005). The bacterial growth and emulsion index % as the indicator of biosurfactant production (Verma *et al.,* 2006) were determined throughout 12 days of incubation.

DNA extraction and RAPD-PCR analysis

One loop of each bacterial isolate was inoculated into 50 ml of nutrient broth PYG medium in 250 ml Erlenmeyer flasks and incubated at 30^{o}C with shaking at 150 rpm for 48 hr. After incubation, cells were separated by centrifugation at 10,000×*g* for 30 min. One gram fresh biomass of each bacterial strain was used for DNA extraction as described by Dellaporta *et al.* (1983). Five random primers were applied for PCR reaction according to Williams *et al.* (1990). List of primers and their corresponding nucleotide sequences were as the following: OP-11, 5′-GAC AGG AGG T -3′;OP-13, 5′-GTC AGA GTC C -3′; OP-14, 5′-AGC ATG GCT C -3′; OP-15, 5′-TGG CGT CCT T -3′; OP-17, 5′-GGC TTA TGC C -3′. Twelve µl of DNA amplified product of each positive producer strain were loaded on each well and run at 75 voltage for about (45-75) min at room temperature. The bands were scanned using Gel Doc-2001 Bio-Rad system. The gels of DNA were visualized and photographed by gel documentation system (Gel-Doc Bio-Rad 2000) under UV transilluminator. RAPD patterns were calculated according to the method of Sambrook *et al.* (1989) by using RAPD distance software package, version 1.04 (Armstrong *et al.*, 1994).

Results and discussion

Isolation, identification and screening of biosurfactant-producing bacteria

Different oil-contaminated soil samples with different degrees of oil contamination were collected in order to isolate biosurfactant-producing bacteria. Thirty different bacterial colonies were isolated from soil samples polluted with oil. The preliminary investigation showed that only ten isolates produced relatively active biosurfactant on mineral basal medium containing 0.5% v/v crude oil, detected by the formation of clear zone on blood agar, by measuring the emulsion index and surface tension reduction assays (Table 1). The biosurfactant-producing isolates were

identified as different strains of *Bacillus licheniformis.* The strains that showed high hemolytic activity reduced by good means the surface tension of the medium comparing with the control (uninoculated medium). *B. licheniformis* str. No. 4, 5 and 6 were preceding in most of the biosurfactant production markers. The surface tension of the medium was reduced from 63.85 to 50.31, 47.08 and 49.02 mN/m by *B. licheniformis* str. No. 4, 5 and 6, respectively. Correlation coefficient at $p<0.05$ indicated a moderate positive correlation (substantial relationship) between the growth of the strains and hemolytic activity (0.478). In addition, high positive correlations (marked relationship) between the growth and emulsion index (0.858) was recorded. However, a high negative correlation (-0.866) was recorded between the growth of the strains and surface tension measurements. It means that the hemolytic and emulsification activities were enhanced as the growth increased, while the surface tension decreased.

TABLE 1. Screening of different strains of *B. licheniformis* for growth and biosurfactant production after 12 days of incubation at 30°C ± 2°C.

Bacterial strains	Cell dry weight (g/l)	Biosurfactant production		
		Hemolytic clear zone (cm)	Surface tension (mN/m)	Emulsion Index (E_{24}%)
Bacillus licheniformis str. No. 1	0.164±0.00	2.8±0.10	55.12±0.05	1.52±0.03
Bacillus licheniformis str. No. 2	0.203±0.01	2.7±0.15	55.33±0.01	1.82±0.03
Bacillus licheniformis str. No. 3	0.196±0.00	3.2±0.16	55.35±0.03	1.85±0.02
Bacillus licheniformis str. No. 4	0.852±0.03	3.3±0.20	50.31±0.02	8.33±1.82
Bacillus licheniformis str. No. 5	0.641±0.04	4.4±0.39	47.08±0.04	12.72±1.95
Bacillus licheniformis str. No. 6	0.590±0.07	3.8±0.13	49.02±0.04	11.16±0.79
Bacillus licheniformis str. No. 7	0.266±0.01	1.5±0.11	55.40±0.06	1.87±0.02
Bacillus licheniformis str. No. 8	0.310±0.01	2.5±0.06	54.90±0.01	3.05±0.062
Bacillus licheniformis str. No. 9	0.357±0.00	3.2±0.53	53.53±0.02	5.10±0.67
Bacillus licheniformis str. No. 10	0.284±0.01	2.7±0.24	54.22±0.00	3.75±0.02

Results are expressed as the means ± standard error of three replicates.

