Enzymatic Degradation of Azo Bonds and Other Functional Groups on Commercial Silk Dyes by *Streptomyces coelicoflavus* CS-29

Wai Phyo Mon[1], Phongphan Jantaharn[2], Sophon Boonlue[1], Sirirath McCloskey[3], Somdej Kanokmedhakul[2], and Wiyada Mongkolthanaruk[1*]

[1]*Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[2]*Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[3]*Natural Products Research Unit, Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

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*** Corresponding author:**
E-mail: wiymon@kku.ac.th

ABSTRACT

Azo dyes are used for silk textile manufacture, where their decolorization and detoxication are necessary after initial dying in the craft industry. The bio-decolorization efficiency of *Streptomyces coelicoflavus* CS-29 toward commercial azo blue and red dyes was investigated, analyzing the degradation and adsorption of dye molecules. *S. coelicoflavus* CS-29 showed reductions of 70% and 51% in red and blue dyes, respectively, after seven days. Morphological observation by light microscopy showed that dye molecules were adsorbed onto *S. coelicoflavus* CS-29 cell surface to form a dense cell pellet. Moreover, peroxidase and laccase activity were detected as extracellular enzymes, but no azo-reductase was detected. From the enzymatic activity, changes of dye profiles in HPLC showed differences between control dyes (untreated dyes) and metabolized products of dyes treated with *S. coelicoflavus* CS-29. The presence of main functional azo groups (-N=N-) in both blue and red silk dyes was indicated by FTIR analysis, in the untreated azo dyes. The azo bonds seemed to disappear in metabolites after *S. coelicoflavus* CS-29 treatment and other functional groups were changed compared to the control dyes. The treated dyes showed no significant effect on seed germination, root length, and shoot length of mung beans during phytotoxicity analysis. The red dyes showed a more negative effect on shoot lengths than the blue dyes. The overall results showed that *S. coelicoflavus* CS-29 is an effective and promising tool for the treatment of dye contaminated wastewater and the permanent elimination of recalcitrant commercial azo dye pollutants.

1. INTRODUCTION

Dyes play a very important role all over the world as an essential material for multiple manufacturing purposes ranging from small to industrial scale usage. Dyes are used in the textile, pulp and paper, dye and dye intermediate industries, in pharmaceutical, tannery and kraft bleaching capacities. Among these industries, the textile sector utilizes many classes of dyes such as azo dyes, nitro dyes, indigo dyes, anthraquinone dyes, phthalein dyes, triphenyl methyl dyes, and nitrated dyes, with choice based on the dye's chemical structure and chromophore, the dyeing process used, the color index and application (Benkhaya et al., 2020). Azo dyes are one of the largest families of textile dyes used. These dyes are xenobiotic compounds containing electron withdrawing groups, generating electron deficiency in the dye molecule, making them resistant to degradation (Singh et al., 2014). Azo dyes are recalcitrant compounds due to a structure of (-N=N-) heterocyclic and aromatic amine which can cause both toxicity (carcinogenic and mutagenic) and a non-biodegradable nature (Sinha and Osborne, 2016).

Silk cottage in Chonnabot istrict, Chaiyaphum, Thailand, is a successful craft industry producing silk products; as such, dyes, particular from Phua Kiam Seen Company, are utilized for the silk dying process in high levels. Disposal of contaminated dyeing

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effluents in natural water has the potential to severely affect environments and create health hazards, including to freshwater organisms. When this water is in turn used for agriculture, it can affect germination rates and biomass of several ecological crops, with consequences also for wildlife habitats and soil fertility (Saratale et al., 2013). Effective effluent treatment is an important step towards reducing pollutants in natural water and conserving water resources. Bioremediation is considered one possible way to decontaminate wastewater, based on natural technologies using microorganisms, plants and enzymatic system. Many organisms are able to decolorize and metabolize dyes, such as bacteria, fungi, yeasts, actinomycetes and algae (Garg and Tripathi, 2017). This occurs through an enzymatic transformation reaction in which reductive cleavage of azo bond leads to the formation of colourless aromatic amines or adsorption of dye onto the biomass of cells. Toxic amines in dyes can further oxidize to simpler non-toxic forms only under aerobic conditions. The end products of azo dye degradation are dependent on enzymatic system and growth condition of microorganisms; therefore, toxicity of metabolites should be detected using phytotoxicity which is a common tool for monitoring whether compounds are toxic on seed germination and growth of monocotyls or dicotyls, particularly compounds contaminated in environments, such as, metal compounds contaminated in soil, secondary metabolites produced by organisms. The phytotoxicity test is a criteria to detect toxic compounds (end products/by-products) before releasing into environments for safety.

