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Biodesulfurization of Dibenzothiophene by a Newly Isolated *Agrobacterium tumefaciens* LSU20

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Abstract. Organic sulfur compound of fossil fuel are too resistant to be removed by the conventional desulfurization processes. This study aimed to investigate the best growth conditions of *Agrobacterium tumefaciens* strain LSU20 on desulfurized of dibenzothiophene (DBT) compound in the *n*-tetradecane as model of oil. The experiments were performed with the medium two-phase system, aqueous phase: mineral salt sulfur free (MSSF) medium and the oil phase: *n*-tetradecane containing 200 ppm of DBT in the ratio of oil/water (1: 5). The culture of LSU20 that has been aged 4 days (OD_{660} 5) of 0.1 ml inoculated in a test tube containing 5 mL of MSSF medium and 1 ml model of petroleum, grown at temperature variations incubation as follows: 33°C, 37°C, 41°C, 45°C, and 49°C; variations in the initial pH of medium: pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; and variations of carbon sources such as glucose, sucrose, glycerol and citric acid. The experiments were conducted using a water bath shaker at 150 rpm for 96 hours of incubation. The results showed that the highest rate of degradation of DBT by LSU20 occurs at a temperature of 37°C, media pH of 7 and glucose as the carbon source, ie with the growth rate reached 0.91 (OD_{660}) and DBT compounds degraded until 76.9% (w/v).

Introduction

Petroleum is still the principal energy source for global energy demands. However, natural petroleum reservoirs are currently depleting and the oil quality is deteriorating due to increasing sulfur content and density [1]. The process of oil combustion causes the emission of certain hazardous pollutants into the atmosphere. Among these pollutants, the sulfur dioxide (SO_2), which causes environmental problems, such as air pollution, acid rain, as well as problems for human health [2, 3, 4]. Aiming to reduce these emissions, the pollutant concentration is controlled during the refining process through physical-chemical methods known as hydrodesulfurization (HDS). However, this process is of high cost for the industries [5]. An alternative process and also complementary to this technology is the use of microorganisms capable of metabolizing the sulfur residues contained in fossil oil by a specific metabolic pathway [6]. Aromatic sulfur compounds such as bezotihophene, dibenzothiophene and others very difficult to desulfurized by HDS process caused such compounds to react very saturated. Public awareness for the use of environmentally friendly fuels sets sulfur content in petroleum must have a sulfur content of below than 200 ppm [7].

Biodesulfurization (BDS) is a promising method to remove sulfur compounds from diesel and gasoline [8]. Biodesulfurization (BDS) is used to reduce the sulfur content as low as possible. BDS is a biotechnology utilizing bacteria or its enzymes as biocatalysts for aromatic sulfur desulfurization [7]. Various studies have been conducted to find bacteria that are potentially in

sulfur desulfurization including *Rhodococcus* sp. IGST8 strain [9], *Gordonia* sp. CYSK1 strain [10] and *Sphingomonas subarctica* T7b [2]. *Agrobacterium tumefaciens* LSU20 strain have isolated from soil contaminated with petroleum for many years in the area of Langkat, North Sumatra. The temperature, initial pH of media and carbon source are an important component in the growth of bacteria, especially bacteria that degrade organic sulfur compounds of oil [5]. In this paper, we describe the basic growth properties of LSU20 strain on various temperature, initial pH, carbon sources and the effect of glucose on growth and desulfurization of DBT.

Materials and Methods

Chemicals. Dibenzothiophene (DBT) was purchased from Sigma-Aldrich, USA, *n*-tetradecane (Merck), light gas oil was kindly provided by the PT Pertamina Indonesia, The concentrated fraction of aromatic compounds (CA) was produced by the fractionation of commercial light gas oil, according to the method of Gunam *et al.* [2]. Other chemicals were of analitical grade, commercially available and used without further purification.

Bacterial strains and culture media. *Agrobacterium tumefaciens* LSU20 strain was isolated from oil-contaminated soil by enrichment culture in previous study [11].

The culture medium was a mineral salts sulfur free (MSSF)-CA medium was prepared by dissolving KH_2PO_4 11.4 g, Na_2PO_4 28.85 g, NH_4Cl 10 g, NaCl 0.375 g, $MgCl_2.6H_2O$ 10.1650 g, $CaCl_2$ 3.6746 g, $FeCl_3$ 1.3510 g, $CuCl_2.2H_2O$ 0.0085 g, $MnCl_2.4H_2O$ 0.0495 g, at 1 L aquades. The final pH was 7.0. Glucose was added as carbon source with a final concentration of 1% (w/v), and concentrated aromatic sulfur compounds (CA) of 0.005%. MSSF-CA agar medium used for refresh of strain was supplemented with 1% Agarose. MSSF-TD was the standard medium for the desulfurization assay, and consisted of *n*-tetradecane: MSSF at a ratio of 1:5, with 200 ppm of DBT dissolved in *n*-tetradecane was used as the model of oil [2].

