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Biological treatment of phenol using microorganisms: *Pseudomonas,Acinetobacter* and *Chlorella*

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Abstract

This research work addressed laboratory study for biological treatment of phenol. Figuring phenol disintegration will give an idea of biodegradation of most organic chemicals since it is included in the composition of most of them.

Biological treatment is considered one of the effective and economic ways to address phenol treatment by biodegradation as compared with high cost methods of physical and chemical processing. The method is of low efficiency when applied to high concentrations, in addition to formation of secondary compounds that may be more dangerous than the target material.

The study focused on use of three isolates, i.e. *Pseudomonas*, *Chlorella* and *Acentobacter*. They were taken from the Microbiology Studies Laboratory at Allepo University, and characterized by its resistance to phenol at concentrations that reached up to 600 mg/L. It was found that algae *Chlorella* genus is the best sample in the disintegration of phenol and the most resistant. It has been able to disintegrate more than 90% of the concentration of phenol in its media within two weeks. The study showed that a vaccine of 4% of the size of the media was enough to resist inhibiting effect to phenol. Best results were achieved the in biodegradation rates of phenol at the temperature range of 30 to 35°C, moderate pH (7 = pH) and ventilation at a rotation speed of 125 rev/min, coupled with the best rate of growth after 48 hours of incubation.

It is concluded from the study of the effect of different sources of nitrogen and carbon on the biological rate of disintegration of the phenol that the organic nitrogen sources were semi-retardant to biodegradation while it has the best impact on bacterial growth rate. Likewise, ammonium chloride was found to be the best nitrogen source to improve rates of growth and disintegration of studied microbial type at concentration of 0.99 g/L.

The research recommended use of microorganisms that are characterized by their resistance to phenol and its compounds and most capable of its disintegration of using it as the sole source of carbon and energy such as species of *Pseudomonas*, *Acentobacter* and *Chlorella*.

Keywords: Phenol, bacteria, biological treatment, microbiology, *Pseudomonas, Acinetobacter, Chlorella.*

1. Introduction

Environment in recent decades gained many Xenobiotic compounds from biological systems. This negatively affected the ecosystems through what it has caused from physical and chemical changes (Abd-El Hameidshalaby, 2003). It has created an imbalance in the ecosystem because they contain molecular combinations that do not exist in nature which makes it toxic to living organisms and decomposer living beings. This is in addition to the difficulty in removing them from the environment (Press-Kristensen, 2007).

Industrial and agricultural products are regarded as sources of most pollution in most countries of the world (Sabri, 2003). They produce relatively high amounts of contaminants that have proven their toxic effect, caused genetic mutations and cancers and for their potential bio-accumulation.Chemical contaminants spread (including persistent organic and inorganic pollutants) in the atmosphere, water and ground cover. These contaminants tend to transform into other compounds that may be even more toxic than their first stage. This is because of their

exposure to many physical, chemical and biological processes in the environment.

Water pollution is considered one of the most intractable problems faced by the countries of the world, without exception. The need to remove contaminants has led to the development of several new technologies for treatment and removal of toxicity for a healthy environment. This is instead of the traditional methods (landfill or throw away or burning (Abd-El Hameidshalaby, 2003)). Biological treatment is one of the ways to deal with treatment of contaminants. It transfers chemicals in the environment to carbon dioxide, water and various inorganic materials (Press-Kristensen, 2007).

Fate of chemical compounds in the environment is assessed by its easiness of mineralization by microorganisms to weak disintegrated compounds, weak compounds disintegrated or recalcitrant compounds and compounds persistent to disintegration.

Phenol and its derivatives constitute basic elements to a wide range of dangerous synthetic organic aromatic materials commonly found in industrial wastewater. Likewise, phenolic compounds are naturally present in the environment. These are aromatic substances dissolved in water and easily dispersed within components of the environment. Phenol hazard is related to its resistance to disintegration, toxic nature even at low concentrations and for the formation of chloro-phenolic compounds upon treating wastewater effluent carrying phenol by chlorination as these compounds have low thresholds to be recognizedby taste and smell.

