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Immobilised *Rhodococcal* Cells: A Competent Approach for Phenol Degradation by *Rhodococcus* sp. NAM 81

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The objective of this current work is to study about phenol degradation ability of the immobilised *Rhodococcus* sp. NAM81. The present investigation evaluates the suitability of five matrices; gellan gum, calcium alginate, agarose, agar-agar and polyacrylamide upon the immobilisation of *Rhodococcus* sp. NAM81 for phenol degradation. Among all of these matrices, gellan gum was proven to be the most effective and suitable matrix as it exhibits higher phenol degradation (99.9%) compared to other matrices studied. Maximum phenol degradation has been achieved at gellan gum with the concentration of 0.7% (w/v), bead size of 4 mm diameter (estimated surface area of 50.26 mm^2), 4 g/Lof cell loading and 350 numbers of beads per 100 mL medium. Both free and immobilised bacteria exhibited similar rates of phenol degradation at phenol concentration of 100 mg/L, the immobilised bacteria exhibited a higher degradation rate of phenol at higher phenol concentrations. The immobilised cells completely degraded phenol within 108, 216, and 240 h at 1100, 1500 and 1900 mg/L phenol, respectively, whereas free cells took 240 h to completely degrade phenol at 1100 mg/L.However, the free cells were incapable of completing phenol degradation at higher concentrations. Overall, the rates of phenol degradation by both immobilised and free bacteria decreased gradually as phenol concentration increased. It also proved that inhibition by heavy metals and respiratory inhibitors decreased by gellan gum encapsulated the cells. The immobilised cells showed no sign of losses in phenol degrading activity after being used repeatedly for 50 times of 18 h cycle and still stable after storing at 4°C for 28 days. The study has showed that the effectiveness of phenol degradation was greater using immobilised cells in gellan gum compared to free living cells. Presumably, this is the first attempt of phenol degradation using the immobilised *Rhodococcus* sp in gellan gum. The gellan gum-immobilised cells showed potential candidature for phenol degradation.

Key words: *Rhodococcus* sp.,encapsulation, gellan gum, phenol, biodegradation.

Phenol is an important chemical compound with a large number of applications and it has been estimated to have about six million tonnes of annual global production achieved worldwide[1]. However, it continues to be a major contaminant in the ground water and marine environment as a result of accidental spilling and extended leakage in the industrial waste treatment plant. Therefore, treatment is very crucial for the conservation of human health and environment due to the toxicity of this poisonous compound by ingestion, contact, or inhalation even at low concentrations.

The conventional method of coping with phenol contamination was limited to physicochemical approaches and it is very expensive. Their by-products may cause

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secondary contaminants of soil and water resulting from the process that is in the need for additional post treatment. Biodegradation using microbes has been widely studied because it leads to a complete degradation of complex hydrocarbons to carbon dioxide and water[2]. Trickling filter, oxidation ponds, lagoons and activated sludge are among the biological methods that have been employed for the treatment of phenolic wastewater[3].

Biotechniques that commonly apply microbes which comprising various strains isolated from phenol-contaminated soil or water have been shown to have phenol-degrading capabilities. However, high concentrations of phenols are toxic to degraders and resulted in low biodegradation rate of phenol. The maximum dilution rate that can be achieved is limited because of cell washout. Therefore, cell immobilisation is a promising approach in biodegradation of toxic compound. It could overcome the problems of using free suspended cells[4]. Cell immobilisation is defined as the physical confinement of the whole cells while preserving its viability. It has gained significant results in many applications such as in agricultural, environmental decontamination, wastewater treatment and food industries. One of the applications for environmental decontamination was to treat phenol-containing wastewater with little sludge production[5-7]. Some of the advantages of cell entrapment are, the matrix protect the cells from the inhibitions of toxic compounds or as a shield from biotic stresses such as predation by protozoa and bacteriophage as well as its ability to alter physiological features in metabolism of living cells[8,9]. Immobilisation has been reported to increase degradation activity by altering metabolic features of the living cells such as cell growth and enzyme induction[10]. Thus, this method could enhance survival ability, improves physiological activity and increases the cells density, thereby increasing the speed of the catalysed processes[11-13]. Other advantages of cells immobilisation are, as an ease of cells separation from the medium, a decrease in costs due to cells reuse in subsequent reaction cycles, and the reducing of contamination possibilities. In the previous studies, most of immobilised cells have showed the ability to degrade phenol that is greater than 1000 mg/L[14]. The entrapment of cells in Ca-alginate, gellan gum, agarose, polyethane, polymide, polyvinyl alcohol, polyprolene and polyacrylonitrile have been applied for microbial degradation of toxic substances in recent years[15-17]. The matrices used are not toxic to the cells, inert, inexpensive and practical[18].

Biodegradation of phenols using microorganism is gaining a huge attention because of its eco-friendly characteristics and cost-effectiveness. One of the potential microorganisms is known as *Rhodococcus* bacteria. *Rhodococcus* is a genus of aerobic, gram positive, non-sporulating and mycolate-containing bacteria. The ability of rhodococci to utilise and it tolerance towards difficult substrates is a physiological basis for choosing this bacterium to be applied in biodegradation of phenol that initiated by phenol hydroxylase as the phenol degrading enzyme. For that reason, *Rhodococcus* sp. strain NAM 81 a bacterial strain that could effectively degrade phenol even at high concentration (1000 mg/L), was applied in this study. In order to enhance biodegradation activity by immobilised cells, it is imperative to establish an optimum immobilisation protocol. Parameters that are crucial to be optimised are the type and size of matrix, gelling components, and initial cell loading as these could affect the performance of the immobilised cells. Hence, the immobilisation of *Rhodococcus* sp. NAM81 by determining the suitable matrix, the size of matrix, optimum matrix concentration/condition and optimum initial concentration of cells on biodegradation capabilities of immobilised strain NAM81 cells has been started. The investigation is essential to explore the feasibility of the immobilised cells for treating wastewater-containing phenol that fixes high densities of microorganism on the carrier and maintain the biodegradation capabilities in an optimal condition.

