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Phenol removal by newly isolated *Acinetobacter baumannii* strain Serdang 1 in a packed-bed column reactor

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ABSTRACT

A newly isolated *Acinetobacter baumannii* strain Serdang 1 was explored for its potential in phenol remediation in batch and continuous system. An immobilization cell system has been successfully developed to remove phenol in a batch system as high as 2,000 mg/L in 12 d at a rate of 6.04 mg/L/h. Repeated use of immobilized cells as many as five cycles was shown without any loss of activity. The continuous system in a packed-bed reactor achieved 65–77% phenol removal at the rate of 38.4 mg/L/h for 200 mg/L influent, which was almost three fold higher than the batch system. Low influent flow rate at 1.5 mL/min and bed height-to-diameter ratio of 15.2 reached steady state faster than the higher flow rate, and the percentage of phenol removal was also higher.

Keywords: Acinetobacter baumannii; Phenol; Bioremediation; Packed-bed column reactor

1. Introduction

Phenol and its derivatives, distributed throughout the environment as artificial or natural mono-aromatic compounds, are some of major hazardous compounds in industrial wastewater and could pose serious eco-logical problems [1]. Being water-soluble, phenol is likely to eventually reach irrigation water and down-stream drinking water sources and can cause severe odor and taste problems even at low concentrations, causing health hazards to humans, animals, plants, and micro-organisms [2–4]. A concentration of 1 μg/L has been described as permissible for drinking water [1], concentrations greater than 50 mg/L are toxic to

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some form of aquatic life, and ingestion of 1 g of phenol can be fatal in human beings [5].

Different methods to remediate sites contaminated with phenols include the use of activated carbons and adsorption [6,7], chemical oxidation, solvent extraction or irradiation, oxidation by ozone, ion exchange, and biodegradation. Biodegradation has become an increasingly popular strategy for *in situ* remediation as it is environmentally friendly, more economical, and offers the possibility of complete mineralization of phenol [8]. Phenol toxicity studies suggest that bacteria can adapt to low phenol concentrations, but the overall phenol biodegradation drops with increasing phenol concentrations. At toxic concentrations, phenol inhibits microbial growth and cause cell lysis [1,8,9].

Immobilization of biomass has several advantages including increasing the biodegradation rate, enhancing bioprocess control, improving biocatalyst stability, and advancing tolerance against harsh environmental conditions [9]. Immobilization technique such as cell entrapment within calcium alginate, in which the living cells are enclosed in a polymeric matrix allowing the diffusion of substrates into the cells and products away from the cells, is one of the most used techniques. Calcium alginate gel is cheap and readily available, and forms rapidly in mild conditions to provide suitable media for the entrapment of whole microbial cells [10]. As fermentation parameters may affect reproducibility, the effect of important parameters on phenol degradation should be determined and optimized.

The objectives of this study were to determine important parameters for phenol removal in a batch and continuous, packed-bed system. Comparison of the activities of phenol removal by Ca-alginate immobilized and freely suspended cells were also made.

2. Materials and methods

2.1. Bacterial Isolation

The bacterial strain was provided by Bioremediation Laboratory, Universiti Putra Malaysia. It was isolated from the soil contaminated with petrol wastes from a car workshop in Serdang, Selangor, Malaysia. The strain was initially assigned Isolate Serdang 1. The morphology of the bacteria was observed by Gram staining and microscopy. Important features such as the shape, elevation, and pattern of colonies were observed and recorded [11].

Microbial isolation strategies usually involve a period of enrichment in liquid culture, followed by separation of organisms in or on solid media where they are allowed to grow as colonies [12]. Bacteria capable of utilizing phenol as a sole carbon source was isolated by enrichment in mineral salts medium containing 500 mg/L phenol as carbon source. The isolation of microbial strains was done by plating technique as prescribed in APHA 9215 [13]. This enables observation and enumeration of colonies. A series of dilutions was made and selected, transferred to the Petri dishes and then the molten phenol–agar medium was poured in. When the agar solidified, the plate was incubated for 48 h at room temperature. Colonies would grow within as well as on the surface of the agar plate. Single colony was plated out and bacterial colonies obtained. In order to obtain pure cultures, a small portion of an individual colony was picked up using sterile inoculating loop and then streaked out on fresh agar plate. In this way, mixed culture can be separated into single colonies and grown up individually as culture stocks.

2.2. Culture media

The medium used in all experiments was as follows (g/L) [14]: 1.0 $(NH_4)_2SO_4$ (R and M Chemical, China), 0.25 $MgSO_4{\cdot}7H_20$ (Merck, Germany), 0.25 KH_2PO_4 (Merck, Germany), 0.07 $CaCl_2{\cdot}2H_2O$ (Merck, Germany), 0.1 yeast extract, and 0.5 phenol (Hamburg Chemical, Germany). The medium was autoclaved (Hirayama, USA) at 121˚C for 15 min. Phenol solution was filter sterilized using 0.2-μm syringe filter (Sartorius, Germany) and added to the sterilized medium at the beginning of the experiment. For the preparation of phenol solid medium in plates, 20 g/L of agar was added.

2.3. Bacterial stock and working culture

Well-defined colonies on the basis of morphological characteristics of pure *Acinetobacter baumannii* strain Serdang 1 isolates were transferred to a phenol medium slant and preserved at 4˚C in refrigerator for future use.

Working culture was streaked and transferred on fortnightly basis onto phenol agar medium and preserved at 4˚C in refrigerator for ongoing experiments. The plates were incubated at 30˚C for 48 h. Single colony was transferred into a universal bottle containing 5 mL liquid phenol medium at 500 mg/L phenol concentrations. Cultures were incubated on a rotary shaker at 30˚C for 48 h at 100 rpm. One percent of bacterial suspension (Absorbency, $A_{600} = 1.0$) was inoculated into a 250 mL-Erlenmeyer flask (Pyrex) containing 50 mL liquid phenol medium and incubated (100 rpm) at 30˚C. The cultures were used as inoculum for experiments after leaving overnight (~15 h).

