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BIODEGRADATION OF METHYLENE BLUE BY BACTERIA STRAINS ISOLATED FROM CONTAMINATED SOIL

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ABSTRACT

Methylene blue is one of the textile dyes that are commonly used in the textile coloring industry. The discharge of textile wastewater containing residual textile coloring substances into the environment can lead to environmental pollution. Thus, bioremediation can be a solution to reduce dye pollution by using bacterial strains. In the present study, two bacterial strains with the ability to degrade methylene blue dye were isolated from contaminated soil. Both isolated bacteria were further evaluated for the dye decolorization percentage and the effect of abiotic parameters on bacterial growth. The isolated bacteria were incubated in a mineral salt medium added with methylene blue dye for eight days in static aerobic conditions. The dye degradation was examined by using UV-VIS spectrophotometer at 665 nm. The isolated bacteria were identified as *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP) using 16s rRNA sequencing. The biodegradation study showed that *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP) were capable to degrade 10.52% and 11.65% of methylene blue dye after 8 days of incubation, respectively. The present study may provide a basis for biotreatment and bioremediation of dye-contaminated soils.

Key words: Biodegradation, dye, methylene blue dye, soil bacteria

INTRODUCTION

The textile manufacturing industry has been one of the most popular sectors nowadays and is among each country's primary industries. In Malaysia, the textile industry contributes to the Gross Domestic Product (GDP) by about 1.2% which is significantly important for the economy (Ślusarczyk *et al*., 2019). Along with the textile industry's popularity, dyes have played a major role as a textile colorant. It is expected that textile industries have accounted for the extensive consumption of dyestuffs which is nearly 80% (Vimala *et al*., 2015). Dyes exist in different structures that give them other names like azo, reactive, disperse, basic, acidic, metal-complex, and anthraquinone-based dye. According to Semeraro *et al*. (2015), more than 10,000 tons per year and around 100 tons/year of dyes are released into wastewater. Besides being used as a colorant in textiles, these dyes are also widely used in the printing, pharmaceutical, and food industries (Gayathri *et al*., 2014).

Wastewater discharged from textile industries contains many pollutants including textile dyes (Yaseen & Scholz, 2019). Improper discharge of textile dyes into wastewater can lead to pollution. Even at a concentration as low as 1 mg/L (1 ppm), the dyes can easily be visible and detected by naked eyes (Guaratini & Zanoni, 2000). According to Vimala *et al*. (2015), 50% of the textile effluent that has been discharged contains a residual dye and it is challenging to be removed by conventional treatment. Thus, dye pollution will also affect groundwater due to soil leaching (Sodeinde & Eboreime, 2013). Textile dyes can persist in the environment and are difficult to degrade due to their complex structure (Sodeinde & Eboreime, 2013). The biological transformation of these dyes may produce aromatic amines that can be absorbed dermally and are known to have genotoxic and carcinogenic properties (Brüschweiler & Merlot, 2017). This aromatic amine released during the breakdown of the dye linkages will turn the dye colorless (Jaiswal & Gomashe, 2017). Usually, the biological decolorization of the dye is done under anaerobic conditions. However, aromatic amines and other carcinogenic and mutagenic metabolites are still being released from the decolorized dyes (Kausar *et al*., 2016).

Various physical methods (adsorption, membrane filtration, ion exchange, and electrokinetic coagulation) and chemical methods (Fenton's reagent,

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ozonation, photochemical, and electrochemical destruction) have been proposed to remove the dye from textile wastewater effluents. Though all of these methods can efficiently decolorize and remove the dye from wastewater, most of it is not economically feasible for larger-scale use, can generate a large amount of sludge, and need a high disposal cost (Robinson *et al*., 2001). As a viable alternative, microbial decolorization of textile dyes has gained great interest because of their cost-effectiveness, less sludge production, environmentally friendly, and yields non-toxic end products.

Recent studies have shown that various microorganisms can decolorize a broad range of textile dyes (Banat *et al*., 1996; Saratele *et al*., 2011). Over the year, several microorganisms have been tested to decolorize and mineralize the azo dye under certain environmental conditions including fungi, bacteria, yeasts, and algae (Pandey *et al*., 2007). According to Banat *et al*. (1996), fungi decolorization results in slow processing and needs a longer time for optimum decolorization. In addition, some fungi only decolorize the dye color in the same species but do not affect the chromophore center of the dye. Thus, fungal decolorization is limited in large-scale decolorization (Chen *et al*., 2003). In contrast, bacterial decolorization can achieve a higher degree of color removal and complete mineralization of the dye under optimum conditions (Saratele *et al*., 2011).