MATERIALS AND METHODS

Chemicals

All chemicals were being analytically graded; phenol has been obtained from Sigma–Aldrich (Steinheim, Germany). All experiments were conducted in triplicate at room temperature (30°C) under sterile conditions.

Microorganism and inoculum preparation

Rhodococcus NAM81 that are being maintained in UNISEL Culture Collection Unit, Institute of Bio-IT Selangor was used in this study. Bacterial from stock culture maintained in glycerol was transferred into nutrient broth (Merck, Germany) and incubated at 30°C in an incubator shaker (Jeio Tech SI-600R, Korea) agitated at 160 rpm for 24 h. The cultures were being used as the standard inoculums throughout this study.

Cultivation and phenol biodegradation experiments

The biodegradation of phenol was done in a minimal salt media (MSM) with the following composition in g/Lwith pH 7.25; $MgSO_4$ (0.1), K_2HPO_4 (0.4), KH_2PO_4 (0.2), NaCl (0.1), $MnSO_4$ (0.01), $FeSO_4H_2O$ (0.01), $Na_2MoO_4.2H_2O$ (0.01), $(NH_4)_2SO_4$ (0.4), NaCl (0.06) and phenol (0.5). Phenol was sterilised separately using 0.2 ¼m cellulose membrane filter (Sartorius Stedim, Germany) and added subsequently to an autoclaved medium. Exponentially growing *Rhodococcus* sp. strain NAM81 bacterial cells were harvested from culture broth by centrifugation at 7,000 rpm at for 10 min. The supernatant was removed and the pellet (0.3 g) was then washed twice with sterile-distilled water. The resulting bacterial pellet was dispersed in the matrix mixture and constantly stirred after that.

Preparation of immobilised cells

Optimisation of cell immobilisation started with the determination of suitable matrix (gellan gum, agarose, calcium-alginate, agar-agar and acrylamide). The concentrated cells were transferred into the cooled matrix solution (the final bacterial concentration was OD_{600} = 0.5) and homogenised by stirring.

Gellan gum

Cell entrapment in gellan gum was made with a slightly modified method described by Ahmad et al., (2012)[19]. Gellan gum (0.75%; w/v) was initially added to 50 mL deionised water and heated in microwave to dissolve the solution. 0.06% (w/v) $CaCl_2$ was put into the mixture and cooled to 45°C. 0.1 M NaOH was used to adjust the pH of the solution to pH 7.0. Beads were formed by dropping the cell and gum mixture into canola oil containing 0.1% Span 80. The uniformly sized beads were then transferred into 500 mL of 0.1% (w/v) $CaCl_2$. The beads were repeatedly rinsed with 0.1% (v/v) Tween 80 solution after 2 h. These beads were successively washed with distilled water for further applications.

Agarose

For entrapment in agarose, the whole cell pellet has been mixed with 50 mL of 2% (w/v) agarose that was melted in microwave. The homogeneous blend has been dropped into 20 mL sunflower oil at 25°C. Then, the resulting gel beads were cooled, filtered and washed with cool hexane to remove excess sunflower oil and distilled water to free beads from solvent before it is stored at 4ºC till use[20, 21].

Calcium alginate

The process of using calcium alginate as the matrix start with the preparation of 3% sodium alginate solution in 50 mL of distilled water and sterilised by autoclaving. The suspended cells were added to the alginate slurry, and stirred to get a uniform mixture. The suspension was extruded drop by drop into a cold, sterile 0.1 M calcium chloride solution through a syringe. Beads that have been formed were incubated overnight for curing. The cured beads were washed with sterile distilled water 3-4 times. After washing with sterile distilled water, the beads were stored at 4ºC for preservation[22].

Agar

Agar immobilisation of cells was done in 2% (w/v) agar saline solution based on work done by Kocher and Mishra, (2008)[23]. A 2% solution of agar-agar was prepared in 50 mL of 0.9% NaCl and melted in microwave before it was cooled. *Rhodococcal* cells that have been added to the molten agar were sustained at 40°C, poured into sterile flat bottom (100 mm) diameter Petri plate, and solidified before it was cut into equal size using sterile blades. The blocks were then added to 0.1 M phosphate buffer (pH 7.0), and kept for 1 h in the refrigerator for curing. After that, cuboids were successively washed with distilled water before use.

Polyacrylamide

0.45 g ammonium persulphate was added to the mixture of acrylamide (9.0 g) and bisacrylamide (0.24 g) buffered solution (pH 7.0) of 0.1 mM EDTA and 0.1 M Tris-HCl in a beaker. Then, 3 mL TEMED was added to the mixture

followed by cells suspension to initiate polymerisation. The slurry was dispensed into flat bottom petri dish for solidification. Polyacrylamide gel was cut into equal size cubes after polymerisation. The cubes were then transferred to 0.2 M phosphate buffer (pH 7.0) and kept at 4°C within 1 h for curing purpose. The cubes were washed with sterile distilled water and stored at 4°C until use[24].

Screening of different matrices for immobilisation of *Rhodococcus* sp. NAM 81

Immobilised cells prepared by the above methods were transferred to 250 mL conical flasks containing 100 mL of MSM containing 500 mg/L phenol. The flasks were incubated on a rotary shaker (160 rpm) at 30°C for 24 h.

