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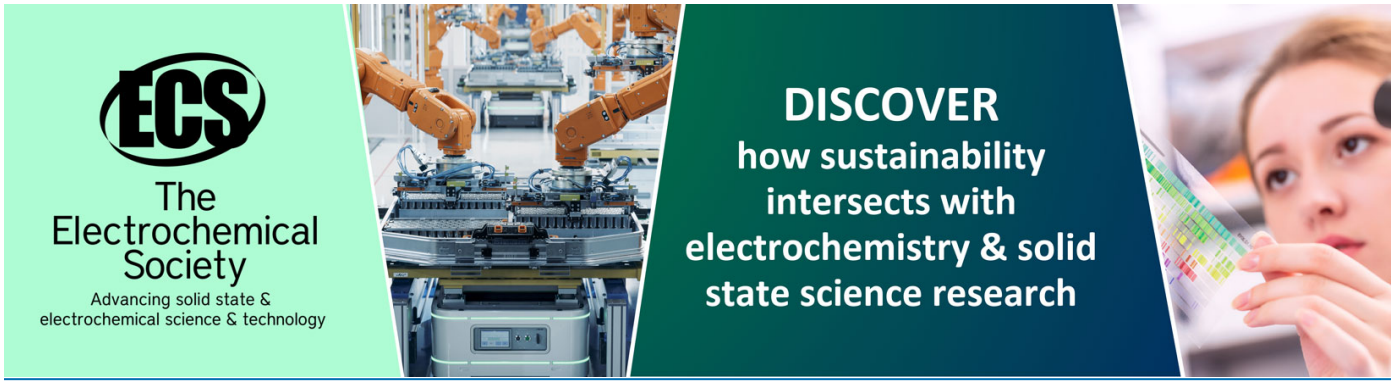

Silica-cellulose material application as the immobilization matrix of *Pseudomonas fluorescens*

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Abstract. Waste management, including heavy metal removal through bioremediation, requires process optimization. One of the important processes in a modern method is bacterial immobilization intended to improve the performance of bioremediation. In this preliminary study, silica material from rice husk processed via sol-gel was used, and cellulose of Nata de Coco was incorporated *in situ* during gelling to give balanced surface properties of a porous matrix. The modified could hold bacteria such as *Pseudomonas fluorescens* to be immobilized for further use. Variation of contact time was done, while temperature and stirring speed were kept constant. Assessment of *Pseudomonas fluorescens* bioremediation activity was carried out in cadmium standard solution. The result showed that the optimum contact time of *Pseudomonas fluorescens* and silica matrices was achieved at 30 min contact time as 99.19% was embedded in the matrices. This system decreased 80% of heavy metal in solution. This result indicates the compatibility of silica-cellulose matrix in *Pseudomonas fluorescens* immobilization, as predicted for other types of bacteria as well.

1. Introduction

Non-degradable (persistent) heavy metal waste accumulated for a long period poses a serious threat to all living creatures [1]. One of those heavy metals considered harmful for human is cadmium since the metal causes various toxic effects, including carcinogenesis, teratogenesis, mutagenesis, and inducing deterioration of endocrine gland as well as reproduction system [2]. Bioremediation refers to the utilization of certain microorganisms cultured in a specific pollutant as an attempt to reduce the concentration of the said pollutant [3–6]. There are several mechanisms exercised by microbes to decrease heavy metal content in aquatic environment, such as detoxification (bioprecipitation), biohydrometallurgy, bioleaching, and bioaccumulation [7]. *Pseudomonas fluorescens* is a species of bacteria potential to be used as a bioremediation agent of one or more types of heavy metal, including Ni, Cd, Cu, Cr, and Pb, to name a few. It also works on radioactive metals, such as Caesium [8–10]. However, the use of microorganisms as bioremediation media exhibits a number of disadvantages; it can only be used once, requires intensive monitoring, and the possibility of the emergence of unknown products [11].

In order to optimize the utilization of *Pseudomonas fluorescens* in the bioremediation process of heavy metals, immobilization method was developed. Immobilization refers to the physical entrapment or localization of microorganisms in the new environment intended to maximize the desired biocatalyst

activity [12]. Bacterial immobilization holds several advantages, such as protecting the microorganisms from an incompatible living environment, easily separated and can be used over multiple times [13]. In cell immobilization process, the technique used is a crucial factor that determines the success of cell embedment into the matrices. Adsorption and entrapment fall to the category of physical immobilization, while covalent bonds and cross-linking belong to the group chemical immobilization. Physical immobilization is preferred among other techniques due to several advantages, including high success rate, rapid process, inexpensive, and feasible in room temperature [14].

