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BIODEGRADATION OF PHENOL BY IMMOBILIZED *PROVIDENCIA RETTGERI*

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ABSTRACT

The cells of locally isolated, phenol-degrading *Providencia rettgeri* strain were immobilized by either entrapment within alginate beads or adsorption on silicate solid supports; celite R-634 and sintered glass rings (siran). Although the cells immobilized by adsorption on celite and siran showed high phenol degradation activity but phenol corrosion effect were observed on celite and siran particles as a release of fine powdery particles to the degration medium. The immobilized cells degraded phenol by 3 times more than free cells in synthetic liquid medium containing phenol as sole carbon and energy source. The shaken cultures of calcium alginate immobilized *P. rettgeri* cells degraded and utilized phenol as carbon and energy source in synthetic liquid medium up to 5 g/L. The cells immobilized with 3% w/v alginate showed the highest rate of phenol degradation and utilization and the optimum phenol degradation and utilization was shown with an inoculum size either with beads seeded with 2.6×10^8 cells/ml or the degradation medium loaded with 15 g seeded aglinate beads per 50 ml synthetic liquid medium in shakn cultures at 30°C for an incubation period 6 days.

INTRODUCTION

There is growing interest in biotransformation of environmental pollutants and in degradative strains because biodegradtaion provides the possibility to remove toxic compounds from contaminated site and water by biological methods[1,2].

In spite of the toxicity and carcinogenicity of phenol and the potential for environmental contamination due to its-scale production, yet there is little available information concerning its presence and persistence in the environment. Phenolic compounds are important plant constituents[3], and phenol is formed by the activity of microorganisms from verious natural compounds like tyrosine or synthetic products[4].

Several attempts were carried out to determine the feasibility of biological treatment for wastewater containing phenol and phenolic compounds. Although phenol and phenolic compounds are toxic pollutants and inhibitors of biodegradation; yet bacterial isolates like *Pseudomonas*[5], *Alcaligenes*[6], that are capable of degradation and growth in presence of phenol as their sole carbon and energy source have been isolated. *Providencia* is one of bacterial isolates that can degrade and grow with phenol concentration up to 1.5 mg/ml. *Providencia* was isolated from phenol-rich wastewater and different factors were studied[7].

The immobilized cells offer several advantages for the treatment of processing and waste streams. These include the retention of catalytic activity, the protection of cells from the effects of inhibitory substrates, and more efficient substrate mineralization through retention of intermediatory products[8,9]. The main goal for this study was :- (i) to test the effect of immobilization on the activity of a phenol-degrading *Providencia* strain, (ii) develop an immobilization technique which would maintain the activity of phenol-degrading *Providencia* strain, and (iii) maintain the long-term survive cells under toxic concentrations of phenol.

MATERIAL AND METHODS

Microorganism:

Locally isolated phenol-degrading *Providencia rettgeri* strain[7] that exhibited high phenol degradation activity was used in this study.

Medium:

Synthetic-Liquid (SL) medium: was used according to Ehrhardt & Rhem[9]; consists of (per liter): NH_4NO_3 1.0 g; $(NH_4)_2SO_4$ $5H_2O$ 0.5 g; NaCl 0.5; $MgSO_4$ $7H_2O$ 0.5g; K_2HPO_4 1.5g; KH_2PO_4 0.5g; $CaCl_2$ 0.01g; $FeSO_4$ $7H_2O$ 0.01g and trace elements 1.0 ml Trace element (freshly prepared) consists of: $MgCl_2$ $4H_2O$ 0.79g; $CuSO_4$ $5H_2O$ 0.64 g; $ZnSO_4$ $7H_2O$ 0.15 g and $FeSO_4$ 7 H_2O 0.11g in 100 ml distilled water. The final pH of the medium was adjusted to 6.9 by sodium hydroxide.

Synthetic-Solid (SS) medium : 2% w/v agar was added before sterilization to SL medium. Phenol was also added after sterilization as a sole carbon and energy source.

Preculture:

The preculture of *Providencia rettgeri* was carried out by growing the cells at 30°C with shaking (50 rpm) in SL medium supplemented with 0.5 gm/L phenol as sole carbon and energy source. After phenol consumption, the cells were recultured in SS medium (slant agar) with 0.5 mg/L phenol and incubated at 30°C for at least two days. Then the cells were harvested by sterile distilled water. For immobilization, the cells titre in the cell suspension was adjusted to approximately 1.7×10^6 cells/ml from phenol degrading *Providencia rettgeri* strain.