Optimisation of immobilised condition

The best matrix was further characterised and optimised for the degradation of phenol by the immobilised bacteria. The optimisation process starts with the determination of the optimum matrix concentration and cross linker concentration, followed by optimisation of bead size, ranging from 1, 2, 3, 4, 5 and 6 mm diameter (equivalent to surface areas of 3.14, 12.56, 28.26, 50.24, 78.50 and 113.04 mm^2, respectively determined by $4Ar^2$) in size. The optimum initial cell loading was then determined followed by the quantity of beads ranging from 100 to 400 beads. 100 mL MSM containing phenol (500 mg/L) was used in the optimisation experiments[19].

Cell leakage

Cell leakage from the gel matrix was determined by measuring the optical density at 600 nm for cell weight. From the standard graph of biomass, the corresponding absorbance was converted into mg of cell leakage from each matrix.

Comparison of phenol degradation between free and immobilised cells

Different phenol concentrations ranging from 100 to 3500 mg/Lwere tested for both the immobilised and free cell systems in separate containers containing 100 mL of MSM with 500 mg/Lphenol. Flasks were incubated in an incubator shaker (Jeio Tech SI-600R, Korea) agitated at 160 rpm and 30°C. Phenol residual was monitored every three hours until complete degradation of phenol was accomplished using phenol media without the presence of bacteria as a control variable. The effects of co-contaminant, represented by heavy metals (Ag, As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) and respiratory inhibitors (cyanide and sodum azide), on phenol degradation ability of the immobilised cells, compared to freely suspended cells in 18 hours incubation have been tested.

Reusability of immobilised cells

300 of immobilised cells were inoculated in 100 mL of liquid MSM containing 500 mg/Lphenol and incubated in an orbital shaker at 160 rpm. Phenol residual was monitored every six hours until complete degradation of phenol was accomplished. The medium was then discarded and the beads were thoroughly rinsed with distilled water before transferred into new fresh MSM containing phenol (500 mg/L). The steps were repeated until phenol-degrading ability of the immobilised cells showed significant decrease.

Analytical procedures

MSM liquid cultures have been withdrawn to monitor phenol degradation ability, and the residual phenol from the spent medium was determined using the 4-aminoantipyrine method[25,26]. Absorbances were measured at 500 nm using BioMate 3 UV-Vis Spectrophotometer, Thermoscientific, USA and subsequent concentrations were determined using a calibration curve. Sample preparation involving centrifugation at 10,000 rpm for 10 min to eliminate interference from biomass before the supernatant was removed and analysed has been made. The estimation of biomass was performed by using predetermined correlation between 660 nm optical density using BioMate 3 UV-Vis Spectrophotometer (Thermoscientific, USA), dry cell weight from filtration and oven dried method[27]. Standard curve for the determination of cells concentration in the sample was plotted.

Statistical analysis

All data were analysed using SPSS v16. Values are means ± standard error. The optimisation data were statistically analysed using One-way ANOVA. $P<0.05$ was considered to be statistically significant.

Scanning electron microscope

Blank and immobilised beads were rinsed to get rid of growth medium with deionised water before fixing. Beads were fixed in fixative

(4% Gluteraldehyde) for 24 h at 4ºC. The bead was then washed with 0.1 M Sodium cacodylate buffer for three changes of 10 min followed by post fixation in 1% Osmium teroxide for 2 h at 4ºC and rewashed with 0.1 M sodium cacodylate buffer for three changes of 10 min. Followed by the stepwise dehydration using acetone, the beads were critically dried. The beads were then examined under scanning electron microscope (LEO 1455 VPSEM) attached with energy dispersive X-ray (EDX).

RESULTS AND DISCUSSION

Hydrogels have received much consideration in the field of biotechnology and biomedical, mainly due to its capability to act as cells carrier and as vehicle for drug delivery[28-32]. Hydrogels are similar to natural extracellular matrix molecules as it could retain great quantities of water. Immobilisation of *Rhodococcus* sp. NAM81cells using different entrapment techniques with hydrogels such as gellan gum, agarose, calcium-alginate, agar-agar and polyacrylamide was investigated. Table 1 represents the comparative phenol degradation by different matrices along with cell leakage. From the result shows in Table 1, all matrixes could be used to immobilise phenol-degrading cells except polyacrylamide. Phenol degradation using immobilised cells in gellan gum is the best matrixes compared to others. 500 mg/Lphenol was completely degraded by gellan gum immobilised cell in 20 h incubation compared to 26, 24, and 24 h by agar, agarose and calcium alginate immobilised cells respectively. Entrapments in polyacrylamide only caused 5% of phenol degradation although after 24 h of incubation. It might due to the toxicity of the acrylamide monomer, the cross-linking agent methylenebisacrylamide and the polymerisation initiator (tetramethylethylenediamine), which decreases the cells viability and enzymes activity.Polyacrylamide shows the ability to act as the entrapment matrix with protocol optimisation. As suggested by Starostina et al., (1982)[33], the decrease of polyacrylamide concentration and the initial temperature of the polymerisation mixture could lower the toxic effects and, consequently, increases the viability of the bacteria cells population. Hence, gellan gum was considered the best matrix for phenol

Table 1. Percentage of phenol degradation and amount of cell leakage using various immobilization matrixes

Matrix	Percentage of phenol degradation within 18 hours incubation (%)	Amount of cells leakage after degradation completed (gL^{-1})	Time required for 100% degradation (hour)
Gellan gum	99.9±0.002	0.005±0.008	20
Agarose	93.1±0.007	0.184±0.002	24
Agar	96.3±0.008	0.087±0.003	26
Polyacrylamide	5.2±0.009	0.488±0.004	400
Sodium alginate	90.5±0.002	0.212±0.009	24

Table 2. Effect of storage stability on percentage of phenol degradation by immobilized and free cells

Storage time (day)	Type of cells	
	Percentage of phenol degradation by immobilized cells	Percentage of phenol degradation by free cells
0	99.5±0.13	98.9±0.09
7	98.8±0.22	97.3±0.12
14	95.7±0.13	90.9±0.07
21	90.5±0.06	80.2±0.13
28	85.4±0.13	70.3±0.13

