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Cite as: AIP Conference Proceedings **2155**, 020009 (2019); https://doi.org/10.1063/1.5125513
Published Online: 06 September 2019

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Transport Phenomena of Carbazole Biodegradation by Immobilized *Thalasosspira profundimaris* Cell and Mechanical Properties

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Abstract. Carbazole is a heterocyclic aromatic compound that imposes threat to the environment when contaminates water source. A marine-isolated bacterium, *Thalassospira profundimaris* shows ability to degrade carbazole. The use of free-cell for bioremediation is inefficient as the cells are exposed to harsh environmental condition. In this study, immobilizations of *T. profundimaris* in gellan gum were investigated to develop robust systems for bioremediation. The mechanical strength and its relationship with transport of carbazole was investigated. The findings proved that concentration of immobilization media affects diffusivity and mechanical strength. Higher media concentration formed a stronger bead with lower diffusivity where lower concentration formed soft bead with higher diffusivity. The optimum concentration of gellan gum was 0.7% (w/v) with 61% carbazole degradation recorded and an optimum diffusivity of 36.8 x 10^{-7} cm^2/s. It has the highest Young's modulus (0.041810 N/mm^2) among other concentrations. The findings of the optimum carbazole degradation, strength and diffusivity were profound to increase the performance of the bacteria entrapped inside the immobilization media for bioremediation and withstand harsh environment.

INTRODUCTION

Heterocyclic aromatic hydrocarbons are well known as environmental contaminants resulted from petroleum industry. The presence of these hazardous compounds in sea water, soil, river sediments, and ground water has become a great threat to the environment due to its toxic and mutagenic properties [1]. The environmental pollution by these toxic compounds has been gaining great concern in the field of environmental remediation. Isolation of marine microorganisms capable of transforming and degrading these compounds has proven that bioremediation technology is potentially effective to treat the pollution in marine environment in an eco-friendly, sustainably and cost-competitively manner [2]. Bioremediation is a process that uses microorganisms and their aggregates to detoxify or eliminate pollutants to their capabilities and is usually used to clean up contaminated soil and

Proceedings of the 2nd International Conference on Biosciences and Medical Engineering (ICBME2019)
AIP Conf. Proc. 2155, 020009-1–020009-7; https://doi.org/10.1063/1.5125513
Published by AIP Publishing. 978-0-7354-1900-1/$30.00

groundwater [3]. Bioremediation involves the growth of certain microbes that use contaminants as a source of food and energy. Carbazole is one of the heteroaromatic compounds containing nitrogen characterized to be a recalcitrant chemical with carcinogenic properties. Isolation and identification of carbazole-degrading microorganisms have been studied [4-6] and these marine isolates are potentially useful for marine water treatment system.

The application of biotechnological processes that involves microorganisms in solving environmental pollution is rapidly growing in recent years. One of the key points for bioremediation is maintaining high biomass of bacterial populations. Degradation of pollutants and toxic compounds in the environment by natural microbial activities are usually limited by the presence of high concentration of xenobiotics that inhibit the growth of microorganisms [7]. Cell immobilization is found to be an attractive strategy to produce a robust cell as a biocatalyst. In comparison with free-living cells, the immobilized microbial cells produce higher biomass and provide high cell stability to promote biodegradation process [8]. This would overcome the limitation of slow growth rate and low biodegradation activity of the free cells. Besides, immobilized cells are feasible for use in continuous processing as they exhibit high recovery at lower cost, high reusability and cells are protected from environmental stresses [9]. These advantages encourage researchers to investigate the applications of immobilized cells in the biodegradation of various toxic compounds [9, 10]. The use of immobilization technique enhances bioremediation of petroleum hydrocarbon contamination, enables reutilization of microorganisms, tolerates harsh temperature and pH and causes easier separation [11].

Various techniques for cell immobilization, such as adsorption on surfaces, covalent bonding to carriers, entrapment in a polymer gel and self-aggregation have been used. Calcium alginate, glass beads, polyacrylamide gel, silanized magnetite, agarose, polyurethane foam and carrageenan are the most commonly used matrices for the immobilization of cells. The choice of immobilization technique and the mechanical properties of the matrix are significant factors affecting the stability of biocatalysts. Matrix must be non-toxic, non-biodegradable, have high cell mass loading capacity and have high mechanical, biological and chemical stability [3]. The strength of the matrix to withstand the harsh environmental condition should also be considered as one of the main subjects when developing the immobilized cells. The concentration of matrix used would subsequently affects mechanical strength as well as diffusivity of the treated compound. Therefore, determining the right properties would ensure optimum performance of the immobilized cells for bioremediation application. In this study, a locally marine-isolated bacterium, *Thalassospira profundimaris* M02 was used to study the biodegradation of carbazole. Cell immobilization by encapsulation in gellan gum was performed, then transport phenomena and mechanical properties of the immobilized cells were discussed.