2.4. Cell immobilization

For the preparation of calcium alginate beads, sodium alginate was first dissolved in boiling water and autoclaved (Hirayama, USA) at 121˚C for 15 min. The bacterial liquid cultures were aseptically centrifuged (Selecta, Spain) at room temperature at 7,000 rpm for 5 min and the supernatant discarded. The pellet was resuspended in sodium alginate (BDH Chemical, Australia) at 35 mg wet weight for 1 mL sodium alginate solution. The mixture was then extruded drop wise by gravity into a sterile calcium chloride solution using sterile syringe (12 mL). The alginate droplets subsequently gelled, upon contacting with calcium chloride solution to form uniformly sized spheres. The beads were kept in calcium chloride solution at 30˚C for 2 h to complete the gel formation. This way, insoluble and stable immobilized beads were obtained [12]. Beads were kept overnight at 4˚C before being harvested by filtration, and finally, washed with sterile distilled water to remove excess calcium ions and free cells [15].

2.5. Optimization of immobilization protocols

Immobilization effectiveness was tested by varying sodium alginate solution between 2 and 5% (w/v), with cell suspension added to sterile calcium chloride solution at 2–5% (w/v). Samples were taken out at fixed time intervals, every 24 h and analyzed for phenol concentration. The effect of initial cell loading (ICL) was tested by varying the number of beads from 100 to 600 beads/flask using 3% (w/v) of both sodium alginate and calcium chloride. The effectiveness was determined with respect to phenol removal. The effects of bead sizes at 2, 3, 4, 5, and 6 mm were determined.

2.6. Comparison of phenol-degrading activities between freely suspended and immobilized cells

Comparison of phenol-degrading activities between immobilized and free cells were carried out using equivalent initial biomass concentrations of 180 mg wet weight for each. Batch cultures were cultivated for 5 d with 50 mL of liquid phenol medium at 500 mg/L phenol concentrations, in 250 mL-Erlenmeyer flask. One milliliter of samples was taken out every 24 h and analyzed for phenol concentration. All experiments were carried out in triplicates. Results were shown as the average of triplicate data with standard error.

For optimum microbial activity in the environment, the preferred range of pH is between pH 6 to 8 [16]. In this study, liquid phenol medium at pH 6, 6.5, 7, 7.5, and 8 were prepared. pH was adjusted using HCl and 0.1 M NaOH. The culture temperature was tested at 10, 25, 30, 35 40, 50, and 60˚C. Liquid phenol medium was prepared by varying phenol concentration from 100 mg/L to 3,000 mg/L.

2.7. Reusability of immobilized cells

After 5 d of culture period for the first cycle, the spent medium was removed by filtering out aseptically the alginate beads containing the cells. The beads were washed and used in the next cycle of biodegradation with initial phenol concentration of 500 mg/L in each cycle.

3. Analyses

3.1. Bacterial growth determination

Bacterial growth population was determined using a serial dilution technique to enumerate the colony-forming unit (CFU). The suspensions (100 μl) were spread onto phenol media agar and mixed by rotation. The plates were incubated for 48 h at 30˚C and the resulting colonies were counted.

3.2. Phylogenetic analysis

Methods for genomic DNA extraction, detection and sequencing were as described before [11]. A multiple alignment of 22 16S rRNA gene sequences which closely matched Phenol-degrading strain was retrieved from GeneBank and was aligned using clustal-W with the PHYLIP (phylogeny inference package) output option [17]. The alignment was visually checked for any obvious misalignments. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed using PHYLIP, version 3.573 (J.Q. Felsenstein, PHYLIP-phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle, http://evolution.genetics.washington.edu/phylip.html), with *Bacillus* strain as the out group in the cladogram.

3.3. Phenol concentration

Colorimetric method based on 4-aminoantipyrine (4-AAP) was used to determine phenol concentrations using phenol solution as a standard [13].

3.4. Packed-bed column reactor

The up-flow, continuous mode of phenol biodegradation was carried out in a packed-bed bioreactor. The set up is shown in Fig. 1. Glass column

(Pharmacia, USA) of 2.5 cm diameter and 40 cm height was used as a reactor column and packed to a required bed height with *Acinetobacter baumannii* strain Serdang 1 immobilized in alginate beads (2 mm bead size).

A known concentration of synthetic aqueous phenolic solution and all other nutrients were pumped into the reactor inlet from the bottom, using a peristaltic pump (Perista Pump, Germany) at a desired flow rate. Influent feed consisting of phenol solution and growth medium (pH 7.5) was pre-aerated with air at 0.1 mL/min. Sterile air filtered through a sterile 0.2 μm-cellulose acetate filter (Sartorius, Germany), was supplied to provide the dissolved oxygen necessary for the bacteria. The reactor outlet and effluent was at the top of the reactor. The experiments were carried out at room temperature and the pH was not controlled. The entire closed system consisting of the packed column, feed vessel, waste vessel, and connecting tubing and filter vents were sterilized by autoclaving. Samples were collected from the outlet for the determination of residual phenol at fixed intervals during start-up until steady state when phenol concentration in the effluent remained constant for a period of 10 h. The experiments were repeated for different conditions by changing the liquid flow rates, bed heights and initial phenol concentrations.

3.4.1. Effects of flow rates and bed height-to-diameter ratio

The effects of feed flow rates at 1.5, 2, 2.5, 3, and 4 mL/min were tested on phenol biodegradation rate. The effects of height to diameter ratio of the packing 15.2 (38 cm/2.5 cm), 14 (35 cm/2.5 cm), 12 (30 cm/2.5 cm), 11.2 (28 cm/2.5 cm), and 10 (25 cm/2.5 cm) were tested.