Table 3. Effect of initial phenol concentration on phenol degradation ability of immobilized and free suspended cells of *Rhodococcus* sp. NAM81 within 12 days of cultivation period

Performance	Initial phenol concentration (g/L)															
	0.1	0.3	0.5	0.7	0.9	1.1	1.3	1.5	1.7	1.9	2.1	2.3	2.5	2.7	2.9	3.1
Immobilized cells																
Residual phenol (g/L)	0.001 ±0.04	0.004± 0.004	0.005± 0.006	0.010± 0.009	0.017± 0.11	0.025± 0.04	0.033± 0.17	0.050± 0.03	0.059± 0.15	0.061± 0.15	0.072± 0.08	0.085± 0.11	0.095± 0.22	1.923± 0.13	2.856± 0.28	3.092± 0.15
Phenol degraded (g/L)	0.099	0.296	0.495	0.690	0.883	1.075	1.267	1.45	1.641	1.839	2.028	2.215	2.405	0.777	0.044	0.008
Percentage of phenol degradation (%)	99.0	98.67	99.03	98.5	98.1	97.72	97.46	96.67	96.53	96.79	96.57	96.3	96.2	28.77	15.17	0.258
Duration for complete degradation (hr)	6	9	18	18	36	54	72	84	90	120	180	240	252	288	288	288
Phenol degradation rate (g/L/hr)	0.017	0.033	0.028	0.038	0.024	0.019	0.018	0.017	0.018	0.015	0.011	0.009	0.009	0.003	0.00015	0.00003
Free cells																
Residual phenol (g/L)	0.003 ±0.13	0.006± 0.06	0.021± 0.07	0.027± 0.14	0.035± 0.08	0.059± 0.61	0.047± 0.01	0.023± 0.20	0.192± 1.20	1.896± 0.009	2.097± 0.089	2.298± 0.040	2.499± 0.067	2.699± 0.291	2.899± 0.139	3.098± 0.058
Phenol degraded (g/L)	0.097	0.294	0.479	0.673	0.865	1.041	1.253	1.477	1.508	0.004	0.003	0.002	0.001	0.001	0.001	0.001
Percentage of phenol degradation (%)	97.0	98.0	95.8	96.14	96.11	94.64	96.39	98.47	88.12	0.211	0.143	0.087	0.04	0.037	0.034	0.032
Duration for complete degradation (hr)	7	9	24	27	192	215	240	252	288	300	300	300	300	300	300	300
Phenol degradation rate (g/L/hr)	0.014	0.033	0.019	0.025	0.0045	0.0048	0.0052	0.0059	0.0052	0.00001	0.00001	0.00001	0.000003	0.000003	0.000 003	0.00 00003

degradation by *Rhodococcus* sp. NAM 81 and used as the immobilisation biomatrix in the subsequent experiment.To the best of our knowledge, this is the first report on gellan gum application of immobilising *Rhodococcus* sp. for phenol degradation. Gellan-gum is a bacterial exo-polysaccharide that is being widely applied in bioremediation of environment and also in pharmaceutical and food industries[34]. It is a linear polysaccharide that produced by *Sphingomonas elodea* or *Sphingomonas paucimobilis*, which contains glucose (60%), glucuronic acid (20%) and rhanmnose (20%)[35]. The gelation mechanism could be through thermal or ionotropic gelation in the presence of cations. Work by Ahmad et al., (2012)[19] also proved that a locally isolated *Acinetobacter* sp. Strain AQ5NOL 1 from Malaysia encapsulated in gellan gum was able to degrade phenol up to 1900 mg/Lin 240 h. It also has been reported that gellan gum beads are more robust (zero breakages) than the beads from calcium alginate[36].

It has been also recommended the in fermentation process as it is mechanically stable and can withstand high temperature[37-39]. This is proven by the determination of cell leakage in this study that could represent its suitability for operational stability. Average cell leakage after complete phenol degradation from *Rhodococcus* sp. NAM81 cells entrapped in agarose, agar, polyacrylamide, gellan gum and calcium alginate was 0.184, 0.087, 0.488, 0.005 and 0.212 g/L,respectively.

The optimised conditions of immobilised

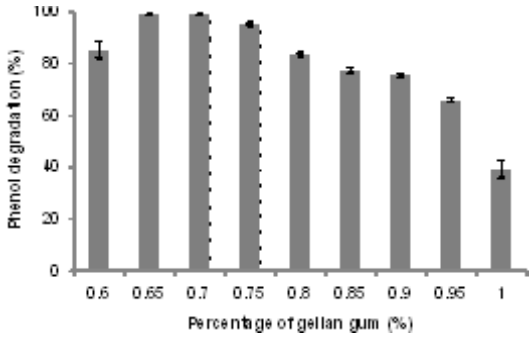

Fig. 1. Effect of percentage of gellan gum on phenol degradation by immobilized *Rhodococcus s*p. strain NAM81. Error bars represent standard error between three determinations

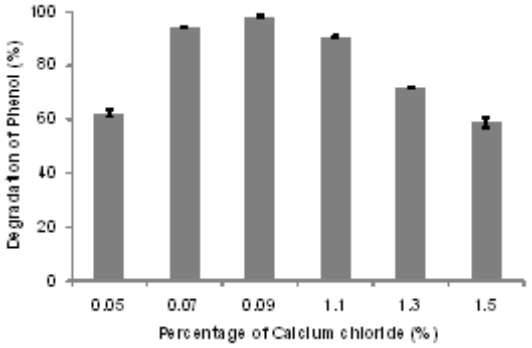

Fig. 2. Effect of percentage of calcium chloride on phenol degradation by immobilized *Rhodococcus s*p. strain NAM81. Error bars represent standard error between three determinations