The previous study by Jaiswal and Gomashe (2017), stated that discharging colored effluent into the water bodies has become a great deal since the dye effluent contains highly toxic material that can be carcinogenic and mutagenic to living organisms. In addition to the dye toxicity, pollution of the colored effluent on the water surface can reduce light penetration into inner layers interrupting the photosynthetic activities, lowering the oxygen content, and harming the aquatic life (Pandey *et al*., 2007; Abo-state *et al*., 2017). Thus, removing dye from wastewater has been a significant concern as they are generally challenging to degrade naturally and reductive cleavage of the dye structure can lead to the formation of aromatic amines that can be carcinogenic to living organisms (Pandey *et al*., 2007). To overcome the environmental pollution caused by textile dye contamination in the wastewater, textile dye degrading bacteria should display the ability to completely decolorize and mineralize textile dyes.

The bioremediation process uses the bacterial strain that can help reduce dye toxicity and pollution into the environment. Microorganisms used in the bioremediation process will feed on the aromatic amines as their carbon sources (Alabdraba & Bayati, 2014). Throughout this study, biodegradation by bacteria can reduce the environmental pollution caused by releasing textile dyes into wastewater. Biodegradation is the chemical dissolution process by using bacteria to degrade the dye structure and used the compound for their energy requirement and growth (Rumky *et al*., 2013). Thus, bacterial degradation of textile dye can help to mineralize and reduce the toxicity of the dye. Furthermore, this method is low in cost, has lower sludge production, and is environmentally friendly. Therefore, this study aims to isolate and identify bacterial strains with the ability to degrade textile dye, methylene blue. The bacterial strain that possessed the ability to degrade methylene blue dye will be selected for further study to determine their dye degrading activity.

MATERIALS AND METHODS

Preparation of the dye

The textile dye that used methylene blue (chemical formula: $C_{16}H_{18}ClN_3S_3H_2O$, molecular weight: 373.9 g/moL, solubility in water: 40 g/L) obtained from brand Sigma- Aldrich. The dye is in a powder state and the concentration of the dye stock solution used in this experiment was 1 g/L.

Soil sample collection

The soil sample was taken from Habibi Batik House in Kampung Pak Tuyu, Kuala Nerus, Terengganu (GPS location 5.4156624,103.083565) as shown in Figure 1. The soil sample was collected near the effluent wastewater channel using a sterile spatula for about 10 cm depth from the soil surface. The sample was then placed in sterile plastic and stored at 4 °C. The temperature and pH of the soil were measured.

Isolation of dye degrading bacteria

For the isolation of the bacteria, 10 g of soil sample was mixed in 100 mL of nutrient broth added with 50 mg/L methylene blue dye for 48 h at 37 °C. Serial dilution was performed after the incubation period to isolate the bacterial colony that decolorized the methylene blue dye. Then, 0.1 mL of the sample was pipetted on the nutrient agar supplemented with 0.1 g/L of methylene blue and the spread plate technique was applied using a sterile glass spreader. The bacterial colony with decolorizing ability was observed after 48 h of incubation. The bacterial colony that can decolorize dye has produced a clearance zone around the colony. The bacterial colony with a clearance zone was streaked on the nutrient agar plate to obtain a single colony for the bacterial identification and dye degrading assay. The pure bacterial isolates were stored at 4 °C on a nutrient agar slant.

Identification of the bacterial isolates

The bacterial isolates were observed by their colony morphological characteristics and biochemical activities. Gram staining was performed then followed

Fig. 1. Sampling site, Kampung Pak Tuyu, Kuala Nerus, Terengganu (Google Maps, 2021)

by SIM (Hydrogen- Sulphide, Indole, Motility) test, oxidase test, citrate utilization test, nitrate reduction test, and triple sugar Iron test (Advanced Microbiology Lab Manual, 2021). BBL Crystal Identification Kit from Becton Dickinson Microbiology Systems, Cockeysville, Md., was also used to identify the bacterial isolates.

