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Optimization of Operative Different Conditions Affecting Phenol Degradation by Free and Entrapped *Acinetobacter johnsonii* Cells

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Abstract: Optimization of cultural condition that facilitates the degradation of phenol by isolated bacterial strain *Acinetobacter johnsonii* (from Makkah Al-Mukarramah region) free and entrapped cells was attempted. Bacterial cells were entrapped in agar-alginate beads. Initial pH of the medium that gave the highest rate of degradation was pH 7 and incubation temperature of 30°C. Supplementing the medium with glucose, yeast and meat extract did not improve degradation rate, but it showed an adverse effect. Optimum concentration of ammonium salts, potassium salts and magnesium sulphate were 1.5, 1.2 and 0.3 g L⁻¹, respectively. Optimum concentration of trace elements was 2 ml L⁻¹ and its omission reduced the rate of degradation. Phenol degradation in the air bubble reactor was superior to that of shaken cultures where phenol concentration up to 5 g L⁻¹ were degraded. The dried biocatalyst lost about 73% of its original phenol degradation activity but repeated cultivation restored some of its activity. The same biocatalyst kept 58% of its activity after a 12 weeks storage, while the wet beads lost 62.5% after the same storage period. Small amounts of phenol were fed batch wisely at a high frequency resulting in a phenol degradation higher than that obtained when large amounts were used.

Key words: Optimization, phenol degradation, entrapment, *Acinetobacter johnsonii*, semicontinuous culture

INTRODUCTION

Phenols are distributed either as natural or artificial mono-aromatic compounds in various environmental sites. As major pollutants, their existence in industrial waste water treatment plants, such as oil refineries, petrochemical plants, coking plants and phenol resin industry plant, has been well established^[1]. Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents and landfill run off waters^[2]. The toxicity of phenolic compounds often results in the reduction of wastewater biotreatment even at relatively low concentrations^[3].

In wastewater treatment, reactors with immobilized microbial cells may offer several advantages over processes with suspended biomass. These advantages include: retention of a high concentration of microorganisms in the reactor, protection of cells from toxic substances, prevention of suspended particles from the effluent^[4]. As a result, the immobilized cell reactors are favored because they provide high efficiency treatment with somewhat compact reactors.

Recent studies showed that optimizing the culture medium could enhance the biodegradation of xenobiotics. A positive impact on biodegradation of aliphatic chlorinated xenobiotics has been observed when the

culture medium supplemented with minerals^[5]. It was also showed that yeast extract, vitamins and trace elements could significantly enhance the aerobic degradation rate of chlorobenzoic acid isomers^[6,7].

Some workers studied the biodegradation of phenolic wastes in semicontinuous cultures using either entrapped or adsorbed cells as the biological active catalyst in different types of bioreactors^[8-12].

The present study focused on the optimization of bioremediation process using agar-alginate entrapped *Acinetobacter johnsonii*, as well as drying up the biocatalyst, which may improve its handling and delivery, which make it more feasible and compatible with the current practices. The activity and viability of the agar-alginate beads was studied before use in semi-continuous cultures.

MATERIALS AND METHODS

Microorganism: *Acinetobacter johnsonii* was isolated among others from garden soil samples (from Makkah Al-Mukarramah region) enriched with phenol and found to be the most active organism in phenol degradation and was used throughout this study. It was identified by the help of Dr. Susanne Verberg, DSMZ-Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH.

Phenol assay: The method described by Korenman *et al.*^[13] was used. It is based on rapid condensation with 4-aminoantipyrine followed by the oxidation with potassium ferricyanide under alkaline conditions to give a red coloured product, which immediately measured at 492 nm using a spectrophotometer.

A standard curve was prepared with pure phenol. The measurement was found to be linear with substrate concentrations between 0.1 and 10 mg L⁻¹ for phenol. It had a sensitivity least ±0.1 mg L⁻¹.

Growth determination: Determination of growth or cell leakage was performed by measuring the turbidity (OD) of the culture at 546 nm using a spectrophotometer (Bechman). The dry weight was indirectly determined by means of prepared calibration curve where in the dry weight measurements were plotted versus relevant turbidity.

