Optimization of Immobilization Media of Thalassospira Profundimaris: Diffusion and Strength Applications

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Abstract*: *Many petroleum samples have abundant heterocyclic compounds. One example of petroleum samples is diesel where nitrogen, sulphur and aromatic compounds are the major impurities present in it. Heterocyclic compounds are inextricably into life processes in which a vast number of active heterocyclic compounds are being used. One example of heterocyclic compounds is carbazole which is known to be highly cancinogenic. Bacteria called Thalassospira Profundimaris could potentially degrade the carbazole compounds. The bacteria are immobilized inside media to offer high mechanical strength, high metabolic activity, and resistance to toxic chemicals causing damage to the cell. The media used widely are gellan gum, Ca-alginate and yeast. The finding of the maximum carbazole degradation, optimum strength and diffusivity of the media are profound to increase the performance of the bacteria entrapped inside as well as withstanding the harsh environment around it. This project has proven that the concentration affects the porosity and strength of the media. Increasing the concentration of media would form stronger media with lower diffusivity where lower concentration forms soft media with higher diffusivity.

Keywords*: *Optimization, Immobilization, Thalassospira Profundimaris, Diffusion

I. INTRODUCTION

The expansion of biotechnology and its development has revitalized enthusiasm on cells or enzymes immobilization. Immobilization is a term describing the entrapment or attachment of cell or particles [1]. Immobilization can be applied to all types of biocatalyst including cellular organisms, animal and plant cells [2]. Immobilization was greatly utilized in many applications, not only in the field of biotechnology but also in food, environment and other industries [3].

Free cell would offer a multitude of disadvantages such as substrate inhibition when using free cell suspension in wastewater [4]. It also offers low stability, lower degradability of the cell in the presence of high concentration of pollutants from the systems [5]. Cell immobilization offers high mechanical strength, high metabolic activity, and resistance to toxic chemicals causing damage to the cell. There are many types of media used for cell immobilization such as gellan gum, Ca-alginate and others.

The mechanical strength of the media is related to the formation of pore size which alters diffusion limits that depended on the concentration of both media and its cation, calcium chloride. Higher concentration of media causes smaller pore size of the media primarily due to condensed polymer mesh making the pore size and forming hard and brittle beads [5]. Meanwhile, low concentration of media forms larger pore size that could lead to bacteria leakage [6]. However, the diffusivity of the media is high in lower media concentration having soft and fragile properties [7].

II. METHODOLOGY

The execution of the project was divided by three parts which are bacteria preparation, Preparation of Ca-alginate and gellan gum, small-scale biodegradation experiment, mechanical strength test and diffusion data analysis.

A. Bacteria Preparation

The culture was prepared prior to grow the bacteria via sub-culturing. The ONR7a and concentrations were prepared prior to sub-culturing the bacteria into petri dish for grow. The sub-culturing is important to prepare as many as required for the small-scale biodegradation experiment. Two 100 ml conical flasks were prepared for the growth of the bacteria using marine agar inside the petri dishes. 100 ml of ONR7a was prepared inside the conical flask and it was added into 100 ml of Marine agar to make a solution of first layer. Continuously, 100 ml of ONR7a prepared was added with 1 ml of Carbazole so the final concentration of Carbazole was 0.1%. 100 ml of ONR7a was autoclaved before the substrate being added into the solution. These solutions were then added into 100 ml of Marine agar for the second layer. The bacteria were cultured inside the petri dish for growth purposes.

B. Harvesting Bacteria

100 ml of ONR7a was prepared using similar ingredient abovementioned before adding it together with marine broth. 20.125 grams of Zobell Marine Broth was added in 500 ml of distilled water before they were stirred using magnetic stirrer.

Revised Manuscript Received on February 08, 2020.

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The bacteria inside the petri dish were then transferred to the marine broth and being shaken for one day by using orbital shaker at 150 rpm. The colour of the solution inside the conical flask shaken for one day was observed to analyze the growth of the mentioned bacteria. The solution turned cloudy as to indicate its growth. The solution was then centrifuged using Kubota 2800 to obtain the pigment or the bacteria. The centrifuge ran for 10 minutes to obtain the bacteria. Suspension of *Thalassospira Profundimaris* cultures were centrifuged using Hercuvan Centrifuge for 10 min at 7,000 rpm at room temperature to obtain the pellet.

