Decolorization of selected azo dye by *Lysinibacillus fusiformis W1B6*: Biodegradation optimization, isotherm, and kinetic study biosorption mechanism

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Abstract
This study reports on the evaluation of biodegradation and biosorption mechanism of indigenous bacteria on azo dye decolorization. The bacterial strains were screened for their ability in decolorizing of selected toxic azo dyes in aqueous solution. *Lysinibacillus fusiformis W1B6* showed the highest decolorizing of about 96% of methyl red within 2 h, with the specific growth rate of 0.273/h. The optimum decolorization under aerobic condition was achieved at pH 7.5, 30 ± 2°C, inoculum 10% (v/v), and initial dye concentration of 100 mg/L. Spectrum analysis confirmed biodegradation as the principal mechanism of decolorization, although biosorption also occurred. Extracellular and intracellular azoreductase, laccase, and lignin peroxidase plays a role in the oxidoreductive mechanism of decolorization. The absorption isotherms fitted with the Langmuir equation and the reaction kinetics of methyl red decolorization followed a pseudo first-order.

Keywords
Azo dyes, decolorization, degrading bacteria, kinetic absorption, mutagenic compound, polluted wastewater

Submission date: 26 February 2018; Acceptance date: 1 April 2019

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Introduction
Azo dyes are the most commonly utilized synthetic dyes in industries. They are widely applied in the production of textiles, leathers, plastics, cosmetics, additives, and foodstuffs, with the largest application being in the textile industries for dyeing processes. This is due to the simple synthetic processes, abundant structural diversity, high molar extinction coefficient, and quite fastness to light and the wetness of azo dyes (Bafana et al., 2011). Azo dye produced from textile wastewater causes discoloration of natural water in the environment. It negatively impacts water quality due to the presence of color, its toxicity, and mutagenicity (Khan et al., 2015). Therefore, effective color removal is very important and should be emphasized in the treatment of wastewater before it can be released into the environment.

Compared to chemical degradation processes, biological technique has been described as a promising route for the decolorization of most problematic contaminants originating from dyes. Microbiological decolorization is particularly environmentally friendly and economically feasible (Yang et al., 2011). Several microorganisms have been selected for dye decolorization and their effectiveness was dependent on their activities and adaptability to the environment. Bacteria were reported to be more efficient than fungi in most studies of azo dye biodegradation. The benefits of using bacteria as biodegradation agents include: (1) removing color over a wide range of pH, in contrast with a narrow pH range of fungi, (2) ability to decolorize a wide range of azo dyes, (3) utilization of azo compounds as the sole carbon and energy sources, and (4) production of oxidoreductive enzymes for decolorization of synthetic azo dyes (Shah et al., 2013).

Methyl red (MR) is a typical azo dye, which contains a mono-azo bond, lacks a sulfonate group, and is classified as mutagenic to the environment (Seesuriyachan et al., 2007). Studies have been conducted on MR biodegradation, but most of such studies did not include the kinetic study of the growth of live cells and kinetic biosorption of dead cells. In this work, adsorption isotherm study was conducted to evaluate the biosorption process by Lysinibacillus fusiformis strain W1B6. Adsorption kinetics investigation was conducted in order to elucidate the mechanism of adsorption of MR onto bacterial cells.

Bacterial strains were screened on three types of azo dye and potential strain was identified using molecular method. The performance of MR decolorization based on physicochemical condition was conducted using randomized design. The spectrum analysis was performed to confirm the biodegradation pattern. Studies on the activity of enzymes involved in biodegradation were conducted while a suitable kinetic model was employed to describe the biosorption mechanism.

Materials and methods

Dyes and chemicals
MR, methyl orange (MO), and Congo red (CR) were purchased from BDH Chemical Ltd., England. Bradford reagent, NADH, syringaldazine, and tartaric acid were purchased from Sigma-Aldrich, USA. All reagents and chemicals were of the analytical grade and highest purity available.
Media and culture conditions for screening of dye degrading bacteria

Six bacterial strains used in this study had been isolated previously from raw textile wastewater. Pure cultures of the strains were maintained on agar slants at 4°C and were sub-cultured onto fresh nutrient agar monthly. Cryo-preservation was also done in sterile glycerol stock (40% v/v). The pure cultures were reactivated in nutrient broth (NB) at 30 ± 2°C for 18 h before being transferred into fresh NB and incubated under similar conditions with agitation of 120 r/min for 24 h. The grown cell culture with an optical density of 0.6 (at 600 nm) was used as an inoculum (10% v/v) for the screening test. Bacterial strains were observed on the feasibility of growth and utilization of the three dyes i.e. MR, MO, and CR. Medium without bacterial inoculum was used as a control.

