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Biodegradation of Phenol by *Microbacterium terregenes* Isolated from oil field NORM SOIL

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Abstract Phenol is highly toxic and carcinogenic compound and its biodegradation is very important to meet the environmental regulations. The paper presents the main results obtained from the study of phenol biodegradation process by pure culture of *Microbacterium terregenes* isolated from NORM soil collected from oil field. The environmental factors affecting phenol degradation such as pH of mineral salt media (MSM), temperature, immobilized biomass weight and stirring were studied. The experimental work was carried out by using bacterial cells immobilized within calcium alginate (CA) gel beads in batch experiments. The optimum conditions for phenol degradation were found to be; pH 7.0, stirring 120 rpm and temperature 30 °C. The strain was highly efficient for phenol degradation. It could degrade phenol at maximum concentration of 700 mg/l within three days also, the effect of heavy metals (cobalt and copper) on bacterial growth and the rate of phenol degradation were studied.

Keywords: *biodegradation-phenol-bacteria-immobilization-calcium alginate*

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1. Introduction

Phenol is highly toxic organic compounds even at low concentration and its presence in aqueous media is severely limited by regulations, however it has adverse effect on aquatic life, plant and toxic to bacterial growth, but some microorganism can tolerate and use it as a carbon and energy source [1]. Moreover phenol has been on the list of priority pollutant for long time, due to cause many adverse effect to human health (toxic to nervous system, the heart, the kidney, and the liver and is rapidly absorbed through the skin and mucosa [2,3],) and the environment [4,5]. Phenol and its derivatives are widely used in many industrial branches like petrochemical, pulp, paper, tannery and coal refining industries. Thus, phenol is generally present in wastewater coming from these industries [6]. Efficient treatment of these wastewaters can be conducted by either hybrid process [7] electrocatalytic degradation [8] or biological techniques [9,10,11,12,13]. Bioremediation, being environmentally sound and relatively cost-effective approach, has emerged as the most advantageous clean-up technique for sites contaminated with organic pollutants [14,15]. Many aerobic bacteria are capable of using phenol as the sole source of carbon and energy [16,17,18,19]. Numerous other types of bacteria and biosorbents were reported to be utilized for the biodegradation or the removal of phenol. These include: *Rhodococcus erythropolis* [20]; *Bacillius* sp. [21]; *Alcaligenes faecalis* [22]; rhizobium *Ralstonia taiwanensis* [23]; *Nocardia hydrocarbonoxydans [24]*; *Candida tropicalis [25]* and activated sludge [26]. Immobilization of bacterial biomass for the degradation of phenol is an important and effective technique that is usually employed to serve several purposes, including protection of the bacteria from high phenol concentrations as well as ease of separation and reutilization of the biomass. In the present study bacterial species isolated from NORM soil of oil companies field was investigated for their ability to degrade phenol. Calcium alginate immobilized bacteria was tested in batch experiments. The immobilized bacterial biomass beads used to determine the optimum conditions for phenol degradation.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of analytical grade. Phenol, cobalt and copper solution were prepared by dissolving distinct amount in one liter of distilled water to form concentrated solution and other concentrations were prepared by dilution. Sodium alginate 4% was prepared by dissolving 4 gm of Na alginate in 100 ml distilled water. Sodium hydroxide and hydrochloric acid were used to adjust pH.

2.2. Instruments

2.2.1. Incubators

Two types of incubators were used, the first was static WTB Binder incubator model 720 and used for incubation of bacterial agar plates and slants. The second incubator was Lab Line orbital environmental shaker model (3527-1) provided with temperature and shaking control and used for production of bacterial biomass.

2.2.2. Centrifuges

Two types of centrifuges were used in this study, the first used for harvesting of bacterial biomass it has centrifugation rang up to 5000 rpm under cooling conditions(Hitachi universal 32 R). The second centrifuge used to obtain clear sample for UV/visible and atomic absorption spectrophotometer measurements.