2. Research importance and objectives

Since phenol enters in the production of many industries, it is regarded as one of the most common environmental pollutants, especially in industrial waste water. Phenol is characterized by its risks, resistance to disintegration and toxicity even at low concentrations. Therefore, it is necessary to get rid of it in various ways, of which biodegradation is the best.

The objective of this research work is the use of isolates of *Pseudomonas*, *Acentobacter* and *Chlorella* that disintegrate phenol, testing ability of these isolates towards biodegradation of phenol and selection of the best isolate.

3. Research methodology and materials

The research study addressed thetheoretical approach for the treatment of phenolic pollutants, and a laboratory study to use isolates of *Pseudomonas*, *Acentobacter* and *Chlorella* for phenol disintegration, testing ability of isolates towards phenol biodegradation to choose the best isolate.

3.1 Theoretical study for treatment of phenolic pollutants

The contamination of soil, surface and ground water by organic pollutants in general and phenol in particular, is a serious problem facing the world (Shourian et al., 2009). Thus, it had to be removed before it is introduced into the environment (Al-Khalid and El-Naas, 2012). Ways and methods of phenol removal include the following:

1) Physico-chemical methods: These methods rely on use of chemical oxidation of pollutants using Hydrogen Peroxide (Oturan, 2014), chemical solvents, ion exchange, sedimentation and flotation, ultrafiltration, electrochemical disintegration, adsorption on porous materials like activated carbon and sawdust, burning, in addition to many non-biological methods (Kotresha and Vidyasagar, 2008). These methods are associated with many problems, particularly high costs and composition of hazardous secondary materials that may be more dangerous than phenol itself (Al-Khalid and El-Naas, 2012).
2) Biodegradation methods: where biological treatment is considered a promising method and an alternative to physico-chemical techniques. They are less expensive and able to convert phenol and other pollutants into its inorganic mineral components. These aspects makebiodegradation one of the world's favorite techniques.
3) Pulsed electrochemical oxidation processfor oxidation of organic compounds and treatment of organic pollutant are influenced by different factors such as: anodic film formation (passivation), electrode life span, high cost of efficient electrodes, energy utilization, current efficiency, economic viability, electrochemical oxidation efficiency and operating costs (Mu'azu et al., 2016).

This work study adopted biological treatment using living organisms (plants, microbes and fungi) for the treatment and removal of toxic pollutants (organic and inorganic materials and heavy metals) from contaminated soil, water and landfills.

Of distinguished microorganisms that have high ability to disintegrate phenol are the genera *Pseudomonas*. This is regarded as one of the most famous microorganisms that are capable of phenol disintegration at high concentrations. This is besides some types of algal living species that are capable of consuming phenol as a source of carbon and energy.

a) *Pseudomonas*isolate:members of the genus *Pseudomonas* are featured by their simple food requirements and ease isolation. This because they grow on many organic and inorganic media, at moderate acidity level and temperatures. The degree of optimum temperature for the growth of most types is 28 to 30°C. It also features as one of the fastest organisms that are adapted in different environs with difficult or harshconditions. Many of its members are resistant to antibiotics, disinfectants, detergents, heavy metals and organic dissolvent. Therefore, the use of its members is preferred in many special biological treatment research studies.

   Several studies were conducted with various types and concentrations. Among these studies is the one conducted by the researcher Kotresh and Vidyasagar(2008) where they detected an isolate from pulp and paper factory wastewater. This isolate showed that it is able to disintegrate 1300 mg/L of phenol. In another study carried out by the researchers (2013) on the isolate showed that it can disintegrate 1000 mg/L when the optimum conditions are provided for their growth. In their study on one species of this type isolated from the wastewater of drugs factory showed that the isolate was able to disintegrate 100 mg/L (2013).

b) *Chlorella* isolate:*Chlorella*grows in fresh water as it appeared from ancient time. *Chlorella* is a microscopic organism derived from “chloros” which means green and “ella” small. It contains the maximum amount of chlorophyll. It is highly efficient in wastewater treatment. Algae turned out to be able to grow within 500mg to 700mg without a clear obstruction up to 1500 mg with decreasing and impeding growth within a time period of seven weeks.

c) *Acentobacter* isolate:It is a genus of gram-negative bacteria and is not mobile. It can be isolated from the soil for to its high presence. It is characterized by its simple food requirements and easy isolation since it grows on many media at moderate degree of acidity and temperatures. The optimum temperature of growth for most types is 28 to 30°C.