Screening of the potential strains was performed in mineral salt medium (MSM) containing (g/L): NaCl, 2; MgSO₄·7H₂O, 0.4; CaCl₂·2H₂O, 0.5; KH₂PO₄, 0.3; (NH₄)₂SO₄, 0.5 supplemented with yeast extract (0.1% w/v) and 100 mg/L of each dye with the final pH adjusted to 6.8. The flasks were incubated at 30 ± 2°C, 120 r/min for 24 h. Samples from the culture were harvested at 4 h intervals and centrifuged at 4000 r/min for 15 min. The cell-free supernatant was used for the determination of dye decolorization. The strains that gave the highest percentage of dye degradation and rapid decolorization on the selected azo dye were used for further studies.

Molecular identification of selected strain

The selected strain from the above screening was further identified using molecular methods. The bacterial DNA was extracted using i-Genomic BYF DNA Mini Kit (Intron Biotechnology Inc.) and the quality (purity and concentration) was checked using a Nanodrops 2000 spectrophotometer (Thermo Scientific). The bacterial 16S rRNA gene was amplified using 27 F: 5'-AGA GTT TGA TCA TGG CTC AG-3' and 1492 R: 5'-TAC GGT TAC CTG TTA CGA CTT-3' and then subjected to gene sequencing. The partial 16S rRNA gene taxonomic identity was determined from the EzTaxon-e database. The phylogenetic tree was constructed using MEGA6 software by employing Neighbor-Joining algorithm. The 16S rRNA gene sequence of the selected bacteria has been deposited in the NCBI nucleotide sequence databases.

Effect of various physicochemical parameters on biodecolorization of MR

Strain W1B6 and MR were selected for further investigation in the shake flask condition. Study on MR biodecolorization was conducted on selected key variables i.e. initial pH (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10), dye concentration (mg/L) (10, 100, 1000), temperature (°C) (25, 30, 37), and inoculum load (% v/v) (5, 10, 15, 20). All experiments were carried out under similar conditions and using a medium with the same composition as the screening medium except for experiments involving the variation of selected parameters. Sampling was done after 4 h incubation and cell-free supernatants were used for the determination of dye decolorization.

Decolorization profile and kinetic study of the growth of strain W1B6 under optimized parameters were performed in MSM medium with YE (0.1% w/v) according to the results obtained from the above studies (initial pH 7.5; 100 mg/L of MR, 30°C and 10% v/v of inoculum size). The decolorization (%) and biomass (cfu/mL) were also observed in the buffer liquid medium with MR as the sole carbon source for growth.
Determination of MR decolorization mechanism

In order to understand the mechanism of MR decolorization as being due to either biodegradation or bioaccumulation, spectrum analysis was performed using UV-Vis Jusco v-630 spectrophotometer.

Enzyme analysis was conducted in further investigation on the role of enzymes in the biodegradation process. The possibility of biosorption mechanism was also conducted using dead cell of strain W1B6.

Preparation of crude enzyme source

The grown bacterial sample from shake flask cultivation was centrifuged at 10,000 r/min at 4°C for 20 min and the supernatant was used as an extracellular source of enzymes. The cells mass was re-suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication (Probe Ultrasonic Sonifier – Branson Digital Sonifier, Model 450, USA) with the sonifier output at 40 A and giving 8 stroke each of 40 s with a 2 min interval at 4°C. Supernatant recovered was served as the intracellular enzyme.

Enzyme activity assays

Azo reductase activity was assayed in 50 mM potassium phosphate buffer (pH 7.5) according to Zhao et al. (2014) with some modification. The decrease of MR concentration in the sample was monitored in a total 3.0 mL reaction mixture containing 100 μM NADH, 50 μM MR, and 100 μL enzyme. The MR reduction was calculated by using an extinction coefficient of 23.36/mM/cm. One unit of enzyme activity was defined as a microgram of MR reduced protein per minute, per mg.

Lignin peroxidase activity was determined by measuring the released propanaldehyde in a 2.5 mL reaction mixture containing 100 mM n-propanol, 250 mM tartaric acid, and 10 mM H2O2 at 300 nm. The molar extinction coefficient of n-propanol was 0.02/mM/cm. One unit of activity was defined as the amount of lignin peroxidase, which converted one micromole of n-propanol to propanaldehyde (Yadav and Yadav, 2006).