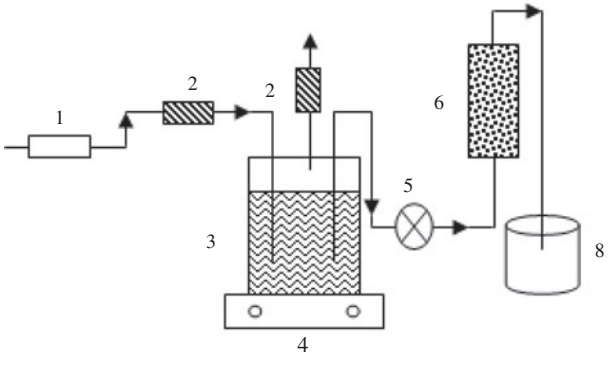

Fig. 1. Continuous system: (1) air pump; (2) air filters; (3) feed reservoir; (4) magnetic stirrer; (5) peristaltic pump; (6) column reactor; (7) sampling/effluent and (8) treated water collection.

3.4.2. Effects of initial phenol concentrations

The effects of initial phenol concentrations at 200, 400, 500, and 1,500 mg/L were investigated. Samples were taken out at fixed time intervals and analyzed for phenol concentration.

4. Results and discussions

4.1. Phylogenetic analysis

Microscopic examination under light microscope with 100X magnification showed that the cells were cocci in shape and formed chains of 3–4 bacterial cells per chain. The pink–red color from gram staining indicated that the isolates were Gram-negative (Fig. 2). The PCR product was sequenced using M13 forward and reverse universal primers on both strands and the complete sequence revealed that the gene of interest consists of 1,501 nucleotides [11]. Based on a BLASTN search of GenBank, the complete sequences of the bacteria shares 98% similarity with *Acinetobacter baumannii* which is confirmed by a neighbor-joining phylogenetic tree (Fig. 3) based on the alignment of 16S rRNA gene sequence of Isolate Serdang 1 with 16S rRNA sequence of the described *Acinetobacter* type strains available in GenBank databases and rooted by using *Bacillus*. Based on Biolog TM Identification System, the sequence alignment and phylogenetic tree analysis, the Isolate Serdang 1 has been redesignated *Acinetobacter baumannii* strain Serdang1 with GenBank accession number 897973.

4.2. Cell immobilization

In our preliminary study with cell immobilization, a total of 57×10^5 CFU/mL/bead was obtained.

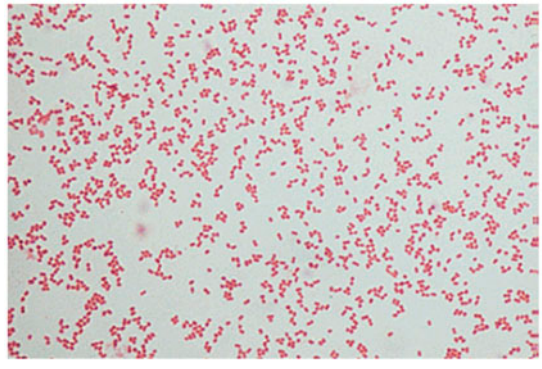

Fig. 2. Micrograph of *Acinetobacter baumannii* strain Serdang 1.