C. Preparation of Gellan Gum and Ca-alginate

The entrapment of cell by Gallen Gum was done by dispersing 0.70% (w/v) in sterile de-ionized water and heating it to 75˚C to let the pre-gel solution form. Calcium chloride was added at about 60mM or 0.66% (w/v) and left at room temperature to cool at 45˚C [8]. The pH of the solution was adjusted to 7.0 using 0.1M NaOH. Beads were formed by using syringe and dropping the gum mixture into oil. The beads were then separated from the oil by transferring them into 500 mL of 0.1% (w/v). After 2 hours, the beads were rinsed repeatedly with sterile 0.1% (v/v) Tween 80 (polyoxyethylene (20) sorbitan monooleate) solution.

The preparation of sodium alginate beads was initiated by dissolving 3% (w/v) sodium alginate in 0.10L of water and added to a 0.10L of suspension of *Thalassospira Profundimaris* [9]. The solution was mildly shaken. 0.15L of $CaCl_2$ solution with 60mM or 0.66% (w/v) final concentration was prepared in separate beaker. The mixture containing sodium alginate was added dropwise to 150 ml of $CaCl_2$ solution using 0.01L syringe [9]. The beads were hardened in solution for 1h.

D. Bacteria Performance Experiment

To determine the optimum immobilization media concentration, five different concentrations ranging from 0.3% to 1.1% (w/v) was used for Gellan Gum beads which were 0.3%, 0.5%, 0.7%, 0.9% and 1.1% (w/v) meanwhile 2.0% to 6.0% (w/v) for Ca-alginate beads preparation which were 2.0%, 3.0%, 4.0%, 5.0% and 6.0% (w/v). The performance of bacteria degrading carbazole was observed inside conical flask shaken for 3 days. 100mL conical flask was used whereby it was filled with 200ml of ONR7a with carbazole with constant concentration which was 1000 mg/L. The samples were then taken for every 6h interval for 36 hours. The concentration of substrate was analyzed using Gas Chromatography Flame Ionization Detector (GC-FID) analysis.

E. Diffusion Data Analysis

The data obtained from the biodegradability experiment was used to study the diffusion study. The model used was linear fitting model where it involves in diffusion in a large diffusion time, t. The formula used as shown in Eq (1) [10].

$$\ln\left(\frac{C_s(1+\propto)}{C_{so}\propto}-1\right)=\ln\left(\frac{6(1+\propto)}{9+9\propto+q_n^2\propto^2}\right)-\left(\frac{D_e q_n^2}{a^2}\right)t \quad (1)$$

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, De is the effective diffusivity, n is the number of the beads and qn is the positive nonzero root, Cs is concentration of solute, Cs0 is initial substrate concentration. The effective diffusivity, De can be calculated once the ratio of volume to beads, α [10]. From the biodegradability experiment, α ratio is 2:1, Cs0 was 1000ppm and diameter of the beads, a was 3mm average.

Mechanical Strength Test

For this testing, the sample of gel was poured onto the mold. The Gellan Gum was let to harden. The molded Ca-alginate gel was hardened by curing it in calcium chloride for 2 hours. The samples were then stored in distilled water at room temperature for 2h. 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 15kN range and gauge length at 30 mm.

III. RESULTS AND DISCUSSION

There were two media used to study the performance of *Thalassospira Profundimaris* namely Gellan Gum (GG) and Calcium Alginate (AG). These two media were then compared for its different concentration.

A. Effect on the carbazole degradability of *Thalassospira Profundimaris* by different concentration of Gellan gum (GG) and Calcium Alginate (AG)

Comparatively, the best carbazole degradability was summarized in Table- I. It was important to study both Gellan Gum and calcium alginate concentration for its contribution to the efficiency of carbazole degradation by *Thalassospira Profundimaris*.

Table- I: The percentage of carbazole degradation by Gellan Gum and calcium alginate

Concentration	Percentage of Carbazole Degradation	Carbazole Left (ppm)	Standard Deviation
GG-0.3*	44.00%	558.618	0.0191
GG-0.5*	39.00%	608.513	0.0398
GG-0.7*	61.00%	387.030	0.0088
GG-0.9*	32.00%	677.223	0.0599
GG-1.1*	33.00%	667.175	0.0054
AG-2*	57.47%	425.298	0.0223
AG-3*	81.23%	187.728	0.0096
AG-4*	63.55%	364.537	0.0784
AG-5*	40.92%	590.755	0.0090
AG-6*	48.46%	515.421	0.0294