Laccase activity was measured according to Razak and Annuar (2014). The assay mixture contained 2.5 mL of phosphate-citrate buffer (0.1 M), 0.1 mL of syringaldazine (5 mM) and 0.5 mL of the enzyme solution. The oxidation rate of syringaldazine was measured at 525 nm using the extinction coefficient 65/mM/cm. One unit activity was defined as the amount of laccase that oxidized one micromole of syringaldazine per minute.

Enzyme activities were calculated as shown in the following equation

\[ \text{Activity} = \frac{\Delta Abs}{t} \times \frac{l}{\varepsilon} \times \frac{\text{total assay enzyme}}{\text{enzyme sample}} \]  

where \( \Delta Abs \) is the change of absorbance, \( t \) is the incubation time (min), \( \varepsilon \) is the extinction coefficient, and \( l \) is the light path length (1 cm).

Biosorption isotherms experiments

The late log phase of cell growth, cultivated in NB was harvested and centrifuged at 10,000 r/min for 20 min at 4°C. The pellet was collected and autoclaved at 121°C for 20 min prior
to use for the study of biosorption mechanism. Then the biosorption test was initiated by adding 0.2 g/L of the dead cells were added into phosphate buffer medium (pH 7.4) containing different concentrations of dye (10, 30, 50, 70, and 100 mg/L). They were continuously stirred at 120 r/min and the temperature was kept constant at 30 ± 2°C for 12 h in order to ensure that adsorption equilibrium was reached. Samples were centrifuged at 4000 r/min for 15 min at 1 h intervals. The cell-free supernatant obtained was used for determination of dye decolorization.

The amount of adsorbed dye in the cell (mg/g) was calculated from the following mass balance relationship

\[ q_e = \frac{(C_0 - C_e)V}{M} \]  \hspace{1cm} (2)

where \( V \) is the volume of dye (L), \( M \) is the mass of cell biosorbent used (g), \( C_0 \) and \( C_e \) are the initial dye concentration and dye concentration at equilibrium (mg/L), respectively.

Adsorption experiment at equilibrium allows relating to the concentration absorbed that remains in the solution and the adsorption capacity of the adsorbent. Thus, Langmuir and Freundlich isotherm models were used to describe the adsorption equilibrium process and the adsorption capacity of dye onto cell biomass. The Langmuir model considers that the adsorption process is in a monolayer, where all of the adsorption sites are of the same type and they are all occupied at the end of the process.

The Langmuir model was calculated using the following equation

\[ \frac{1}{q_e} = \frac{1}{Q_M} + \left( \frac{1}{Q_M K_L} \right) \frac{1}{C_e} \]  \hspace{1cm} (3)

where \( q_e \), the amount of dye adsorbed per unit mass of adsorbent (mg/g) and \( C_e \), concentration of dye at equilibrium (mg/L). The Langmuir relationship was linearized by plotting \((C_e/q_e)\) vs. \( C_e \), which intercept and slope in the linear equation correspond to \( K_L \) and \( Q_M \). The coefficient \( K_L \) represented the Langmuir constant (L/mg), while \( Q_M \), the maximum amount of biosorption per unit mass of biosorbent (mg/g), which is similar to the complete monolayer surface coverage.

While the derivate of Freundlich assumed the multilayer sorption due to the heterogeneous active sites for biosorption. The Freundlich model was calculated using this equation

\[ \ln q_e = \ln K_F + \frac{1}{n} \ln C_e \]  \hspace{1cm} (4)

where the slope and the intercept of linear plot \( \ln C_e \) vs. \( q_e \) represent the value of \( 1/n \) and \( \ln K_F \), respectively. The \( K_F \) approximately showed the biosorption capacity and \( n \) represented the biosorption intensity.

**Analytical methods**

**Determination of cell biomass and kinetic of cell growth.** The viable cell count was determined by determining the number of colonies forming units (CFU) on the solid medium. For cell mass determination, samples were centrifuged at 4000 r/min for 15 min. The pellet was
resuspended in distilled water, and its optical density was measured using a spectrophotometer at 600 nm wavelength. The standard growth curve of cell dry weight (CDW) (g/L) vs. OD 600 nm was prepared and used for the determination of the cell mass produced.

