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Removal of heterocyclic compound carbazole using cell immobilization of *Thalassospira profundimaris* strain M02

Remoción del compuesto heterocíclico carbazol usando inmovilización celular de *Thalassospira profundimaris* cepa M02

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Abstract

Cell immobilization offers promising potential to maximize microbial biodegradation activity and overcome limitations involved in bioremediation. *Thalassospira profundimaris* strain M02 was used to determine the effectiveness of cell immobilization for carbazole biodegradation. Effects of different matrices, effective diffusivity, mechanical strength, matrix concentration, cell mass load and reusability were investigated in this study. Results revealed that calcium alginate (Ca-Al) was better compared to gellan gum as higher carbazole biodegradation rate was observed. Diffusion analysis and mechanical strength analyses also revealed that Ca-Al possessed superior characteristics as immobilization matrix for carbazole biodegradation compared to gellan gum. Ca-Al works best at 4% (w/v) with 1.25 g of cell mass loading. In addition, immobilized strain M02 retained carbazole biodegradation activity after 6 cycles of usage.

Keywords: Bioremediation, calcium alginate, carbazole, cell immobilization, gellan gum.

Resumen

La inmovilización celular ofrece un futuro prometedor para maximizar la actividad microbiana de biodegradación y superar las limitaciones involucradas en la biorremediación. La cepa M02 de *Thalassospira profundimaris* se usó para determinar la efectividad de la inmovilización celular para la biodegradación de carbazol. En este estudio se evaluó el efecto de diferentes matrices, el efecto de difusividad, la resistencia mecánica, la concentración de matriz, la carga de masa celular y reusabilidad. Los resultados revelaron que el alginato de calcio (Ca-Al) fue mejor en comparación con la goma gelana, ya que se observó una mayor tasa de biodegradación de carbazol. Los análisis de difusión y resistencia mecánica también revelaron que el Ca-Al en comparación con la goma gelana, presentó características superiores como matriz de inmovilización para la biodegradación de carbazol. Ca-Al funciona mejor a 4% (p/v) con 1.25 g de carga de masa celular. Además, la cepa M02 inmovilizada mantuvo la actividad de degradación de carbazol después de 6 ciclos de uso.

Keywords: Biorremediación, alginato de calcio, carbazol, inmovilización celular, goma gelana.

1 Introduction

Carbazole (CAR) is a member of a group of chemicals known as heterocyclic compounds. Distinct from typical hydrocarbons, heterocyclic compounds possess one or more atoms, which are not carbon or hydrogen. Well-studied members of this group are dioxins, which have been shown to have adverse biological activities. Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCB) have been demonstrated to be associated with carcinogenic, genetic, reproductive, and developmental effects in animal studies (Mikolajczyk *et al*., 2020). Carbazole is the major N-heterocyclic hydrocarbons in fossil fuels and also found in cigarette smoke, as well as from coal and wood combustion (Salam *et al*., 2015).

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Industrially, carbazole is being utilized as starting material for production of dyes and pigments (Azri *et al*., 2015).

Bioremediation utilizes the metabolic potential of microorganisms to remove xenobiotics from the environment as they are able to transform hazardous compounds into less harmful substance suitable for natural biogeochemical cycle (Zakaria *et al*., 2020; Martínez-Prado and Soto-Álvarez, 2019; Cisneros-de la Cueva *et al*., 2016). In comparison to physical and chemical approaches, bioremediation provides effective environment treatment in a cost competitive and eco-friendly manner (Vikrant *et al*., 2018). Despite the apparent advantages, there are undeniable limitations, which may reduce the overall effectiveness of bioremediation. Unfavourable conditions of the environments, such as low temperature, high salt concentration and alkaline or acidic pH reduce performance of microorganisms in metabolizing recalcitrant xenobiotics (Darham *et al*., 2019; Roslee *et al*., 2020; Abdulrasheed *et al*., 2020). High levels of xenobiotics present in the contaminated environment was also identified as an inhibition factor for microbial growth, which impacts bioremediation performance (Ahmad *et al*., 2017; Karamba *et al*., 2020; Lee *et al*., 2018; Ibrahim *et al*., 2020).

