# Decolorization of Reactive Black 5 using Soil Bacterial Consortium under a Combined Dynamic and Static Condition

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# การกำจัดสีรีแอคทีฟแบล็ค 5 ด้วยกลุ่มแบคทีเรียดินภายใต้สภาวะผสมแบบพลวัตและสถิต

นางดิตา ปุสปิตา ซาริ



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ดิตา ปุสปิตา ซาริ : การกำจัดสีรีแอคทีฟแบล็ค 5 ด้วยกลุ่มแบคทีเรียดินภายใต้สภาวะผสมแบบพลวัตและสถิต. ( Decolorization of Reactive Black 5 using Soil Bacterial Consortium under a Combined Dynamic and Static Condition) อ.ที่ปรึกษาหลัก : ดร.เสริมพงศ์ สายเรี่ยม, อ.ที่ปรึกษาร่วม : ดร.ภูมิศร์ ทับทิมแดง

งานวิจัยนี้ศึกษาการกำจัดสีรีแอ็คทีฟ แบล็ค 5 (RB-5) ด้วยกลุ่มแบคทีเรียดิน โดยมีวัตถุประสงค์เพื่อศึกษาผลกระทบของสภาวะการทดลองและศึกษาจลนพลศาสตร์ของการกำจัดสี RB-5 ด้วยแบคทีเรียสายพันธุ์เดี่ยวและกลุ่มแบคทีเรีย สายพันธุ์แบคทีเรียที่คัดแยกได้จากดินและใช้ในงานวิจัยนี้คือ *Micrococcus* sp., *Janibacter* sp. และ *Rhodococcus* sp. ดำเนินการทดลองโดยใช้ระยะเวลาการกำจัดสี 5 วัน โดยใช้น้ำเสียสังเคราะห์ สภาวะของการทดลองที่ศึกษา ได้แก่ สภาวะพลวัต (เขย่า) สภาวะสถิต (ไม่เขย่า) ปริมาณแบคทีเรีย ค่าพีเอชเริ่มต้น อุณหภูมิ และความเข้มข้นสีเริ่มต้น ผลการทดลองแสดงให้เห็นว่าการกำจัดสี RB-5 จำเป็นต้องใช้สภาวะผสมทั้งแบบพลวัตและสถิตเพื่อเพิ่มประสิทธิภาพ พบว่าแบคทีเรียสายพันธุ์เดี่ยวมีประสิทธิภาพการกำจัดสีสูงที่สุดที่พีเอช 7 และสภาวะเขย่า 2 วัน ต่อเนื่องด้วยสภาวะไม่เขย่า 3 วัน โดยแบคทีเรียที่มีประสิทธิภาพการกำจัดสีสูงที่สุดคือ *Janibacter* sp. (94.8% ที่ความเข้มข้น 100 มิลลิกรัม/ลิตร) และสามารถกำจัดสีได้ที่พีเอชสูงถึง 11 สำหรับการทดลองเพื่อศึกษาจลนพลศาสตร์นั้น พบว่าการกำจัดสีด้วยแบคทีเรียสายพันธุ์เดี่ยวเป็นไปตามปฏิกิริยาอันดับที่หนึ่ง (ความเข้มข้นสี 200 ถึง 1,000 มิลลิกรัมต่อลิตร) ถึงแม้ว่าการกำจัดสีย้อมด้วยกลุ่มแบคทีเรียจะไม่ได้มีประสิทธิภาพสูงที่สุด (88%) เมื่อเทียบกับการใช้แบคทีเรีย *Janibacter* sp. แต่การใช้กลุ่มแบคทีเรียนั้นสามารถลดเวลาของการเขย่าลงเหลือเพียง 1 วัน ในสภาวะพีเอชเท่ากับ 11 แสดงให้เห็นว่าการใช้กลุ่มแบคทีเรียเพื่อกำจัดสีในน้ำเสียนั้นสามารถลดเวลาการเติมอากาศลงได้ เหมาะแก่การนำไปประยุกต์ใช้บำบัดน้ำเสียโรงงานย้อมสิ่งทอซึ่งมีพีเอชอยู่ในช่วงเบสได้



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KEYWOR D: Decolorization, Reactive Black 5, Soil Bacterial Consortium



Dita Puspita Sari : Decolorization of Reactive Black 5 using Soil Bacterial Consortium under a Combined Dynamic and Static Condition. Advisor: Sermpong Sairiam, Ph.D. Co-advisor: Pumis Thuptimdang, Ph.D.



Decolorization of Reactive Black 5 (RB-5) dye by isolated soil bacterial consortium had been conducted in this research. This study aims to determine the effect of operating conditions and kinetics of RB-5 decolorization by single strains and the consortium. Bacterial strains capable of RB-5 decolorization were isolated and identified as *Micrococcus* sp., *Janibacter* sp. and *Rhodococcus* sp. The RB-5 synthetic wastewater was used in the experiments with five days of incubation time. The studied conditions include combinations of shaking (dynamic) and static period of incubation, bacterial concentration, initial pH, temperature, and initial dye concentration. The results showed that the combined dynamic and static condition was important to RB-5 decolorization by the isolated bacteria. The decolorization with highest efficiency for all cultures was obtained at pH 7 with 2-day shaking period continued with 3-day static period for single strains and 1-day continued with 4-day for the consortium. The highest efficiency was given by *Janibacter* sp. (94.8% at 100 mg/L), which exhibited a good performance up to pH 11. All the decolorizations of RB-5 by single strains followed the first-order kinetics (4/-5 concentrations of 200 to 1,000 mg/L) but this was not observed with the consortium. Even though the decolorization efficiency of the consortium (88%) was not as high as the standalone *Janibacter* sp., the shaking period could be reduced from 2 days to 1 day without losing high ability to decolorize at pH 11. This suggests that working with consortium could give a benefit of reducing the aeration period compared to working with single strain, which will be useful for wastewater treatment application.




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Dita Puspita Sari

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# CHAPTER 1
# INTRODUCTION

## 4.1 Background

Pollution in the surface water is often generated by the careless discharge of industrial wastewater, which pollutes the environment via its toxic components. Without a proper treatment, those toxic components may be harmful to any organism in the surface water and aquifer, and eventually become a serious threat to human health (Konig et al., 2017). One of the industries contributing to the rise of environmental pollution is the textile industry (Pandey et al., 2007).

Textile industry is well known for the huge amount of water required for its industrial processes including bleaching, dyeing, rinsing, washing, and finishing (Shaikh, 2009). Dyeing is the process that should become a main concern due to the dye released in the wastewater ranging from 10 up to 50% (Starling et al., 2017). Azo dyes are commonly used in the textile industry and accounted for 50% of all dyes produced (J.-S. Chang et al., 2001). Azo dyes are listed as the hazardous substance (de Souza et al., 2010) due to their stable and bulky structure, which makes them hard to be degraded by light, water and chemicals (Wang et al., 2013). The International Agency for Research on Cancer (IARC) had classified several azo dyes and their intermediates into Group 1, 2A or 2B, which respectively are defined as: as known, probable, and possible human carcinogens (Brantom, 2005). Moreover, once azo dyes are degraded, they have an ability to generate carcinogenic byproducts such as the colorless aromatic amines (Husain, 2006).



Reactive Black 5 (RB-5) is a common dye used in the textile finishing process (Deive et al., 2010), which could possibly become the main compound in the dye wastewater. Gottlieb et al. (2003) reported that the parent structure of RB-5 has the EC50 of 27.59±4.01 mg/L. Surprisingly, the EC50 value of 11.49±3.68 mg/L was higher for the hydrolyzed form of RB-5, which is the form of RB-5 found in the dyeing process. RB-5 with the concentration of 100 ppm was reported to have 100% toxicity on Daphnia magna (Meriç et al., 2004). This concentration then becomes the main concern for treatment.

Studies have been conducted to find the suitable methods for removing the azo dye and its color in wastewater. Table 1 summarizes the pros and cons of physical and chemical methods (Pearce, 2003) compared with biological methods (Husain, 2006); (Kornaros et al., 2006). Three main methods based on physical, chemical and biological technologies to treat the azo dye-containing wastewater have their own advantages and disadvantages. According to Table 1, physical and chemical methods are often stated as the costly method that produces the large amount of sludge; therefore, biological methods are preferable in terms of the cost effectiveness and possibility to undergo a complete mineralization. The biodegradation of azo dye often utilizes microorganisms from environment based on the fact that environment could be the good source of bacteria, both the culturable and unculturable ones. Soil bacteria have been known to effectively degrade environmental pollutants such as polychlorinated biphenyl (Sierra et al., 2003) and pentachlorophenol (Khessairi et al., 2014); likewise, some soil bacteria should possess the capability to degrade azo dyes.

**Table 1.** The Overview of Wastewater Treatments

| Treatment methods | Advantages | Disadvantages |
|---|---|---|
| Coagulation-flocculation | Inexpensive process | Large amount of sludge production |
| Adsorption | Removal of wide range of dye types | Regeneration or disposal requirement of adsorbent |
| Oxidation | Fast process | Costly and formed byproducts |
| Membrane | Suitable for any types of dyes | Production of sludge with high concentration |
| Decolorization by microorganisms | Complete mineralization of dye | Detoxification requirement |
| Trickling filters | Feasible process | Limited time available to do the process due to non-sterile bioreactors |

The biodegradation of azo dye using bacteria has been widely applied and the results seem promising. (Deive et al., 2010) worked with thermophilic bacteria isolated from mud and water of geothermal sites in Spain and obtained 80% decolorization of RB-5 azo dye in 24 hours under aerobic condition. Under saline environment, Khalid et al. (2012) the isolated bacteria from Arabian seawater sediment that capable of decolorizing RB-5 around 80% in 48 hours. (Franciscon et al., 2012) had successfully utilized the strain from activated sludge of textile wastewater treatment plant in Brazil to decolorize and reduce the toxicity of azo dyes, including RB-5, under static condition followed by aeration. The decolorization reached as high as 99% in two weeks of incubation time. Similarly, (Wang et al., 2013) worked with soil bacteria isolated from textile factory that was able to tolerate RB-5 dye concentration up to 500 mg/L with the decolorization ability around 95% in 120 hours. Another research from Khan et al. (2015) showed that the isolated dye degrading bacteria from wastewater and soil around textile industrial area in India achieved 93% decolorization in 120 hours.

Among all the successful research utilizing a single bacterial strain, several studies conducted the decolorization with bacterial consortium. The decolorization with bacterial consortium provided an enhancement of degradation rate (Saratale et al., 2009). Another advantage of bacterial consortium is the possible synergistic work among the bacteria composing the consortium, leading to more complete degradation (Dejonghe et al., 2003). Based on the natural condition that many species of bacteria in the environment live and work together, the bacterial consortium would be more adaptable to the real wastewater treatment.

This study aims to isolate soil bacteria capable of decolorizing RB-5 dye and construct a bacterial consortium to enhance the decolorization efficiency. Subsequently, the effect of environmental conditions of RB-5 decolorization is studied, and the kinetics of the decolorization is determined.

## 4.2 Objectives

1. To determine the effect of environmental conditions on the decolorization of RB-5 by the isolated soil indigenous bacteria and the consortium.
2. To study the kinetics of RB-5 decolorization.

## 4.3 Hypotheses

1. Highest RB-5 decolorization by the isolated native soil bacteria is achieved under the combined dynamic and static conditions.
2. RB-5 decolorization by the isolated bacteria follows the first-order kinetics.

## 4.4 Scope of the study

This research focused on the isolation of soil bacteria and biodegradation of RB-5 in laboratory-scale experiments. The RB-5 synthetic wastewater was used. The studied environmental conditions include combinations of shaking and static periods of incubation, bacterial concentration, initial pH, temperature, and initial dye concentration.

## 4.5 Expected outcomes

1. Bacterial strains capable of decolorizing RB-5 under various environmental conditions.
2. The constructed consortium from the bacterial strains, which can be further developed for RB-5 wastewater treatment.

# CHAPTER 2
# THEORETICAL BACKGROUND AND LITERATURE REVIEW

## 2.1 Reactive Black 5 dye

Reactive Black 5, also known as Remazol Black B (CASRN 17095-24-8), is a dye containing diazo groups as one of its chromophore (Pearce, 2003). As shown in Figure 1, RB-5 can be divided into several components as follows:

1. Chromogen

   Chromogen is a compound structure that is being colored by the specific substituent attached, which are the benzene rings in RB-5.

2. Chromophore

   Chromophore is a chemical group responsible for coloring the dye. The azo group (N=N) act as the chromophore in RB-5

3. Solubilizing group

   Solubilizing group, which is the sulfonate group in RB-5, is a chemical group responsible for turning the dye into more water soluble.

4. Reactive site

   Reactive site consists of a compound that reacts with fibre or cellulose in the dyeing process. Reactive site consists of electron poor substituent ready to be attacked by nucleophilic group of cellulose. In RB-5, the reactive site is the alkane between sulfate and sulfonate salt as illustrated in Figure 1.

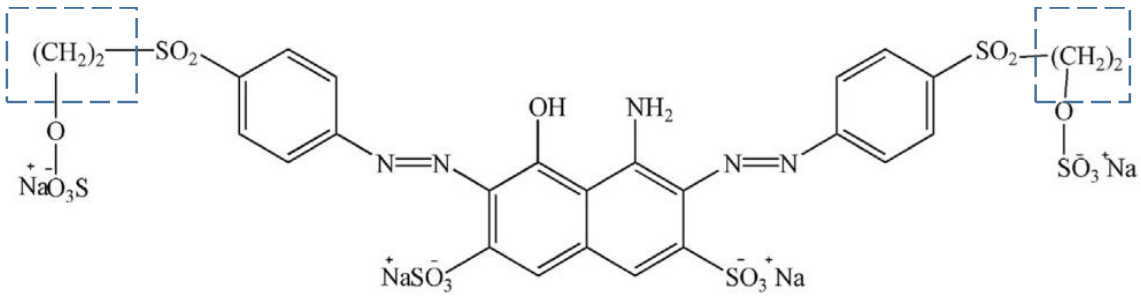



**Figure 1.** Reactive site of Reactive Black 5 dye
Source :El Bouraie et al. (2016).

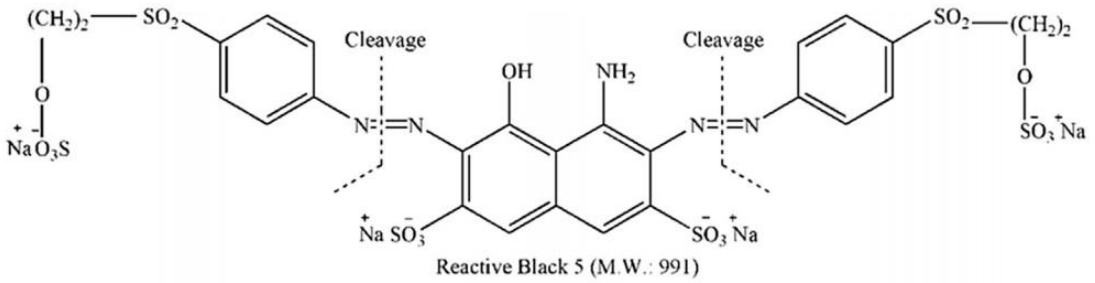



**Figure 2.** Proposed initial azoreduction process of Reactive Black 5 dye
Source: El Bouraie et al. (2016).

The degradation for azo colorants is called decolorization. The process occurred in the decolorization is known as azoreduction. In the azoreduction process, the azo bond, which consists of two nitrogen atoms connected with double bond, is broken (Figure 2). As a result, the color of a dye disappears.

Generally, azo dyes have very low vapor pressure in the range of $8.3 \times 10^{-18}$ up to $1.2 \times 10^{-5}$ Pa (Baughman et al., 1988 ). Reactive dyes are included in the group of soluble dyes with solubility more than 100 mg/L (Ollgaard et al., 1998). The low value of vapor pressure and high value of solubility indicated that RB-5 could possibly be widespread once it contaminated the environment, since this chemical could be very hard to be eliminated by the nature vaporization into gas form.



The toxicity of RB-5 could be expressed with an $EC_{50}$ value (median effective concentration), a value at which the RB-5 concentration causing 50% reduction in algae growth rate or immobilization in *Daphnia magna*. The $EC_{50}$ value of RB-5 as the parent compound was 27.59±4.01 mg/L, and it decreased slightly by a hydrolysis process to 11.49±3.68 mg/L (Gottlieb et al., 2003), which means the toxicity of the hydrolyzed RB-5 became greater. In the same research, (Gottlieb et al., 2003) also examined the effect of decolorization in the toxicity and it was conducted anaerobically by utilizing *Enterococcus faecalis* and *Clostridium butyricum.* Surprisingly, the toxicity became even higher than the hydrolyzed RB-5 that didn't undergo the decolorization, which were 0.2±0.03 mg/L for decolorization with *E. faecalis* and 0.3±0.01 mg/L with *C. butyricum.*

RB-5 as the part of azo dyes could contaminate river and groundwater in the surrounding envionment of dyeing industries (Riu et al., 1998). As RB-5 contains naphtalene in its formula, it can be accumulated in *Glichthus mirabilis* and *Fundulus similes* (Puvaneswari et al., 2006). Due to the toxicity of RB-5 metabolites and the possible bioaccumulation by organisms in the food chain, there should be a serious concern of eliminating this dye from wastewater before discharging it into the environment.

### 2.2 Physical and chemical treatments of RB-5

Physical treatment of RB-5 is mainly by the adsorption of the dye into the adsorbent such as powdered activated carbon (PAC) and fly ash (Eren et al., 2006). The PAC, compared to fly ash, is more expensive and requires regeneration, which become the disadvantages of physical decolorization method even though the result showed that PAC had seven times higher adsorption capacity than fly ash (Eren et al., 2006). The initial RB-5 concentration was the main concern due to the adsorption efficiency of the adsorbents. Both PAC and fly ash reached lower adsorption efficiency as the initial dye concentration increased. Inversely, the adsorption capacity of both adsorbents increased by the enhancement of the initial dye concentration (Eren et al., 2006).

Color removal of RB-5 by the chemical method is commonly from using an oxidizing agent such as hydroxyl radical formed from hydrogen peroxide and ferrous ions in the Fenton's oxidation (FO) process (Meriç et al., 2004). Temperature is one of the essential parameters in the decolorization by FO process where increasing the temperature up to 40°C could increase both COD and color removals, which were 71% and 99% respectively, and further temperature enhancement could decrease both removals due to the flocs' destabilization (Meriç et al., 2004). Another essential parameter is the mixing time, where the prolonged mixing time by only 10 minutes could decrease the COD removals due to the flocs' destabilization (Meriç et al., 2004). The FO process works better at pH lower than 3.5 due to the stable form of ferrous ions. As a result, the redox system with hydrogen peroxide is more stable and the decolorization process initiated by hydroxyl radical is efficient. Even though the organic matters in the wastewater can possibly undergo oxidation to the end products,

FO process is not widely applicable due to the formation of sludge containing high concentration of Fe (III), which have to be further managed before disposal.

The research utilizing a combination between physical and chemical color removal, which combined coagulation, adsorption, and membrane, has been conducted (Lee et al., 2006). In that research, complete RB-5 decolorization of 250 mg/L concentration was achieved within 5 hours of decolorization with the dose of coagulant and adsorbent as much as 250 mg/L and 252 mg/L respectively (Lee et al., 2006). The membrane acted as a separator of PAC particles from the water, possibly making the reusable water after treatment (Lee et al., 2006). Even though the chemical treatment of RB-5 seems promising, but from the cost perspective, the method can be an expensive option especially for the small and developing factories.

### 2.3 Biodegradation of azo dyes

Biological degradation or biodegradation is the breakdown of any substance by utilizing microorganisms or other biological methods. In biodegradation of a pollutant, the microorganisms use the target pollutant as the carbon source or additional substrates other than the target substance itself. The microorganisms that require additional substrates as the carbon source is known to perform the cometabolic reductive cleavage, which is the mechanism of enzyme production to reductively cleaved the target pollutant as they grow in the additional carbon source (Stolz, 2001). RB-5 is one of the substances that can hardly act as the sole carbon source for any microorganism in the biodegradation process according to previous studies, which requires alternate substrates as the nutrient for the microorganisms (El Bouraie et al., 2016 ; Khan et al., 2015; Wang et al., 2013). Biodegradation of azo dyes can be carried out in the presence of oxygen, without oxygen, and or anoxic condition.

#### 2.3.1 Aerobic decolorization

Aerobic decolorization by bacteria have been widely explored. Bacteria that aerobically decolorize dye tend to require an organic carbon source for growth (Stolz, 2001). This is because the presence of oxygen would be a strong competitor to the azo compounds

to receive the electron from reduced electron carriers, thus the azo compound cannot undergo any breakdown and the microorganism could not be able to digest the structure as the carbon source. Aerobic bacteria that decolorize RB-5 can utilize various carbon sources to grow such as trypticase and yeast extract (Deive et al., 2010), or glucose and pyruvate (Franciscon et al., 2012). Several studies used rich media such as Nutrient Broth for growing aerobic bacteria and for being a carbon source during the decolorization process (Khan et al., 2015; Wang et al., 2013).

Aerobic bacteria use oxidative and reductive enzymes that possibly symmetrically or asymmetrically break the dye molecule or carry out desulfonation, deamination, or hydroxylation (Khandare et al., 2015). This becomes an advantage of aerobic decolorization due to the fact that deamination can eliminate carcinogenic aromatic amines.

(Kalyani et al., 2009) proposed the aerobic degradation pathway of Reactive Red 2 (RR-2) due to the peroxidase activity of the isolated *Pseudomonas* sp. SUK1 as shown in Figure 3. In this degradation pathway, the decolorization of RR-2 dye was initiated with the asymmetric cleavage of the heterocycle compound in the RR-2 structure instead of azo bond cleavage. The azo bond cleavage took place in the second step, which also was the reduction step to stabilize the negative charges in the nitrogen atom to be the amino group. The two last steps were desulfonation and deamination that changed the acid group into alcohol group.