Biodesulfurization assay. The Biodesulfurization assays were performed in a two-layer system of MSSF (Mineral Salts Sulfur Free) medium and an organic layer of *n*-tetradecane containing DBT. Seed culture was carried out in a test tube containing 5 ml of MSSF-CA medium with reciprocal shaking at 150 strokes per min at 37°C for 4 days. Six milliliters of MSSF-TD medium containing DBT compounds was inoculated with 0.1 ml of the seed culture (LSU20) and cultured under the same conditions as in the seed culture. After the incubation, the organic layer and the water layer were separated by centrifugation at 5,000×g for 10 min at 4°C. An uninoculated medium, which was treated in the same manner was used as a control [2, 12].

Analytical methods. Growth of LSU20 strain was determined by measuring the OD_{660} of the water layer. The concentration of DBT in growth culture were analyzed by GC-MS (Agilent Tech. 6890N) with Mass Selective (MS) detector (Agilent Tech. 5973) equipped with a column HP 5 MS (30 m x 0.32 mm x 0.25 μm; J&W Scientific) and was used under the following conditions: the initial temperature of the column oven was 160^{o}C, which was heated from 160^{o}C to 270^{o}C at a rate of 10^{o}C min-1. The injection and detector temperature were maintained at 250^{o}C [13].

Results and Discussion

Effect of temperature on the growth and desulfurization activity. Determination of temperature conditions, the growth of *A. tumefaciens* LSU20 was observed for 96 hours with a temperature variations of 33, 37, 41, 45, and 49°C at medium pH of 7, and glucose as carbon source, with shaking speed at 150 rpm. Growth medium consists of two phases: 1 mL of *n*-tetradecane containing 200 ppm of DBT and 5 mL of MSSF. Cells growth rate observed in the water phase using spectrophotometer (OD_{660}), and DBT degradation of the oil phase was analyzed by GC-MS [13]. Biodesulfurization activity of LSU20 strain on temperature variations can be seen in Fig. 1.

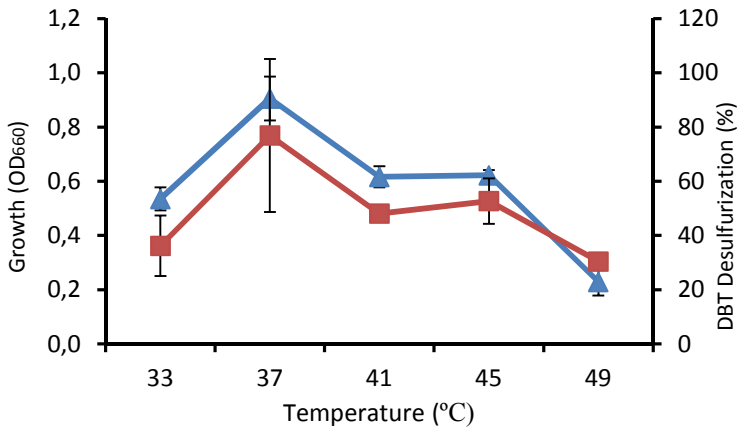

Fig. 1 Effect of temperature variations on the growth and desulfurization activity of *A. tumefaciens* sp. LSU20. Filled Triangle, cells growth; filled square, DBT desulfurization. Cultures were carried out in test tube containing 6 mL of MSSF-DBT medium (5 mL MSSF medium and 1 mL of *n*-tetradecane containing 200 ppm of DBT). The strain was grown at various temperature for 96 hours incubation with shaking at 150 rpm with initial pH of medium of 7 and glucose as a carbon source.