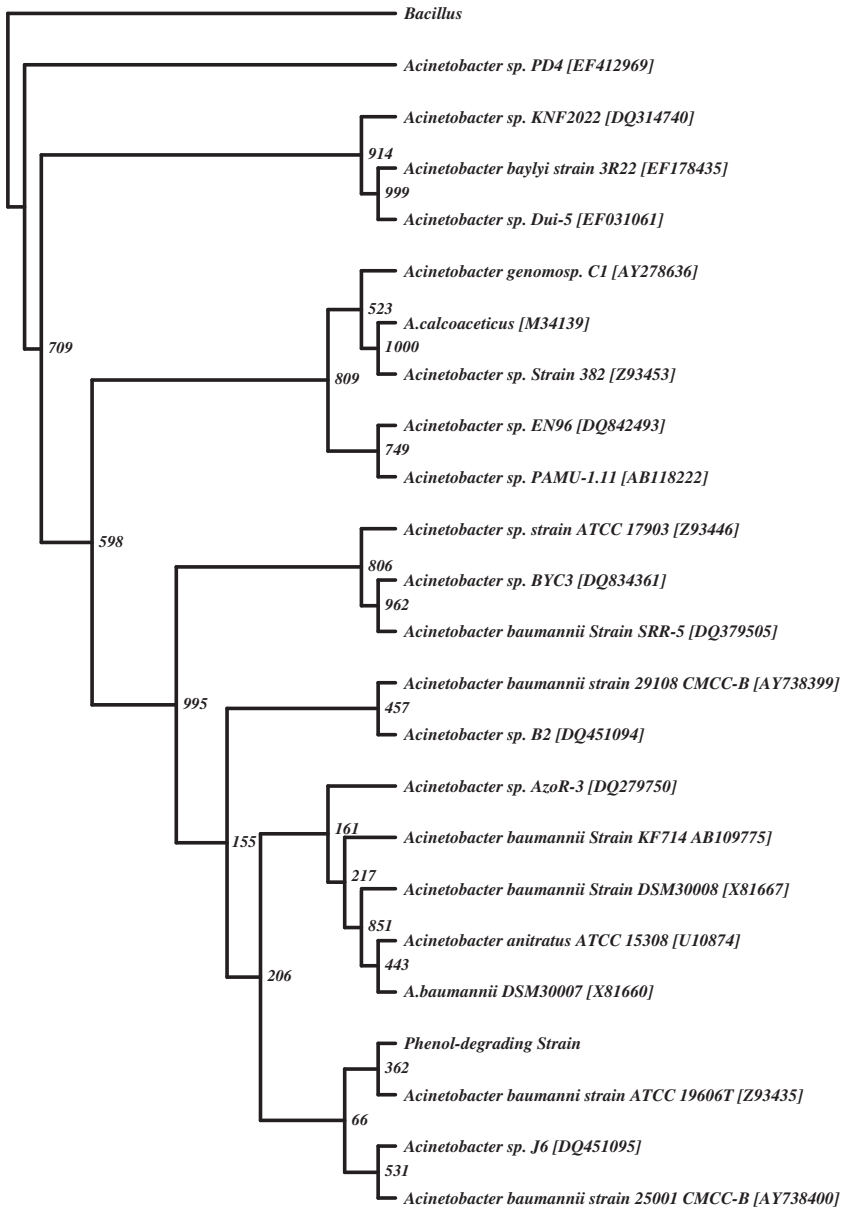

Fig. 3. Neighbor-joining method cladogram showing phylogenetic relationship between phenol-degrading strain and other related reference micro-organisms based on the 16s rDNA gene sequence analysis.

Fig. 4(a) shows that phenol removal of 77.2% was the highest at 3% (w/v) composition of both sodium alginate and calcium chloride. This was achieved by retaining ICL at 300 beads/flask with 2 mm bead size. When the numbers of beads were varied from 100 to 600 beads/flask using 3% (w/v) composition at 2 mm bead size (Fig. 4(b)), the highest phenol removal of 83.5% was achieved at 400 beads/flask. Bead size of 2 mm was optimum for phenol removal of 81.5% (Fig. 4(c)) when gelling composition was retained at 3% (w/v) and 400 beads/flask. These results suggest that more and larger beads do not necessarily translate into optimum phenol removal, and the major limiting factor could be the encapsulating matrix.

In an immobilized system, the physical structure of the immobilizing material can inhibit degradation, due to restriction on oxygen and nutrient flow. The strength can be improved by changing the composition of gelling components. It is important to achieve acceptable number of cells/bead as insufficient number could lead to zonation of the microenvironment within the bead that may alter intraparticle growth,

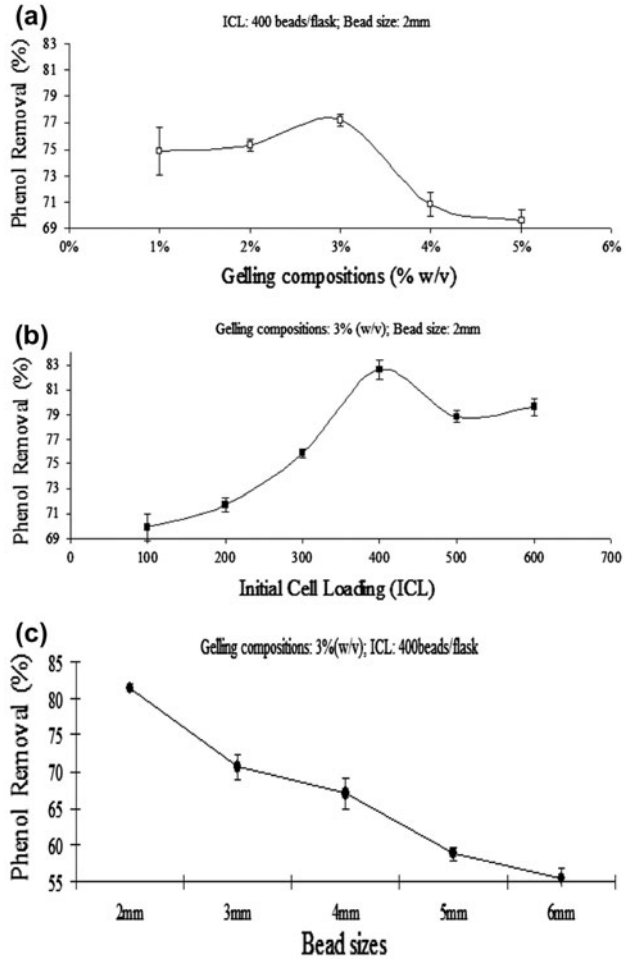

Fig. 4. The effects of (a) gelling compositions, (b) initial cell loading (ICL) and (c) bead sizes, on effectiveness of immobilized cells for phenol removal at basic conditions.

metabolism, and product formation [18,19]. While low cell numbers at the center will generate unproductive regions and influence particle density, too high the number of cells may result in the accumulation of cells on the bead surface that may rupture the gel surface, causing outgrowth and leakage [16]. Furthermore, non-homogenous distribution of cells can cause insufficient diffusivity of solutes [18].

The matrix structure determines the reaction capabilities of the cell population. The binding strength represented by the pore size, affects the diffusion of substrates or products, and could promote or inhibit the production or breakdown of products. Too small a pore size only allows low-molecular-weight substrates and products to diffuse through, while too large a pore size may result in cell leakage [20]. The pore size may be altered via a closely linked, stronger matrix, but as shown in Fig. 2(a), an increase in the concentration of both sodium alginate and calcium chloride solutions beyond 3% (w/v) reduced the effectiveness for phenol removal. A study on immobilized *Pseudomonas putida* MTCC1194 similarly shows reduced phenol removal from 99 to 92% and 85% when gelling components are increased to 4–5% (w/v) [21]. Porous polymer bead of polyvinyl alcohol (PVA) and Xanthan gum have been found to be the best entrapment for phenol degradation by *Pseudomonas fluorescence* in terms of bead shape (spherical form), bead strength, non-agglomeration, phenol degradation rate, and cell holding inside the bead. Activated carbon is co-immobilized with the micro-organism in the bead, which readily adsorb phenol to decrease initial phenol concentration [22].