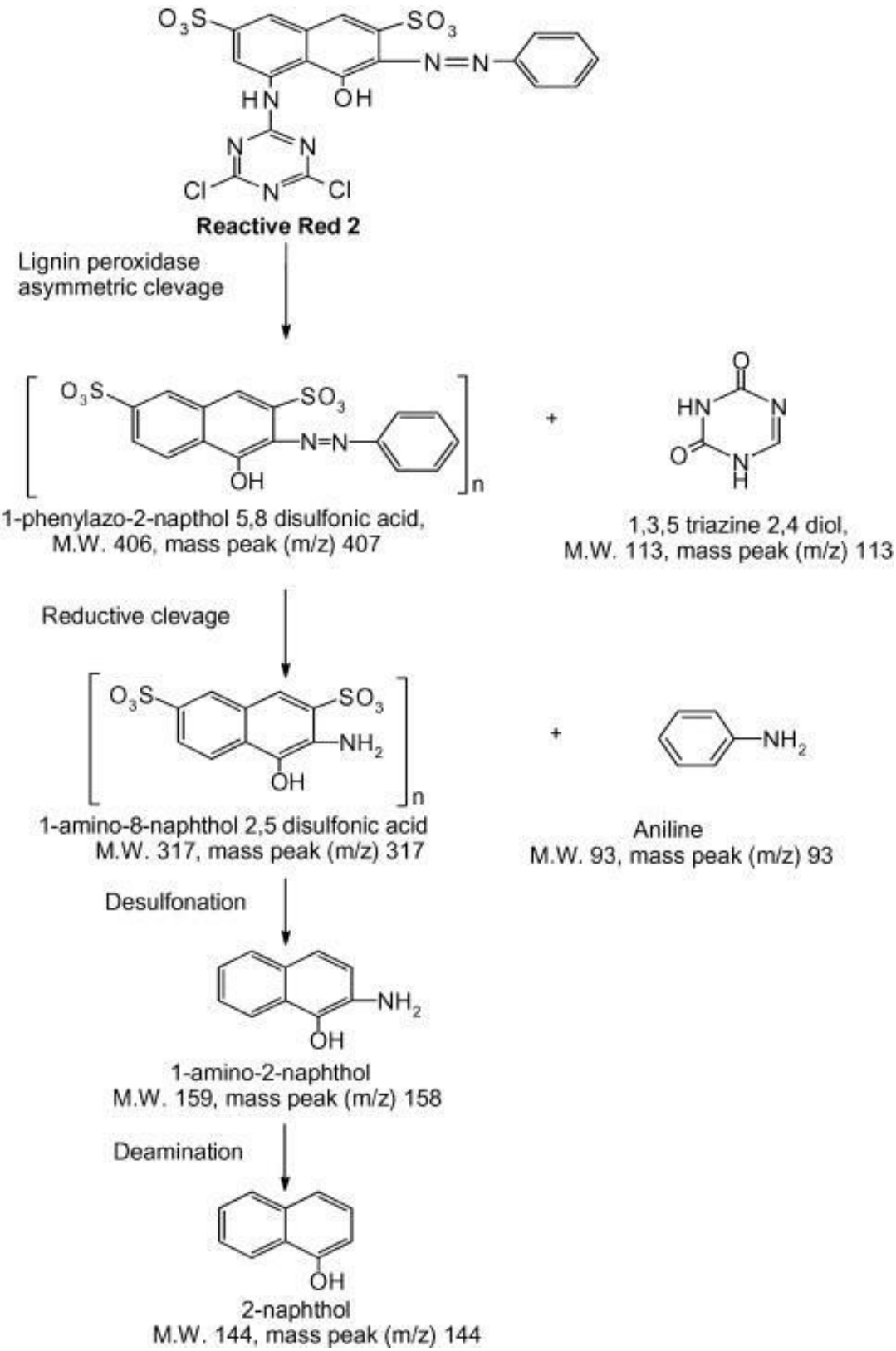



**Figure 3.** Proposed aerobic degradation pathway of RR-2 dye
Source: Kalyani et al., (2009).

### 2.3.2 Anaerobic decolorization

The main role of anaerobic decolorization is held by azoreductase, which breaks the azo bonds in the dye structure (Khandare et al., 2015). This reduction occurs at varied groups of azo compounds, showing nonspecific reactions. The decolorization rate in anaerobic condition depends on dye structure and type of organic carbon source, but it does not have any correlation with molecular weight of the dye, which is another proof that the decolorization is a nonspecific reaction (Pandey et al., 2007). As an example, mostly sulfonate group is attached to the azo dye structure, which resulted in high molecular weight and the limited molecular passage through the cell membrane of bacteria. However, this condition is compensated by the intracellular uptake of the dye that occurred with the formation of linkage between bacteria's intracellular electron transport system with the dye molecules (Myers et al., 1992).

Figure 4 shows the anaerobic biodegradation pathway of general azo dye. The anaerobic azoreduction is known as a redox mediator dependent reduction (Pearce, 2003). The dehydrogenase enzyme is synthesized in the cytoplasm and secreted out of the cell (Bragger et al., 1997), which then acts as the redox mediator to supply electrons into the enzymatic reaction involving azoreductase (Yoo et al., 2001). As being stated above, the azoreductase plays the main role in anaerobic decolorization with the azo bond cleavage as the primary step. The overpass concept about anaerobic decolorization was due to the activity of methanogens (Razo et al., 1997), but further research showed that the acidogenic bacteria play that role as well (Bras et al., 2001).

The main concern of anaerobic decolorization, which becomes the disadvantage of this method, is the formation of carcinogenic aromatic amines (Khandare et al., 2015). The aromatic amines that are the potential products of azo bond cleavage were divided into two chemical categories: aromatic azo-based substances (containing at least one aromatic compound with at least one of its substituents having azo bond) and benzidine-based substances.

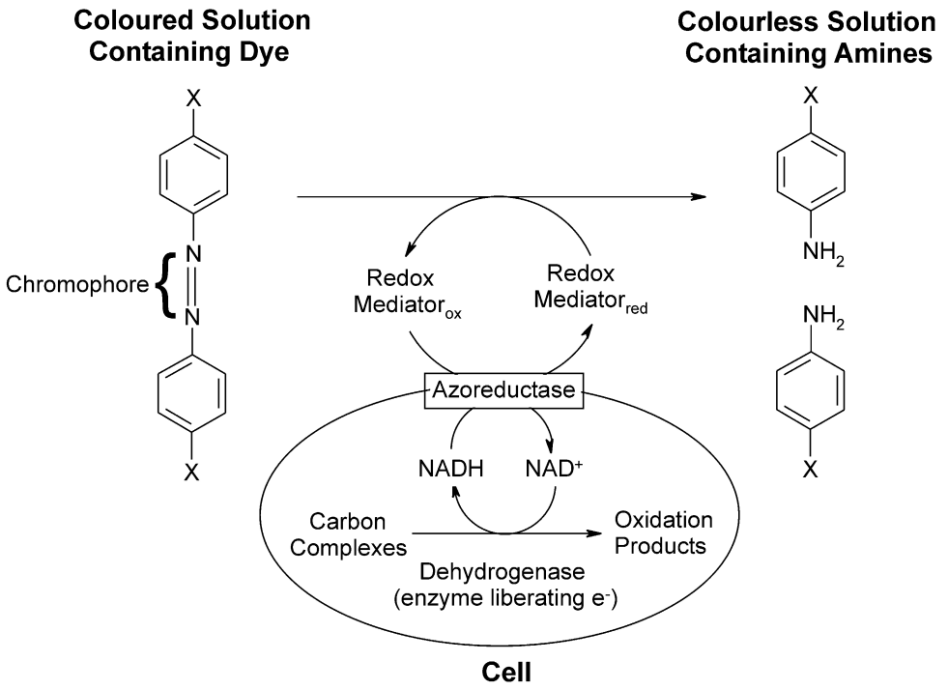



**Figure 4.** Proposed mechanism of anaerobic degradation of azo dye
Source: Keck et al. (1997).

### 2.3.3 Anoxic decolorization

Anoxic condition is similar to the anaerobic condition, but it consists of the limited amount of oxygen in the system, which is less than 0.5 ppm of dissolved oxygen (Khandare et al., 2015). Varied complex organic sources are required in the anoxic condition depending on the bacterial strain (Pandey et al., 2007). Research showed that anoxic decolorization has a low effectiveness but is still practical for azo dye removal from wastewater (Gottlieb et al., 2003). The aeration and agitation could inhibit the color removal of either Reactive Red 198 or Reactive Black 5 by *Aeromonas hydrophila*, thus the anoxic static condition was preferred to conduct the decolorization (Chen et al., 2003). The switched condition into static anoxic could increase the decolorization efficiency more than 90% within 8 days incubation at the initial dye concentration less than 3000 mg/L and the working temperature was 30°C with neutral pH (Chen et al., 2003).

### 2.3.4 Decolorization by bacterial consortium

The defined consortia are often constructed for the purpose of decolorization rate enhancement. As reported in Saratale et al. (2009), a developed consortium composed of *Proteus vulgaris* and *Micrococcus glutamicus* showed significantly higher decolorization rate of various dyes than the individual strains themselves. The consortium required 3 hours to achieve complete decolorization of Scarlet R while the individual bacteria composing the consortium, which were *P. vulgaris* and *M. glutamicus*, required 14 hours and 20 hours, respectively.

In addition to the rate enhancement, phytotoxicity test on the extracted metabolite after underwent complete decolorization with bacterial consortium provided the result of a good germination rate, suggesting that the detoxification could be another benefit of working with bacterial consortium (Saratale et al., 2009).

Another research on decolorization by bacterial consortium used the naturally built consortia from the textile industry sludge samples (Tony et al., 2009). By utilizing these types of consortium, an advantage of natural adaptation in the toxic dye environment (Chen et al., 2003) that had been passed by the consortium could increase the decolorization efficiency as it depends on the enzyme's activity and ability to adapt produced by the microorganisms in the consortium (Senan et al., 2004). The result showed that between two consortia, named SKB-I and SKB-II, applied in the synthetic wastewater consisted of either individual dye or mixed dyes, SKB-II consortium gave a better decolorization performance with the maximum decolorization of 94.8% with initial Congo red's concentration of 10 mg/L within 120 hours (Tony et al., 2009). The 16S rDNA analysis revealed five bacteria composing the SKB-II consortium as follows: *B. vallismortis*, *B. pumilus*, *B. cereus*, *B. subtilis* and *B. megaterium*, which were then individually utilized to decolorize various dyes. The range of Congo red (10 mg/L) decolorized by individual strain was 72.2 up to 92.6 %, which were lower than the decolorization result from the consortium. In conclusion, the bacterial consortium could increase the probability to achieve a better decolorization since the consortium composed of diverse microbes is able to degrade many types of pollutants (Watanabe et al., 2000).

## 2.4 Environmental factors affecting RB-5 biodegradation

A wide range of bacteria with RB-5 decolorization ability has been isolated. Bacteria isolated either from a textile processing plant (Wang et al., 2013) or an extreme sampling site (Deive et al., 2010) gave various maximum decolorization percentages based on their optimum condition, which means these bacteria worked under a certain condition in order to achieve the desired decolorization percentage within a specific incubation time. Generally, factors affecting decolorization during incubation time are oxygen, initial dye concentration, temperature, and pH.

### 2.4.1 Oxygen

The presence of oxygen in the system affects the growth of bacterial cells. Aerobic bacteria definitely require oxygen for their growth, and vice versa for the anaerobic bacteria. However, the presence of abundant oxygen itself could inhibit the color removal mechanism due to the availability of the electron acceptor with high redox potential (J. S. Chang et al., 2004).

The decolorization of RB-5 is often conducted in the static (Khalid et al., 2012); (Wang et al., 2013) and slowly aerated conditions (Deive et al., 2010) to limit the oxygen in the system. Different decolorization percentage could be observed between the limited oxygen and rapidly shaking conditions, which was up to 60% lower decolorization (Wang et al., 2013).



### 2.4.2 Initial dye concentration

The toxicity of dye and also possible additional toxicity from the dye degradation products could affect the survival ability of bacteria. Research on RB-5 dye decolorization showed that the decolorization percentage declined gradually by increasing the initial RB-5 concentration in the system. In the system with 50 ppm RB-5, *Pseudomonas entomophila* BS1 could decolorize as much as 90% compared to 80% in the system with 500 ppm RB-5 (Khan et al., 2015). Another study showed that double incubation time was needed to decolorize 300 ppm compared to 50 ppm of RB-5 (Wang et al., 2013).

### 2.4.3 Temperature

Temperature is regarded as an important factor for bacterial growth. Each bacterial strain has a different optimum temperature for its growth. Outside the optimum temperature, the bacterial cells could lose their viability. Temperature is also important for the working of enzyme produced by bacteria, including the enzyme for color removal process. Enzyme that contains protein as the building blocks could be denatured at too high temperature as the covalent and hydrogen bonds conforming its shape might be broken from the substrate collision with active sites at higher frequency (Reece et al., 2010). Results on the RB-5 decolorization research showed that the optimum temperature between 35°C to 40°C for different bacterial strains: 35°C for *Aeromonas hydrophila* (El Bouraie et al., 2016), 37°C for *Pseudomonas entomophila* BS1 (Khan et al., 2015), and 40°C for *Bacillus* sp. YZU1 (Wang et al., 2013).

### 2.4.4 pH

The decolorization performance could be enhanced by adjusting the pH of either the liquid medium or the dye wastewater itself. The pH value affects the protein structure of the enzyme that could lead to a change in its shape under too acidic or too alkaline condition. The excess or lack of hydrogen ions interacting with amino acids could alter its degree of ionization, which is important for binding the specific substrate (Lehninger, 1982). Research showed that the neutral up to slightly alkaline pH was the suitable pH for RB-5 decolorization, which ranges from 5 to 9 depending on the bacteria that produce different types of enzyme to decolorize RB-5 as each of them has different optimum working pH (El Bouraie et al., 2016 ; Khan et al., 2015; Wang et al., 2013).



### 2.4.5 Detoxification of azo dye biodegradation products

As one of the azo colorants, RB-5 is biologically active through its metabolites (Golka et al., 2004). Colorless aromatic amine is one of the metabolites that often being a concern due to its carcinogenicity (Husain, 2006). Previous research reported that the aromatic amines found in the extracted metabolite of RB-5 were the derivatives of benzene and naphtalene (El Bouraie et al., 2016 ; Khan et al., 2015; Wang et al., 2013). Figure 5 showed the proposed degradation pathway of RB-5 by *Aeromonas hydrophila* that formed several types of aromatic amines after undergoing decolorization process

(El Bouraie et al., 2016) but no further study was conducted to determine the toxicity of these biodegradation products.

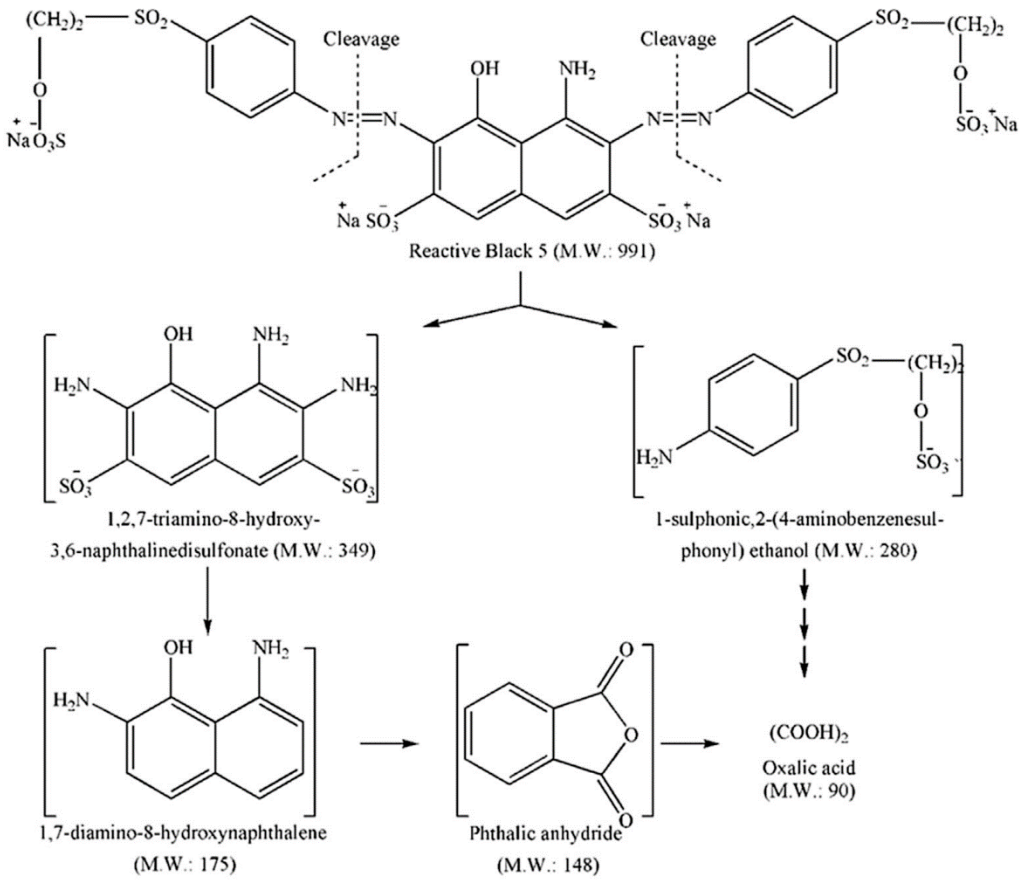



**Figure 5.** RB-5 decolorization mechanism by *Aeromonas hydrophila*
Source: El Bouraie and El Din (2016).



Decolorization of azo dyes not always resulted in the detoxified metabolite product. (Gottlieb et al., 2003) compared the toxicity values of RB-5, Procion Navy, Procion Yellow and Procion Crimson as their parent compounds, hydrolyzed forms and both of them after decolorization process. The decolorization of these dyes were conducted anaerobically by *Enterococcus faecalis* and *Clostridium butyricum*. The results (Table 2) suggested that decolorization of either parent compound or hydrolysed azo dyes could increase the toxicity of the dyes as the $EC_{50}$ values decreased. Therefore, this research put a concern on the toxicity of the RB-5 degradation products.

Since RB-5 has sulfonic acid groups, this dye along with its metabolites are water soluble. Thus, the generated colorless aromatic amine has a possibility for exposure through inhalation or skin in case of water contamination occurred. Various biomonitoring studies have been conducted to measure the toxicity of the metabolites in the water. Among the organisms used for the toxicity study, *D. magna* is the most common one especially for testing the liquid chemical due to the convenient handling procedure (Adema, 1978), low cost and less space needed. Previous study showed correlation between metals' acute toxicity tested with *D. magna* and the result on $LD_{50}$ tested on mammals (Khangarot et al., 1988).

**Table 2.** The toxicity values of azo dyes

| **Azo dyes** | **Treatments** | **$EC_{50}$ (mg/L)** | |
|---|---|---|---|
| | | **Parent** | **Hydrolyzed** |
| Reactive Black 5 | Control | 27.5 ± 4.01 | 11.4 ± 3.68 |
| | *E. faecalis* | 0.7 ± 0.09 | 0.2 ± 0.03 |
| | *C. butyricum* | 2.2 ± 0.71 | 0.3 ± 0.01 |
| Procion Navy | Control | 18.9 ± 5.65 | 27.9 ± 3.28 |
| | *E. faecalis* | 0.5 ± 0.17 | 0.5 ± 0.07 |
| | *C. butyricum* | 9.1 ± 0.53 | 8.6 ± 1.19 |
| Procion Yellow | Control | 71.0 ± 6.00 | 66.4 ± 0.93 |
| | *E. faecalis* | 0.8 ± 0.17 | 0.5 ± 0.28 |
| | *C. butyricum* | 9.1 ± 1.04 | 5.6 ± 0.89 |
| Procion Crimson | Control | 34.7 ± 0.27 | 37.7 ± 1.72 |
| | *E. faecalis* | 19.9 ± 0.10 | 18.2 ± 2.3 |
| | *C. butyricum* | 8.1 ± 0.66 | 9.1 ± 1.04 |

Source: Gottlieb et al. (2003).



### 2.4.6 Isolated bacteria capable of RB-5 decolorization

In this research, six bacterial strains that showed the ability to degrade RB-5 were isolated from soil. These strains have also been reported to be the environmental pollutants' degrader, which are as follows:

1. *Gordonia terrae*

   *G. terrae* has been reported on its ability to degrade dibenzothiophene (DBT) with the desulfurization (cleavage of C–S bond) pathway (Akhtar et al., 2016). Theoretically, this strain should possess the ability to degrade RB-5 with the clear proposed mechanism, but several reports on the pathogenicity of *G. terrae* (Blanc et al., 2007; Grisold et al., 2007) suggest a concern for application of this strain.

2. *Rhodococcus* sp.

   Different *Rhodococcus* sp. strains have been widely known for polychlorinated biphenyls (PCB) degradation (Yang et al., 2011). The strain of R04 was isolated from oil contaminated soil and proven to degrade PCB via ring cleavage and dechlorination (Yang et al., 2011). The latest research (Vignali et al., 2018) utilized the lignin peroxidase produced by *Rhodococcus jostii*, which showed the improvement of dye-decolorizing activity by the addition of $Mn^{2+}$, yielding more than 95% decolorization of Remazol Brilliant Blue R and also 11% and 22% decolorization of Azure B and RB-5, respectively.

3. *Bacillus flexus*

   *B. flexus* was known as an alkaliphilic bacteria (Sanchez-Gonzalez et al., 2011) with the optimum growth rate at basic pH, and this strain was able to synthesize active enzymes under extreme pH conditions. The strain of XJU-4 was reported to utilize 3-nitrobenzoate as the sole carbon source (Mulla, Manjunatha, et al., 2011). *B. flexus*, worked together with other *Bacillus* species as a consortium, was reported to degrade Direct Red 31 and Direct Blue 151 with the efficiency more than 90% at the concentration of 300 mg/L for five days incubation (Lalnunhlimi et al., 2016).

4. *Microbacterium* sp.

   *Microbacterium* sp. secreted dehalogenase, dehydrogenase, dehydro-chlorinase and hydrolase as the responsible enzymes for biodegradation of the pesticide isoxaben (Umadevi et al., 2017). This strain also produced bioemulsifier that

could remove the residue of Cd and Zn in the industrial wastewater (Aniszewski et al., 2010). The strain of B12 was reported to decolorize Reactive Blue 160 with the work of azoreductase, NADH-DCIP reductase, and laccase (Roat et al., 2016). The best condition was achieved at pH 5 with the efficiency of 94% for 96 hours of incubation time (Roat et al., 2016). Although this strain looks promising for performing decolorization, some species such as *Microbacterium nematophilum* has been reported for its pathogenicity on the nematode *Caenorhabditis elegans* (Parsons et al., 2014).

5. *Micrococcus* sp.

   *Micrococcus* sp. has been widely known to degrade many types of environmental pollutants. This bacterium could utilize 2-nitrotoluene as sole source of carbon and energy (Mulla, Hoskeri, et al., 2011). As a consortium, *Micrococcus* sp. along with other types of bacteria could degrade and use several hydrocarbons as the substrates such as toluene, o-xylene, n-tetradecane, octanol, or even the crude oil itself (Roy et al., 2002). Malachite Green (MG), a type of biocide widely used in aquaculture as well as a common dye used in textile industry, was successfully degraded by *Micrococcus* sp. with laccase and NADH-DCIP reductase as the enzymes responsible for the degradation (Du *et al*., 2013).



6. *Janibacter* sp.

   *Janibacter* sp. were reported to effectively degrade two types of persistent pollutants: pentachlorophenol (PCP) and polychlorinated biphenyl (PCB) (Khessairi et al., 2014 ; Sierra et al., 2003). During the biodegradation of PCP, *Janibacter* sp. could adapt with the salinity fluctuation at less than 10% (Khessairi et al., 2014 ). *Janibacter* sp. had a high capacity to degrade biphenyls in liquid medium but when the biphenyls were trapped in the soil, it could only degrade the small amount due to the less bioavailability of the compound (Sierra et al., 2003). So far, there are no report on this strain for its dye decolorization performance.

# CHAPTER 3
# METHODOLOGY

## 3.1 Research framework

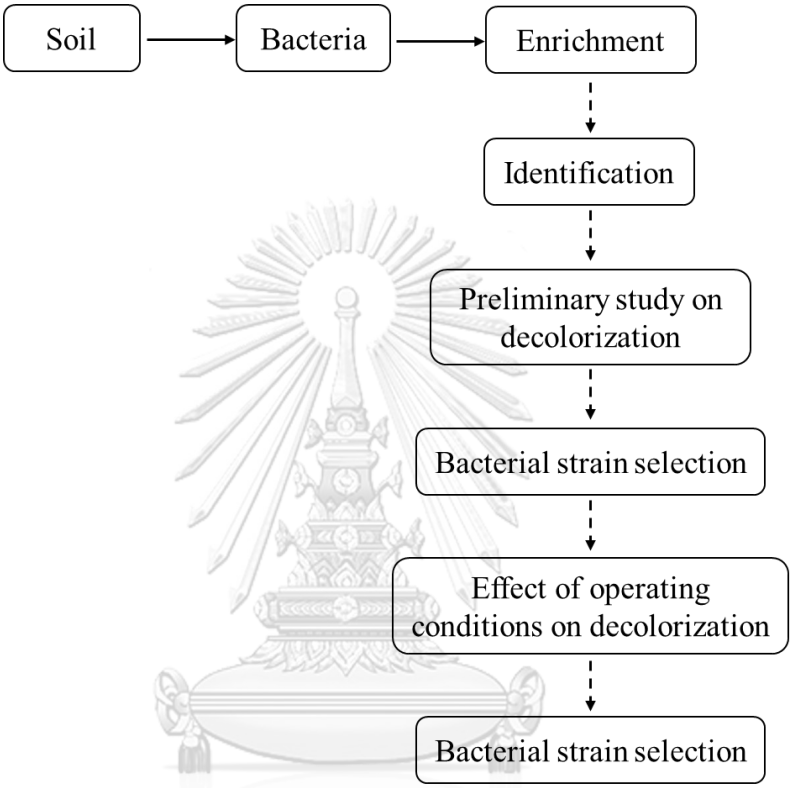



**Figure 6.** The research framework.



This research is divided into four parts: bacterial isolation, preliminary study of decolorization, decolorization experiment, and study of the kinetics. More detailed steps of the research are provided in Figure 6. The first part, bacterial isolation, covers the area of bacterial screening by enriching bacteria with the presence of RB-5 before isolating and identifying the strains able to decolorize the dye using 16S rRNA analysis. In the second part, preliminary study is conducted to confirm and determine the minimum requirements for RB-5 decolorization conditions. The strains showing RB-5 decolorization were selected, and the consortium from the selected strains was constructed. The third part is the study of RB-5 decolorization under different incubation and environmental conditions to ensure the possible application of the

strains. The last part of this research is the kinetic study of the RB-5 decolorization process.

