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ABSTRACT

The importance of this study is that it is deals with the Barada River pollution with hydrocarbons (crude oil and its derivatives), as well as evaluating the ability of different types of Micrococcus sp. to bioremediation of hydrocarbons. Ten strains of Micrococcus sp. were isolated from the water samples from Barada River in Damascus, and these isolated were classified depending on Bergey's manual to four species: M. luteus, M. lylae, M. flavus, and M. antarcticus. The crude oil dependent growths of these isolates were assessed for 15 days at 30°C by monitoring the gradient fluxes in pH, optical density OD and total viable count TVC in the medium. The result was statistically supported by applying the One-way ANOVA test and the Paired sample t-test. The results showed that activity of species was best at the concentration of 2% of crude oil, and the species of M. lylae was best in dealing with crude oil, although this superiority between species was not supported at the statistical studying. In general, the different species of Micrococcus sp. have a good ability to deal with the different concentrations (1, 2 and 3%) of crude oil. And this supports research which encourages the use of this genus in the bioremediation. This research was indicated to the ability of M. lylae to deal with different concentrations of crude oil, which was not mentioned in previous research

KEYWORDS

Hydrocarbons, Crude Oil, Micrococcus sp., Barada River, Biodegradation.

1. INTRODUCTION

Hydrocarbons pollution is one of the most significant contamination problems on the continent because it contains a lot of toxic compounds, which have a toxic effect on all organisms, especially in aquatic environments, as well as their ability to increase values biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solid (TTS), in addition to the petroleum contaminated water contains hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons and they are potentially toxic, mutagenic and carcinogenic, and they are fixed in the environment, so they are dangerous if not controlled [1-6]. Remediation of oil-contaminated system could be achieved by either physicochemical or biological methods. However, the attendant negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives, which can support ecosystems and not harm their living or non-living content [7-9]. The bioremediation processes have long been desired in the world because they would be cost effective and efficient in terms of acclimation time [9, 10].

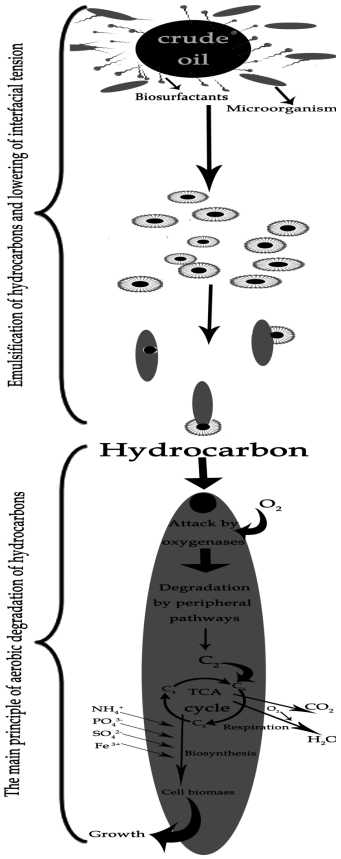


Figure 1: Role of biosurfactants and aerobic degradation of hydrocarbons

This method depends on microorganisms because they have many enzymatic systems that allow them to adapt in different limited conditions, which was confirmed by many research findings in the world [11-13]. *Micrococcus* sp. is one of that microorganisms which is widespread in the environment (water, soil, air, Antarctica, active sludge, human body, whey, roots of plants …) [14-16]. The spread of this genus makes it vulnerable to the development of enzymatic systems capable of dealing with different conditions in environments, as well as its ability to produce biosurfactant, which have important role in bioremediation of hydrocarbons, Figure 1 shows role of biosurfactants in emulsification of hydrocarbons and lowering of interfacial tension [17, 18].

Micrococcus sp. was used in bioremediation of many hazardous compounds such as dye, and pesticide, and 4-chlorobiphenyl [19-22]. Species of *Micrococcus* showed heavy metal resistance [23, 24]. And they are using gold as metal center in enzymes [25]. in addition to their ability to absorb thorium, as well as their capability to bioremediation of benzene, toluene, xylenes and naphthalene [26-30]. Researches did not study *Micrococcus* sp. in Syria despite its importance in bioremediation treatments, so this research came in an attempt to harness the vital processes of this genus in the biological treatment of hydrocarbons.

