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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(4):3533-3544. **OPEN ACCESS**

Isolation and identification of biosurfactants producing bacteria capable of degrading Topsin from Hydrocarbon burned oil

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This research was conducted to investigate the isolation of biosurfactants producing bacteria having the ability to degrade Topsin from hydrocarbon burned oil. Two segregates were distinguished as *Bacillus cereus* strain AM1260 and *Serratia marcescens* strain NBRC 102204 by comparing its 16S rRNA sequence with those available in GATC Center in Germany. The strains were selected due to their capacities to create extracellular products (biosurfactants) able to reduce surface tension. To prove the potency of these isolates to produce biosurfactant, a screening method including emulsification was applied. The emulsification index E24 was 65%.significance and impact of study: Results demonstrated that the biosurfactant activity was steady at elevated temperature with wide domain of pH

Keywords: Biosurfactents, Zea mays, *Bacillus, Serratria*, E24

INTRODUCTION

Wide kinds of microorganisms can produce biosurfactants that are amphiphilic surface active agents containing hydrophilic and hydrophobic moieties. These biosurfactants are able to lower surface and interfacial tensions through their accumulation at the interface between two non-mixable fluids such as water and oil (Nitschke and Coast, 2007). They own a much structural diversity, ranging from glycolipids, lipoproteins and lipopeptides to neutral lipids, fatty acids, phospholipids, polymeric and particulate biosurfactants. Different microorganisms are capableof producing biosurfactant eg. *Pseudomonas aerogenosa, Bacillus subtilis, Brevibacterium brevis, Bacillus licheniformis, Candida bombicola, Candida apicola, Arthrobacter species* and *Acinetobacter species* but this biosurfactant producing ability get affected by carbon, nitrogen source used in medium, salinity, temperature, pH, pressure etc. (Rahman et al., 2002). According to a classification proposed by Neu 1996, the term "biosurfactants" should be correctly used to identify low-molecular-weight microbial surfactants (Neu, 1996).

In general, these are glycolipids, such as trehalose lipids, rhamnolipids, phsopholipids and fructose lipids, or lipopeptides, such as polymixin, gramicidin S and surfactin. In contrast, high-molecular-weight polymers can be collectively defined as bioemulsifiers (Rosenberg and Ron, 1997 and Smyth et al., 2010a).In point of fact, bioemulsifiers contain molecules that efficiently lower surface and interfacial tension, however the latest consists of amphiphilic and polyphilic polymers which are more influential, in stabilizing oil-in-water emulsions but not reducing the surface tension as much.

The highmolecular-weight bioemulsifiers are amphiphilic or polyphilic polysaccharides,

proteins, lipopolysaccharides and lipoproteins (Smyth et al., 2010b). Over the previous years, biosurfactants have been investigated substitutes for synthetic surfactants and are expected to have many potential industrial and environmental usage attached to foaming, emulsification, wetting, detergency, dispersion and solubilization of hydrophobic component, composition, synthesis (Luna et al., 2013).

Among the various potential substitutes for synthetic surfactants, biosurfactants, bioemulsifiers and exopolysaccharides attract because of their constitutional and Functional dissimilarity (Sousa and Bhosle, 2012). Some explorations have demonstrated that surface activity of biosurfactants is comparable to surface activity of synthetic surfactants.

As a result of their physicochemical properties, low toxicity, excellent surface activity, high specificity, effectiveness under exaggerated circumstance and biodegradability, bioemulsifiers and biosurfactants are excessively applied in environmental protection techniques, e.g., water, oil spill removal and soil remediation, etc. (Xu et al., 2011). The latter properties of bioemulsifiers and biosurfactants mirror their potential for commercial applications (Sivapathasekaran et al., 2010).

Biosurfactants produced microorganisms can be successfully applied to remove heavy metals and hydrocarbons (Lee et al., 2007). Because it is known that biosurfactants stimulate bioavailability and perform biodegradation of nonpolar components, various technologies, such as clean up combined technology and soil washing technology, use biosurfactants for the effective disposal of metals and hydrocarbons, respectively (Liu et al., 2010).

The biosurfactants can be exceedingly used in agricultural areas to promote the biodegradation of pollutants to enhance the goodness of agriculture soil, for indirect plant propagation advancement through antimicrobial activity and to increase the plant-microbe relationship that benefit plants (Sachdev and Cameotra, 2013). Pesticide pollution is counted as one of the important problems about polluted land.

Concerns about pesticide pollution have led to global efforts to find substitutioal biological control techniques. New investigations have revealed that a surfactant perhaps utilized to promote the processes that convert the pollutants into more accessible form for the microorganisms. These studies focused on the application of surfactant technology for environmental remediation have on the dual solubilization and biodegradation of HOC's (hydrophobic organic compounds).

The main concern with the utilization of biosurfactants instead of chemical surfactants is that the values of production, which can be kept low by choosing economical strains of microorganisms that produce biosurfactant, thereby optimizing the surroundings constituent and using various cheap renewable substrates. The main purpose of the current study is to isolate microbial strains from 2 fuel oil station at Giza, Egypt through enrichment culture technique. The isolates were capable of producing biosurfactants and biodegrading fungicide Topsin.