Direct sequencing of 16S rRNA gene sequence was done by PCR-amplified 16S rDNA. The bacterial 16S rDNA, full-length of 1.5 kb was amplified using universal primers 27F and 1492R. The PCR was performed as follows: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by the standard method and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Effect of abiotic parameters on the bacterial growth

Two types of bacterial strains with potential decolorizing ability were further investigated under different abiotic parameters. Screening of the dye degrading was performed to determine the best culture condition for the bacteria to grow and decolorize the dye. The screening process will help to identify the bacteria's ability to decolorize methylene blue dye under suitable pH, concentration, and temperature. Each of the bacterial isolates with potential dye decolorizing ability was evaluated by testing the ability of the selected bacteria to degrade dye at different pH (pH 4, pH 7, & pH 8), dye concentrations (50 mg/L, 100 mg/L, 150 mg/L, 250 mg /L and 400 mg/L), and temperatures (28 °C, 37 °C, & 50 °C). The screening process was conducted one factor at a time (OFAT). It is performed by supplementing methylene blue dye in 10 mL of the mineral salt medium under (MSM) preferred condition. The MSM contained the following components: 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4.7H_2O$ in 1 L of distilled water (Zajic & Supplisson, 1972). For all treatments, the bacterial density was checked after 24 h of static incubation using a spectrophotometer at a wavelength of 600 nm. The bacterial culture condition that gives the highest growth rate was used for the dye degradation analysis.

Dye degradation analysis

After the screening process was conducted on the effect of abiotic parameters on bacterial growth, the bacterial isolates from the stock culture were re-cultured in the enrichment culture consisting of 100 mL of MSM and 400 mg/L methylene blue dye. A control experiment was conducted containing 100 mL MSM and 400 mg/L methylene blue dye without bacterial isolate. The mediums were incubated under static conditions for 8 days and the absorbance of each medium was observed every 24 h of culture. For all treatments, 10 mL of the sample and control were centrifuged at 5000 rpm for 10 min. The supernatants were analyzed using a spectrophotometer at a wavelength of 665 nm (Eslami *et al*., 2017). Each of the experiments was carried out in triplicate.

The calculation for percentage of dye degradation activity was calculated using the following formula (Zhuang *et al*., 2020):

$$\% \text{ Dye degradation} = \frac{\text{O.D (initial) - O.D (final)}}{\text{O.D (initial)}} \times 100$$

RESULTS AND DISCUSSION

Isolation of dye degrading bacteria

In this study, after the combination of 10 g of contaminated soil with 100 mL of nutrient broth added with 50 mg/L methylene blue (MB) dye, the results revealed that the MB from blue color (Flask A) was decolorized and formed in the Flask B (Figure 2). The medium showed color changed from dark blue to brown after 48 h of incubation. Then, 0.1 mL of the sample was spread on a nutrient agar plate supplemented with methylene blue dye. The agar medium has shown a clearance zone of dye around the bacterial colonies, which proved their decolorization abilities (Figure 3). two bacteria colonies showed decolorization abilities named Isolate 1 and Isolate 2.

These observations indicated that the isolates bacteria were able to decolorize dye in static conditions even during low oxygen. This support by Cao *et al*. (2019) was conducted decolorization of Direct Blue 2B by indigenous bacterial consortium experiments under static conditions. A study by Lade *et al*. (2015), stated that 99% decolorization of Trypan Blue dye occurred under microaerophilic conditions within hour using native microbial consortium and only 12% found decolorization under aerobic conditions.

Identification of isolated bacteria

The cell morphology magnification with immersion oil shows that the isolated bacteria turn to be Gram-negative with rod shape for both Isolate 1 and Isolate 2. These bacteria were undergone biochemical tests to further identify the dye-degrading bacteria. The biochemical tests for Isolate 1 indicated positive results for lactose and carbohydrate fermentation which showed the ability to reduce nitrate. Negative results are shown for citrate fermentation, oxidase, and SIM (Sulphide-Indole-Motility) test. Additionally, the result for BBL Crystal Kit proves that Isolate 1 is *Klebsiella pneumoniae.* Meanwhile, Isolate 2, shows that the bacterial strain is a non-lactose and carbohydrate fermenter. Moreover, it also gives negative results for the SIM test and nitrate reduction test. Positive results are shown in citrate utilization and oxidase test. Hence, BBL Crystal Kit indicates that Isolate 2 is *Pseudomonas aeruginosa* as shown in Table 1.