## 3.2 Research methods

### 3.2.1 Bacterial screening

#### 3.2.1.1 Bacterial isolation and enrichment

Soil from Chulalongkorn University area (Figure 7) was sampled with the depth of 20 cm below the topsoil (Wang et al., 2013) and placed in the sterile conical tube. After adding 0.9% of NaCl solution to 10 g of soil with the total volume of 100 mL, the soil suspension was undergoing serial dilution up to $10^{10}$. One milliliter of the soil suspension from each dilution was inoculated in 100 mL nutrient broth (NB) amended with 100 mg/L of RB-5 dye (DyStar Thai Ltd.) under static condition at room temperature. The NB media as complex media was used because the complex media has a high possibility to provide the growth of the unknown bacteria (Todar, 2006) (Todar, 2012). The RB-5 concentration of 100 mg/L was used due to the toxicity of RB-5 that was reported to have 100% toxicity on *Daphnia magna* (Meriç et al., 2004 ). The cultures were being transferred (1 mL) right after the color of the solution changing from black to yellow, suggesting the degradation of RB-5. The transferring processes were conducted continuously for two weeks to enrich the bacteria with RB-5 degrading ability.

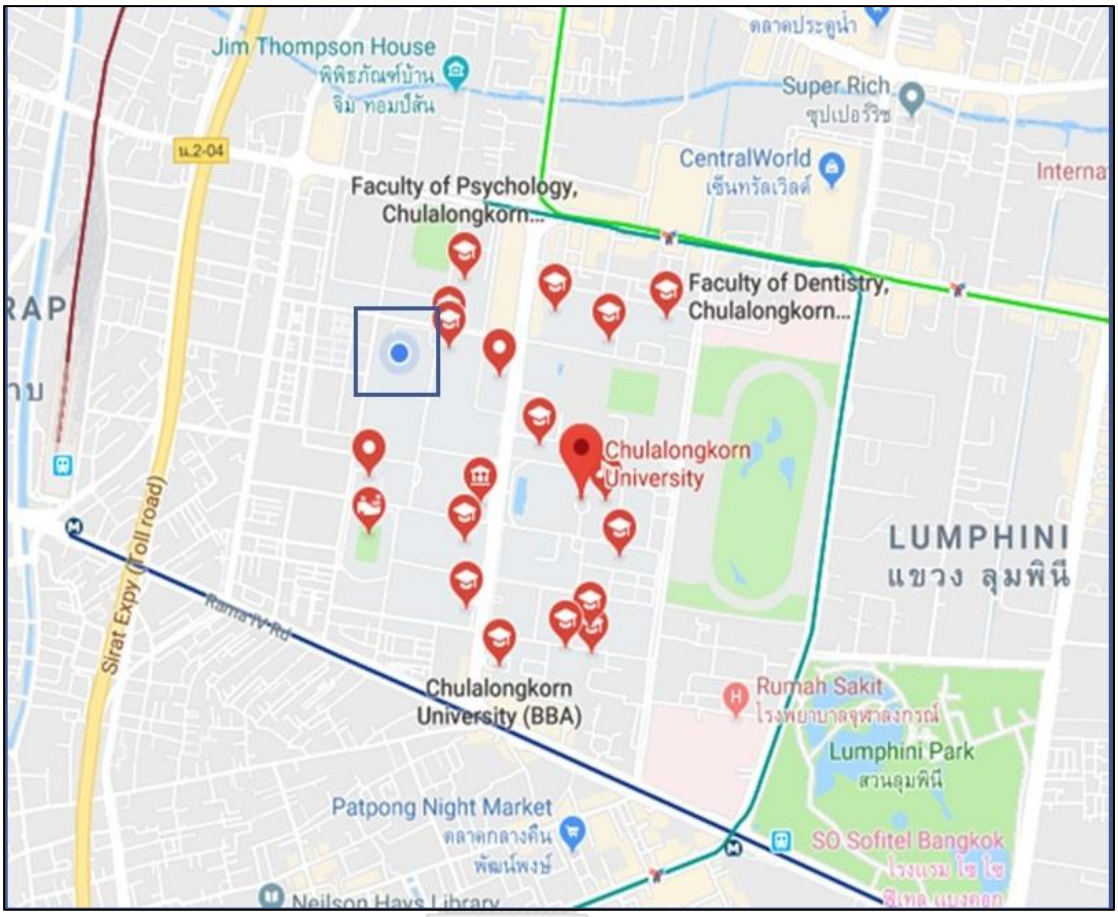



**Figure 7.** Soil sampling area for bacterial isolation.
Source: Google Map (2019)



#### 3.2.1.2 Bacterial purification and identification

Cultures with good performance of decolorization were being plated in nutrient agar amended with 100 mg/L of RB-5. Each type of colonies grown in the plate with the clear zone was streaked in a new plate with the same composition continuously until the pure culture is obtained. Then, pure bacterial cultures were grown in liquid NB containing RB-5 to confirm the decolorization ability. Bacteria that completely reduced the color of RB-5 were identified for their species by 16S rRNA analysis (Macrogen Corp.).

### 3.2.2 Preliminary study on RB-5 decolorization

The decolorization efficiency for each culture was primarily studied to determine whether the aeration is required for RB-5 decolorization. Bacteria were grown in 100

mL of NB (Wang et al., 2013) amended with 100 mg/L of RB-5 in static condition at room temperature for 120 hours and 28°C (room temperatures were between 28°C – 32°C). The visual inspections were conducted to choose the best pure cultures by counting the days required for eliminating the black color. The decolorization of consortium was assessed by mixing the selected bacteria with 1:1 ratio and redoing the previous step.

### 3.2.3 Decolorization experiments

The decolorization experiments were conducted at 100 mg/L of RB-5 dye amended in NB media. Every decolorization experiment was done in triplicate. From Figure 8, the experiments were conducted by adjusting the $OD_{600}$ of each bacterium to 0.3 after overnight grown in 100 mL of NB at 150 rpm and 28°C. Each pure culture and consortium was inoculated in 100 mL of NB amended with RB-5 under shaking condition at 150 rpm and 28°C or static condition at the room temperature. The samples were taken every 24 hours by pipetting 1 mL of culture into a microtube and centrifuged at 8000 g for 10 minutes (Wang et al., 2013).

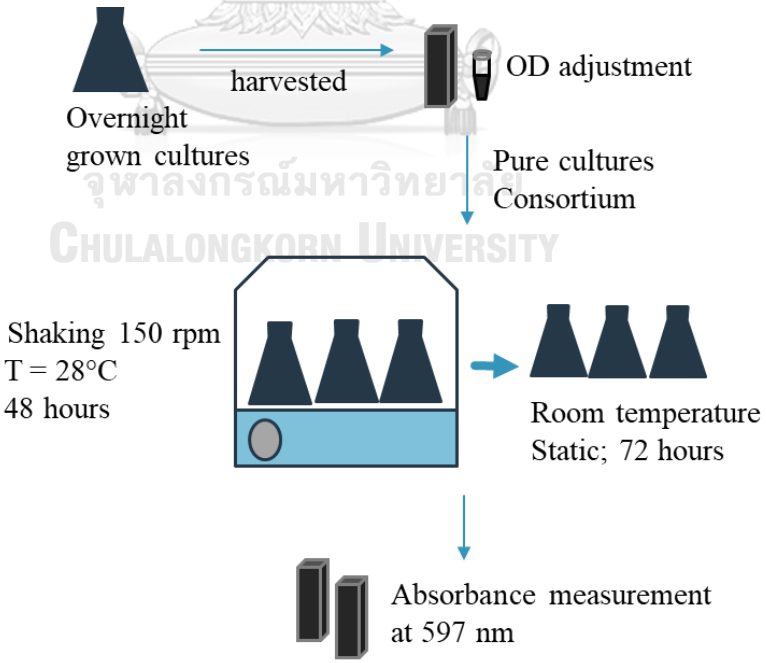



**Figure 8.** The main methods used for the decolorization experiment.

The absorbance of supernatant was then measured at 597 nm ((Lucas et al., 2006) from incubation at day 0 up to day 5. Decolorization percentage was calculated with the following formula (Wang et al., 2013):

$$Decolorization\ (\%) = \frac{Absorbance\ at\ day\ 0 - Absorbance\ at\ day\ x}{Absorbance\ at\ day\ 0} x100$$

**3.2.3.1 Effect of environmental conditions on the decolorization process**

The environmental conditions for RB-5 decolorization were determined based on shaking condition, temperature, pH and initial dye concentration. The shaking condition was studied between static (no shaking) and dynamic (shaking at 150 rpm) conditions and the combination of them. The bacterial concentrations were varied from $10^{7}$ up to $10^{12}$ CFU/mL. The studied pH values were 7, 9 and 11. These pH values were picked based on the textile dyeing wastewater that used the reactive dyes, which could reach pH 11 ((Racyte et al., 2009). The effect of dye concentration was studied at the initial RB-5 concentration from 100 up to 1000 mg/L. The decolorization percentage was monitored as described above. In the end, the optimum condition of decolorization was selected.

**3.2.3.2 Degradation kinetics**



After determining the optimum conditions, the decolorization experiments with different RB-5 concentrations were conducted to study the degradation kinetics. The concentration of RB-5 during decolorization was determined by converting the A597 values using the standard curve. The first-order rate constants for different RB-5 concentrations were determined using the following equation:

$$ln\left(\frac{C_t}{C_0}\right) = -kt$$

Where, $C_t$ is the concentration of RB-5 at time t, $C_0$ is the concentration of RB-5 at day 0, k is the rate constant, and t is the experimental time (day).

Also, the Michaelis-Menten kinetics were used to determine the relationship between the dye concentration and RB-5 decolorization (Das et al., 2017 ). From the first-order rate constants, the data was transformed into the rate of decolorization. These rates then changed into their reciprocal values along with the dye initial concentration data. After that, the double reciprocal values of rate and initial concentration were plotted into a graph. Finally, the Michaelis-Menten constant ($K_m$) and the maximum decolorization rate ($V_m$) can be determined from the graph

# CHAPTER 4
# RESULTS AND DISCUSSIONS

## 4.1 Bacterial screening and isolation

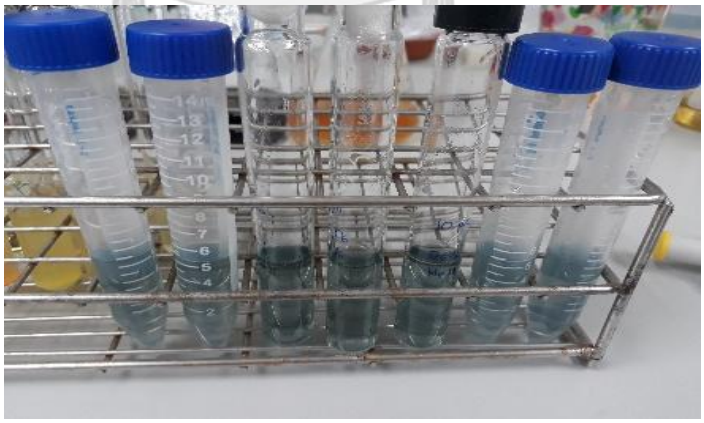
RB-5 is an organic compound even though it is a toxic dye, so it could possibly be remediated by bacteria found in the soil (Glick, 2010). Thus, soil seems to be a promising source of bacteria for biodegradation of this environmental pollutant. In the first step of this research, the soil samples from around Chulalongkorn University area were taken and screened for RB-5 degrading bacteria. The cultures that had the ability to grow under the presence of RB-5 were purified. During the purification steps, six bacterial colonies with different morphologies created clear zones in the agar plates containing 100 mg/L of RB-5. These six pure cultures were analyzed for 16S rRNA and their strains were identified as follows: *Gordonia terrae*, *Rhodococcus* sp., *Bacillus flexus*, *Microbacterium* sp., *Micrococcus* sp. and *Janibacter* sp. Each of these species were known to have its own record on the degradation of environmental pollutants as described in Chapter 2.

Using these six isolated bacteria, the preliminary experiments on RB-5 degradation was conducted. The efficiency of the degradation, reported as time (in days) needed for RB-5 color disappearance, was determined qualitatively by visual inspection. In the first trial, the overnight grown pure cultures (1 mL) were utilized to degrade 10 mg/L of RB-5 in static condition at room temperature, resulting in three days decolorization for *Janibacter* sp. and *B. flexus* as the fastest ones. Other bacteria required five days to decolorize, except *Microbacter* sp. that performed the slowest decolorization up to 11 days. As this research focuses on constructing bacterial consortium, the second visual inspection's trial utilized mixed cultures to degrade RB-5 with the concentration ranged from 10 to 100 mg/L (Figure 9 – 14). At 10 mg/L of RB-5, all consortia completely decolorized the dye at day 2 except for the consortium of *G. terrae* and *Rhodococcus* sp. that required 4 days (Figure 9). At 20 mg/L of RB-5, the consortium of *Janibacter* sp. and *Micrococcus* sp. completely decolorized the dye and for the rest of consortia required 4 days' incubation (Figure 10). At 30 mg/L of RB-5, all of the consortia required 6 days to completely decolorize the dye (Figure 11). At 40 to 100 mg/L of RB-

5, different consortia required different number of days to decolorize RB-5 (Figure 12, Figure 13, and Figure 14).

For the consortia, the results showed that the time for decolorizing RB-5 ranged from two days at 10 mg/L up to two weeks at 100 mg/L (Table 3). Even though the isolated bacteria and the consortium showed promising results of RB-5 decolorization, *Gordonia terrae*, *Bacillus flexus*, *Microbacterium* sp. were excluded from further experiments due to their pathogenicity, and only *Janibacter* sp., *Microbacter* sp., and *Rhodococcus* sp. were used.

From the results, the consortium comprising *G. terrae*, *Rhodococcus* sp., *Micrococcus* sp. and *Janibacter* sp. showed better decolorization efficiency than the others, especially at the higher RB-5 concentration up to 100 mg/L, which yielded six days of decolorization period without any additional adjustment in the system. However, since *G. terrae* showed possibility of pathogenicity as mentioned before, it was excluded from this study. Thus, this study focused on the utilization of *Rhodococcus* sp., *Micrococcus* sp. and *Janibacter* sp. as both single and mixed cultures for RB-5 decolorization.

**(a)** 

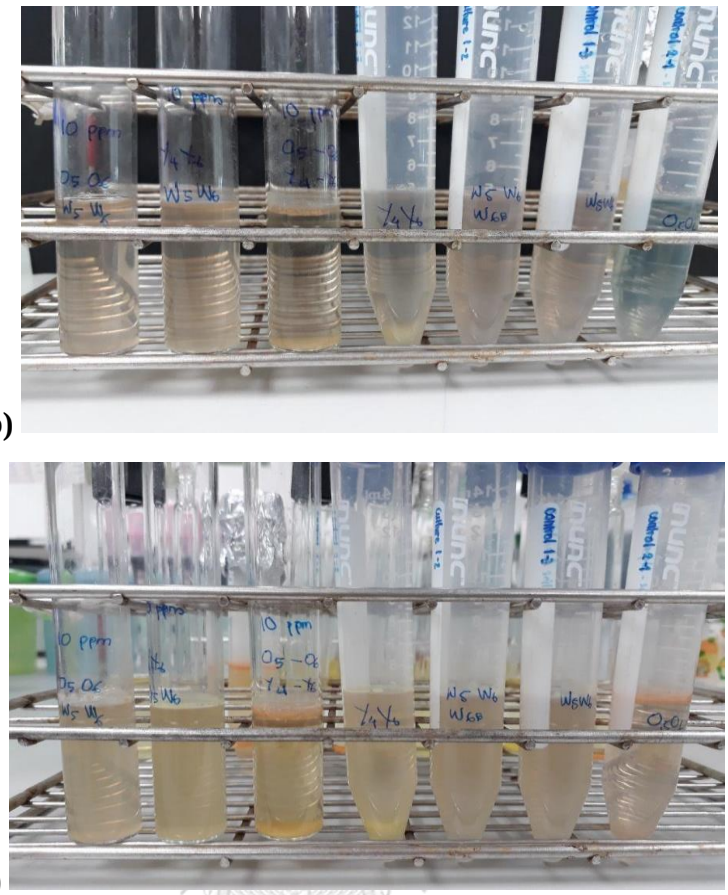
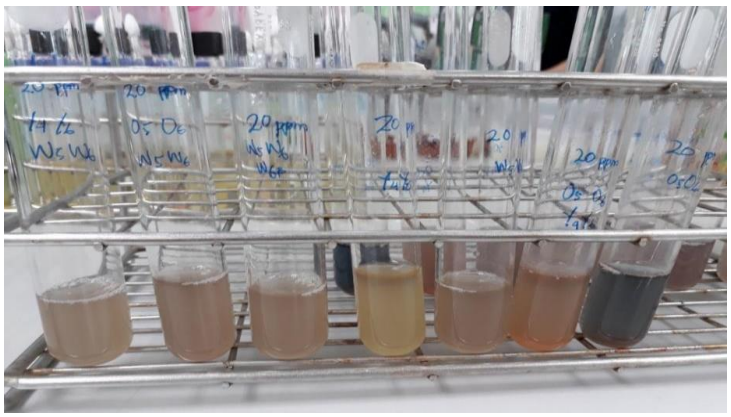


**Figure 9.** The qualitative test of RB-5 decolorization at 10 mg/L: (a) 0 day of incubation, (b) 2nd day of incubation, and (c) 4th day of incubation.

**(b)**



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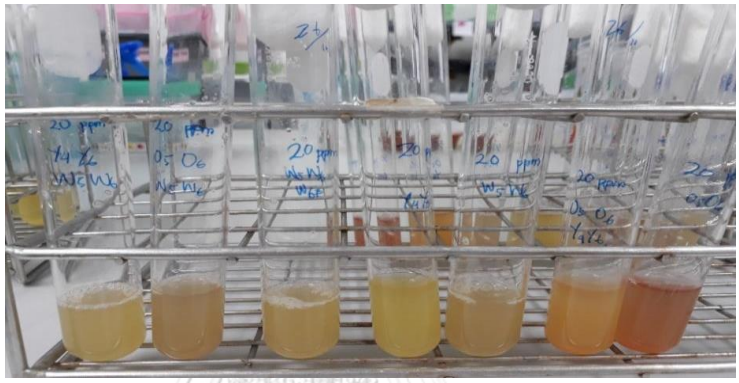
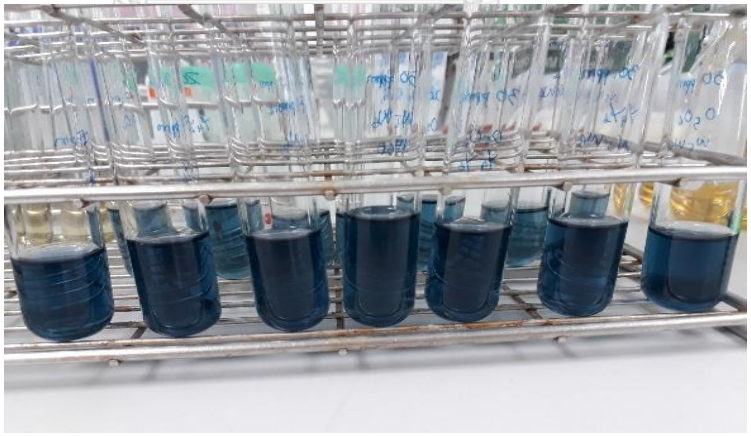


**Figure 10**. The qualitative test of RB-5 decolorization at 20 mg/L: (a) 0 day of incubation, (b) 3rd day of incubation, and (c) 4th day of incubation.

**(a)**



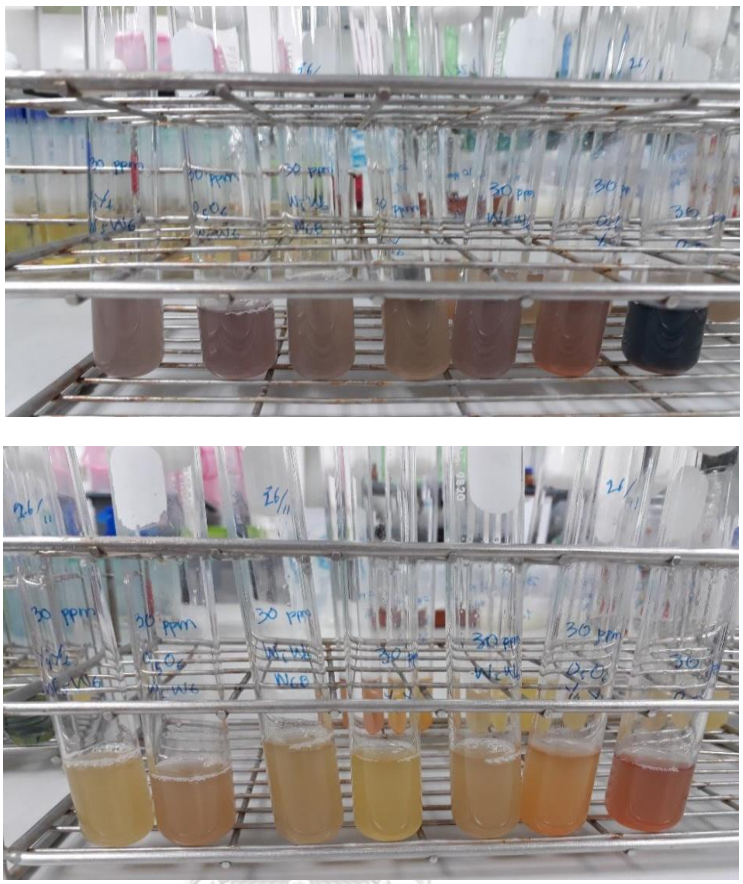


**Figure 11.** The qualitative test of RB-5 decolorization at 30 mg/L: (a) 0 day of incubation, (b) 3rd day of incubation, and (c) 6th day of incubation.