The spreading of bacteria in the soil contaminated with oil has been explained on the basis of the adaptation of the strains to the oil contaminated soil/liquid environment (Sugiura *et al.*, 1997 and Rahman *et al.*, 2003). Furthermore, Ijah & Antai (2003) reported that *Bacillus* spp. were predominant among the crude oil utilizing bacteria in soil contaminated with 30–40% crude oil. They also found that, among five strains compared, *Bacillus* sp. was most effective. Likewise, Ghazali *et al.* (2004) postulated that *Bacillus* spp. were more tolerant to high levels of hydrocarbons in soil owing to their resistant endospores. In this work, thirty different bacterial colonies were isolated from oil-contaminated soil samples with different degrees of oil contamination. The choice of localities for soil sampling was because the microbial communities in these contaminated samples are expected to produce

biosurfactant (Lin, 1996). The hemolytic activity was regarded by many authors as an indicative biomarker for the biosurfactant production and used as a rapid method for bacterial screening (Banat, 1995 and Lin, 1996). In a similar study, Rismani *et al.* (2006) used such method to detect the biosurfactant production by *B. licheniformis* isolated from the Persian Gulf. Although the hemolytic activity has been reported as an initial criterion for biosurfactant producers, other more conclusive tests such as surface tension measurement were carried out for confirmation of the obtained results. The reduction of surface tension may be due to the biosurfactant availability. It was reported that the growth of microorganisms on hydrocarbon containing medium is limited by a decrease of interfacial tension and increase in the degree of medium emulsification as a critical factor (Palittapongarnpim *et al.,* 1998). The differences in emulsifying activity of the isolated *B. licheniformis* strains may be related to the hypothesis of that a minor variation in biosurfactant isoforms produced by these strains might result in a large variation of the emulsification property (Das & Mukherjee, 2007).

Time course of biomass production and biosurfactant accumulation

This experiment was carried out to monitor the growth and biosurfactant biosynthesis by the most efficient strains of *B. licheniformis* throughout 12 days of incubation period. Chosen bacterial strains proved to grow on oil slurry in addition to produce the biosurfactant which was strain dependent (Fig. 1, 2). *B. licheniformis* str. No. 4, 5 and 6 grew slowly during the twelve days of incubation. The most growth (0.929 g/l) was obtained after eight days of incubation by *B. licheniformis* str. No. 4. The most growth of *B. licheniformis* str. No. 5 and 6 were 0.729 and 0.698 g/l, respectively after 9 days. These results were consistent with biosurfactant production. *B. licheniformis* str. No. 4 and 6 attained their highest emulsification indexes after 7 days of incubation (8.48%, 11.5%, respectively), while, *B. licheniformis* str. No. 5 attained its highest emulsification index (15.12%) after 9 days of incubation.

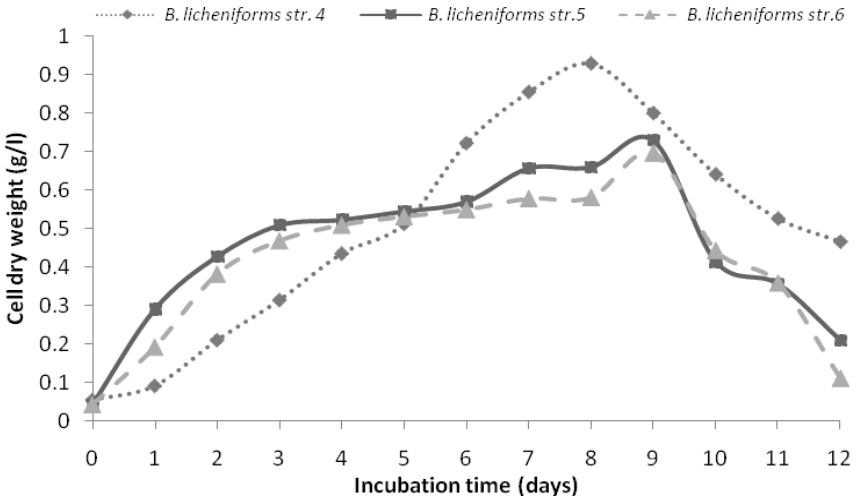


Fig. 1. Time course of biomass production by the most biosurfactant-producing strains of *B. licheniformis.*