It is to be noted herein that both aerobic and anaerobic microorganisms can disintegrate phenol. However, aerobic microorganisms are more capable of disintegration (Lika et al., 2009). They are the fastest growing, this is in addition to the low cost and ease of application compared to anaerobic disintegrating microorganisms (Mittal, 2011).

The disintegration of phenol aerobically is achieved by a number of microorganisms including *Acentobactercalcoceticus*, *Candida tropicalis* and species of *Pseudomonas* genus (Basha et al., 2010), which is considered the most famous for disintegration of phenol and other pollutants.

A group of biological or environmental factors (Al-Khalid and El-Naas, 2012) affect biological disintegration of phenol. These factors can stimulate or inhibit the disintegration process by its reflection on the growth of the organism responsible for this process. These factors include: temperature, degree of acidity, oxygen content and availability, in addition to concentration of phenol which at high concentrations inhibit organism metabolic activity and biodegradability. To achieve optimization of biodegradation these conditions must be appropriate to this organism. Likewise, the use of pure and mixed media affects the disintegration rate. Mixed media farms are capable of disintegrating phenol more efficiently in this process.

3.2 Laboratory study

a) Sampling: Three samples were collected for identified organisms from the neighborhoods of the Microorganisms Studies Laboratory at Faculty of Science at University of Aleppo. This is besides a blank sample drawn from the soil, as shown in Table (1).

Table (1) Samples collected for identified organisms

Sampling #	1	2	3	4
Living organisms	*Pseudomonas*	*Chlorella*	*Acento-bacter*	Soil sample as a blank

b) Isolating microbes of genus *Pseudomonas*for phenol biodegradation:
   - ✓ Select-salt mineral media (MS) containing phenol only as a sole source of carbon to isolate the bacteria disintegrating phenol as follows:
   - ✓ Take 5 ml of each sample (after shaking) and place it in 50 ml of (MS) media enriched with phenol at concentration of 100 mg/L within tubes

of capacity of 250 ml, and incubate at a temperature of 30°C for 48 hours, at ventilation speeds of 125 rev/min using an incubator with rotating vibrator (Figure 1).

Fig. (1): Rotary vibrators

- ✓ Take 5 ml of former suspension from each sample and add to the other tube for the same media and graded concentrations of phenol, starting from 100 to 600 mg/L, and consecutively in the same conditions of the previous incubation.

c) Measuring concentration of phenol:
The 4-Aminoantipyrine reagent method is used as a colour detector to measure the concentrations of phenol. This method relies on reaction of phenol with 4-Aminoantipyrine at pH of 7.9±0.1, which gives pigment in the aqueous solution with Potassium Ferricyanide$K_3Fe(CN)_6$. Color is measured by using spectrophotometer apparatus at a wavelength of 500 nm.

In order to determine the concentration of phenol remaining in the media, the researchers drew a graph chart that connects known concentrations of phenol (in mg/L) and the value of optical density (OD) as follows:

1. Add 2.5 ml of ammonium hydroxide NH_4OH (0.5 N) for each of tube containing the series of concentrations and the blank.
2. Adjust solution pH values to 7.9 ± 0.1 using phosphate buffer, then add 1 ml of 4-Aminoantipyrine solution (2%) and mix well.
3. Add 1 ml of $K_3Fe(CN)_6$ (8%) and mix well, then measure the optical density (OD) after 15 minutes.
4. Draw a chart (Table 2) (Figure 2), which connects concentrations of known phenol information in mg/L and the value of absorbance Absorption. From equation of slope of straight line a relationship is drawn to calculate the concentration of remaining phenol.

