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BACTERIAL DESULFURIZATION OF DIBENZOTHIOPHENE BY *PSEUDOMONAS* SP. STRAIN KWN5 IMMOBILIZED IN ALGINATE BEADS

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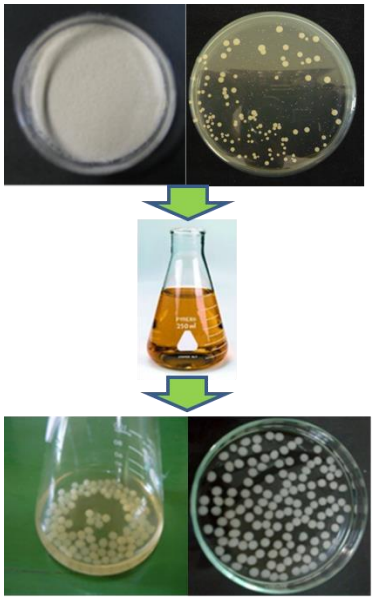
Graphical abstract



Abstract

Biodelfurization of petroleum has emerged as a potential alternative to the hydrodesulfurization and oxidative desulfurization processes. However, the main obstacle in its commercial application is the efficiency and practicality of using bacterial cells. *Pseudomonas* sp. strain KWN5 was tested for the ability to use dibenzothiophene (DBT) in *n*-tetradecane as the sole sulfur source with two phase oil-water system. The biodesulfurization ability of strain KWN5 was evaluated by immobilized cells with dibenzothiophene as substrates. The cells immobilized by entrapping them with sodium alginate (SA) had high DBT biodesulfurization activity and could degrade 100 mg DBT/L in *n*-tetradecane of 46.76–100%, depended on concentrations of sodium alginate and cells within 24 h at 37°C with shaking at 160 rpm. The combination of SA concentration of 3% (w/v) with bacterial cells OD_{660} 40 (25.52 mg DCW/mL) has an optimal biodesulfurization activity on 100 mg DBT/L in *n*-tetradecane, which is equal to 71.85% biodesulfurization. The immobilized cells of *Pseudomonas* sp. strain KWN5 in alginate beads were more efficient for the degradation of DBT and can be reused for five cycles (220 h) without any loss in their activity. The results of this study clearly show the role of the effects of cell immobilization in increasing the process of biodesulfurization.

Keywords: Biodesulfurization, dibenzothiophene, immobilized cells, alginate, *Pseudomonas* sp.

1.0 INTRODUCTION

At present, people still need a lot of energy derived from the conversion of fossil energy sources, for example in factories, power plants, and transportations [1]. One type of fossil energy that is commonly used is petroleum [2]. Up to the present day, although the petroleum supply has decreased, but it has a very broad benefit in human life, however, the combustion of petroleum produces emissions that have negative impacts on the environment, human health, and other living things [3, 4].

Sulfur oxide (SO_x) compounds are one of the pollutants from the combustion of petroleum that is hazardous. The presence of these emissions in the atmosphere causes serious environmental problems such as air pollution, acid rain, corrosion, crop damage, and various other problems [3, 5, 6, 7]. In order to reduce these emissions, the levels of organic

sulfur compounds in petroleum must be reduced during the refining process [8, 9].

Crude oil has a high aromatic sulfur content, consist of benzothiophene (BT), dibenzothiophene (DBT), and its derivatives [10]. It is difficult to remove aromatic sulfur in crude oil through the process of hydrodesulfurization (HDS) because it was harder to desulfurize heterocyclic aromatic sulfur compounds (Polyaromatic Sulfur Heterocycles; PASHs) found in larger fractions [11, 12]. This system uses a chemical catalyst consisting of metal at high temperatures and pressures therefore, this technique requires high investment and high operating costs. Because of these reasons, researchers try to find out more effective and efficient new approaches such as "biodesulfurization" [2, 9].

According to Nassar *et al*. [13] biodesulfurization (BDS) using microbes or enzymes as biocatalysts; these have attracted much attention in the recent years, that has bright prospects because the process is cheap, it does not require complex installations, and is environmentally friendly. In addition, the BDS is beneficial because it uses specific enzymes specifically to degrade benzothiophene (BT), dibenzothiophene (DBT), and its derivatives.

Some strains of bacteria can degrade aromatic sulfur compounds with long-chain alkyl including the bacteria *Rhodococcus* spp. [13, 14, 15, 16], *Bacillus* [6, 17], *Mycobacterium* spp. [18, 19], *Pseudomonas* spp. [20, 21, 22], *Sphingomonas* spp. [3], *Pantoea* sp. [9], *Klebsiella* [23], *Agrobacterium* sp. [24], and *Stenotrophomonas* sp. [25] have demonstrated their ability to separate organic sulfur compounds such as those found in oil models or petroleum. As the KWN5 isolate, this bacterial strain is isolated from the tropical regions of Indonesia shows a high ability to desulfurize the sulfur content of DBT which is soluble in *n*-tetradecane. In addition, this bacterium with growing cells can degrade 100 mg DBT/L up to 96% at a higher temperature of 37°C for 96 h with shaking at 190 rpm [26]. The bacterium follows 4S pathway for BDS that leads to the formation of 2-Hydroxybiphenyl (2-HBP). This isolate will be used as a sulfur degrading biocatalyst in the petroleum model in the form of an *n*-tetradecane solvent containing DBT.

It has been reported that the biodesulfurization process in both the oil model and petroleum is a "triphasic system" which consists of cells, water phase, and oil phase [27, 28]. It is very difficult to separate oil and water phase from the emulsion, furthermore, cell recovery is also difficult [29]. Besides, biocatalysts are short-lived and it is difficult to use repeated biocatalysts. To overcome these problems by making cells in an immobilized state which is one of the promising approaches to improve cell efficiency, can be used repeatedly, and reduce operating costs [30]. Biodesulfurization of oil with immobilized cells also makes it easier to separate biocatalysts from oil and can reduce the occurrence of contamination during the BDS process [31].