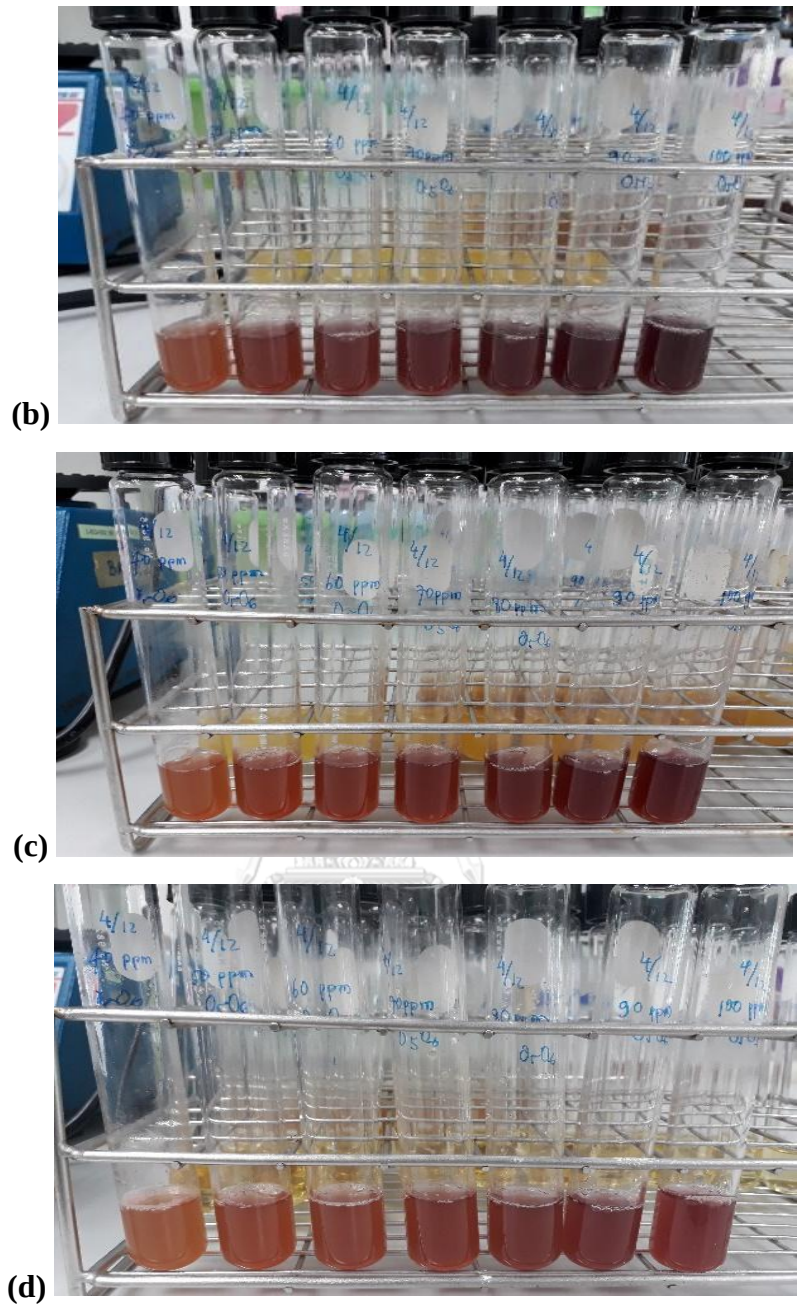


**Figure 12.** The qualitative test of RB-5 decolorization from 40 to 100 mg/L (left to right) by the consortium of *G. terrae* and *Rhodococcus* sp.: (a) 0 day of incubation, (b) 8$^{th}$ day of incubation, (c) 10$^{th}$ day of incubation, and (d) 14$^{th}$ day of incubation.

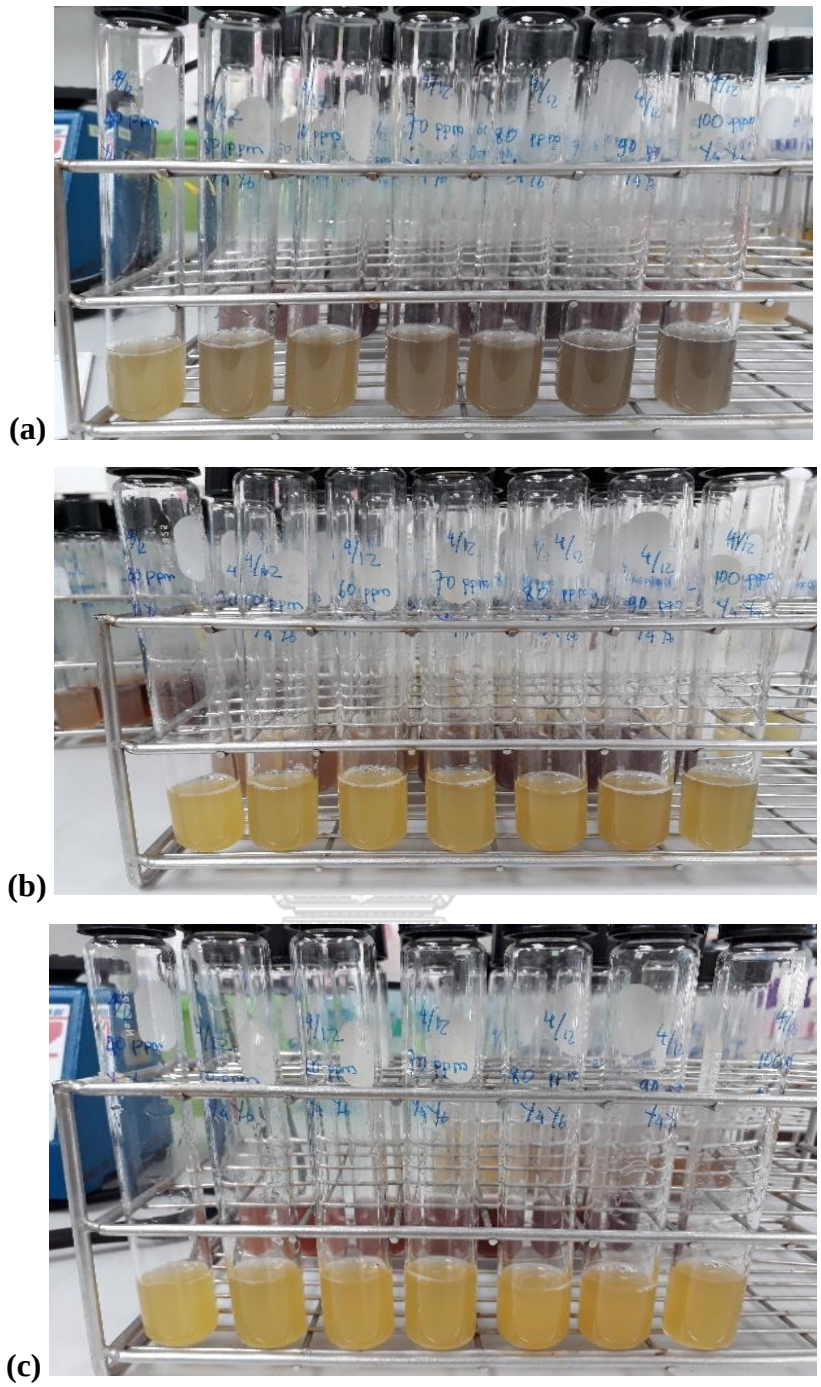


**Figure 13.** The qualitative test of RB-5 decolorization from 40 to 100 mg/L (left to right) using the consortium of *Janibacter* sp. and *Micrococcus* sp.: (a) 3rd day of incubation, (b) 8th day of incubation, and (c) 14th day of incubation.

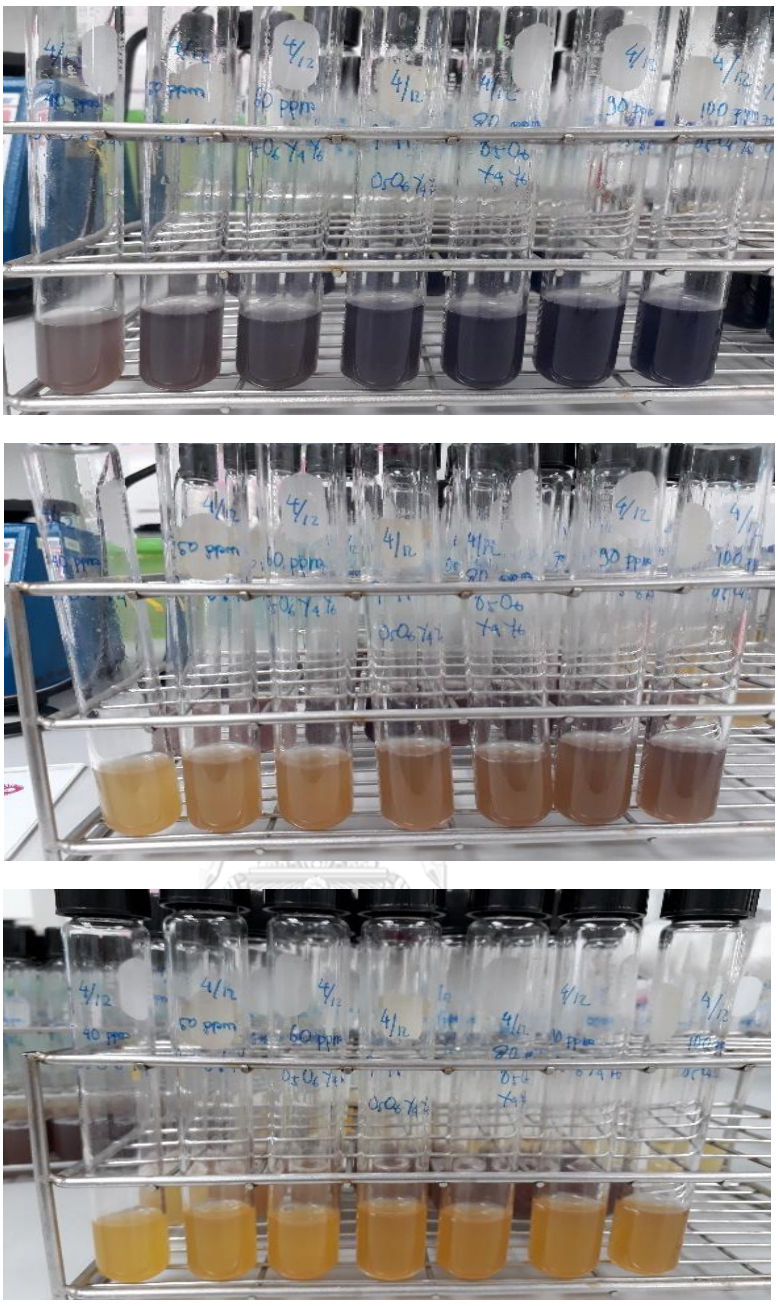


**Figure 14.** The qualitative test of decolorization from 40 to 100 mg/L (left to right) using the consortium of *G. terrae*, *Rhodococcus* sp., *Janibacter* sp., and *Micrococcus* sp.: (a) 2nd day of incubation, (b) 4th day of incubation, and (c) 6th day of incubation.

**Table 3.** Qualitative screening on RB-5 decolorization by bacterial consortia constructed from the isolated strains. The numbers are time required for RB-5 color disappearance (days).

| Consortium | Initial RB-5 concentrations (mg/L) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **10** | **20** | **30** | **40** | **50** | **60** | **70** | **80** | **90** | **100** |
| *G. terrae*<br>*Rhodococcus* sp. | 4 | 6 | 6 | 8 | 10 | 10 | 10 | 14 | 14 | 14 |
| *B. flexus*<br>*Microbacterium* sp. | 2 | 4 | 6 | 8 | 14 | 14 | 14 | 14 | 14 | 14 |
| *Micrococcus* sp.<br>*Janibacter* sp. | 2 | 3 | 6 | 6 | 8 | 8 | 8 | 8 | 8 | 8 |
| *G. terrae*<br>*Rhodococcus* sp.<br>*B. flexus*<br>*Microbacterium* sp. | 2 | 4 | 6 | 8 | 14 | 14 | 14 | 14 | 14 | 14 |
| *B. flexus*<br>*Microbacterium* sp.<br>*Micrococcus* sp.<br>*Janibacter* sp. | 2 | 4 | 6 | 4 | 10 | 10 | 10 | 10 | 10 | 10 |
| *G. terrae*<br>*Rhodococcus* sp.<br>*Micrococcus* sp.<br>*Janibacter* sp. | 2 | 4 | 6 | 4 | 6 | 6 | 6 | 6 | 6 | 6 |



### 4.2 Determination of operating conditions for RB-5 decolorization

The decolorization of RB-5 could be performed either in dynamic (shaking) or static (not shaking) condition. Since all the isolated strains in this research are aerobic bacteria (Vignali et al., 2018); (Du et al., 2013); (Khessairi et al., 2014 )), the dynamic condition should be able to enhance their growh and activity. *Rhodococcus* sp. performed the decolorization by utilizing lignin peroxidase (Vignali et al., 2018) and *Micrococcus* sp. by laccase and NADH-DCIP reductase (Du et al., 2013), so these enzymes could use oxygen as an electron acceptor rather than RB-5 if too much aeration is provided. Also, azoreductase enzymes work better in the limited oxygen condition (Khandare et al., 2015 ), so the static condition is also important to the decolorization system.

The experiments were conducted in two different conditions, which were static (without shaking) and dynamic (shaking). The selected bacterial strains from the preliminary study were tested to decolorize the RB-5 dye at the fixed concentration of 100 mg/L and temperature of 28°C. The results in static condition (Figure 15) showed highest decolorization for *Janibacter* sp., and 76.6% at day 5. On the other hand, *Micrococcus* sp. and *Rhodococcus* sp. showed only 37.8% and 31.8% of decolorization within 5 days. Since there has been no report on the decolorization performance by *Janibacter* sp., this study could be a good start to optimize the efficiency of this bacteria since it is easily cultivable and no specific requirement is needed to decolorize the dye.

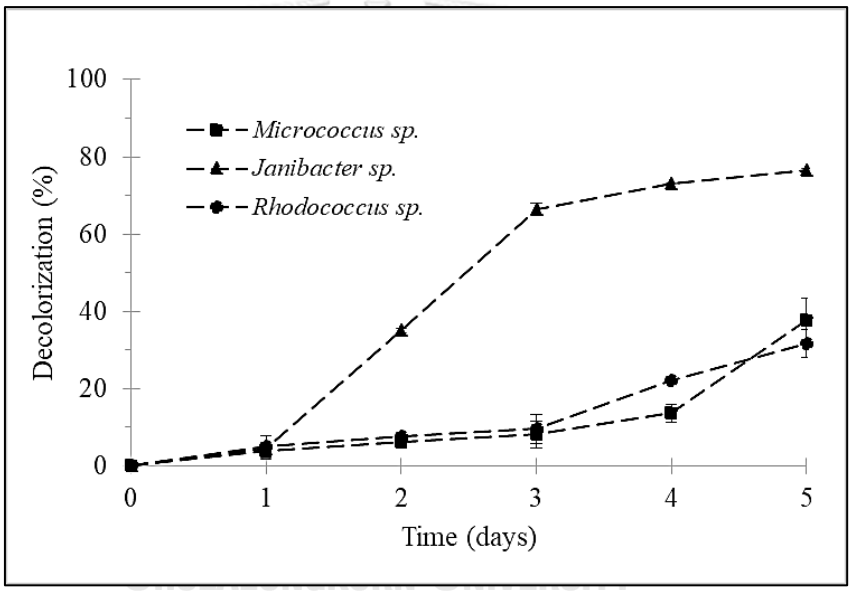



**Figure 15.** RB-5 decolorization under static condition at 28°C, pH 7 and 150 rpm with the RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization efficiency as percentage are represented in y-axis.

The dynamic condition at shaking speed of 150 rpm was tested for each bacterial strain on RB-5 decolorization. The objective of applying the dynamic condition was due to the types of aerobic bacteria isolated in this study. With the abundance of oxygen given to the system, the bacteria would grow more and thereby the decolorization percentage could be enhanced. However, the decolorization efficiencies obtained from the shaking condition were much lower than in the static condition (Figure 16). The highest performance was also given by *Janibacter* sp., but only with 61.3% decolorization. Less

decolorization under dynamic condition could be explained by the bacterial preference for oxygen in the system to be utilized as an electron acceptor rather than the RB-5 dye as the redox potential of oxygen is higher than the RB-5 dye, which is, in V vs SCE, the potential (V) that is measured against the electrode in standard calomel electrode (SCE), 0.988 (Bacardit et al., 2007) compared to 0.205 (Costa et al., 2012).

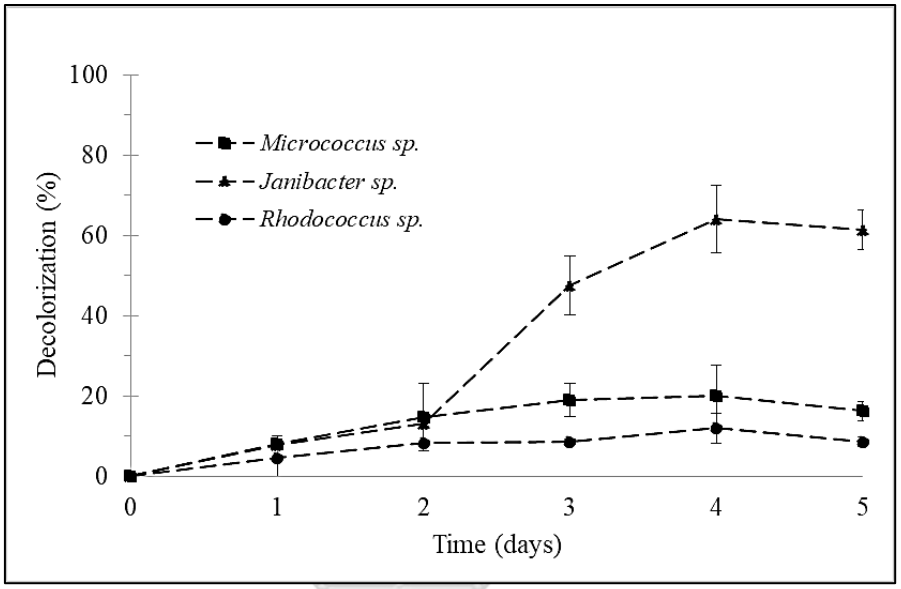



**Figure 16**. RB-5 decolorization under dynamic condition at 28°C, pH 7 and 150 rpm with the RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization efficiency as percentage are represented in y-axis.



Due to the limitations of using only single condition, the operating condition that combines both dynamic and static may be the key to enhance RB-5 decolorization. Therefore, another decolorization experiment was set up with the fixed 2-day shaking (at 150 rpm) and 3-day static conditions. The purpose of combining both conditions was to accelerate the bacterial growth by the presence of enough oxygen, and then to initiate the better activity of azoreductase enzyme produced by these bacteria by applying the oxygen limitation condition in the last three days. The shaking time was fixed at 2 days to give sufficient bacteria in the cultures for the efficient work up to day-5 incubation.

The result showed that, with the combined conditions (Figure 17), the decolorization percentages obtained by all bacteria were higher than using static or dynamic condition

alone (Figure 15 and 16). The huge decolorization enhancements were obtained for *Micrococcus* sp. and *Rhodococcus* sp. that gave 82.2% and 69.8% decolorization, respectively, in the combined system compared to the static system alone that gave only 37.8% and 31.8% decolorization, respectively. *Janibacter* sp. still showed the best performance with 86.7% of RB-5 decolorization. The results proved the hypotheses that the shaking condition was important to provide enough oxygen for bacterial growth. After that, the decolorization was allowed at static condition to limit the oxygen and thus initiate the presence of azoreductase enzyme, which works best in the anaerobic condition (Khandare et al., 2015 ).

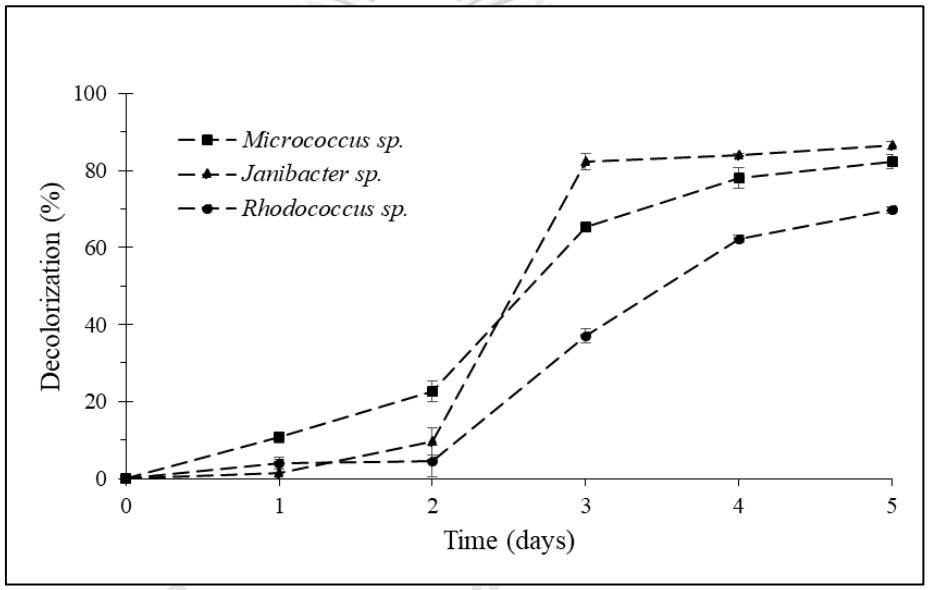





**Figure 17.** RB-5 decolorization under combined dynamic-static condition at 28°C, pH 7 and 150 rpm for the dynamic stage with the RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization efficiency as percentage are represented in y-axis.

Likewise, the comparison between combined and single operating conditions was applied to the decolorization of RB-5 by the bacterial consortium. Different from pure cultures, the change of the system's condition did not greatly affect the decolorization efficiency by the bacterial consortium (Figure 18). The increase in decolorization efficiency was observed only when compared with the complete dynamic condition, which gave only 34.7% decolorization. When static condition was applied entirely, the consortium had a good decolorization performance, resulting in 81.5% decolorization.

This was only a slight difference from the combined condition that gave 85% decolorization. The results proved that using the bacterial consortium can be beneficial in the conditions where single culture cannot perform the decolorization of RB-5.

Since the combined dynamic-static conditions showed the best decolorization for both single culture and consortium, this proves the point of mixing both static and dynamic conditions that the bacteria needed oxygen to grow at the beginning of incubation stage and the enzyme that they produced during that stage could work well later under the limited amount of oxygen in the system. Thus, this condition has been chosen for further decolorization experiments.

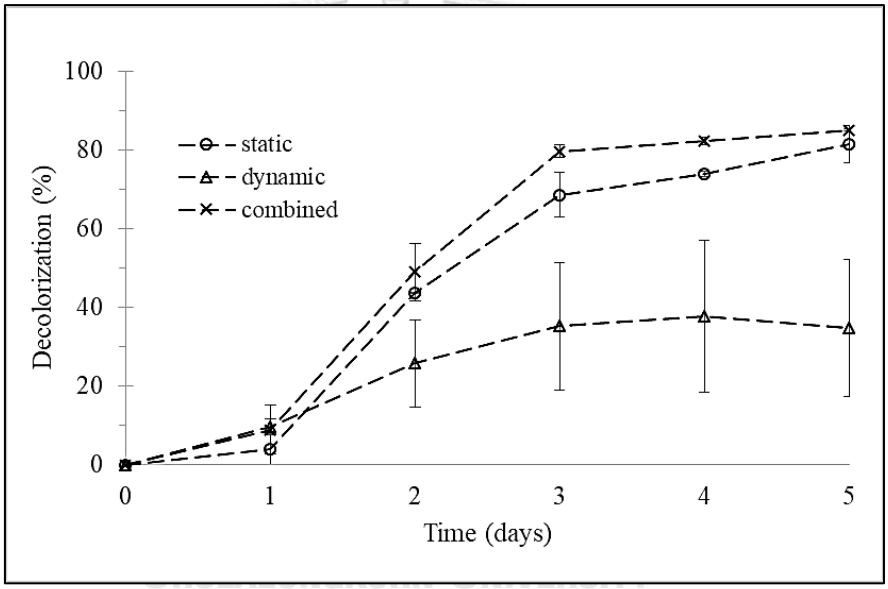



**Figure 18**. RB-5 decolorization by bacterial consortium under different operating conditions at 28°C, pH 7 and 150 rpm for the dynamic stage with the RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization efficiency as percentage are represented in y-axis.