MATERIALS AND METHODS

Isolation of microorganisms

Samples of burned oil were obtained from 2 fuel station at Giza, Egypt. Six- fold serial dilution was done, pour plate technique was utilized for isolation; One (1ml) of 10^{-6} dilution was inoculated on sterile Petri dishes, after which the nutrient agar medium was poured aseptically on the inoculated plates. The plates were incubated at 30˚C for 24hours.

Morphologically various colonies saw on the plates after incubation, were subculture on a supplemented agar plates to culture of the pure colonies then moved into a nutrient agar slants. The slants were preserved in the fridge at 4°C as stock culture to maintain these microorganisms. 40 microorganisms were separated from the obtained oil samples. Just five isolates were chosen to be characterized.

Characterization and Identification of isolates

Pure cultures of the isolates were set depend on their cultural, morphological characteristics according to the taxonomic scheme of Barrow and Feltham (1993) and reference to Holt et al. (1994). The tests performed include Gram stain, spore form, motility test and identified by comparing its 16S rRNA sequence with those available in GATC center in Germany (Saitou and Nei, 1987).

Biosurfactant test

Just five isolates were given pronounced results for biosurfactant test and only two were the most efficient isolates in biosurfactants production.

Screening for biosurfactant and bioemulsifier production

Isolates were cultivated on the richness medium including burned or pure oil as the sole

carbon source at 30°C for 7days by using the richness conditions. The emulsification index (E24) were determined, as described by Cooper and Goldenberg, 1987, by adding 5 ml of the cultures to test tubes filled with 5 ml of the hydrocarbons (crude oil), homogenized by vortex at highly velocity lasts for 2min and let to stand for 24 h. The E24 was determined as the height percentage of the emulsified layer cm divided by the total height of the liquid column (cm). Emulsification activity was estimated by E24 using Eq. (E24% = HEL/HS x 100%) where, HEL is the emulsified layer height cm and HS is the total solution height (Smyth et al., 2010a).

Effect of temperature and pH on isolates emulsification production

The selective two isolates were mature in nourishing stock medium at 30°C, 40°C to test the best temperature of growth. The cells in nourishing stock medium were removed by centrifugation and supernatant was used to calculate the emulsification index (E24%). For testing the suitable pH, the isolates were mature in liquid nourishing stock which was adjusted at different pH values at 5, 6, 7, and 8 and incubated for 3 days at 30°C.

Study the impact of several pesticides on the growth of isolates

The impact of three pesticides types such as Topsin, Rizoliex and Ridomil on the isolates growth was investigated by adding different doses of pesticides 0.5, 1.0, 1.5, 2 and 2.5 g/l to nutrient agar medium in Petri dishes. Then these petri dishes incubated at 30°C for 48 h.

In order to study the biodegradation of Topsin by selected isolates, the bacterial inoculum was prepared from stock cultures nutrient broth kept at -20°C, which were transferred to plates including nutrient agar and incubated at 30°C for 24 hours. With the aid of a platinum handle, an aliquot containing approximately 10^6 CFU ml^{-1} bacterial suspensions was transferred to 15 ml Falcon tubes containing 3 ml of nutrient broth and were incubated at 30°C and under agitation (250 rpm) for 4 hours. The resultant culture was centrifuged (1792 rcf) for 10 minutes.

The supernatant was throw away and the cell pellet suspended with mineral salts medium (MMS). Bacterial biomass was diluted in MMS to obtain O.D. equal to 1 absorbance at 610 nm, which corresponds to a population of 10^6 CFU ml^{-1}. In the biodegradation tests were used aliquots (1 ml) of bacterial inoculum suspension. In the consortium of selected bacteria, were utilized aliquots (500 µL) of each bacterial isolate (Santos et al.,2017).

Biodegradation test

Biodegradation test was done with the two finest strains selected after preliminary tests for evaluation of bacterial growth measured after 72 hours of culture by optical density. Concurrently, the degradation was quantified by high-performance liquid chromatography. The biodegradation experiment with each isolate was performed in triplicate. Aliquots (1 ml) of the bacterial suspension (O.D equal to 1) were inoculated in 125 ml Erlenmeyer flask containing MMS (50 ml) and S00 mg L^{-1} Topsin (97% purity, Sigma-Aldrich) diluted in dimethylsulfoxide (DMSO) and incubated at 30°Cunder agitation (125 rpm) for 21 days. The positive control group (MMS only and bacteria) was used to monitor the bacterium viability.

Extraction of Topsin

The extraction of Topsin was performed using ethyl acetate. To each Erlenmeyer flask, 50 mL of ethyl acetate was added. The material was carried to a 125 ml separatory funnel and undergoes a strong agitation for 1 minute. The organic layer was grouped and the aqueous layer was extracted two more time, by adding 50 ml of ethyl acetate.

Extraction end was observed by thin layer chromatography (TLC). In the collected organic phase, anhydrous sodium sulfate was added and after filtration the samples were undergo vacuum rotary evaporator at 40°C until reduction of the volume of the sample to approximately 10 ml. The sample was conveyed to 10 ml volumetric flask containing 10g ml^{-1} of an internal standard solution. The volume of 1µL of the sample was added to HPLC. (Santos et al., 2017)

Biodegradation data analysis

The chromatographic area of the analyte was corrected by extraction recovery rate, which was obtained by the formula: [(CAs/CAis] x 100, where CAis corresponds to the chromatographic area of the internal standard and the CAs for the chromatographic area of the treatment.