Chemicals:

Pure white crystals of phenol was obtained from Jessen; Belgium. Sodium alginate as purified powder was supplied from Kelco, Hamburg, Germany. Celite R-634 particles was supplied from Manville Crop. Denver, USA, sintered glass rings (siran) were provided from Schott, Mainz, Germany. Other chemicals used analytical reagent grade.

Immobilization techniques:

Entrapment in calcium alginate beads: sodium alginate powder (3 g) was dissolved in 98 ml hot distilled water with the aid of a magnetic stirrer. The viscous solution was sterilized by autoclaving at 110°C for 10 min[10]. A measured volume (2 ml) of the previously prepared preculture of phenol-degrading *P. rettgeri* was mixed well with the previously sterilized sodium alginate solution. The obtained suspension was extruded dropwise through a narrow glass tube into 2% w/v sterilized calcium chloride solution, using a peristaltic pump (Watson - Marlow limited, Falmouth, England), to form beads about 1.5-2.0 mm in diameter. The formed calcium alginate beads were held in calcium chloride solution for a gelation period of about 1 hr. After filtering and washing the beads on a sterile Buchner's funnel, the alginate beads were used to inoculate the degradation and growth medium.

Adsorption on Celite R- 634 and sintered glasss rings (Siran):

Celite R-634 was washed out and sterilized in hot air oven at 200°C for 6 hrs. Wet sintered glass rings were autoclaved at 121°C for 30 min[11]. A measured volume (300 ml) of the previously prepared preculture of phenol-degrading *P. rettgeri* was added to 75 gm of either celite R-634 particles or sintered glass rings. The mixture was gently shaken for a contact period of 110 minutes. After this period, the liquid supernatant was decanted, and the solid supports loaded with phenol-degrading cells were added to the culture medium.

Figure 1 showed the rate of adsorption of *P. rettgeri* into celite and siran particles. The quantity of immobilized microbial cells attached to the surface of the celite R-634 and siran was dependent on the immobilization time. After about 80 and 90 minutes for celite and siran respectively, an adsorption balance was reached between free and immobilized cells as there is no sharp decrease in the O.D. of non-adsorbed supernatants. The loaded supports was then added to the culture medium.

Preparation of inoculum:

About 12.5 g from either calcium alginate beads or celite R-634 or siran loaded with *P. rettgeri* were used to inoculate about 50 ml SL medium containing phenol as sole carbon and energy source.

Phenol assay:

Residual phenol was colorimetrically assayed according to Martin [16]; accurately measured, 1.0 ml of phenol-containing sample was mixed with 0.3 ml of 2% (w/v) 4-aminoantipyine, 1.0 ml of 2N ammonium hydroxide, and 1.0 ml of 2% (w/v) potassium ferricyanide. The resulted red colour was measured in the spectrophotometer at $\lambda_{max.}$ = 541nm.

RESULTS

Phenol Degradation by Immobilized *P. rettgeri* cells:

In a 250 ml Erlenmyer flask, about 12.5 gm from each washed seeded al
634 or siran was inoculated to 50 ml SL medium containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 and 2.0 mg/ml phenol. Each concentration was carried out in triplicate and all flasks were incubated with shaking (50 rpm), at 30°C for 6 days. About a 1.0 ml sample was withdrawn daily and diluted (up to 100 times), and the residual phenol was measured spectrophotometrically according to Martin[12].

It was noticed that, during incubation period (6 days), cultures with 0.1, 0.2, 0.6 and 0.8 mg/ml were completely degraded after the elapse of (2-4) days of incubation (table 1). At higher concentrations, 1.0, 1.2, 1.5 and 2.0 mg/ml, there was a residual phenol in the medium as show in the results except in the case of cells immobilized by adsorption on solid supports (celite and siran) which showed complete degradtion of 1.0 mg/ml phenol. In addition, there is more than 85, 80 and 70% of phenol were degraded at concentrations 1.2, 1.5, and 2.0 mg/ml in cells immobilized in the alginate beads or by adsorption on celite and siran. However, the residual phenol which was entrapped within the alginate beads or adsorbed on siran or celite particles were not determined except that entrapped within alginate beads, The beads were dissolved in about 50 ml phosphate buffer, pH 7.0, with gentle heating and constant stirring, till no beads were observed in the solution. The residual undegraded phenol concentrations were 0.05, 0.10, 0.15 and 0.19 mg/ml at the end of the incubation period, within the beads of medium with 1.0, 1.2, 1.5 and 2.0 mg/ml phenol, respectively, but in SL medium with lower concentrations, phenol was degraded completely.