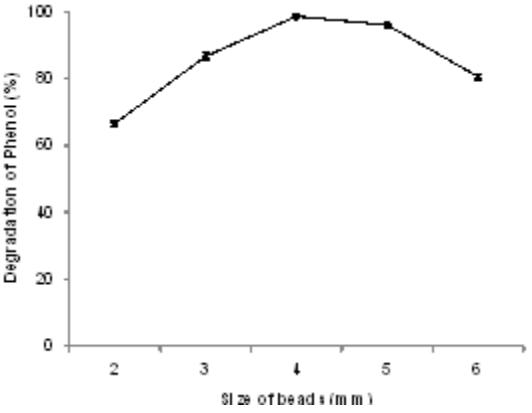

Fig. 3. Effect of size of beads on phenol degradation by immobilised *Rhodococcus s*p. strain NAM81. Error bars represent standard error between three determinations

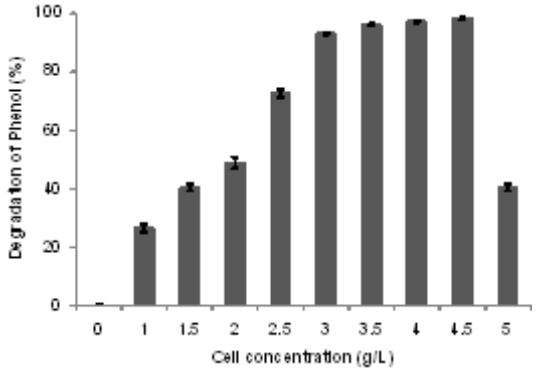

Fig. 4. Effect of cell concentration on phenol degradation by immobilized *Rhodococcus* sp. strain NAM81. Error bars represent standard error between three determinations

cells was further characterised based on effects of entrapment matrix concentration, crosslink, bead size, initial cell loading and beads number. The gellan gum concentration was varied from 0.6% (w/v) to 1.0% (w/v) to determine the suitable concentration that effects the pore size and beads mechanical strength, which controls the diffusion of substrates and leakage of cells from the beads. Fig 1 shows the effect of gellan gum concentration on phenol degradation by the immobilised *Rhodococcus* sp. NAM81. Phenol degradation was observed to be highest at 0.7% (w/v) of gellan gum concentration but has no significant differences with 0.65% and 0.75%. Low phenol degradation was observed for beads with 0.6% (w/v), 0.8% to 1.0% (w/v). In this study, 0.7% was chosen as the right concentration that promotes the best phenol degradation. The result was achieved using 4 mm bead with 300 beads per 100 mL phenol media.

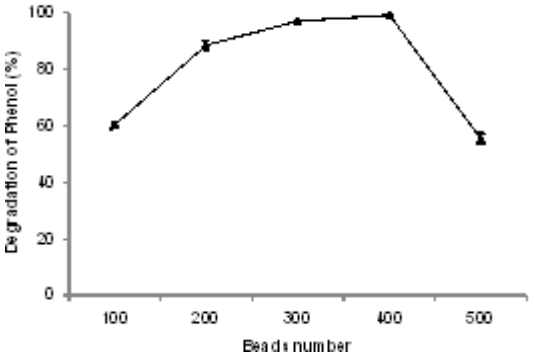

Fig. 5. Effect of beads number on phenol degradation by immobilized *Rhodococcus* sp. strain NAM81. Error bars represent standard error between three determinations

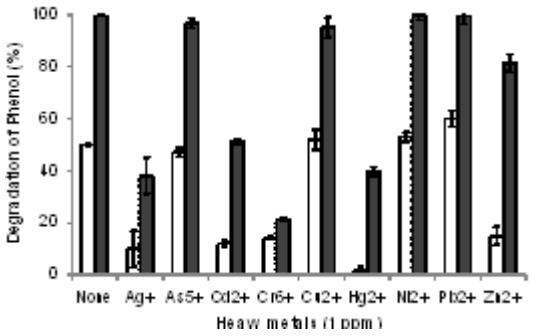

Fig. 6. Effect of heavy metals on phenol degradation by immobilized (black) and free cells (white) of *Rhodococcus* sp. strain NAM81. Error bars represent standard error between three determinations

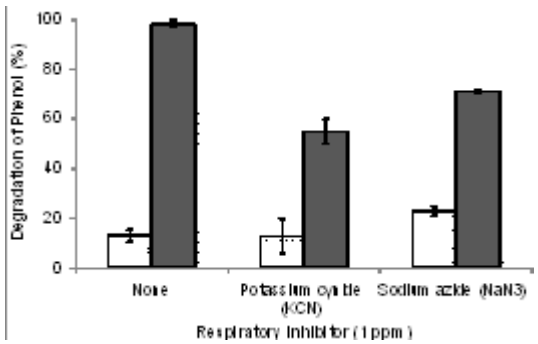

Fig. 7. Effect of respiratory inhibitor on phenol degradation by by immobilized (black) and free cells (white) of *Rhodococcus* sp. strain NAM81. Error bars represent standard error between three determinations

Besides affecting the mechanical strength of beads, the correct gellan gum composition was also very important to control the stability and rigidity of the beads which can provides optimum diffusion since the small pore only limit the diffusion of certain molecules with larger size. However, a larger pore size may caused leakage[40-42]. This could be also ascribed to the reduction efficiency with the reduction of oxygen and nutrient into the gel matrix or to the limitation of phenol hydroxylase release due to its high rigidity[43].