The Topsin concentration was obtained by a calibration curve using different Topsin concentrations: 100, 200, 300, 500, 700, 900 and 1200 mgL^{-1}. The Topsin degradation proportion was calculated by the formula: [(CC - CE)/CC] x 100, where CC is the Topsin concentration in the control group and CE the remaining concentration of Topsin from experiment. Finally, it was

calculated the average and the standard deviation of the biodegradation rate with a view to obtain greater accuracy of Topsin concentration (Santos et al., 2017).

HPLC analysis

HPLC analyses of Topsin was performed in a Shimadzu (CBM-20A) Prominence® UFLC system, coupled with high-pressure binary pump (LC-20AD); auto injector (SIL-20AHT), detector UV-Vis (SPD-20A); column oven heating; CTO-74. The column used was Phenomenex Luna C18 (250 mm x 4.6 mm, 5u). The Solvent A: ultrapure water/0.1% formic acid and the Solvent B: acetonitrile grade HPLC/0.1% formic acid. Flow: 1.0 ml min-1.

Gradient: 0-2 min, linear gradient from 60 to 85% B; 2-8 min, isocratic at 85% B; 8-9 min, linear gradient from 85 to 60% B. Wavelength set at 285 nm.

Greenhouse pot experiment

A greenhouse pot experiment was done to set particularly the effectiveness of the selected bacteria and Topsin fungicide on morphological growth of Zea mays cultivation next 30 days and 60 days. Sandy soil was mixed with compost and placed in a plastic pots (25 cm diameter and 30 cm depth) filled with 10 kg of soil. Shoot length, shoot fresh and dry weight, also root length, root fresh and dry weight were measured.

Identification of bacterial isolates by 16S rRNA gene sequencing

The16S rRNA gene fragments of the most effective 2 bacterial isolates were amplified using the universal primers 16s rRNA using PCR machine (Arktnk). The PCR products were examined by agarose gel electrophoresis then purified using gel extraction kit and sequenced by GATC (Germany).

Phylogenic analysis of bacterial isolates

The evolutionary history was derived using the Neighbor-Joining technique as represented by Saitou and Nei (1987). The tree was computed using the Maximum Composite Likelihood method.

The analysis concerned 18 nucleotide sequences of that 2 sequences of 16S rRNA gene amplified from bacterial isolates of current study whereas 16 sequences representing the most similar hits were obtained from the NCBI gene bank data base. Evolutionary analyses were dole out in MEGA5 software.

Statistical analysis

The recorded results and information were submitted to the proper statistical analysis for the complete randomized block design according to Snedecor and Cochran (1980). Least significant difference (L.S.D.) at 5% level of significance was applied to treatment means comparison. All statistical analysis was done using analysis of variance technique by means of (MSTAT) computer software package.

RESULTSAND DISCUSSION

Morphological characters of burned oil isolates

This process was done by using the streaking method to get pure cultures. Isolates K, H1, H2, M, and C were chosen to identified and characterized. Table (1) shows the morphological characteristics of these isolates.

Results showed that most of all isolates are G+ and have a turbid shape in the culture. In regard to cell shape, most of the cells are not spore formers. Colonies in culture plates are different in colors such as pink, pale yellow and beige or light red.

Emulsification % of five isolates against different incubation time

Figure (1) shows the influence of various incubation periods (24, 48 and 120 h) for the isolates of burned and pure oil on emulsification %.

As expected, while the incubation period of burned oil isolates emulsification increased, the improvement in emulsification % was seen and it continued till 120 h of incubation for most isolates. For the pure oil isolates, the results have not obvious trends. Noticeably, Isolates C and H2 record the high Emulsification %. So, these isolates will be studied. The lowest Emulsification % was obtained by K isolate for both burned and pure oil, also for all different incubation periods.

Emulsification % of two isolates against different temperature and time

Figure (2) illustrate the emulsification % of two chosen isolates C and H2 at different temperature 25-30 or 35-40°C and time, 24, 48 and 72 hrs.

Table (1): Morphological characters of isolates

Isolates Characters	K	H1	H2	M	C
Cell shape	Cocci	Cocci	Long rods	Short rods	Rods
	Single or in pairs	Single or conjugated	In chains	Single or in pairs	Single or Conjugated
	Non-spore formers	Non-spore formers, Capsulated	Spore formers	Non-spore formers, Capsulated	Non-spore Formers
Color on the culture	Pink	Pale yellow	Beige	Beige	Light red
Color on the Petri plate	Pink	Yellow	Off white	Dirty white (off-white)	Dark red
Gram stain	G+	G+	G+	G+	G-
Shape in the culture	Turbid	Turbid	Turbid	Turbid	Turbid
Shape in the Petri plate	Conjugated with uneven edges.	Single cells with uneven edges.	-conjugated with uneven edges.	Single or conjugated with uneven edges.	Conjugated with uneven light-colored edges.