Streptomyces is one of the most plenteous genera of Gram-positive bacteria, which have a superior sorption capacity from mycelia structure consisting of carboxyl, phosphonate, amine and hydroxyl groups for dye interaction. *Actinomyces* used as an adsorbent was able to adsorb and decolorize anthroquinone, phalocyanine and azo dyes effluents without dye degradation (Zhou and Zimmermann, 1993). Meanwhile, the role of peroxidase, laccase, veratryl alcohol oxidase, tyrosinase and azo reductase has been reported for degradation and detoxification of textile dyes (Kurade et al., 2016), particularly laccase likely in *Streptomyces ipomoeae* CECT 3341 (Blánquez et al., 2019). This study focused on the decolorization and detoxification of silk azo dyes using *S. coelicoflavus* CS-29 by observing bacterial morphology changes and dye chemical changes. Biosorption and detoxification are criteria for wastewater treatment systems including dyes and metals. The mycelial bacteria, *Streptomyces*, are good candidates as adsorbents in this system as following removal of biomass after treatment, they can perhaps be re-used.

2. METHODOLOGY

2.1 A bacterium strain and cultivation

The bacterium, *Streptomyces coelicoflavus* CS-29 was a candidate as it had the potential for removal of azo textile dyes from wastewater in previous study (Mon et al., 2020). This strain was isolated from dye-contaminated soil in Chonnabot District, Khon Kaen Province, Northeastern Thailand. The cells grew in minimal broth medium [containing (g/L) Na_2HPO_4 (5.3), KH_2PO_4 (1.98), $MgSO_4 \cdot 7H_2O$ (0.2), NaCl (0.2), yeast extract (2.5) and 1 mL of trace metal solution (g/L) which contained $CaCl_2 \cdot 2H_2O$ (0.05), $CuSO_4 \cdot 5H_2O$ (6.4), $FeSO_4 \cdot 7H_2O$ (1.1), $MnCl_2 \cdot 4H_2O$ (7.9), $ZnSO_4 \cdot 7H_2O$ (1.5)]; the medium was incubated with shaking at 37°C for 2 days and then was used as an inoculum.

2.2 Determination of dye removal

The bacterium grew in the minimal broth medium containing 300 mg/L of commercial textile dyes, blue dye (No. 21) and red dye (No. 34) from Phua Kiam Seen, Co., Ltd, and incubated at 37°C under shaking speed of 150 rpm. The bacterial culture was collected at three, five, seven and 10 days to separate cells and supernatant by centrifugation; the experiment was performed in triplicate. Color residue was measured from the supernatant by UV-VIS spectrophotometer at 554 and 506 nm for blue and red dyes, respectively. The cells were washed with methanol (98%) until no color remained in the cell pellets; the dyes dissolved in methanol solution were measured at 554 and 506 nm for blue and red dyes, respectively; and then the dye concentration (mg/L) was calculated from standard curves with equations of each dye below.

$$\text{Blue dye concentration (y)} = 0.3149X - 0.0029$$

$$\text{Red dye concentration (y)} = 0.2777X + 0.4303$$

Where; X is absorbance values at 554 and 506 nm for blue and red dyes, respectively.

2.3 Enzyme assay for biodegradation

The cell free supernatant of *S. coelicoflavus* CS-29 was collected by centrifugation at 12,000 rpm for 5 min after culturing by inoculating into nutrient broth [(g/L) beef extract (3), peptone (5), sodium chloride (5)] and incubated at 37°C for 3, 5, and 7 days. The supernatant was used as the crude extracellular enzyme for determination of enzyme activity. Each enzyme activity was calculated using the equation below based on the definition of each enzyme.

$$\text{Enzyme activity} = \frac{\text{absorption x test volume x dilution factor}}{\text{absorption coefficient x enzyme volume x time}}$$

2.3.1 Peroxidase activity

The crude enzyme (500 µL) and 1 mL of pyrogallol (0.013 M) in 0.1 M potassium-phosphate buffer (pH 7.0) were mixed into a cuvette. The potassium-phosphate buffer was used as a blank and 1 mL of pyrogallol substrate in 500 µL of potassium phosphate buffer was used as the control sample. 50 µL of hydrogen peroxide (3% v/v) was added into the control and enzyme sample to start the reaction. Color change (the yellow to dark brown) was measured by using UV-Vis spectrophotometer at 420 nm. This showed the change of the pyrogallol to purpurogallin (Buntić et al., 2017). One unit of enzyme was defined as the amount of enzyme that expressed 1 µmol of purpurogallin/min. The molar extinction coefficient of purpurogallin is 12 M/cm^{-1}.

2.3.2 Laccase activity

The bacterium was cultivated in nutrient broth containing 0.1% guaiacol; the activity of laccase in supernatants was determined by observing the oxidation of ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). The aliquots of the crude enzyme (200 µL) and 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) were mixed into a final volume of 1 mL. The reaction was held at 32°C for 10 min; and then was stopped by adding 0.5 mL of 80% trichloroacetic acid. The green formation of oxidized ABTS was examined at A_{420} nm. The substrate blank (200 µL of nutrient broth instead of crude enzyme) and enzyme blank (without ABTS) were performed. The absorbance of samples was calculated by subtracting from the absorbance of both blanks. One unit of enzyme was defined as the amount of enzyme oxidizing 1 µmol of ABTS/min. The molar extinction coefficient is 36,000 M/cm^{-1} (Mongkolthanaruk et al., 2012).