MATERIALS AND METHODS

Microorganism and cultivation

Previously isolated *Thalasospira profundimaris* strain M02 was maintained in double layer agar of ONR7a supplemented with 0.1% of carbazole. The bacteria was cultured in 100 ml of ONR7a medium enriched with 0.1% carbazole and marine broth at 9:1 ratio in a shake flask. The culture was shaken at 150 rpm using orbital shaker and incubated for 24 hours at room temperature. After 24 hours, the broth was centrifuged at 7000 rpm for 10 minutes at room temperature to obtain cell pellets. All media were sterilized by autoclaving at 121 ˚C for 15 minutes prior to cultivation.

Preparation of gellan gum and immobilization

Gellan gum was mixed sterile distilled water to obtain final concentration of 0.70% (w/v) and heated to 75 ˚C to dissolve the gum. Calcium chloride ($CaCl_2$) was added to the mixture at about 0.66% (w/v), then mixed and left at room temperature to cool to approximately 45˚C [13]. The pH of the solution was adjusted to 7.0 by using 0.1 M NaOH. Harvested bacteria cells pellet was dispersed into the solution and mixed continuously. Beads were formed by withdrawing the mixture solution using a syringe and dropping it into oil. The beads were then separated from the oil by transferring them into 500 mL of 0.1% (w/v) $CaCl_2$ solution. After 2 hours, the beads were rinsed repeatedly with sterile 0.1% (v/v) Tween 80 (polyoxyethylene (20) sorbitan monooleate) solution. All procedures were performed in aseptic condition. To determine the optimum immobilisation media concentration, five different concentrations ranging from 0.3% to 1.1% (w/v) was used for gellan gum and 2.0% to 6.0% (w/v) for Ca-alginate.

Carbazole biodegradation study

The performance of bacteria in degrading carbazole was observed. The immobilized bacteria were added into 200 ml of artificial seawater media ONR7a as the medium containing 0.1% (w/v) carbazole in a shake flask. The mixture was incubated at room temperature for 36 hours with shaking at 100 rpm. The samples were taken every 6 hours interval. The concentrations of carbazole in the samples were analyzed by using Gas Chromatography Flame Ionization Detector (GC-FID) analysis. All the experiments were done in triplicates.

Diffusivity study

The data obtained from the biodegradation experiment were used to study the diffusivity. The data analysis of diffusion was mainly conducted by assuming the second 6 hours of the experiment where all substrate was beginning to obtain its equilibrium concentration. The model used was linear fitting model where it involves in diffusion in a large diffusion time, t. The formula used was as follows [14];

$$ln\left[\frac{C_s(1+\alpha)}{C_{S0}\alpha}-1\right]=ln\left[\frac{6(1+\alpha)}{9+9\alpha+q_1^2\alpha^2}\right]-\left(\frac{D_e q_1^2}{\alpha^2}\right)t \quad (1)$$

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 is the positive nonzero root, C_s is concentration of solute, C_{s0} is initial solute concentration. The effective diffusivity, D_e was calculated from the slope of the linear plot. From the biodegradation experiment, α was 2, C_{s0} was 1000 ppm and diameter of the beads, a was 3 mm in average.

Mechanical strength test

Five different concentrations of gellan gum beads ranging from 0.3% to 1.1% (w/v) were subjected to mechanical strength test. The gel samples were poured into a mold. The gellan gum was let to harden. The molded Ca-alginate gel was hardened by curing it in $CaCl_2$ solution for 2 hours. The samples were then kept in distilled water at room temperature for 2 hours. The samples dimension was set to be 4 mm x 100 mm (w x h). The stress-strain measurement was obtained by using Tensile/Universal testing machine (Shimadzu) at 10mm/min with 15 kN range and gauge length at 30 mm.

Gas chromatography flame ionization detector (GC-FID) analysis

Quantitative analysis of carbazole degradation was determined using gas chromatography with flame ionization detector (SHIMADZU GC 14B). Sample of known carbazole concentrations with no bacterial culture are used as standards and a calibration curve was plotted. 1 ml sample was pre-treated using 1 ml ethyl acetate. The resulted inorganic layer, observed as the upper layer was acquired for analysis using GC-FID. The liquid was transferred into a 1 mL vial tube for processing. Detection of the compound was done by using HP-5 fused silica capillary column (50mm x 0.32 mm x 0.25 µm) with temperature of 250 ˚C at the injector, 300 ˚C at the detector, with column heated to 200 – 250 ˚C at 5 ˚C per minute and split less column with helium as the carrier gas.