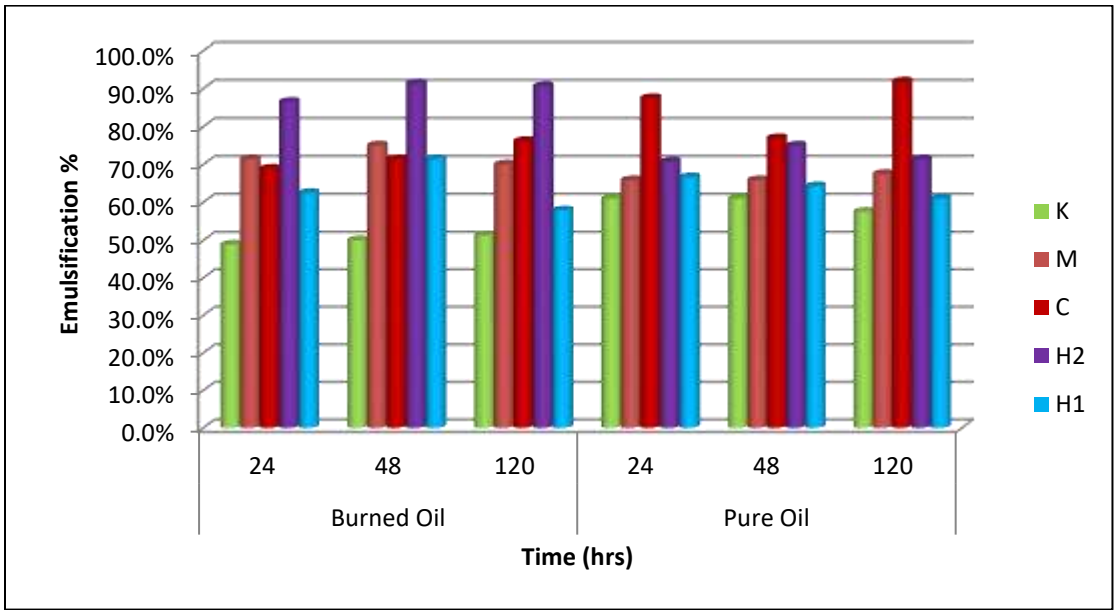

Figure 1; Emulsification % of five isolates against different incubation time for burned and pure oil

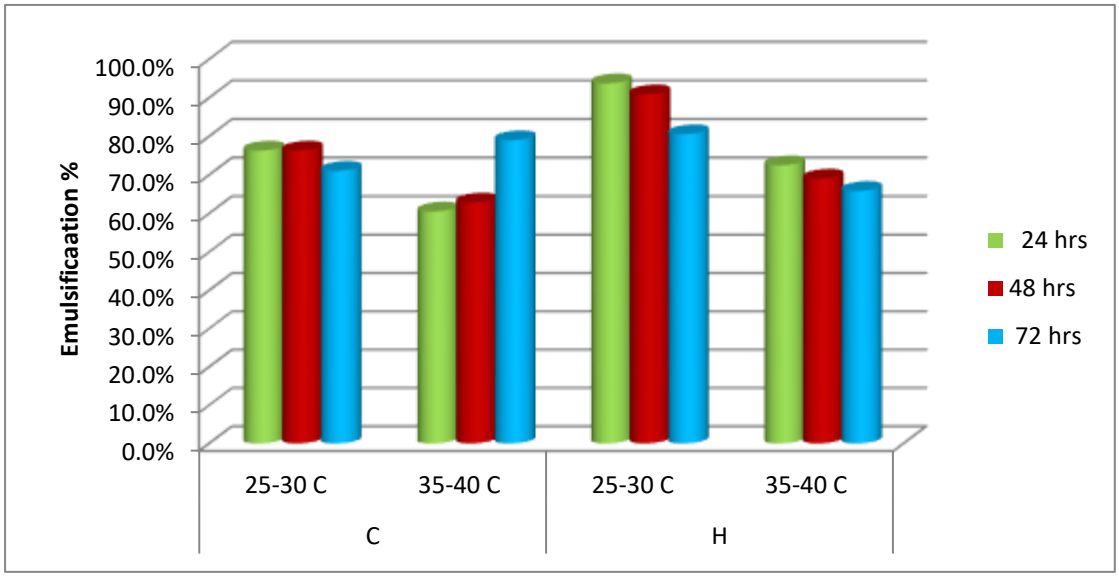

Figure 2 ; Impact of several temperatures and incubation periods on emulsification % of two isolates

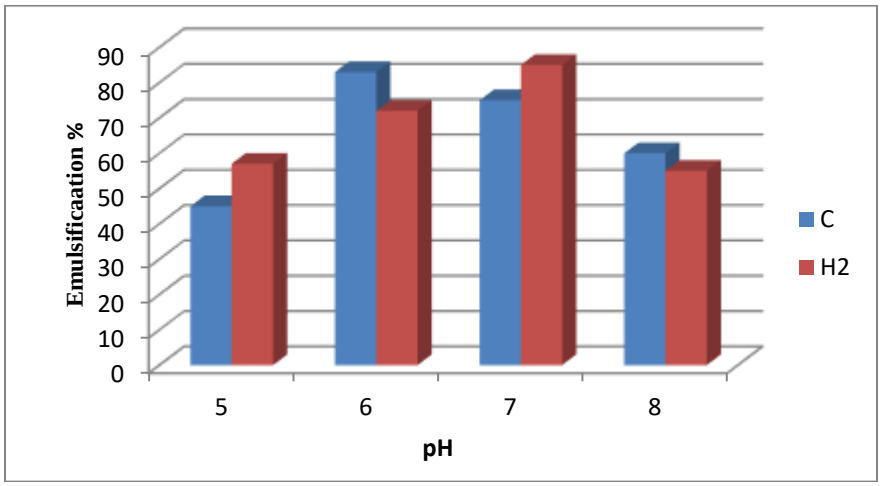

Figure 3 ; Impact of several pH on emulsification% of two isolates.