The kinetics of cell growth in the flask system were calculated by Malthus law as below (Lee, 2006)

\[
\frac{dX}{dt} = \mu X \\
\int_{X_0}^{X} \frac{dx}{x} = \mu \int_0^t dt \\
\mu = \frac{\ln \left( \frac{X_t}{X_0} \right)}{t - 0} \\
\ln \left( \frac{X_t}{X_0} \right) = \mu t
\]

where \(dX/dt\) is the rate of cell growth (mg/L), \(\mu\) is the specific growth rate (/h), and \(t\) is the incubation time (h).

**Determination of dye decolorization.** The dye decolorization assay was done using Jusco v-630 spectrophotometer at the maximum wavelength resulting from the spectrum analysis i.e. 437 nm for MR, 464 nm for MO, and 498 nm for CR. The efficiency of decolorization was expressed as the percentage ratio of the decolorized dye concentration to the initial dye concentration based on the following equation (Simarani et al., 2016)

\[
\% \text{Decolorization} = \frac{OD_0 - OD_t}{OD_0} \times 100
\]

where \(OD_0\) is the initial dye concentration and \(OD_t\) is the dye concentration (mg/L) after predetermined in the incubation time.

**Determination of biosorption kinetics.** These kinetic studies aimed to evaluate the biosorption performance, i.e. the efficiency of the biosorbent and the mechanism of biosorption with the equations according to Simarani et al. (2016). Two common kinetic models were selected in this study to describe the adsorption process: pseudo first-order and pseudo second-order. The linear form of first-order model and the second-order model were described as in equations (7) and (8), respectively (Samarghandi et al., 2012).

\[
\ln \frac{C_0}{C_e} = k_1 t
\]

\[
\frac{1}{C_e} - \frac{1}{C_0} = k_2 t
\]

where \(C_0\) and \(C_e\) (mg/L) is the dye concentration at the initial time \(t_0\) and at various time \(t\), \(k_1\) (L/min) is the rate constant of the pseudo first-order kinetic, and \(k_2\) (L mg/min) is the rate
constant of the pseudo second-order kinetics. The $k$ value is evaluated from the slope of a straight line between the left side of equations (7) and (8) versus time.

**Scanning electron microscope analysis**

The microbial interaction during dye degradation was determined using SEM. Morphological changes of the dead and live bacterial cells as biosorbent were observed after 2 h incubation at pH 7.5, temperature 30°C with dye concentration of 100 mg/L under the agitation rate of 120 r/min. Bacterial cells in the absence of dye were used as a comparison. The samples were fixed with glutaraldehyde 4% (v/v) then dehydrated with a series of ethanol. The dehydrated samples were air-dried at room temperature, then mounted on stainless steel SEM stubs and coated with gold in an ion counter. Observation was carried out using field-emission scanning electron microscope model FEI QUANTA FEG 650.

**Statistical analysis**

All experiments were performed at least in triplicate. The numerical results are expressed as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test using IBM SPSS Statistics 23. Readings were considered significant when $P$ was ≤0.05. Statistical analyses were also performed using Sigma Stat 12.0.

**Results and discussion**

**Screening and identification of azo dyes decolorizing bacteria**

Six indigenous bacterial strains (W1B1, W1B6, W3B2, W3B4, W3B5, and W3B8) were screened for their ability to decolorize three synthetic azo dyes (MR, MO, and CR) in liquid MSM. The performance and efficiency of all strains in decolorizing three types of azo dyes are illustrated in Figure 1. The results indicated that all strains could grow in the medium containing azo groups as substrate. Strain W1B6 was clearly the most efficient in decolorizing the MR where 94% of decolorization was achieved within 4 h under aerobic condition. Thus, the decolorization rate of 23.5/h was considered as the fastest decolorization of MR compared to the other dyes (MO, 2.5/h and CR, 14.5/h).

Culture medium with CR was the second preferable growth substrate for strain W1B6 with 58% decolorization achieved after 4 h of incubation. This might be due to the possession of the polyaromatic sulfonate group by CR, which made the structure more complex compared to MR that possesses mono-azo bond without sulfonate group (Seesuriyachan et al., 2007). However, the capability of this strain in decolorizing the CR was not significantly different ($P > 0.05$) compared with W1B1, W3B2, and W3B4.