2.2.3. UV/visible Spectrophotometer

Measurement of phenol was carried out using Shimadzu -160 UV/visible at wave length 269 nm using quartz cells at room temperature with light path length equal 1 cm.

2.2.4. Atomic Absorption Spectrophotometer

The quantitative determination of cobalt and copper was carried out using atomic absorption spectrophotometer (Buck) model 210 VGP.

2.2.5. High Pure Germanium Detector

The radioactivity analysis of the soil samples was performed using high pure germanium detector.

2.2.6. Biolog

The identification of bacterial isolates was carried out using Biolog GEN III instrument which is automated system for bacterial identification.

2.3. Isolation of Bacterial Strain

The soil samples were mixed thoroughly, then 15 gm were weighted from each sample and put in 50 ml of sterile mineral salt media (MSM). Its gradient showed in Table 1 [27] with phenol as sole carbon source. The mixture was incubated for 48 h at 37 ºC, after incubation 1 ml of soil suspension was put in Petri dish and nutrient agar was added on it, then mixed thoroughly and incubated for 24 to 48 h at 37 ºC. The obtained separated colony was taken for purification by streaking plate method [28]. The purified the bacteria were maintained on slants of agar containing (in g/l): beef extract, 1; yeast extract, 2; peptone, 5; bacteriological agar, 15; NaCl, 5; as was reported in a previous work [29].

2.4. Accommodation of Bacteria for Phenol Uptake

Mineral salt media was prepared with gradual concentration of phenol and glucose as showed in Table 2. The first MSM was inoculated with bacterial isolate suspension and incubated at 37 °C in orbital shaker for 72 h, then an inoculum was taken from the culture media to the second MSM media. The process was repeated until MSM containing phenol only as sole carbon source. An inoculum was taken from the last MSM to nutrient agar slants with 0.1 g/l phenol and kept refrigerated for use.

Table 1. The composition of mineral salt media

Component	Concentration (mg/l)
$MgSO_4 \cdot 7H_2O$	300
K_2HPO_4	250
$CaCl_2 \cdot 2H_2O$	150
$(NH_4)2CO_3$	120
$FeSO_4 \cdot 7H_2O$	3.5
$ZnSO_4 \cdot 7H_2O$	1.3
$MnCl_2 \cdot 4H_2O$	0.13
$CuSO_4 \cdot 5H_2O$	0.018
$CoCl_2 \cdot 6H_2O$	0.015
$Na_2MoO_4 \cdot 2H_2O$	0.013

Table 2. gradual concentrations of phenol and glucose

No.	Glucose conc.	Phenol conc.
1	20 gm/l	0.025g/l
2	15 gm/l	0.5g/l
3	10 gm/l	0.1g/l
4	5 gm/l	0.15g/l
5	Zero	0.2g/l

2.5. Identification of Bacterial Strain

The bacterial isolate was identified using Biolog GEN III in Cairo Mircen, Ain-Shams University, Faculty of Agriculture. The instrument analyze the ability of the cell to metabolize all major classes of biochemical's, in addition to determining other important physiological properties such as pH, salt, and lactic acid tolerance, reducing power, and chemical sensitivity [30]. The Biolog GEN III Micro Plate which analyzes a microorganism in 94 phenotypic tests: 71 carbon source utilization assays (column1-9) and 23 chemical sensitivity assays (column 10-12). The test panel provides a **"**Phenotypic Fingerprint" of the microorganism that can be used to identify it at the species level. Testing is performed very simply. The isolate to be identified is grown on agar medium and then suspended in a special "gelling" inoculating fluid A (IF) at the recommended cell density (90-98%). The obtained cells suspension is inoculated into the GEN III Micro Plate, 100 µl per well, and the Micro Plate is incubated at 33 °C to allow the phenotypic fingerprint to form. All of the wells start out colorless when inoculated. During incubation there is increased respiration in the wells where cells can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple color. Negative wells remain colorless, as does the negative control well (A-1) with no carbon source. Wells with extremely faint color, or with small purple flecks or clumps are best scored as "borderline" (\). There is also a positive control well (A-10) used as a reference for the chemical sensitivity assays in columns 10-12. After incubation, the phenotypic fingerprint of purple wells is compared to Biolog's extensive species library. If a match is found, a species level identification of the isolate is made.