2.3.3 Manganese peroxidase activity

Enzyme assay is based on the oxidation of phenol red with or without peroxidase. For manganese dependent peroxidase, the mixture was prepared with 1 mL of sodium succinate buffer (0.25 M, pH 4.5), 700 µL of phenol red (0.71 mM), 400 µL of $MnSO_4$ (1.25 mM), 1 mL of sodium lactate buffer (0.25 M, pH 4.5), 1 mL of gelatin (0.5%), and 500 µL of the crude enzyme. In parallel, the mixture of manganese independent peroxidase was prepared without addition of $MnSO_4$. Distilled water was used instead of enzyme for control sample. The reaction was started by adding 400 µL of hydrogen peroxide (0.62 mM) with the incubation temperature at 30°C for 20 min; 1 mL of the assay mixture was added into 40 µL of NaOH (5 M) to stop the reaction. All samples were measured at A_{610} nm. Manganese dependent peroxidase activity was calculated by subtracting the extinction value of phenol red-oxidizing activity in the absence of $MnSO_4$ from the extinction value of the activity obtained in the presence of manganese. One unit of manganese dependent peroxidase activity was expressed as an absorbance increase of 0.1 units/min/mL of the enzyme sample (Buntić et al., 2017). The molar extinction coefficient of phenol red is 22 mM/cm^{-1}.

2.3.4 Azo-reductase activity

The mixture of 400 µL of crude enzyme, 100 µL of methyl red (500 mg/L) and 50 mM of sodium phosphate buffer in a final volume of 1 mL was prepared, resulting in a final concentration of methyl red at 50 mg/L. The addition of 8 µL of NADH (50 mM) was added to start reaction. Control was all reaction mixture, except the crude enzyme. The reaction was held at 37°C for 20 min. Enzymatic activity was determined by following the disappearance of methyl red (ε = 8.5 mM/cm^{-1}) at its maximum absorbance wavelength (430 nm). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmol of methyl red per min under the assay condition (Leelakriangsak and Borisut, 2012).

2.4 Chemical analysis of commercial synthesis textile dyes and degraded dyes from bacterial treatment

The bacterium grew in the minimal broth medium with the dyes (300 mg/L) at 37°C for seven days and the medium containing dyes without bacteria was prepared as controls. The supernatants were

collected and the dye metabolites were extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated using rotary evaporator. The pellets were dissolved in methanol. The crude extracts of the treated dyes (medium with dyes and bacteria) and the crude extract of the untreated dyes (medium with dyes) were analyzed by High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopic (FTIR). Both commercial textile dyes (blue and red dyes) were prepared in methanol and also determined by HPLC and FTIR as original dyes. The HPLC (Shimadzu) unit was equipped with UV-vis detector and was used to study the degradation of dye. The samples were injected into a C8 column; the mobile phase used was 80% methanol, 20% deionized water with 0.05% acetic acid at a flow rate of 1.0 mL/min with 100 µL of injection volume. The samples were detected at respective wavelengths of 554 nm for the blue dye and 254 nm for the red dye. FTIR analysis of all samples was carried out using a spectrophotometer in the mid-infrared region of 600-4,000 cm^{-1} with 16-scan speed for examination of main functional groups (Bankole et al., 2018).

2.5 Phytotoxicity assay

Phytotoxicity of the treated dyes and the untreated dyes was studied using *Vignabean radiata* (Mung bean) seeds. Healthy seeds of uniform size were selected. Seeds were surface sterilized with 1.5% sodium hypochlorite solution and then washed five times with sterilized distilled water before transferring to the petri plates layered with two sheets of tissue paper (Sarayu and Sandhya, 2010). The crude extracts of the treated and untreated samples were dissolved in 1 mL of methanol first to dissolve well and added distilled water to final concentration of 200 mg/L. All of samples were performed in 30 individual seeds including control without dyes (distilled water) and with commercial dye solution at concentration of 50, 100, and 200 mg/L. Germination of mung bean was performed at 30°C in dark and tissue paper was damped with 5 mL of respective dye solution for all replications. The assay was carried out triplicate. The results were recorded after four days by determination of shoot and root length. The percentage of germination index (GI) and percentage of phytotoxicity were expressed by the following equations (Rahman et al., 2018).

$$\text{Germination Index (\%)} = \frac{(\text{\% Relative seed germination}) \times (\text{\% Relative root growth})}{100}$$

$$\text{Phytotoxicity (\%)} = \frac{\text{Radical root length of control} - \text{Radical root length of sample}}{\text{Radical root length of control}} \times 100$$

2.6 Statistical analysis

All experiments were performed in triplicate. The phytotoxicity test was designed in a completely randomized design (CRD). Data were analyzed using analysis of variance and Tukey HSD all-pairwise comparisons test at $p<0.05$ to compare the means of each experiment. All statistical analyses were conducted using Statistix 10.0.

