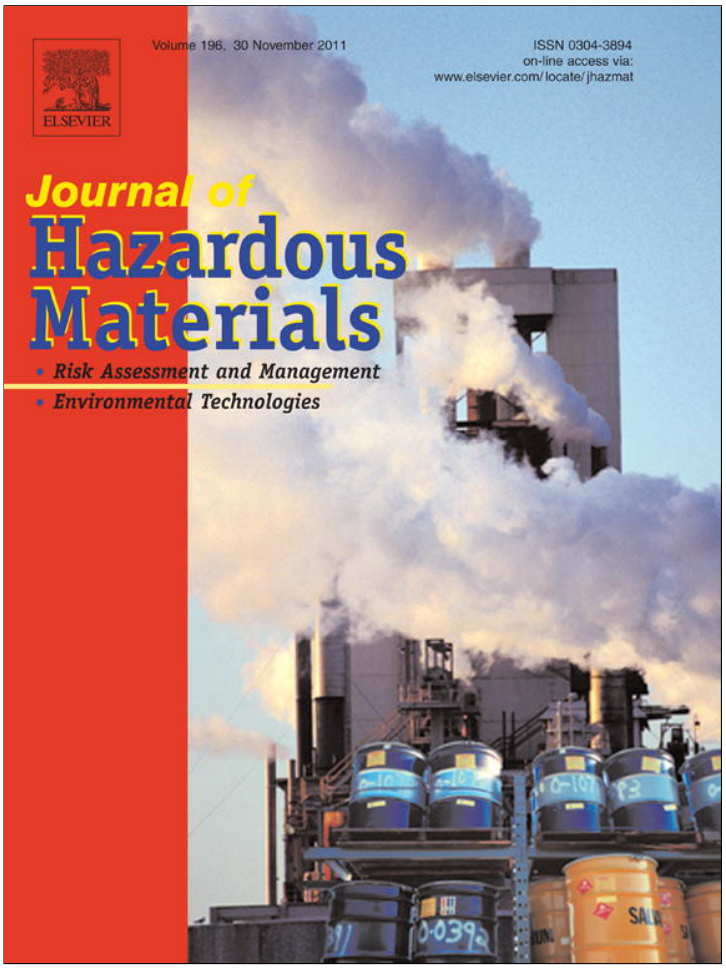

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Decolorization characteristics and mechanism of Victoria Blue R removal by *Acinetobacter calcoaceticus* YC210

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ABSTRACT

Acinetobacter calcoaceticus YC210 has been isolated and its ability to remove Victoria Blue R (VBR) from aqueous solution was assessed. The effects of various factors on decolorization efficiency were investigated in a batch system. The decolorization efficiency was found to be optimal within a pH of 5–7 and increased with VBR concentration up to 450 mg/l with high efficiency (94.5%) in a short time. The decolorization efficiency was significantly affected by cell concentrations. The decolorization of VBR by *A. calcoaceticus* YC210 followed first order kinetics. The apparent kinetic parameters of the Lineweaver–Burk equation, $R_{VBR,max}$ and K_m, were calculated as 6.93 mg-VBR/g-cell/h and 175.8 mg/l, respectively. Based on the biodegradation products, VBR degradation by *A. calcoaceticus* YC210 involves a stepwise demethylation process to yield partially dealkylated VBR species. To our knowledge, this is the first report using microbes to remove VBR. It clearly demonstrates the dealkylation pathway of VBR degradation.

1. Introduction

Triphenylmethane dyes are used extensively in the textile and fish farming industries, paper and leather industries, food and cosmetic industries, and in medicines [1]. In the study of Black et al. [2], it was noted that triphenylmethane dyes could be mutagenic and carcinogenic to biota. Some triphenylmethane dyes have been shown to be potent clastogens, possibly responsible for promoting tumor growth in some species of fish [3,4]. The discharge of triphenylmethane dye solution into the biosphere will negatively impact the exposed aquatic organisms. Such dyes are not easily degradable so their discharge will cause serious problems to the environment, and achieving legal purification levels is very difficult. Furthermore, triphenylmethane dyes could cause strong coloration and contribute to the organic load and toxicity of wastewater [5]. As there is a significant health risk to humans and aquatic organisms, it is very important to devise an efficient method to remove this substance from wastewater.

A number of studies have been carried out in which in physicochemical methods, such as adsorption, precipitation, flocculation, oxidation, and photodegradation, have been used to treat triphenylmethane dyes [4]. However, none has been found to be completely successful because present methods have proved to be methodologically demanding, relatively inefficient, and time-consuming [1,6]. Recent focus has therefore turned to biological processes as viable alternatives as such systems are environmentally friendly, cost-competitive, and produce less sludge [7]. The triphenylmethane dye, crystal violet has been successfully degraded by bacteria [7]. Theoretically, Victoria Blue R, another triphenylmethane dye, should be degraded by bacteria. However, there has been no published work on VBR decolorization or biodegradation by this means till now. *Acinetobacter* is a gram-negative genus of *Bacteria* belonging to the *Gammaproteobacteria*. Non-motile, *Acinetobacter* species are known to be involved in the biodegradation of aromatic pollutants such as biphenyl, chlorobiphenyl, and aniline [8]. *Acinetobacter calcoaceticus*, which belongs to the genus of *Acinetobacter*, was able to degrade crude oil such as alkanes and aromatics [9]. Although there are no published data currently available on its efficiency to biodegrade VBR, preliminary experiments carried out in our laboratory revealed that *A. calcoaceticus* is easily able to degrade VBR. In addition, *A. calcoaceticus* can be cultured easily in culture medium.