Table (2): Phenol concentration and absorbance

Phenol concentration, mg/L	0.1	0.2	0.3	0.4	0.5
Absorbance (A)	0.14	0.28	0.43	0.56	0.72

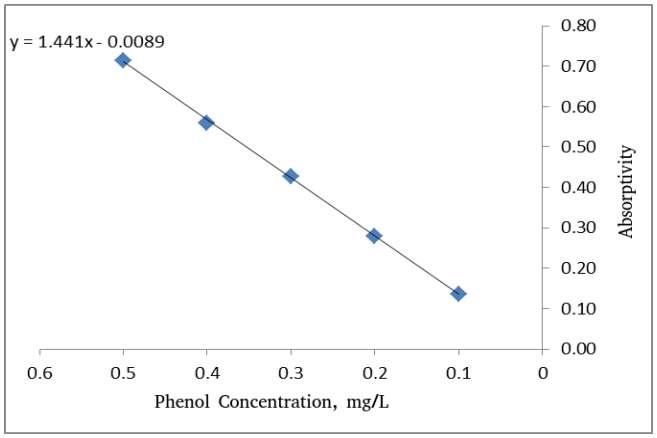

Fig. (2): A diagram linking absorbance and concentration of phenol.

5. Determine concentration of remaining phenol from the relationships presented in equation 1.

The concentration of phenol remaining (x) = $\frac{y+0.0089}{1.441}$ (1)

Where:
y = absorbance and
x = concentration of phenol

6. Find the proportion of disintegration from the relationship depicted in equation 2.

Biodegradation ratio = [(initial phenol concentration-remaining phenol concentration)/ initial phenol concentration]*100 (2)

7. After placing phenol detectors and waiting for a quarter-hour, detector positive coloration is noticed, which contains phenol in red color, and the negative blank which does not contain phenols in yellow as shown in the photo (1).

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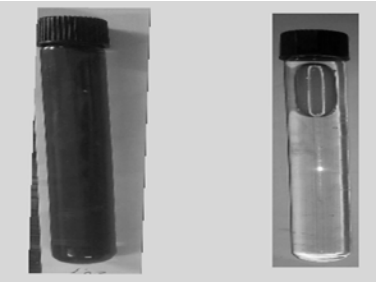


Photo (1): Color identification.

4. Results and discussions

After placing phenol at initially consecutive concentrations 100 and 200 mg/L for previous living organisms, waiting and incubation for the duration of the specific period (3, 6 and 9 days), 1 ml of raw suspension for isolates was taken. Mentioned detector reagents were then added and after waiting for a period of a quarter of an hour coloration of samples is noticed with different colors depending on the amount of phenol remaining in the sample without disintegration, as shown in photos (2) and (3). Samples were then placed in the spectrophotometer and perform measurements of concentrations of phenol as described in tables (3) and (4).

Phenol at initial concentrations of 400 mg/L were placed as well previous organisms, waiting and incubation for the period specified (3) days, 6 days, a week and two weeks. After which 1 ml of basic suspension was taken for the isolates. Previously mentioned detector reagents were then added and after waiting for a period of a quarter of an hour is noticed the coloration of samples with different colors depending on the amount of remaining phenol in the sample without disintegration, as shown in photo (4). Then it was placed in the device and measurements were performed for theconcentrations of phenol as described in table (5).

Phenol was placed at concentrations of 600 mg/L initially for previous microorganisms, waiting and incubation for the specified period of three days, six days, a week and two weeks. 1 ml of original suspension was then withdrawn for the isolates and aforementioned detector reagents were then added, waiting for a quarter of an hour, and then placed in the device where measurements were taken and performed for the concentrations of phenol as described in table (6).

In choosing the best isolate for biodegradation of phenol the following steps were undertaken:

Photos (2, 3 and) shows coloration of samples after three, six and nine days at concentrations of 100, 200 and 400 mg/L, respectively.

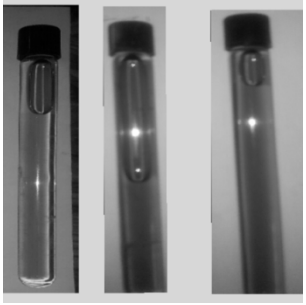
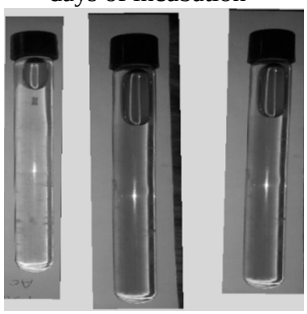
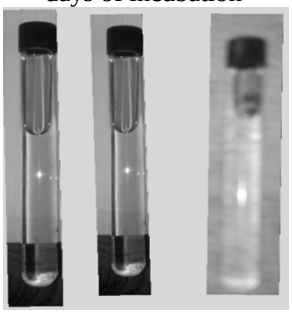
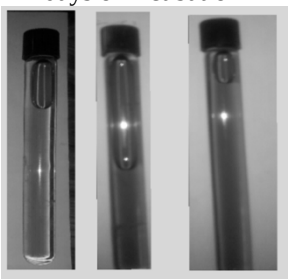
Photos of samples after three days of incubation	Photos of samples after six days of incubation
	