Moreover, siran and celite showed phenol corrosion effects, especially at high phenol concentrations which led to the release of fine particles from siran and celite and powdery appearance in the medium was observed. Consequently, it was thought that the release of fine particle led to an increase in the surface area that might have adsorbed phenol and decreased the free phenol molecules that were available for degradation

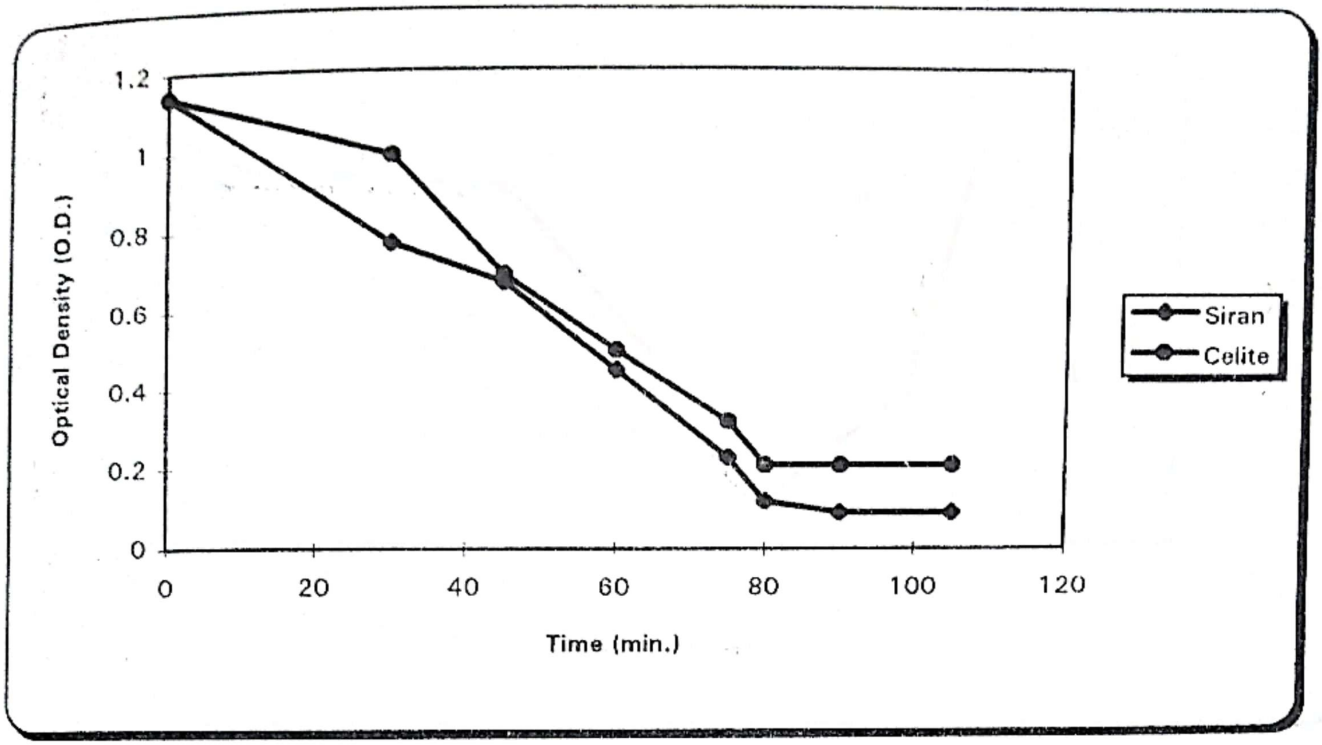

Fig. 1. Time course adsorption of *P. rettgeri* cells on the solid supports; sintered glass rings (Siran) and Celite R-634.