To overcome factors limiting bioremediation performance, significant efforts have been invested in the isolation of resilient microorganisms from a wide phylogenetic distribution namely phyla Proteobacteria, Actinobacteria, Cyanobateria, Bacteriodetes and Firmicutes (Louvado *et al*., 2015). Alternatively, cell immobilization may be an attractive strategy in combating bioremediation performance loss. Cell immobilization potentially reduce abiotic and biotic stress from the cells, which will lead to more advantages such as protection from toxic effects of hazardous compounds. This should result in increased cell survivability as well as metabolic activities (Karamba *et al*., 2017). It was also reported that immobilized cells showed greater catalytic stability than of free cells, and some immobilized microorganism showed higher toleration towards toxic compounds than they do in non-immobilized state (Ahmad *et al*., 2017). Immobilization also allows more operational flexibility when used in reactors as it prevents biomass washout and making higher cell densities possible (Mendoza-Madrigal *et al*., 2017). Different matrices can be used to immobilized cells, namely polyurethane foam (Rajendran *et al*., 2015), calcium alginate (Upendar *et al*., 2017), cinder beads (Huang *et al*., 2016), activated carbon powder (Jorfi *et al*., 2018) and gellan gum (Yusuf *et al*., 2019). To date, there is no specific study on the optimum matrix and cell loading of bacterial immobilization for an improved removal of carbazole. Hence, this study aims to investigate the optimum conditions for CAR removal using bacterial immobilization for future development of heterocyclic compounds removal bioreactor.

2 Materials and methods

2.1 *Microorganism*

Isolated strain of *Thalassospira profundimaris* strain M02 (accession no.: KX180913.1) was obtained from Environmental Biotechnology Laboratory, Universiti Malaysia Sarawak. Strain M02 was isolated from marine sediment and was confirmed to be able to utilize various polyaromatic hydrocarbons and heterocyclic compounds as sole carbon source. *T. profundimaris* strain M02 was cultivated in 10 mL ONR7a medium enriched with 0.1% (w/v) CAR. Prior to immobilization, *T. profundimaris* strain M02 was cultivated in 100 mL marine broth. After 24 h of cultivation, the broth was centrifuged at 7000 rpm for 10 min to obtain cell pellets.

2.2 *Cell immobilization*

Cell immobilization using gellan gum was performed according to method described by Yusuf *et al*. (2019). Gellan gum was added into 100 mL dH_2O to final concentration of 0.75% (w/v) and heated at 75 ºC to dissolve the gellan gum completely. Upon reaching 75 ºC, $CaCl_2$ (final concentration 0.06% (w/v)) was added to the mixture and then left to cool until approximately 50 ºC and prior to pH adjustment to 7.0. When the temperature reached between 40 ºC and 30 ºC, *T. profundimaris* strain M02 cell pellets were added to the mixture. Immobilization beads were formed by expelling the mixture into oil using syringe. The beads are separated from oil by repeatedly washing it using Tween 80 and sterile distilled water. Immobilization using calcium alginate (Ca-Al) was done according the method described by Hui *et al*. (2020).

2.3 *Optimization of cell immobilization conditions*

The conditions determined for the optimization were types of matrices, matrix concentrations and cell mass loading. The experiments were done one factor at a time. Gellan gum and Ca-Al were used to study the effects of different matrices on the removal of carbazole. Matrix concentrations ranging from 0.55% to 0.95% (w/v) was used for gellan gum, while concentration of Ca-Al varies from 3% to 5% (w/v) in this study. While for the cell mass loading, cell weight ranging from 1.25 to 5.0 g per 100 mL of mixture were used. These experiments were conducted in 100 mL ONR7a medium supplemented with 0.1% carbazole for 48 h and the concentration of CAR in the media were measured every 6 h. All experiments were conducted in triplicates.

Comparison of CAR removal rate for immobilized cell and free cells were conducted using best conditions determined after optimization. For reusability experiment, the beads are collected and washed using ONR7a thrice to remove remaining substrate. The washed cells are then added into fresh ONR7a medium with the same initial amount of CAR for reusability test. The experiment was repeated for 5 cycles to determine the life span of the immobilized cells

2.4 *Residual carbazole analysis*

Quantitative analysis of CAR removal was determined using gas chromatography with flame ionization detector (Shimadzu GC 14B, Japan). One mL aliquots were sampled from each experiment. Sample of known CAR concentrations with no bacterial culture are used as standards. The samples were extracted using 1.0 mL ethyl acetate and the inorganic layer of were collected for GC-FID analysis using HP-5 fused silica capillary column (50 mm x 0.32 mm x 0.25 μm) at 250 ºC at the injector, 300 ºC at the detector, with column heated to 200 -250 ºC at 5 ºC per min and split-less column with helium as the carrier gas. In this study, the residual CAR remaining was expressed in percentage and calculated using the equation (1).