Note. GG is Gellan Gum; GG-0.3 is gellan gum at 0.3% (w/v), GG-0.5 is gellan gum at 0.5%(w/v), GG-0.7 is gellan gum at 0.7% (w/v), GG-0.9 is gellan gum at 0.9%(w/v) and GG-1.1 is gellan gum at 1.1%(w/v). AG is Ca-alginate; AG-2 is calcium alginate at 2%(w/v), AG-3 is calcium alginate at 3%(w/v), AG-4 calcium alginate at 4%(w/v), AG-5 is calcium alginate at 5%(w/v) and AG-6 is calcium alginate at 6% (w/v)

From Table- I, the best carbazole degradation was observed to be GG-0.7 and AG-3. Both GG-0.7 and AG-3 was recorded with 387.03ppm and 187.728ppm carbazole left, respectively. Among GG concentration, 61% was the highest carbazole degradation observed with 387.03ppm carbazole left after 36 hours of experiment. For the rest of GG concentration, the carbazole concentration was observed to be more than 500ppm. Comparatively, the best concentration of GG for carbazole degradation by *Thalassospira Profunsimaris* was at GG-0.7 [11]. Among AG, the best carbazole degradation was observed to be AG-3 where carbazole left was as low as 187.728ppm. Meanwhile, the carbazole left for the rest of Ca-alginate was observed to be more than 350ppm. Comparatively, the best concentration of AG for carbazole degradation by *Thalassospira Profunsimaris* was at AG-3 [6].

According to the previous research [6], higher concentration of media and calcium chloride affected the pore size of the beads. When comparing GG-0.3 and AG-2 with higher concentration at GG-0.9 and AG-6, lower bacteria activity was observed similar to the bacteria activity at GG-0.9 and AG-6. This might be due to the high cross-linking that could lead to the formation of hard and brittle with small pore size of the beads [3]. Smaller pore size limited the diffusion rate into the beads. Lower diffusion rate would limit the oxygen and nutrient amount in the center of the beads. As consequences, the bacteria died inside the beads during the cultivation [12]. Lower concentration of alginate led to the formation of soft and fragile beads with large pore size which could increase bacteria leakage [7]. The beads with high porosity diffused more substrate. However, it could lead to bacteria leakage. This could be observed at GG-0.3, GG-0.5 and AG-2 where the bacteria activity was lower due to bacteria leakage.

B. Diffusivity of Gellan gum (GG) and Calcium Alginate (AG)

From Table- II, the best effective diffusivity for GG and AG was 36.8×10-7cm^2/sec and 576×10-7cm^2/sec. The best effective diffusivity of GG was at GG-0.7 where its carbazole degradation was 61%. Two parameters had proven that the best concentration of GG was at GG-0.7 where its degradation and diffusivity was at its peak at this concentration. However, among AG, it was contrary to the first discussion in Subtopic 3.1, where its best concentration was at AG-3. However, AG-3 diffusivity was 195×10-7 cm^2/sec difference to the highest one at AG-4. To ensure the optimum condition could be achieved, the strength test was the final parameter to be carried out.

The decreasing trend of diffusivity was observed in Table-II where the lowest effective diffusivity was 8.92×10-7cm2/sec for GG-0.9, GG-1.1, AG-5 and AG-6. This was due to the pore size of the beads limited by high concentration of GG. This phenomenon was caused by the gel concentration in three-dimensional network that formed harder and smaller pore size [13]. The pore size of the gel was found to be reduced as GG decreasing which was due to the fact that the polymer mesh became more condense at higher concentration [5].

Among GG, the effective diffusivity was also low when GG-0.3 and GG-0.5 were having 17×10-7cm2/sec and 13.2×10-7cm2/sec, respectively. Soft and fragile beads were formed due to low concentration of GG [7]. However, maintaining the concentration of calcium chloride at 60mM or 0.66% (w/v) caused GG-0.3 and GG-0.5 reached beyond its critical region. Critical region was observed where the hardness of the bead would decrease by higher amount of calcium chloride [13]. Lower hardness meant softer that could form a larger pore size for both GG-0.3 and GG-0.5 where larger pore size increased the effective diffusivity. Among AG, the diffusivity of the alginate at AG-2 and AG-3 should be high enough due to its high porosity. However, the situation was opposite as it showed slow diffusivity. Lower effective diffusivity at these concentrations was mainly due to its soft properties where most of the beads were broken.