Another parameter that determines the success of immobilization process is the immobilization matrix (carrier). The matrices may be derived from organic materials, inorganic materials, or the combination of both strong enough to restrain degradation processes, physically, chemically and biologically. These matrices should be of non-toxic category, do not induce pollution, possess constant quality, and available in low-price [15]. This study employed silica-cellulose material as the matrix for *Pseudomonas fluorescens* immobilization. The material was obtained through the modification of silica from rice husk incorporated with cellulose of Nata de Coco.

Modification of silica using cellulose resulted in silica-cellulose matrices. Not only the procedure reduces surface polarity of the material, it also works on enhancing the pore size and surface area of the material, which leads to an increase in adsorption capacity [16,17]. The silica-cellulose matrix was reported to have a high adsorption capacity indicated by its iodine adsorption capacity that reached 3.3%. Furthermore, SEM analysis also indicates that silica-cellulose matrices possess an evenly distributed porosity, with a mean pore size around 60 nm. In those researches, silica-cellulose matrix was implemented as an adsorbent of synthetic colourings such as rhodamine B and tartrazine [16]. In addition, silica modified with cellulose has also resulted in material of 7.1% iodine solution adsorption capacity, which was further used to separate large molecules, including chlorophyll and curcumin [18]. Based on the descriptions above, this study developed another potential of silica-cellulose material which as the immobilization matrix of *Pseudomonas fluorescens* for heavy metal bioremediation.

2. Materials and Methods

2.1. Material and analysis instrument

All chemicals used in this study were purchased from Merck and of pro-analysis quality, while the glasswares were of Iwaki pyrex. All instruments used in the research include atomic absorption spectroscopy (AAS) Varian AA240, UV-Vis spectrophotometer smartplus Biorad, XD-1700 M and Naberthem furnace.

2.2. Silica-cellulose matrices preparation

Rice husks were incinerated at 350 °C and the ash immersed in alkaline solution. During gelling, the cellulose of hydrolized Nata de Coco was incorporated with the filtrate obtained through the immersion process to generate cellulose nanoparticles and stirred until the pH reaches 2. The hydrolysis process was conducted through immersion in strong acid while continuously heated for several hours. Afterward, ammonium hydroxide was added dropwise until the pH of the solution reaches 8 and silica-cellulose particles formed gradually. This procedure has been patented per February 14, 2018 with Indonesian Patent Number of IDP000049626 [16]. The reaction can be seen at (1) and (2):

$$SiO_2(s) + 2\ NaOH(aq) \rightarrow Na_2SiO_3(aq) + H_2O(l) \quad (1)$$

$$Na_2SiO_3(aq) + H_2SO_4(aq) \rightarrow SiO_2(s) + Na_2SO_4(aq) + H_2O(l) \quad (2)$$

2.3. Bacterial regeneration of pseudomonas fluorescens

Pseudomonas fluorescens bacterial regeneration was started by transferring the pure culture preserved on agar slants using inoculating loop into new NA slants. The culture was incubated at 37 ˚C for 24 h. All treatments were carried out in a sterile environment inside a laminar airflow [9].

2.4. Immobilization of Pseudomonas fluorescens

The silica-cellulose powder was put into Nutrient Broth (NB) growth media containing *Pseudomonas fluorescens* isolate and stirred until thoroughly homogenized. Afterward, the suspension was filtered using Buchner funnel and the filtrate was measured for the absorbance using UV-Vis spectrophotometer at 600 nm. Bacterial cell counting was conducted with Hamocytometer Neubauer and observed under microscope with 400x magnification. The residual materials were silica-cellulose matrices successfully incorporated with *Pseudomonas fluorescens.*[19]

2.5. Bioremediation assessment of Pseudomonas fluorescens incorporated in silica-cellulose matrices against Cadmium (Cd)

A series of experiments were carried out in a fixed volume (100 mL) of Cd standard solution in reaction flasks. Silica-cellulose matrices, which have been incorporated with *pseudomonas fluorescence,* were added into 50 mL of Cd solution and incubated in a shaker incubator at 100 rpm for 3×24 h. Measurement of cadmium content was conducted once every 24 h. Cadmium reduction was calculated by comparing metal content before and after bacterial treatment using Atomic Absorption Spectrophotometer (AAS).