## 4.3 Factors affecting RB-5 decolorization under the combined dynamic-static condition

### 4.3.1 Effect of temperature on RB-5 decolorization

To ensure that the system could be applied in a wastewater treatment plant under varying temperatures, the decolorization experiment, with the fixed combined condition between two-day shaking and three-day static, was conducted at different temperatures. The temperature of the system was varied based on the ambient and outdoor temperature in Thailand, ranging from 25°C to 40°C. The results showed that this range of temperature did not much affect the decolorization performance of each pure cultures as well as the consortium (Figure 19), which still yielded more than 80% of decolorization for both pure cultures and the consortium at all temperatures except for *Rhodococcus* sp. at 25°C, which was less than 70%. Temperature plays a role in the decolorization process as an important factor for bacterial growth. Each bacterial strain could have a different optimum temperature for its growth and the working enzymes produced by bacteria including the ones responsible for color removal process. Other studies on the RB-5 decolorization showed that the optimum temperature of 40°C for *Bacillus* sp. YZU1 (Wang et al., 2013) and 35°C for *Aeromonas hydrophila* (El Bouraie et al., 2016 ). The data from this study suggested that the isolated soil bacteria could be useful for the decolorization of real RB-5 wastewater under varying temperatures. As changing temperature did not much affect the efficiency, further decolorization experiments would be conducted at the room temperature, which was ranging from 28°C to 32°C.

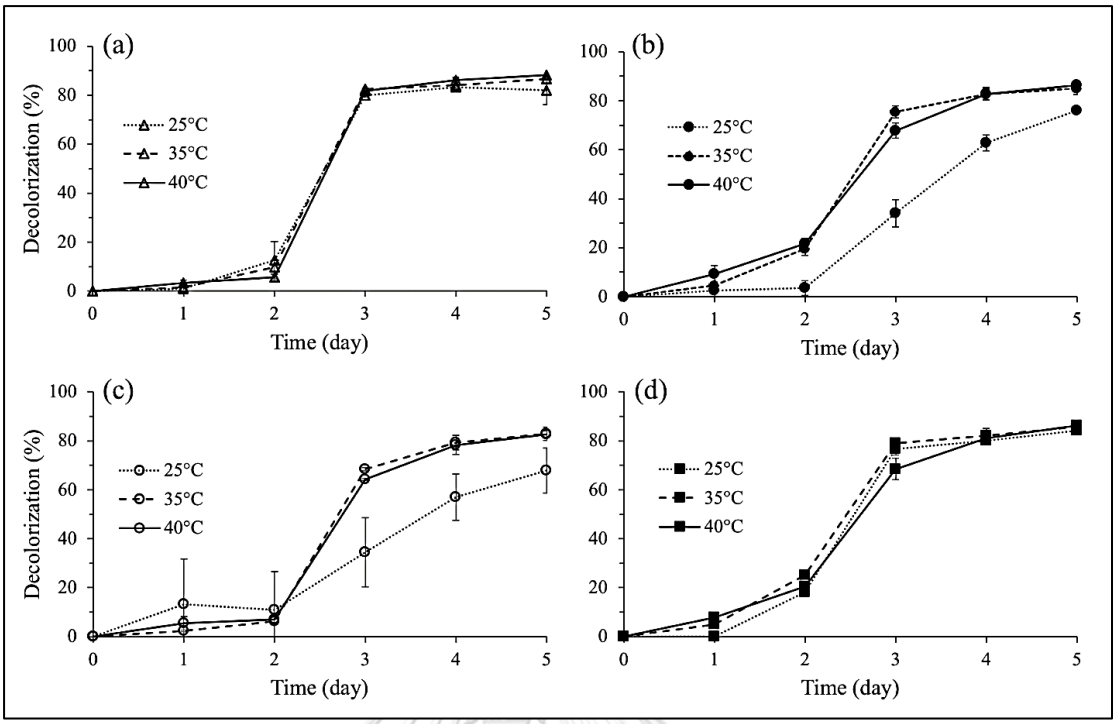



**Figure 19.** Effect of the temperature on RB-5 decolorization efficiency: (a) *Janibacter* sp., (b) *Micrococcus* sp., (c) *Rhodococcus* sp., and (d) Consortium. The operating system was 2-day shaking and 3-day static conditions with pH 7 and the RB-5 concentration of 100 mg/L. The x-axis represents the types of bacteria and the y-axis represents the decolorization efficiency as percentage at day 5.

### 4.3.2 Effect of initial cell concentration on RB-5 decolorization

More initial cells in the media were expected to produce more enzymes for decolorization, thus the efficiency could become higher. This part observes the effect of different cell number on RB-5 decolorization efficiency. In previous experiments, the bacterial concentration was around $10^7$ CFU/mL by adjusting the $OD_{600}$ at 0.3. In this experiment, the initial bacterial concentrations for each single species were adjusted to different levels before using in the decolorization experiments.

For *Micrococcus* sp., cells were adjusted to the concentrations of $10^8$, $10^{10}$, and $10^{12}$ CFU/mL before using in the experiment. Surprisingly, each of this concentration resulted in slight differences (85% up to 86%) in the decolorization percentage obtained at the 5$^{th}$ day, as shown in Figure 20. The multiple *t*-test analysis showed no statistical

differences between the decolorization efficiencies of any pair of the experiments with the P value more than 0.05 (Table A1, Appendix).

According to the data on bacterial growth during the decolorization period (Figure A1, Appendix), at day 1 after the dynamic condition had started, the cell numbers of *Micrococcus* sp. in all conditions were at between $10^8$ to $10^9$ CFU/mL. The difference between initial cell concentration and the concentration at day 1 might be due to the initial lag phase from the adjustment to new media, which normally resulted in slow growth, no growth, or even death in the toxic conditions with RB-5. After day 2, the experiment with the initial cell concentration at $10^8$ CFU/mL still showed elevated growth while the others were slightly decreased. These three cultures seemed to have the same highest growth at the cell number of $3 \times 10^9$ CFU/mL. With the difference in growth less than 1 order of magnitude, it might be the reason for the similar decolorization efficiencies obtained by these three experiments (Figure 20).

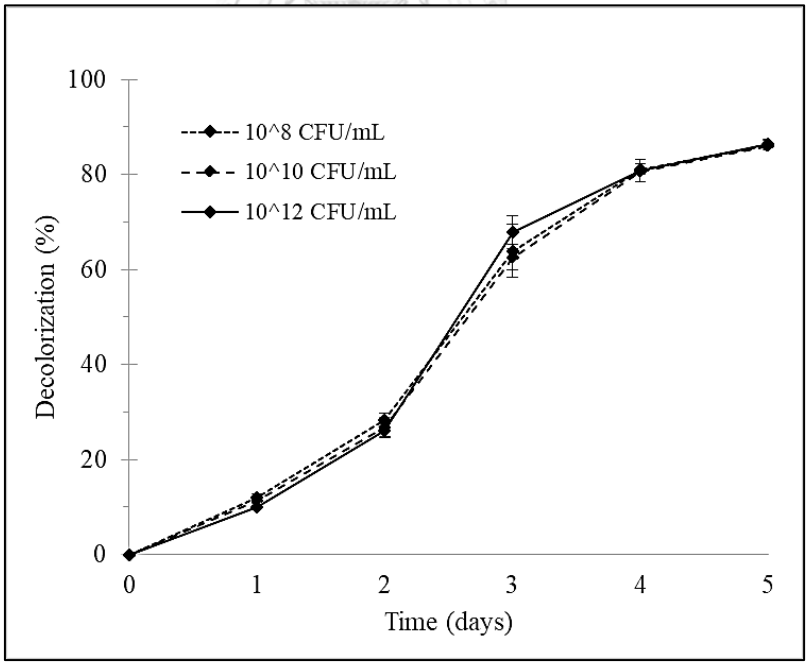



**Figure 20.** Effect of initial cell concentration of *Micrococcus* sp. on RB-5 decolorization efficiency. The operating system was 2-day shaking and 3-day static conditions with pH 7, 150 rpm for the dynamic stage and the RB-5 concentration of 100 mg/L. The x-axis represents the types of bacteria and the y-axis represents the decolorization efficiency as percentage.

For *Janibacter* sp., the cell concentrations were adjusted to the same range as *Micrococcus* sp., and the results showed slightly different trends (Figure 21). The results indicated that the best performance was at 94.8% decolorization with the initial cell concentration of $10^{10}$ CFU/mL. However, the final decolorization percentage obtained were statistically different from the initial concentration of $10^{8}$ CFU/mL (86.7%) and $10^{12}$ CFU/mL (90.7%) (Table A1, Appendix).

During the decolorization, the growth of *Janibacter* sp. was higher when the initial concentrations of cell increased to $10^{10}$ and $10^{12}$ CFU/mL (Figure A2, Appendix), which was around 1-log higher with the initial cell concentration of $10^{8}$ CFU/mL. This could explain the faster decolorization in both conditions, which can be observed from day 1 to day 3 of the decolorization (Figure 21). The cell concentration dropped at day 1 might be the effect of the initial lag phase from the adjustment to new condition as described before.

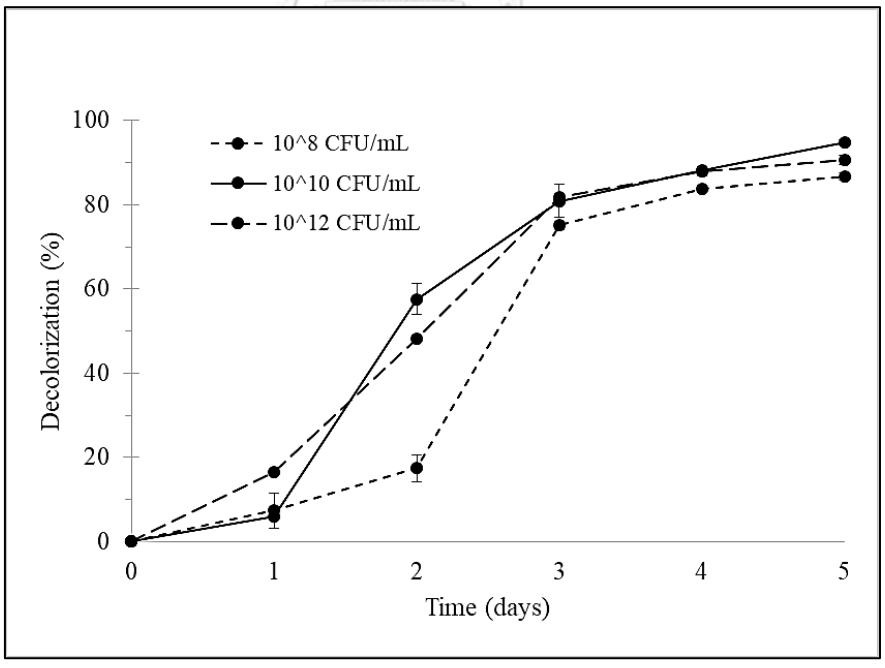



**Figure 21.** Effect of initial cell concentration of *Janibacter* sp. on RB-5 decolorization efficiency. The operating system was 2-day shaking and 3-day static conditions with pH 7, 150 rpm for the dynamic stage and the RB-5 concentration of 100 mg/L. The x-axis represents the types of bacteria and the y-axis represents the decolorization efficiency as percentage.

For *Rhodococcus* sp., the cell number was adjusted to the initial concentrations of $10^7$, $10^8$, and $10^9$ CFU/mL. Increasing the cell concentration to $10^9$ CFU/mL resulted in the highest decolorization percentage up to 74.9% while the cell concentrations of $10^7$ and $10^8$ CFU/mL gave 61.1% and 63.4% decolorization (Figure 22). However, there were no statistical difference on the decolorization efficiencies. (Table A1, Appendix)

The cell concentration of *Rhodococcus* sp. during RB-5 decolorization kept increasing until it reached the maximum number at day 5 of the decolorization period (Figure A3, Appendix), which explains the decolorization efficiency that also gradually increased to the maximum at day 5 (Figure 22). Interestingly, highest initial cell concentration of $10^9$ CFU/mL showed slowest cell growth in the last day of the experiment.

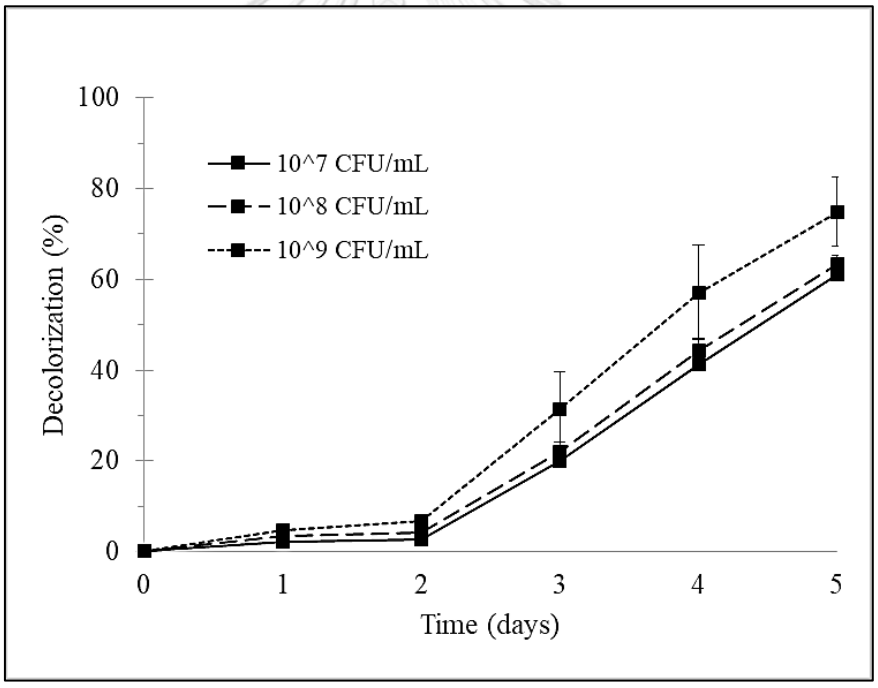



**Figure 22.** Effect of initial cell concentration of *Rhodococcus* sp. on RB-5 decolorization efficiency. The operating system was 2-day shaking and 3-day static conditions with pH 7, 150 rpm for the dynamic stage and the RB-5 concentration of 100 mg/L. The x-axis represents the types of bacteria and the y-axis represents the decolorization efficiency as percentage.

The results suggested that the initial cell concentration did not provide much effect on the decolorization efficiency. This might be due to the limitation of maximum cell

growth at the same concentration of carbon source provided in the culture although different strains were used. Previous research on the decolorization of RB-5 using soil bacteria with similar culturing method did not report the effect of cell concentration (Wang et al., 2013); (El Bouraie et al., 2016 ), thus the cell concentration might not have an important role in the RB-5 decolorization.

### 4.3.3 Effect of shaking time on RB-5 decolorization

The purpose of shaking in the combined dynamic-static decolorization is to provide enough oxygen for bacterial growth and therefore enhances the decolorization of RB-5. In a wastewater treatment plant, oxygen is normally provided by aeration. This aeration process obviously requires an extra operation cost. Since the cost-effective treatment is more preferred, the experiments varying the shaking period were conducted to determine whether the shaking time could be reduced while the decolorization efficiency was still obtained. With the total of 5-day decolorization, the initial shaking times were varied into one, two and three days. After shaking, each condition was followed by the static incubation until day five.

For *Micrococcus* sp., the variation in the shaking period did not result in a huge difference in the decolorization efficiencies obtained within five incubation days (Figure 23). The highest decolorization percentage was observed in two-day shaking condition (86.3%), which was slightly higher than one-day shaking condition (77.5%), followed by three-day shaking condition (74.5%). This could mean that the oxygen supply was enough even in just one day of shaking. But, the P value from t-test between one-day and two-day shaking period suggested the statistical difference (Table A2, Appendix). The decolorization rate for two-day shaking was higher than one-day shaking as well (Table A3, Appendix).

It can be seen that increasing the shaking time to 3 days resulted in faster growth, which can be observed on day 3 data with the cell number of $10^{10}$ CFU/mL (Figure 24). However, at the end of experiments, cultures from all three conditions still showed

similar cell concentrations at the range of $10^9$ CFU/mL (Figure 24). This could explain the similar efficiencies for RB-5 decolorization (Figure 23).

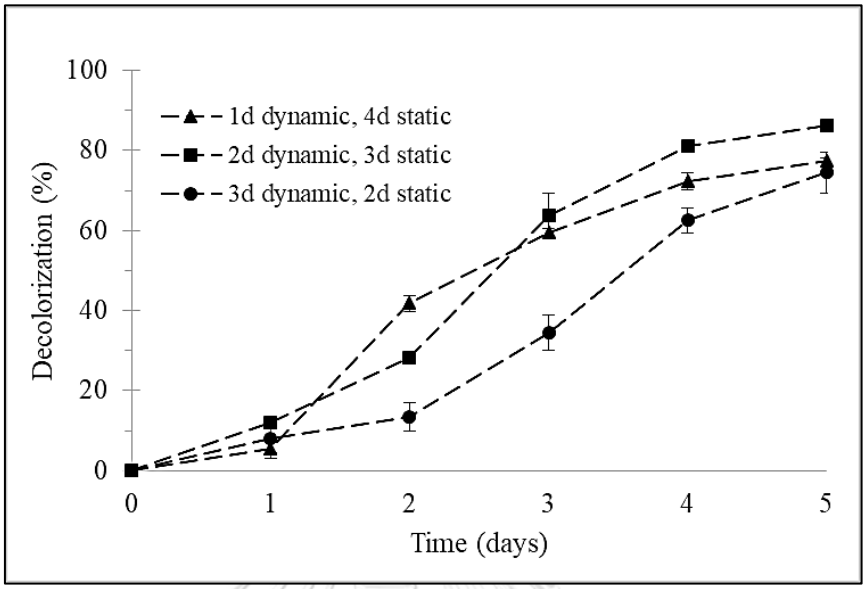



**Figure 23.** Effect of shaking time on RB-5 decolorization by *Micrococcus* sp. at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

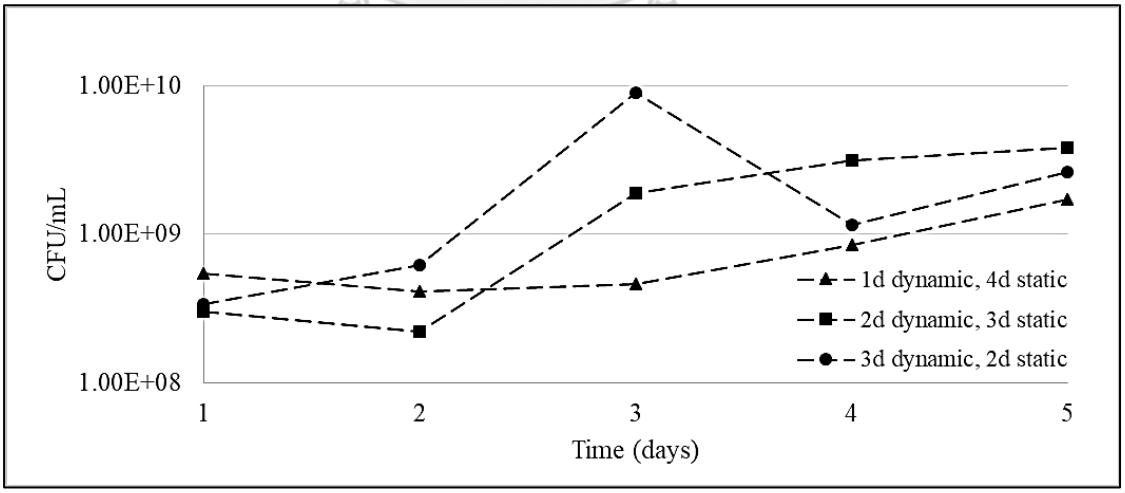



**Figure 24.** Growth of *Micrococcus* sp. under different shaking times during RB-5 decolorization at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the number of cells in CFU/mL in y-axis.

For *Janibacter* sp., RB-5 decolorization increased with longer shaking period applied to the system. The highest decolorization of 89% was obtained with 3-day shaking compared with 1-day and 2-day shaking, which resulted in 77.7% and 86.7% decolorization, respectively (Figure 25). The t-test result showed that all the efficiencies were statistically different with the P value less than 0.05. The rate of decolorization for 3-day shaking was the highest as well, compared with the 1-day and 2-day shaking rates (Table A4, Appendix).

Figure 26 shows the change of cell concentration over time. The highest cell concentration during RB-5 decolorization was obtained with 3-day shaking. However, the cell number at the end of experiment was similar in all conditions, ranging between $10^8$ to $10^9$ CFU/mL.

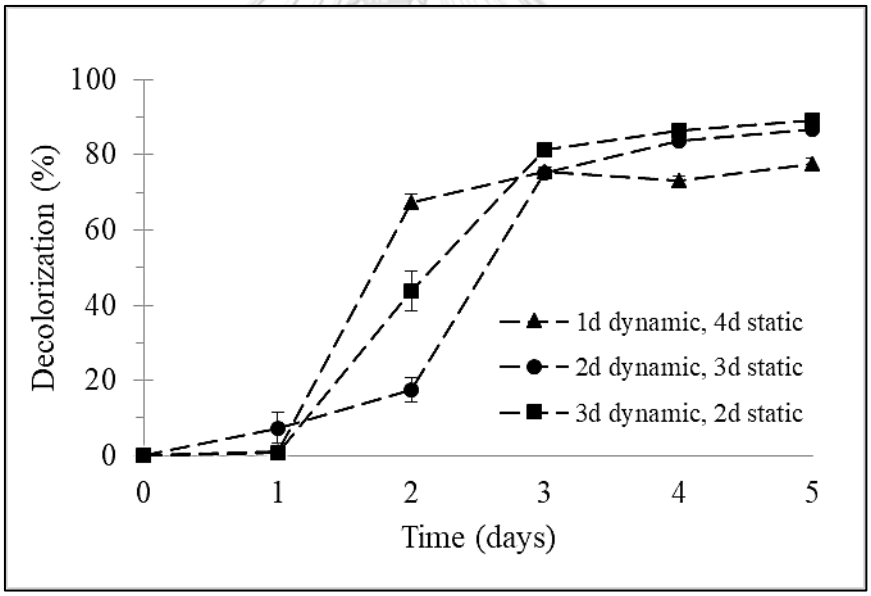



**Figure 25.** Effect of shaking time on RB-5 decolorization by *Janibacter* sp. at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

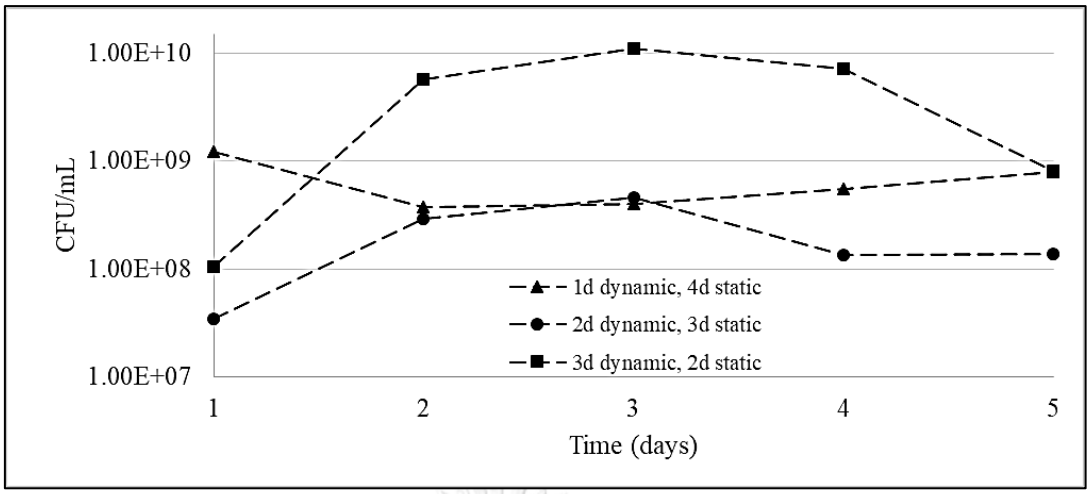



**Figure 26.** Growth of *Janibacter* sp. under different shaking times during RB-5 decolorization at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the number of cells in CFU/mL in y-axis.

