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Evaluation of the ability of *Micrococcus* sp. isolated from Barada river to biodegradation of vegetable oil wastes

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Abstract

Fifty six strains of *Micrococcus* sp. were isolated from the water samples from Barada River in Damascus. Ten isolates of *Micrococcus* sp. species were taken for the present study for investigate biodegradation ability of vegetable oil wastes under the influence of this genus. The vegetable oil wastes dependent growths of these isolates were assessed for 15 days by monitoring the gradient fluxes in pH, optical density OD and total viable count TVC in the medium. Results showed an increase in OD and TVC as well as fluctuations in pH values. The relationship between these values was studied and determined if the relationships were statistically significant, and values were compared with the results in the positive control to the same strain. Biodegradation activity of different *Micrococcus* sp. species was tested in vegetable oil wastes which showed a good activity with 1% concentration of vegetable oil wastes, but decreased in 2% and 3% concentration. The genus *Micrococcus* sp. showed a good ability to grow in different concentrations of vegetable oil wastes, that refers to the ability of the genus to use vegetable oil wastes as the only carbon source.

Introduction

Oils have been in use by human since ancient times. They are usually extracted from plant seed. Many vegetable oils are used directly as ingredients in food. Thus production, use and transportation of vegetable oils is growing fast around the world. Tons of cooking oils are daily subjected to high temperatures (160 – 200°C) for relatively long periods of time, and as a result of that, oxidation of fatty acids and their subsequent transformations may cause unpleasant flavors and odors, which severely limits long-term repeated use (Cvengroš and Cvengrošova 2004). At the same time the frequency of chocking up of sewage lines because of oily wastes of vegetable origin generated from houses, canteens, restaurants, pharmaceutical industries and various other industries, and when poured down drains, they result in coating and eventual occlusion of drainage and sewage pipes which leads to increase in foul smell and environmental problems, which has serious effects on terrestrial and aquatic environments (Takeno *et al*., 2005).

The wastes also reduce light penetration and the ability of algae to produce food and oxygen (Bala *et al*., 2014). Oil contamination is one of the pollution factors known today, because it has toxic and persistent components in terrestrial and aquatic environments, which endanger the environment if not controlled (Bharathi *et al*., 2012). For example in China, more than 10 million tons of wasted frying oils are dumped to the sewage every year (Zhang *et al*., 2009).

Several physical- chemical methods of decontaminating the environment have been established and employed but they are usually expensive and require intensive labor; however, biological degradation is a safe, effective and an economic alternative method. Biodegradation refers to site restoration through the removal of organic contaminants by microorganisms, which aim to obtain efficient products capable of minimally affecting the environment. Effective biodegradation requires an appropriate population of degraders that have adequate tolerance to environmental changes, and the environment should be conducive to potential activation microorganisms (Pandey and Jain, 2002; Abu- baker *et al*., 2003; Graciela *et al*., 2011).

Vegetable oil biodegradation occurs in a similar way both in the presence or absence of oxygen, through hydrolysis of triglycerides and beta oxidation of released fatty acids (Domĭnguez *et al*., 2010). If complete biodegradation takes place under aerobic conditions, the organic carbon of the material will be converted to carbon dioxide and water, whereas under anaerobic conditions biogas results, which is a mixture of carbon dioxide and methane. The organic carbon of the material is in both cases also partly

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converted to new biomass (Mistriotis and Briassoulis, 2014).

Common *Micrococcus* species are positive gram and oxidase aerobic cocci, non-motile and non-spore forming, present in soil, water, dust, Activated sludge, and the skin of humans and animals (Goodfellow *et al*., 2012); it is recognized that *Micrococcus* species can be representation of the genus play important roles in the biodegradation of xenobiotics, bioremediation processes, production of biotechnologically important enzymes or bioactive compounds, cells usually are provided of plasmids conferring antibiotic resistance, the ability to degrade aromatic compounds, and which confer putative advantageous capabilities, such as antibiotic or heavy metal resistances, and has ability to utilize pyridine, herbicides, di-n-butyl phthalate and oil (Santhini *et al*., 2009; Dib *et al*., 2013; Hu *et al*., 2015). This study was the first in Syria, which was interested in genus *Micrococcus* and aimed to evaluate ability of *Micrococcus* sp. isolated from Barada River to biodegradation of vegetable oil wastes.