$$\text{Residual CAR (\%)} = \frac{\text{CAR concentration at } T_0}{\text{CAR concentration at } T_n} \times 100\% \tag{1}$$

The rate of CAR removal by immobilized cells was also calculated using the equation (2).

$$\text{Rate of CAR removal} = \frac{[(\text{CAR concentration at } T_0 - \text{CAR concentration at } T_n)]}{[(T_n - T_0)]} \tag{2}$$

2.5 *Diffusion analysis*

The data obtained from the CAR removal experiments were used to study the diffusion effect in different matrices. Linear fitting model were selected where it involves large diffusion time as follows (3);

$$\ln\left(\frac{c_s(1+\alpha)}{C_{s0}\alpha} - 1\right) = \ln\left(\frac{6(1+\alpha)}{\left(9+9\alpha+q_1^2\alpha^2\right)}\right) - \left(\frac{D_e q_1^2}{a^2}\right)t \tag{3}$$

where t is the diffusion time; a is the diameter of the beads; α is the ratio of the volume of the solution to the volume of the beads; D_e is the effective diffusivity; n is the number of the beads and q_n are the positive non-zero roots; C_s is concentration of solute; C_{s0} is initial substrate concentration. The effective diffusivity, D_e can be calculated using the ratio of volume to beads, α (Hui *et al*., 2020). From the biodegradability experiment, α ratio is 2:1, C_{s0} is 1000 ppm and diameter of the beads, a is 3 mm average.

2.6 *Tensile strength analysis*

Mechanical characteristic of immobilization matrices were evaluated by tensile strength analysis. Three different concentrations ranging from 3.0% to 5.0% (w/v) for Ca-Al were used. The mixture of gel was poured into dog bone shaped mold. The mold dimensions were set to be 4 mm x 100 mm. The molded Ca-Al gels were hardened by curing it in calcium chloride for 2 h before stored in distilled water at room temperature for 2 h. The stress-strain measurements were obtained by using Tensile/Universal testing machine (Shimadzu, Japan) at 10 mm/min with 15 kN range and gauge length at 30 mm. The Young's modulus (E) and tensile strength (TS) were then calculated from the stress-strain plot.

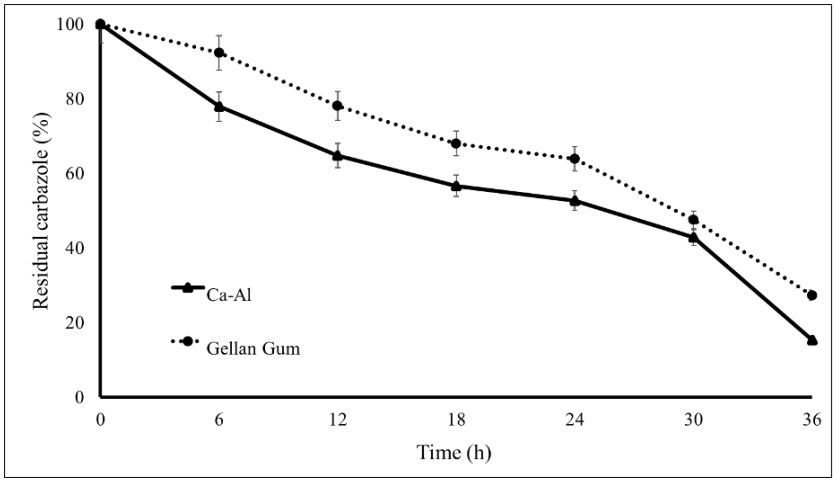

Fig 1. Residual carbazole after removal by immobilized *T. profundimaris* strain M02 using gellan gum and Ca-Al as immobilization matrices.

3 Results and discussion

3.1 Carbazole removal and effective diffusion comparison between gellan gum and Ca-Al

Carbazole removal performance was evaluated when gellan gum and Ca-Al were used as immobilization matrices. Removal rates were measured based on residual CAR in the media after 36 h period (Figure 1). Both matrices exhibited similar CAR removal pattern with almost linear reduction of residual carbazole. The Ca-Al matrix exhibited higher rate of removal at 2.35% h^{-1} compared to gellan gum matrix at 2.02% h^{-1}. Ca-Al also showed lower CAR residual concentration at 15.29% while gellan gum shows 27.28% after 36 h of biodegradation. In a similar study, phenol biodegradation efficiency by *Pseudomonas putida* strain P53 and *Arthrobacter scleromae* strain P69 was higher when cells were immobilized with Ca-Al compared to free cell system (Abarian *et al*., 2019). However biodegradation for strain P53 were improved significantly more than strain P69 when immobilized, indicating possibility of matrix compatibility with different strains.