Entrapment in agar-alginate: Two gram agar and 0.75 g. sodium alginate were dissolved in 80 mL water and stirred well before sterilization. Twenty milliliter cell suspension was added and mixed well. The formed mixture (3 mg dry weight cells/ml gel) was aseptically extruded dropwise through a needle into a cold solution of 2% CaCl₂. The formed beads were transferred to a phosphate buffer solution (1.46% K₂HPO₄ and 0.22% KH₂PO₄) for 2 h, to remove the alginate from the beads. Beads were then washed and transferred to the desired cultivation medium.

Mineral medium: Contained (g L⁻¹): (NH₄)₂SO₄, 0.5; NH₄NO₃, 1.0; MgSO₄·7H₂O, 0.5; K₂HPO₄, 1.0; KH₂PO₄, 0.5; NaCl, 0.5; CaCl₂, 0.02; 2 mL trace elements solution^[14] consisting of (per l) FeSO₄·7H₂O, 5 g; H₃BO₃, 25 mg; CuSO₄·5H₂O, 5 mg; KI, 5 mg; CoSO₄·7H₂O, 0.3 g; MnSO₄·4H₂O, 3 g; ZnSO₄·7H₂O, 5 g; NaMoO₄·7H₂O, 12 mg. The medium was adjusted to pH 7.0. After sterilization by autoclaving at 121°C for 20 min, the phenol was added with the desired concentration.

Optimization of cultural conditions: The concentration of different chemical constituents of the chosen cultural medium were varied to obtain the optimum concentration of each component which will yield the maximum rate of phenol degradation.

To study the effect of initial pH, the reaction of the medium was adjusted with few drops of concentrated NaOH or HCl solution to obtain a pH range of 4-8. Also the effect of incubation temperature was studied in the range (20-40°C).

The study involved supplementing the medium with different glucose from 0.0-5.0 g L⁻¹, yeast and meat extract from 0.0-2.0 g L⁻¹ concentrations.

To study the effect of nitrogen source on phenol degradation and cell leakage different concentrations of NH₄NO₃ from 0.0-1 g L⁻¹ and (NH₄)₂SO₄ from 0.0-0.5 g L⁻¹ were used in a proportion of 2:1 w/w, respectively.

To study the effect of mineral salts on phenol degradation, different concentrations of each of the following ingredients were successively tested: K₂HPO₄ from 0.0-1 g L⁻¹; KH₂PO₄ from 0.0-0.5 g L⁻¹ and MgSO₄·7H₂O from 0.0-0.5 g L⁻¹; trace elements solution from 0.0-4 ml L⁻¹.

Fermentation: Cultivation was carried out in 250 mL Erlenmyer flasks, each containing 100 mL of a mineral medium, were either inoculated with 20 mL agar-alginate beads loaded with *Acinetobacter johnsonii* cells (biocatalyst) or with a cell suspension of an equal quantity of cells (free cells) and incubated at 30°C in an incubator shaker (180 rpm). In all experiments the residual phenol concentration was determined in the culture at different time intervals, until complete phenol consumption was attained.

Cultivation was performed in an air bubble reactor (a glass column of 5 cm diameter and 30 cm height) was filled with 100 mL phenol mineral medium and inoculated with 20 mL agar beads entrapping *Acinetobacter johnsonii* cells or with an equal quantity of free cells. Sterile air was pumped (1.5 l min⁻¹) through a perforated glass plate at the bottom of the column (Fig. 1).

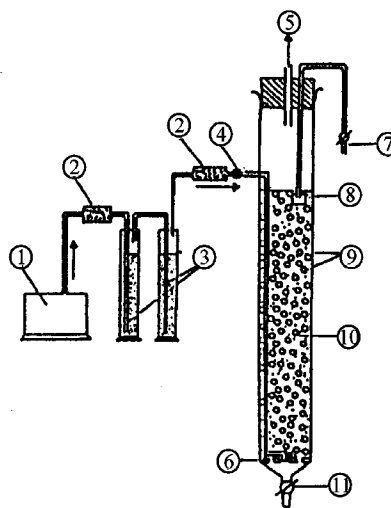


Fig. 1: Schematic drawing of the bed aerated bioreactor (1) Air pump; (2) Sterile air filters; (3) Sterile distilled water; (4) Air inlet; (5) Air outlet; (6) Perforated glass plate; (7) Sampling outlet; (8) Sintered glass large sieve cylinder to avoid bead passage; (9) Bio catalyst beads; (10) Sterile medium; (11) Medium outlet.