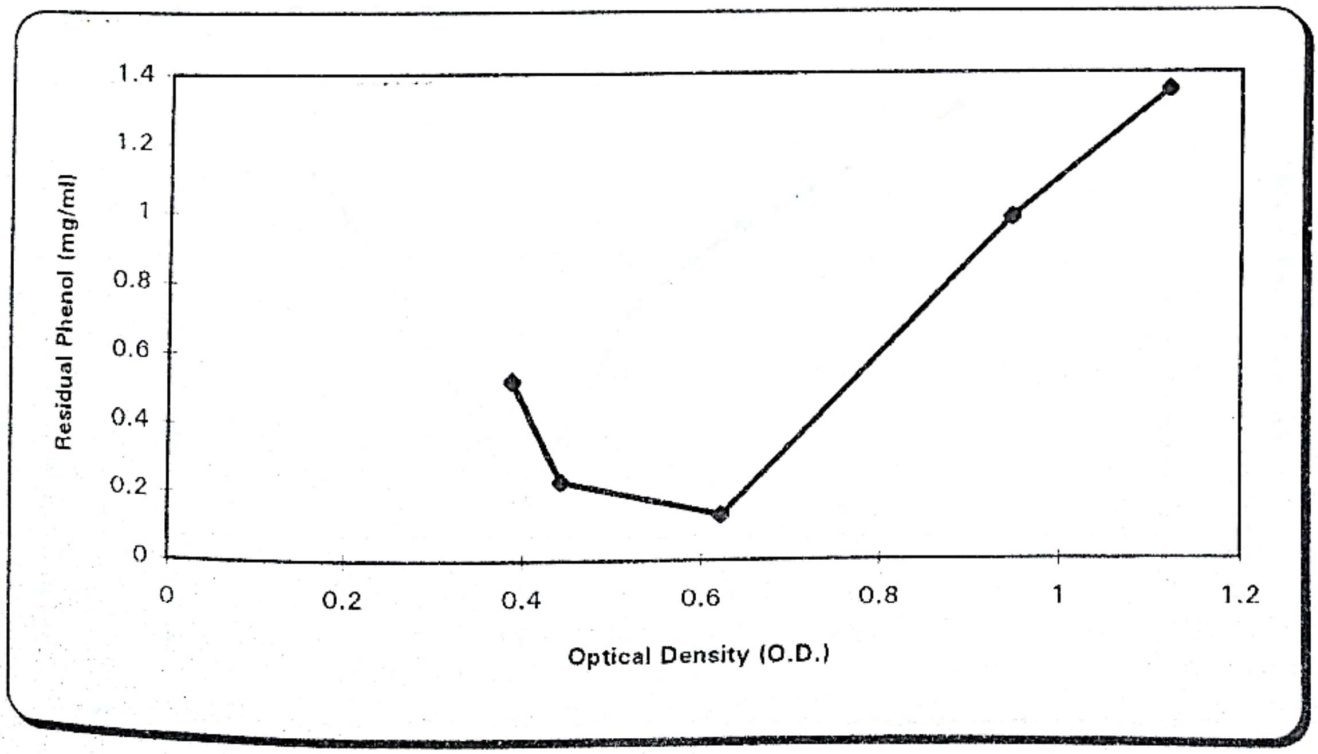

Fig. 2. Residual phenol in shaked cultures of *P. rettgeri* immobilized in calcium alginate beads seeded with different cell densities.