For *Rhodococcus* sp., increasing the shaking time resulted in higher decolorization efficiency from 28.8% for 1-day shaking to 63.4% for 2-day shaking (Figure 27). However, increasing the shaking time to 3 days did not enhance any further decolorization (55%). Among the three bacterial strains used in this study, this stain showed lowest RB-5 decolorization in terms of both efficiency and rate.

Other research reported the performance of *Rhodococcus* sp. to decolorize Remazol Brilliant Blue R (RBBR, a.k.a. Reactive Blue 19) and RB-5 with the addition of $Mn^{2+}$ as the catalyst, showed only 22% decolorization for RB-5 compared to 95% on RBBR (Vignali et al., 2018). Compared to RB-5, RBBR has a simpler chemical structure that might be the reason for the higher decolorization performance by *Rhodococcus* sp.

According to the cell concentration of *Rhodococcus* sp. during the decolorization, highest growth was observed in the condition of 2-day shaking (Figure 28). This might well be the reason for highest decolorization in this condition. It should be noted that the cell number of *Rhodococcus* sp. is at similar level to that of other strains while the decolorization efficiency is relatively different. This could mean that the decolorization

of RB-5 not only depends on the cell concentration of the culture but also the capability possessed by the cell itself.

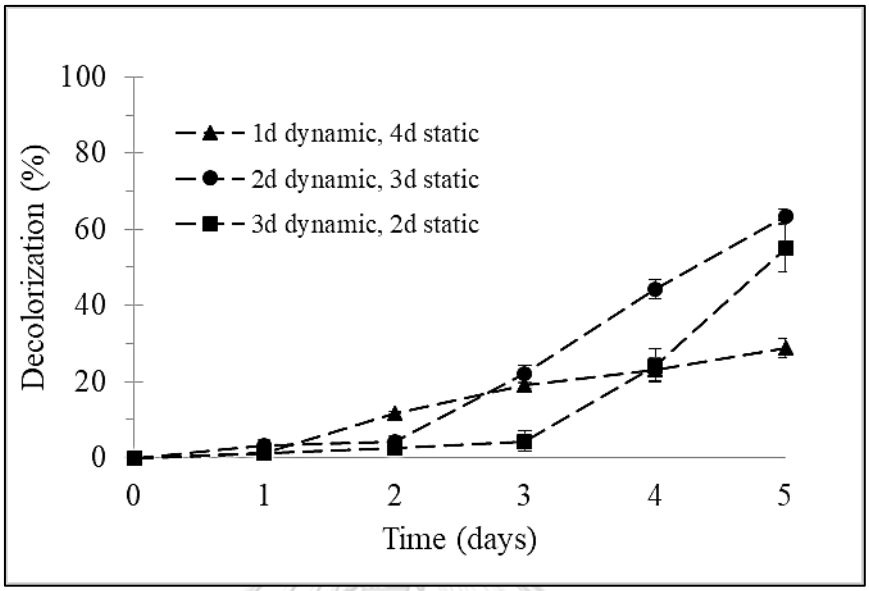



**Figure 27.** Effect of shaking time on RB-5 decolorization by *Rhodococcus* sp. at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

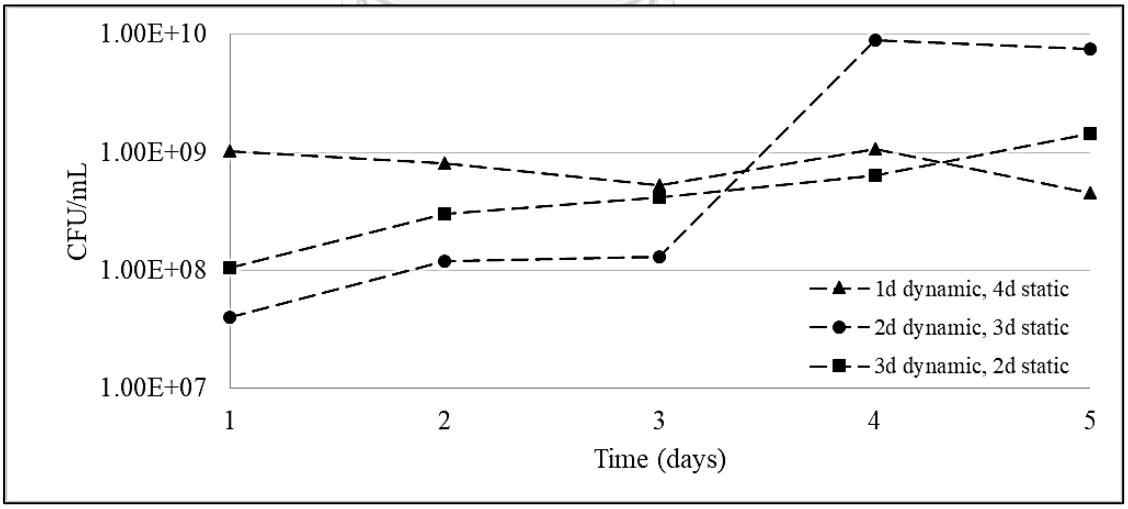



**Figure 28.** Growth of *Rhodococcus* sp. under different shaking times during RB-5 decolorization at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the number of cells in CFU/mL in y-axis.

Table 4 showed the optimum shaking time for each strain. Numbers of days required for highest decolorization are 3 days for *Janibacter* sp. and 2 days for *Micrococcus* sp. and *Rhodococcus* sp., which were 89.14%, 86.27% and 63.39% respectively.

The effect of shaking time on RB-5 decolorization by the consortium was also observed. Surprisingly, the consortium only required 1-day shaking to achieve the fastest decolorization of 75% at day 2 and the highest decolorization efficiency of 86% (Figure 29). Even though the efficiencies at the end of experiment were similar in all three conditions, using the consortium could reduce the time required for aeration. Faster decolorization of bacterial consortium compared to its single strains of *Proteus vulgaris* and *Micrococcus glutamicus* was also achieved with Scarlet R dye up to seven-time shorter for complete decolorization (Saratale et al., 2009 ).

**Table 4.** Summary of the decolorization efficiencies at different shaking periods

| **Bacterial strains** | **Decolorization efficiency at day 5** | | |
|---|---|---|---|
| | 1 day shaking | 2 days shaking | 3 days shaking |
| *Janibacter* sp. | 77.73 | 86.69 | **89.14** |
| *Micrococcus* sp. | 77.47 | **86.27** | 74.50 |
| *Rhodococcus* sp. | 28.80 | **63.39** | 55.13 |

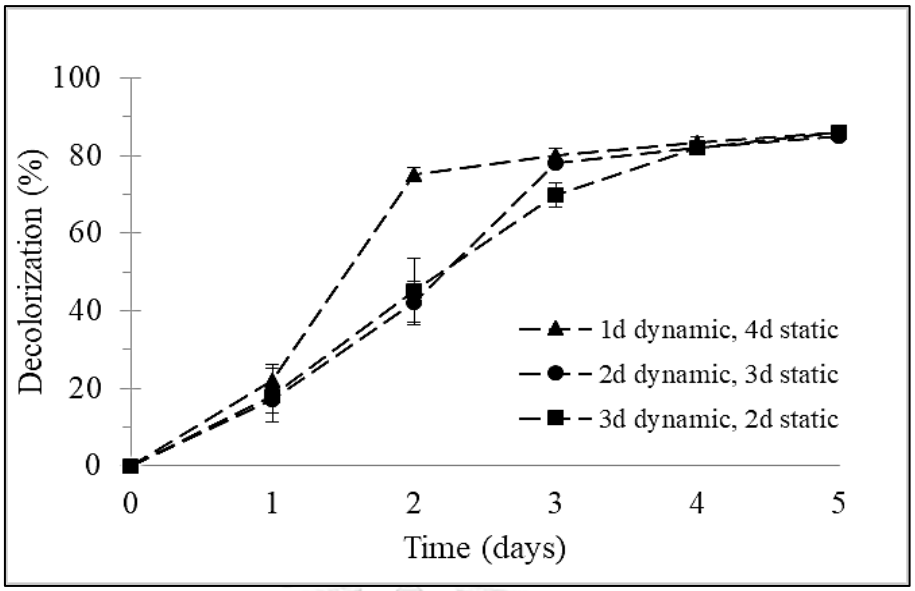



**Figure 29**. Effect of shaking time on RB-5 decolorization by bacterial consortium at room temperature, pH 7, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

### 4.3.4 Effect of pH on RB-5 decolorization

This part aims to study the effect of initial pH for RB-5 decolorization. Previous research showed the different optimum pH ranging from 5 to 9 for the decolorization of dye wastewater (El Bouraie et al., 2016 ; Khan et al., 2015; Wang et al., 2013). Another research reported that the textile dyeing wastewater that used the reactive dyes could have pH up to 11 (Racyte et al., 2009). In this experiment, the pH was adjusted from neutral to 11 to adopt the condition of dye wastewater.

Using the combined condition of 2-day dynamic and 3-day static, the highest decolorization was obtained at pH 7 for all cultures. The decolorization efficiencies were found as 86.3% for *Micrococcus* sp. (Figure 30), 89.1% for *Janibacter* sp. (Figure 31) and 63.4% for *Rhodococcus* sp. (Figure 32).

From the previous experiments on varying the shaking period, the optimum shaking time for both *Micrococcus* sp. and *Janibacter* sp. were 1-day shaking and 4-day static.

Therefore, the effect of pH on the decolorization at this condition was also conducted for those two strains. Interestingly, the reduction of shaking period resulted in highest decolorization at pH 9, which were 82.3% and 88.3% for *Micrococcus* sp. and *Janibacter* sp., respectively (Figure A4 and A5, Appendix). At pH 7, these two strains only gave around 77% decolorization; moreover, at pH 11, the decolorization efficiencies were less than 20%.

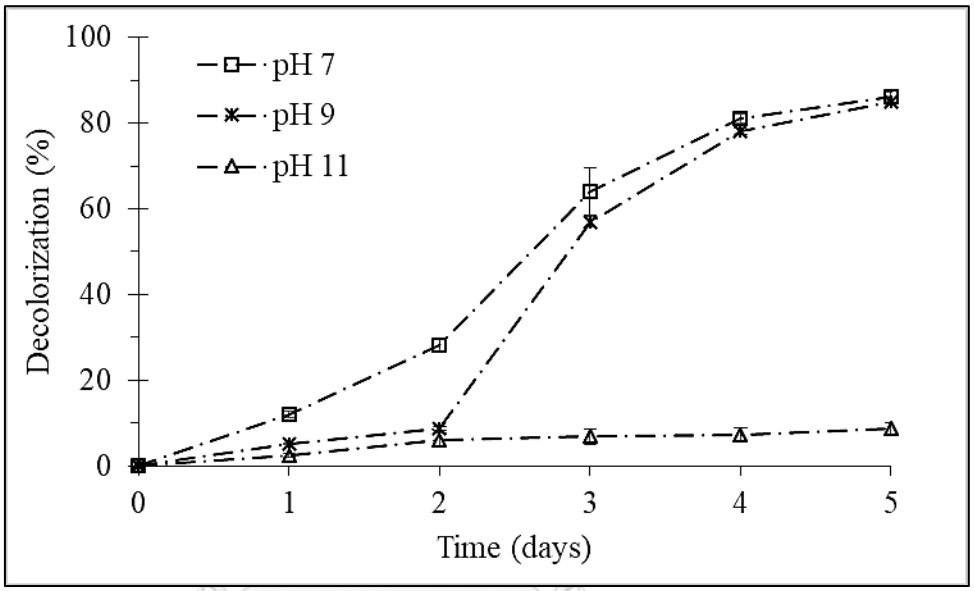



**Figure 30.** Effect of pH on RB-5 decolorization by *Micrococcus* sp. at room temperature, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

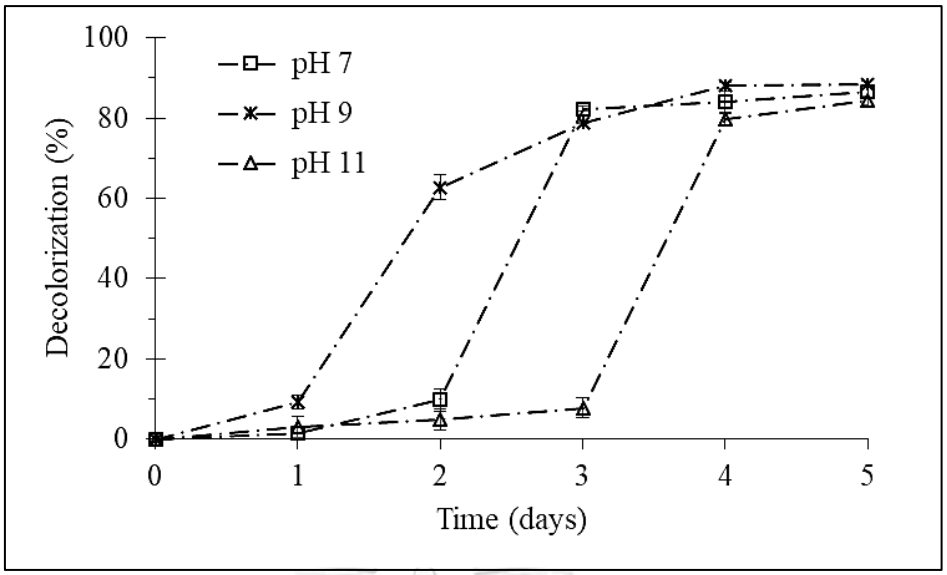



**Figure 31.** The effect of pH to the decolorization performance by *Janibacter* sp. at room temperature, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

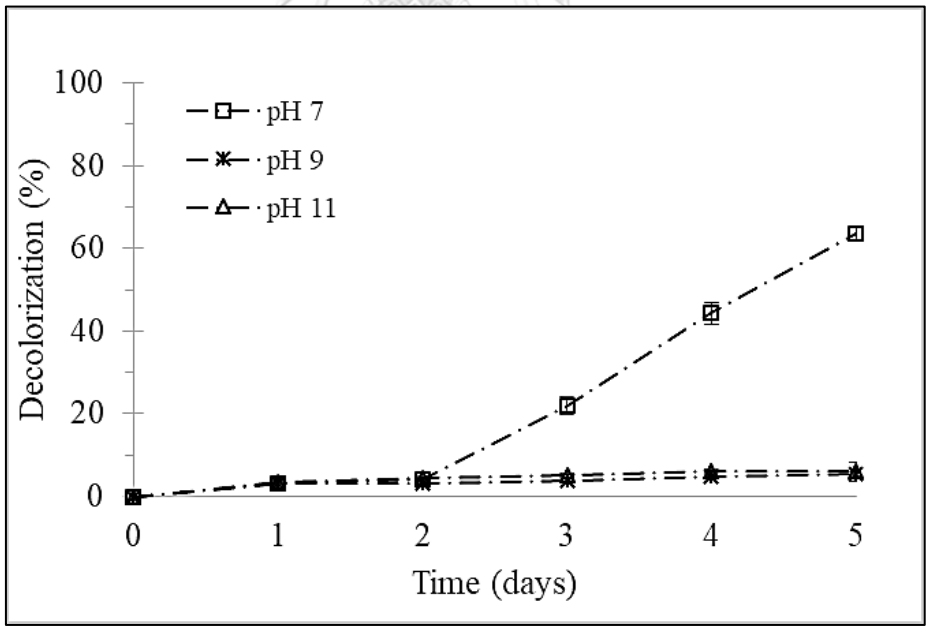



**Figure 32.** The effect of pH to the decolorization performance by *Rhodococcus* sp. at room temperature, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

*Janibacter* sp. showed a good performance for all pH values that have been applied in the system. Even at pH 11, surprisingly this strain could give the efficiency up to 84.5% at day 5 after given its optimum shaking period, compared to 17% decolorization only with 1-day shaking (Figure A5, Appendix). By the fact that real textile wastewater is at basic pH and there is no report on the decolorization ability of this strain yet, this finding could become a good potential for the real application of wastewater treatment plant.

The consortium did not yield as high efficiency as *Janibacter* sp., but with only one-day shaking period, this culture could give 87.9% decolorization at pH 9 as the highest efficiency (Figure 33). The consortium also provided high efficiency at pH 11, which was 82.4% even though only with one-day shaking period, while other strains as being mentioned before could not work well with one-day shaking only. This could be another advantage of working with consortium and the results indicated a good future application for a wastewater treatment plant because the consortium only need one-day aeration and could adapt with more basic range of pH.

Figure 34 summarized the optimum condition for each culture. Based on the obtained results for all the cultures, the best conditions were selected to be used for the kinetic study.

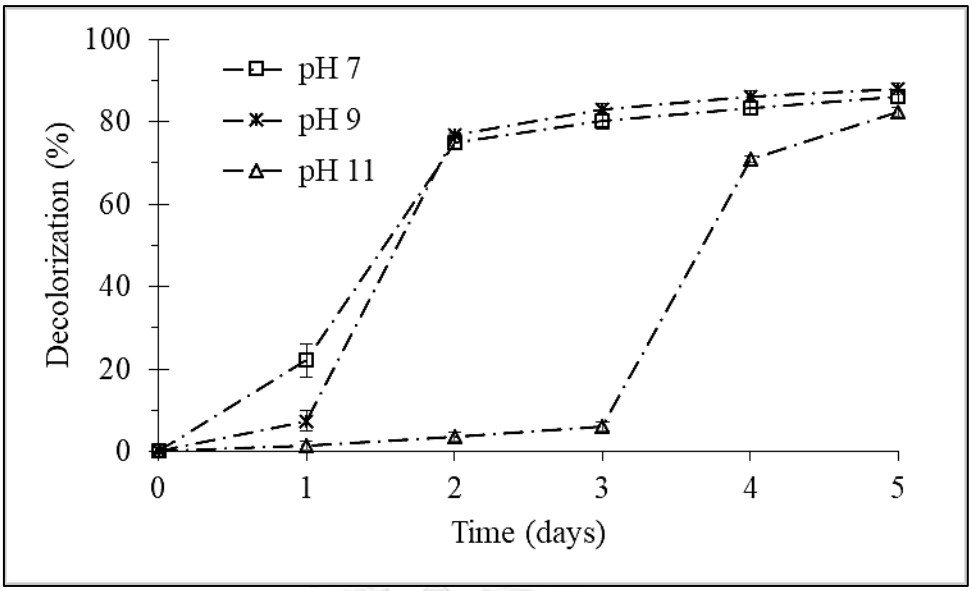



**Figure 33.** The effect of pH to the decolorization performance by consortium at room temperature, 150 rpm for the dynamic stage, inoculum concentration of $10^8$ CFU/mL and RB-5 concentration of 100 mg/L The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

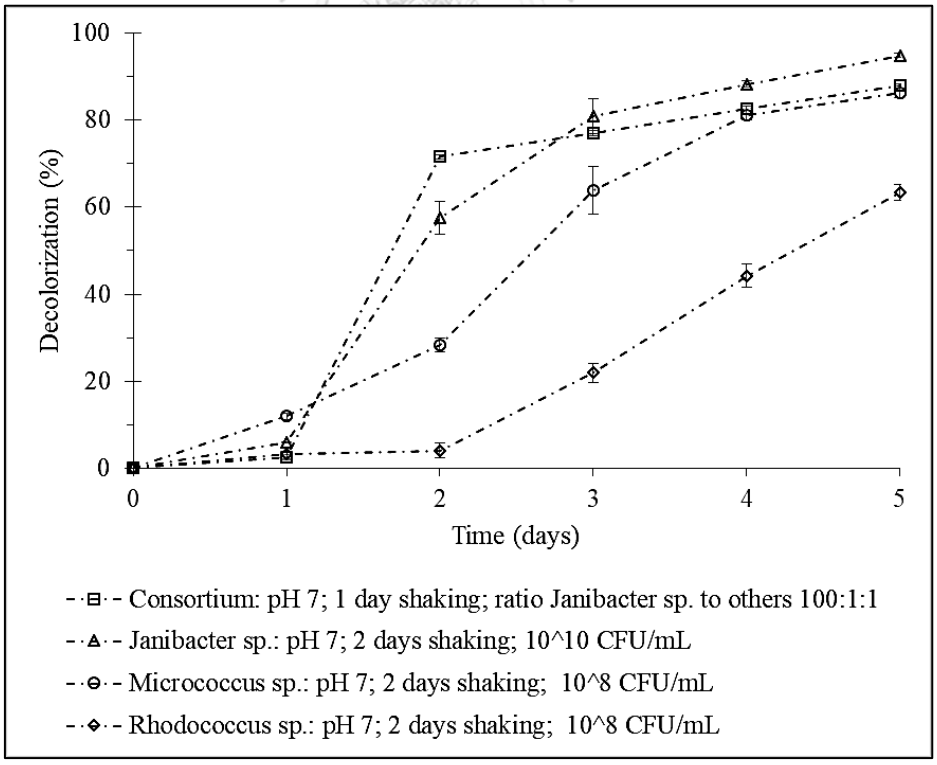



**Figure 34.** Optimum conditions of RB-5 decolorization by each culture. The incubation period expressed as days are represented in x-axis and he decolorization performances as percentage in y-axis.

### 4.4 Effect of RB-5 concentration and kinetic study

The initial RB-5 concentration could affect the decolorization performance due to the toxicity of RB-5 dye, which reduces the viability of the bacteria in the toxic environment. Figure 35 showed the effect of initial dye concentration on the efficiency of RB-5 decolorization. *Micrococcus* sp. could decolorize up to 85.9% for the RB-5 concentration of 400 mg/L at day 5, also *Janibacter* sp. gave the highest decolorization at the same concentration and incubation time with the efficiency of 92%. *Rhodococcus* sp. gave the lowest decolorization efficiencies among all as the RB-5 dye concentration increased with the maximum decolorization only 40% for dye concentration of 200 mg/L. The consortium did not only show similar decolorization efficiency to those of *Micrococcus* sp. and *Janibacter* sp., but it also yielded highest efficiency at the dye concentration of 1000 mg/L (84.35%) with only 1-day shaking period. Moreover, the consortium showed fastest decolorization, which is more than 60% decolorization in 2 days, compared with the pure cultures.

The varied dye concentrations could be used to determine the rate constants of the RB-5 decolorization according to the first-order kinetics (Das et al., 2017 ). By plotting the values of ln [$C_t/C_0$] against time, the rate constant can be obtained from the slope. Table 5 showed the first-order kinetic constants that mostly declined as the concentration increased up to 1000 mg/L, but the correlation coefficients ($R^2$) for *Janibacter* sp. at the concentration of 1000 mg/L and for all concentrations of the consortium indicated that these two types of cultures did not follow first-order kinetics during RB-5 decolorization.