Cross linker is one of the important components in preparation of immobilised cells as its concentration affects the mechanical strength, density and the activity of immobilised cells[44,45]. Ca^{2+} from $CaCl_2$ has been chosen as the cation in this study because the degree of cross-linking of gelling agent affects the pore size of the beads to achieve the highest immobilisation efficiency. The mechanical strength of beads is depending on the cation concentration of the gelation solution. Thus, the concentration of calcium chloride is important for the stability and pore size of the beads. In order to evaluate the best percentage of calcium cloride ($CaCl_2$) as the cross linker with 0.7% of gellan gum, immobilised cells have been prepared using different percentage of $CaCl_2$, ranging from 0.05% (w/v) to 1.5% (w/v). The effects of calcium chloride concentration on the rigidity of the beads are shown in Fig 2. It is shown that calcium chloride at a concentration of 0.09% registered the highest phenol degradation with 97.17% of

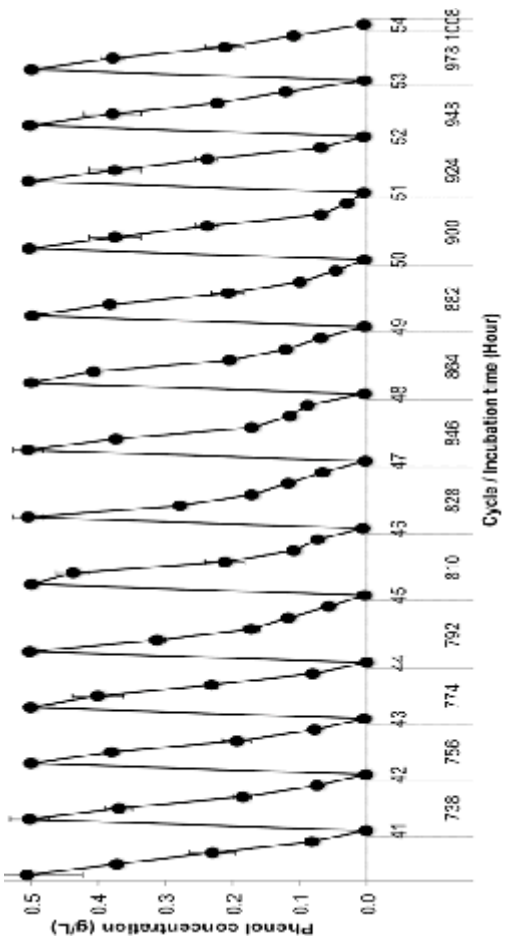

Fig. 8. Repeated usage of immobilized *Rhodococcus sp*. strain NAM81 in initial phenol concentration of 500 mgL^{-1} for cycle 41–54. Error bars represent standard error between three determinations

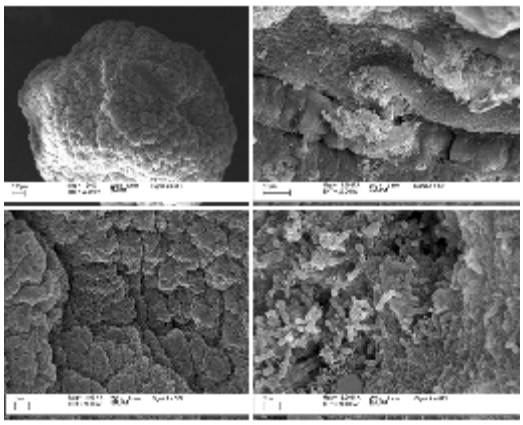

Fig. 9. Scanning electron microscope of blank gellan gum bead (a) and cells immobilized in gellan gum bead (b) after degrading phenol 18 hours under optimum condition

immobilisation percentage value. However, 0.07 g of calcium chloride also produces 96.88% phenol degradation. This was significant ($p<0.05$) when compared to the beads which prepared using 0.05, 1.1 and 1.3 g calcium chloride, revealing an entrapped phenol degradation of only 62.3%, 90.8% and 71.9%, respectively. These concentrations also form irregular shape of beads. As for gellan gum, lower concentrations of calcium chloride may possibly result in the increasing of cells leakage. The finding 0.09% calcium chloride concentration was found to be optimum for the immobilisation of *Rhodococcus* cells that can be correlated with the reports of Ahmed et al., (2011)[46] who stated that immobilisation of cells using 0.3 M calcium chloride was found to be the best for enzyme invertase from *Bacillus macerans*. This result was supported with the finding by Daniela, et al. reporting that, the hydrogel response was strongly influenced by the presence of cations in the solution through swelling kinetics assay[47]. They studied the effects of varying the concentration ions during the fabrication process of gellan gum that influence the entanglement of hydrogel network. Higher amount of Ca^{2+} ions used in the fabrication have significantly decreases the mechanical properties of gellan gum because the disproportionate crosslinking of hydrogels leads to the formation of a brittle hydrogel.

The effect of size that controls the surface area of the beads on phenol degradation by 0.7% gellan gum immobilised cells was assessed by measuring phenol degradation over a range of gellan gum bead sizes of 2 to 6 mm diameter (Fig 3). As a result, the highest phenol degradation rate has been achieved at 4 mm beads (approximately 50.26 mm^2 surface area). Phenol degrading activity was markedly reduced over the larger surface area tested. Since the quantity of bacteria was kept constant during the preparation of the gellan gum beads, the size of beads can also be related directly to the amount of bacteria per flask. The result showed that phenol degradation was reduced at higher cell densities and microspheres were found to be mechanically robust than macrocapsules[48,49]. This observation may attribute to the limitation of oxygen in beads that are larger than 4 mm[50]. Besides, smaller beads allow efficient diffusion of nutrients into the

microspheres and diffusion of metabolites out of the microsphere. This finding aligns with the report by Guieley, (1989)[51]that suggested the best size of bead for microbe encapsulation was between 500-1000 µm.