Material and Methods

Collection and preparation of samples

Vegetable oil wastes samples were collected from many houses, canteens, and restaurants in Damascus 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 15-20 minutes, centrifuged at 3500 revolutions per minute (rpm) for 15 minutes (Vinitha *et al.*, 2011). Sediments were deposed and buoyant liquid was stored at 4°C for further use and studies.

Microorganisms

To isolate the *Micrococcus* genus, Furazolidone-Peptone (FP) medium was used (kocur *et al.*, 2006). 56 bacterial strains of *Micrococcus* sp. were isolated from Barada River which is contaminated with vegetable oil wastes and other contaminants. Ten of these strains were found to have a good capability to degrade vegetable oil wastes; they were identified according to Bergey's manual (2012), and Rieser (2013) as *Micrococcus* sp. Strains code symbol Mb numbered from one to ten (M: from name of genus, b: from name of river). 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.

Inoculum preparation

A single colony of each of the isolates was inoculated into 10 ml of nutrient broth incubated at 30 C^o (Ajao *et al*., 2011) for 24 h (Darsa *et al*., 2014), centrifuged for 15 minutes at 3500 rpm (Santhini *et al*., 2009), 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) (Nwinyi *et al*., 2014).

Detection of ability strains of Micrococcus *to biodegradation of vegetable oil wastes*

Tow ml of bacterial inoculum (OD_{600} = 0.4) were transferred into tubes containing Bushnell-Hass medium (8 mL) supplemented with vegetable oil wastes (Nwinyi *et al*., 2014) (1, 2 and 3% v/v) as the only carbon source. They were incubated at 30°C (Darsa *et al*., 2014) at 150 rpm (Bahig *et al*., 2008) for 15 days (Darsa *et al*., 2014). A negative control was Bushnell-Hass medium and vegetable oil wastes devoid of microorganism, and a positive control was supplement with glucose (Bushnell-Hass medium, vegetable oil wastes, different concentration of glucose 1, 2 and 3% and microorganism) and incubated at the same conditions (Bahig *et al.*, 2008). Measurements of the OD, pH and TVC were carried out at 0, 4, 8, 12 and 15 days interval (Darsa *et al*., 2014). 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.

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 to 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 day using the SPSS. 20 computer software program were statistically significant or not. Those differences were determined at the $P<0.05$ level.

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.

Results and Discussion

Isolation of Micrococcus *species*

Fifty six 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* by 73%, *M. lylae* by 14%, *M. flavus* by 11%, and *M. antarcticus* by 2% of the isolates. This percentage is somewhat similar to the one in Al-Musawy and Al-Sammak (2013), where *M. luteus* was ranked first in the number of strains followed by *M. lylae*. Ten strains were chosen to represent all the species, and according to their ability to degrade vegetable oil wastes. Table 1 shows strains and some of biochemical characteristics of *Micrococcus* species which were taken for the this study.

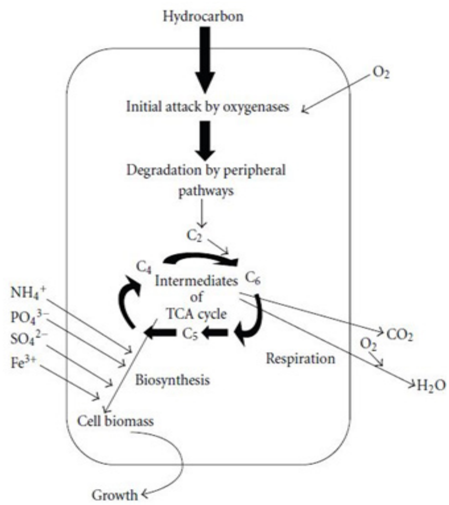
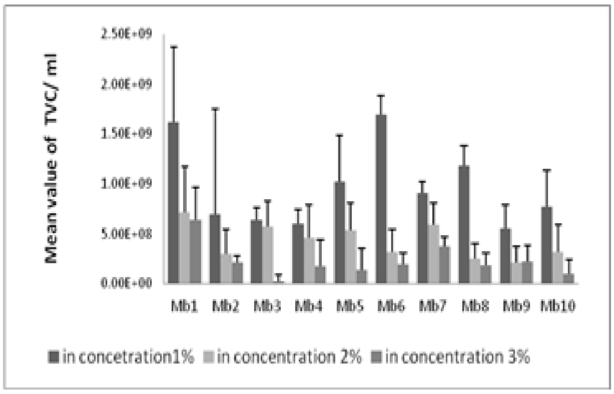