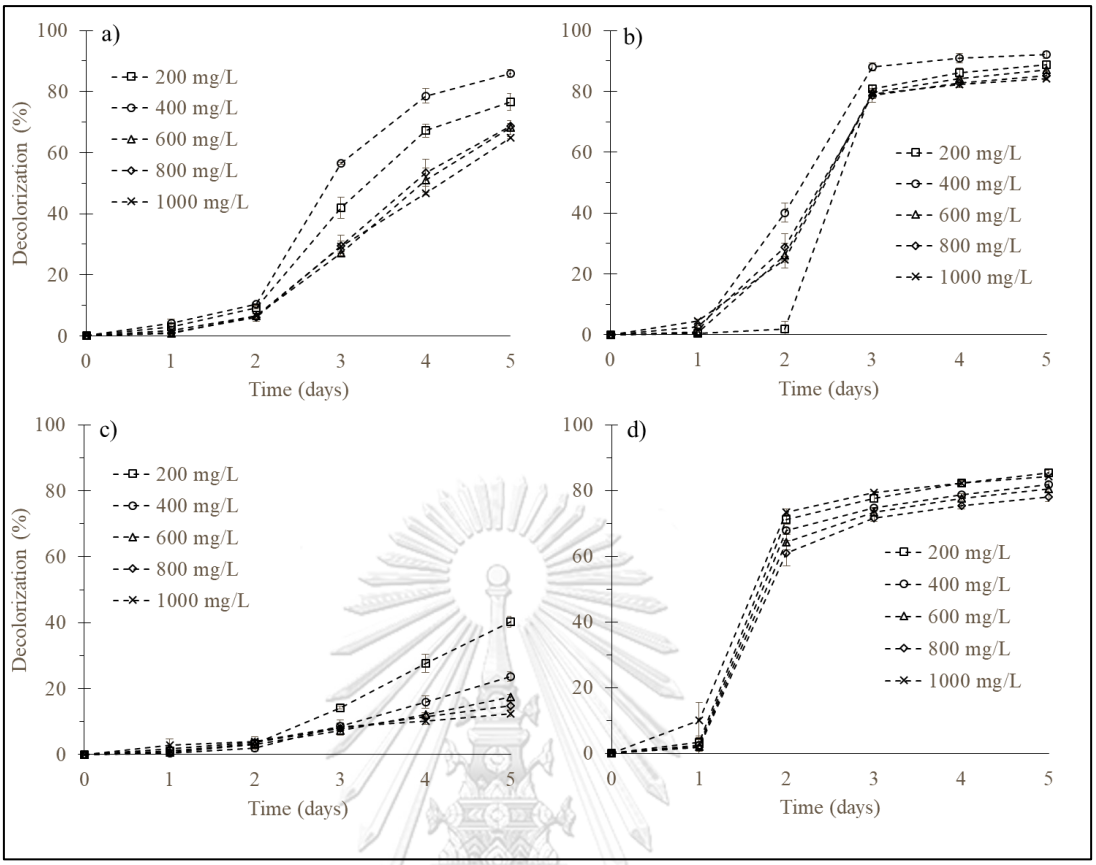



**Figure 35.** Effect of initial dye concentration on RB-5 decolorization by: a) *Micrococcus* sp. b) *Janibacter* sp. c) *Rhodococcus* sp. and d) consortium at room temperature, pH 7, 150 rpm for the dynamic stage and inoculum concentration of $10^8$ CFU/mL. The incubation period expressed as days are represented in x-axis and the decolorization performances as percentage in y-axis.

**Table 5.** The rate constants and decolorization rates of the RB-5 decolorization obtained by first-order kinetics

a) *Micrococcus* sp.

| Values | Concentrations | | | | |
|---|---|---|---|---|---|
| | 200 mg/L | 400 mg/L | 600 mg/L | 800 mg/L | 1000 mg/L |
| k (constants) | 0.529 | 0.6584 | 0.3149 | 0.3373 | 0.2848 |
| $R^2$ | 0.9456 | 0.9534 | 0.9312 | 0.9379 | 0.9377 |
| v (rate) | 105.8 | 263.36 | 188.94 | 269.84 | 284.8 |

b) *Janibacter* sp.

| Values | Concentrations | | | | |
|---|---|---|---|---|---|
| | 200 mg/L | 400 mg/L | 600 mg/L | 800 mg/L | 1000 mg/L |
| k (constants) | 1.4069 | 1.125 | 0.6528 | 0.6416 | 0.5908 |
| $R^2$ | 0.9431 | 0.9375 | 0.9135 | 0.9056 | 0.8748 |
| v (rate) | 281.38 | 450 | 391.68 | 513.28 | 590.8 |

c) *Rhodococcus* sp.

| Values | Concentrations | | | | |
|---|---|---|---|---|---|
| | 200 mg/L | 400 mg/L | 600 mg/L | 800 mg/L | 1000 mg/L |
| k (constants) | 0.1413 | 0.0734 | 0.0492 | 0.0395 | 0.0291 |
| $R^2$ | 0.9358 | 0.9524 | 0.9769 | 0.9907 | 0.9728 |
| v (rate) | 28.26 | 29.36 | 29.52 | 31.6 | 29.1 |

d) Consortium

| Values | Concentrations | | | | |
|---|---|---|---|---|---|
| | 200 mg/L | 400 mg/L | 600 mg/L | 800 mg/L | 1000 mg/L |
| k (constants) | 0.5387 | 0.4545 | 0.415 | 0.4092 | 0.4604 |
| $R^2$ | 0.866 | 0.8318 | 0.8404 | 0.846 | 0.7935 |
| v (rate) | 107.74 | 181.8 | 249 | 327.36 | 460.4 |



Figure 36 showed the relationship between the decolorization rate and concentration of RB-5. For *Janibacter* sp. and consortium, the data suggested that higher the RB-5 concentration resulted in faster the decolorization. *Micrococcus* sp. seemed to have similar trend but with only the slight increase. Both strains had high decolorization rates at the RB-5 concentration of 400 mg/L, making the rate at 600 mg/L seemed declined. *Rhodococcus* sp. gave the lower rate of decolorization and even declined at the concentration of 1000 mg/L.

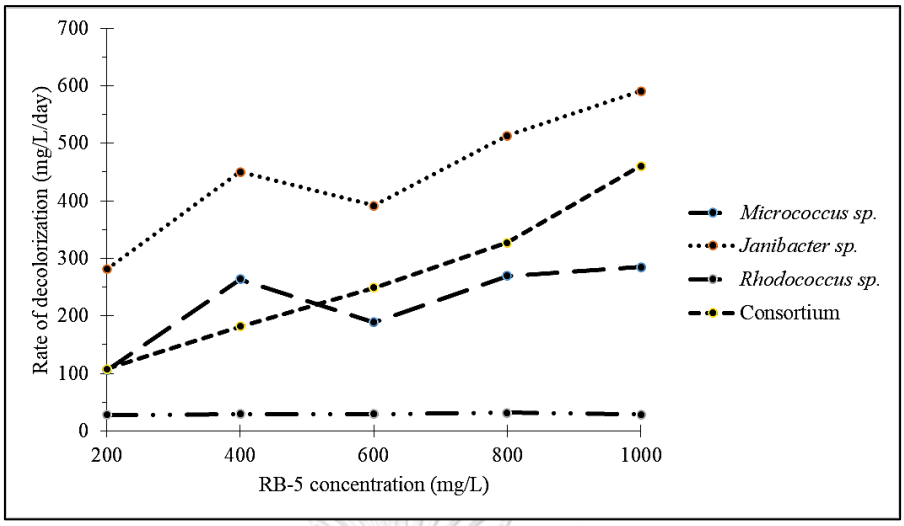



**Figure 36.** The relationship between the rate of RB-5 decolorization and RB-5 concentration. The values of RB-5 concentrations are represented in x-axis. The RB-5 decolorization rates are represented in y-axis.

To find the maximum value of decolorization rate, the Michaelis-Menten type rate model was used. The Michaelis-Menten type rate model could help to understand the work of the system by determining the value of Michaelis-Menten constant ($K_m$) and the maximum decolorization rate ($V_m$). Figure 37 showed the double reciprocal plot of the rate constants with the RB-5 concentrations.

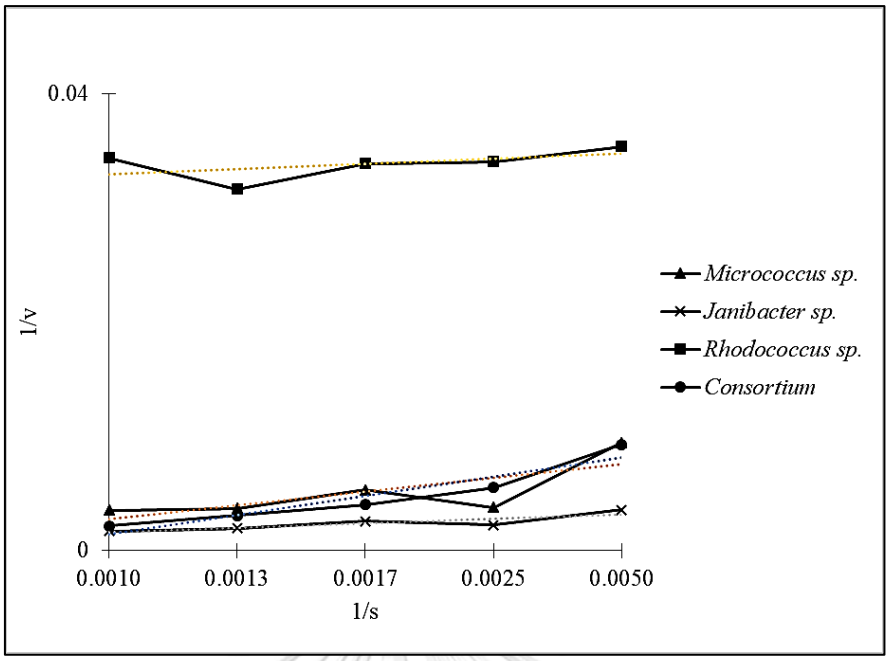



**Figure 37.** The Michaelis-Menten kinetics for the RB-5 decolorization. The reciprocal values of RB-5 concentrations are represented in x-axis and rates are represented in y-axis.

From Figure 37, the intercept and slope were obtained and used for calculating the $K_m$ and $V_m$ of the RB-5 decolorization. The result (Table 6) showed the maximum decolorization rates ($V_m$) that could be achieved when the concentration of RB-5 was higher than different levels ($K_m$) for each bacterial strains. However, these results seemed unpromising because the linearity of the graph was very low. To get the better linearity, it is suggested that the RB-5 initial concentrations should be adjusted to be higher than 1000 mg/L and the gap of concentrations observed should be lowered to obtain the clear decolorization trend.

**Table 6.** The Michaelis-Menten constants and maximum decolorization rates of RB-5

| | ***Micrococcus* sp.** | ***Janibacter* sp.** | ***Rhodococcus* sp.** | **Consortium** |
|---|---|---|---|---|
| Intercept | 0.0016 | 0.0012 | 0.0325 | 0.0002 |
| $V_m$ | 625 | 833.3333 | 30.7692 | 5000 |
| Slope | 0.0012 | 0.0004 | 0.0004 | 0.0017 |
| $K_m$ | 0.75 | 0.3333 | 0.0123 | 8.5 |

For the RB-5 decolorization observed in this study, a specific mechanism might be used to explain the work of each bacterium in the cultures. *Rhodococcus* sp. could decolorize the dye with the help of dye-decolorizing peroxidases (DyPs) as *Rhodococcus* sp. was classified as the lignin degrader (Vignali et al., 2018); . Figure 38 explained the work of DyPs in the decolorization of Reactive Blue 5 ((Sugano et al., 2006).

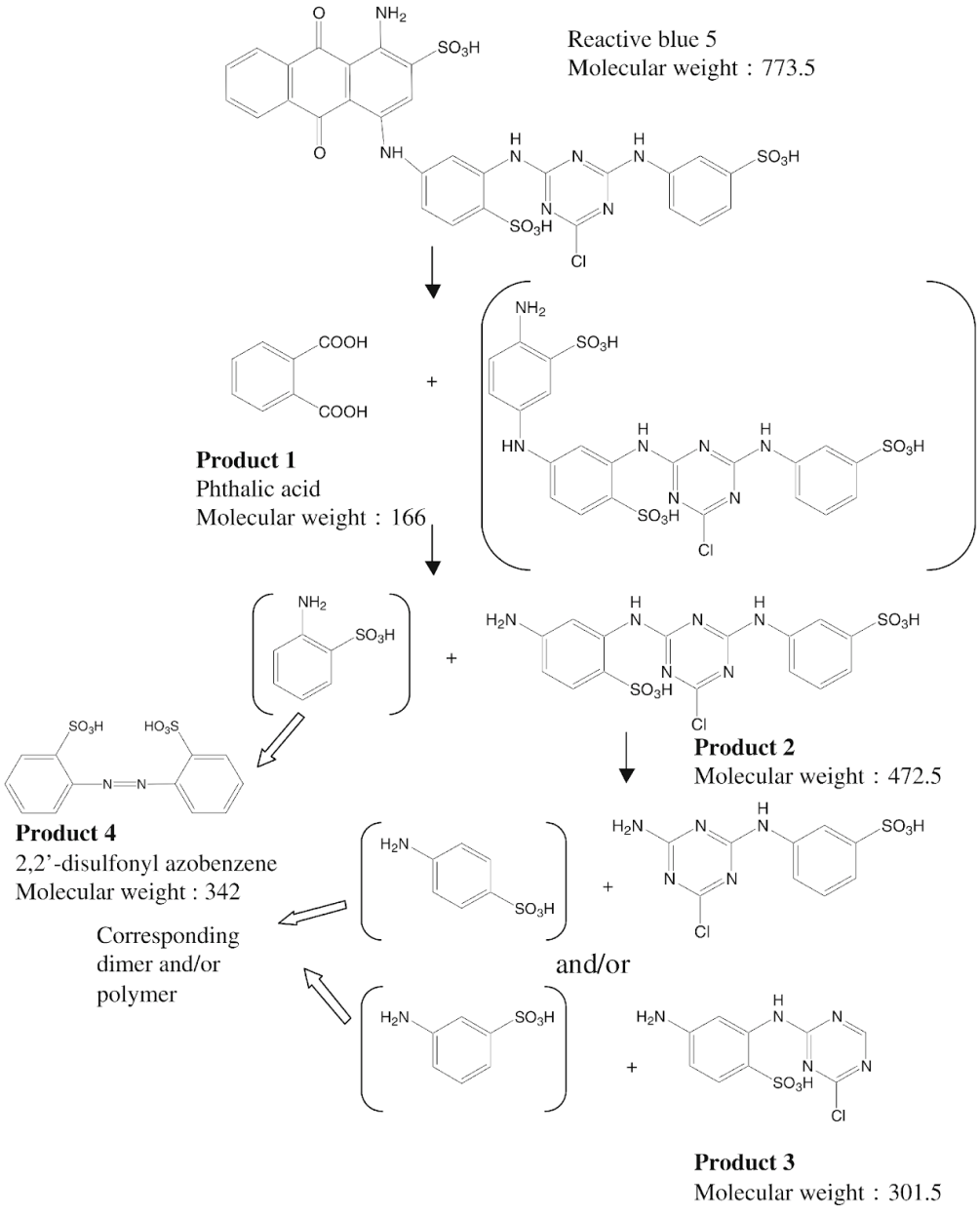



**Figure 38.** The mechanism of Reactive Blue 5 decolorization by DyP
Source: Sugano et al. (2006).

*Micrococcus* sp. might utilize laccase as dye-degrading enzyme, reported in Malachite Green decolorization (Du et al., 2013), the mechanism was shown in Figure 39. The characteristic of the enzyme's work was the cleavage at the C–C bonds (Du et al., 2013).

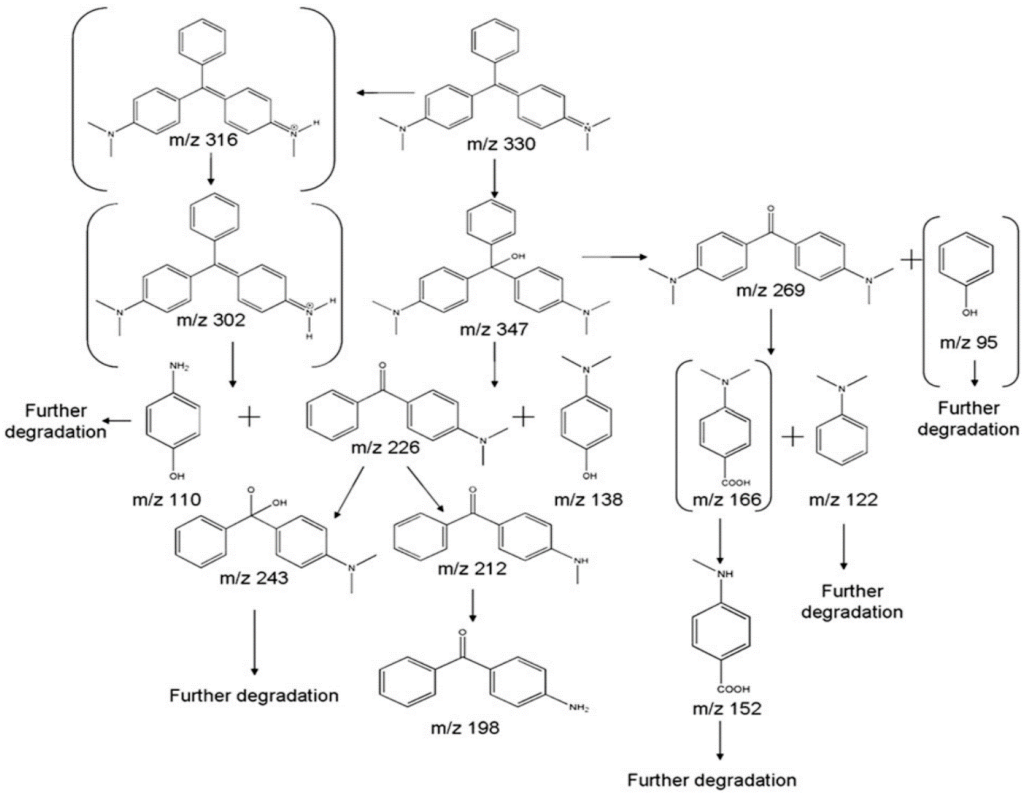



**Figure 39.** The proposed degradation pathway of Malachite Green by laccase enzyme from *Micrococcus* sp.
Source: Du et al. (2013).

The similar sulfonated azo dye, Reactive Red dye, was reported to be degraded with laccase as well, produced by *Pseudomonas aeruginosa* ((Jadhav et al., 2011)). The mechanism was shown in Figure 40.

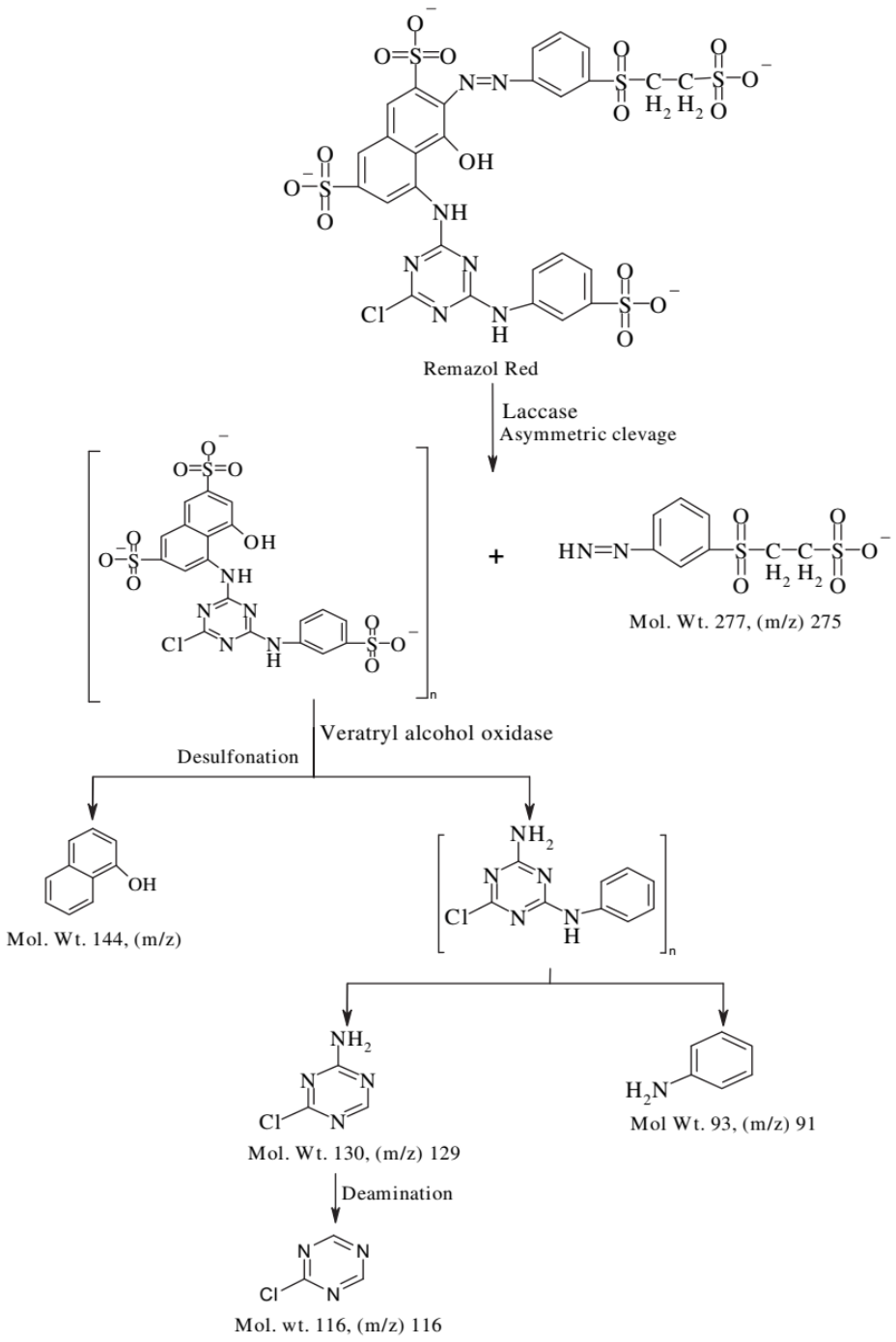



**Figure 40.** The mechanism of Reactive Red degradation by laccase enzyme from *Pseudomonas aeruginosa*
Source: Jadhav et al. (2011).

Different with the other two bacteria, *Janibacter* sp. so far has no report on the mechanism of dye decolorization activity. Thus, finding the possible decolorization pathway can be made by understanding the main character of *Janibacter* sp., which was

a halotolerant bacterium (Khessairi et al., 2014). *Planococcus* sp., which share the same characteristic as a halotolerant bacterium as *Janibacter* sp., had been reported to degrade the sulfonated azo dye, Orange I (Ma et al., 2013). Figure 41 showed the proposed degradation mechanism (Ma et al., 2013).

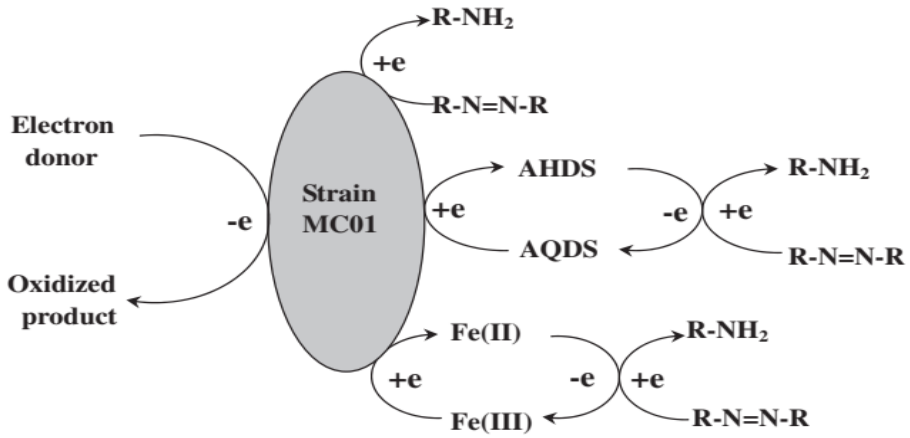



**Figure 41.** The proposed degradation mechanism of Orange I
Source: Ma et al. (2013).

To validate all the decolorization results shown in this research, the control samples of both single cultures and consortium as well as the abiotic one were measured to determine the chance of bacterial cells absorption and/or degradation by light. Figure 42 showed the result that there were almost no decolorization reported in each of the control sample.