2.6. Biomass Production

Bacterial slants were eluted in 2 ml distilled water, then the bacterial suspension were added to 250 conical flask containing 100 ml nutrient broth and incubated at 37 °C for 24 h in environmental shaker. Each 100 ml was distributed into three one liter conical flasks containing 200 ml nutrient broth and incubated at 37 °C for 24 h. The bacterial biomass finally harvested by cooling centrifugation for 15 min at 5000 rpm and kept frozen.

2.7. Immobilization

Sodium alginate was used for immobilization of bacterial cells [31]. 4 gm of sodium alginate was dissolved in 100 ml distilled water to obtain 4% sodium alginate gel, then it was autoclaved and kept in refrigerator for use. The desired weight of bacterial biomass was added to 5 ml sodium alginate gel and kept refrigerated for about 15 min before injected to 100 ml 2% $CaCl_2$ solution using peristaltic pump to form Calcium alginate (CA) immobilized bacterial beads which were left in $CaCl_2$ solution for one hour and washed with distilled water three times. Each 5 ml sodium alginate gel form approximately 150 beads with diameter 2-2.5 mm .

2.8. Analytical Methods

The uptake experiment performed using 50 ml of sterile phenol or sterile MSM containing phenol as sole carbon source. The certain volume of beads (150 beeds) was added to the phenol solution, then the mixture was incubated at 30 °C and pH 7.0 at 120 rpm in orbital environmental shaker. Samples were taken both in the beginning and at the end of experiments for phenol measurement. The samples were centrifuged for 5 min for separation of supernatant from any bacterial growth and media. The phenol concentration was determined using a shimadzu-160 UV spectrophotometer. All measurements were carried out at room temperature using quartz cells with light path length equal to 1 cm. Control experiment was done and used as reference in measurements, also each experiment was done in triplicate.

3. Results and Discussion

3.1. Radioactive Analysis of Soil Samples

Samples were collected from oil contaminated soil from Petrol Palaeem Company and khalda Petrol Company in the west desert, Egypt. After collection of soil samples, it was analyzed for radioactivity using high pure germanium detector. The analysis showed that the soil samples containing naturally occurring radioactive materials (NORM) as showed in Figure 1. Radioactive analysis of soil sample A showed that the total activity of (U-238) daughter was 14976 Bq/kg, while the total activity of (Th-232) daughter was 4899 Bq /kg. Radioactive analysis of soil sample B showed that the total activity of (U-238) daughter was 4705.94 Bq/kg, while the total activity of (Th-232) daughter was 1010.5 Bq/kg.

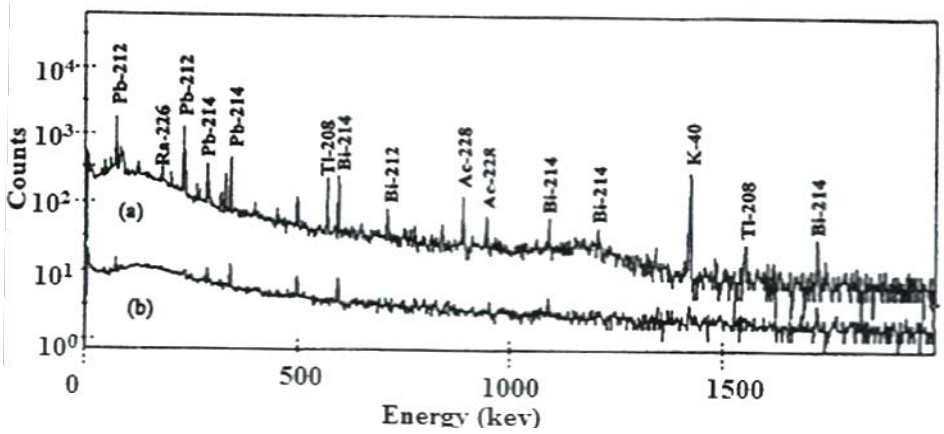

Figure 1. spectrum of soil sample; a) samples A & B , b) background

3.2. Identification of Bacterial Isolate

According to Biolog GEN III Micro Plate interpretation the isolated species was identified as *Microbacterium terregens.*