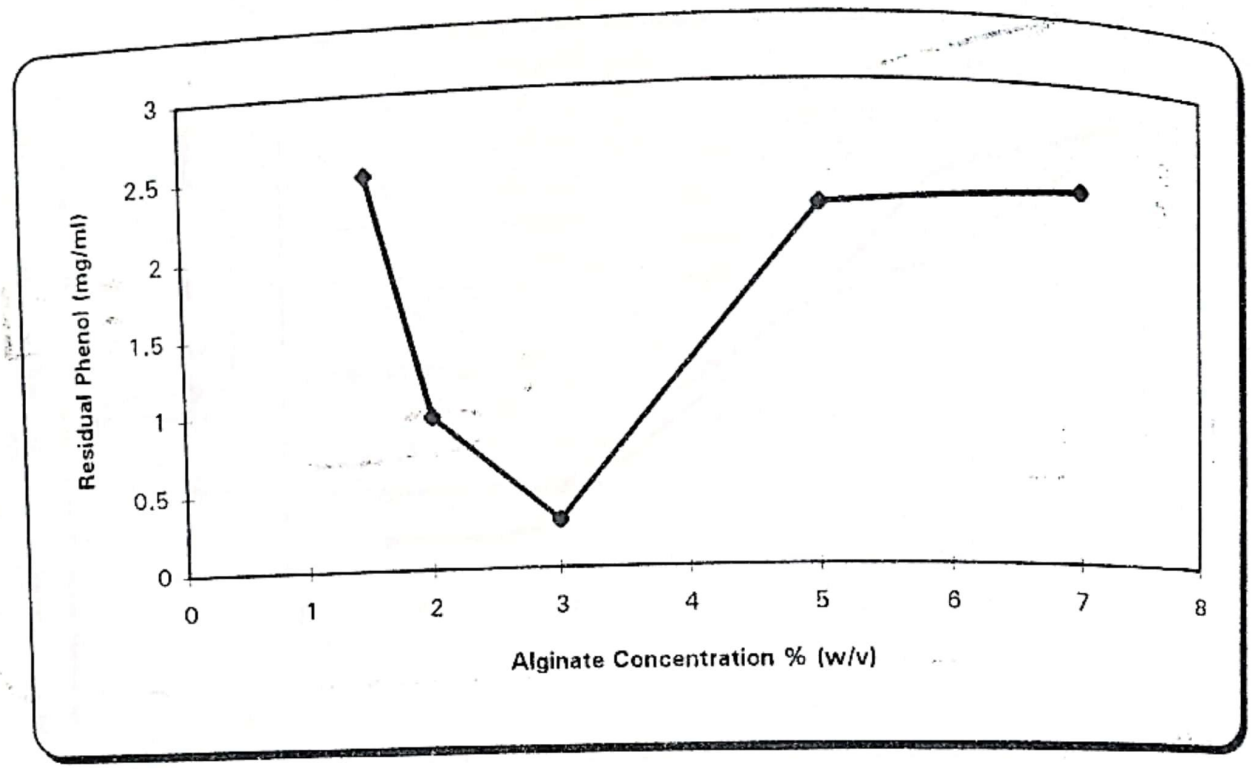

Fig. 3. Residual phenol in shaked cultures of *P. rettgeri* immobilized in beads of different calcium alginate concentrations.

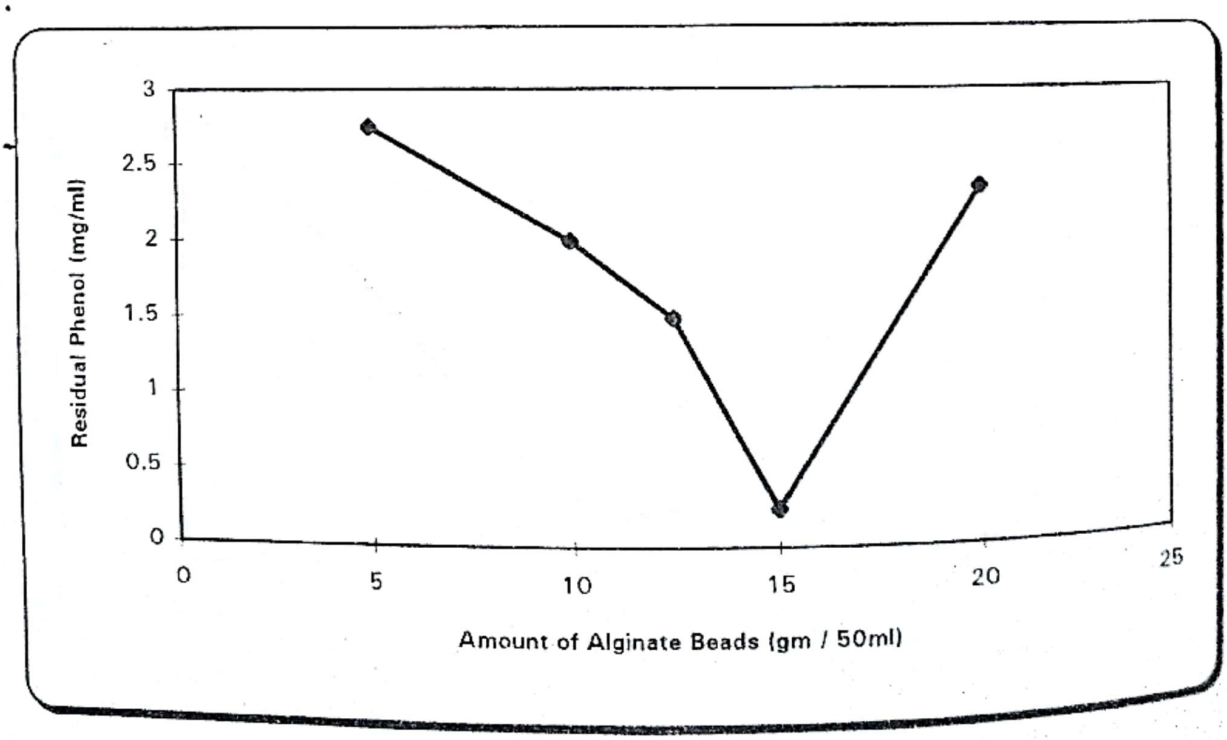

Fig. 4. Effect of the amount of alginate beads (gm / 50ml SL medium) on the phenol degradation and utilizaiton by calcium alginate immobilized *P. rettgeri*.

At the same time, the release of such fine particles affected the area of immobilization and the immobilized cells were released to the medium and they became free instead of immobilized. Also it might also affect the metabolism of phenol and nutrients by the degradable cells. These are the reasons that pushed in to avoid the immobilization by adsorption onto celite and siran from more studies.