Entrapment is one of the many immobilization techniques that has been widely used. The living cells are trapped in a matrix polymer which has enough pores for the substrate to diffuse into cells and the products are distance from cells [32]. Besides preventing cell loss during biodesulfurization, the entrapment technique also guarantees immobilized the life of the cells [28]. Many potential immobilization supporting materials with entrapment methods that have been used by previous researchers including sodium alginate (SA) [5, 19, 31], polyurethane foam [16], polly(vinyl) alcohol (PVA) [28, 33, 34], K-carrageenan [35], agar [32, 36], chitosan [37], and gelatin [38].

PVA and SA are good materials used for cell immobilization by entrapment techniques. PVA has a strong and stable polymer structure used in petroleum biodesulfurization. Immobilized biodesulfurization with 12% PVA material can be used up to 8 biodesulfurization cycles performed in 24 h for each process, but its activity is slightly lower than sodium alginate [28]. So is the case with SA, is an organic polymer that is environmentally friendly and easy to conduct cells immobilization with entrapment technique. Many biodesulfurizations with immobilized cells using SA has been carried out, including SA with concentrations ranging from 1–4% and combined with cells at concentrations of 12.4–31.0 mg/mL (dry cells weight) [19, 20, 27, 31]. In addition, the use of a mixture of these two polymers has also been conducted by previous researchers in desulfurizing DBT [28] using 10% PVA (w/v) mixed with 2% SA (w/v) in DBT biodesulfurization, but the structure is weaker compared to the PVA [20, 34] also use a mixture of PVA and SA in biodesulfurization of DBT produces a strong bead structure, but the activity of the cells in the beads is low. According to Gunam *et al*. [28], SA is one of the immobilized materials which is quite good and its preparation is very easy to be carried out.

Referring to previous research, it is necessary to desulfurize petroleum by cell immobilization using SA. In this study, the optimization of SA concentration has been conducted that can obtain the best-immobilized cells based on the shape and stability of beads and determine the combination of SA and KWN5 cells concentrations that have optimal activity and stability in desulfurizing DBT compounds in *n*-tetradecane.

2.0 MATERIALS AND METHODS

2.1 Chemicals

The dibenzothiophene (DBT) was purchased from Aldrich, *n*-etradecane (TD) and sodium alginate were supplied by Wako Pure Chemical, Osaka, Glycerol (99%) was purchased from Honeywell, Germany, ethyl-acetate (99.8%) was purchased from Sigma-Aldrich. The $NaHCO_3$, glucose, hexan, and ethanol supplied by Merck, and $CaCl_2$ (Medis). All other chemicals were of available analytical grade, which were obtained from various commercial sources.

2.2 Bacterial Strain and Culture Medium

The bacterial strain used in this work was a culture of *Pseudomonas* sp. strain KWN5, which was previously isolated from an oil-contaminated soil, around the Kawengan petroleum mine site, Bojonegoro, East Java [26]. The strain was grown in a mineral salts sulfur free (MSSF) enriched with concentrated aromatic sulfur compounds (CA) as the sole of sulfur source, as reported in our previous paper [3, 28]. While the media for testing the biodesulfurization of dibenzothiophene compounds was carried out in a two-phase system, namely medium containing saline solution (NaCl 0.85%) and as an organic phase was *n*-tetradecane (TD) which contained DBT 100 mg/L as petroleum model [27, 28].

2.3 Rejuvenation of Cells Culture and Mass Cells Production

A starter culture was prepared by inoculating strain KWN5 from 0.5 mL stock culture into 3 test tubes each containing 5 mL MSSF media and 10 µL concentrated aromatic sulfur (CA), incubated at 37°C for 96 h with shaking at 190 rpm [26]. After the incubation period, 2 of the test tubes containing cell suspensions were grown again on new MSSF-CA media with a larger volume, while the remaining one tube was made into a stock culture. A total volume of 10 mL cell suspension was grown again on Erlenmeyer containing 200 mL MSSF and 0.26 mL CA, incubated at 37°C for 96 h with a shaker rotation speed of 190 rpm until a starter culture was obtained.

To produce a large quantity of cells, 20 mL cell suspensions from starter culture were grown in 10 pieces of Erlenmeyer flasks, with each containing 300 mL MSSF enriched media and 0.4 mL CA, incubated at 37°C for 96 h with shaking at 190 rpm. After the incubation period, cells were harvested by centrifugation (3,500 rpm, for 20 min), washed with saline solution (NaCl 0.85%), and then cells concentration was determined by measuring the OD_{660} of seed culture [26].

2.4 Cell Immobilization Methods

Sodium alginate with various concentrations [1–4% (w/v)] was dissolved in distilled water, then stirred it using a magnetic stirrer and sterilized (the solutions were subsequently stirred for 10 min). After cooling, as much as 6 mL SA solution with variations in concentration of 1, 2, 3, and 4% (w/v) were mixed with 3 mL cell suspensions with variations in concentrations (OD_{660}) 20, 30, and 40 or equal to 12.20, 19.15, and 25.52 mg dry cell weight (DCW)/mL, respectively, then it was extruded drop-wise into a cold 4% calcium chloride ($CaCl_2$) solution (4°C) with an injector with a diameter of 2 mm, so the immobilized beads were produced. The beads were allowed to harden in the $CaCl_2$ solution for 6 h at 4°C to complete replacement of sodium ions by calcium ions. Then the beads were washed with saline solution (NaCl 0.85%) twice, next, stored at 4°C in a refrigerator for further use [39].

2.5 Determination of Stability and Reusability of Immobilized Cells

To obtain immobilized material that produced the best beads, immobilized cells were tested for repeatedly biodesulfurization rate. To test the reusability of immobilized cells, the beads were repeatedly used in several consecutive DBT biodesulfurization processes, and the DBT degradation rate was detected by GC-FID. Before the immobilized cells were used, the immobilized cells beads were activated in a minimal medium of 20 mL saline solution (0.85% NaCl) and 1% glucose (w/v), incubated at 37°C for 20 h with shaking at 160 rpm as one cycle [40].