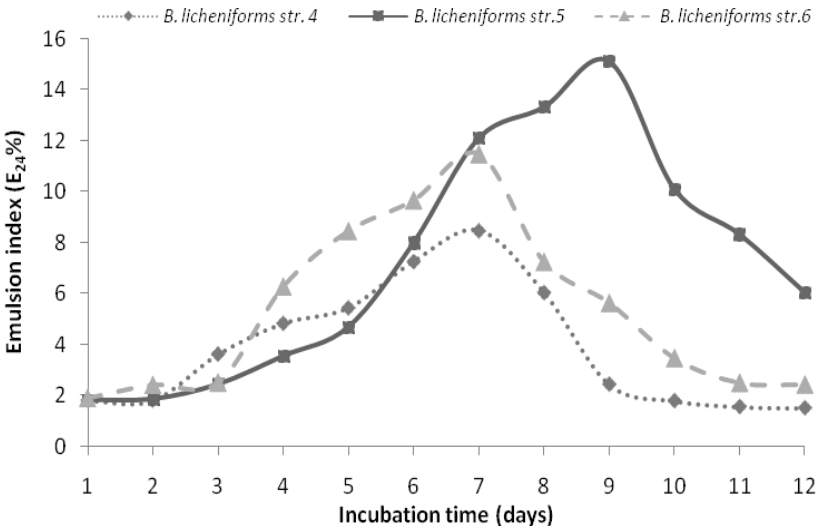
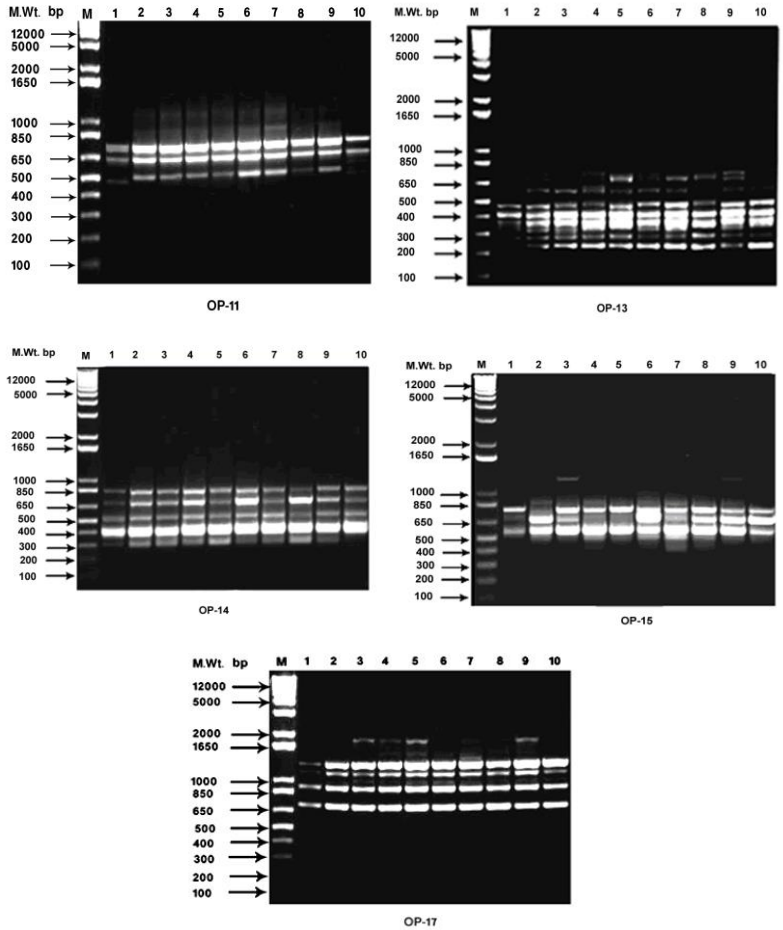