With greater number of beads per flask, and larger bead size, one would expect an increase in the total surface area, which eventually should facilitate the mass transfer to and from the Ca-alginate beads. Instead, more beads per flask and larger bead size only resulted in decreased phenol removal (Fig. 4(b)). The major limitation with immobilized cells appears to be the diffusion of oxygen and nutrients through the gel matrix [18,19]. At median ICL (400 beads/flask) and correct bead size, there may be no oxygen/nutrient diffusion limitation to the cells. The liquid layer surrounding the beads could facilitate easy transport of oxygen/nutrients to the cells and optimum number of cells keeps oxygen/nutrient demand manageable. However, diffusion becomes increasingly difficult as size of the bead increases, inhibiting the transfer of oxygen and nutrients through the gel matrix [18]. With increasing cell numbers in the bead and with more number of beads per flask, oxygen is consumed faster than it can diffuse. There will be greater competition for oxygen [23] and the nutrient becomes limiting as the nutrient/bead ratio decreases [15].

4.3. Effects of temperature and initial pH

The effects of temperature on phenol removal for free and immobilized cells studied at pH 7.5 (Fig. 5(a)), suggest that there is a minimum temperature where activity does not occur, an optimum temperature at which activity is the best, and a maximum temperature at which activity ceases. The optimal temperature was determined at 30˚C for free and immobilized cells, with phenol removal of 69.9–99.8%, respectively. The effects of pH on free and immobilized cells were studied by varying pH between 6 and 8 (Fig. 5(b)). Immobilized cells achieved higher phenol removal than free cells at all pH range with the highest removal almost 100% at pH 7.5 as compared to

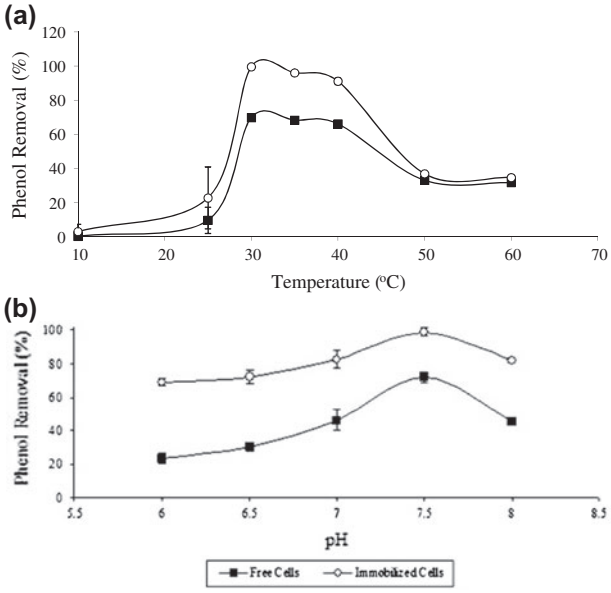

Fig. 5. The effect of (a) temperature at pH 7.5 and (b) pH at 30˚C, on phenol removal.

71.7% for free cells. This optimum pH was similar to that reported for *Acinetobacter radioresistens* [24].

Alginate gel matrices are negatively charged, so that the pH of the surrounding environment influences the diffusion of substrates and the excretion of products [19]. Results from control experiments suggest that volatilization and immobilizing agent were not the causes of these phenol removals. As the environment becomes increasingly basic (from pH 6–7.5) with the addition of OH^- group, there could be a pH gradient that drives phenolic compounds to leave the environment and diffuse into the gel matrices. Alternatively, more positive ions or H^+ diffuse out or have the tendency to neutralize the negative charges on the matrices, which subsequently attract the phenols onto the gel surface and into the cellular environment. As the pH moves to 8, the negatively charged environment could possibly repel the negatively charged gel matrices.

pH affects the activity of enzymes such that enzyme activity may only be active over a certain pH range. Variations in medium pH change the ionic form of the active site and change the enzyme activity and the reaction rate [25]. The solubility of a compound at different pH values will also determine the rate of substrate degradation [26]. This in turn influences the microbial growth rate, cellular functions, cell membrane, and protein transport. Our study confirmed that mesophilic temperature between 30 and 37˚C was suitable for phenol removal as has been reported for a number of mesophilic bacteria, fungi, and yeast [27–29]. The immobilized cells could tolerate more drastic changes in pH and temperature than the free cells [30].

4.4. Effects of initial phenol concentrations and reusability of immobilized cells

The effects of initial phenol concentrations from 100 to 2,000 mg/L on phenol removal by free and immobilized are as shown in (Fig. 6(a–e)). At 100 mg/L phenol, complete phenol removal with free cells was achieved after 16 d as compared to 24 d for immobilized cells. Infact, immobilized cells showed a lag period of 12 h. The shift in performance can be seen, as phenol concentration was increased to 200 mg/L and higher. Whilst the removal rate was negligible for free cells at 2,000 mg/L phenol, complete removal was attained within 12 d by immobilized cells. At low phenol concentration, internal mass transfer of phenol or oxygen could be the limiting factor in immobilized cells [30]. At high phenols, freely suspended cells are not able to tolerate phenol toxicity [9,28]. *Pseudomonas putida* cannot tolerate phenol toxicity at concentrations between 800 and 1,000 mg/L [9], and *Candida tropicalis* at level above 1,500 mg/L [31].