Immobilization using Ca-Al involves entrapping bacterial cells without exposing cells to extreme changes in physicochemical conditions as the process can be conducted at ambient temperatures. On the contrary, the process of immobilization using gellan gum requires bacterial cells to be added to solution at higher temperatures (approximately 45 ℃) prior to solidification. This exposes bacterial cells to unfavourable heat, which eventually affect the initial cell viability in the produced beads. Cell viability after the immobilization process was not investigated in this study. Both Ca-Al and gellan gum were reported as excellent matrices for removal of toxic compounds (Jin *et al*., 2019, Maniyam *et al*., 2019, Fang *et al*., 2020).

Further studies are required to investigate the effects of different matrices on bacterial growth and will be investigated in future studies. Immobilization of microorganisms to inert and porous solid matrices provide several advantages for increasing bioremediation rate while reducing cost, specifically by increasing cell concentration, reducing start-up time and minimizing cell loss.

Effective diffusivity values were calculated to investigate mass transfer rate of CAR and/or oxygen within matrices. (Table 1). Calculation results showed Ca-Al possess significantly higher effective diffusivity value of 5.76×10^{-5} cm^2/s compared to gellan gum which was 3.68×10^{-6} cm^2/s. This result revealed that effective diffusivity value for Ca-Al was higher compared to gellan gum. Carbazole removal performance of strain M02 can be greatly influenced by the availability of CAR as substrate in the vicinity of the entrapped cells, and O_2 diffusion since CAR metabolism is an aerobic process. Optimum effective diffusivity at 4% (w/v) Ca-Al concentration. This can be interpreted that higher concentration of alginate would cause the diffusivity to decrease as pore size of the beads are getting smaller and hinder the substrate to enter the beads (Wang *et al*., 2019).

Table 1. The effective diffusivity of carbazole at 3%, 4% and 5% (w/v) concentration of Ca-Al.

Matrix type	Concentration (% w/v)	Diameter, D	q_1 (cm)	Effective Diffusivity (D_e) (cm^2/s)
Gellan gum	0.75	0.3	1.37	3.68×10^{-6}
Ca-Al	3	0.3	0.58	3.81×10^{-5}
	4	0.3	0.58	5.76×10^{-5}
	5	0.3	0.58	3.13×10^{-5}

On the other hand, at low concentration, softer beads are easily damaged by shaking/agitation, causing broken beads and bacterial cell leakage, thus reducing overall CAR removal. The decreased diffusion rate is due to the degree of crosslinking, where the diffusion coefficient decreases as cross-linking density increases (Xu *et al.*, 2019). The optimal Ca-Al concentration for the removal of CAR was at 4% as it shows the highest amount of residual CAR concentration as well as best effective diffusivity.

3.2 Effects of Ca-Al concentrations and cell mass load on carbazole removal

Carbazole biodegradations by immobilized strain M02 were investigated using different concentration (from 3% to 5% (w/v)) of Ca-Al and the initial concentration of CAR was kept constant in every experiment. It was observed that after 36 h, Ca-Al concentration 3% showed the highest residual CAR (Figure 2). While 4% and 5% concentrations initially showed comparable removal rate, 4% (w/v) concentration Ca-Al immobilized cells showed the highest CAR biodegradation as it presented the lowest residual carbazole at the end of 36 h. The Ca-Al concentration could affect mass transfer rate of CAR and/or oxygen within matrices hence effective diffusivity values were also calculated for different concentration of Ca-Al (Table 1).

Effects of cell mass loading towards the CAR removal were investigated by varying the immobilized cell mass. Generally, results showed that as the cell mass increases, removal of CAR decreases (Figure 3). It can be seen that 1.25 g of cells produced the lowest residual CAR at 15.29%, indicating that 84.71% of CAR has been removed after 36 h. On the contrary, the highest cell mass used in this experiment was at 5.0 g showed the lowest removal at 46%.