Obviously, it seems to be that 25-30°C is the best temperature to give the highest emulsification % to both isolates after 24 hrs of the incubation period. Emulsification %recorded about 70% and 90% for C and H2 isolates, respectively after 24 hrs. As generally, results showed that as the incubation period increased, % of emulsification decreased..(Kim et al.,1997) found that strains reduced surface tension in tested temperatures but the best temperature for selected strains was between 30- 40°C. Also Abu-Ruwaida et al.,1991 found that the optimum biosurfactant production of *Rhodococcus. sp* at 37ºC.Prasadet al., (2017) showed that the isolate, identified as *Pseudomonas aeruginosa*. showed optimum growth at pH 7.0 and temperature 37ºC.

PH effect on the emulsification % of selected isolates

Data in Figure (3) indicate that the best pH value on the emulsification of isolate C and H2 was 6 and 7, respectively. Kim et al., (1997) found that the surface tension reducing the activity of *Bacillus subtillisC9* was stable to pH over the range of pH of 5.0-9.5, where the reduction of surface tension indicates the production of surface-active compounds. Also Abu-Ruwida et al., (1991).observed biosurfactant production of

Rhodococcus was at 6.5-7.2 that determined by surface tension.

Effect of different pesticides on the growth of C and H2 isolates

Table (2) reveals that both selected isolate can be grown only on Topsin pesticide at different concentration 500, 1000, 1500, 2000, 2500 ppm. Clearly, as Topsin pesticide concentration decreased, the growth of these isolates increased. So, the use of 0.5 g/l was the best growth of used isolates C and H2.

Table (2): Impact of many pesticides on the two isolates growth

D1.500ppm; D2, 1000 ppm;D3, 1500ppm ; D4, 2000 ppm; D5, 2500 ppm

Effectiveness of varied Topsin concentration and C and H isolated on vegetative and root characters of mays

Data in Tables (3 & 4) show the effect of Topsin at different concentration on two selected isolates H2 and C at 30 and 60 days from cultivation on vegetative growth characteristics in this investigation, i.e., shoot length (cm), fresh and dry weight of shoot/plant (g), also on root length (cm), fresh and dry weight of root/plant (g). Table (3) illustrated that shoot length ranged between 59.4 and 66.3 cm. Use of Topsin either alone or in combination with isolate H2 or C gave significant increases in shoot length over control. From the mean values, the highest increase was 64.08 cm by the use of Topsin + isolate C, whereas it was 63.79 and 63.00 cm by Topsin + H2 and Topsin, respectively.

Regarding the influence of Topsin or Topsin + H2 or C, results revealed that shoot fresh and dry weight, also root length and fresh weight under this study, significantly increased by using of Topsin + C which represented 15.7g, 2.78 g for shoot fresh and dry weight, respectively and 56.19 cm and 9.48g for root length and root fresh weight, respectively. While this treatment give non-significant elevation in the previous parameters for the root dry weight.

With respect to the effectiveness of Topsin concentration, results obviously declared that the use of lowest concentration of Topsin, 0.5g/l led to the highest values of most of the parameters used by Topsin treatment or Topsin + H2 isolate. Whereas applying the recommended concentration of Topsin, 2.0 g/l resulted in the highest values of the shoot and root parameters.

Topsin + isolate H2 or C led to a significant rise in all used parameters after 60 days from cultivation(Table 4). Topsin without any isolate caused a significant decrease in most of the used parameters. The use of Topsin + C isolate gave the highest values of shoot length, fresh weight and dry weight also root length, fresh weight, and dry weight, where these values represented 98.28 cm, 36.19 g, 10.13 g, 60.79 cm, 10.21 g and 3.82 g, respectively. While these values of control were 90.2 cm, 27.16 g, 6.08 g, 51.6 cm, 9.63 g and 3.06 g, respectively.

Regarding the effectiveness of Topsin concentration on these parameters, data in Table 3 and 4 shows that applying the lowest concentration, 0.5 g/l caused the highest values of most parameters as only Topsin was used. While the use of the recommended dose of Topsin, 2.0 g/l gave the biggest values of most parameters when Topsin was used with H2 or C isolate. This result is due to the biodegradation of this fungicide in this study, as shown in Table (5), to nutrient sources which can use in plant growth. As regard to this point, Many studies have been performed to investigate the plant uptake of hydrophobic compounds like polychlorinated biphenyls, dioxins, furanes and polycyclic aromatic hydrocarbons from soil (Sawhney and Hankin, 1984; Webber et al., 1994) and O'Connor et al., (1990) from the greenhouse.