Determination of degradation activity as well as the toxic concentration of phenol on *P. rettgeri* cells immobilized in calcium alginate beads:

In previous studies[7], it was shown that the toxic concentration of phenol on *P. rettgeri* was, 1.5 mg/ml, a maximum activity was at 1.0 mg/ml and an optimum acticity was at 0.8 mg/ml. In this experiment, different high concentrations of phenol were added as carbon and energy source to SL medium for determining the maximum, and optimum activities, and toxic concentration of phenol on *P. rettgeri* cells.

The phenol concentrations 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/ml were added to 50 ml SL medium in 250 ml Erlenmyer flasks. All flasks were shaken (50 rpm) and incubated at 30°C for 6 days. About 1 ml sample was withdrown daily for determining the residual phenol and consequentely the rate of phenol degradation for every concentration. Each phenol concentration was carried out in triplicate and the net result represents the mean. Table (2) representing the residual phenol that was undergraded and not utilized by *P. rettgeri* at the end of incubation period. From the table, it is clear that the toxic concentration was 6.0 mg/mL (6 g/L) phenol and the maximum activity of phenol degradation by calcium alginate immobilized cells of *P. rettgeri* was 5 g/L. While the optimum activity was obtained at 3 g /L phenol. This was confirmed by the values of viable count; the original viable cells imbedded in the alginate beads was 1.7×10^6 cells/ml in about 100 ml alginate solution while the maximum viable count obtained was 1.6×10^{19} cells/ml at concentration 3.0 g /L phenol. Moreover, the viable count at concentration 5.0 g /L phenol which considered the maximum degrading activity of phenol was 2.4×10^{12} cells/ml and at the toxic phenol concentration, 6.0 g /L was 2.0×10^8 cells/ml .

This signifies that, the 3.0 g/L phenol is the optimum concentration, that the immobilized cells of *P. rettgeri* can efficiently degrade and utilize it as sole carbon and energy source in SL medium, while 5.0 g/L phenol concentration, representing the maximum degradation activity of *P. rettgeri* calcium alginate immobilized cells.

Effect of the cell-density within the alginate beads:

The cells of *P. rettgeri* were cultured on slants of SS medium containing 100 µg/ml phenol, incubated at 30°C for 48 hr. The cells were harvested with sterile distialled water, the obtained cell-suspension was subjected for dilutions to be used as inoculum for seeding the alginate beads. About 2.0 ml from each cell-suspension, was added to 98 ml sodium alginate solution, mixed-well and then extruded through the peristaltic pump to 2% w/v calcium chloride solution to form seeded alginate beads. About 12.5 g seeded beads were inoculated to 50 ml SL medium containing 3 g/L phenol, and all cultures were incubated with shaking (50 rpm) at 30°C for 6 days. Figure 2, shows the results, the optimum cell suspensin for phenol degradation was 2.6×10^8 cells/ml (A = 0.6) that showed the lowest residual phenol in the medium at the end of incubation period and the maximum degradation activity then 3.1×10^6 cells/ml (A = 0.4) then 1.3×10^5 (A = 0.3). Inoculation with higher cell number than 2.6×10^8 cells/ml, showed low degradation activity and less phenol utilization as carbon and energy source.

Effect of the alginate concentration:

The cells of *P. rettgeri* were cultured in SS medium slants, then harvested and the absorbance was adjusted to A = 0.6 at λ_{max} = 541 nm with viable count of 2.6×10^8 cells/ml. About 2.0 ml cell suspension was added to 98 ml solution of 1.5%, 3%, 5% and 7% (w/v) sodium alginate. Each flask containing 50 ml SL medium with 3 g /L phenol as sole carbon and energy source, was inoculated by 12.5 g alginate beads of each alginate concentration. Each alginate concentration was inoculated in triplicate and the net results respresent the mean. All flasks were incubated at 30°C with shaking (50 rpm) for 6 days. Figure (3), shows that the most appropriate alginate concentration was 3% w/v, it showed the maximum phenol degradation and utilization under the sited conditions followed by 2% w/v alginate. While lower and higher alginate concentrations showed little degradation and less phenol utilization activity by immobilized *P. rettgeri* cells. It was also observed that 1.5% alginate solution resulted in soft beads which moderately dissoluted in the degradation medium. These might affect the degradation and utilization activity of the immobilized cells.

Effect of the amount of alginate beads:

The cell suspension of *P. rettgeri* of viable count 2.6×10^8 cell/ml (A = 0.6) was immobilized within 3% w/v alginate beads. Different amounts of seeded alginate beads, 5, 10, 12.5, 15 and 20 gm were inoculated to 50 ml SL medium containing 3 g/L phenol as carbon and energy source. The cultures were shaken (50 rpm) /at 30°C for 6 days. Figure (4) shows that the most suitable alginate amount was 15 g/50 ml SL medium which represented 1/3 of medium followed by 12.5 g and the lowest degradtiation rate was 5 g followed by 20 g beads inoculum size.