3. Results and Discussion

Silica modification of rice husks using cellulose of Nata de Coco was initiated by heating the husks in the oven for an hour. The procedure was intended to transform the phase of the silica from crystalline to amorph. Amorph silica exhibits a porous structure which led to a larger surface area compared to crystalline silica [20]. The next step is the immersion of rice husk ash in strong alkaline solution to acquire sodium silicate extract. A yellow paste of sodium silicate was then combined with cellulose of Nata de Coco which has been hydrolized in advance using acid solution. After white colloids present, NH_4OH was added to remove sulphuric ions contained within the pores of silica matrices. The suspension was filtered and the residue dehydrated in the oven to acquire white powders of silica cellulose matrix.

In reality, the application of silica as an adsorbent has been widely studied as well as the utilization of silica as an immobilization matrix [19,21,22]. However, there were no reports denoting silica as a good adsorbent. Thus, there has to be an alteration performed on silica, specifically regarding the surface area of the material. Modification of silica using cellulose might induce alterations on its surface area which facilitates the interaction between the surface area of silica and the organic materials [23]. This phenomenon occurred due to the change in surface polarity of silica, resulting from the interaction between the silanol group (Si-OH) of silica and the hydroxyl and ether group of cellulose. These groups contain electron-rich oxygen atoms that serve as the active site of various polar compounds as the carbon atoms of cellulose for non-polar compounds, due to the formation of single bonds [24].

The change in surface polarity did not only improve the physical interactions, but it also increased the pore size of silica. A larger pore size provides certain advantages on silica-cellulose matrices as an adsorbent, including the potential of the matrices to be used as the immobilization matrix of *Pseudomonas fluorescens,* since the biocatalyst system of the a forementioned microorganism tends to be stable on matrix pores [25]. In addition to a suitable matrix, immobilization is determined by the age of the cells. The process requires a fresh suspension of bacterial inoculum to acquire cells with good viability [26]. The result of the study demonstrates that contact time between silica-cellulose matrices and *Pseudomonas fluorescens* affects the percentage of total bacteria immobilized within the matrices, as displayed at table 1.

The percentage of total bacteria *Pseudomonas fluoroscens* immobilized is directly proportional to the increase of contact time between the inoculum and matrices. At 15 min variation, the number of bacteria before contacted with silica-cellulose matrices reached 312.8×10^6cells/mL. Whereas, after contact, the number of bacteria decreased to $216.8\text{x}10^6$ cells/mL or if converted into a percentage, 31% bacteria have been immobilized in the matrices. This number rises as the contact time increases.

Eventually, the percentage of immobilization, which was around 70% at 20 min contact time, was mounting to 98% at 25 min contact time.

Table 1. Percentage of bacterial immobilization in silica-cellulose matrices.

No.	Contact time	ABS		Number of bacteria (cell/mL)		Percentage of immobilization
		Before	After	Before	After	
1.	15 min	1.497 ± 0.007	1.357 ± 0.007	312.8 x 10^6	216.8 x 10^6	**30.69%**
2.	20 min	1.635 ± 0.024	0.467 ± 0.011	1130.4 x 10^6	336 x 10^6	**70.28%**
3.	25 min	1.958 ± 0.002	0.532 ± 0.002	3152 x 10^7	43.52 x 10^7	**98.62%**
4.	30 min	2.072 ± 0.013	0.536 ± 0.001	5808 x 10^7	47.04 x 10^7	**99.19%**
5.	45 min	0.872 ± 0.019	0.526 ± 0.008	128 x 10^7	17.2 x 10^7	**86.56%**
6.	60 min	2.384 ± 0.002	1.699 ± 0.001	792 x 10^7	728 x 10^7	**8.08%**

It can be seen from the table 1 that the optimum percentage of immobilization of bacteria was achieved at 30 min contact time, as the percentage of *Pseudomonas fluorescens* embedded in the matrices reached 99% or nearly all bacteria have been successfully immobilized into silica cellulose matrices. The result indicates that *Pseudomonas fluorescens* requires sufficient contact time to interact with the surface of the material and occupy the pore of the matrices. In more than 30 min, the number of bacteria immobilized significantly decreased as the matrices could not accommodate any more bacteria. At 45 min contact time, no more than 86% of bacteria incorporated, while after an hour contact time, the percentage declined drastically. The data is presented in Figure 1.