3.3. The Effect of Environmental Conditions

The environmental conditions such as, pH, temperature, phenol concentration, immobilized weight and stirring were studied to optimize phenol degradation process.

3.3.1. Effect of pH

The effect of pH on phenol degradation was studied at pH range between 2.5 and 8. The other environmental factors remain constant and phenol concentration was 0.3 g/l MSM. The results showed in Figure 2. indicates that at low pH (2.5) the phenol degradation was inhibited, while at pH range 3.5- 8 the degradation was 100% after 72 hour. It was found that the degradation rate was fast at pH 7.0, where it reached 80% within 24 hour. These behaviors are consistent with those reported in the literature for the biodegradation of phenol [32].

3.3.2. Effect of Temperature

The experiments were done at temperature rang from 25 to 45 °C using phenol solution 0.3 g/l (MSM) at pH 7.0 and stirring 120 rpm. Results showed in Figure 3. clears that the activity of *M. terregens* and consequently its ability to degrade phenol, is optimized at about 30 °C. Higher temperatures seemed to inhibit the activity of the bacteria and hence inhibit its biodegradation capabilities.

It is believed that sudden exposure to temperatures higher than 35 °C may have detrimental effect on the bacterial enzymes that are usually responsible for the benzene ring cleavage, which is the main step in the biological degradation process. On the other hand, exposure to lower temperatures is expected to slow down the bacterial activity.

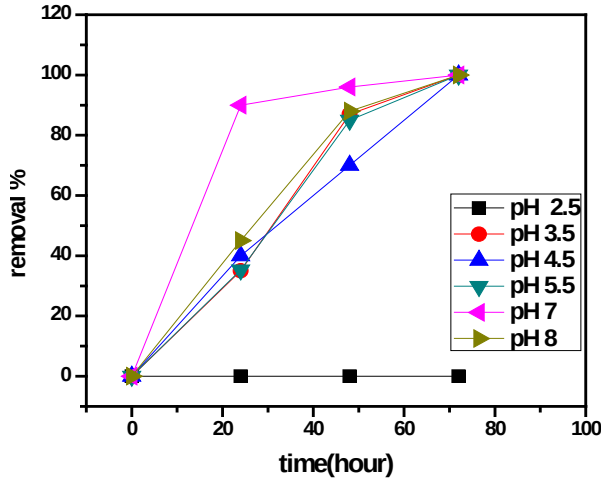

Figure 2. Removal% of phenol by *M. terregenes* at different pH values, T= 30 °C, phenol concentration 0.3 g/l and stirring 120rpm

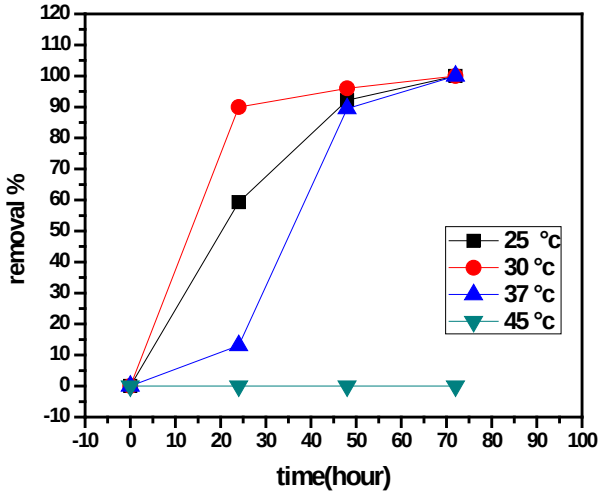

Figure 3. Removal% of phenol by *M. terregenes* at different incubation temperature values, pH 7.0, phenol concentration 0.3 g/l and stirring 120 rpm