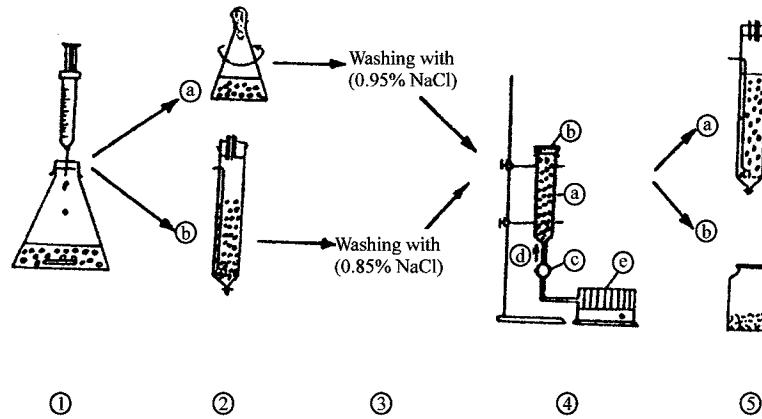


Fig. 2: Schematic representation of the process used for the preparation of the dried biocatalysts (dried agar gel beads entrapping *A. johnsonii* cells)

1) formation of the agar gel beads; 2) Activation of the beads by cultivation either in shaken flasks or in the air bubble reactor; 3) Washing; 4) Drying using sterile air over night, (a) sterile syringe, (b) sterile sheath cloth (2 layers with a thin cotton pad between them), (c) sterile air filter, (d) air stream, (e) air pump, 5) The obtained dried biocatalysts are either (a) cultivated or (b) stored in a sterile container.

Semi continuous cultivation (feeding) was performed in the air bubble column. Phenol was fed to the culture in doses of different magnitudes (from 0.2 to 0.6 g L⁻¹). When phenol was substantially degraded a further dose was fed to the culture.

Drying up the biocatalyst: Agar-alginate beads entrapping *Acinetobacter johnsonii* cells were first activated to reach their maximum phenol degradation activity. The resulted active biocatalyst were washed several times with saline solution (0.85% NaCl), then aseptically transferred to a sterile syringe. The drying process was carried out by blowing a sterile air stream into the syringe over night, after which dried agar-alginate beads were obtained and stored in a sterile container or used directly for phenol degradation.

Storage of the biocatalyst: The wet agar-alginate beads as well as the fresh dried beads of *Acinetobacter johnsonii* were washed several times then suspended in a saline solution (0.85% NaCl) and stored at 10°C for the desired period. Both types of beads were kept in a sterile closed container. After different storage periods both biocatalysts were cultivated in the bioreactor to evaluate their phenol degradation activity (Fig. 2).

RESULTS AND DISCUSSION

The pH range of 7 to 8 seems appropriate for both free and entrapped cell cultures to degrade phenol.

However, pH 7 was the optimum value in giving the highest rates of degradation in both cultures (Fig. 3). The results are in accordance with those reported by others. At pH 4 the rate of degradation was reduced to 5 and 57% by free and entrapped cells, respectively and cell leakage was increased probably due to the effect of acidity on the stability of the biocatalyst, such results are in agreement with those reported by other researchers^[15,16].

The effect of incubation temperature showed that a maximum activity in phenol degradation in both free and entrapped cell cultures of *A. johnsonii* was recorded in the

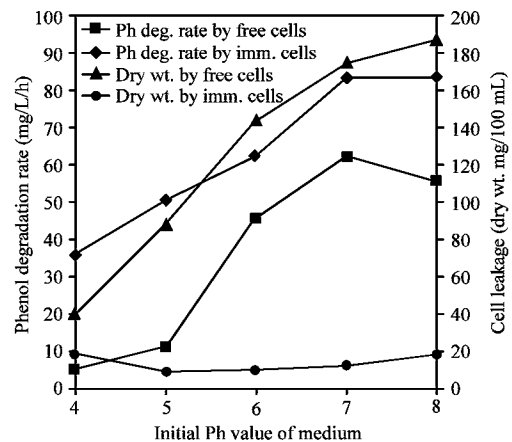


Fig. 3: Effect of initial pH value on phenol (0.5 g L⁻¹) degradation by free and immobilized cells of *A. johnsonii*

range 30-35 and 30-40°C, respectively (Fig. 4). The decrease of temperature to 20°C reduce the rate of degradation to 67 and 75% in both free and entrapped cell cultures, respectively higher temperature (40°C) reduce the rate of degradation to 80% by free cells. These results are in agree with those reported for the degradation of aromatic compounds by other microbial strains^[16-18].