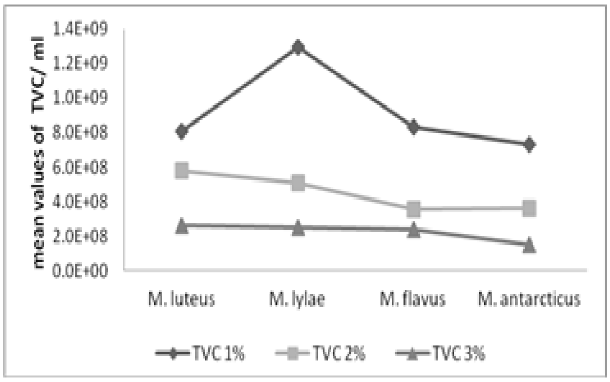
Figure 1. Main principle of aerobic degradation of hydrocarbons by microorganisms (Das and Chandran, 2011)

Biodegradation of vegetable oil wastes

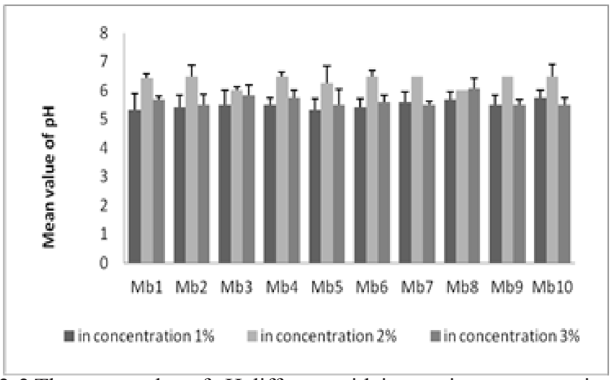
The most degradation of the majority of organic contamination is brought about under aerobic conditions. Figure 1 shows the main principle of aerobic degradation of hydrocarbons (Das and Chandran, 2011). This study would help in understanding the role of bacteria-especially aerobic bacteria and heterotrophic- 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 vegetable oil wastes by genus Micrococcus different species showed different results, which can be attributed to the difference in their ability to withstand the accumulated toxic compounds by biodegradation, and this is referred to in Horvath 1972. In it, the ability of biodegradation was decreased with concentration increasing, as shown in Figure 2, and this is expected because of what



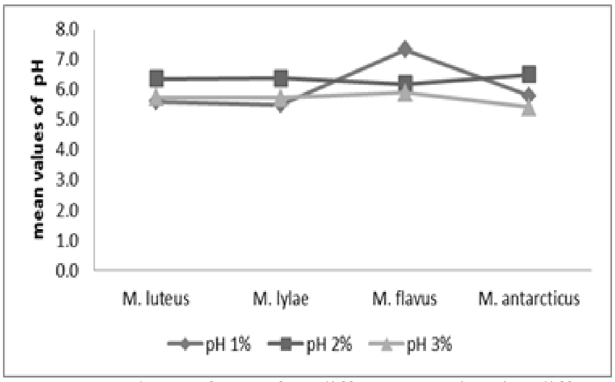
2-1 The mean value of TVC different with increasing increasing concentration of the vegetable oil wastes



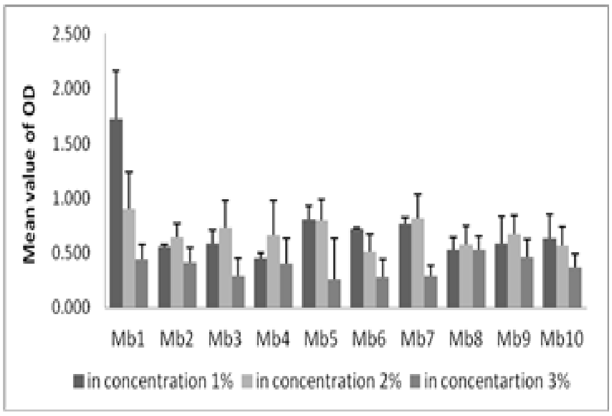
3-1 mean values of TVC for different species in different concentration



2-2 The mean value of pH different with increasing concentration of the vegetable oil wastes