Photos of samples after nine days of incubation	
	

Photo (2): Coloration of samples after three, six and nine days at concentration of 100 mg/L

Photos of samples after three days of incubation	Photos of samples after six days of incubation
	

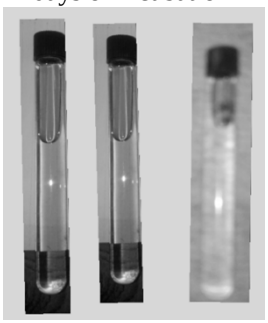
Photos of samples after nine days of incubation	
	

Photo (3): Coloration of samples after three, six and nine days at concentration of 200 mg/L

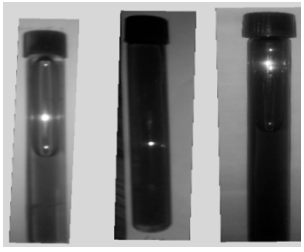
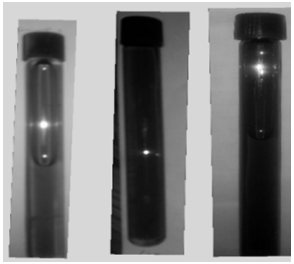
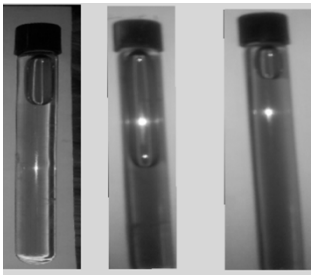
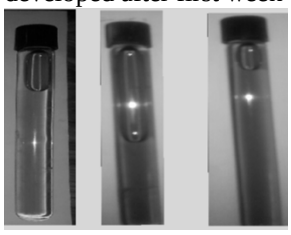
Three days after incubation, no distinct difference was observed in colors	Six days after incubation, no distinct difference was observed in colors
	
Photos of samples after two weeks of incubation	Two weeks after incubation, no distinct difference was observed in samples colors that developed after first week
	

Photo (4): Coloration of samples after three, six and nine days at concentration of 400 mg/L

Table (3): Phenol at concentration of 100 mg/L

Sample type	*Chlorella*			*Acento-bacter*			*Pseudo-monas*		
# of days	3	6	9	3	6	9	3	6	9
Absorbance	0.47	0.19	0.1	1.09	0.96	0.37	1.13	0.95	0.33
Concentration of remaining phenol	33	13	7.5	76	67.2	26.2	79.03	66.5	23.5
% disintegration	67	87	92.5	24	32.8	73.7	20.97	33.5	76.5

Table (4): Phenol at concentration of 200 mg/L

Sample type	*Chlorella*			*Acentobacter*			*Pseudomonas*		
# of days	3	6	9	3	6	9	3	6	9
Absorbance	1.95	1.15	0.28	2.39	1.59	1.07	2.46	1.76	1.11
Concentration of remaining phenol	136	80	19.4	166.05	110.4	74.3	171.19	118.3	77.2
% disintegration	32	60	90.18	16.97	44.8	62.85	14.40	40.85	61.4

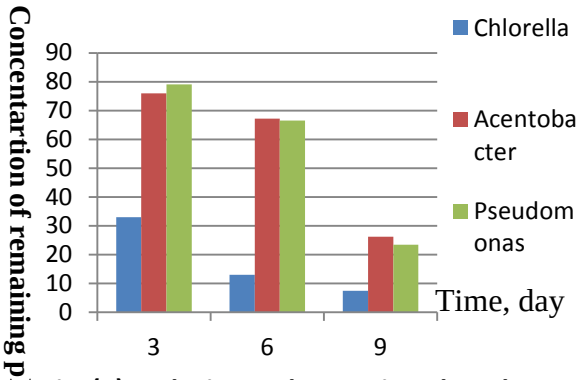


Fig. (3) Reducing and removing phenol in relation to type of bacteria and time at concentration of 100 m/L