In this study, it was observed that all the strains decolorized MO poorly (less 10%). This suggests that the strains probably require longer contact time for effective decolorization of the dye. Moreover, the low MO decolorization might also be due to the formation of an enzyme-substrate complex that was inhibited by the *ortho* position of the benzene ring in the methyl group. (Seesuriyachan et al., 2007). The sulfonated group that attached to the structure of the mono-azo bond made MO resistant to degrade under aerobic condition (Ng
et al., 2017). Besides, the effectiveness of microbial decolorization may be influenced by many factors such as their survival, adaptability, varying activities of enzymes secreted by microorganisms, and the chemical structures of the azo dyes (Khalid et al., 2010).

Strain W1B6 after 16S rRNA analysis was identified as *L. fusiformis*. Figure 2 shows the phylogenetic position of W1B6 and other *Lysinibacillus* species found in the Gen Bank database. Comparison of 16S rRNA sequence of W1B6 (accession no: KP233829) with other related strains showed very high similarity (99.93%) with *L. fusiformis* sp.

![Figure 1](image1.png)

**Figure 1.** Percentage decolorization of various types of azo dyes by indigenous bacterial strains after 4 h cultivation.

![Figure 2](image2.png)

**Figure 2.** Phylogenetic analysis of 16S rRNA gene sequence of strain W1B6. Bar, 0.005 substitutions per nucleotide position and numbers in bracket represent GenBank accession number.
A study on the effect of selected key variables on decolorization of MR in the shake flask was performed based on the adjustment of inoculum load, initial pH, temperature, and dye concentration. Figure 3(a) shows the effect of inoculum size on the efficacy of MR degradation by *L. fusiformis* strain W1B6. The use of 5% (v/v) inoculum resulted in 60% decolorization of the dye. Higher percentage of decolorization was obtained when the inoculum size was increased up to 10% (v/v). This indicated that the increasing of initial biomass leads the increasing of dye removal capacity significantly. Further increase in inoculum size ranging from 10% to 20% (v/v) did not yield any significant increase in the decolorization. The maximum decolorization of 95% was achieved within 4 h from shake flask containing 20% (v/v) inoculum. Similar findings also reported that the increasing inoculum size gave no significant increase in the decolorization rate of Reactive Black 5 (RB 5) (Bhimani, 2011).

The efficiency of dye decolorization was also affected by initial pH of the medium. Figure 3(b) shows that this strain was capable of decolorizing MR from 90% to 97% within the pH range of 5.5 to 8.5 within 4 h. Decolorization declined significantly at pH 5, 9, and 10 to 63%, 34%, and 30%, respectively. Similar results were reported by Zhao et al. (2014) where decolorization rate was decreased at acidic (<pH 6) and extremely alkaline (>pH9) conditions. The ability of *L. fusiformis* strain W1B6 grow in the wide range of pHs is a significant aspect, demonstrating the strain’s adaptability to different environmental conditions.

**Biodecolorization of MR by *L. fusiformis* strain W1B6 on different physicochemical parameters**

Figure 3. The effects of inoculum size (a), initial pH (b), temperature (c) and dye concentration (d), on methyl red decolorization by *L. fusiformis* strain W1B6 cultivated in MSM for 4 h.
range of pH indicated this strain’s tolerance to acidic and strongly alkaline conditions, thus making this bacterium a promising candidate for degradation of dye pollutants.

The most suitable temperature for MR decolorization by *L. fusiformis* strain W1B6 in this study was within 25–37°C (Figure 3(c)). Maximum decolorization was achieved up to 93% at 30°C within 4 h. This might be related to the optimum temperature for the microbial growth and the degradation process. This result is similar to what was reported by Jadhav et al. (2008) on the optimum temperature of MR decolorization by *G. geotrichum*.

The effect of MR concentration on decolorization is presented in Figure 3(d). The MR decolorization increased with an increase in the dye concentration from 10 to 100 (mg/L) but decreased rapidly at the highest concentration (1000 mg/L). This might be due to the limited inherent ability of the strain to the bio-elimination process at the higher concentration.

**Growth and dye decolorization profile of *L. fusiformis* strain W1B6 on MR**

The growth of *L. fusiformis* strain W1B6 and the rate of MR decolorization were monitored under optimized conditions, i.e. inoculum 10% (v/v), initial pH 7.5, temperature 30°C with dye concentration of 100 mg/L under agitation rate of 120 r/min after 2 h (Figure 4).