DISCUSSION

The grown cells of *P. rettgeri* like other bacteria as *Psudomonas* and *Alcaligines* before being immobilized, were subjected to a major disruption in their physical and nutritional environments at a time when they were most efficient in degrading the desired toxic compounds.

Table 1. Phenol degradation activity of *P. rettgeri* immobilized in 3% (w/v) calcium alginate beads, and adsorbed on celite R-634 and sran after incubation at 30°C with shaking for 6 days.

Immobilization method	Phenol conc. in SL medium (mg/ml)	Residual phenol (mg/ml) / time (day)					
		1	2	3	4	5	6
Entraped within calcium alginate beads	0.1	0.01	0	0	0	0	0
	0.2	0.02	0	0	0	0	0
	0.4	0.01	0	0	0	0	0
	0.6	0.2	0.1	0	0	0	0
	0.8	0.27	0.2	0.13	0.1	0	0
	1.0[a]	0.5	0.3	0.21	0.18	0.10	0.05
	1.2[b]	0.44	0.32	0.22	0.19	0.15	0.11
	1.5[c]	0.59	0.47	0.44	0.38	0.29	0.2
	2.0[d]	0.72	0.69	0.57	0.5	0.4	0.31
Adsorbed on celite R-634	0.1	0.03	0	0	0	0	0
	0.2	0.11	0	0	0	0	0
	0.4	0.14	0	0	0	0	0
	0.6	0.21	0.13	0.08	0	0	0
	0.8	0.19	0.17	0.06	0	0	0
	1.0	0.31	0.21	0.15	0.09	0	0
	1.2	0.69	0.52	0.35	0.33	0.21	0.16
	1.5	0.77	0.59	0.48	0.44	0.35	0.26
	2.0	1.3	0.81	0.68	0.63	0.52	0.5
Adsorbed on siran	0.1	0	0	0	0	0	0
	0.2	0	0	0	0	0	0
	0.4	0	0	0	0	0	0
	0.6	0.25	0	0	0	0	0
	0.8	0.27	0.15	0.09	0	0	0
	1.0	0.32	0.28	0.17	0.13	0	0
	1.2	0.46	0.42	0.3	0.26	0.2	0.1
	1.5	0.63	0.52	0.41	0.32	0.25	0.18
	2.0	0.81	0.72	0.62	0.4	0.36	0.25

a, b, c, and d represent the residual undegraded phenol inside the alginate beads; 0.05, 0.1, 15, and 19 (mg/ml) respectively at the end of incubation period and the phenol was assayed after dissolving the beads in phosphate buffer, pH 7.0.

Table 2. Determination the optimum and maximum phenol degradation activity as well as the toxic concentraion of phenol on the degredation activity and growth of *P. rettgeri* immobilized in 3% (w/v) calcium alginate beads.

Phenol concentration (mg/ml)	**Residual phenol (mg/ml)/time (day)**					
	1	**2**	**3**	**4**	**5**	**6**
1.5	0.92	0.69	0.48	0.33	0.10	0.10
2.0	1.10	0.90	0.70	0.50	0.20	0.20
3.0	2.40	1.80	0.70	0.52	0.10	0.10
4.0	3.10	2.60	1.89	0.55	0.49	0.30
5.0	3.40	2.70	1.22	0.66	0.40	0.40
6.0	5.90	5.90	5.80	5.80	5.70	5.57

The cells of *P. rettgeri* were able to degrade and grow in phenol up to 1.5 g/L in batch cultures, while the immobilized cells pocesses a higher potential for phenol degradation and utilization by about 3 times; up to 5 g/L; as shown in table 2, more than free cells. Moreover, the phenol concentration in the medium decreased clearly when the immobilized microorganism started growing, mostly after 2-4 days incubation. This might be due to the phenol is the only carbon and energy source in the medium, and the cells of *P. rettgeri* has the activity to degrade or transform it to the most available degradable and utilizable form.

This study indicates also a high affinity of some porous silicate supports like celite R-634 and siran to the cells of *P. rettgeri*. The favourable adsorption capacity of siran could be attributed to the large mean pore diameter of this support along with it porosity compared with the type of celite used. Siran is characterized by double pore structure which is determined by micro- and macropores. This structure may help in the adsorption of different microorganisms[13,14]. A disadvantage of using these supports is the week electrostatic interaction between the solid particles and the adsorbed cells[15]. This led to desorption of the cells from supports to outgrow. In this work, the residual phenol in the cultures of *P. rettgeri* adsorbed on celite and siran is low as indicated in table 1. This might be due directly to the degradation and utilization activity of the adsorbed cells or mostly due indirectly to the adsorption of phenol molecules to the surface of solid supports, especially phenol corrosion effect on such silicate solid supports was observed. This corrosion effect might have increased the phenol adsorption surface area and retared the degradation activity of the adsorbed cells through disruption of bacterial metabolism and decreasing the phenol molecules as well as other growth factors that should be available to be degraded by the immobilized cells.