Fig. 2. Time course of biosurfactant accumulation by the most biosurfactant-producing strains of *B. licheniformis.*

RAPD-PCR analysis

The RAPD-PCR of *B. licheniformis* strains produced bands which ranged in sizes of 236 to 1284 bp. The results in Fig. 3 and Table 2 showed that the number of amplified fragments differed with different primers. Also, the number and sizes of amplified fragments differed from one strain to another for the same primer. The number of bands for each primer varied from 3 to 9. The results of primer OP-11 indicated the production of two monomorphic bands with 729 and 626 bp and one polymorphic band (shared) with 500 bp that present in all strains except isolate No.10. Primer OP-13 resulted in the appearance of four monomorphic bands with 477, 410, 365 and 287 bp. Five polymorphic bands were scored. One of them was genotype specific band of isolate No. 9 with 745 bp. The results of primer OP-14 indicated the presence of two monomorphic bands (with 853 and 430 bp) and five polymorphic bands. Two bands of 717 and 558 bp were present in all strains except No. 1, 2 and 3. On the other hand, two bands with 688 and 526 bp were present only in isolate No. 1, 2 and 3 and absent in other strains. Only band with 329 bp was present in all strains except isolate No.10. There are two monomorphic bands (with 786 and 566 bp) and only one polymorphic band (with 696 bp which was present in all strains except No. 5) was scored in primer OP-15. Primer OP-17 resulted in the production of only four monomorphic bands with 1284, 1122, 892 and 685 bp. No polymorphic bands were observed using this primer. The results presented in Table 3 indicated that a total number of twenty six amplified fragments were obtained, out of which fourteen were monomorphic bands, eleven were shared bands and only one unique band was presented by using primer OP-13. The similarity between the ten strains of *B. licheniformis* was ranging from 78 to 100 % (Table 4). Clustering of all strains was determined and the results of phylophenetic tree were given in Fig 4. The data indicated the presence of two major related clusters (A, B). Cluster A included *B.*

licheniformis str. No. 1, 2 and 3 and cluster B included *B. licheniformis* str. No. 4, 5, 6, 7, 8, 9 and 10. Cluster A contained two sub clusters. *B. licheniformis* str. No.1 placed in one sub cluster with a similarity of 90.5 and 92.7% to *B. licheniformis* str. No.2 and No. 3, respectively. The other sub cluster included *B. licheniformis* str. No. 2 and 3 with a similarity degree of 97.8 %. Cluster B contained two sub clusters. *B. licheniformis* strain No.10 fell in one sub cluster with similarity of 90 % to *B. licheniformis* str. No. 4 & 6; and an 87.2 % with *B. licheniformis* str. No. 5, 7; and 92.3 & 87.8 % with *B. licheniformis* str. No. 8 & 9, respectively. The other sub cluster contained *B. licheniformis* str. No. 4, 5, 6, 7, 8 and 9. The similarity between *B. licheniformis* str. No. 4 and 6 reached 100%. The degree of similarity was comparatively low among most of the strains, showing that there is a large degree of heterogeneity among some of these local strains.



M=marker **1-10=** *B. licheniformis* strains

Fig. 3. 1.2% agarose gel electrophoresis show DNA polymorphisms of RAPD-PCR analysis of ten *B. licheniformis* strains (Lanes 1-10) using OP-11, OP-13, OP-14, OP-15 and OP-17 primers.

TABLE 2. DNA polymorphism of *B. licheniformis* strains amplified with OP-11, OP-13, OP-14, OP-15 and OP-17 primers.