Isolate	Pesticide concentration ppm	Pesticide		
		Topsin	Rizoliex	Ridomil
C	D1*	++++	-	-
	D2	+++	-	-
	D3	++	-	-
	D4	++	-	-
	D5	++	-	-
H2	D1	++++	-	-
	D2	+++	-	-
	D3	+++	-	-
	D4	+++	-	-
	D5	++	-	-

Selective mineral salts medium (MMS)containing Topsin as a sole carbon source

Enabled the isolation of 2 kinds of bacteria (H2 and C).Figure (8) shows the familiar bacteria identified by rDNA sequencing. The predominant genera were H2 identified as *Bacillus cereus strain AM1260* and C identified as *Serratia marcescens* strain NBRC 102204 by comparing its 16S rRNA sequence with those available in GATC center in Germany. Among the identified isolates, the genus Bacillus (H2) was the most representative, with 99.74% removal after 3 days incubation. This removal decreased to 99.68% after 5 days. While, genus *Serratia* (C) was the

most representative, with 94.35% removal after 3 days incubation and increased up to 99.09% after 5days.(Table 5 and Figure 4,5,6 and 7).

Phylogenic tree of bacterial isolates

Based on the sequences of 16S rRNA gene (about 900 bp) a phylogenetic tree was constructed. The phylogenetic analysis showed that the two bacterial isolates shared a 16S rRNA sequence similarities and clustered in two different clusters. Members of cluster 1 consisted of *Bacillus cereus* group with the clustered hit to the strain IAM 12605.

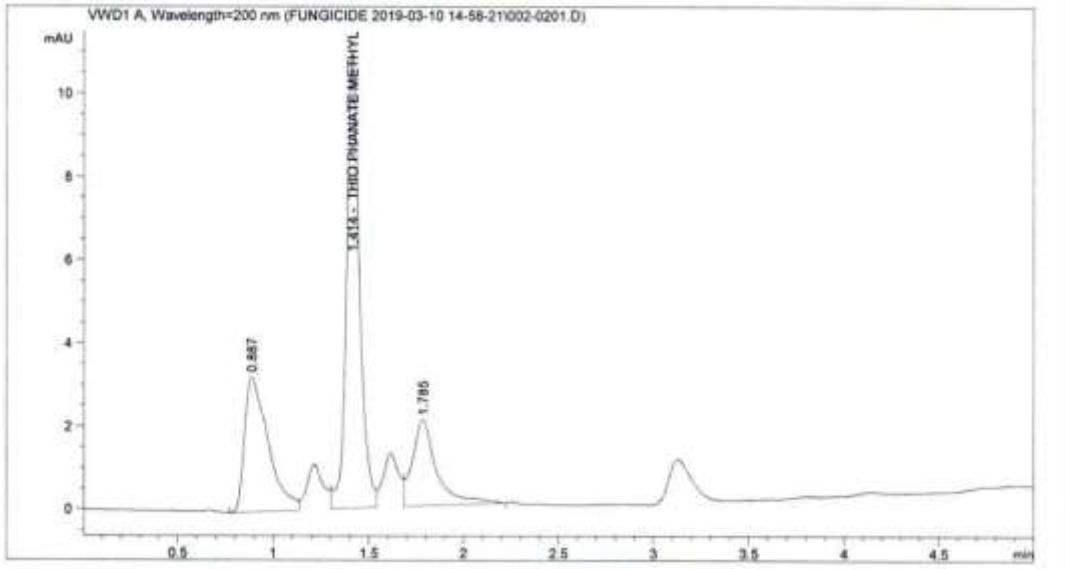

Figure 4; Results of biodegradation of Topsin by strain H2 after 3days incubation by HPLC.

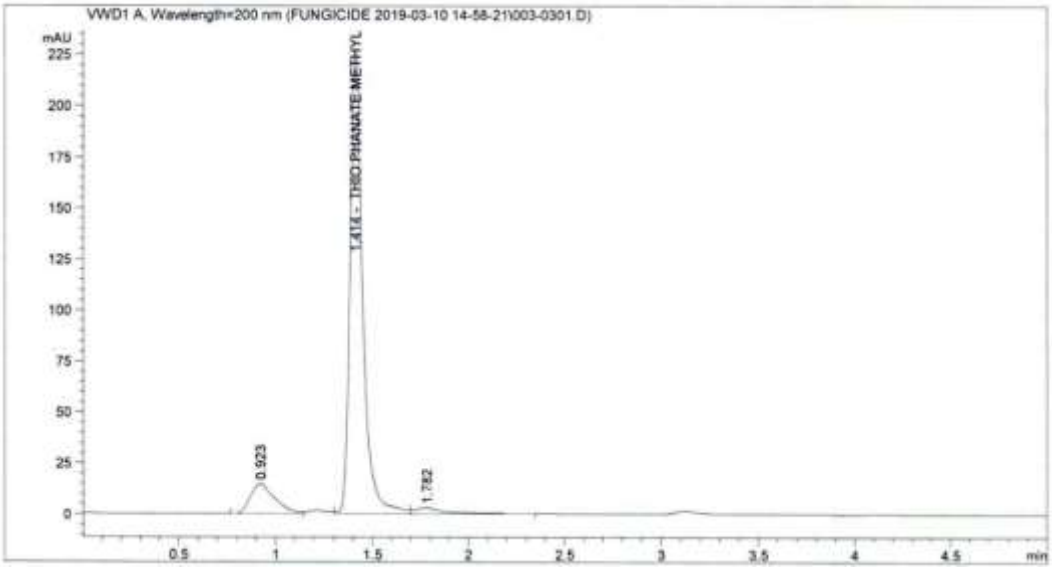

Figure 5; Results of biodegradation of Topsin by strain C after 3 days incubation by HPLC.