3.3.3. Effect of Phenol Concentration

Initial phenol concentration plays an important role in the biodegradation process, since some hydrocarbon contaminants, including phenol are known to have inhibitory effect on the activity of the biomass. Experiments were carried out at different initial phenol concentrations ranging from 150 to 1000 mg/l. The results showed that the rate of phenol degradation decreased with the increase in phenol concentration till reached 1000 mg/l, where complete inhibition of phenol degradation occurred Figure 4a. The maximum degradation capacity for 1 gm dry weight of the bacteria was found to be 693.6 mg phenol per gm dry weight Figure 4b.

3.3.4. The Effect of Immobilized Weight

These experiments assayed to detect the best weight of biomass giving better biodegradation rate. Different biomass weights (0.1-0.2-0.3-0.4 and 0.5gm) were used with fixed temperature, pH and initial phenol concentration. The results showed that the rate of biodegradation increased with the increase in biomass weight as showed in Figure 5a. However the biodegradation capacity of CA immobilized bacteria (mg/g dry wt.) decreased with the increase in the immobilized bacterial biomass Figure 5b.

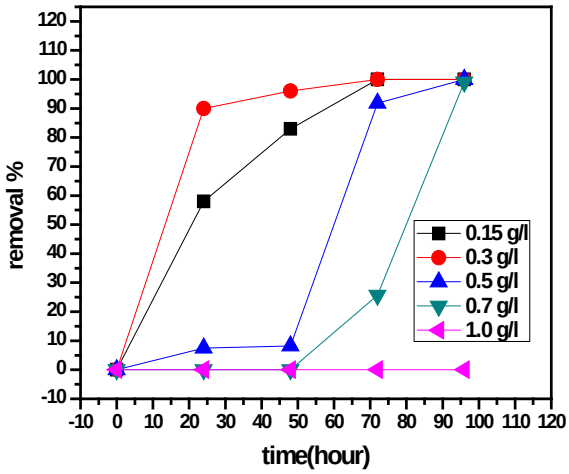

Figure 4a. Removal % of phenol by CA immobilized *M. terregenes* at different phenol concentrations, T=30°C, pH 7.0 and stirring 120 rpm

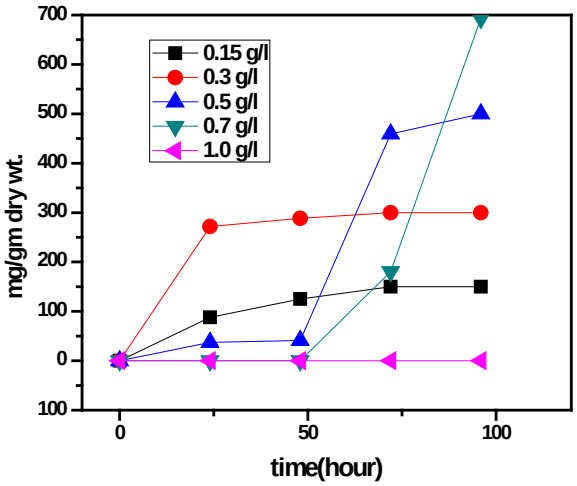

Figure 4b. Phenol degradation capacity in mg/g dry wt. at different phenol concentrations, pH 7.0, T= 30 °C and stirring 120 rpm