Primer's name	No.	M. Size (bp)	*Bacillus licheniformis* strains									
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
OP-11	1	729	1	1	1	1	1	1	1	1	1	1
	2	626	1	1	1	1	1	1	1	1	1	1
	3	500	1	1	1	1	1	1	1	1	1	0
	Total		3	3	3	3	3	3	3	3	3	2
OP-13	1	745	0	0	0	0	0	0	0	0	1	0
	2	689	0	1	0	1	1	1	1	1	1	0
	3	589	0	1	1	1	1	1	1	0	1	0
	4	477	1	1	1	1	1	1	1	1	1	1
	5	410	1	1	1	1	1	1	1	1	1	1
	6	365	1	1	1	1	1	1	1	1	1	1
	7	326	0	1	1	0	0	0	0	0	0	0
	8	287	1	1	1	1	1	1	1	1	1	1
	9	236	0	1	1	1	1	1	1	1	1	1
	Total		4	8	7	7	7	7	7	6	8	5
OP-14	1	853	1	1	1	1	1	1	1	1	1	1
	2	717	0	0	0	1	1	1	1	1	1	1
	3	688	1	1	1	0	0	0	0	0	0	0
	4	558	0	0	0	1	1	1	1	1	1	1
	5	526	1	1	1	0	0	0	0	0	0	0
	6	430	1	1	1	1	1	1	1	1	1	1
	7	329	1	1	1	1	1	1	1	1	1	0
	Total		5	5	5	5	5	5	5	5	5	4
OP-15	1	786	1	1	1	1	1	1	1	1	1	1
	2	696	1	1	1	1	0	1	1	1	1	1
	3	566	1	1	1	1	1	1	1	1	1	1
	Total		3	3	3	3	2	3	3	3	3	3
OP-17	1	1284	1	1	1	1	1	1	1	1	1	1
	2	1122	1	1	1	1	1	1	1	1	1	1
	3	892	1	1	1	1	1	1	1	1	1	1
	4	685	1	1	1	1	1	1	1	1	1	1
	Total		4	4	4	4	4	4	4	4	4	4
Total No. of bands			19	23	22	22	21	22	22	21	23	18

TABLE 3. Number and types of amplified DNA bands as well as the percentage of the total polymorphism generated by five primers of *B. licheniformis* strains.

Primer code	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism %
		Shared bands	Unique bands		
OP-11	2	1	-	3	33.3%
OP-13	4	4	1	9	55.5%
OP-14	2	5	-	7	71.4%
OP-15	2	1	-	3	33.3%
OP-17	4	-	-	4	0%
Total No. of bands	14	11	1	26	-

TABLE 4. Percentage of phylogenetic relationship between *B. licheniformis* strains based on RAPD-PCR analysis using 5 primers.

***Bacillus licheniformis* strains**	**str. No. 1**	**str. No. 2**	**str. No. 3**	**str. No. 4**	**str. No. 5**	**str. No. 6**	**str. No. 7**	**str. No. 8**	**str. No. 9**	**str. No. 10**
str. No. 1	100									
str. No. 2	90.5	100								
str. No. 3	92.7	97.8	100							
str. No. 4	82.9	88.9	86.4	100						
str. No. 5	80	86.4	83.7	97.7	100					
str. No. 6	82.9	88.9	86.4	100	97.7	100				
str. No. 7	80	86.4	83.7	97.7	95.2	97.7	100			
str. No. 8	85	86.4	83.7	97.7	95.2	97.7	95.2	100		
str. No. 9	81	87	84.4	97.8	95.5	97.8	95.5	95.5	100	
str. No. 10	81.1	78	80	90	87.2	90	87.2	92.3	87.8	100

Conclusions

The present work confirmed the relationship between the contamination with crude oil and enumeration of the soil with biosurfactant-producing bacteria. The isolated bacteria were able to produce biosurfactant on mineral basal medium containing 0.5% v/v crude oil. The bacterial isolates were identified as different strains of *B. licheniformis*. The similarity between the strains was ranged from 78 to 100 %. Further investigations will be carried out to optimize either the nutritional and environmental factors to enhance the production of biosurfactant.