Table (5): Phenol at concentration of 400 mg/L

Sample type	*Chlorella*				*Acentobacter*				*Pseudomonas*			
# of days	3	6	1 week	2 weeks	3	6	1 week	2 weeks	3	6	1 week	2 weeks
Absorbance	5.43	5.15	3.2	2.02	5.64	5.57	4.89	3.69	5.68	5.62	4.99	3.73
Concentration of remaining phenol	378.1	358.3	222.13	140.9	392.4	387.24	340.243	257.25	395.13	391.16	347.32	259.80
% disintegration	5.4	10.4	44.2	64.7	1.9	3.19	41.9	35.7	1.21	2.21	13.17	35.05

Table (6): Phenol at concentration of 600 mg/L

Sample type	*Chlorella*				*Acentobacter*				*Pseudomonas*			
# of days	3	6	1 week	2 weeks	3	6	1 week	2 weeks	3	6	1 week	2 weeks
Absorbance	8.55	8.30	6.73	5.01	8.61	8.49	8.40	8.18	8.6	8.53	8.43	8.21
Concentration of remaining phenol	594.15	577.3	468.23	348.3	598.3	590.4	584.12	568.9	598.1	592.7	586.03	570.4
% disintegration	0.9%	3.78%	21.9%	41.9%	0.28%	1.6%	2.6%	5.1%	0.3%	1.21%	2.32%	4.93%

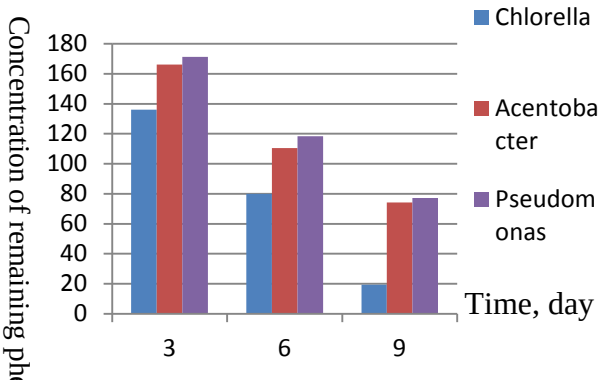


Fig. (4) Reducing and removing phenol in relation to type of bacteria and time at concentration of 200 m/L

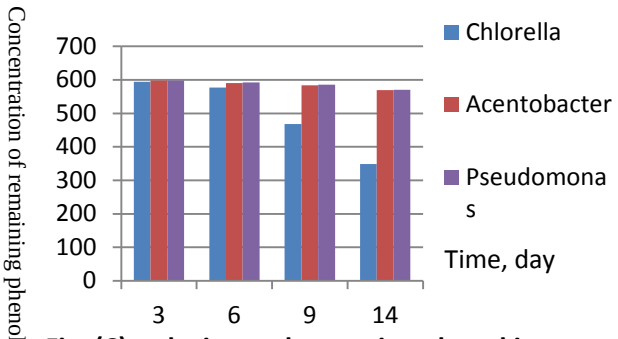


Fig. (6) reducing and removine phenol in relationship to bacteril type and time at concentration of 600 mg/L

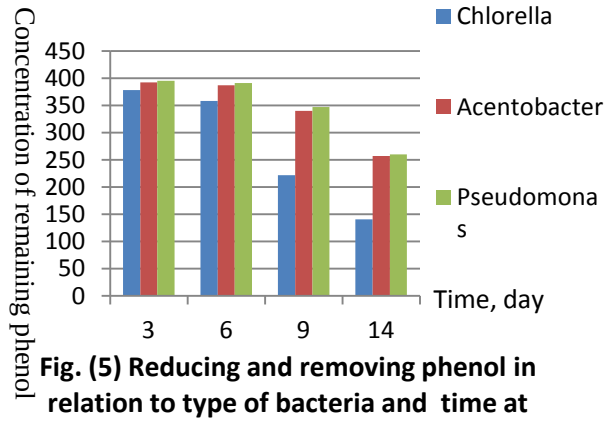


Fig. (5) Reducing and removing phenol in relation to type of bacteria and time at concentration of 400 m/L