After the reactivation period, each immobilized cell bead was washed with saline solution twice, and then the bead stability was tested. Biodesulfurization tests were carried out in a 100 mL Erlenmeyer containing 17.5 mL saline solution and 2.5 mL *n*-tetradecane containing 100 mg DBT/L, incubated at 37°C and shaken at 160 rpm for 24 h. After the biodesulfurization period was completed, the beads were separated from the oil and water phase and then washed twice using distillate water and twice using saline solution. Further, the beads were reactivated again, and then the next biodesulfurization was done as the previous procedure. This process was repeated until the bead's ability to maintain its overall structure remains stable. When the beads had passed the three immobilized stages if there were any of them have broken out, it showed the capability limit of the immobilized beads [28].

2.6 Biodesulfurization by Alginate-Entrapped Cells

Immobilized cells were tested for DBT desulfurization rate. At this stage, 100 mg DBT/L biodesulfurization was carried out in *n*-tetradecane by combining the SA immobilized material with 4 variations in concentration [1–4% (w/v)] with 3 variations in concentration of cell suspensions (OD_{660} 20-40), previously explained in the forming of immobilized cells.

Before the biodesulfurization was carried out, immobilized cells were activated first as in the previous experiments. After the incubation period, the beads were separated from the activated media then washed twice with saline solution, further the biodesulfurization activity was tested. Biodesulfurization with immobilized cells was carried out in a 100 mL Erlenmeyer containing 17.5 mL saline solution and 2.5 mL *n*-tetradecane containing 100 mg DBT/L, at 37°C with the shaker rotation speed of 160 rpm for 24 h [28, 41].

After the biodesulfurization testing period was completed, the beads were further separated from the water and oil phases, and then the beads were

washed twice using distilled water and twice using saline solution. Next, the beads were reactivated and desulfurized on the new 100 mg DBT/L media. This process was carried out repeatedly to obtain a combination of SA and bacterial cells concentrations that had optimum activity and stability in the desulfurizing DBT.

2.7 Analitycal Procedures

The bacterial growth was observed by measuring the optical density (OD) of cell suspension using a UV/Vis spectrophotometer, at λ660 nm and uninoculated MSSF was used as a blank. The culture pH was observed at certain time intervals using a pH-meter, based on the method prescribed by Gunam *et al*. [28]. The culture was then acidified with 6 N HCl to reach pH 2.0 and extracted with the same volume of ethyl acetate for quantitative estimation of DBT removal using a GC-FID.

All samples extracted with ethyl acetate were analyzed to measure the concentration of DBT residues by GC (GC8000TOP-CE instruments) equipped with a DB-17 HT column (30 m x 0,25 mm x 0,15 m; J&W Scientific, Yokogawa) with a flame ionization detector (GC-FID). The analysis was carried out under the following conditions: the initial temperature of the column oven was 200°C, and heated to 270°C at a rate of 10°C/min. The carrier gas was helium, and both the injector and detector temperatures were set at 250°C. When the temperature of the column was increased from 200°C to 270°C at 10°C/min, the retention times observed were 11.20 min for DBT. In addition, samples are MSSF and cell free [25, 42]. All experiments and measurements were carried out in duplicate and the mean were used throughout the data analysis [13].

3.0 RESULTS AND DISCUSSION

Previous research had conducted the selection of cell immobilization materials, namely sodium alginate (SA), poly(vinyl alcohol) (PVA), a mixture of PVA with sodium alginate, agar, and carrageenan. The results showed that sodium alginate was a relatively better cell immobilization material than other materials, especially in terms of cell activity, uniformity of beads, and the ease of preparation [26]. All treatment combinations were investigated on biodesulfurization of 100 mg DBT/L in *n*-tetradecane to obtain the treatment combination that produced the most optimal biodesulfurization activity.

3.1 Cell Immobilization in Alginate

The homogenization process of sodium alginate with bacterial cells suspension required different times at each concentration. A mixture of 1% SA and bacterial cells were successfully mixed and became easily and quickly homogeneous. This was due to the mixture had a low level of viscosity so that it was easily mixed evenly. A 2% SA mixture with bacterial cells was also easy to homogenize but the timing was slightly slower than a 1% SA mixture. The 3% SA mixture was increasingly difficult to homogenize even the 4% SA mixture, the more difficult to homogenize it because the level of viscosity of sodium alginate increased in line with the increased of its concentration. The process of beads formation also required different time each concentration and the shape of the beads produced were also different. When it was dropped wise into a $CaCl_2$ solution, a mixture of lower concentration falls into the solution very quickly but produces an imperfect round shape bead. This is due to the low concentration of sodium alginate unable to wrap the cell suspension which is half of its volume. Conversely, the more concentrated the concentration of the solution the rate of fall of the mixture to the $CaCl_2$ solution is also slower and the more perfect round shape is produced. Besides, the texture of the bead is also getting tougher. According to Peng and Wen [33] immobilized cell beads which resembles round form like balls were formed because of the reaction between sodium alginate and liquid containing Ca^{2+} ions, which were formed initially floated on the surface of the solution, after a while, the beads dropped to the bottom of the solution. Beads produced from the 4% SA mixture quickly left the surface to the bottom of the solution, as well as with the 3% SA mixture was slightly slower. However, the lower the concentration of the mixture, it took more time to get to the bottom of the solution, especially the 1% SA mixture.

After all the beads were formed, then they were placed in the $CaCl_2$ solution in the refrigerator to harden the structure of the beads, further washed them using saline solution (NaCl 0.85%). According to Göksungur and Zorlu [43] washing immobilized beads using sterile saline solution (0.85% NaCl) was intended to reduce the excess Ca^{2+} ions in the bead because excess Ca^{2+} ions would cause the beads porosity to decrease due to contraction. All beads that had been solidified with $CaCl_2$ solution did not show any turbidity in the solution. This meant that the cells were still completely bound to the SA polymer, in other words, none of the cells was detached. The washed immobilized cells were stored at 4°C before testing for biodesulfurization activity. The appearance of the beads from a mixture of KWN5 cells OD_{660} 20, 30, and 40 with sodium alginate concentrations of 1, 2, 3, and 4% (w/v) can be seen in Figure 1.

From the four concentrations of sodium alginate used to entraped the strain KWN5 cells, it was found that the higher the concentration of the mixture, the better the forms of the beads were obtained, while the lowest concentration could not produce a good bead form. In addition, the higher the concentrations of sodium alginate resulted in stronger polymer structures. According to Hou *et al*. [20] in their research, mechanically sodium alginate 1.5% is very soft, 2% is soft, 3% is strong, while the concentration of 4% is very strong.