The addition of glucose, yeast and meat extract with different concentrations did not improve the rate of degradation of either free or entrapped cell cultures, but it showed an adverse effect on the rate of degradation (Fig. 5-7), probably because of the presence of these substances interferes with the metabolism of phenol in the cells by inhibiting the production of the oxidative enzyme responsible for its degradation^[15].

The absence of ammonium salts from the medium not only decreased cell leakage from the biocatalyst by about

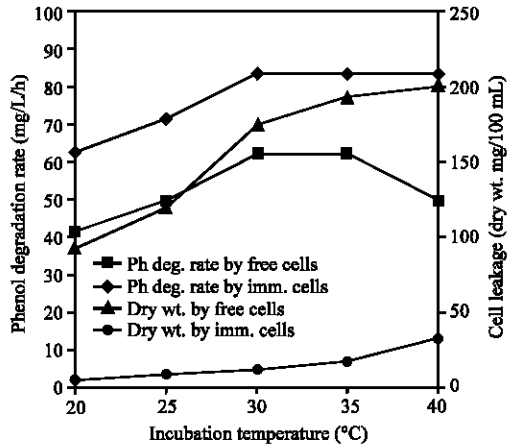


Fig. 4: Effect of temperature on phenol (0.5 g L⁻¹) degradation by free and immobilized cells of *A. johnsonii*

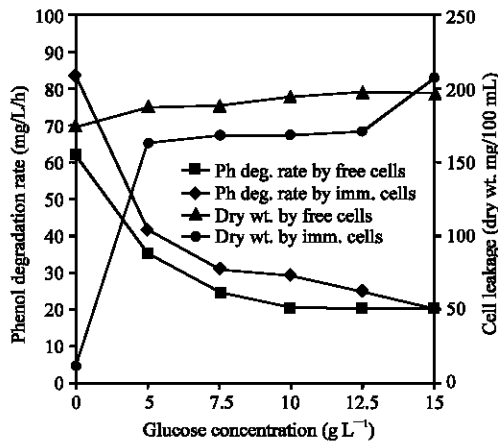


Fig. 5: Effect of different concentrations of glucose on phenol (0.5 g L⁻¹) degradation by free and immobilized cells of *A. johnsonii*

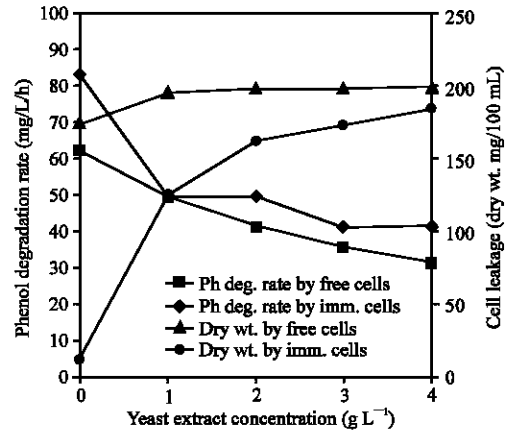


Fig. 6: Effect of different concentrations of yeast extract on phenol (0.5 g L⁻¹) degradation by free and immobilized cells of *A. johnsonii*

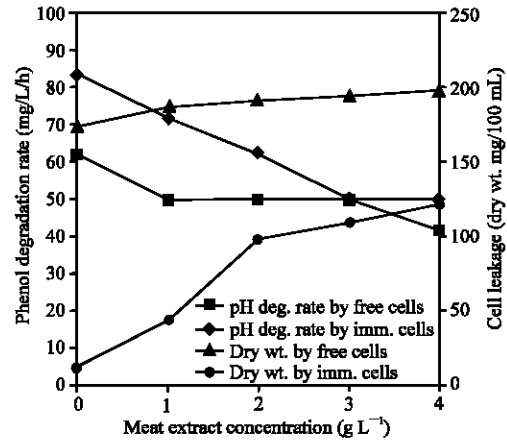


Fig. 7: Effect of different concentrations of meat on phenol (0.5 g L⁻¹) degradation by free and immobilized cells of *A. johnsonii*