2. MATERIAL AND METHODS

2.1 Collection and preparation of samples

crude oil samples were collected from Banyas treatment station in Syria in sterile vials and transferred to a laboratory in Damascus University for further studies. Samples were mixed together then 300 ml of dilution was taken and subjected to different temperatures such as 20, 40, 50 and 60°C for 20 ± 2 minutes, centrifuged at 3500 revolutions per minute (rpm) for 15 minutes [31]. Sediments were deposed, and buoyant liquid was stored at 4°C for further use and studies.

2.2 Microorganisms

To isolate the *Micrococcus* genus, Furazolidone-Peptone (FP) medium was used [32]. Ten bacterial strains of *Micrococcus* sp. were isolated from Barada River which is contaminated with oil derivatives and other contaminants. These strains were found to have a good capability to degrade crude oil wastes; they were identified according to Bergey's manual and Rieser as *Micrococcus* sp. Strains code symbol Mb numbered from one to ten (M: from name of genus, b: from name of river) [15,33]. Pure bacterial colonies were transferred on Tryptic Soy Agar (TSA) (Avonchem. En) to determine the colonies morphology. Pure cultures were stored on nutrient agar slants at 4°C.

2.3 Inoculum preparation

A single colony of each of the isolates was inoculated into 10 ml of nutrient broth incubated at 30 Cº for 24 h, centrifuged for 15 minutes at 3500 rpm, the cell pellet was washed twice with Bushnell-Hass medium (containing of g/l: magnesium sulfate: 0.2, calcium chloride: 0.02, monopotassium phosphate: 1, diammonium hydrogen phosphate: 1, potassium nitrate: 1, ferric chloride: 0.05, pH 7.0 ± 0.2) until optical density at 600 nm (OD_{600}) was equivalent to 0.4 by using Spectrophotometer (Vis- 7220. UK) [3,34-36].

2.4 Detection of ability strains of *Micrococcus* to bioremediation of crude oil.

Two ml of bacterial inoculum (OD_{600} = 0.4) were transferred into tubes containing Bushnell-Hass medium (8 mL) supplemented with crude oil (1, 2 and 3% v/v) as the only carbon source [36]. They were incubated at 30°C at 150 rpm for 15 days [3,24]. A negative control was Bushnell-Hass medium and crude oil devoid of microorganism, and a positive control was

supplement with glucose (Bushnell-Hass medium, crude oil wastes, different concentration of glucose 1, 2 and 3% and microorganism) and incubated at the same conditions [24]. Measurements of the OD, pH and TVC were carried out at 0, 4, 8, 12 and 15 days interval [3]. From each tube 1 ml of broth was removed and serial dilutions ranging from 10^{-1} to 10^{-6} were prepared. Three plates from each dilution were inoculated and incubated at 30°C for 24 h, and then colony forming units were recorded.

2.5 Statistical analysis

All the samples were studied in three duplicates. Statistical tests (mean and standard deviation) and graphs were performed using MS Excel (2010) computer software program.

One-way ANOVA was performed for the parameters pH, OD and TVC for determination of the best species in degradation. Statistically significant differences between means were determined by LSD multiple range tests. Paired-sample t-test was carried out to determine if OD and TVC value which were raised after 15 days using the SPSS. 20 computer software programs were statistically significant or not. Those differences were determined at the $P < 0.05$ level.

3. RESULTS AND DISCUSSION

3.1 Isolation of *Micrococcus* species

Ten strains of *Micrococcus* sp. were isolated in Furazolidone-Peptone (FP) medium and were classified depending on many modern references and Bergey's manual, and according to the biochemical tests, to four species: *M. luteus*, *M. lylae*, *M. flavus* and *M. antarcticus* [15]. Table 1 shows strains and some of biochemical characteristics of *Micrococcus* species.

Table 1: Some of biochemical characteristics of Micrococcus species.

Identification characters	Mb1	Mb2	Mb3	Mb4	Mb5	Mb6	Mb7	Mb8	Mb9	Mb10
	M. luteus				*M. lylae*			*M. flavus*		*M. antarcticus*
Gram Staining	+	+	+	+	+	+	+	+	+	+
Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Motility	-	-	-	-	-	-	-	-	-	-
Oxidase test	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	+	-	-	+
Voges proskauer	-	-	-	-	-	-	-	-	-	+
Citrate utilization	+	+	-	-	+	-	+	-	-	+
H_2S production	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	-	+	+	+	+	+	+
Coagulase test	-	-	-	-	-	-	-	-	-	-
Hydrolysis of										
Urea	+	+	-	-	+	+	+	-	-	-
Starch	-	+	-	-	-	-	-	+	+	-
Gelatin	-	-	-	-	-	-	-	-	-	W
Carbohydrate fermentation test										
Glucose	+	W	-	-	+	+	+	-	-	-
Maltose	W	-	W	-	+	+	+	-	-	+
Sucrose	W	-	-	-	+	W	+	-	W	+
Mannitol	-	-	-	-	+	+	+	-	-	+
Galactose	-	-	-	-	-	-	+	-	-	+