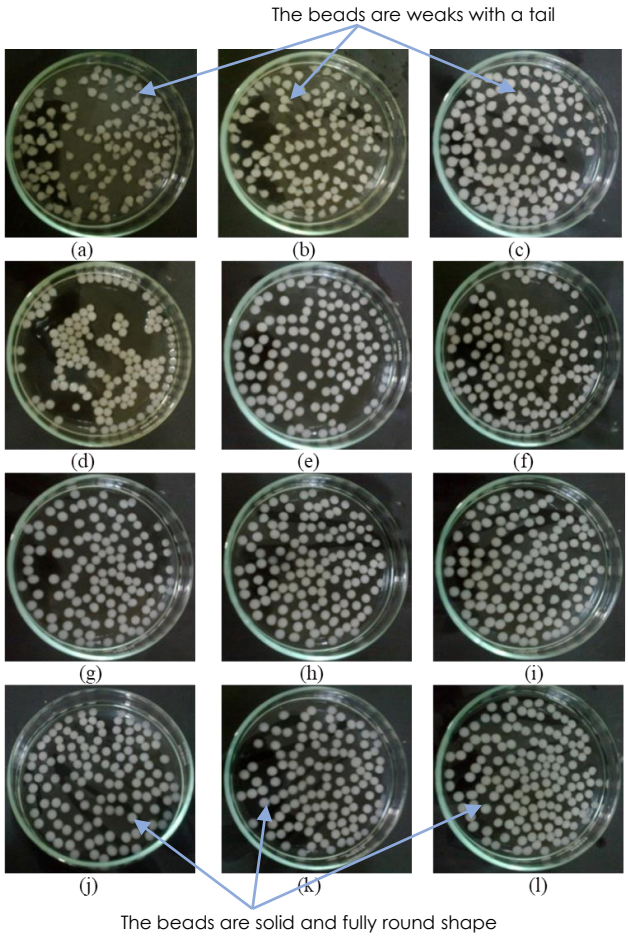


Figure 1 The appearance of beads from SA concentration of 1, 2, 3, and 4% (w/v) combined with bacterial cells of KWN5 OD_{660} 20, 30, and 40. (a) SA 1% and OD 20; (b) SA 1% and OD 30; (c) SA 1% and OD 40; (d) SA 2% and OD 20; (e) SA 2% and OD 30; (f) SA 2% and OD 40; (g) SA 3% and OD 20; (h) SA 3% and OD 30; (i) SA 3% and OD 40; (j) SA 4% and OD 20; (k) SA 4% and OD 30; (l) SA 4% and OD 40.

It should be noted that the ability of sodium alginate to trap cells and produce a round bead also depended on the volume of the sample trapped in it. According to Peng and Wen [33], a good concentration of sodium alginate to form beads are 4% (w/v). Meanwhile, Derikvand and Etemadifar [31] used sodium alginate 2%, 4%, 6% (w/v) for cell immobilization and mixed with cells in the same volume, resulting in a final concentration of 1%, 2%, and 3% (w/v) with a ratio of 1:1 and obtained beads with good shape and elasticity and were quite stable on beads made from 2% sodium alginate and cell concentration OD_{660} was 40 (25.52 mg DCW/mL).

3.2 DBT Biodesulfurization Using KWN5 Immobilized Cells

The data on the results of 100 mg DBT/L biodesulfurization at different bacterial cell concentrations and different sodium alginate concentrations can be seen in Table 1.

Table 1, shows that the combination of SA 1% with cells OD_{660} 20 and SA 1% with cells OD 30, has the highest biodesulfurization activity, from 2.5 mL *n*-tetradecane containing 100 mg DBT/L can be degenerated by 100% and 94% consecutively. followed by SA 4% and OD 30 (74.63%), SA 3% and OD 40 (71.85%), SA 3% and OD 30 (70.96%), SA 1% and OD 40 (69.88%), SA 2% and OD 20 (69.68%), SA 4% and OD 40 (63.40%), SA 2% and OD 40 (62.65%), SA 3% and OD 20 (59.16%), SA 4% and OD 20 (47.30), and SA 2% and OD 30 with the lowest activity (46.76%).

From the results of the study, the average combination of 1% SA with bacterial cell suspensions had a very high biodesulfurization activity that was degradation without residue (SA 1% and OD_{660} 20). This is due to the low concentration of sodium alginate, which was 1% plus the cell suspensions of strain KWN5 which was relatively not solid, resulting in very low viscosity so that it formed beads with high porosity, but it was weak. High porosity allowed smooth diffusion of the substrate into the beads so that cells regeneration in the beads when reactivation occurred properly, as well as when biodesulfurization of all DBT substrates diffused smoothly into the beads and could be utilized by strain KWN5. The results of this study were supported by Guobin *et al*. [32] which stated that immobilized cells in a porous polymer matrix tended to have higher biodesulfurization activity. This is supported by the research conducted by Derikvand and Etemadifar [31] that at lower sodium alginate concentrations the biodesulfurization activity is higher, but the beads formed are weaker and more easily to break.

The combination of 2% SA with cell concentrations, namely OD_{660} 20, 30, and 40, showed the results of biodesulfurization which was rather low compared to all 3% SA combinations. The combination of SA 2% and OD 20 with SA 2% and OD 40 has a high biodesulfurization activity that can degrade 100 mg DBT/L, respectively as follows: 69.68% and 62.65%. However, SA 2% and OD 30 only desulfurize 100 mg DBT/L up to 46.76%. Individuals and groups of SA 3% have higher biodesulfurization activities than SA 2%. The three combinations of SA 3% treatments with cell suspensions have biodesulfurization activity, namely: 59.16; 70.96; and 71.85%. When the SA combination of 3% compared to the SA combination of 4% obtained the results that the individual SA 4% had a relatively higher biodesulfurization activity of SA 4% and OD 30 with 74.63%, but on average the activity was lower than the SA combination of 3%, they are SA 4% and cell concentrations of OD 20: 47.30% and SA 4% and OD 40: 63.40%.