Figure 1 shows the greatest desulfurization rate occurs at a temperature of 37ºC, LSU20 strain could degrade DBT up to 76.9%. The initial concentration of DBT was 200 ppm, and after 4 days incubation only remaining 46.1 ppm of DBT. Whereas smallest desulfurization activity occurs at a temperature of 49ºC. At this temperature the concentration of DBT only reached 139.1 ppm (30.5%). In the previous studies of the bacteria used for the desulfurization process which strain *Gordona* CYKS1 [10] and *G. rubropertinctus* strain T08 [14] have the optimum temperature of 30˚C, *R. erythropolis* strain sp. IGTS8 at a temperature of 35˚C [15], *Encherichia coli* [16] and *B. subtilis* strain WU-S2B [17] at a temperature of 37˚C. According to Ohasiro *et al* [18], there are a variety of enzymes capable of degrading substrate as a catalyst in the desulfurizing bacteria. Enzymes of various desulfurizing bacteria such as DszA, DszB, and DszC have an optimal temperature of 37˚C in the desulfurization process. One of the bacteria that have these enzymes is strains of *B. subtilis* WU-S2B [18]. Temperature affects on two different things, namely the increasing temperature of the chemical properties and enzymatic reactions in cells activated at a faster rate. However, if the ambient temperature is the growth of microorganisms at low levels or lower than 4°C then a microorganism will be in a position of inactive, and will be active again as rising temperatures growth environment [19]. The visual observation of oil-water mixing after 4 days incubation can be seen on Fig. 2.

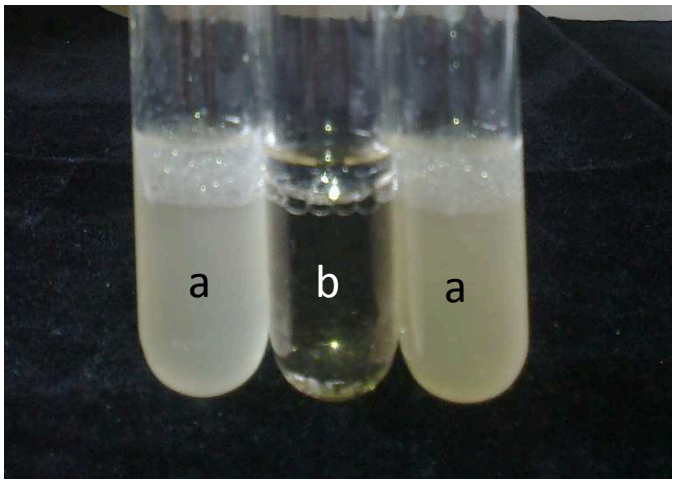

Fig. 2 Visual observation of oil-water mixing condition after 4 days incubation **a** and control **b,** biodesulfurization of DBT by *A. tumefaciens* LSU20.

Effect of initial pH on the growth and desulfurization activity. To determine the effect of initial pH of medium on the growth condition and desulfurization activity of *A. tumefaciens* LSU20, the strain was grown in MSSF medium with initial pH variation among others; pH 5, pH 5.5, pH 6, pH 6.5, pH 7, pH 7.5 and pH 8 at 37°C for 96 hours incubation and glucose as a carbon source, with shaking at 150 rpm. The growth and desulfurization activity of LSU20 strain on initial pH variation can be seen in Figure 3.

Fig. 3 shows the highest rate of degradation by *A. tumefaciens* LSU20 that are in the initial pH of medium of 7 with a degradation rate of DBT by 66.1% and the lowest in pH 8 was 29.4%, or left only 141 ppm of DBT. Various sulfur degrading bacteria has an optimum a pH at pH 6 to 7 [6]. The growth of *A. tumefaciens* LSU20 strain on the initial pH medium of 5 that the turbidity level only of 0.4 (OD_{660}). The bacteria do not degrade DBT optimally. It also ocurred on the research conducted by Kim et al. [20], which the *Gordona* sp. CYKS1 strains was inoculated at the beginning of the growth medium of pH 5 was not significantly desulfurized DBT. In the previous study that pH 7 is also the optimal pH sulfur degrading bacteria growth, with the bacterium *S. subartica* T7b [2, 12]], and Akhtar [21] reported that *Rhodoccocus* sp. Eu-32 strain using media initial pH 7 on an aromatic sulfur desulfurization process through the method of cell growth (growing cells). Bacteria are very sensitive to the concentration of hydrogen ions contained in the growth environment. Proteins such large quantities of the enzyme whose activity is greatly influenced by the optimal conditions of pH, the enzyme catalyst function and metabolism have been lost immediately stopped. At the end of the metabolic pathways of sulfur forming BDS sulfur degrading bacteria, especially bacteria that have 4s pathway in the biodesulfurization process [22].