Antibiotic sensitivity test										
Furazolidone 0.03% (w/v)	R	R	R	R	R	R	R	R	R	R
Oxolinic acid 2mcg	R	R	R	R	R	R	R	R	R	R
Gentamicin 10mcg	S	S	S	S	R	S	S	S	S	S
Tetracycline 30mcg	R	Nd	Nd	Nd	R	R	R	S	S	Nd
Amikacin 30mcg	S	S	S	S	S	S	S	R	S	S

+ = positive
- = negative
W = weakly
R = resistant
S = sensitive
Nd = not determined

3.2 Biodegradation of crude oil

The most degradation of the majority of organic contamination is brought about under aerobic conditions. Figure 1 shows the main principle of aerobic degradation of hydrocarbons. This study would help in understanding the role of bacteria –especially aerobic bacteria and heterotrophic– in biological treatment of hydrocarbons, which directly interact with the changing microenvironment. Through evolution, microorganisms have developed effective mechanisms that help to regulate their cellular function in response to changes in their environment. Biodegradation of crude oil by genus *Micrococcus* different species showed different results (Figure 2), but these differentiations were unpretentious and not statistically significant ($P > 0.05$) when One Way ANOVA test was applied to TVC values after 15 days of incubation, this difference can be attributed to the variation in their ability to withstand the accumulation of toxic compounds by bioremediation of crude oil, and this is referred to a studies [37, 38].

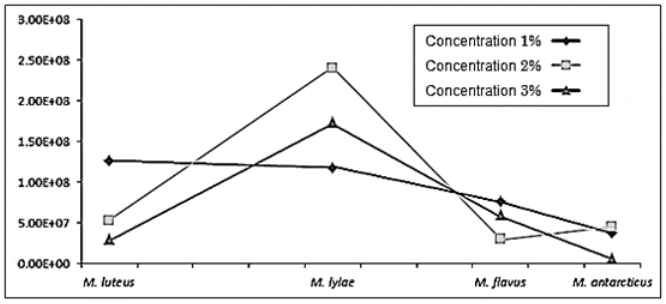
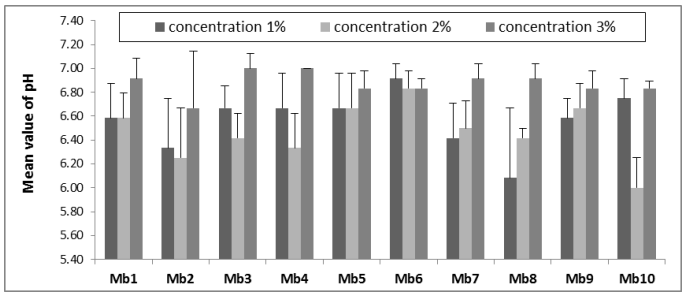
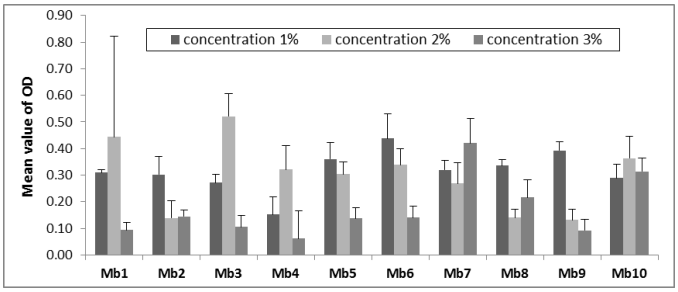


Figure 2: The mean values of TVC for different species in different concentration.