Table 1 Biodesulfurization activity of KWN5 immobilized cells

Sodium Alginate (SA) Concentration	Cells Concentration (OD_{660})	pH after Desulfurization	OD_{660} after Desulfurization	DBT Residue (mg/L)	Desulfurization Rate (%)
1%	20	6.16	0.047	0	100
	30	6.47	0.048	5.88	94.12
	40	6.52	0.042	30.12	69.88
2%	20	6.57	0.044	30.32	69.68
	30	6.14	0.047	53.24	46.76
	40	6.52	0.047	37.35	62.65
3%	20	6.48	0.042	40.84	59.16
	30	6.56	0.050	29.04	70.96
	40	6.64	0.039	28.15	71.85
4%	20	6.09	0.045	52.70	47.30
	30	6.65	0.028	25.37	74.63
	40	6.47	0.033	36.60	63.40

The isolate was grown in Erlenmeyer 100 mL containing 17.5 mL saline solution and 2.5 mL *n*-tetradecane containing 100 mg DBT/L. Incubated for 24 h at 37°C with shaking at 160 rpm [26]

3.3 Cell Stability Testing with Repeated Beads Biodesulfurization

In the biodesulfurization process with repeated KWN5 immobilized cells, the results showed that SA 3% and SA4% remained stable until the 5th biodesulfurization. This was indicated by the structure of the beads that were still intact or no bead from both concentrations that are damaged. Meanwhile, the combination of 1% SA with cells could be used only one-time biodesulfurization, then the bead from the combination of three cell concentrations was damaged. Besides SA 2% with bacterial cells had better stability compared to SA 1%, where the combination of SA 2% with OD_{660} 20, 30, and 40 cell concentrations on average could be used up to 3 times in the biodesulfurization process, then most of the beads damaged during the fourth biodesulfurization. Unlike the case with SA 3% and SA 4%, all combinations of the two concentrations remained stable until they were used to the 5th biodesulfurization. This was indicated by the structure of the bead that was still intact or none of the beads from both concentrations was damaged. When measuring the level of turbidity (OD_{660}) of the water phase from the two concentrations, a low absorbance result was obtained and was not much different, so most bacterial cells that were bound to the polymer walls could still be maintained. Absorbance of water phase immobilized cells with 3% SA and OD_{660} cell concentrations 20, 30, and 40 were; 0.022; 0.018; and 0.021. Meanwhile, SA 4% with OD_{660} cell concentrations of 20, 30, and 40 had an absorbance or turbidity of water phases consecutively: 0.020, 0.023, and 0.018. According to Gunam *et al.* [28] the turbidity in the aqueous phase in this system indicated that the immobilized material had damaged into small particles and cell leakage occurred.

Thus, it appeared that the combination of SA 3% had better activity and stability compared to SA 1% and SA 2%. Even though the stability of SA 3% was almost the same as SA 4%, but the average desulfurization activity of SA 3% was higher than SA 4% (Table 2). Therefore, SA3% is recommended as a combination of cell immobilization which has the best activity and stability in the sulfurization of 100 mg DBT/L in TD. Besides, SA 3% has a strong structure to carry out repeated biodesulfurization [20].

The appearance of beads that used sodium alginate had no difference in terms of the shapes and sizes of the beads before and after the use; the difference was only on the color, the beads which had used sodium alginate were paler. This happened because, after the use, there was the part that forms the color was released or dissolved in the water phase or activation medium, so that the color of the beads were lighter (Figure 2).

Table 2 Bead stability at different concentrations of SA and strain KWN5 based on bacterial cells and material released from the beads by measuring their absorbance (OD_{660})

Sodium Alginate (SA) Concentration	Cells Concentration (OD_{660})	Repeated Biodesulfurization				
		1	2	3	4	5
1%	20	0.047	1.155	-	-	-
	30	0.048	1.066	-	-	-
	40	0.042	1.242	-	-	-
2%	20	0.044	0.010	0.014	0.368	-
	30	0.047	0.070	0.199	0.612	-
	40	0.047	0.062	0.136	0.581	-
3%	20	0.042	0.004	0.016	0.018	0.022
	30	0.05	0.008	0.015	0.015	0.018
	40	0.039	0.008	0.010	0.017	0.021
4%	20	0.045	0.008	0.012	0.015	0.020
	30	0.028	0.007	0.014	0.019	0.023
	40	0.033	0.006	0.008	0.012	0.018

Cell leakage was determined based on turbidity level. Biodesulfurization was carried out up to 5 repeated batch; SA 1% bead was damaged after the first biodesulfurization; SA 2% was damaged after biodesulphurization 3 times; SA 3% and SA 4% were remained stable until the 5th biodesulfurization

The higher the concentration of sodium alginate produced beads with a stronger structure, in other words, the porosity of the beads turned out to be narrower, thus the rate of DBT substrate became longer and less volume of substrate entered the beads. Therefore, the utilization of DBT by strain KWN5 in the beads lasted longer so that the biodesulfurization of DBT did not succeed optimally at 24 h incubation time. It was due to the stability of the strain KWN5, the higher concentration of sodium alginate inhibited the reactivation of the isolate because the slow rate of nutrients into the beads caused not all substrates to be used by cells to reactivate. This also greatly affected the results of DBT biodesulfurization. According to Samia and Ahmed [44] and Lincoln [45] low sodium alginate concentrations produced high porosity bead, where higher sodium alginate concentrations caused a decrease in bead porosity thereby limiting nutrient supply and oxygen diffusion into the beads. Besides the concentration of sodium alginate, the concentration of bacterial cells has an effect on the biodesulfurization of 100 mg DBT/L dissolved in TD. The higher concentration of bacterial cells allowed the

porosity of the bead to decrease because there were too many cells to be trapped by the polymer causing cells to be tightly arranged within the polymer wall. This will also result in inhibition of the rate of nutrients and DBT substrates into the polymer [16]. So, it was possible for some cells to die due to lack of food supply when used to desulfurize DBT, their ability was low. Thus, the use of high KWN5 cell concentrations does not necessarily result in high DBT degradation as well. This is in line with what was stated by Gunam *et al.* [28] that the higher the concentration of cells, the results of biodesulfurization will also be higher.