Reusability of immobilized cells was tested at 500 mg/L initial phenol concentration (Data not shown). Immobilized cells could be reused up to five cycles with 5 d duration per cycle, achieving phenol-removal activity of 97–100%. The 6th cycle showed the activity was reduced to 80%. We therefore have successfully developed an immobilization system that could remove phenol at as high as 2,000 mg/L initial concentration, and shown the potential for continued use of immobilized cells for *in situ* or *ex situ* remediation. This requires further improvement such as ease of application, robustness, and speed of remediation and the use of microbial consortium at the contaminated site. Microbial consortium consisting of two *Pseudomonas*, and two *Acinetobacter* species used within a two-phase partitioning bioreactor (TPPB) with solid polymer beads (comprised of ethylene vinyl acetate) as the second-phase, is capable of degrading as high as 2,000 mg/L phenol, with decreased lag time (10 h) and increased specific rate of phenol degradation (0.71 g phenol/g cell/h) [32]. It has been reported that *P. aeruginosa* degrades phenol up to 500 mg/L in 50 h as free cell and 900 mg/L in 80 h when immobilized in the calcium alginate beads. However, for 1,200 mg/L concentration, the immobilized cells took much more time (290 h) for a complete degradation. The reuse of these beads in different

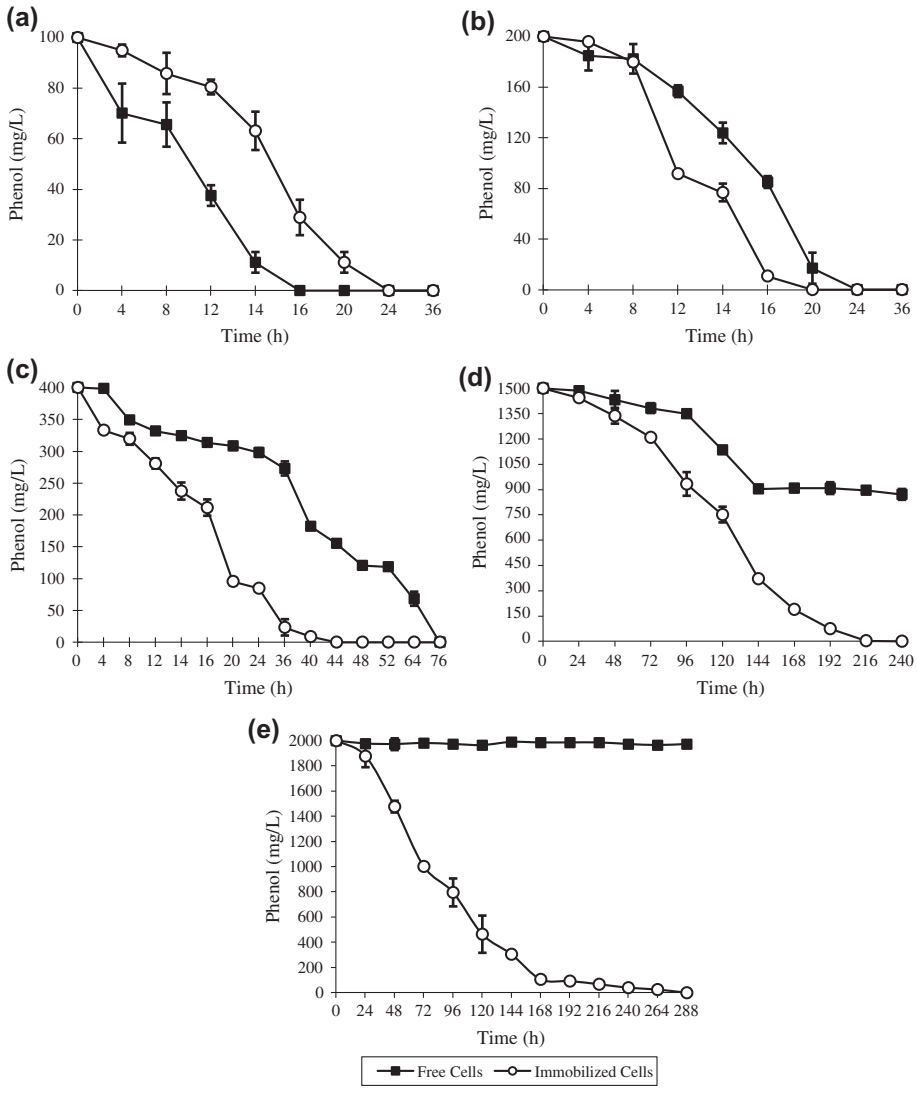

Fig. 6. The effects of phenol concentration (mg/L): (a) 100, (b) 200, (c) 400, (d) 1,500 and (e) 2,000 on phenol removal by free and immobilized cells.

concentrations of phenol (100–900 mg/L) shows that the cells keep their phenol degradation ability up to 900 mg/L in 78.5 h with 99% removal efficiency [10].

4.5. Packed-bed column reactor

The effect of flow rate and bed height-to-diameter ratio (H/D_c) at 1.5 mL/min flow rate and 200 mg/L initial phenol concentration was investigated (Table 1). This was achieved by retaining the height-to-diameter ratio at 11.2 (28/2.5 cm), at 200 mg/L initial phenol concentration. Phenol removal of 71% could be achieved at 1.5 mL/min, while about 65% removal was achieved at 4 mL/min but the time taken to reach steady state however increased (data not shown). At 1.5 mL/min flow rate, the phenol concentration in the reactor decreased significantly within the first 8 h, whilst at 4 mL/min, it took 20 h to achieve almost the same level of removal.