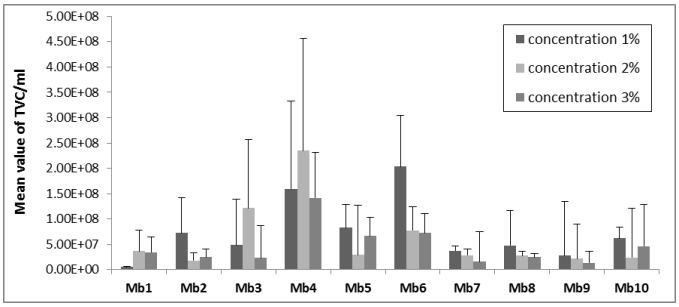
In addition, the genetic differences between the species which can degradation of hydrocarbon that may be at least 10%, especially in plasmids, which are the main responsible for resistance to antibiotic and other stress conditions, which was confirmed by [16, 28, 39]. In general, the ability of biodegradation was decreased with concentration increasing, as shown in Figure 3, which is compatible with results of Tazeena and his team in 2013, and this is expected because crude oil contains many toxic compounds [2, 40, 41], that pose a real hazard on all organisms, especially decomposers in water. When crude oil exists in high concentration means increase in insoluble substances, and increase in concentration heavy metals, all this has adverse effects on bioremediation of hydrocarbons [4, 23, 30, 42]. Results showed raise on TVC values with a raise in incubation period until the 12th day, which agrees with [3, 43].



3 (a) The mean value of pH different with increasing concentration of the crude oil.



3 (b) The mean value of OD different with increasing concentration of the crude oil.



3 (c) The mean value of TVC different with increasing concentration of the crude oil.

Figure 3: A different ability of study strains to biodegradation of crude oil.

However, TVC values receded in the 15th day to some strains, which is also compatible with some research [45-47]. This can be explained by a number of hypotheses. One of them: the toxic compounds which were formed have more toxicity from original compounds, which may be the reason for inhibition or stopping of bioremediation [2]. Another hypothesis suggests that the decrease in TVC can be attributed to the mineral nutrient lower in the medium with increased incubation time [34, 48]. When nutrients available to the microorganisms are depleted, they came in contact with the compounds that need longer time to break down, this reduces viable cell bacterial number because they need longer time to adapt with new substrate [40, 49, 50]. The statistical study showed that there is no statistically significant correlation between the means values of OD and TVC with the time in all the concentrations studied ($P > 0.05$), and no statistically significant correlation between the means values of OD with TVC.

However, both of the TVC and OD appeared to increase with time (Figure. 3), because they refer to biomass within the medium, and this is consistent with the results in [43, 51]. Therefore, the pH values showed decrease with time, and this is consistent with [3, 34, 47]. This can be explained by the composition of organic acids [34, 47]. However, the pH value showed a slight rise in concentration of 3%, and this was mentioned in some reference research [36, 52]. This can be explained by the ability of bacteria to develop different methods of metabolism of hydrocarbons, or as a result of an exceptional reaction by the bacteria when used high concentration of crude oil [36]. The Mb4 Strain of *M. lylae* was the most efficient at three concentrations, but this efficient was not statistically supported when applying One Way ANOVA test on average TVC values ($P > 0.05$).

The explanation of superiority one of strains *M. lylae* needs further study, because this species rarely exists in environments contaminated with crude oil. Unlike *M. lutes* which are common in these environments. However, a comparison of values of TVC to all strains after 15 days in concentrations 1%, 2% and 3% of crude oil by values of TVC for positive control after 15 days, it was observed that TVC was greater in positive control. By applying Paired-sample t-test to determine if the superiority was statistically significant, P-value was greater than 0.05. This can be explained by the fact that glucose runs out faster than other sources of

carbon like crude oil, which means it runs out in a matter of days. After 15 days, the glucose will be completely consumed by bacteria, and that reduced the number of TVC in medium, which was the reason for the differences between positive control and experiment of crude oil being not statistically significant.

4. CONCLUSION

The genus *Micrococcus* different species are widespread in the Barada River, which is indicative of the organic pollution in the River. The existence of *M. flavus* confirms the issue of river pollution by wastewater because it is usually isolated from activity sludge. Increasing in crude oil leads to a reduction in the efficiency of bacteria treatment process. Bioremediation ability of the *Micrococcus* species varies depending on the concentration. The statistical study did not support the superiority of species or strains, but it can be said that the Mb4 strain was the most efficient in bioremediation of crude oil in all concentrations. We can consider *Micrococcus* as one of the bacteria which can be used in the bioremediation of hydrocarbon pollutants, which can lead to further research on genus and identification of genes responsible for their adaptation to these pollutants.

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