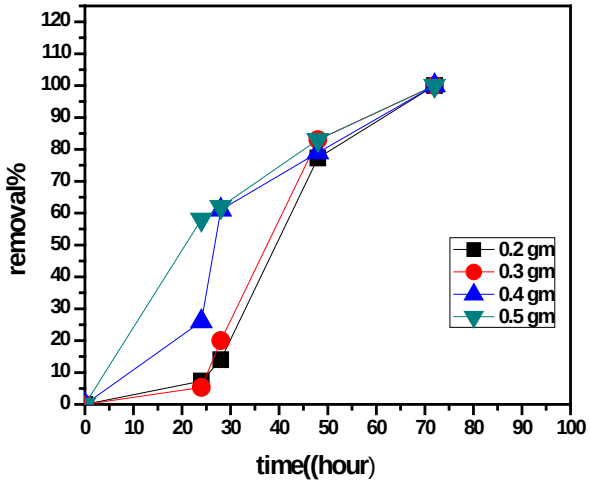

Figure 5a. Removal % of phenol by CA immobilized *M. terregenes* at different immobilized weights using phenol concentration 0.3 g/l, T= 30 °C, pH 7.0 and stirring 120 rpm

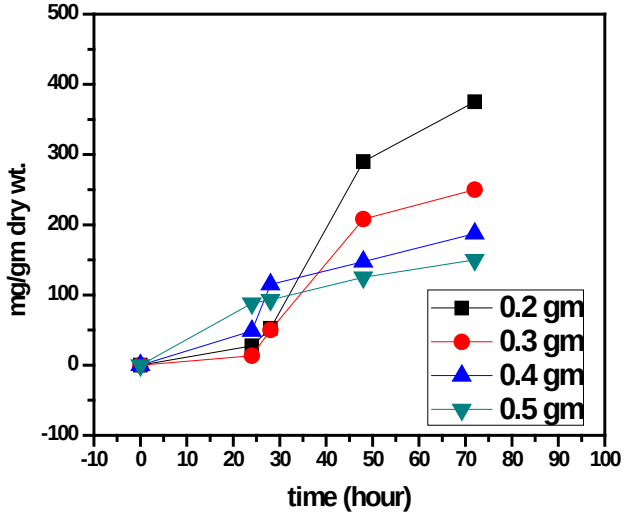

Figure 5b. Phenol degradation capacity in mg/g dry wt. at different immobilized biomass weights, pH 7.0, T= 30°C and stirring 120 rpm

3.3.5. Effect of Heavy Metals

3.3.5.1. Effect of Cobalt

Cobalt is required as a trace element in prokaryotes and eukaryotes to fulfill a variety of metabolic functions. Even though, this metal is less frequently encountered in metallo enzymes than iron, manganese, copper or zinc, it is an important cofactor in vitamin B12-dependent enzymes and in some other enzymes in animals, yeast, bacteria, Archaea, and plants [33]. High intracellular concentration of the redox active metal ion Co^{+2} is highly toxic, but mechanisms of this toxicity are largely unknown. The study performed using different concentrations of cobalt (10-25-50-100 ppm). The results showed that, there were variation in the effect of cobalt on biodegradation rate where 10 ppm delayed phenol degradation for 48 h, while other concentration (25-50-100ppm) delayed the degradation for 24 h as showed in Figure 6.

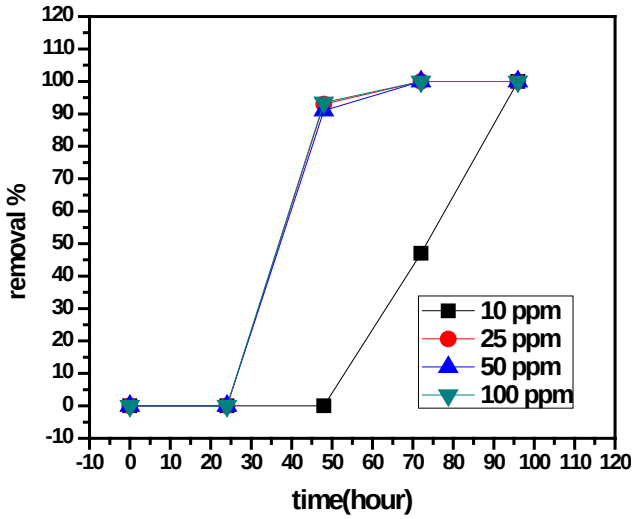

Figure 6. Removal % of phenol by CA immobilized *M. terregenes* in the presence of different Co^{+2} concentrations using phenol concentration 0.15g/l, pH7.0, T=30°C and stirring 120rpm

3.3.5.2 Effect of Copper

The experiments were carried out to study the effect of copper on the biodegradation of phenol using different concentrations 10-25-50-100 ppm. The results showed that copper had complete inhibitory effect as it blocks the biodegradation of phenol .