3. RESULTS AND DISCUSSION

3.1 Dye removal and observation of cell aggregation with dye molecules

The dye concentration was determined from the supernatant (residual dyes) and the adsorbed dyes (washed out from cells by methanol) to confirm the mechanism of decolorization by *S. coelicoflavus* CS-29. The results showed clearly that the dyes were aggregated with the cell, with higher dye concentrations detected in the cell pellets (Table 1); the residual dyes in the supernatant were at low concentrations, indicating a percentage of decolorization of 70% for red dye and 51% for blue dye. This result was the opposite of that from previous study (Mon et al., 2020), which reported that the blue dye gave higher percentage of dye removal than the red dye. These results could be explained by Figure 1 in the previous study (Mon et al., 2020). The graph showed that the red dye absorbance was higher than the blue dye absorbance at the same concentration. It is possible that the red dye type is more color-rich at low concentrations as many dyes are visible in water at a concentration of 1 mg/L (Pandey et al., 2007). Thus, if more of the red dye was adsorbed to cell, the residual dye in the supernatant should be at low concentration. However, the dye was still visible, suggesting high absorbance.

Table 1. Concentration of dyes that were adsorbed to cell and remained in medium for decolorization of bacteria

Day	Blue dye concentration (mg/L)				
	3	5	7	10	Average
Control	249.00±0.03	239.00±0.06	241.00±0.04	214.00±0.05	236.00±0.09
Supernatant	128.00±0.13	132.00±0.08	96.00±0.02	105.00±0.02	115.00±0.10
Cell pellet	199.00±0.09	167.00±0.15	318.00±0.05	261.00±0.06	236.00±0.39
Day	Red dye concentration (mg/L)				
	3	5	7	10	Average
Control	261.00±0.02	231.00±0.06	151.00±0.15	204.00±0.05	212.00±0.24
Supernatant	64.00±0.01	59.00±0.03	63.00±0.01	67.00±0.01	63.00±0.02
Cell pellet	378.00±0.06	326.00±0.06	280.00±0.24	316.00±0.14	325.00±0.21

The blue dye particles aggregated with mycelia of the bacterium to form dense cell pellets (Figure 1). The bacterial filaments grew and protruded from the outer edge of older pellets. Interestingly, the red dye was more attracted to mycelia for the adsorption process and aggregated with the cells, resulting in opaque dense pellets with red color. The new mycelial fragments could establish new pellets in minimal broth medium, increasing the number of dye pellets. However, pellet size was not significantly different over long incubation; this might be caused by a limitation of nutrients (minimal broth medium with/without dyes). In *Streptomyces*, mycelia adhere to one-another in a process called germling aggregation which requires adhesive glycan. With an absence of new nutrients, hyphae protrude from the periphery of aging pellets. This event may be due to weak adhesive forces between hyphae prone to fragmentation as a result of mechanical stress (Zacchetti et al., 2018). Similarly, the dyes were adhered to extracellular glycan of *S. coelicoflavus* CS-29 that associated with cell surface, showing adsorption mechanism on the surface of cell pellet. This result matched that found in *Streptomyces* (Zacchetti et al., 2018). Likely, lactic acid bacteria adsorbed Dorasyn Red azo dye showed smoother cell surfaces from dye coating pores of biomass (Sofu, 2019); also in fungi, the surface of *Aspergillus niger* filaments was coated with small particles (0.1-1.0 µm) after adsorbing acid dyes (Li et al., 2019). According to the results, the decolorization mechanism of *S. coelicoflavus* CS-29 may occur by adsorption of dyes in the cell pellets. The enzyme system may be necessary for next step mechanism.

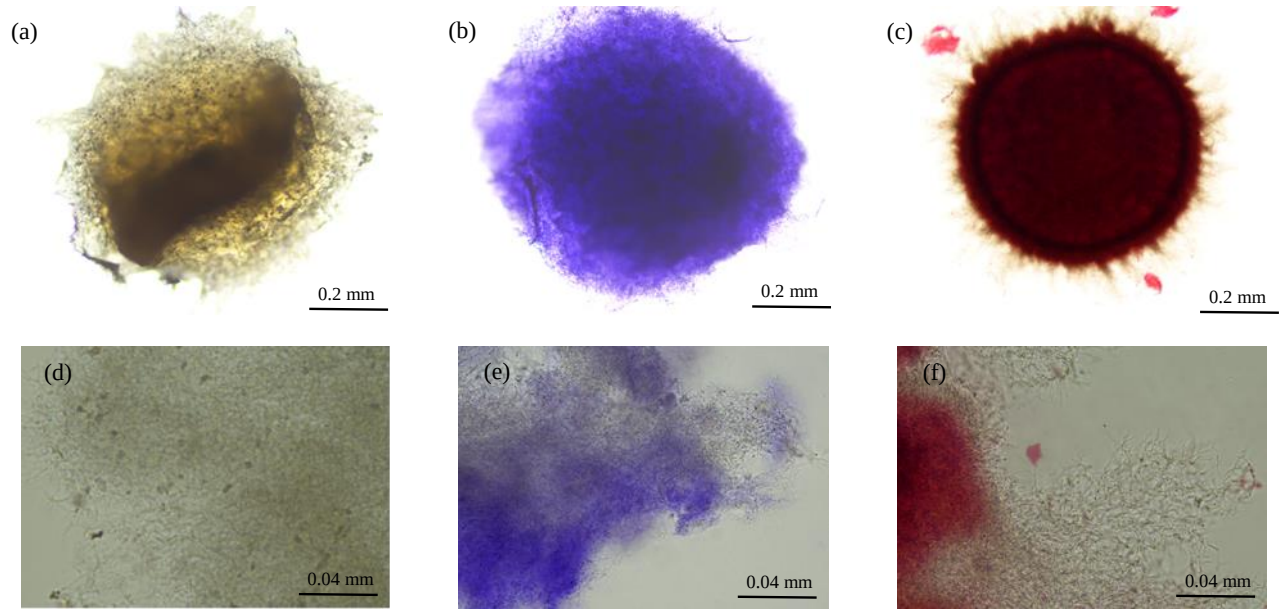

Figure 1. Pellets consistency of *Streptomyces coelicoflavus* CS-29 during growth in minimal broth medium with 300 mg/L dyes for five days observed under light microscope; dense pellets of filamentous bacteria that grew in the medium without dye (a, d), with blue dye (b, e), and with red dye (c, f)