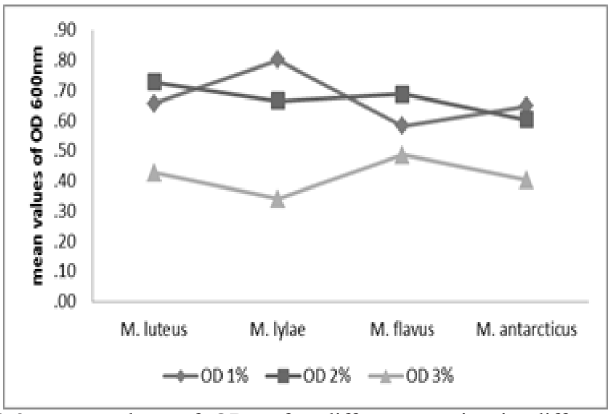


3-2 mean values of pH for different species in different concentration



2-3 The mean value of OD different with increasing concentration of the vegetable oil wastes

Figure 2. A different ability of study strains to biodegradation of vegetable oil wastes



3-3 mean values of OD_{600} for different species in different concentration

Figure 3. The vital signs indicator to biodegradation of vegetables oil wastes for studied species

happens during frying, a complex series of various chemical reactions take place, such as hydrolysis, polymerization and fission (Andrikopoulos *et al.*, 2002), and this is what leads to the formation of many toxic compounds, which in turn operate as materials restraining the growth of organisms (Tamada *et al.*, 2012). Thus when vegetables oil wastes concentration increases, the ability bacteria on growth decreased. Therefore, the higher toxicity of the sample containing these hydrocarbons over time can be attributed to the secondary compounds formed by microbial action. In addition to correlation between each of the means value of OD and TVC with time in all the concentrations studied, and this relationship was statistically significant (P = 0.00001). There was also a correlation between the mean value of OD and mean value of TVC and was statistically significant (P = 0.00001). As for the relationship between each of the means value of OD and TVC with mean value of pH at three concentrations were not statistically significant (P > 0.05). Strain Mb1 was the most efficient at three concentrations. By applying Paired-sample t-test to determine if the alteration in OD, TVC and pH value after 15 days were real, the results showed real differences between the means values of the studied (Sig < 0.05). Generally, microbial vegetable oil degradation is considered to occur as a result of hydrolysis of vegetable oil by secretion of some enzymes (oil degradation enzymes), which degrades the vegetable oil to organic acids and volatile fatty acids or reduces it to a low molecule via beta oxidation this was in agreement with (Bala *et al*., 2014) .

By comparing TVC value after 15 days for the strains at concentrations of 1% and 3% of vegetable oil wastes with a TVC value for positive control after 15 days, it was noticed that TVC for vegetable oil wastes was greater than TVC for positive control; whereas by the application of paired sample t-test showed these differences were not significant ($P > 0.05$), which means the increase was not significant. This refers to the ability of *Micrococcus* sp. to grow on vegetable oil wastes similar to its grow on glucose. But the increase was significant in concentration 2%.

The species have most ability to degrade and also identify by applying one way ANOVA test, which found that species *M. lylae* is the most efficient at the concentration of 1%, while *M. luteus* was the best in concentration 2%, and in concentration 3% *M. flavus* was the most efficient where it had the highest values of OD and TVC, pH value was receded which can be attributed to metabolism of vegetables oil wastes which leads to increase of the free fatty acid in the medium, as shown in Figure 3. This result can be explained by the ability of vegetables oil wastes to act as enzymes production inducer by the microorganism, and this is referred to in Domĭnguez and colleagues (2010). Ability of the genus to grow on vegetables oil wastes may be explained by Alkane hydroxylases which play an important role in the microbial degradation of oil, hydrocarbons, and many other compounds, and this agrees with Beilen and Funhoff (2007) results.

Conclusion

The genus *Micrococcus* different species are widespread in the Barada River, which refers to the organic pollution in the River. We can consider *Micrococcus* as one of the bacteria which can be used in the biodegradation of hydrocarbon pollutants. Increasing in vegetable oil wastes leads to a reduction in the efficiency of bacteria treatment process. Biodegradation ability of the *Micrococcus* species vary depending on the concentration. The existence of *M. flavus* confirms the issue of river pollution by wastewater, because it is usually isolated from activity sludge. It was active in the degradation process when there was an increase in vegetable oil wastes concentration.

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