As shown in Figure 4, the bacterial cell mass increased rapidly from 0 to 4 h as the dye decolorization was increased in the medium containing 0.1% (w/v) of YE as a supplementary. The specific growth rate of 0.273/h was recorded with the $R^2 > 0.94$.

The results showed that dye was almost completely reduced (96%) within 2 h without any changes in the pH value (data not shown). After 2 h, the decolorization rate was almost stable. This observation might be due to the limiting capacity of the cells to decolorize the dye. Also the bacterial growth tended to decrease due to the death phase after 4 h.

![Figure 4. Decolorization of methyl red and the biomass of *L. fusiformis* strain W1B6. Symbols: filled black triangle (▲) is the decolorization of dye with yeast extract as an additional nutrient source, unfilled black triangle (△) is decolorization with dye as the sole source for nutrient. Filled with black circles (●) is biomass in dye with yeast extract as an addition nutrient source, un-filled black circle (○) is the biomass with dye as the sole source for nutrient.](image-url)
Meanwhile, the low amount of cell mass and dye decolorization were observed in the medium with MR as the sole source for nutrient. It indicated that the decolorization rate of dye-containing culture media without any other nutrient source was low due to poor cell growth despite the growth increasing slightly from 0 to 2 h. It might be caused by the absorption of the dye due to the decolorization of the medium and/or utilizing new by-product metabolite resulting from the reductive cleavage of azo bond for bacterial growth as a carbon and energy source.

The decrease of viable cell number (cfu/mL) was probably due to the accumulation of a large quantity of intermediate metabolites, which may also be toxic, carcinogenic, or mutagenic. The dye accumulated on the bacterial cell membrane could disturb its structural and functional system, which causes the inhibition of the bacterial cell growth (Xu et al., 2010). It was also reported that azo dyes inhibited the biosynthesis of nucleic acid during the multiplication process and have an adverse effect on the enzymes involved in the decolorization (Saratale et al., 2011).

The decolorization of dye could occur by (1) biodegradation (the bacteria cell degrades the dye and produce the metabolite by-product), (2) bioaccumulation (the active uptake of the dye by the bacteria across the cell membrane or through the cell metabolic cycle), or (3) biosorption (passive uptake of dye by dead cell or inactive biomass) (Zaman et al., 2016; Wang and Hu, 2008). The studies in biodegradation process and biosorption efficiency were further conducted in order to determine the mechanism mode of the dye decolorization by *L. fusiformis* strain W1B6.

**Determination of mechanism of MR decolorization**

In order to elucidate the mechanism of biodecolorization either by biodegradation and bioaccumulation, the UV vis spectra of the supernatant of dye-containing culture media were recorded as shown in Figure 5.

![Figure 5. Profile of UV-vis spectra of MR (100 mg/L) biodecolorization by *L. fusiformis* strain W1B6 before and after 2 h incubation time.](image-url)
As shown in Figure 5, the spectrum profile of the culture supernatant revealed the main peak with a maximum absorbance of MR at 437 nm was almost completely disappeared. It was indicated that the dye decolorized and decreased rapidly after degradation. The decrease of the absorbance peak described that the dye has been degraded. The appearance of an extra absorbance peaks (300–350 nm) after 2 h incubation, might be due to the presence of metabolic by-products or compounds originating from dye degradation. The characteristic spectra appearing after incubation can be attributed to new by-products produced by the growing cells. This indicates biodegradation rather than bioaccumulation as the active mechanism of decolorization of the dye. Biodecolorization that occurs as a result of biodegradation is characterized by either the disappearance of a major absorbance peak or the appearance of a new peak (Chen et al., 2008). The red color of culture medium consequently turned to colorless due to the reductive breakdown of azo (−N = N−) bond, while the presence of new peak was probably due to the production of metabolites (Cinar et al., 2009; Shah et al., 2013).

**Enzymatic analysis on decolorization**

Several microorganisms have the potential for azo dye degradation due to their ability to develop enzyme systems for the decolorization and mineralization under specific conditions (Pandey et al., 2007). In order to understand the role of enzymes in the oxidoreductive mechanism involved in MR decolorization, the activities of extracellular and intracellular enzymes from *L. fusiformis* strain W1B6 after 2 h and 24 h were investigated (Table 1).