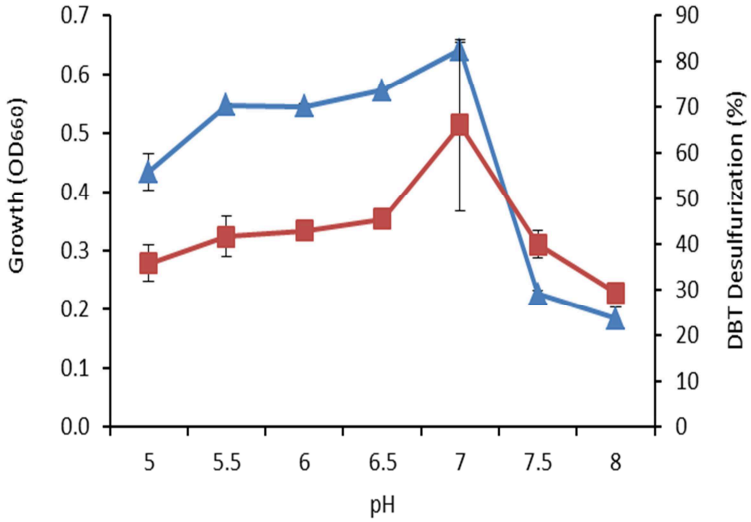

Fig. 3 Effect of innitial pH on the growth and desulfurization activity of *A. tumefaciens* LSU20. The reaction conditions were same as those described in Fig. 1. Filled triangle, cells growth ; filled square, DBT desulfurization. The strain was grown at various innitial pH of medium for 96 hours at 37°C incubation with shaking at 150 rpm with glucose as a carbon source.

Effect of various carbon sources on the growth and desulfurization activity. To test the effect of carbon source on the growth characteristic of LSU20 strain, four different sources of carbon: glucose, sucrose, citrate and glycerol were used to supplement MSSF medium. All these experiments have been conducted using an initial carbon source concentration of 1% (w/v). The rate of growth and desulfurization of DBT is highest with glucose, and the lowest with citrate. The degradation rate of DBT using glucose and citrate as carbon source were 68.9% and 3.4%, respectively (Fig. 4). Glucose was appropriate carbon sources for growth and desulfurization activity. Therefore, the following studies of growing cells of LSU20 strain were performed using glucose as a sole of carbon source.

Carbohydrates are converted from glucose by bacteria will form the DNA (deoxyribonucleic acid) during the growth process, but the main function of carbohydrates as an energy source is aktitvitas cell itself [23]. In the previous study of glucose was also used as the main carbon source

for various DBT degrading bacteria such as *Paenibacillus* [24], *Sphingomonas* sp. [2, 12, 25], and *R. erythropolis* strain IAWQ [26]. From all of these bacteria has been determined that glucose was the best carbon source for DBT desulfurization. LSU20 can not grow well without carbon source, these result indicate that this strain utilized DBT as a sole of sulfur source but not as a carbon source.

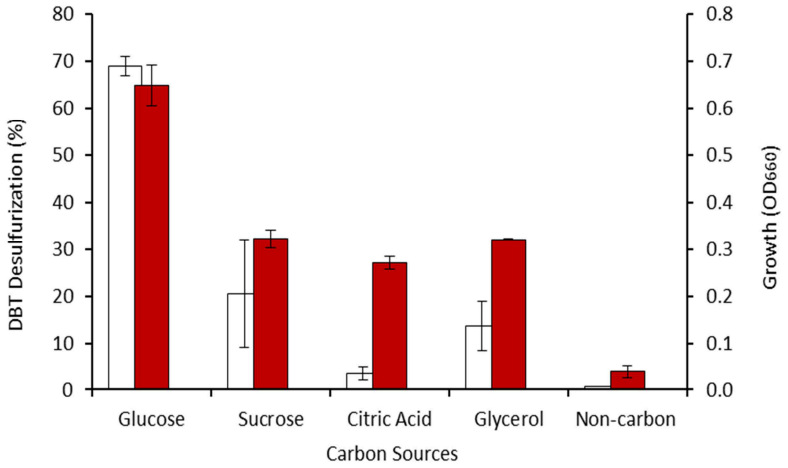

Fig. 4 Effect of various carbon source on the growth and desulfurization activity of *A. tumefaciens* LSU20. Cultures were carried out in test tube containing 6 mL of MSSF-DBT medium (5 mL MSSF medium and 1 ml of tetradecane containing 200 ppm of DBT). The strain was grown at 37ºC for 96 hours incubation with shaking at 150 rpm with initial pH of medium of 7 and each carbon source using a 0.1% (w/v). Red colloms, cells growth; White columms, DBT desulfurization.

Conclusions

The research that has been done, then the specified conditions of growth and desulfurization activity of *A. tumefaciens* LSU20 strain was 37ºC, with initial pH of medium of 7, and glucose as a carbon source. In that condition the strain growing well with high desulfurization activity, generate a high growth rate 0,91 (OD_{660}) following the degradation rate of DBT 200 ppm of 76.9%.

Acknowledgments

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