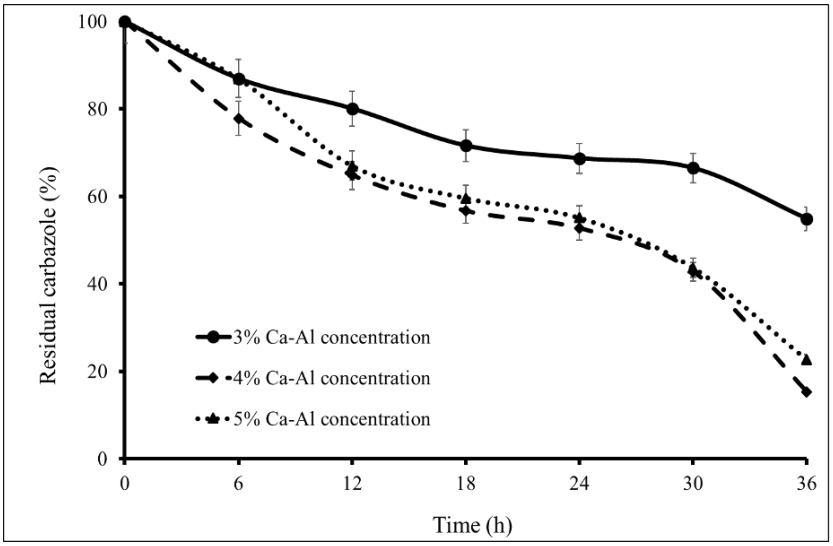

Fig 2. Residual carbazole after removal by immobilized *T. profundimaris* strain M02 using different concentrations of calcium alginate.

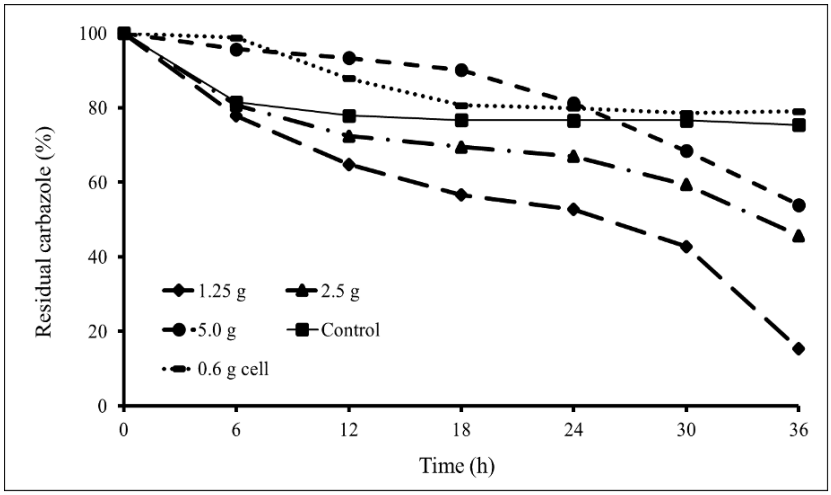

Fig 3. Residual carbazole after removal by immobilized *T. profundimaris* strain M02 using different cell mass loading.

Higher mass of cell immobilized causes higher competition among bacterial cells thus limiting the CAR removal. This can be seen when 5.0 g of cell required longer time for removal. A highly dense immobilized bead with cells may lose its efficiency as most cells encapsulated at the surface of the beads tends to get the nutrients from the media, leaving most of the cells embedded at the centre of the beads to starve and eventually die. As a result, only part of the cells inside the beads are functional thus lowering the removal rate. Increasing cell mass loading also introduces geometrical constraints, as there is limited capacity of the immobilization beads to encapsulate more cells.

3.3 Immobilized cells reusability and mechanical strength analysis of matrices

Reusability experiments were conducted in repetitive batch to assess the CAR removal activity retention and durability once cells were immobilized. The results showed significant decrease (32%) in CAR removal rate between the first and second cycle (Figure 4). From the third cycle onwards, the CAR removal activity reduction was no greater than 10% after each cycle indicating activity retention. In a similar study, Ferreira *et al*. (2015) reported Ca-immobilized beads were reusable without losing degradation activity for 3 cycles. However, beads begin to dissolve in the 4th cycle when used for removing phenanthrene and fluoranthene. It is unclear at this point, whether different types of aromatic compounds may contribute to the reusability of immobilized beads. Further studies are required to determine factors affecting reusability of immobilized bacteria in CaAl beads.