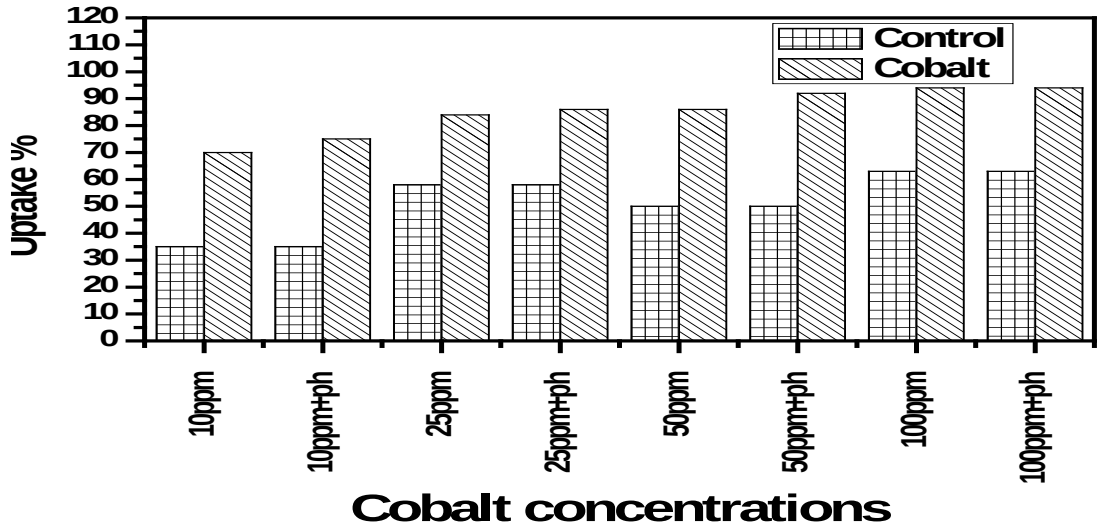

Figure 7. The uptake % of Co^{2+} by control CA-beads, CA immobilized *M. terregens* in absence and presence of phenol 0.15g/l at different Cobalt concentrations after equilibrium (2 h).

3.3.6. The Effect of Phenol on Biosorption of Heavy Metal

The interaction effect between phenol and heavy metals in wastewater during bioremediation should be studied to determine the best biomass immobilized system for treatment process of wastewater containing both organic and heavy metals. The effect of phenol (organic waste) present in wastewater on the biosorption of heavy metals by the CA immobilized *M. terregens* was studied using different concentrations 10-25-50-100 ppm of Co^{+2} and Cu^{+2}. The results showed in Figure 7 &Figure 8. indicats that phenol had little effect on biosorption of Co^{+2} and Cu^{+2}, where the biosorption increased in the presence of phenol.