However, cells with too high concentrations caused a decrease in porosity from the immobilized beads so that the diffusion of the substrate into the cell also decreased so that the ability to desulfurize DBT also decreased (Figure 3).

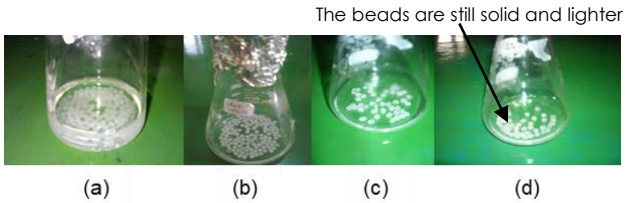


Figure 2 *Beads* with sodium alginate before use (a), after activation (b), the first biodesulfurization (c), and the second biodesulfurization (d)

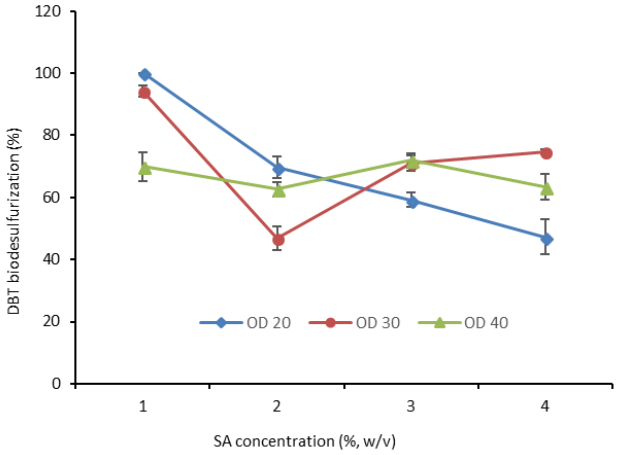


Figure 3 Biodesulfurization of 100 mg DBT/L in *n*-tetradecane at SA concentrations of 1, 2, 3, and 4% and strain KWN5 OD_{660} 20, 30, and 40 were incubated at 37°C, shaken at 160 rpm for 24 h

4.0 CONCLUSION

In this study, sodium alginate is an immobilized material that produces beads with good shape and stability. The combination of 3% (w/v) SA concentration with KWN5 cells OD_{660} 40 (25.52 mg DCW/mL) has an optimal biodesulfurization activity at DBT 100 mg/L in *n*-tetradecane, which is equal to 71.85% biodesulfurization. Immobilized cells of strain KWN5 are potential to desulfurize DBT in *n*-tetradecane and can be used repeatedly, up to five times; in which its activity has not changed much, but further research needs to be done on the stability and activity of strain KWN5 to desulfurize DBT in kerosene or diesel oil.

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References

[1] Gray, D. J., and R. N. Trigiano. 2011. Towards a More Sustainable Agriculture. *Critical Reviews in Plant Sciences.* 30(1-2): 1. DOI: 10.1080/07352689.2011.553147.

[2] Sohrabi, M., H. Kamyab, N. Janalizadeh, and F. Z. Huyop. 2012. Bacterial Desulfurization of Organic Sulfur Compounds Exist in Fossil Fuels. *Journal of Pure and Applied Microbiology*. 6(2): 717-29.

[3] Gunam, I. B. W., Y. Yaku, M. Hirano, K. Yamamura, F. Tomita, T. Sone, K. Asano. 2006. Biodesulfurization of Alkylated Forms of Dibenzothiophene and Benzothiophene by *Sphingomonas subarctica* T7b. *Journal of Bioscience and Bioengineering.* 101(4): 322-327. DOI: 10.1263/jbb.101.322.

[4] Gunam, I. B. W., Y. Setiyo, N. S. Antara, I M. M. Wijaya, I W. Arnata, I W. W. P. Putra. 2020. Enhanced Delignification of Corn Straw with Alkaline Pretreatment at Mild Temperature. *Rasayan Journal of Chemistry*. 13(2): 1022-1029. DOI: 10.31788/RJC.2020.1325573.

[5] Xu, P., J. Feng, B. Yu, F. Li, and C. Ma. 2009. Recent Developments in Biodesulfurization of Fossil Fuels. *Adv Biochem Engin/Biotechnol*. 113: 255-274. DOI: 10.1007/10_2008_16.

[6] Buzanello, E. B., R. P. Rezende, E. M. O. Sousa, E. D. L. S. Marques, and L. L. Loguercio. 2014. A Novel Bacillus Pumilus-Related Strain from Tropical Landfarm Soil Is Capable of Rapid Dibenzothiophene Degradation and Biodesulfurization. *BMC Microbiology*. 14(1): 1-10. DOI: 10.1186/s12866-014-0257-8.

[7] Abdullah, N. R., M. Syarifuddin, M. Zaharin, A. Mohd, I. Bin, and M. R. Nawi. 2015. Effects of Ethanol Blends on Gasoline Engine Performance and Exhaust Jurnal Teknologi. *Jurnal Teknologi.* 76(11): 107-112. DOI: 10.11113/jt.v76.5920.

[8] Maghsoudi, S., M. Vossoughi, A. Kheirolomoom, E. Tanaka, and S. Katoh. 2001. Biodesulfurization of Hydrocarbons and Diesel Fuels by *Rhodococcus* sp. Strain P32C1. *Biochemical Engineering Journal.* 8(2): 151-56. DOI: 10.1016/S1369-703X(01)00097-3.

[9] Bhatia, S., and D. K. Sharma. 2010. Biodesulfurization of Dibenzothiophene, Its Alkylated Derivatives and Crude Oil by a Newly Isolated Strain *Pantoea agglomerans* D23W3. *Biochemical Engineering Journal.* 50(3): 104-9. DOI: 10.1016/j.bej.2010.04.001.

[10] Su, T., J. Su, S. Liuc, C. Zhang, J. He, Y. Huang, S. Xu, and L. Gu. 2018. Structural and Biochemical Characterization of BdsA from *Bacillus subtilis* WU-S2B, a Key Enzyme in the '4S' Desulfurization Pathway. *Frontiers in Microbiology*. 9(FEB): 1-11. DOI: 10.3389/fmicb.2018.00231.