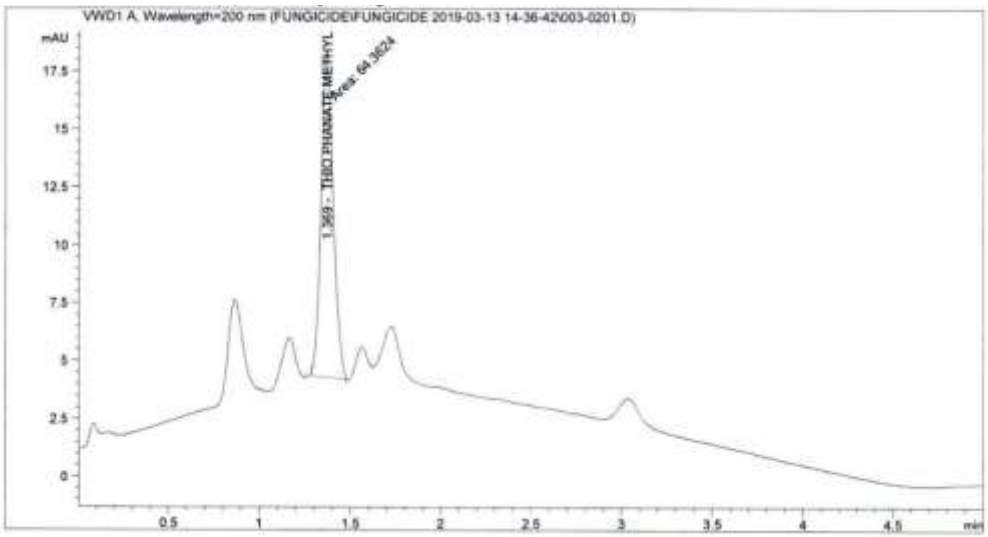

Figure 6; Results of biodegradation of Topsin by strain H2 after 5 days incubation by HPLC.

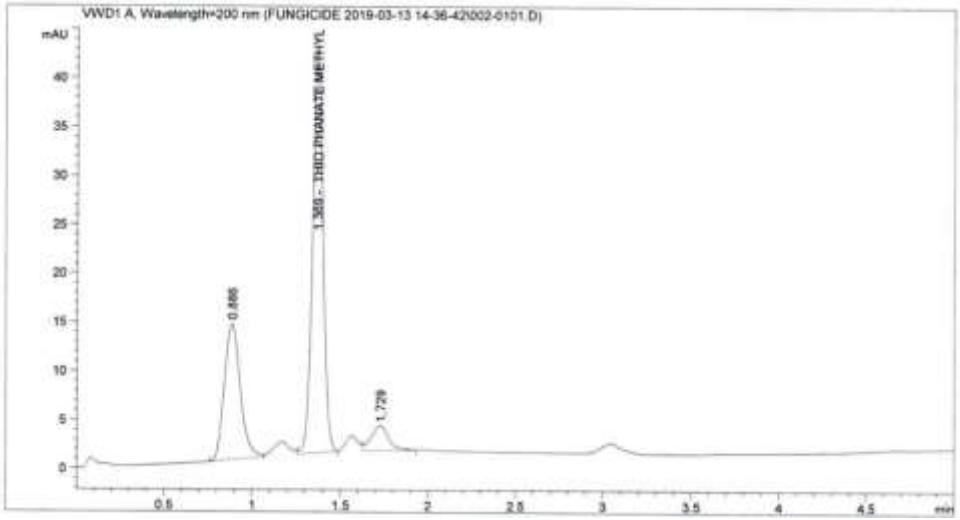

Figure 7; Results of biodegradation of Topsin by strain C after 5 days incubation by HPLC.

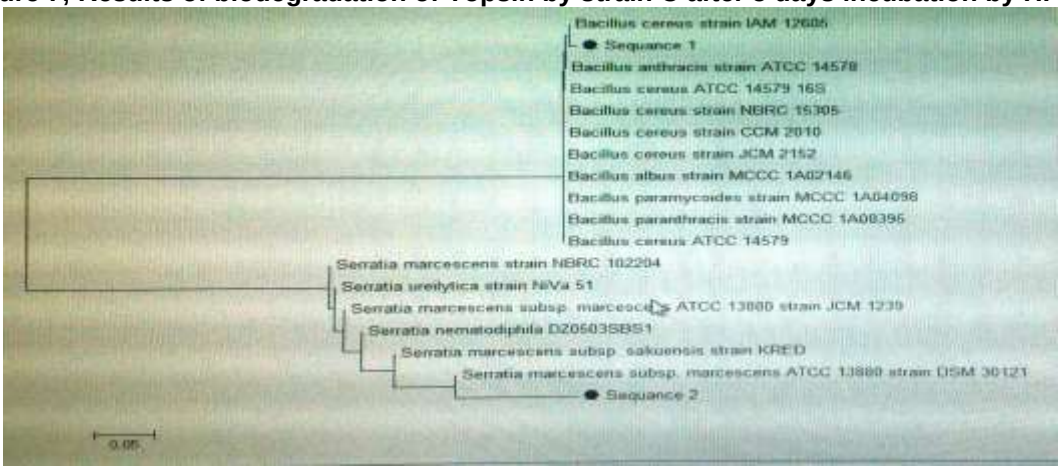

Figure 8; Aneighbor-joining phylogenetic tree based on approximately 900 bp segment of the 16S rRNA gene sequences of 2 bacterial isolates (Dark circles) with the closest hits obtained from the