Table- II: Effective diffusivity and percentage of carbazole degradation for each concentration of GG and AG

Concentration	Effective Diffusivity, D_e X10^{-7} (cm^2/sec)
GG-0.3	17.0
GG-0.5	13.2
GG-0.7	36.8
GG-0.9	8.92
GG-1.1	8.92
AG-2	520
AG-3	381
AG-4	576
AG-5	313
AG-6	256

C. Tensile Strength of Gellan gum (GG) and Calcium Alginate (AG)

From Table- III, the standard deviation on the observation of each sample was ranging from 0.809711 to 3.205976 and 0.033705 to 0.102713 for elongation and force, respectively. Table 3 was observed that the elongation was increasing when concentration of GG was at GG-0.3 to GG-0.7 but decreasing in trend to GG-0.9. Similar situation occurred to the force applied. The area of the gauge was 32mm^2 based on the standard used. Higher concentration of GG might lead to the high cross-linking that could lead to the formation of hard and brittle with small pore size of the beads [7]. However, the Young's modulus decreased above GG-0.7 concentration of GG while maintaining the concentration of calcium chloride of 60mM or 0.66% (w/v). However, the Young's modulus was increasing as concentration of AG was increasing. Among GG, the Young's Modulus increased exponentially to its peak at GG-0.7. The lower Young's modulus when GG at GG-0.3 and GG-0.5 was due to maintaining concentration of calcium chloride that could eventually drop the Young's modulus.

The "optimal hydrogel" for GG-0.5 was when calcium chloride was at 10mM [14]. This could be concluded that, by maintaining calcium chloride at 60mM, the GG-0.5 had gone beyond its critical region. Similar trend was observed from AG when the concentration of AG was at AG-2 and AG-3. The lower concentration of GG should possess elastomer properties [6]. The elastomer properties were mainly due to the decreasing charge density within the molecular backbone, which would promote the stabilization of the double helix that leads to increased elasticity of the gelled system [15]. The concentration of calcium chloride did not affect the "optimal hydrogel". This could be observed at AG-5 and AG-6, where it has highest Young's modulus among AG. This meant that AG-5 and AG-6 were having hard but brittle properties. Different situation when GG concentration at GG-0.9 and GG-1.1 where its Young's modulus decreased exponentially. From the previous research [13], the hardness of the media increased 166% when CaCl2 increased from 0.005 to 0.01% (w/v) of calcium chloride while maintaining the GG concentration at 0.2%. While maintaining concentration of 60mM or 0.66% (w/v) calcium chloride, the GG at GG-0.9 and GG-1.1 fell within the critical region. The properties of GG at GG-0.9 and GG-1.1 were observed to possess hard and brittle properties [16]. GG-0.7 and AG-4 showed the highest Young's modulus.

Table- III: The elongation, strain, force, engineering stress and Young's modulus of various concentration of GG and AG

Conc.	Average Elongation (L) (mm)	Standard Deviation	Strain (ϵ)	Average Force (F) (N)	Standard Deviation	Engineering Stress (σ) (N)	Young Modulus (E) (N/mm^2)
GG-0.3	9.48	1.889	0.316	0.202	0.034	0.0063	0.019976
GG0.5	11.638	2.14	0.388	0.315	0.038	0.0098	0.025375
GG-0.7	19.508	3.206	0.65	0.87	0.082	0.0272	0.04181
GG-0.9	11.067	1.617	0.369	0.343	0.054	0.0107	0.029013
GG-1.1	13.823	0.81	0.461	0.25	0.103	0.0078	0.016955
AG-2	43.309	1.957	1.444	1.627	0.151	0.0508	0.035212
AG-3	41.934	6.656	1.398	2.533	0.513	0.0792	0.056636
AG-4	39.283	6.405	1.309	3.875	0.64	0.1211	0.092479
AG-5	39.591	7.041	1.32	4.833	0.666	0.151	0.114451
AG-6	33.332	3.883	1.111	5.033	0.694	0.1573	0.141568

D. Optimization of Different Media Concentration by Performance of Degradation, Diffusivity and Tensile Strength

It is the best to fit all the parameters; percentage of degradation, effective diffusivity and Young's modulus into one discussion to decide the optimum concentration of media. This study was to investigate which concentration with higher degradation had relationship with effective diffusivity and young's modulus. Each concentration of media was chosen so it could be compared to choose the best media for the immobilization of *Thalassospira Profundimaris*. Table- IV showed the percentage of carbazole degradation, effective diffusivity and Young's Modulus of various concentration of gellan gum and Ca-alginate.