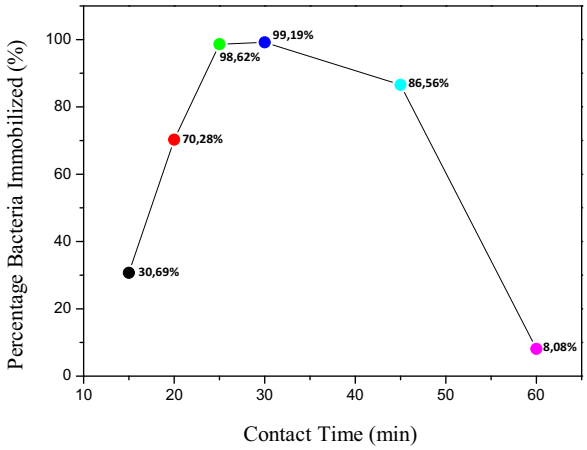

Figure 1. Graph describing percentage of bacterial immobilization in silica-cellulose matrices.

Silica-cellulose matrices which have been incorporated with *Pseudomonas fluorescens* were later used as the heavy metal bioremediation agent of cadmium, to assess the activity of the bacteria. The matrix was applied to cadmium standard solutions of 10 ppm and the reduction of cadmium content was measured using AAS, based on Lambert Beer's Law.

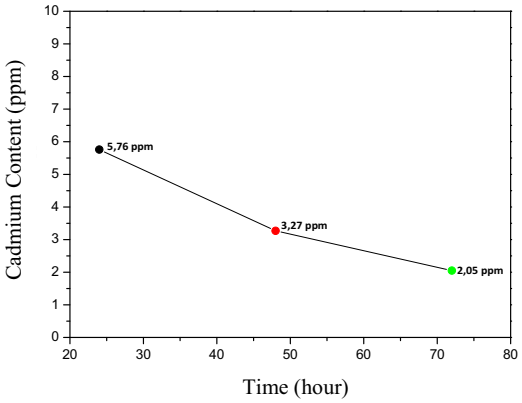

Figure 2. Graph depicting the reduction of cadmium content in the standard solution.

Bacteria is considered a prominent factor in nature as the microorganisms can be found almost everywhere, available in abundant and has various functions, one of which is as biosorbent to absorb metal ions in aquatic environment [27]. This attribute is supported by the availability of biological materials and the habitat environment, which in this case is the standard solution that contains heavy metals of high affinity, which makes the metals easily absorbed by absorbents. Heavy metal absorption in aqueous solution is feasible through ion-exchange, in which the ions that reside in the cell wall of the microorganisms are exchanged with heavy metal ions [28]. The surface of the cell membrane or the inside of extracellular polymers (e.g. protein and polysaccharide) contains a negatively-charged active center that will interact with positively-charged heavy metal ions [29]. The absorption process of heavy metal ions by microorganisms is divided into biosorption and bioaccumulation [30,31]. While biosorption refers to a non-metabolic sorption process which is commonly known as adsorption, bioaccumulation is identified as an absorption process, a form of a resistance mechanism. The mechanism involves excretion of a chelate compound out of the cell and the bonding of metal ions by intracellular molecules such as metallothionein or the storing of metal ions in specific organelles such as mitochondria and vacuole [30].

The result indicates the presence of bacterial activity capable of reducing cadmium (Figure 2). On the first day, the reduction of heavy metal content reached 40% of the initial concentration prior to the addition of matrices incorporated with bacteria. The significant increase of heavy metal reduction was observed on the second day, and later on the third day as well, in which the system decreased 80% of the initial heavy metal concentration from 10 ppm to 2 ppm. The observation revealed that cadmium reduction reaches optimum at 48 h contact time. Beyond that, the reduction of cadmium content experienced a significant decrease. The trend presumably occurred because the surface of silica-cellulose matrices has achieved maximum absorption capacity of heavy metal beyond 48 h contact time, which leads to the decrease in heavy metal absorption by *Pseudomonas fluorescens*. The result ultimately demonstrates that *Pseudomonas fluorescens* retains its metabolism activity albeit embedded in the matrix.

4. Conclusion

The optimum contact time for the immobilization of *Pseudomonas fluorescens* was achieved at 30 min variation, as 99% of bacteria was successfully embedded in silica-cellulose matrices. *Pseudomonas fluorescens* incorporated in silica-cellulose matrices exhibits high potential as bioremediation agent, indicated by the reduction of 80% cadmium content in standard solution. The result shows the prospect of silica-cellulose material as the immobilization matrix of *Pseudomonas fluoroscens.*

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