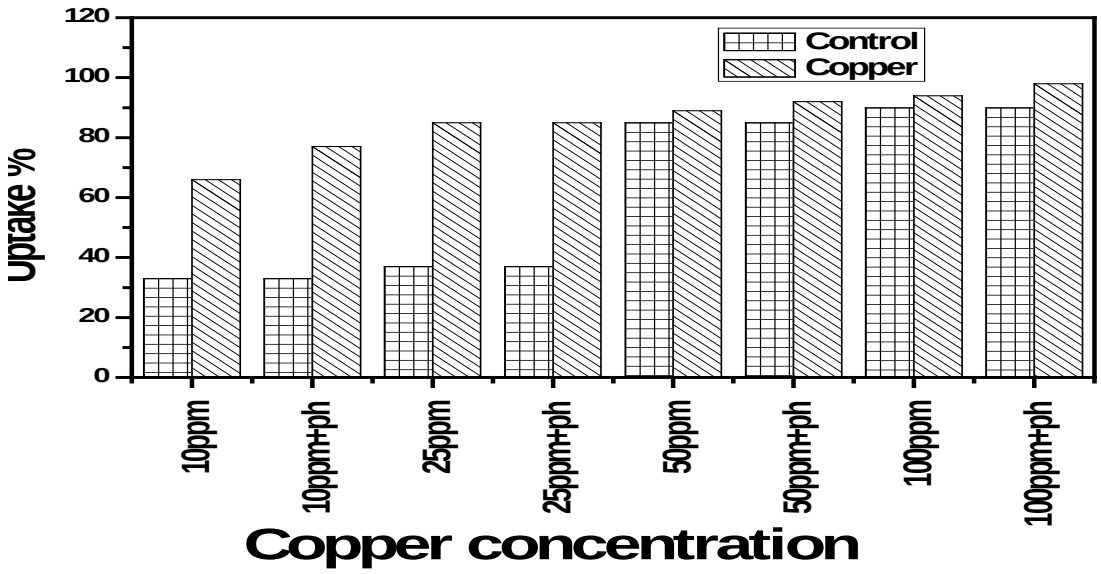

Figure 8. The uptake % of Cu^{2+} by control CA-beads, CA immobilized *M. terregens* in absence and presence of phenol 0.15g/l at different Copper concentrations after equilibrium (2 h)

4. Discussion

Phenol in wastewater even in low concentration can be toxic to aquatic organism and also, cause taste and odor problems in drinking water [34]. Biodegradation had been received attention during the last decade, where many type of bacteria and biosorbents were reported; rhizobium *Ralstonia taiwanensis* [23], *Nocardia hydrocarbonoxydans [24]*, *Candida tropicalis[25]* and activated sludge [26]. In the present study the bacterial species *Microbacterium terregenes* isolated from oil field soil was acclimated to phenol degradation using MSM enriched with phenol and glucose. The phenol degradation and optimizing conditions were studied using CA immobilizing *M. terregens* beads. The optimum pH was 7.0, where the degradation reached 80% within 24 h, the results correlated with the previous studies which showed that phenol degradation usually carried out at/or near neutral pH [32]. Also, the results showed that the optimum temperature was 30 °C, while the exposures to high temperature inhibit the degradation and low temperature slow down the degradation rate. Previous studies indicated that maximum degradation of phenol (85%) was recorded at 35 °C and the elevation of temperature to 40 °C resulted in decrease in phenol degradation to (80%), then decline phase occurred due to cell worsen [35]. The increase in immobilized weight increased the rate of degradation and decreased the degradation capacity. The initial phenol concentration play an important role in the biodegradation rate, where the degradation increased with the increase in initial phenol concentration until reaching maximum degradation at 75 mg/l [27]. For the studied species the maximum degradation capacity was 693.6 mg phenol/gm dry weight, at initial phenol concentration (700 mg/l). The effect of heavy metal that may be found in wastewater was studied using two metal ions Co^{+2} & Cu^{+2}. Previous studies stated that the presence of heavy metals reduced the degradation rate [36]. Results of the present study showed that the reduction in degradation rate decreased with the increase in cobalt concentration, where at 10ppm the degradation delayed 48 h while at 25, 50 and 100 ppm the degradation delayed 24 h. On the other hand, copper caused complete inhibition for phenol degradation. It has been found that phenol had little effect on biosorption of heavy metals, where biosorption increased in the presence of phenol. The obtained results indicated that, *M. terregens* had fast phenol degradation rate and its maximum degradation capacity was promising.

List of Abbreviations

CA: Calcium Alginate
MSM: Mineral Salt Media.
NORM: Naturally Occurring Radioactive Material.

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