Azo dye reduction mediated by enzyme can be located either intracellularly or extracellularly that involved with various oxidoreductive enzymes. Owing to the high polarities and complexities of azo dyes, it was difficult for them to diffuse through cell membranes. Thus, azo reductase enzyme released extracellularly to reduce the azo compound. Meanwhile, extracellular oxidative enzymes are relatively nonspecific on catalyzing of the dyes (Cinar et al., 2009). As for nonsulphonated dyes, the reduction can permeate through the cell membrane by soluble cytoplasmic azo reductase (Chacko and Subramaniam, 2011).

Bacterial cell could produce enzymes in the absence of dye as a constitutive enzyme. Also, the presence of dye stimulates the cell to produce more of these enzymes as inducible enzyme. The increase of azo reductase activity during decolorization indicated that an initial

| Enzymes      | Extracellular | Intracellular |
|--------------|---------------|---------------|
|              | 0 h           | 2 h           | 24 h          | 0 h           | 2 h           | 24 h          |
| Azoreductase | 2.28 ± 0.27   | 23.51 ± 0.29*** | 21.93 ± 0.14*** | 22.64 ± 8.7E-15 | 24.79 ± 0.72** | 23.07 ± 0.43 |
| Laccase      | 0.90 ± 1.4E-16 | 1.80 ± 0.03*  | 6.31 ± 0.70*** | ND           | 2.67 ± 0.15*** | 12.41 ± 0.15*** |
| LiP          | 13.55 ± 0.01  | 12.27 ± 0.02  | 27.97 ± 1.20*** | 2.69 ± 0.12  | 12.81 ± 0.07*** | 24.95 ± 0.02*** |

ND: not detected.

Values are means of three replicates ± SEM. Significantly different from control cells at *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with Tukey–Kramer multiple comparisons test.

*a*Units min⁻¹ mg protein⁻¹.

*b*μmol of syringaldazine oxidized/mg of protein min⁻¹.

*c*μmol of n-propanol oxidized/mg of protein min⁻¹.
step of biodegradation has occurred. Azo reductase enhanced the reductive cleavage of azo bonds (–N = N–) resulting in intermediate metabolites, which were then further degraded by aerobic or anaerobic mechanisms (Pandey et al., 2007). Other than reductive enzymes, there are also oxidative enzymes, which are involved in the bacterial degradation and decolorization, i.e. laccase and LiP. Laccase catalyzes the oxidation of aromatic and inorganic compounds, while LiP plays a role in mineralizing many recalcitrant aromatic compounds. Azo reductase and laccase seem to be the most promising enzymes in enzymatic bioremediation.

Azo reductase, laccase, and lignin peroxidase (LiP) were detected during enzymatic remediation of MR. Within the log phase of bacterial growth, both intracellular laccase and LiP increased very significantly \((p < 0.001)\), as well as extracellular azo reductase. As for both the intracellular azo reductase and extracellular laccase, significant differences were found at \(p < 0.01\) and \(p < 0.05\), respectively. The activity of extracellular LiP was not significant \((p > 0.05)\) within the log phase, but this enzyme was produced continuously during the death phase \((24\ h)\), giving very significant change \((p < 0.001)\). In addition, extracellular laccase as well as both intracellular LiP and laccase also showed a very significant increase, during this death phase \((p < 0.001)\).

In this study, the intracellular activities of azo reductase and laccase were higher than the extracellular while for LiP, and it was higher in the extracellular form. The intracellular enzyme might play role in another physiological mechanism due to the toxicity of metabolite products from the reduction of azo compounds. The significant increase of azoreductase and laccase were also previously reported by Zhao et al. (2014) in MR biodegradation by Bacillus sp strain UN2. It was also found that laccase was induced significantly (up to 3.6 fold) in MR decolorization by G. geotrichum at 30°C incubation (Jadhav et al., 2008).

Kinetic study on modeling biosorption

The biosorption isotherms are important tools to understand the distribution of adsorbate molecules among the cell mass and dye solution at equilibrium. Thus, isotherms could describe the dye interaction with the cell mass, including the biosorption features and types.

According to the calculation and plotting shown in Figure 6(a) and Table 2, the Langmuir modeling was fit to reflect the sorption system. It assumed that the adsorption onto the surface was restricted to a monolayer or uniform strategies when the process reaches its equilibrium state. The linear plot of Figure 6(a) showed the initial dye MR confirmed the applicability of the Langmuir isotherm model \((R^2 > 0.98)\) where the coefficient \(Q_M\) reading was 0.01 mg/g.