The highest H/D_c ratio of 15.2 showed the highest phenol removal at 77%, and the time taken to reach steady state was three times shorter than that achieved with H/D_c ratio of 10, and the rate of phenol removal at 0.28 mg/L/h/mL of beads was two-fold higher than the latter (Table 1). With an increase in H/D_c ratio (at constant D_c = 2.5 cm), there are greater number of beads and cells available to degrade phenol [14]. However, the percentage of phenol removal in the range of 78–86% was not significantly different as the influent phenol concentration was increased from 200 to

Table 1
The effects of flow rate, bed height-to-diameter ratio (H/D_c), and initial phenol concentration on packed-bed column reactor performance for phenol removal

	Flow rate (mL/min)					Bed height-to-diameter ratio (H/D_c)					Initial phenol concentration (mg/L)			
Kinetic parameter/performance	1.5	2.0	2.5	3.0	4.0	10	11.2	12	14	15.2	200	400	500	1,500
Rate of phenol removal (mg/L/h)	17.7	13.6	9.51	8.15	6.46	11.5	17.9	24.1	38.1	38.4	38.9	28.5	13.4	23.4
Rate of phenol removal of beads (mg/L/h/mL)	0.18	0.14	0.09	0.08	0.07	0.13	0.18	0.23	0.31	0.28	0.29	0.21	0.1	0.24
Time to reach steady state (h)	8	10	14	16	20	12	8	6	4	4	4	12	24	40
Residual phenol at steady state (mg/L)	58	64	66	69	70	62	52	56	48	46	44	58	178	204
Phenol removal (%)	71.0	68.0	67.0	65.5	65.0	69.0	74.0	72.0	76.0	77.0	78.0	85.5	64.4	86.4

1,500 mg/L, at the same flow rate of 1.5 mL/min and the same H/D_c ratio of 15.2. The time taken to reach steady state for 1,500 mg/L influent at 40 h was eight times longer than at 200 mg/L, and the removal rate of phenol was also lower (Table 1). Some effects of inhibition was observed at 500 mg/L phenol concentration but the residual concentrations prevailing in the reactor at steady state at all times were lower than the inhibitory concentrations.

During steady state, the cell population is at a dynamic equilibrium where cells utilize phenol at the level at which the cell populations could grow and be maintained. In a packed-bed bioreactor study of *Pseudomonas fluorescence* and 1% activated carbon co-immobilized in the bead, the readily adsorbed phenol reduces adaptation time of the cells to stably reduce phenol. The start-up period is shortened by 40 h and the removal efficiency is increased by 28% than the case with only micro-organism [22]. As the flow rate increases, the dissolved oxygen carried through per pass from the reservoir to the reactor increases, and this may increase the reaction rates. The mass transfer resistance solid/liquid across the biofilm is reduced, and this may further increase the reaction rates [33]. Our study, on the other hand, showed that phenol removal rate at 1.5 mL/min flow rate was 0.18 mg/L/h/mL of beads (Table 1), which was almost three times the removal rate achieved at 4 mL/min flow rate. With an increase in flow rate, the residence time in the reactor is reduced. This could have resulted in reduced cell growth, a decrease in percentage of phenol removal and longer time taken to achieve a steady state [34]. It is possible that with up-flow mode of influent, low flow rate allows longer residence time for the immobilized cells to make contact with the substrate. Hence, the shorter time to achieve steady state.

With increasing cell numbers in the bead, it is possible that oxygen is consumed faster than it can diffuse into the beads, and so cells have to compete for oxygen [33]. Whilst 1.5 mL/min flow rate may be the lowest flow rate, it appears to be high enough to eliminate the external mass transfer resistance. Plus, the cells may already reach the state of substrate saturation, and with a fixed number of beads, increase in flow rate does not improve the rate of substrate removal. This saturation has been suggested, can be relieved when the total population size is increased such as using more beads [35].

5. Conclusions

Based on the sequence alignment and phylogenetic tree analysis, a new isolate capable of degrading phenol had been redesignated *Acinetobacter baumannii* strain Serdang1 with GenBank accession number 897973. The optimal conditions for growth and phenol removal by immobilized cells were found to be 3% (w/v) of both sodium alginate and calcium chloride solution, 2 mm bead diameter and ICL of 400 beads/flask, at 30°C and pH 7.5, respectively. Repeated uses of immobilized cells suggest as many as five cycles of use without loss of activity. The immobilized cell system was more effective than free cell system in removing phenol at initial concentration as high as 2,000 mg/L in 12 d at 6.94 mg/L/h. With the packed-bed column reactor, about 77% removal of 200 mg/L phenol was achieved at 1.5 mL/min flow rate and bed height-to-diameter ratio of 15.2. The major parameter to assess efficiency of phenol removal in a continuous system was the time taken to achieve steady state, whilst the influent flow rate and bed height-to-diameter ratio, when the D_c remained constant, being the most important factors affecting the performance.