NCBI gene bank. The sequences obtained in this study are highlighted in dark circles. The scale bar indicates a 5% divergence.

Table 3; Vegetation characteristics of Zea maize treated with several concentrations of pesticide after 30 days of cultivation.

Character Treatments	Topsin Concentration (ppm)	Shoot			Root		
		length	Fresh weight	dry weight	Length	fresh weight	dry weight
Control	--	60.50	13.08	2.07	51.9	9.26	1.19
	500	65.4	13.97	2.26	52.3	9.43	1.17
Topsin	1000	64,6	13.17	2.23	48.7	9.33	1.21
	1500	64.2	12.98	2.07	49.6	8.85	0.85
	2000	60.7	12.00	2.16	51.4	9.10	1.37
	2500	60.1	11.87	1.74	50.3	8.45	0.95
Mean		63.00	12.80	2.09	50.51	9.03	1.11
H+Topsin	500	65.7	14.40	3.13	53.5	9.42	1.74
	1000	64.5	13.56	2.60	53.1	9.22	1.43
	1500	65.4	13.18	2.46	52.8	9.65	1.29
	2000	63.7	13.20	2.34	52.8	9.50	1.33
	2500	59.4	12.40	2.22	48.2	8.34	1.26
Mean		63.79	13.35	2.55	52.13	9.23	1.41
C+ Topsin	500	66.2	15.87	2.88	57.9	9.43	1.84
	1000	64.4	15.78	2.74	56.7	9.55	1.83
	1500	63.2	15.64	2.46	54.4	9.32	1.41
	2000	66.3	16.80	3.05	58.3	10.28	1.91
	2500	60.2	14.42	2.78	53.4	8.83	1.35
Mean		64.08	15.70	2.78	56.19	9.48	1.67
LSD at 5%		1.10	1.39	0.49	1.39	0.76	n.s.

Table 4 ; Vegetation characteristics of Zea maize treated with varying concentrations of pesticide after 60 days of cultivation.

Character Treatments	Topsin Concentration (ppm)	Shoot			Root		
		Length	Fresh weight	Dry weight	Length	fresh weight	Dry weight
Control	--	90.2	27.16	6.08	51.6	9.63	3.06
Topsin	500	95.5	35.28	9.79	58.6	12.46	3.94
	1000	96.5	36.80	9.08	53.0	9.13	3.53
	1500	95.4	39.09	7.90	52.6	9.44	3.12
	2000	95.6	30.57	8.13	65.3	9.48	3.02
	2500	94.6	30.41	6.57	49.6	8.71	2.86
Mean		95.55	34.43	8.29	55.862	9.84	3.29
	500	98.3	41.33	10.39	58.0	10.88	4.45
	1000	95.0	34.11	9.41	57.3	9.57	4.21
	1500	93.6	32.52	9.74	57.3	9.29	3.64
	2000	102.6	44.35	10.62	58.6	11.33	3.10
	2500	93.1	26.6	8.73	55.3	7.31	2.95
Mean		96.56	35.78	9.78	57.33	9.68	3.67
C+ Topsin	500	97.6	33.87	10.02	62.6	10.99	3.82
	1000	99.0	37.33	10.67	62.0	9.96	3.62
	1500	95.5	36.07	9.89	59.3	8.45	3.27
	2000	108.6	40.82	10.19	63.0	12.17	4.51
	2500	90.6	32.84	9.87	57.0	9.48	3.86
Mean		98.28	36.19	10.13	60.798	10.21	3.82
LSD at 5%		8.49	4.13	1.70	3.31	1.76	0.45

Table 5 ; Results of biodegradation of Topsin by isolated strains.

Isolate	0 days	3 days	% Biodegradation	5 days	% Biodegradation
Control	500 ppm	500 ppm	0	500 ppm	0
H2	500 ppm	1.284 ppm	99.74	1.6 ppm	99.68
C	500 ppm	28.263 ppm	94.35	4.564 ppm	99.09

The member of cluster two were much more diverse however all assigned to the genus *Serratia.* The most similar hit was *Serratia marcescens* sub sp. *marcescens* ATCC 13880 (Figure 8)

CONCLUSION

Results demonstrated that the biosurfactant activity was steady at elevated temperature with wide domain of pH.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMEN

The authors would like to acknowledge Soil, Water and Environmental Research Institute A.R.C., Giza, Egypt for funding research project. Also acknowledgment extends to Professor Dr. Mohamed Z. Sedik at Department of Microbiology, Faculty of Agriculture, Cairo University for his assistance in our research project.

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