3.2 Mechanism of commercial textile dyes biodegradation by bacterial enzyme

The active enzymatic system of biodegradation in commercial dye (Azo dyes) is peroxidases and azo-reductases which were determined in *S. coelicoflavus* CS-29. The bacterium produced peroxidases of high significance in seven days, as opposed to laccase which produced the highest activity after three days (Table 2). Mn-peroxidase was produced consistently during seven days of incubation. The azo-reductases could not be detected in cell free culture as an extracellular enzyme. The presence of peroxidase, Mn peroxidase and laccase enzyme could be confirmation that *S. coelicoflavus* CS-29 secreted enzymes to oxidize structurally different organic compounds, including polymeric dyes of both silk dyes in this study. Similarly, to *Streptomyces chromofuscus* A11 oxidized azo dyes by extracellular peroxidase (Pasti-Grigsby et al., 1996). The functions of Mn-dependent peroxidase and laccase are the oxidation of phenolic compounds with a requirement of Mn^{3+} and oxygen, respectively (Stolz, 2001). The possible degradation mechanism of the silk dyes were oxidation of phenolic structures and conversion of azo bond by additional methyl or methoxy substituents or by breaking down into two components. In the case of azo-reductase, no activity was detected in the cell free culture of *S. coelicoflavus* CS-29; it may be produced as an intracellular enzyme. This enzyme is involved in the reductive cleavage of the azo bond as the initial stage in degradation of azo dyes and transforms the parent dye molecule into compounds with reduced toxicity (Buntić et al., 2017).

Table 2. Enzyme activities related to dye reduction by *Streptomyces coelicoflavus* CS-29 in various time interval

Incubation time (day)	Peroxidase (U/mL)	Manganese peroxidase (U/mL)	Laccase (U/mL)
3	0.022±0.030^{b}	0.093±0.060^{a}	0.045±0.030^{a}
5	0.022±0.010^{b}	0.077±0.020^{b}	0.034±0.020^{b}
7	0.032±0.050^{a}	0.097±0.020^{a}	0.026±0.010^{c}

Values are mean for triplicate readings using Analysis of variance and Tukey HSD All-Pairwise Comparisons Test. Different letters in each column show significant values of enzyme activity.

3.3 Analysis of commercial textile dyes and degraded dye products by FTIR

The FTIR analysis of commercial blue dye exposed the presence of the main functional group, azo bond (–N=N–) between 1,576-1,597 cm^{-1} corresponding to N-H bending, peaks at 1,716-1,737cm^{-1} possible alkenes conjugation with C=O, C-N bond at 1,033.19 cm^{-1}, and nitro group (NH_2) at 1,500-1,660 cm^{-1} (Figure 2(a)). The blue dye treated with bacteria (bacterial metabolites) showed a clear absence of peak at 1,580 cm^{-1} of azo bond and showed the change of peaks around C-N stretching and C-O stretching. The thioethers group, CH_3-S- (C-S stretch), in the region of 660-630 cm^{-1} disappeared in the bacterial metabolite of the blue dye after treatment.

Interpretation of commercial red dye found the existence of aromatic amine at 1,551 cm^{-1}, N-H bond at 1,598 cm^{-1} corresponding to azo bond (-N=N-), C=C bond at 1,619 cm^{-1}, C-N bond between 1,042-1,200 cm^{-1}, C=C-H bending between 701-868 cm^{-1} (Figure 2(b)). The absence of peak at 1,598 cm^{-1} for azo bond indicated the cleavage of red dye in bacterial metabolite; the changes at 1,327 cm^{-1} and 893 cm^{-1} of untreated dye corresponded to C-N stretch of aromatic amines (1,290-1,310 cm^{-1} in bacterial metabolite) and C-H deformation (780-910 cm^{-1}), respectively.

The dyes were prepared in the minimal broth medium without bacterial cell designed as untreated controls which showed a different pattern from commercial dyes. This may be due to the high temperature and pressure of the sterilization process (Autoclave); the C-H, O-H bonds (2,800-3,300 cm^{-1}) were changed and aromatic compounds obtained C=O at 1,723 cm^{-1} differed from those in the untreated control dye. Another possibility, is that the commercial dyes were not extracted with ethyl acetate, but they were impurities containing various compounds with a complex structure. According to the results, the main functional groups (–N=N–) in both of blue and red dyes indicated a type of azo dye. The general structure of azo dyes is R_1–N=N–R_2; where R_1 brings electron withdrawing substituents and R_2 bears electron releasing substituents, mostly sulfonic (SO_3^-) substituent. It can be assumed that bacteria could break some compounds in dyes by oxidation and cause reduction via enzymatic activity.