RESULTS AND DISCUSSION

Carbazole degradation by *Thalassospira profundimaris* immobilized in different concentration of gellan gum

Different concentrations of the gellan gum were tested to find the best concentration for immobilization of *T. profundimaris* by measuring carbazole degradation level. Carbazole degradation by *T. profundimaris* immobilized in different concentrations of gellan gum is illustrated in Figure 1. In this experiment, calcium chloride concentration was fixed at 60 mM or 0.66% (w/v). It was observed that different concentration of gellan gum resulted in variations of carbazole degradation by *T. profundimaris.* Among all gellan gum concentration tested, 0.7% (w/v) concentration resulted the highest carbazole degradation, which was 61.0% (Figure 1a). At this gellan gum concentration, 387.03

ppm carbazole were left after 36 hours of the degradation experiments from initial carbazole concentration of 1000 ppm (Figure 1b). For the rest of gellan gum concentration, the carbazole concentration left was observed to be more than 500 ppm. Time course analysis also shows that the degradation of carbazole was faster at 0.7% (w/v) gellan gum concentration. It needed approximately 18 hours to degrade 50% of the carbazole compared to other concentrations which only degraded carbazole at approximately 30% at the same duration. Degradation rate was more rapid as shown by the decreasing amount of carbazole at 0.7% (w/v) gellan gum, while others achieved near stagnant degradation as early as 6th hours. The same optimum concentration of gellan gum within 0.7 to 0.75% (w/v) was also reported for phenol degradation by *Acinetobacter* sp. [7].

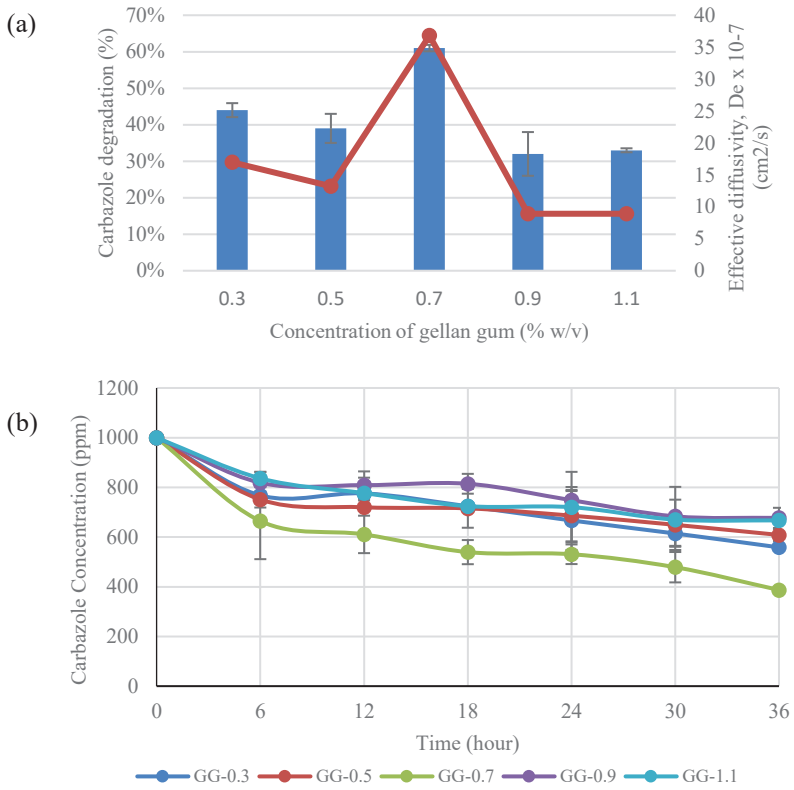

FIGURE 1. (a) The percentage of carbazole degradation and effective diffusivity at different concentrations of gellan gum. (b) Carbazole concentration level within 36 hours of treatment. Error bars indicate standard deviation.