[11] Aburto, J, N. Akhtar, M. A. Ghauri, M. A. Anwar, S. Heaphy, U. Arellano, J. M. Shen. 2014. Desalination and Water Treatment Kinetic Evaluation and Modeling for Batch

Degradation *Corynebacterium variabilis* Sh42. *Process Biochemistry*. 5(4): 37-41. DOI: 10.1080/19443994.2012.744950.

[12] Alkhalili, B. E., A. Yahya, N. Abrahim, and B. Ganapathy. 2017. Biodesulfurization of Sour Crude Oil. *Modern Applied Science*. 11(9): 104. DOI: 10.5539/mas.v11n9p104.

[13] Nassar, H. N., S. F. Deriase, and N. S. El-Gendy. 2017. Statistical Optimization of Biomass Production and Biodesulfurization Activity of *Rhodococcus erythropolis* HN2. *Petroleum Science and Technology*. 35(20): 1951-59. DOI: 10.1080/10916466.2017.1373129.

[14] Tao, F., P. Zhao, Q. Li, F. Su, B. Yu, C. Ma, H. Tang, C. Tai, G. Wu, and P. Xu. 2011. Genome Sequence of *Rhodococcus erythropolis* XP, a Biodesulfurizing Bacterium with Industrial Potential. *Journal of Bacteriology*. 193(22): 6422-23. DOI: 10.1128/JB.06154-11.

[15] Bhasarkar, J. B., P. K. Dikshit, and V. S. Moholkar. 2015. Ultrasound Assisted Biodesulfurization of Liquid Fuel Using Free and Immobilized Cells of *Rhodococcus rhodochrous* MTCC 3552: A Mechanistic Investigation. *Bioresource Technology*. 187: 369-78. https://doi.org/10.1016/j.biortech.2015.03.102.

[16] Agarwal, M., P. K. Dikshit, J. B. Bhasarkar, A. J. Borah, and V. S. Moholkar. 2016. Physical Insight into Ultrasound-Assisted Biodesulfurization Using Free and Immobilized Cells of *Rhodococcus rhodochrous* MTCC 3552. *Chemical Engineering Journal*. 295: 254-67. https://doi.org/10.1016/j.cej.2016.03.042.

[17] Lyu, Y., T. Zhang, B. Dou, G. Li, C. Ma, and Y. Li. 2018. A Lipopeptide Biosurfactant from: *Bacillus* sp. Lv13 and Their Combined Effects on Biodesulfurization of Dibenzothiophene. *RSC Advances*. 8(68): 38787-91. DOI: 10.1039/c8ra06693k.

[18] Furuya, T., Y. Ishii, K. Noda, and K. Kino. 2003. Thermophilic Biodesulfurization of Hydrodesulfurized Light Gas Oils by *Mycobacterium phlei* WU-F1 221: 137-42. DOI: 10.1016/S0378-1097(03)00169-1.

[19] Li, F., P. Xu, J. Feng, L. Meng, Y. Zheng, L. Luo, and C. Ma. 2005. Microbial Desulfurization of Gasoline in a *Mycobacterium goodii* X7B Immobilized-Cell System. *Applied and Environmental Microbiology*. 71(1): 276-81. DOI: 10.1128/AEM.71.1.276-281.2005.

[20] Hou, Y., Y. Kong, J. Yang, J. Zhang, D. Shi, and W. Xin. 2005. Biodesulfurization of Dibenzothiophene by Immobilized Cells of *Pseudomonas stutzeri* UP-1. *Fuel*. 84 (14-15): 1975-79. DOI: 10.1016/j.fuel.2005.04.004.

[21] Larentis, A. L., H. C. C. Sampaio, C. C. Carneiro, O. B. Martins, and T. L. M. Alves. 2011. Evaluation of Growth, Carbazole Biodegradation and Anthranilic Acid Production by *Pseudomonas stutzeri*. *Brazilian Journal of Chemical Engineering*. 28(1): 37-44. DOI: 10.1590/S0104-66322011000100005.

[22] Calzada, J., A. Alcon, V. E. Santos, and F. Garcia-Ochoa. 2011. Mixtures of *Pseudomonas putida* CECT 5279 Cells of Different Ages: Optimization as Biodesulfurization Catalyst. *Process Biochemistry*. 46(6): 1323-28. DOI: 10.1016/j.procbio.2011.02.025.

[23] Bhatia, S., and D. K. Sharma. 2012. Thermophilic Desulfurization of Dibenzothiophene and Different Petroleum Oils by Klebsiella Sp. 13T. *Environmental Science and Pollution Research*. 19(8): 3491-97. DOI: 10.1007/s11356-012-0884-2.

[24] Gunam, I. B. W., M. Iqbal, I W. Arnata, N. S. Antara, A. A. M. D. Anggreni, Y. Setiyo, I. B. P. Gunadnya. 2016. Biodesulfurization of Dibenzothiophene by a Newly Isolated *Agrobacterium tumefaciens* LSU20. *Applied Mechanics and Materials*. 855(October): 143-49. DOI: 10.4028/www.scientific.net/AMM.855.143.

[25] Papizadeh, M., M. R. Ardakani, H. Motamedi, I. Rasouli, and M. Zarei. 2011. C-S Targeted Biodegradation of Dibenzothiophene by *Stenotrophomonas* Sp. NISOC-04. *Applied Biochemistry and Biotechnology*. 165(3-4): 938-48. DOI: 10.1007/s12010-011-9310-3.

[26] Gunam, I. B. W., I G. A. L. Triani, N. S. Antara, A. S. Duniaji, Y. Setiyo, and D. A. Supata. 2012. Biocatalytic Desulfurization of Dibenzothiophene by *Pseudomonas* sp. Strain KWN5. *Proceeding of 4th International Conference on Biosciences and Biotechnology. Advancing Life Sciences for Health and Food Security*. 21st–22nd September, 2012, Udayana University, Denpasar, Bali, Indonesia. 245-248.