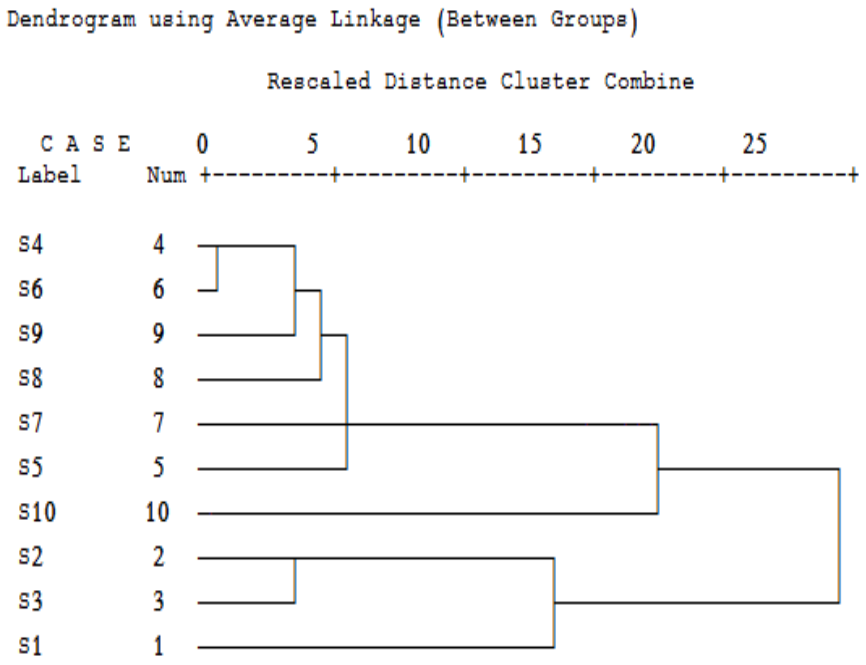


Fig. 4. Phylogenetic tree of *B. licheniformis* strains based on RAPD-PCR analysis using five random primers and by using SPSS13 analysis program, (The number denoted from 1-10 refers to the number of strain).

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(*Received* 31/12/2009;
accepted 28/2/2010)

بكتريا انتاج مخفضات التوتر السطحى الحيوية من التربة المصرية الملوثة بزيت البترول

الشحات محمد رمضان ، زينب محمد خير الله* ، محمد عبد العزيز فؤاد ، طارق سعيد الطيب و ايمان زكريا جمعة****

قسم الميكروبيولوجيا الزراعية - كلية الزراعة - جامعة عين شمس ، *قسم الميكروبيولوجيا - كلية البنات للاداب والعلوم والتربية - جامعة عين شمس و** قسم العلوم البيولوجية والجيولوجية - كلية التربية - جامعة عين شمس – القاهرة – مصر .

إن مخفضات التوتر السطحى الحيوية هى جزيئات يتم تخليقها بواسطة الكائنات الدقيقة. هذه المركبات لها مميزات عديدة عن مخفضات التوتر السطحى الكيميائية وهى ذات أهمية تجارية حيث لها نواحى تطبيقية عديدة فى البيئة. البحث عن بدائلِ أرخص لإنتاجِ مخفضات التوتر السطحى الحيوية جَذبَ العديد من الدِراساتَ الأخيرةَ . تم عَزل البكتيريا المنتجة لمخفضات التوتر السطحى الحيوية من عينات تربة ملوثة بزيت البترول بدرجات مختلفة ، باستخدام وسط الأملاح المعدنية المحتوية على زيت البترول الخام كمصدر وحيد للكربون والطاقة. اختيرت عينات تربةٍ مِنْ محطاتِ مرآب الحافلات في القاهرة ومِنْ مواقع مختلفةٍ حول آبارِ نفط الصحراءِ الغربيةِ بمصر. تم عزل عشَر مزارع بكتيرية نقية منتجة لمخفضات التوتر السطحى الحيوية من التربة الملوثة بزيت البترول. و بعد اجراء خطوات تعريفِ البكتيريا تم الحصول على سلالات مختلفة من *الباسيلس ليشينيفورمز*. تم عمل مسح للبكتيريا المنتجة لمخفضات التوتر السطحى الحيوية اعتمادا على قياس القدرة على تحلل الدم وانخفاض التوتر السطحى والقدرة الاستحلابية. تم الحصول على أعلى نمو وكذلك أعلى انتاجية لمخفضات التوتر السطحى الحيوية بواسطة سلالات *باسيلس ليشينيفورمز* رقم ٦،٥،٤. تم استخدام تقنية تحليل مكونات الحمض النووى (دى ان ايه) لتحديد درجة القرابة بين سلالات *باسيلس ليشينيفورمز* المعزولة .