Fig 4 represents the experimental result on the effect of initial cell loading on the biodegradation process by immobilised cells. At lower cell concentration, phenol degradation was observed to be low but peaks at 0.35 to 0.45 g/L of cell concentration with 0.4 g/L as the maximum. Phenol degradation was observed to be decreased when 0.5 g/L was added, as it is too pack for the cell in 4 mm of bead size. The maximum phenol were eliminated by 4.5 g/L of immobilised cells followed by 4, 3.5, 3, 2.5, 2, 1.5, 5 and 1 g/L with 98.3%, 97.2%, 96.2%, 93.2%, 72.5%, 48.8%, 40.4%, 40.4%, and 26.5% respectively. Cell densities that influence the percentage of phenol degradation might depend on the size of beads. Cheetam et al., (1985)[52], explained that high concentration of cells in bead were less active because the porous structure of the beads was lost. Since 4 and 4.5 g/L does not showed significant differences, therefore 4 g/L was used as the amount of cell loading.

For determination of optimum beads number, 100 mL of the MSM that contained 500 mg/L was added with different amount of beads ranging from 100 to 400 beads. Using 0.7% (w/v) as the gellan gum concentration and 4 mm in size, the effect of bead number on phenol degradation by the immobilised *Rhodococcus* sp. has been evaluated (Fig 5). After 20 hours incubation, phenol degradation has been observed to be increased as the amount of bead has increased and dropped gradually when 450 and 500 beads were used. Phenol degradation was optimum with 350 beads. A similar report was revealed by Ahmad et al., (2011)[19] with 300 beads for phenol degradation by *Acinetobacter* sp. cell density that is essential for optimum degradation of phenol by the immobilised bacteria. A greater competition for oxygen and nutrient might be the constraint for phenol degradation since more beads are equivalent to high cells density per flask[53]. All of the optimised conditions have been applied to further assess the ability of immobilised cells in phenol degradation and revealed that the 100% phenol degradation was achieved within 18 h incubation.

Many studies reported that immobilised cells system is more dominant than free cells system in degrading phenol. Hence, a series of experiment were carried out to compare the ability of free and immobilised *Rhodococcus* sp. cells for their phenol-degrading activity over time at different initial phenol concentrations ranging from 100 to 3100 mg/L (Table 2). To ensure a valid comparison, the same amount (wet weight) of bacterial cells was used in this experiment. Initial phenol concentration is important in biodegradation process in order to know the inhibitory effect son the activity of the biomass. Both free and immobilised bacteria exhibited similar phenol degrading characteristics at 100 mg/L, completely degraded phenol within 6 h. Free cells showed equal efficiency at low phenol concentration that might be attributed to the accessibility of phenol to the cells as it could directly get in contact with the carbon source compared to mass transfer control for immobilised cells. Mass transfer was reported as a control mechanism for biodegradation of heavy metals at low concentration[50]. Hence, our result also expected to have a similar mechanism for phenol biodegradation as phenol are known to have inhibitory effects on the activity of cells. Biodegradation of phenol by *Acinetobacter* sp. AQ5NOL1 was also comparable, as free cells of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 gave higher phenol removal efficiency than immobilised cells at phenol concentration lower than 500 mg/Land 300 mg/L for *Psedomonas putida*[36,54]. However, better degradation efficiency by immobilised cells was observed from higher phenol concentration. The toxicity of high phenol concentration could be inactivated the bacteria and causing the death of free cells but gellan gum acts as a shelter for the immobilised cells since it can survive in high phenol concentration over a longer period. Immobilised *Rhodococcus* sp. completely degraded phenol within 108, 216, and 240 h at 1100, 1500 and 1900 mg/L phenol, respectively, whereas free cells took 240 h to completely degrade phenol at 1100 mg/L. Phenol-degrading activity of the free bacteria was inhibited at higher concentrations. Prieto et al., (2002)[55]reported that immobilisation of *Rhodococcus erythropolis*

UPV-1 had resulted in the shortening of lag phase as observed from this study. Encapsulating the bacteria in gellan gum will afford protection to the bacteria. The pore size of 0.7% beads possibly affects diffusion of phenol into the beads. This positive effect had also been observed on the immobilisation of *Rhodococcus opacus* 1G that is able to degrade 2200 mg/L of phenol[56]. In this study, the immobilised cells of *Rhodococcus* sp. strain NAM 81 were able to tolerate up to 2500 mg/Ldegradation within 252 h.

The simultaneous contamination by organic compounds and heavy metals at industrial areas have been widely reported[57]. Besides, metals may have also been released from agricultural activities and human wastes that were being transported to marine environment by estuaries of river[58,59]. The excessive concentration may harm the living organisms. Heavy metals were also associated with phenol contaminant in the polluted area, which will interfere with the efforts of bioremediating phenols using microorganisms as the heavy metals exert their toxic effects on the microorganisms' cells. However, certain microorganisms have the capability to colonise the polluted environment. Hence, studies were carried out to determine the effects of selected heavy metals toward phenol degrading ability of free suspended and immobilised *Rhodococcus* NAM 81. When both systems (freely-suspended and immobilised cells) were being compared, the inhibition of heavy metals within 18 h incubation time toward free cells were increased when immobilised cells have been applied. From the result obtained (Fig 6), almost 99% of phenol was degraded by immobilised cells in MSM containing As^{5+},Ni^{2+}, $Cu^{2+}$and Pb^{2+} compared to free cells that only degraded 47%, 53%, 52% and 60% of the respective compound. Whereas, the other five heavy metals tested, Cr^{6+}, Cd^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+} inhibited phenol-degrading activities. Cr^{6+} had caused the highest effect with only 21% phenol degraded but there is no significant different ($p>0.05$) with free cells as it could degrade 14% of phenol that might because of 1 ppm gave less effect towards degradation. It has also been reported by de Lima e Silva et al., (2007)[60], that biotransformation and bio-degradation of aromatic compounds were affected by 5 ppm of chromium.Hg^{2+} and Ag^{+}suppressed the biodegradation, which caused 38% of phenol degradation and more inferior free cells with only 1.5% and 10% degradation respectively. The reduction might because of the inhibition of hydroxylase activity[61, 62]. Additionally, 1 mgL^{-1} of Cd^{2+} and Zn^{2+} inhibited 49% and 19% of phenol degradation of immobilised cells and 75% of free cells respectively. $Cd^{2+}$was found to exert a strong inhibitory result on the activity of cathecol 2,3 dioxygenase enzyme[63]. Fijalkowska et al., (1998)[64] also showed a complete reduction in anthracene degradation by *Rhodococcus* with the presence of lead acetate. From the result obtained, it has been evidently showed that the inhibitions were reduced for immobilised cells as the encapsulation could reduces the effect of environmental stress on cells because heavy metals have affected the viability of the microbiota and disturbs the catalysis enzyme which compromisethe biodegradable processes of the aromatic compounds.