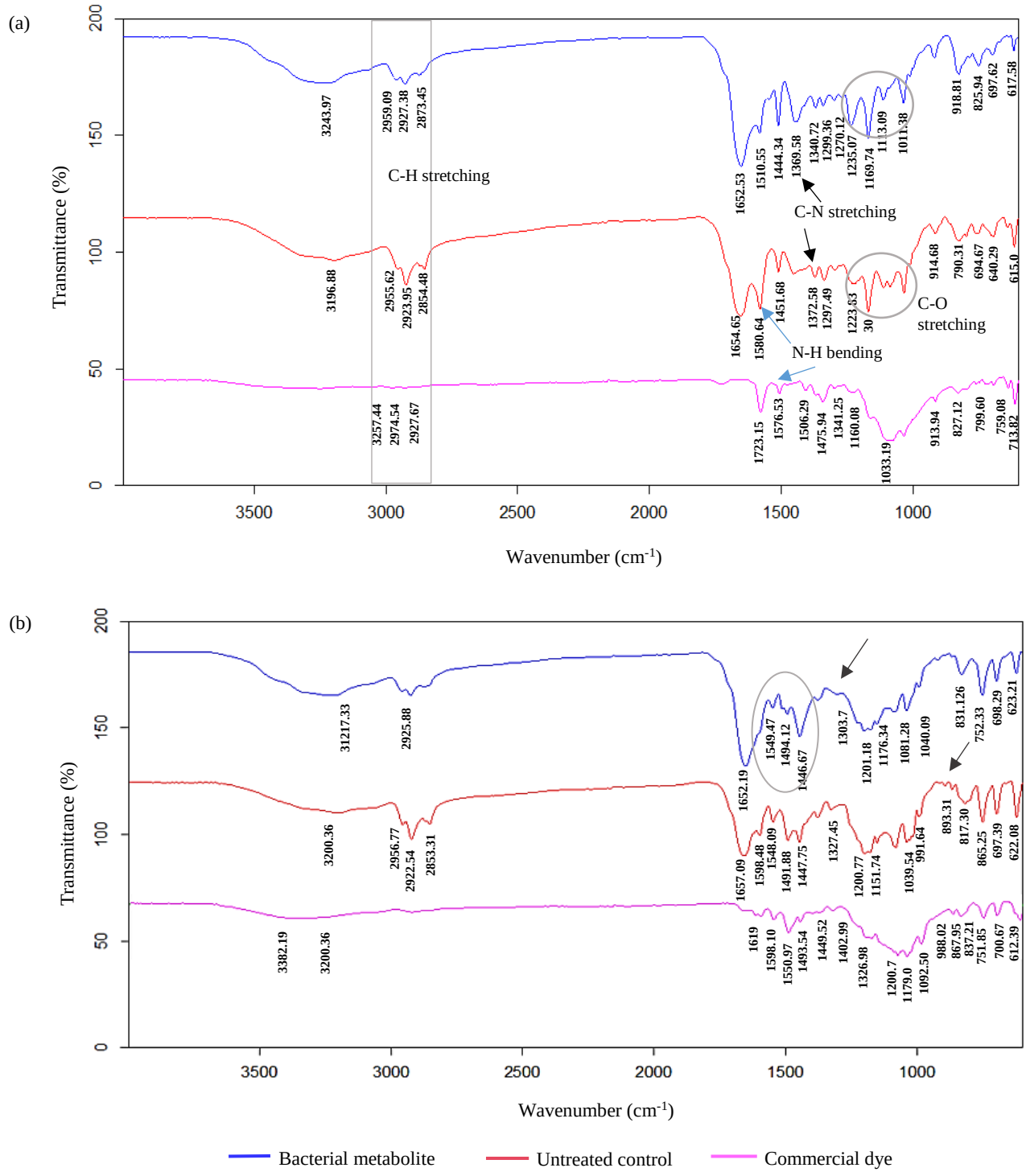

Figure 2. Main functional groups of commercial dyes, untreated dyes, bacterial metabolites for blue dye (a) and red dye (b) by FTIR

3.4 Analysis of bacterial metabolites by HPLC

HPLC analysis of commercial dyes, untreated control dyes, and bacterial metabolites of *S. coelicoflavus* CS-29 is presented in Figure 3. The main peak for commercial blue dye was at 4.171 min of retention time (Figure 3(a)). The profile of the control blue dye was 4.222, 3.198, 2.707 min, and this changed in the treated dye shown at 4.314, 3.281, 2.425 min (Figure 3(b) and (c)). In parallel, the main peak of commercial red dye was at 3.881, 2.701, 2.081 and 1.982 min of retention time (Figure 3(d)); these peaks disappeared from the control dye, which showed a main peak at 1.807 min (Figure 3(e)). This result confirmed that the dye components were treated by autoclave process, particularly in the red dye. The different peaks at the retention times of 1.807, 2.374,

3.354, 3.820 min indicated the degradation of red dye into different metabolites (Figure 3(f)). The HPLC profiles implied that dye adsorption to the cell imparts an approximate decrease of all peaks in proportion to each other, whereas dye removal by biodegradation indicates a complete reduction of the major peaks and the formation of new peaks at the same time.