The identification result was further confirmed by 16S rRNA sequencing as shown in Figure 4 showed the highest similarity with *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and identified and named as *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP). In

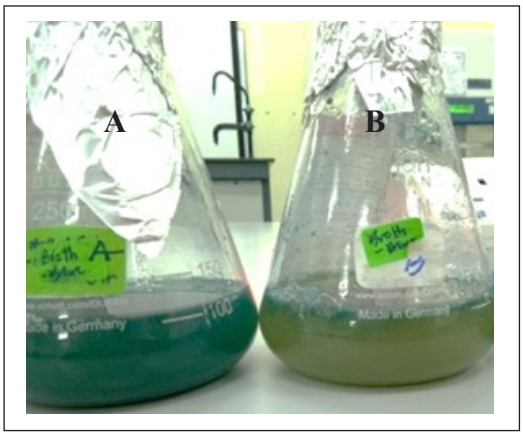

Fig. 2. The color changes from dark blue (Flask A) to brown (Flask B) after 48 h of incubation.

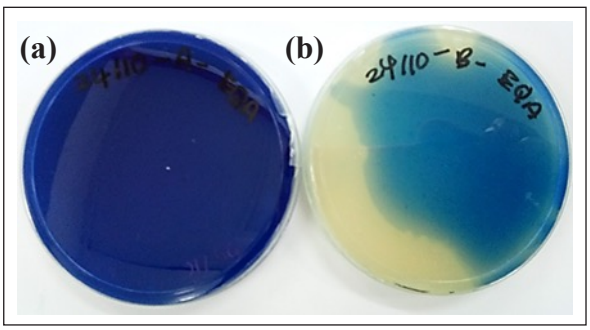

Fig. 3. The clearance zone for the dye decolorization on sample plate (b) compared to the control plate without soil microorganism (a) after 48 h of incubation.

previous studies, *Klebsiella* sp. was reported to have azo dye-decolorizing abilities. The present study shows similar to Holkar *et al*. (2018) which identified a facultative *Klebsiella* sp. C NCIM 5546 was based on the 16 S rDNA gene sequences and this bacterium was able to degrade reactive blue dye (Holkar *et al*. 2018). While a study done by Ariffin and Ariffin (2020), proved that *Pseudomonas aeruginosa* based on the 16 S rDNA gene sequences was the dominant bacteria for biodegradation of textile dye such as Reactive Green 19.

Effect of abiotic parameter on bacterial growth

The effect of pH was varied at pH 4, pH 7, and pH 8. The highest number of bacteria growths was at pH 7 for both isolates after 48 h of incubation, as shown in Figure 5a. It shows that the cells can tolerate a range of pH because they do have some mechanisms to regulate their cytoplasmic pH. However, there are limits to tolerance to their pH range. The bacterial growth is decreased at pH out of this range due to the destruction of the stability of plasma membrane, inhibition of membrane enzymes, and transport protein (Prescott *et al*., 2017)

Both isolates show the highest bacterial growth at 37 °C indicated in Figure 5b. Bacteria need optimum temperature for growth. The higher temperature causes thermal inactivation of proteins and probably affects cell structures such as the membrane cell (Shah, 2013). A similar finding by Eslami *et al*. (2019), had shown that maximum decolorization occurred at 37 °C. A similar effect of temperature (37 °C) was observed in Malachite green dye degradation showed more than 97% by isolated soil bacteria under static conditions (Etezad & Sadeghi-Kiakhani, 2021).

For the effect of concentration, the growth of both isolates was tested in different concentrations in the range of 50 mg/L to 400 mg/L. Figure 5c shows that at the concentration of 400 mg/L, the bacterial growth was the highest for both isolates. When higher or lower concentrations of dye were provided in the enrichment culture, there were decreases in bacterial growth. Too little or too much initial concentration of dye will not be able to induce the enzyme that can break down the azo dye linkage because the enzyme is inductive (Dave *et al*., 2015). The present study showed the isolated bacteria growth was higher at a dye concentration tested of 400mg/L. A similar finding by Roy *et al*. (2018), indicate that *Enterobacter* sp. CV–S1 can decolorize and degrade a relatively higher concentration of the crystal violet dye. Therefore, the effect of dye concentration plays an important role in the process of bioremediation of textile wastewater.