Table- IV: The three parameters of immobilization media concentration

Concentration	Percentage of Carbazole Degradation	Effective Diffusivity De X 10^{-7} (cm^2/sec)	Young Modulus, E (N/mm^2)
GG-0.3	44.00%	17.0	0.019976
GG-0.5	39.00%	13.2	0.025375
GG-0.7	61.00%	36.8	0.041810
GG-0.9	32.00%	8.92	0.029013
GG-1.1	33.00%	8.92	0.016955
AG-2	57.47%	520	0.035212
AG-3	81.23%	381	0.056636
AG-4	63.55%	576	0.092479
AG-5	40.92%	313	0.114451
AG-6	48.46%	256	0.141568

Among GG, the effective diffusivity of both GG-0.3 and GG-0.5 were higher than GG-0.9 and GG-1.1. This was due to the pore size of GG-0.3 and GG-0.5 was bigger. The polymer mesh became more condense at higher concentration of GG making the pore size of higher concentration gellan gum to be smaller and this created hard and brittle beads [5]. This phenomenon could be proved as the hardness of GG-0.3 and GG-0.5 were lower than GG-0.9. The effective diffusivity was inversely proportional to the Young's modulus. The lower percentage of carbazole degradation for GG-0.3, GG-0.5, GG-0.9 and GG-1.1 had two different reasons. GG-0.3 and GG-0.5 which had higher diffusivity was soft and fragile. The soft formation was due to its lower concentration of GG that made it easier to damage during the washing process [7].

Larger pore size also permits the bacteria to leak out and as consequences, the degradation was lower [6]. Meanwhile, GG-0.9 and GG-1.1 were two hard and brittle beads. These beads had smaller pore size than GG-0.3. 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 [12]. GG-0.7 was observed to have the highest percentage of degradation, effective diffusivity and Young's modulus. Three of these parameters were accumulated in GG-0.7. This was due to the fact that the amount of calcium chloride is optimum for the gelation of 0.7% of GG. At this point, GG-0.7 had reached its "optimal hydrogel" [14]. The optimum concentration of GG for the entrapment of *Thalassospira Profundimaris* was GG-0.7 where it has the highest carbazole degradation, effective diffusivity and Young's modulus.

Table- V showed the two optimum concentrations for efficient *Thalassospira Profundimaris* activity on carbazole degradation. The best concentration of GG was GG-0.7 for 61% of carbazole degradation while AG-3% for AG with 81.23%. The Young's modulus of AG was higher than that of GG-0.7. The effective diffusivity of AG-3 was also higher than that of GG-0.7.

Table- V: The percentage of carbazole degradation, effective diffusivity and young's modulus of GG and AG

Concentration	Percentage of Carbazole Degradation	Effective Diffusivity De X 10^{-7} (cm^2/sec)	Young Modulus, E (N/mm^2)
GG-0.7	61.00%	36.8	0.041810
AG-3	81.23%	381	0.056636

From Table- V, the Young's modulus of AG-3 was the highest with moderate effective diffusivity. AG-3 at 3% needed 3g of AG for every 100ml. However, GG-0.7 only needed 0.7g of GG for 100ml. At low weight of GG, the beads could degrade 61% of carbazole. Meanwhile, calcium alginate beads needed higher amount of sodium alginate to degrade the carbazole for 81.23%. However, AG provided higher mechanical strength than GG. Higher mechanical strength was important to withstand the strong sea force that could eventually damage the beads. The effective diffusivity of AG-3 was also higher than that of GG-0.7. This meant that substrate or any pollutant could diffuse into the beads faster.

IV. CONCLUSION

It is necessary to include more parameters to investigate the optimum concentration of GG and AG. The parameters were percentage of degradation, diffusivity and tensile strength. The concentration involved in this investigation is GG-0.3, GG-0.5, GG-0.7, GG-0.9 and GG-1.1 for GG meanwhile AG-2, AG-3, AG-4, AG-5 and AG-6 for AG. The optimum concentration of gellan gum for the entrapment of *Thalassospira Profundimaris* was GG-0.7. GG-0.7 had 61% of carbazole degradation and had optimum diffusivity due to its moderate pore size. The best concentration of Ca-alginate was AG-3 due to its moderate diffusivity and Young's modulus with higher carbazole degradation. The comparability of AG-3 and AG-4 were one hard task due to properties of AG-4 was stronger than AG-3. Comparatively, AG-3 had more advantages than GG-0.7 because it was more efficient for carbazole degradation and higher effective diffusivity and Young's modulus. Three parameters used in this investigation were vital to study the strong media, so it could withstand the force in the sea with higher contaminant degradation. Diffusivity was vital to ensure that the bacteria receive enough oxygen, nutrient and substrate.

ACKNOWLEDGMENT

Authors thank UNIMAS for financial support with Grant number FOR/SpMYRA/1715/2018.

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