Finally, Table 7 summarized the best decolorization efficiency with the limitation of working conditions, compared to the similar research. It is shown that the results from this research seems promising to be applied with RB-5 wastewater treatment.

**Table 7.** The comparison of this research and the previous similar research

| | **Khan *et al*. (2015)** | **This research** |
|---|---|---|
| Highest efficiency | 93% | 94.8% |
| Incubation time | 120 hours | 120 hours |
| Temperature | 30 – 37°C | 28 – 32°C |
| pH | 5 – 9 | 7 – 11 |
| Max. dye concentration | 500 mg/L | 1000 mg/L |
| Incubation condition | static | 2-day shaking + 3-day static |

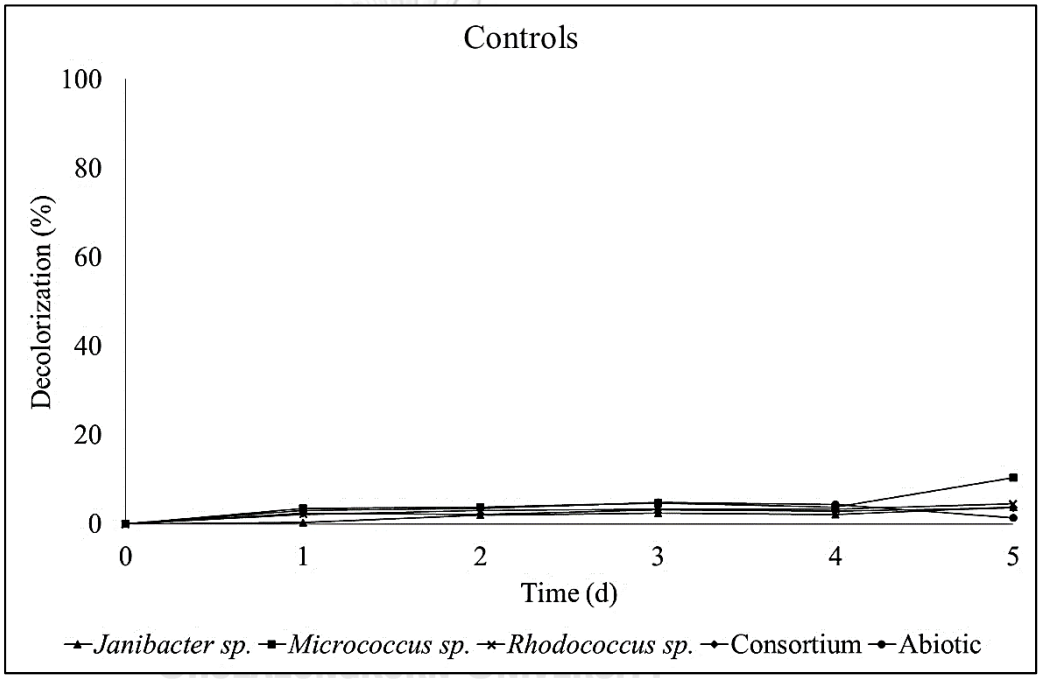



**Figure 42.** RB-5 decolorization of control samples.

# CHAPTER 5
# CONCLUSIONS

1. The highest decolorization efficiencies for all single strain cultures as well as the consortium were obtained with the combination of static and dynamic conditions. For all single strains, the optimum shaking conditions were two days. For the consortium, only one-day shaking period could yield the highest efficiency. The results indicated that working with consortium would be an advantage in the reduction of aeration time.

2. From the study of the effect of environmental conditions on RB-5 decolorization, the optimum conditions for each strain are as follows:

| **Strains** | **Shaking condition** | **pH** | **Temperature** | **Initial cell concentration** |
|---|---|---|---|---|
| *Micrococcus* sp. | 2-day shaking and 3-day static | 7 | 30-35 °C | $10^8$ CFU/mL |
| *Janibacter* sp. | 2-day shaking and 3-day static | 7 | 30-35 °C | $10^{10}$ CFU/mL |
| *Rhodococcus* sp. | 2-day shaking and 3-day static | 7 | 30-35 °C | $10^8$ CFU/mL |
| Consortium | 1-day shaking and 3-day static | 7 | 30-35 °C | $10^8$:$10^{10}$:$10^8$ CFU/mL* |

*for the cell concentration of *Micrococcus* sp., *Janibacter* sp., and *Rhodococcus* sp., respectively.

3. All the decolorization of RB-5 by single strains followed the first-order kinetics, but not the consortium.

4. The next research should be focused on the enhancement of decolorization rate along with the reduction of incubation period. This enhancement could be achieved by using organic waste as the C-source, which could be more beneficial to wastewater treatment. Another enhancement could be done by doing enzyme assays for each bacteria to ensure the specific enzyme responsible for decolorization. By knowing

the specific enzymes, studies could be conducted to avoid the condition that could inhibit the enzyme to work.

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# APPENDIX

## APPENDIX 1

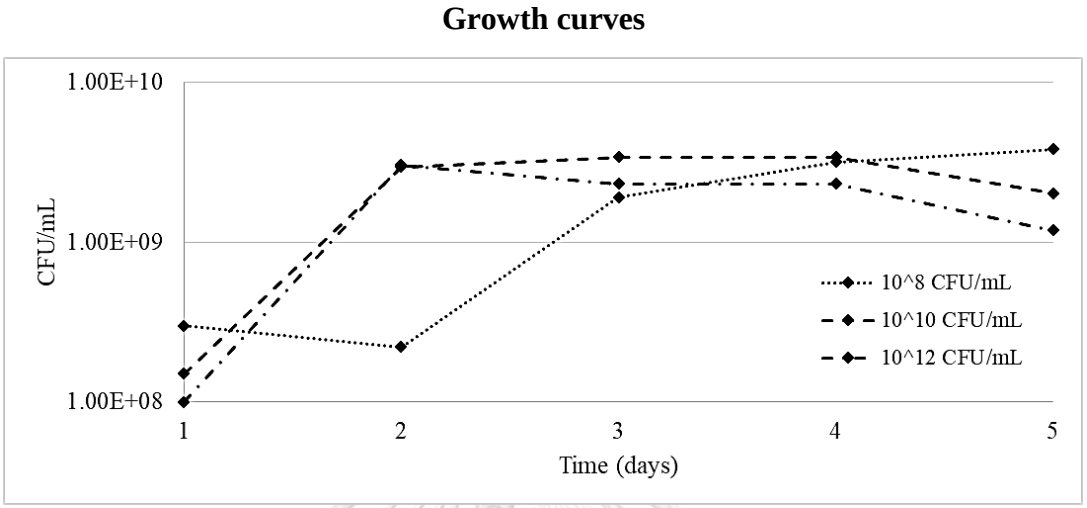


**Figure A1.** Growth of *Micrococcus* sp. with different initial cell concentrations during RB-5 decolorization process. The operating system was 2-day shaking and 3-day static conditions. The x-axis represents the decolorization period in days. The y-axis represents the number of cells in CFU/mL. The concentration of RB-5 dye was 100 mg/L.

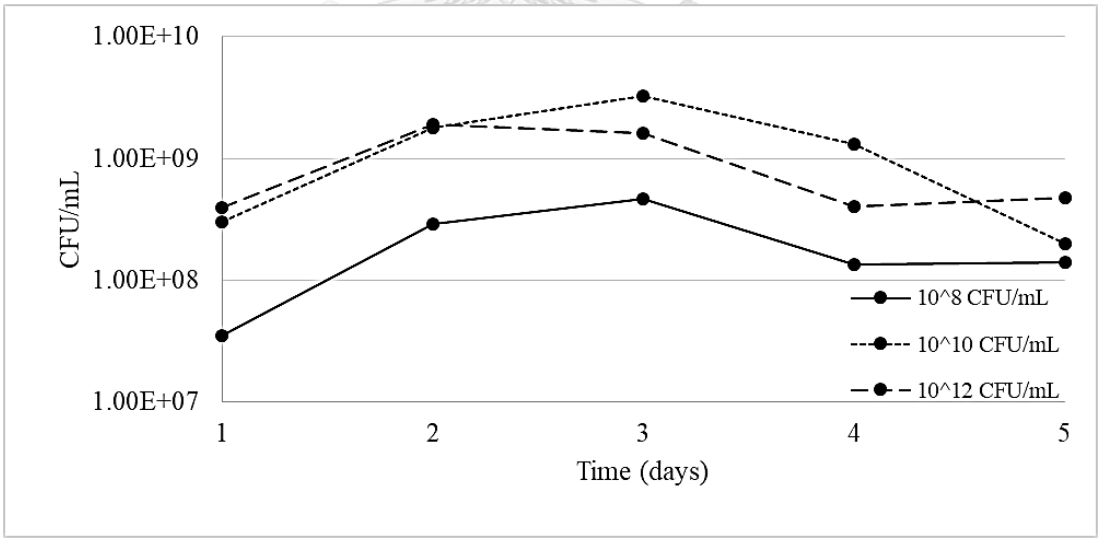


**Figure A2.** Growth of *Janibacter* sp. with different initial cell concentrations during RB-5 decolorization process. The operating system was 2-day shaking and 3-day static conditions. The x-axis represents the decolorization period in days. The y-axis represents the number of cells in CFU/mL. The concentration of RB-5 dye was 100 mg/L.

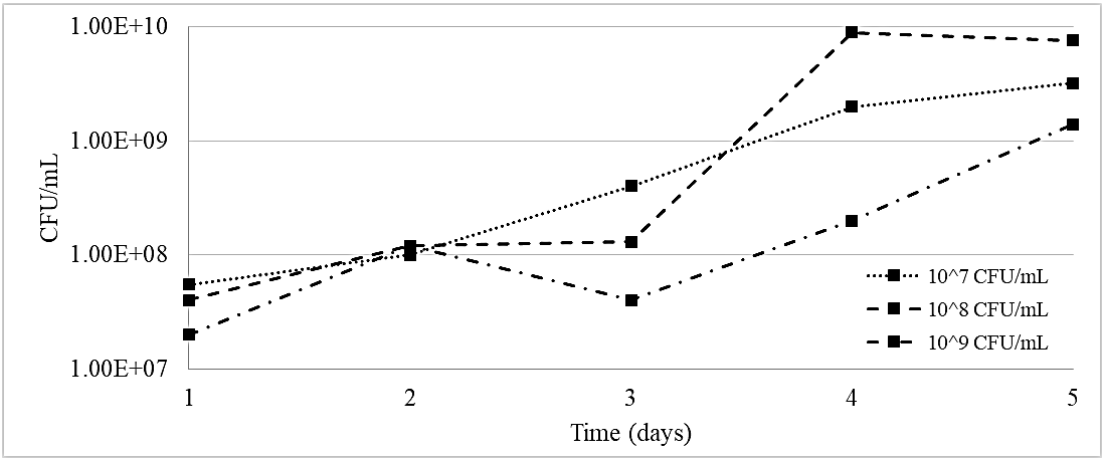


**Figure A3.** Growth of *Rhodococcus* sp. with different initial cell concentrations during RB-5 decolorization process. The operating system was 2-day shaking and 3-day static conditions. The x-axis represents the decolorization period in days. The y-axis represents the number of cells in CFU/mL. The concentration of RB-5 dye was 100 mg/L.

## APPENDIX 2

**pH study**

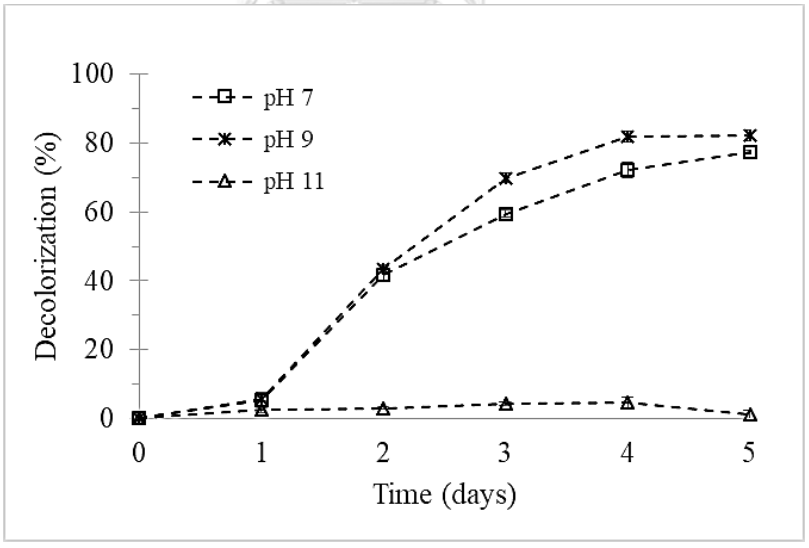


**Figure A4.** Effect of pH on RB-5 decolorization by *Micrococcus* sp. with one day shaking period. The incubation period expressed as days are represented in x-axis. The decolorization performances during incubation period are represented in y-axis. The concentration of RB-5 dye was 100 mg/L. The standard deviations are based on three separate samples.

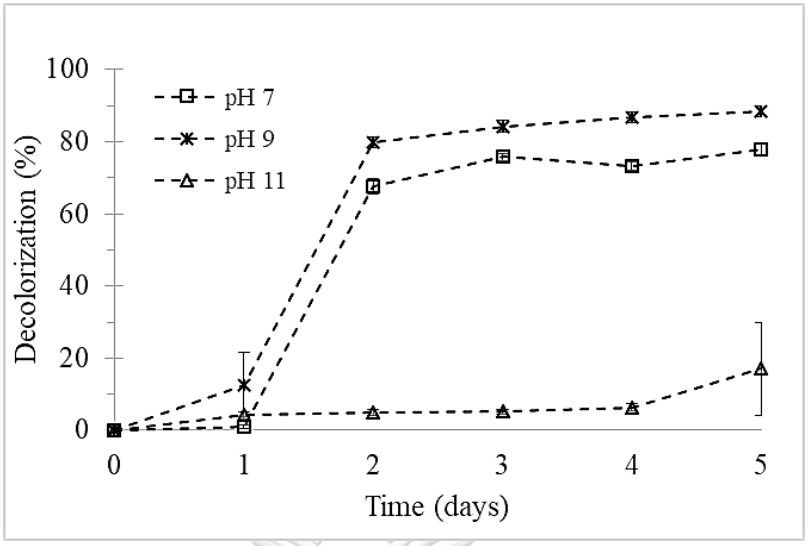


**Figure A5.** The effect of pH to the decolorization performance by *Janibacter* sp. with one-day shaking period. The initial cell concentrations were $10^8$ CFU/mL. The incubation period expressed as days are represented in x-axis. The decolorization performances during incubation period are represented in y-axis. The concentration of RB-5 dye was 100 mg/L. The standard deviations are based on three separate samples.

## APPENDIX 3

### Statistical analysis

**Table A1.** Statistical analysis of the effect of initial cell concentration on the decolorization performance (the P-values from 2-tail t-tests with unequal variances)



| 2-tail unequal | | 10^8 CFU/mL | 10^10 CFU/mL |
|---|---|---|---|
| | **10^8 CFU/mL** | | |
| *Micrococcus* sp. | **10^10 CFU/mL** | **0.6642** | |
| | **10^12 CFU/mL** | **0.8054** | **0.4082** |
| | **10^8 CFU/mL** | | |
| *Janibacter* sp. | **10^10 CFU/mL** | 1.00E-04 | |
| | **10^12 CFU/mL** | 0.007 | 0.0102 |
| | | **10^7 CFU/mL** | **10^8 CFU/mL** |
| | **10^7 CFU/mL** | | |
| *Rhodococcus* sp. | **10^8 CFU/mL** | **0.1323** | |
| | **10^9 CFU/mL** | **0.089** | **0.126** |

**Table A2.** Statistical analysis of the effect of shaking period on the decolorization performance (the P-values from 2-tail t-tests with unequal variances)

| | 2-tail unequal | 1d dyn.; 4d stat. | 2d dyn.; 3d stat. |
|---|---|---|---|
| | 1d dynamic; 4d static | | |
| *Micrococcus* sp. | 2d dynamic; 3d static | 0.0011 | |
| | 3d dynamic; 2d static | **0.4242** | **0.0601** |
| | 1d dynamic; 4d static | | |
| *Janibacter* sp. | 2d dynamic; 3d static | 0.0023 | |
| | 3d dynamic; 2d static | 0.0057 | 0.0141 |
| | 1d dynamic; 4d static | | |
| *Rhodococcus* sp. | 2d dynamic; 3d static | 7.06E-05 | |
| | 3d dynamic; 2d static | 0.0141 | **0.1563** |

## APPENDIX 4

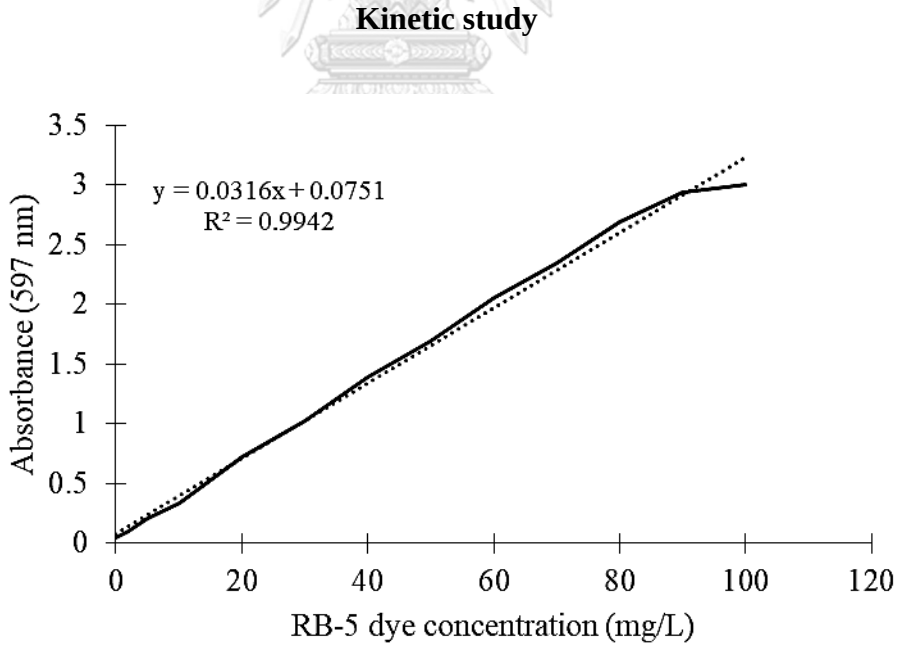


**Figure A6.** Standard curve of the RB-5 decolorization study

**Table A3.** The rate of decolorization by *Micrococcus* sp.

| | 1d dynamic, 4d static | 2d dynamic, 3d static | 3d dynamic, 2d static |
|---|---|---|---|
| $R^2$ | 0.9958 | 0.9654 | 0.9208 |
| k | 0.4981 | 0.8069 | 0.4454 |
| v | 9.962 | 16.138 | 8.908 |

**Table A4.** The rate of decolorization by *Janibacter* sp.

| | 1d dynamic, 4d static | 2d dynamic, 3d static | 3d dynamic, 2d static |
|---|---|---|---|
| $R^2$ | 0.712 | 0.9549 | 0.9858 |
| k | 0.4339 | 0.9224 | 1.1713 |
| v | 8.678 | 18.448 | 23.426 |

## APPENDIX 5

### Sequence data

***Gordonia terrae* strain NIBGE-HS3 16S ribosomal RNA gene, partial sequence**

CCCAATCGCCGATCCCACCTTCGACAGCTCCCTCCCACAAGGGGTTAGGCCACCG
GCTTCGGGTGTTACCGACTTTCATGACGTGACGGGCGGTGTGTACAAGGCCCGGG
AACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATG
GGGTCGAGTTGCAGACCCCAATCCGAACTGAGACTGGCTTTAAGGGATTCGCTCC
ACCTCACGGTATCGCAGCCCTCTGTACCAGCCATTGTAGCATGTGTGAAGCCCTG
GACATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCC
GGCAGTCTCCTGCAAGTCCCCGGCATAACCCGCTGGCAATACAGGACAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCAT
GCACCACCTGTACACCAACCACAAGGGAACGACTATCTCTAGCCGCGTCTGGTGT
ATGTCAAACCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCACATGCTCCG
CCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCC
CCAGGCGGGGTACTTAATGCGTTAGCTACGGCACGGATCCCGTGAAAAGGAACC
CACACCTAGTACCCACCCTTCTACGGCGTGGACTACCAGGGTATCTAATCCTGTT
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CACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCAGTC
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ACCCCACCAACAAGCTGATAGGCCGCGGGCCCATCCCACACCGCAAAAGCTTTCC
ACCAACCACCATGCGACAGTTGGTCATATCCGGTATTAGACCCAGTTTCCCAGGC

TTATCCCAGAGTGCAGGGCAGATCACCCACGTGTTACTCACCCGTTCGCCACTCG
AGTACCCAGCAAGCTGGGGCCTT

***Rhodococcus* sp. PBTS1, complete genome**

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TTTCCCGGGCTTATCCCGAAGTGCAGGGCA

***Bacillus flexus* strain MSBC2 16S ribosomal RNA gene, partial sequence**

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***Microbacterium* sp. PSSUR4 16S ribosomal RNA gene, partial sequence**

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***Micrococcus* sp. HB241 16S ribosomal RNA gene, partial sequence**

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AGGCCGCGAGTCCATCCAAAACCGATAAATCTTTCCAACACCCACCATGCGGTGG
ACGCTCCTATCCGGTATTAGACCCAGTTTCCCAGGCTTATCCCAGAGTTAAGGGC
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TTCATCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATCCTGAGCGAG
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***Janibacter* sp. BSi20546 16S ribosomal RNA gene, partial sequence**

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TTGCGTCTCGTATCCGGTATTAGACGCCGTTTCCAGCGCTTATTCCAGAGT
CCGGGGCAGGTTACTCACGTGTTACTCACCCGTTCG

# VITA

| | |
|---|---|
| **NAME** | Dita Puspita Sari |
| **DATE OF BIRTH** | 18 May 1992 |
| **PLACE OF BIRTH** | Yogyakarta, Indonesia |
| **INSTITUTIONS ATTENDED** | Bachelor's degree: Universitas Gadjah Mada |
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| **PUBLICATION** | Hartono, M., Sari D.P., Petrus, H.T.B.M., Sulistyo, H. dan Budhijanto, W., 2015, Seleksi Bakteri untuk Bioleaching Litium dari Baterai Ion Litium Bekas, Prosiding Seminar Teknik Kimia Indonesia 2015.<br><br>Murti, W., Sari D.P., Sulistyo, H., Petrus, H.T.B.M. dan Budhijanto, W., 2015, Seleksi Jamur untuk Bioleaching Litium dari Limbah Baterai Litium-Ion (LIB) Bekas, Prosiding Seminar Teknik Kimia Indonesia 2015.<br><br>Sari., D.P., Sairiam, S. and Thuptimdang P., 2018, Reactive Black 5 Decolorization by Soil Bacteria using the Combined Dynamic-Static Condition, IWADP 2018 Conference Proceeding.<br><br>Chandra W. Purnomo, Dita P. Sari, Yanisa Laoong-u-thai, and Respati T. Swasono, International Journal of Chemical Engineering and Applications vol. 10, no. 2, pp. 46-49, 2019. |
| **AWARD RECEIVED** | Beasiswa Peningkatan Prestasi Akademik, Universitas Gadjah Mada, 2010<br><br>ASEAN Scholarship, Chulalongkorn University, 2017 |