The effect of abiotic factors such as dye concentration, pH, temperature, time, glucose, and sodium chloride concentrations on decolorization

Table 1. Bacterial identification results for Isolate 1 and Isolate 2

	Isolate 1	Isolate 2
A. Morphological characteristics	Colonies appear large, mucoid, and white on nutrient agar. Rod-shaped, Gram-negative bacteria	Colonies appear large, flat colonies with irregular margins and greenish on nutrient agar. Rod-shaped, Gram-negative bacteria
B. Biochemical Test		
• Mac Conkey Agar	Pink colonies	Colorless colonies
• Nitrate test	Positive	Negative
• Citrate utilization test	Negative	Positive
• Oxidase test	Negative	Positive
• SIM test	H_2S production: Negative Motility: Negative Indole test: Negative	H_2S production: Negative Motility: Negative Indole test: Negative
• Triple Sugar Iron test	Slant: Yellow Butt: Yellow Gas production: Positive H_2S production: Negative	Slant: Red Butt: Red Gas production: Negative H_2S production: Negative
C. BBL Crystal Identification Kit	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
D. Molecular Identification using 16S rRNA sequencing	*Klebsiella pneumoniae* strain UMTFA1 (EK*)*	*Pseudomonas aeruginosa* strain UMTFA2 (EP)

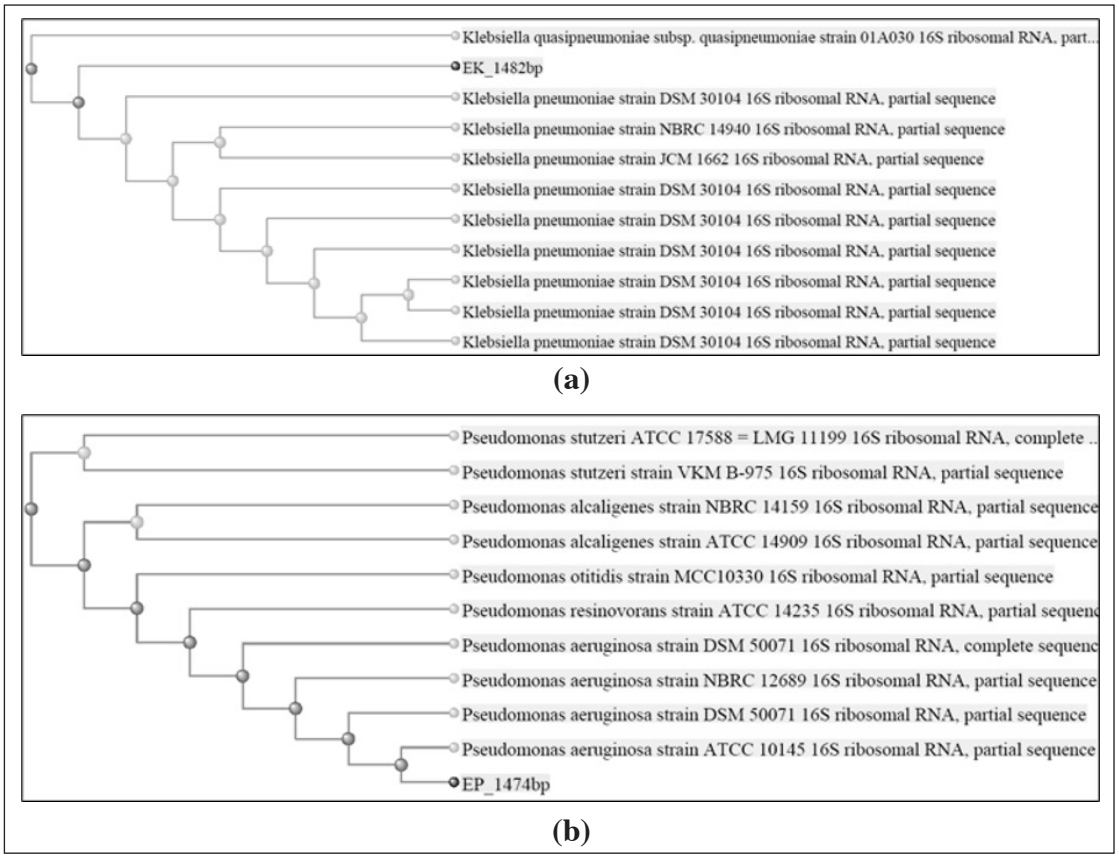

Fig. 4. Phylogram showed a genetic relationship between the isolated bacteria (a) *Klebsiella pneumoniae* strain UMTFA1 (EK) (b) *Pseudomonas aeruginosa* strain UMTFA2 (EP) and other related reference bacteria based on the 16S rRNA gene sequence analysis comparisons with accession numbers.