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References

[1] L. Guozheng, P. Seongjun, B.E. Rittmann, Degradation of reactive dyes in a photocatalytic circulating-bed biofilm reactor, Biotechnol. Bioeng. 109(4) (2012) 884–893.
[2] S.C. Lee, H.O. Lintang, L. Yuliati, Photocatalytic removal of phenol under visible light irradiation on zinc phthalocyanine/mesoporous carbon nitride, International Conference on Enabling Science and Nanotechnology (ESciNano), Johor Bahru, Malaysia, 2012, pp. 1–2.
[3] S.Y. Bashtan, V.A. Bagrii, Electrochemical oxidation of phenol on metaloxide electrodes, J. Water Chem. Technol. 34(1) (2012) 24–27.
[4] A. Haddadi, M. Shavandi, Biodegradation of phenol in hypersaline conditions by *Halomonas* sp. strain PH2-2 isolated from saline soil. Int. Biodeterior. Biodegrad. 85 (2013) 29–34.
[5] S.A. Mahapure, V.H. Rane, J.D. Ambekar, L.K. Nikam, R. Marimuthu, M.V. Kulkarni, B.B. Kale, Novel solar light driven photocatalyst, zinc indium vanadate for photodegradation of aqueous phenol, Mater. Res. Bull. 46(5) (2011) 635–638.
[6] M.L. Soto, A. Moure, H. Domínguez, J.C. Parajó, Recovery, concentration and purification of phenolic compounds by adsorption: A review, J. Food Eng. 105 (1) (2011) 1–27.
[7] J. Han, Z. Du, W. Zou, H. Li, C. Zhang, *In-situ* improved phenol adsorption at ions-enrichment interface of porous adsorbent for simultaneous removal of copper ions and phenol, Chem. Eng. J. 262 (2015) 571–578.
[8] A. Krastanov, Z. Alexieva, H. Yemendzhiev, Microbial degradation of phenol and phenolic derivatives, Eng. Life Sci. 13(1) (2013) 76–87.
[9] R. Surkatti, M.H. El-Naas, Biological treatment of wastewater contaminated with *p*-cresol using *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel, J. Water Proc. Eng. 1 (2014) 84–90.
[10] O. Ali, A. Namane, A. Hellal, Use and recycling of Ca-alginate biocatalyst for removal of phenol from wastewater, J. Ind. Eng. Chem. 19(4) (2013) 1384–1390.
[11] Z.H.M. Yadzir, M.Y. Shukor, M.S. Nazir, M.A. Abdullah, Characterization and identification of newly isolated *Acinetobacter baumannii* strain Serdang 1 for phenol removal, AIP Conf. Proc. 1482 (2012) 223–228.
[12] H. Keweloh, H.J. Heipieper, H.J. Rehm, Protection of bacteria against toxicity of phenol by immobilization in calcium alginate, Appl. Microbiol. Biotechnol. 31(4) (1989) 383–389.
[13] APHA Standard methods for the examination of water and wastewater, seventieth ed., American Public Health Association, Washington, DC (2005).
[14] A. Banerjee, A.K. Ghoshal, Isolation and characterization of hyper phenol tolerant *Bacillus* sp. from oil refinery and exploration sites, J. Hazard Mater. 176(1–3) (2010) 85–91.
[15] U. Beshay, Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads, Afr. J. Biotechnol. 2 (2003) 60–65.
[16] S.P. McLelland, Supporting a ground water and soil natural remediation proposal, Site Remed. News 8(1) (1996) 1–11.
[17] D. Higgins, J. Thompson, T. Gibson, J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[18] E. Doherty Speirs, P.J. Halling, B. McNeil, The importance of bead size measurement in mass-transfer modelling with immobilised cells, Appl. Microbiol. Biotechnol. 43 (1995) 440–444.
[19] A.J. McLoughin, Controlled release of immobilized cells as a strategy to regulate ecological competence of inocula, Adv. Biochem. Eng. Biotechnol. 51 (1994) 1–46.
[20] I. Chibata, T. Tetsuya, T. Sato, Methods of cell immobilization, in: A.L. Demain, N.A. Solomon (Eds.), Manual of Industrial Microbiology and Biotechnology, American Society for Microbiology, Washington DC, 1986, pp. 217–229.
[21] K. Bandhyopadhyay, D. Das, B.R. Maiti, Solid matrix characterization of immobilized *Pseudomonas putida* MTCC 1194 used for phenol degradation, Appl. Microbiol. Biotechnol. 51 (1999) 891–895.
[22] K.H. Kwon, K.Y. Jung, S.H. Yeom, Comparison between entrapment methods for phenol removal and operation of bioreactor packed with co-entrapped activated carbon and *Pseudomonas fluorescence* KNU417, Bioproc. Biosyst. Eng. 32 (2009) 249–256.
[23] B. Gosmann, H.J. Rehm, Oxygen uptake of microorganisms entrapped in Ca-alginate, Appl. Microbiol. Biotechnol 39 (1986) 627–631.
[24] S. Divari, F. Valetti, P. Caposio, E. Pessione, M. Cavaletto, E. Griva, G. Gribaudo, G. Gilardi, C. Giunta, The oxygenase component of phenol hydroxylase from *Acinetobacter radioresistens* S13, Eur. J. Biochem. 270 (2003) 2244–2253.
[25] W. Ying, T. Ye, H. Bin, H.B. Zhao, J.I. Bi, B.L. Cai, Biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12, J. Environ. Sci 19(2) (2007) 222–225.
[26] A. Banerjee, A.K. Ghoshal, Phenol degradation performance by isolated *Bacillus cereus* immobilized in alginate, Int. Biodeterior. Biodegrad. 65(7) (2011) 1052–1060.
[27] M.H. El-Naas, S.A. Al-Muhtaseb, S. Makhlouf, Biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel, J. Hazard. Mater. 164(2–3) (2009) 720–725.
[28] V.L. Santos, V.R. Linardi, Biodegradation of phenol by a filamentous fungi isolated from industrial effluents —Identification and degradation potential, Process Biochem. 39 (2004) 1001–1006.
[29] J. Yan, W. Jianping, L. Hongmei, Y. Suliang, H. Zongding, The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*, Biochem. Eng. J. 24 (2005) 243–247.

[30] A.Y. Dursun, O. Tepe, Internal mass transfer effect on biodegradation of phenol by Ca-alginate immobilized *Ralstonia eutropha*, J. Hazard. Mater. 126 (2005) 105–111.
[31] K.C. Chen, Y.H. Lin, W.H. Chen, Y.C. Liu, Degradation of phenol by PAA-immobilized *Candida tropicalis*, Enzyme Microb. Technol. 31 (2002) 490–497.
[32] G.P. Prpich, A.J. Daugulis, Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor, Biodegradation 16 (2005) 329–339.
[33] F.S. Lakhwala, B.S. Goldberg, S.S. Sofer, A comparative study of gel entrapped and membrane attached microbial reactors for biodegrading phenol, Bioprocess Eng. 8 (1992) 9–18.
[34] K.V. Shetty, I. Kalifathulla, G. Srinikethan, Performance of pulsed plate bioreactor for biodegradation of phenol, J. Hazard. Mater. 140 (2007) 346–352.
[35] A. Mordocco, C. Kuek, R. Jenkins, Continuous degradation of phenol at low concentration using immobilized *Pseudomonas putida*, Enzyme Microb. Technol. 25 (1999) 530–536.