Mechanical strength parameters of matrices were measured as these parameters determine the durability of reused immobilized cells. The results of were summarised in Table 2. The average elongation, strain, engineering stress and Young's modulus parameters indicate the strength and flexibility of the matrices. Overall, it is evident that Ca-Al possessed superior mechanical strength characteristics compared to gellan gum. From mechanical strength analysis, Ca-Al showed higher mechanical strength compared to gellan gum. Better mechanical strength characteristic would be advantageous since the ultimate goal for microbial immobilization is utilization in reactors, and stronger matrices may be used repeatedly which would eventually lead to longer retention of biological activity and overall lower operational cost. For the case of Ca-Al matrix, higher mechanical strengths can be achieved with higher concentration gels (Table 2). Despite having highest mechanical strength at 5% (w/v), Ca-Al at 4% (w/v) showed the best CAR removal. A study on phenanthrene and fluoranthene biodegradation using immobilized bacteria found that at 3% (w/v) Ca-Al concentration shows an optimum condition for the biodegradation (Wang *et al*., 2019). However, in this study the removal of CAR was lowest at 3% (w/v).

Table 2. Mechanical strength analysis of calcium alginate at 3%, 4% and 5% (w/v) concentration used for cell immobilization.

Calcium Alginate Concentration	Average Elongation	Strain, (Dimensionless)	Average Force, F (N)	Engineering Stress, (N)	Young's Modulus, E (N/mm^2)
3%	41.93±6.66	1.40	2.53±0.51	0.079	0.06
4%	39.28±6.40	1.31	3.88±0.64	0.121	0.09
5%	39.59±7.04	1.32	4.83±0.67	0.151	0.11

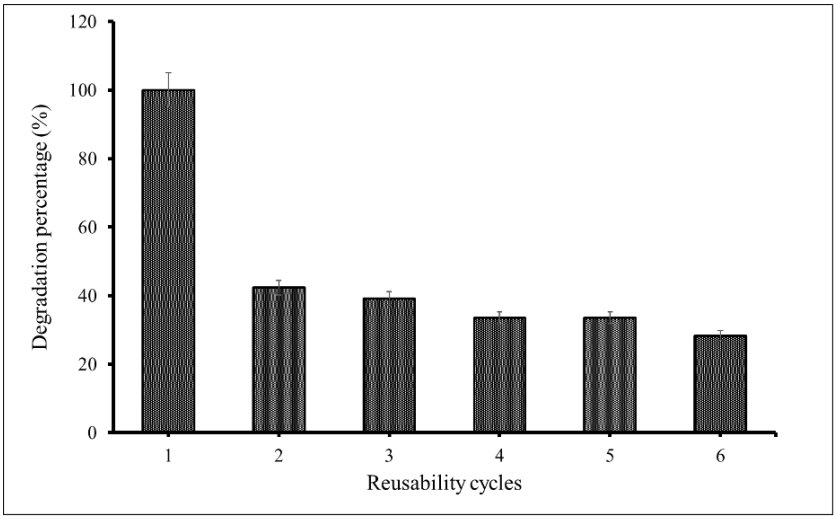

Fig 4. Carbazole removal at the end of 24-hour cycles with reused immobilized beads.

This may due to the different physical and chemical characteristics of carbazole and phenanthrene and fluoranthene. Carbazole is chemically distinct when compared to phenanthrene and fluoranthene as carbazole is a heterocyclic compound, while they may be structurally similar being polycyclic compounds. At lower Ca-Al concentration, the stability of the beads was reduced due to the reduced cross-linking with lower alginate concentrations and ruptured beads directly expose the entrapped cells to the toxic environment, depleting the growth of cells and lowering removal rate. On the contrary, at higher Ca-Al concentrations, the matrix become more rigid and affects the diffusion of CAR into the beads thus affecting the removal of carbazole. Apart from mechanical strength, chemical stability of matrices should also be considered specially when used in environments where reactive chemicals exists. It was reported that Ca-Al beads will gradually dissolve when sodium polyphosphate, sodium carbonate and/or calcium chelating agent is present (Ferreira *et al*., 2015).

Conclusions

Cell immobilization of *T. profundimaris* strain M02 immobilized in Ca-Al at 4% (w/v) concentration and with cell mass loading of 1.25 g provided the most optimum condition for the immobilized cells to remove carbazole. Reusability experiment of using immobilized beads indicated CAR removal activity was retained above 25% even after 6 cycles. These results indicated that with the optimized condition, the usability of immobilized *T. profundimaris* strain M02 could be further extended for effective removal of heterocyclic compounds such as carbazole for bioremediation applications.

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