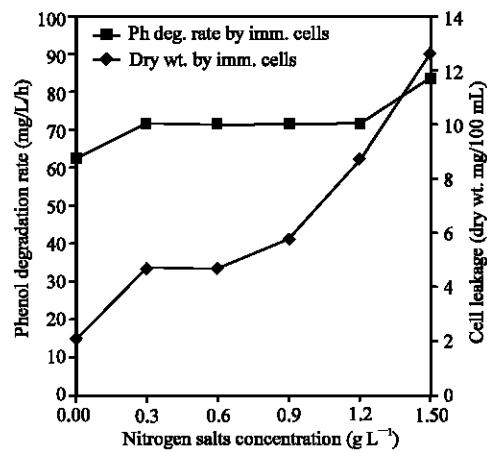


Fig. 8: Effect of different concentrations of nitrogen salt on phenol (0.5 g L⁻¹) degradation by immobilized cells of *A. johnsonii*

83.5%, but also reduced the degradation rate by about 25% while by increasing the nitrogen source to about 1.5 g L⁻¹ ammonium salts, the degradation rate was at its maximum value (Fig. 8). These results indicate the importance of supplementing the medium with nitrogen to improve the degradation rates^[19,20]. On the other hand, cell leakage may not be a problem as most of the cells are still captured in the gel matrix^[21].

Omission of potassium salts from the medium reduced the degradation ability by about 25% and the cell leakage also was reduced by about 82.5%. The same results were obtained at the potassium salts concentration 0.3 g L⁻¹ (Fig. 9). The optimum potassium salts concentration was range from 1.2 to 1.5 g L⁻¹. These results are in partial agreement with those of Kostenbader and Flecksteiner^[22] who reported that when potassium supply was stopped the oxidation of the aromatic compound ceased after a period of time.

The absence of magnesium sulphate from the mineral medium reduced the growth and degradation activity of entrapped cell culture by about 33% (Fig. 10). The rate increased by increasing the magnesium sulphate concentration up to 0.2 g L⁻¹. Higher concentrations did not affect the activity. Dagley and Gibson^[23] proved that Mg⁺² ions stimulated an enzyme involved in the oxidation of phenolic compounds by bacteria.

Omission of trace elements from the medium reduced the degradation rate to about 45% in entrapped cells (Fig. 11). By increasing the trace element concentration the degradation rate increase. Highest value was obtained at 2 mL⁻¹ concentration. Many investigators showed the importance of trace elements on the process especially Fe⁺² ions because of their effect on oxidase enzymes^[24].

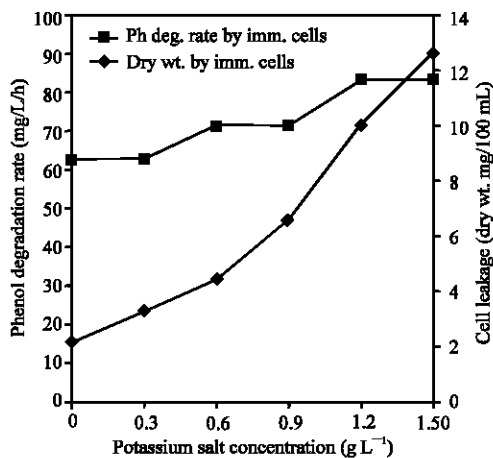


Fig. 9: Effect of reduced potassium salt concentrations of on phenol (0.5 g L⁻¹) degradation by immobilized cells of *A. johnsonii*

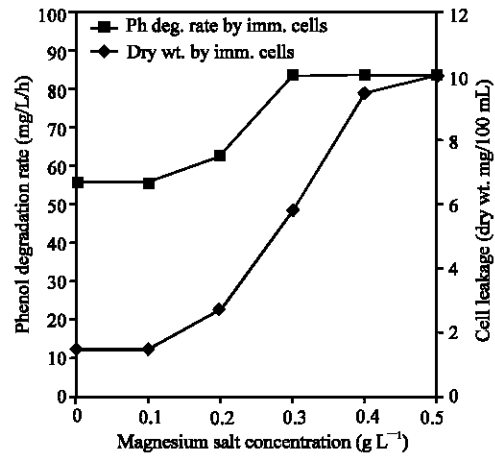


Fig. 10: Effect of reduced magnesium salt concentrations on phenol (0.5 g L⁻¹) degradation by immobilized cells of *A. johnsonii*