The important characteristics of the Langmuir isotherm were described by equilibrium constant \(R_L\), which was represented by

\[
R_L = \frac{1}{1 + K_L C_0}
\]

where \(C_o\) is the initial concentration of dye (mg/L), \(K_L\) is the Langmuir constant. The separation factor \((R_L)\) described the isotherm shape and the favorable of the adsorption: \(R_L > 1\) is unfavorable; \(R_L = 1\) is linear; \(0 < R_L < 1\) is favorable; \(R_L = 0\) is irreversible (Hameed and Daud, 2008). The \(R_L\) value of this work \((0.07)\) indicated a favorable adsorption process between cell and dye MR.
The integrated rate laws were conducted to determine the reaction order for the biosorption. Kinetic coefficients obtained from Table 2 and Figure 6(c) and (d) could explain the behavior of dye biosorption. Linear regression analyses showed that correlation coefficients \( R^2 \) were 0.935 and 0.883 for the first- and second-order model, respectively. First-order kinetic model was found to be more appropriate with the experimental results to describe the kinetic biosorption of MR. The slope of the \( k_1 \) value was 0.151. This model corresponded with previous reports dealing with the anaerobic mixed microbial culture on some azo dyes removal (Karatas et al., 2009). For the second-order kinetic, the experimental data deviated from linearity due to the low value of coefficient determination, which that indicated this kinetic order was not applicable to the system.

**Figure 6.** Linearization plots of modeling biosorption isotherm of dead cell: (a) Langmuir; (b) Freundlich and kinetic coefficients: (c) pseudo-first order; (d) second-first order for the dye biosorption onto *L. fusiformis* strain W1B6 cell biomass.

| Table 2. Biosorption characteristic of dye on *L. fusiformis* strain W1B6 cell biomass. |
|-----------------------------------------------|-----------------------------------------------|
| **Biosorption isotherm modeling** | **Biosorption kinetic study** |
| Langmuir coefficients | Freundlich coefficients | Pseudo-first-order | Pseudo-second-order |
| \( K_L \) | \( Q_M \) | \( R^2 \) | \( K_F \) | \( N \) | \( R^2 \) | Slope | \( R^2 \) | Slope | \( R^2 \) |
| 0.13 | 0.01 | 0.987 | 2.976 | 3.049 | 0.645 | 0.151 | 0.935 | 0.012 | 0.883 |

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**Microbial interaction during degradation**

The descriptions of microbial interaction of live and dead cells during dye decolorization observed under SEM are illustrated in Figure 7(b) and (d). The cells in the absence of dye are shown in Figure 7(a) and (c). Biosorption involves a combination of active and passive transport mechanisms starting with the diffusion of the adsorbed component to the surface...
of the microbial cell (Wang and Hu, 2008). In this study, the dye biosorption on live cell showed no significant change on the cell morphology and the color feature (Figure 7(b)). It showed that the biodegradation process was the main role in the decolorization process than biosorption. In the absence of dye, some endospore at the end shape of the dead cell was released (Figure 7(d)). It might be due to the effect of high temperature during the autoclaving process. As this strain is a Gram-positive bacterium consisting of many layers of peptidoglycan, the shrivelled cell in Figure 7(d) indicates the breakdown of the bacterial cell wall when exposed to the dye. It could be that the absorption of dye caused the plasmolysis of the layers where they play a role in withstanding the turgor pressure exerted on the plasma membrane (Silhavy et al., 2010).

**Conclusions**

*L. fusiformis* strain W1B6 is an effective strain for the decolorization of MR in the mineral salt medium. About 96% of MR was successfully removed within 2 h at 30 ± 2°C, at a wide range of pH 5.5–8.5, inoculum 10% (v/v), and 100 mg/L of initial dye concentration under
120 r/min, with the specific growth rate of 0.273/h. Azo reductase, laccase, and lignin peroxidase for both intracellular and extracellular were detected in the oxidoreductive mechanism involved in the biodegradation of MR. Equilibrium data for biosorption dye/dead cell system were well fitted to Langmuir isotherm. The reaction kinetics of MR was expressed to follow the pseudo first-order with the decolorization rate of 0.151. Biodegradation is more suitable to explain the main mechanism on the decolorization of dye MR using *L. fusiformis* strain W1B6 than biosorption or bioaccumulation mechanism.

**Declaration of Conflicting Interests**
The author(s) declare that there is no conflict of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors thank the IPPP grant of the University of Malaya for its financial support of this work (Project no: PG 057-2013A and RG 048-11B10).

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