is important to determine the optimal conditions required for maximum decolorization or degradation of selected dye (Ikram *et al*., 2020). Results of previous studies have shown that the optimal conditions for the decolorizing dye activity of bacteria were pH 7, the temperature of 37 °C, the glucose concentration of 6 g/L, and the dye concentration of 100 mg/L (Holkar *et al*., 2018).

Dye degradation analysis

The study proceeds for the dye degradation activity by both isolates after eight days of incubation in static conditions. Figure 6 shows the decolorization percentage by *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP) incubated at 37 °C with a concentration of 400 mg/L and pH 7. The decolorization percentages for the *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP) are increasing from Day 0 to Day 8 with 10.52% and 11.65% of decolorization percentages after 8 days, respectively. The presence of this slight decolorization may be correlated to the suitable temperature for the enzymatic activity in many cells, which is normally placed between 35 and 40 °C. However, the incubation time may need to be extended further to see the effect on the decomposition of the dye.

The present study showed that the decolorization rate for both isolates was found to be slower compared to previous studies (Zabłocka-Godlewska *et al*., 2015; Garg *et al*., 2020). This might be due to the isolated bacteria utilizing only the dye in the MSM as a sole source of carbon and energy without supplementing any other carbon source. According to Mishra *et al*. (2020), the addition of some nutrients, such as carbon sources (acetate and glucose) and nitrogen sources (yeast extract & peptone) enhanced the degradation process by the bacteria and maintain the diversity of the microbial community. The effect of abiotic parameters on the extensiveness of dye decolorization was tested by varying the carbon source and nitrogen source added to MSM and the maximum decolorization was observed in MSM supplemented with peptone at pH 7 (Garg *et al*., 2020).

Another reason ineffectiveness of dye degradation could be considered is the mode of cultivation of the microorganism itself. Previous studies reported that static conditions are preferred and are more efficient for azo dye decolorization (Prasad & Aikat, 2014). It is because the aromatic amines of its metabolite products were assumed to resist further degradation compared with shaking cultivation conditions (Pandey *et al*., 2007). However, the effects of different modes of cultivation, either static or shaking conditions on

dye decolorization efficiency, and the mechanisms of azoreductase activity and metabolite production during the dye degradation still need to be further investigated.

Several studies have shown that *Pseudomonas* sp. exhibited high potential for selected dye decolorization. *Pseudomonas stutzeri*, strain AK6 has been studied and decolorized up to 86.2% within 96 h of Acid Blue 113 dye at 300 ppm (Hinsu *et al*., 2020). While the findings reported by Garg *et al*. (2020) showed that *Pseudomonas aeruginosa* decolorized about 85% of 50 mg L^{-1} Reactive Yellow 145 dye within 4 days under static conditions. *Pseudomonas* sp. SUK1 was approximately degraded by 25% of a mixture of ten azo dyes after 4 h and approximately 90 % after 24 h in static conditions (Zabłocka-Godlewska *et al*. 2015).

In previous studies, many *Klebsiella* species were reported to have azo dye-decolourizing abilities. For example, *Klebsiell*a strain Rz7 degraded 89.12 % of Evans Blue dye (Zabłocka-Godlewska *et al*., 2015) while *Klebsiella pneumoniae* strain AHM completely decolorized 50 and 250 mg/L Reactive Orange dye 16 within 150 and 600 min under static condition (Kumar *et al*., 2017). However, there are only a few studies reporting methylene blue dye decolorization by *Klebsiella pneumoniae*.