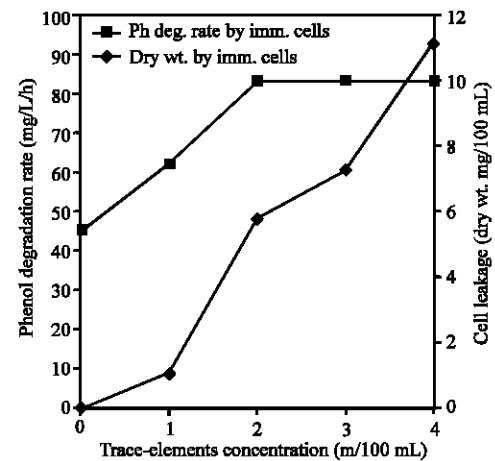


Fig. 11: Effect of different concentrations of trace-elements on phenol (0.5 g L⁻¹) degradation by immobilized cells of *A. johnsonii*

Biodegradation of different concentrations of phenol in the bioreactor by free and entrapped cells was performed. The degradation rate by entrapped cells was 1.3, 1.2, 1.8 and 2.2 fold of that of free cells at phenol concentrations 0.5, 1.0, 1.5 and 2.0 g L⁻¹, respectively (Fig. 12). Phenol degradation rate of free or entrapped cells was higher in the bioreactor than in shaken cultures (Table 1) probably because of better aeration. Entrapped *A. johnsonii* cells were able to degrade phenol concentrations up to 5 g L⁻¹, but with a relatively slow rate (56.82 mg/L/h). Similar results concerning better degradation of hydrocarbons and phenol, were reported upon using air bubble columns^[11,25,26].

The dried agar-alginate beads swelled reaching about 50% of their original size after cultivation in the bioreactor

Table 1: Degradation of different phenol concentrations by free and immobilized cells of *A. johnsonii* in shaken culture and in air lift bioreactor

Phenol concentration (g L ⁻¹)	Phenol consumption time (h)				Phenol degradation rate (mg/L/h)			
	Shaked culture		Air lift bioreactor		Shaked culture		Air lift bioreactor	
	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells
0.5	6	8	6	8	83.33	62.50	83.33	62.50
1.0	12	24	10	12	83.33	41.67	100.00	83.33
1.5	18	40	11	20	83.33	37.50	136.36	75.00
2.0	30	-	13	29	66.66	-	153.85	69.97
2.5	44	-	24	-	56.81	-	104.17	-
3.0	58	-	40	-	51.72	-	75.00	-
3.5	-	-	48	-	-	-	72.91	-
4.0	-	-	60	-	-	-	66.67	-
4.5	-	-	72	-	-	-	62.50	-
5.0	-	-	88	-	-	-	56.82	-

Table 2: Effect of drying the biocatalyst of *A. johnsonii* on phenol degradation

Phenol concentration (g L ⁻¹)	Number of reincubation	Dried beads		
		Phenol consumption time (h)	Phenol degradation rate (mg/L/h)	Relative activity (%)
0.5	1	22	22.73	100.0
	2	12	41.67	183.0
	3	11	45.45	200.0
	4	10	50.00	220.0
	5	10	50.00	220.0
1.0	6	23	43.48	191.0
	7	19	52.63	231.5
	8	19	52.63	231.5
	9	14	71.43	314.0
	10	14	71.43	314.0
1.5	11	25	60.00	264.0

Table 3: Effect of storage (starvation) of wet and dried biocatalyst of *A. johnsonii* on phenol (0.5 g L⁻¹) degradation activity

Storage period (month)	Wet biocatalyst			Dried biocatalyst		
	Phenol consumption time (h)	Phenol degradation rate (mg/L/h)	Relative activity (%)	Phenol consumption time (h)	Phenol degradation rate (mg/L/h)	Relative activity (%)
0	6	83.33	100.00	22	22.73	100.00
4	12	41.67	50.00	29	17.24	75.90
8	13	38.46	46.15	40	12.50	55.00
12	16	31.25	37.50	52	9.61	42.30