Besides the effects of heavy metals, the immobilised cells have also tested with the presence of respiratory inhibitors in the medium containing 0.5 g/Lphenol since industrial wastewater discharge may co-contaminate the compound[65,66]. Respiratory inhibitors blocked the electron transfer in electron transport chain or disturbed the activity of cytocrome oxidase enzyme[67,68]. The effect of 1 ppm potassium cynide (KCN) and sodium azide (NaN_3) in 18 hours incubation can be observed in Fig 7. Immobilisation of *Rhodococcus* NAM81 cells in gellan gum have improved phenol degradation ability from only 12% by free cells to 55% by immobilised cells with KCN and from 22% by free cells to 71% by immobilised cells with the existence of NaN_3. Cynide that is toxic, carcinogenic and mutagenic had been released in wastewater from electroplating, metal finishing and coal coking industries[69]. Sodium azide is also toxic but from this experiment, this strain especially the immobilised cells could tolerate to 1 ppm NaN_3. The registered growth might due to the concentration of KCN and NaN_3 that were too low to inhibit the growth of *Rhodococcus* NAM81 since bacteria from this genus poses the ability to degrade various types of toxic compounds in agreement with previous reports[70,71]. This observation indicated that

respiratory inhibitors inhibited phenol degradation ability of the tested strain but the percentage was increased with the application of immobilised cells as the amount of phenol had been ceased from the medium.

One of the benefits of using immobilised cells is that the cells could be reused for a considerable period due to constant cell regeneration within microspheres[72]. An experiment to test the reusability of immobilised cells in gellan gum was conducted, as it is important to determine the effectiveness of cells after being repeatedly use as well as the total cycle. In this study, the immobilised cells have been tested in 18 hours cycle under the optimum condition and phenol degradation capability remained constant for at least 50 cycles but the degradation period had increased to 24 hours in cycles 51 and 52 and longer starting at cycle 53 with 30 hours of incubation period (Fig 8). In conclusion, the 18 hours of degradation time can be maintain by recycling the immobilised *Rhodococcus* NAM 81 within 50 rotations. It was proven that the tolerance of cells toward toxicity and protection from substrate inhibition has been improved by using immobilisation as reported in numerous researches[73]. The reusable gellan gum gives a great number of advantageous since it is able to decrease cells waste, as well as saving cost and time for cells cultivation. *Rhodococcus* sp. NAM81 was able to grow within the gels as it produces bright orange colour after the 10th cycle, which indicated high cell densities in the gels as it grows on phenol as the carbon source during repeated phenol degradation process. It has been reported that gellan gum could be an alternative to agar for microbiological agar or cultivation of plant tissue[74,75]. The weight of immobilised beads has consistently rose with the increase in number of cycle especially within 10 cycles as the cells were multiplied. The weight of beads decreased when the immobilised cells were reused after 50 cycles and led to cells leakage from the beads (data not shown).

The strength for the long duration of storage and operation is a key factor for functional application of immobilised cells system. Therefore, the investigation about storage stability of phenol degradation activity was done on the free and immobilised *Rhodococcus* cells, which were stored in 100 mL of phosphate buffer under 4ºC. After 7, 14, 21 and 28 days of storage, both type of cells were transferred to MSM containing 500 mg/Lof phenol and phenol degradation experiment has been performed under the optimum condition. As shown in Table 3, the degradation rates of immobilised and free cells does not decreased significantly with the extension storage time of 85% and 70% after being stored for 28 days, respectively, suggesting that storage stability of encapsulated cells were better than free cells as it maintained the physiological stability.

The surface structure of entrapped *Rhodococcus* sp. NAM 81 cells were studied using SEM. Fig 9 shows the scanning electronic micrographs of blank gellan gum beads (Fig 9a) and gellan gum-immobilised *Rhodococcus* sp. NAM81 (Fig 9b) after incubation in MSM containing phenol for 24 hours under optimum conditions. The blank gellan gum surface was scabrous and had multiple porous structures that could allow the diffusion of oxygen, substrates and metabolite. Gellan gum beads depicted a random distribution of *Rhodococcus* sp. NAM 81 rod coccus cells. There were pockets of high cells density at a scale of 10 µm, which were the areas of cell growth of the porous matrix. The result further suggested that gellan gum was able to become an ideal carrier of immobilised *Rhodococcus* sp. as the carrier offers ample space to support the growth of cells. To the best of our knowledge, we have not come across any report regarding the SEM of phenol degrading gellan gum entrapped *Rhodococcus* cells. Though in literature, SEM of alginate entrapped cells available were showing similar observations of random cell distribution[40,53,76].

CONCLUSION

From this study, the results obtained proved that the immobilised cells exhibited higher degradation efficiency. Phenol degrading abilities of a locallyisolated *Rhodococcus* sp. NAM 81 entrapped in gellan gum has been optimised and their candidature has been warrant for remediation of phenol and phenolic compounds in wastewaters.Research on application of immobilised *Rhodococcus* sp. NAM 81 in the

treatment of industrial effluents containing phenols is in the pipeline.

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