From Figures (3, 4.5, 6) and tables (3, 4.5, 6) the following points could be drawn:

- Best isolate in disintegration of phenol at concentration of 100 mg/L:
  From Fig. (3) and Table (3) it is shown that for biodegradation rate of 100 mg/L best isolate in disintegration of phenol at named concentrationreached about 93% nine days later (the concentration of remaining phenol is approximately 7 mg) for the *Chlorella* isolate. This is followed by *Pseudomonas* isolate with a disintegration ratio of about 77% (the concentration of phenol remaining is approximately 23 mg). Yet to be followed by *Acentobacter* isolate for a disintegration proportion of 74% (the concentration of phenol remaining is 26 mg). Color of reagent detector taken from the media

containing algae closely approached the color of the media which does not contain phenol. This suggests that algae have disintegrated the whole amount and no inhibitory effect was observed for this concentration on bacterial and algal growth.

- Best isolate in disintegration of phenol concentration at 200 mg/L:

Figure (4) and Table (4) show that disintegrating rate for a concentration of 200 mg/L nine days later, reached about 91% (the concentration of phenol remaining is approximately 20 mg) for the *Chlorella* insolate. This is followed by *Pseudomonas* isolate for disintegration ratio of 63% (concentration of phenol remaining is approximately 75 mg). *Acentobacter* isolate followed by a rate of disintegration of about 62% (the concentration of phenol remaining is approximately 78 mg). Color of reagent detector taken from the media containing the algae closely approached the color of the media which does not contain phenol. This is evidence that algae may have disintegrated whole quantity and no inhibitory effect had been observed for this concentration on bacterial and algal growth.

- Best isolate in disintegration of phenol at concentration of 400 mg/L:

Figure (5) and Table (5) show that disintegration rate of 400 mg/L after two weeks has reached 65% (concentration of phenol remaining is 140 mg) for *Chlorella* isolate. This is followed by *Acentobacter*isolate with disintegration proportion of 36% (concentration of phenol remaining is 257 mg). Yet to be followed by *Pseudomonas* isolate with disintegration rate of 35% (concentration of phenol the remaining 259 mg). Disintegration period has been increased to two weeks with this concentration due to inability of isolates for full disintegration of phenol. Decrease in density of organisms was observed for all three types due to the inhibitory effect of this concentration. Consequently, incubation period was increased.

- Best isolate in disintegration of phenol at concentration of 600 mg/L:

Figure (6) and table (6) show that percentage of disintegration of 600 mg after two weeks reached 42% (concentration of phenol remaining 348 mg) for the *Chlorella* isolate. To be followed by *Acentobacter*isolate disintegration ratio of 6% (concentration of phenol remaining 568 mg). This is followed by *Pseudomonas* isolate by disintegration rate of 5% (concentration of phenol remaining 570 mg). Required disintegration period has been increased for this concentration to two weeks due to the inability of the isolates to complete total biodegradation of added phenol. A decrease was observed in the density of living cells for each of the three isolates due to inhibitory effect at such concentration. This is why further study did not go beyond the concentration of 600 mg/L.

- Bacteria's ability to reduce and remove the various initial concentrations of phenol in relationship with time

Three types of microorganisms were used: *Chlorella*, *Acentobacter* and *Pseudomonas* to disintegrate initial different concentrations of phenol (100, 200, 400 and 600) mg/L, as shown in Figures (7.8, 9). It is noticed from Figures (7, 8 and 9) that for initial low different concentrations of phenol algal cells managed to disintegrate phenol better than for high concentrations. Nevertheless, phenol biodegradation occurred even for initial concentrations of 600 mg/L only after giving time more than a week.