To summarize for both blue and red dyes, peaks that appeared in the control dyes differed from the bacterial metabolites; there might be two degradation mechanisms such as the degradation of main compounds into intermediate products or the mineralization of parent compounds. According to enzyme activity, *S. coelicoflavus* CS-29 released the peroxidase and laccase enzymes to oxidize dyes molecules, mineralizing dye compounds which showed in HPLC profiles. The small dye compounds interacted with the filamentous surface of *S. coelicoflavus* CS-29 obtaining negative charges and then aggregating into the cells as shown in SEM. The results showed that *S. coelicoflavus* CS-29 plays the role of bio-adsorption in decolorization of textile dyes and degrade dye molecules. Similarly, *Streptomyces* sp., removed triphenylmethane dyes via biosorption and biodegradation (Adenan et al., 2021).

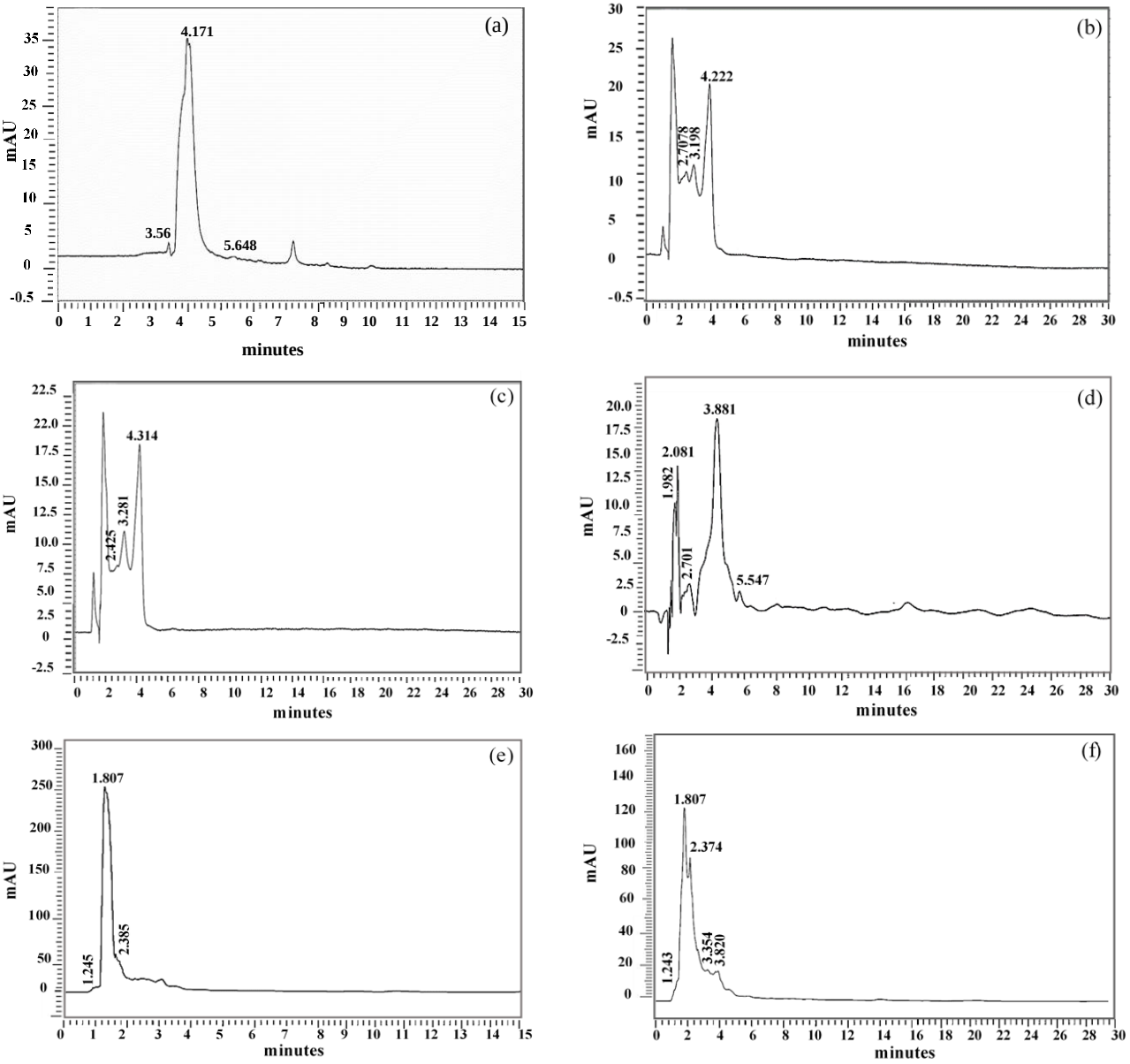

Figure 3. HPLC Chromatogram of commercial dyes, blue dye (a), red dye (d); untreated control dyes, blue dye (b), red dye (e); bacterial treated dyes, blue dye (c), red dye (f). The dyes were treated by *Streptomyces coelicoflavus* CS-29 for seven days.