[27] Naito, M., T. Kawamoto, K. Fujino, M. Kobayashi, K. Maruhashi, and A. Tanaka. 2001. Long-Term Repeated Biodesulfurization by Immobilized *Rhodococcus erythropolis* KA2-5-1 Cells. *Applied Microbiology and Biotechnology*. 55(3): 374-78. DOI: 10.1007/s002530000527.

[28] Gunam, I. B. W., K. Yamamura, I N. Sujaya, N. S. Antara, W. R. Aryanta, M. Tanaka, F. Tomita, T. Sone, and K. Asano. 2013. Biodesulfurization of Dibenzothiophene and Its Derivatives Using Resting and Immobilized Cells of *Sphingomonas subarctica* T7b. *Journal of Microbiology and Biotechnology*. 23(4): 473-482, DOI: 10.4014/jmb.1207.07070

[29] Yu, L-Q, T. A Meyer, and B. R. Folsom. 1998. United States Patent (19), no. 19.

[30] Chang, J. H., Y. K. Chang, H. W. Ryu, and H. N. Chang. 2000. Desulfurization of Light Gas Oil in Immobilized-Cell Systems of *Gordona* sp. CYKS1 and *Nocardia* sp. CYKS2. *FEMS Microbiology Letters*. 182(2): 309-12. DOI: 10.1016/S0378-1097(99)00604-7.

[31] Derikvand, P., and Z. Etemadifar. 2014. Improvement of Biodesulfurization Rate of Alginate Immobilized *Rhodococcus erythropolis* R1. *Jundishapur Journal of Microbiology*. 7(3): 1-8. DOI: 10.5812/jjm.9123.

[32] Guobin, S., X. Jianmin, G. Chen, L. Huizhou, and C. Jiayong. 2005. Biodesulfurization Using *Pseudomonas delafieldii* in Magnetic Polyvinyl Alcohol Beads. *Letters in Applied Microbiology*. 40(1): 30-36. DOI: 10.1111/j.1472-765X.2004.01617.x.

[33] Peng, Y., and J. Wen. 2010. Modeling of DBT Biodesulfurization by Resting Cells of *Gordonia* sp. WQ-01A Immobilized in Alginate Gel Beads in an Oil-Water-Immobilization System. *Chemical and Biochemical Engineering Quarterly*. 24(1): 85-94. https://hrcak.srce.hr/49485.

[34] Cheng, Y., H. Y. Lin, Z. Chen, M. Megharaj, and R Naidu. 2012. Biodegradation of Crystal Violet Using *Burkholderia vietnamiensis* C09V Immobilized on PVA-Sodium Alginate-Kaolin Gel Beads. *Ecotoxicology and Environmental Safety*. 83: 108-14. DOI: 10.1016/j.ecoenv.2012.06.017.

[35] Shan, G., J. Xing, H. Zhang, and H. Liu. 2005. Biodesulfurization of Dibenzothiophene by Microbial Cells Coated with Magnetite Nanoparticles Biodesulfurization of Dibenzothiophene by Microbial Cells Coated with Magnetite Nanoparticles. *Applied and Environmental Microbiology*. 71(8): 4497-4502. DOI: 10.1128/AEM.71.8.4497.

[36] Malik, M., and M. Ghosh. 2012. Immobilization Parameters Statistically Optimized for Whole Cells of *Pseudomonas putida* G7 to Enhance Limonin Biotransformation. *Journal of Advanced Laboratory Research in Biology*. 3 (4): 266-75.

[37] Quek, E., Y. P. Ting, and H. M. Tan. 2006. *Rhodococcus* sp. F92 Immobilized on Polyurethane Foam Shows Ability to Degrade Various Petroleum Products. *Bioresource Technology*. 97(1): 32-38. DOI: 10.1016/j.biortech.2005.02.031.

[38] Selvaraj, P. T., M. H. Little, and E. N. Kaufman. 1997. Analysis of Immobilized Cell Bioreactors for Desulfurization of Flue Gases and Sulfite/Sulfate-Laden Wastewater. *Biodegradation*. 8(4): 227-36. DOI: 10.1023/A:1008200411998.

[39] Bergero, M. F., and G. I. Lucchesi. 2013. Degradation of Cationic Surfactants Using *Pseudomonas putida* A ATCC 12633 Immobilized in Calcium Alginate Beads. *Biodegradation*. 24(3): 353-64. DOI: 10.1007/s10532-012-9592-3.

[40] Mohanty, S. S., and H. M. Jena. 2017. Biodegradation of Phenol by Free and Immobilized Cells of a Novel *Pseudomonas* sp. NBM11. *Brazilian Journal of Chemical Engineering*. 34(1): 75-84. DOI: 10.1590/0104-6632.20170341s20150388.

[41] Li, G. Q., S. S. Li, S. W. Qu, Q. K. Liu, T. Ma, L. Zhu, F. L. Liang, and R. L. Liu. 2008. Improved Biodesulfurization of Hydrodesulfurized Diesel Oil Using *Rhodococcus erythropolis* and *Gordonia* sp. *Biotechnology Letters*. 30(10): 1759-64. DOI: 10.1007/s10529-008-9748-8.

[42] Mohamed, M. E. S., Z. H. Al-Yacoub, and J. V. Vedakumar. 2015. Biocatalytic Desulfurization of Thiophenic Compounds and Crude Oil by Newly Isolated Bacteria. *Frontiers in Microbiology*. 6(FEB). DOI: 10.3389/fmicb.2015.00112.

[43] Göksungur, Y., and N. Zorlu. 2001. Production of Ethanol from Beet Molasses by Ca-Alginate Immobilized Yeast Cells in a Packed-Bed Bioreactor. *Turkish Journal of Biology*. 25(3): 265-75.

[44] Samia, A., and F. A. Ahmed. 2010. Production of *Bacillus licheniformis* ATCC 21415 Alkaline Protease in Batch, Repeated Batch and Continuous Culture. *Malaysian Journal of Microbiology*. 6(2): 156-60. DOI: 10.21161/mjm.20209.

[45] Lincoln, L. 2018. Invertase Production by *Bacillus macerans* Immobilized on Calcium Alginate Beads. *Journal of Food Biochemistry*. 42(5): 18903-10. DOI: 10.4236/aim.2013.35057.