According Ertan et al. (2007), higher concentration of media affects the pore size of the beads [12]. When comparing with optimum concentration, higher concentration of gellan gum caused lower bacteria activity. This might be due to the high cross-linking that could lead to the formation of hard and brittle beads with small pore size [15]. Smaller pore size limits the diffusion rate into the beads. Lower diffusion rate would limit the oxygen and nutrient amount in the center of the beads. As a consequence, the bacteria could not survive inside the beads during the cultivation [16]. This could be observed from the percentage of carbazole degradation which was less than 50% at high media concentration. Lower concentration of gellan gum led to the formation of soft and fragile beads with large pore size, which could increase bacteria leakage [15]. The beads with high porosity diffused more substrate but it could lead to bacteria leakage. This can be observed from lower bacteria activity at lower media concentrations.

Diffusivity of carbazole in gellan gum beads

Diffusivity of carbazole in different concentrations of gellan gum was investigated to further confirm the theory. As shown in Figure 1a, the best effective diffusivity for gellan gum was 36.8×10^{-7} cm^2 /sec at 0.7% (w/v) gellan gum concentration where the carbazole degradation was 61%. Two parameters have proven that the best concentration of gellan gum was at 0.7% (w/v) where its degradation and diffusivity was at its peak at this

concentration. At this concentration, carbazole was diffused at its maximum level and subsequently degraded by the encapsulated bacteria. Because the oxygen and substrate diffusions were optimum, the growth of bacteria in the gellan gum was maintain, hence promoting carbazole degradation.

Lower effective diffusivity values were recorded at higher concentrations. At higher gellan gum concentration, small pore size mostly contributed to the lower diffusivity of carbazole. This phenomenon was caused by the gel concentration in three-dimensional network that formed harder and smaller pore size [17]. According to Dewan et al. (2017), the pore size of the gel was found to be reduced as the gellan gum increased which was due to the fact that the polymer mesh becomes more condense at higher concentration [18].

At lower gellan gum concentration, the diffusion of carbazole might be higher due to the bigger pore size of the gellan gum. But, because of the cell leakage, the diffused carbazole was slowly degraded by the remaining encapsulated cells. This explained the lower degradation and lower diffusivity at lower gellan gum concentration. Soft and fragile beads were formed due to low concentration of gellan gum [15]. However, maintaining the concentration of calcium chloride at 0.66% (w/v) caused 0.3% and 0.5% (w/v) gellan gum beads reached beyond its critical region. Beyond critical region, the hardness of the bead would decrease although higher amount of calcium chloride was added [17]. Lower effective diffusivity at these concentrations were mainly due to its soft properties where most of the beads were broken.

Mechanical properties of the gellan gum beads

Mechanical strength of gellan gum beads were indicated by Young's modulus from the stress/strain plot. Table 1 shows the elongation, strain, force, engineering stress and Young's modulus of various concentration of gellan gum beads. It was observed that the elongation was increasing when concentration of gellan gum increased from 0.3% to 0.7% (w/v) but decreased from 0.9% to 1.1% (w/v). Similar trend was observed to the average force applied. As a result, all strain, stress and Young's Modulus follow the same pattern according to the gellan gum concentration described. Gellan gum beads with 0.7% (w/v) concentration recorded the highest Young's modulus which indicates the highest mechanical strength among the other concentrations.

TABLE 1. The elongation, strain, force, engineering stress and Young's modulus of various concentration of Gellan gum

Concen-tration* (% w/v)	Average Elongation (L) (mm)	Standard Deviation	Strain (ϵ)	Average Force (F) (N)	Standard Deviation	Engineering Stress (σ) (N)	Young Modulus (E) (N/mm^2)
0.3	9.480	1.889	0.316	0.202	0.034	0.0063	0.019976
0.5	11.638	2.140	0.388	0.315	0.038	0.0098	0.025375
0.7	19.508	3.206	0.650	0.870	0.082	0.0272	0.041810
0.9	11.067	1.617	0.369	0.343	0.054	0.0107	0.029013
1.1	13.823	0.810	0.461	0.250	0.103	0.0078	0.016955

*gellan gum concentration

Lower Young's modulus were recorded at 0.3 and 0.5% (w/v) gellan gum. Meng et al. (2013) reported the effects of calcium chloride concentration on the gellan gum gel hardness [17]. Increasing the calcium chloride concentration increased the gel hardness which indicates the viscoelastic properties. This is because calcium ion connects the double helices helix of gellan gum molecules to form a stable network structure. However, beyond the critical point, more calcium added will disturb the charge of the system, hence decrease the hardness at the last of gelation. According to Lopez et al. (1997), the "optimal hydrogel" for 0.5% gellan gum was obtained when calcium chloride was at 10 mM [19]. Therefore, increasing the calcium chloride at 60 mM in this study would make the hydrogel go beyond its critical region. Maintaining concentration of calcium chloride when lowering gellan gum concentration could therefore drop the Young's modulus.