CONCLUSION

In this study, two isolated bacteria from contaminated soil were successfully identified known as *Klebsiella pneumoniae* strain UMTFA1 (EK) and *Pseudomonas aeruginosa* strain UMTFA2 (EP). These isolated

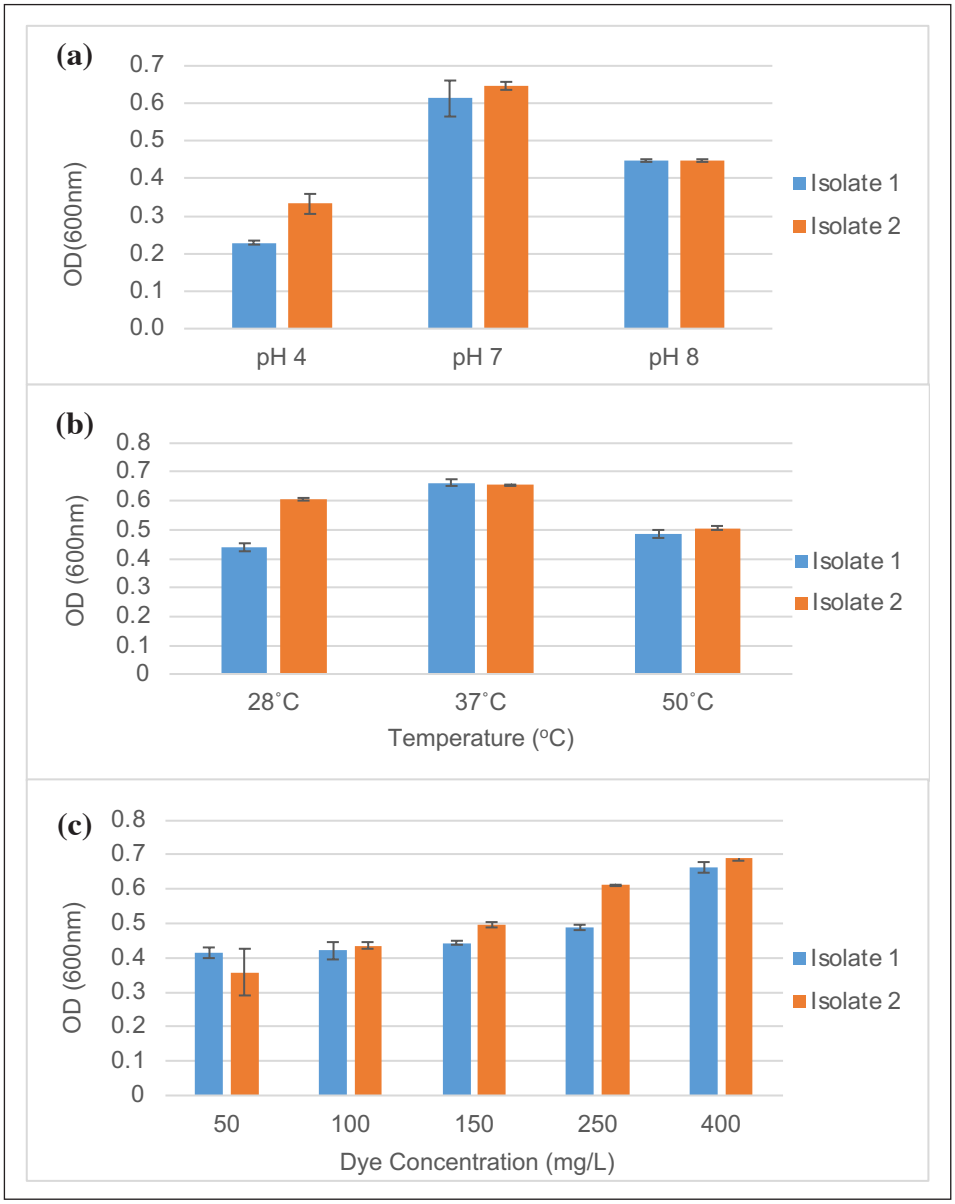

Fig. 5. Bacteria growth of Isolate 1 and Isolate 2 at different pH (a) different temperatures (b) different concentrations (c) after 48 h incubation in MSM supplemented with Methylene Blue dye.