3.5 Phytotoxicity study of degraded dye products by bacteria

This study focused on the actual consequences of significant wastewater discharge from Chonnabot District, Khon Kaen Province, Northeastern Thailand which causes harm to the living environment. Thus, a phytotoxicity test was carried out on the degraded silk dye products after treatment with *S. coelicoflavus* CS-29. This test confirmed that there was no significant difference in root length between control, untreated dyes and treated dyes extracts at 50 and 100 mg/L (Table 3). Of note, the treated blue dye showed greater shoot length than control, but in the treated red dye shoot length was less than control. In the case of the extracted metabolites might perform some nutritive role in seed germination and plant growth. Meanwhile, the red dye was evidently more toxic than the blue dye; it can be inferred that the red dye contains toxic components. The germination index (GI) was low in all dye treatments, showing lower GI percentage in higher dye concentrations according to the percentage of phytotoxicity. Comparison of phytotoxicity between untreated dyes and treated dyes revealed no difference in all parameters; this might be caused by an extraction method which recovered specific compounds. Moreover, the phytotoxicity effect of degraded dye products depended on the types and chemical structure of the dyes and types and tolerances of the seed cultivars (EPPO, 2007). The tolerance of untreated methyl red to seed germination and plant growth showed different levels in *Vigna radiata*>*Arachis hypogaea*>*Vigna unguiculata* (Sari and Simarani, 2019). The results implied that the bacterial metabolites still had low levels of toxicity after dyes biosorption with *S. coelicoflavus* CS-29. Similarly, the degraded metabolites of methyl red by *Lysinibacillus fusiformis* strain W1B6 were less toxic to the plants (Sari and Simarani, 2019). To conclude, *S. coelicoflavus* CS-29 has potential for the decolorization of silk dyes via enzymatic activity and adsorption as a biomass.

Table 3. Phytotoxicity analysis of the azo blue dye from control and bacterial degraded products tested in mung bean for four days after inoculation

Sample/parameters	Germination index (%)	Root (cm)	Shoot (cm)	Phytotoxicity (%)
Control (distilled water)	100.00±0.00^{a}	3.40±0.77^{a}	1.40±0.44^{b}	0.00±0.00^{e}
Blue dye				
Untreated dye extract				
• 50 mg/L	62.00±0.21^{c}	2.20±0.95^{ab}	2.20±0.38^{a}	33.00±0.25^{c}
• 100 mg/L	62.00±0.05^{c}	2.10±0.77^{ab}	2.10±0.12^{ab}	38.00±0.05^{bc}
• 200 mg/L	50.00±0.09^{d}	1.90±0.81^{ab}	1.90±0.20^{ab}	43.00±0.09^{b}
Treated dye extract				
• 50 mg/L	76.00±0.34^{b}	2.10±0.89^{ab}	2.10±0.24^{ab}	33.00±0.32^{c}
• 100 mg/L	62.00±0.08^{c}	1.90±0.63^{ab}	1.90±0.28^{ab}	43.00±0.05^{b}
• 200 mg/L	45.00±0.05^{d}	1.90±0.42^{ab}	1.90±0.12^{ab}	44.00±0.05^{b}
Red dye				
Untreated dye extract				
• 50 mg/L	72.00±0.21^{b}	2.40±0.66^{ab}	0.60±0.41^{c}	26.00±0.19^{d}
• 100 mg/L	66.00±0.23^{c}	2.10±0.62^{ab}	0.50±0.32^{cd}	34.00±0.23^{c}
• 200 mg/L	50.00±0.20^{d}	1.60±0.61^{b}	0.50±0.25^{cd}	50.00±0.20^{ab}
Treated dye extract				
• 50 mg/L	76.00±0.22^{b}	2.50±0.76^{ab}	0.50±0.24^{cd}	24.00±0.22^{d}
• 100 mg/L	62.00±0.07^{c}	2.10±0.72^{ab}	0.70±0.32^{c}	38.00±0.07^{bc}
• 200 mg/L	45.00±0.22^{d}	1.50±0.80^{b}	0.40±0.24^{d}	54.00±0.21^{a}

Values are mean of triplicate readings using analysis of variance and Tukey HSD All-Pairwise Comparisons Test at $p<0.05$. Different letters in each column show significant value of germination index, root length, shoot length and phytotoxicity.

4. CONCLUSION

Synthetic silk dyes are azo dyes with main functional groups, such as aromatic amine with double bonds of nitrogen. These dyes do not easily degrade and present a hazard to the natural environment. Biosorption is an effective system to treat contaminated dye molecules, leading to a reduction in both dye color and toxicity. *S. coelicoflavus* CS-29 was selected for

cell absorbent as it produces mycelium, giving it a high biomass. This strain was able to aggregate dye molecules with mycelium or adsorbed dye on the cell surface, showing dense cell under light microscope. Moreover, it produced laccase and peroxidase enzymes to mineralize dye structures. FTIR and HPLC were used to confirm the changes of functional groups and structure in the treated dyes. Finally, the metabolites of treated dyes were found to be non-toxic for seed germination, and not deleterious to root and shoot length of mung bean.

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