As described by Nawaz et al. (2015), higher concentration of gellan gum leads to the high cross-linking that leads to the formation of hard and brittle beads with small pore size [15]. The Young's modulus in this study decreased above 0.7% (w/v) concentration of gellan gum. This is because concentration of calcium chloride was maintained at 0.66% (w/v). To form a strong beads at higher gellan gum concentrations, more calcium ion is needed for the crosslinking process. Therefore, in this study, 0.7% (w/v) gellan gum with 0.66% (w/v) of calcium chloride led to a formation of strong beads with high viscoelastic properties.

Optimum media concentration from performance of degradation, diffusivity and mechanical properties

It is best to fit all the parameters; percentage of degradation, effective diffusivity and Young's modulus to find the optimum concentration of gellan gum for immobilization of *T. profundimaris*. Table 2 summarizes the percentage of carbazole degradation, effective diffusivity and Young's modulus of immobilized bacteria in different gellan gum concentrations. From the table, it can be concluded that the effective diffusivity is proportional to the percentage of carbazole degradation. Young's modulus pattern forms a bell shape as gellan gum concentration increases (at constant calcium chloride concentration), but it is proportional to the diffusivity and carbazole degradation. At 0.7% (w/v) gellan gum and 0.66% (w/v) calcium chloride, the gel has reached its critical point. So, further addition of gellan gum will reduce the strength of the gel. Diffusivity of carbazole was highly affected by gellan gum concentration. The polymer mesh became more condense at higher concentration of gellan gum making the pore size of higher concentration gellan gum to be smaller, hence increased the mass transfer resistance. This explained the lower diffusivity phenomena at concentration higher than 0.7% (w/v).

TABLE 2. Percentage of carbazole degradation, effective diffusivity and Young's modulus of immobilized bacteria in different gellam gum concentrations.

Gellan gum concentration (% w/v)	Percentage of Carbazole Degradation	Effective Diffusivity (D_e)X10^{-7} (cm^2/sec)	Young Modulus (E) (N/mm^2)
0.3	44.00%	17.0	0.019976
0.5	39.00%	13.2	0.025375
0.7	61.00%	36.8	0.041810
0.9	32.00%	8.92	0.029013
1.1	33.00%	8.92	0.016955

Gellan gum beads of 0.3 and 0.5% (w/v) with high diffusivity has soft and fragile properties. The soft formation was due to its lower concentration of gellan gum that made it easier to damage during the washing process. Larger pore size also permits the bacteria to leak out and as consequences, the degradation is lower. Meanwhile, 0.9 and 1.1% (w/v) gellan gum were hard and brittle beads. These beads had smaller pore size. As for its properties having hard and smaller pore size, the reason for lower carbazole degradation was its diffusion limits. The substrate transported into the beads at low rate. The diffusion limitation also slowed the oxygen and nutrient to the centre of the beads. Gellan gum beads with 0.7% (w/v) was observed to have the highest percentage of degradation, effective diffusivity and Young's modulus. This was due to the fact that the amount of calcium chloride is optimum for the gelation of 0.7% of gellan gum. At this point, the gel has reached its "optimal hydrogel" [20]. Therefore, the optimum concentration of gellan gum for the entrapment of *T. profundimaris* was 0.7% (w/v) where it has the highest carbazole degradation, effective diffusivity and Young's modulus.

CONCLUSION

The transport phenomena studied showed that the gellan gum concentration highly affects diffusion of carbazole and its degradation. It was proven that higher degradation of carbazole by *T. profundimaris* immobilized in 0.7% (w/v) gellan gum was corroborated with high effective diffusivity and Young's modulus (mechanical strength). At this gellan gum concentration, the gel reached its optimal hydrogel properties. The beads showed performance with 61% of carbazole degradation and optimum diffusivity due to its moderate pore size. Three parameters used in this investigation are vital to study the strong media, so it could withstand the harsh environmental condition for application in bioremediation of carbazole in wastewater. Diffusivity was vital to ensure that the bacteria receive enough oxygen, nutrient and substrate. It is necessary to include more parameters in the future to investigate the optimum degradation such as calcium chloride concentration, initial carbazole concentration, cell leakage and cell viability.

ACKNOWLEDGMENTS

The authors thanked Universiti Teknologi Malaysia and Ministry of Education Malaysia for the financial support. This project was supported under grant no. Q.J130000.2746.03K27.

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