The approach of the "*in situ* growth" from bacterial cells can be used conveniently to entrap cells in gel matrix without perturbing the later desirable activity[16,17]. Therefore, entrapment of cells of the phenol degrading strain of the locally isolated *P. rettgeri* in calcium alginate was chosen. Alginate concentration influenced the pore size and so the activity of the immobilized cells[17]. At high alginate concentrations (more than 3%), comparable smaller pore-sized beads were formed. This led mainly to retardation of growth of cells inside the gel as well as increased the mass transfer problem within the biological system.

Moreover, the formed beads were firm enough to decrease the availability of phenol and nutrients as well as the oxygen molecules to be utilized by the entrapped cells. For alginate concentration lower than 3%, the mechanical stability of the calcium alginate beads was not sufficient to keep the cells entrapped inside, and a comparable number of cells were grown outside the alginate beads. Therefore, alginate concentration should be kept around 3% as was reported before [10,18,19].

The optimization of immobilization conditions was further continued through changing the inoculum size by two strategies. The first strategy based on using fixed amount of support loaded with different cell densities. The second strategy based on using different amounts of calcium alginate beads with fixed cell density in a fixed degradation and growth medium. The optimum inoculum size was alginate amount of 15 gm per 50 ml medium. Each gram (milliliter) calcium alginate beads should contain about 2.6×10^6 cells. An increase or decrease in the optimum inoculum size led to a decrease in the degradation activity of the immibilized biocatalyst.

Generally, a balance between the availability of the substrate and the degradation activity of *P. rettgeri* cells should have occured. A large inoculum size may lead to insufficiency of the available nutrients to induce cell growth and consequently formation of weak cells that exhibited weak degradation activity. On the other hand, too small inoculum size may induce a non-restricted bacterial growth as a result of still available nutrients. Thus, the presence of still undergraded and non-utilized phenol might by due to depletion of other nutrients like nitrogen and minerals from degradation medium.

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التكسير البيولوجى للفينول بواسطة الخلايا المثبتة من بكتريا البروفدنشيا رتجيرى

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فى هذا البحث تم تثبيت خلايا بكتريا البروفدنشيا رتجيرى، المعزولة محليا والتى تكسر الفينول وتنمو عليه كمصدر وحيد للطاقة والكربون فى الوسط التخمرى التخليقى، داخل حبيبات الكالسيوم ألجينات أو بالإدمصاص على سطح السيلكون الصلب (السيران والسيلايت ر- ٦٣٤). ومع أن الخلايا المثبتة بالإدمصاص على سطح السيلكون (السيران والسيليت) قد أظهرت قدرة عالية على النمو وتكسير الفينول ولكن قد لوحظ تأثير الفينول على نفس درجة الإدمصاص الصلب (تآكل) بظهور حبيبات بودرة فى وسط تخمرى تخليقى. والدراسة أظهرت أن الخلايا المثبتة لها قدرة على النمو والتكسير للفينول أكثر بثلاث مرات من الخلايا الغير مثبتة (الحرة) فى وسط التخمر الذى يحتوى على الفينول كمصدر وحيد للكربون والطاقة. وكذلك إتضح أن الخلايا المثبتة داخل حبيبات الكالسيوم ألجينات فى وسط تخمرى تخليق مهتز تكسر الفينول وتنمو عليه حتى تركيز ٥ جرام/لتر. وبتثبيت الخلايا بداخل حبيبات كالسيوم ألجينات ذات تركيزات مختلفة من الألجينات، إتضح أن أعلى معدل لتكسير الفينول والنمو عليه كان بواسطة الخلايا المثبتة فى تركيز ٣٪ ألجينات. وكذلك قد أظهرت الدراسة أن معدل تكسير الفينول والنمو عليه كان عاليا بواسطة الخلايا المثبتة داخل حبيبات ٣٪ ألجينات التى تحتوى على ٢,٦ × ١٠ ^ خلية أو أن الوسط التخمرى يُزرع بكمية من حبيبات الألجينات قدرها ١٥ جرام/ ٥٠ مل وسط تخمرى مهتز لمدة ٦ أيام عند درجة حرارة ٣٠ °م.