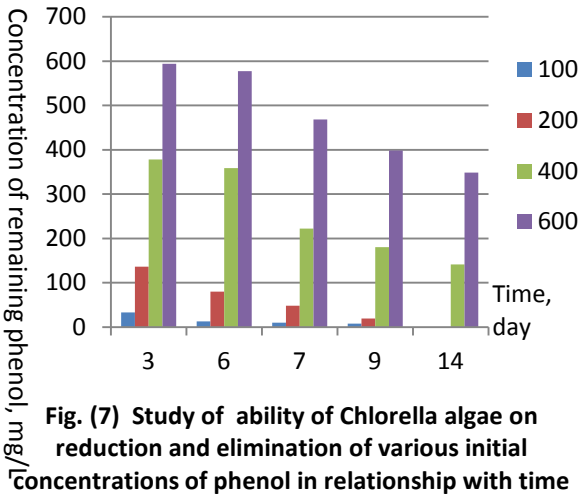


Fig. (7) Study of ability of Chlorella algae on reduction and elimination of various initial concentrations of phenol in relationship with time

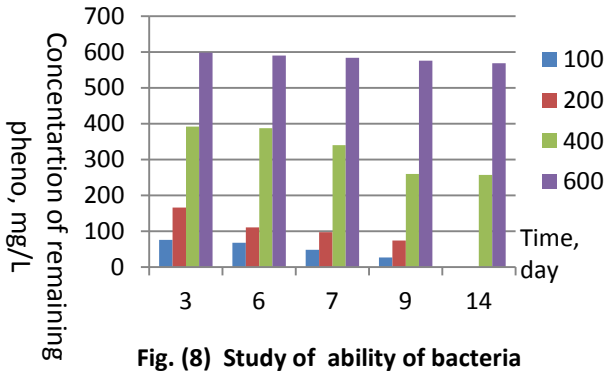


Fig. (8) Study of ability of bacteria (Acentobacter) on reduction and removal of

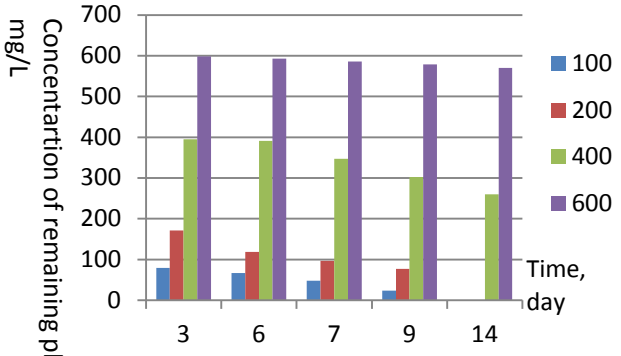


Fig. (9) Study of ability of bacteria (Pseudomonas) to reduce and remove various initial concentrations of phenol in relation with time

5. Conclusions and recommendations

From the research work carried out herein the following conclusions emerged:

- Inoculation results of each of the three isolates on media (MS) enriched with phenol at increasing concentrations from 100 to 600 mg/L indicated that the best growth rates and biodegradation was for*Chlorella* isolate in all studied concentrations.
- *Chlorella* isolate is the best sample in the disintegration of phenol. It has the most resistance and has been able to biodegrade more than 90% of phenol concentration in its media within two weeks.
- A vaccine by 4% of size of media was enough to resist inhibiting effect towards phenol.
- Better results in rates of biological disintegration of phenol were achieved in atemperature range of 30 to 35°C, moderate pH (7 = pH) and ventilation at rotation speed of 125 rev/in, occurring with the best rate of growth after 48 hours of incubation.
- For low initial concentrations of phenol algae was able to biodegrade phenol better for high concentrations. Nevertheless, phenol was disintegrated even for initial concentrations of 600 mg/L but after a time period exceeding a week.
- When studying effect of different sources of nitrogen and carbon on the biodegradation rate of phenol it is observed that organic nitrogen sources were semi-retardant and inhibit biodegradation, while it has the best impact on bacterial growth rate.
- Ammonium chloride was the best nitrogenous source in improving rates of growth and disintegration of the studied bacterial type at concentration of 0.99g/l.
- The research study recommended to use microorganisms, which is characterized by resistance to phenol and its compounds, being the most capable of disintegrating it and using it as the sole source of carbon and energy such as species and genera of *Pseudomonas*, *Acentobacter* and *Chlorella*.

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