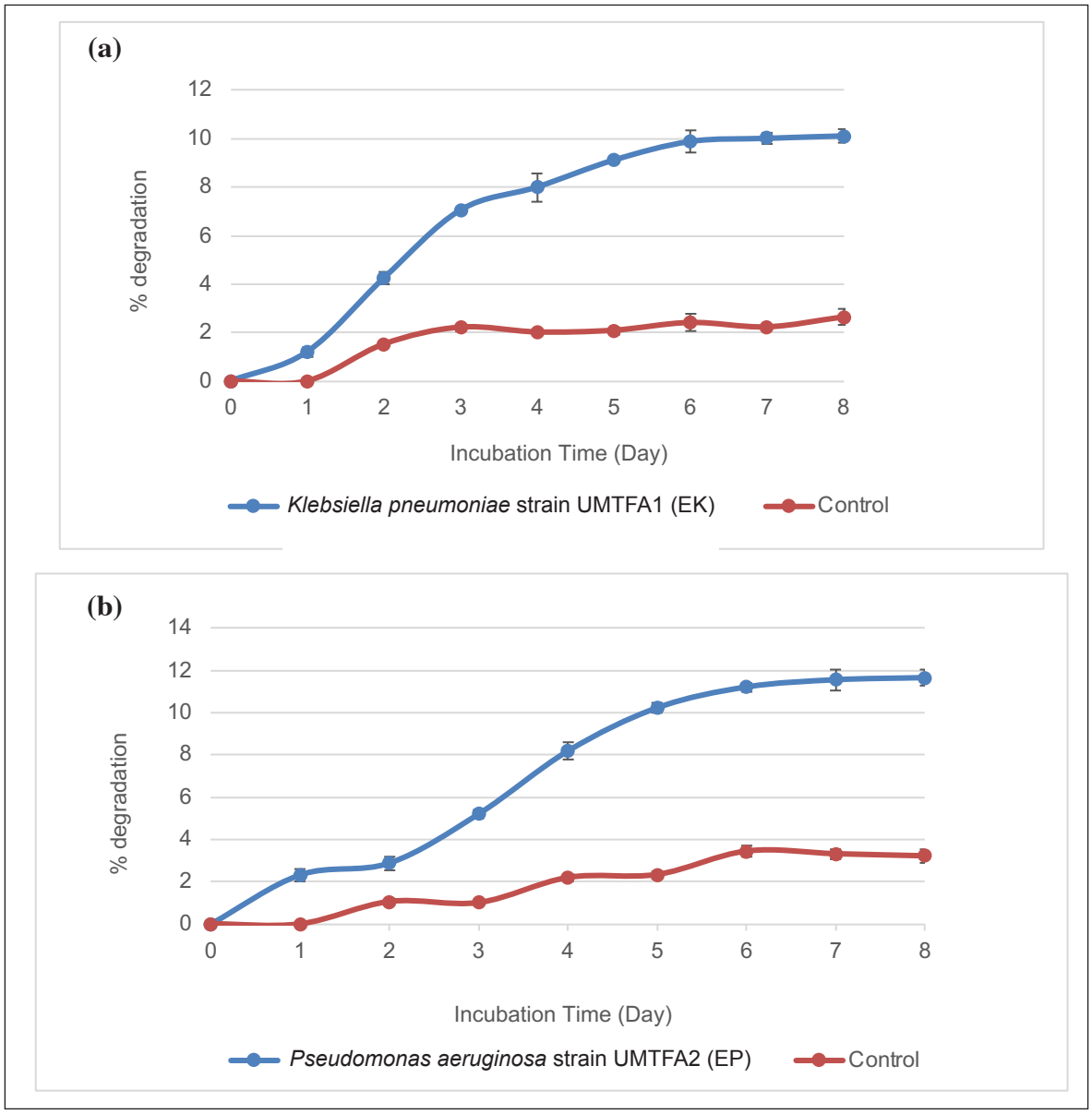

Fig. 6. Decolorization of methylene blue dye by *Klebsiella pneumoniae* strain UMTFA1 (EK) (a) *Pseudomonas aeruginosa* strain UMTFA2 (EP) (b) after 8 days of incubation in MSM (at pH 7, 37 °C, and 400 mg/L concentration of dye).

bacteria have the potential decolorizing 10.52% and 11.65% of methylene blue dye within 8 days. Therefore, it believes that these bacteria have the potential in treating contaminated textile effluent as well as dye-contaminated soil. Further studies need to be carried out, such as factors that influence the performance of the bacteria to maximize the biodegradation process.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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