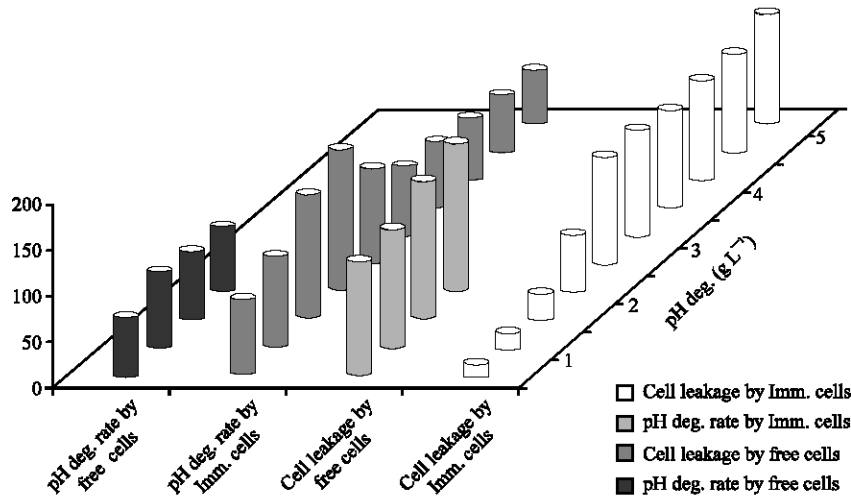


Fig. 12: Degradation of different phenol concentration by free and immobilized cells of *A. johnsonii* in the air lift bioreactor

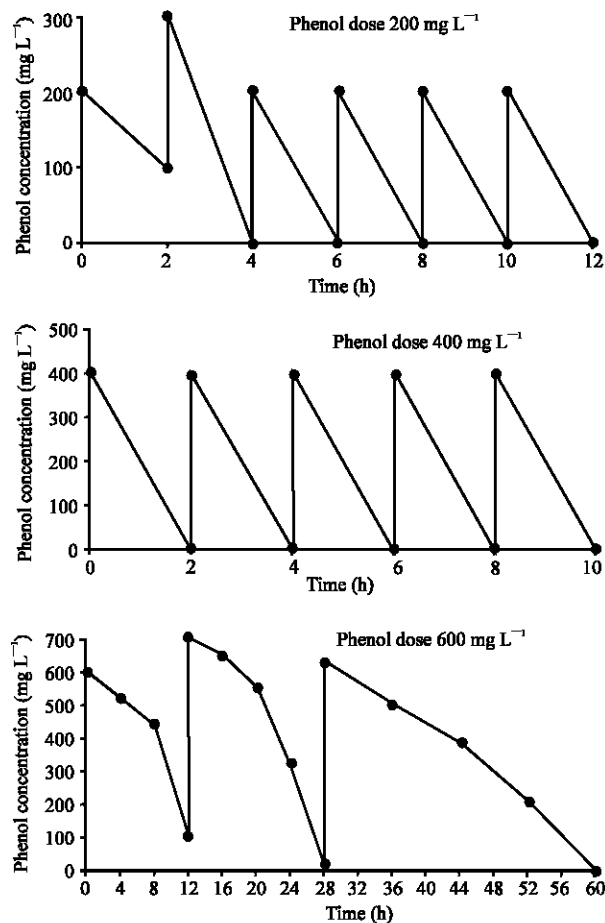


Fig. 13: Semi continuous degradation of phenol by immobilized cells of *A. johnsonii*

and their phenol degradation activity lost 73% of that before drying. However, the dried beads restored 33 and 59% of its activity after 5 successive reincubations at initial phenol concentrations 0.5 and 1.0 g L⁻¹, respectively (Table 2). The previous observations indicate that the viable cells present in the dried beads were grown again. A partial loss of the viability of entrapped microorganisms after drying was also observed^[7,10,27].

Wet entrapped cells of *A. johnsonii* lost 50% of their original phenol degradation activity after 4 weeks storage period (half-life time). The highest resistance for storage was noticed for the dried beads since they lost 50% of their original phenol degradation activity after more than 8 weeks. After 12 weeks storage the dried beads lost 58% of their original activity while wet beads lost 62.5% after the same storage period (Table 3).

The ability of the biocatalyst to degrade phenol in a semicontinuous culture indicates that small amounts of phenol (0.4 g L⁻¹) could be fed at a much higher frequency than greater amounts (0.6 g L⁻¹). The balance

after a degradation time of 82 h showed that high frequency low dosage of phenol (0.4 g L⁻¹) resulted in an increase of phenol degradation rate by 6.7-fold higher than that obtained in low frequency high dosage of (0.6 g L⁻¹) phenol (Fig. 13). This could be explained by the relative toxicity of the fed substrate. Thus it may be assumed that the low dosage feeding of phenol would be the best degradation system^[10,28], also the elevation of phenol concentration in the semicontinuous cultures up to 400 mg L⁻¹ resulted in a 2.4 fold increase in degradation rate.

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