The aim of the study reported here was to isolate VBR-degrading bacteria from contaminated soil near a wastewater treatment plant, determine the basic physiological characteristics of the isolated strain, and provide further details on *A. calcoaceticus* YC210 in terms of its capability to degrade VBR. The effects of initial concentration of VBR, pH, and cell numbers of *A. calcoaceticus* YC210 on VBR removal was evaluated, and the primary metabolic pathway during the biodegradation process was established.

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2. Materials and methods

2.1. Chemicals

All the chemicals used in our experiment were analytical grade. Victoria Blue R was obtained from Sigma–Aldrich, Inc. and confirmed to be a pure organic compound through high-performance liquid chromatography (HPLC) analysis. The formula, molecular weight and solubility in water of Victoria Blue R are $C_{29}H_{32}ClN_3$, 457.5 g/mol, and 0.5%, respectively. Its absorption maximum occurs at 615 nm.

2.2. Microorganisms and cultivation

A. calcoaceticus YC210 was isolated from the soil near a wastewater sewage treatment plant in southern Taiwan. Each soil sample (10 g) was mixed with 200 ml sterile basal mineral medium [BMM (per liter distilled water): 4.8 g K_2HPO_4, 1.2 g KH_2PO_4, 1 g NH_4NO_3, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.04 g $CaCl_2$, 0.001 g $Fe_2(SO4)_3$] in a 350 ml flask and vortexed vigorously for 30 min. A 10 ml aliquot of each suspension solution was pippetted into a 350 ml flask containing 200 ml sterile BMM with 10 mg/l VBR and the solution mixed thoroughly. After 3 days of culture, 10 ml of culture solution was pippetted out and mixed with fresh BMM containing 20 mg/l VBR. The screening cycle was repeated until the major colony could be isolated by the spread plate method in BMM containing agar and VBR. Subsequently, following a 30-day acclimation period, the VBR-degrading bacterium was isolated from BMM containing 100 mg/l VBR. To identify the VBR-degrading bacterium, the cells of the dominant isolate were lysed and the DNA extracted. The 16S rRNA gene sequence of the dominant isolate was compared using BLASTN programs to search for nucleotide sequences in the NCBI website. A phylogenetic tree was constructed using the neighbor-joining method [10], and its topology was evaluated by bootstrap analyses of the neighbor-joining dataset using the SEQBOOT and CONSENSE options from the PHYLIP package. During the culture period, bacterial growth was monitored by spectrophotometry at 600 nm and by the spread plate method.

2.3. Batch decolorization system

The batch decolorization experiments were conducted in 350 ml flasks containing BMM, VBR, and *A. calcoaceticus* cells. To analyze the effects of different environmental factors on the efficiency of *A. calcoaceticus* cells to decolorize VBR, we tested different initial VBR concentrations (8–450 mg/l), different concentrations of bacterial cells (10^4–10^9 cfu/ml), and various pH values (5–9). The pH was adjusted using diluted solutions of NaOH and HCl. The batch experiments were run as a shaking culture at 30 °C and 250 rpm, with 60 mg/l VBR and 10^8 cfu/ml of *A. calcoaceticus* cells, unless stated otherwise. The decolorization efficiency was analyzed after 150 min of reaction time.

2.4. Analysis of VBR degradation by A. calcoaceticus YC210

The amounts of VBR in the culture medium at the end of the various culture periods (=degradation times) were determined by high performance liquid chromatography (HPLC). The samples were concentrated by solid phase extraction (SPE) and the extract identified by HPLC. The HPLC system consisted of an Econosil column (5 μm, 4.6 mm × 250 mm) and an isocratic mobile phase containing 70% (v/v) acetonitrile in an ammonium acetate solution (pH 5.5), λ_{max} = 615 nm. The flow rate was 0.5 ml/min. A Waters ZQ LC/MS system equipped with the 1525 Binary HPLC pump, 2996 Photodiode Array Detector, 717 plus Autosampler and micromass-ZQ4000 Detector were used to identify the intermediates of VBR degraded by *A. calcoaceticus* YC210 in the culture medium. Organic intermediates were analyzed by means of a coupled HPLC–electrospray ionization mass spectrometry (ESI-MS) after readjustment of the chromatographic conditions to make the mobile phases (solvents A and B) compatible with the working conditions of the MS. Solvent A consisted of 25 mM aqueous ammonium acetate buffer (pH = 6.9) and solvent B, methanol. HPLC was carried out on an Atlantis^{TM} dC18 column (250 mm × 4.6 mm, 5 μm). The flow rate of the mobile phase was 1.0 ml/min. A linear gradient was run as follows: t = 0, A = 95, B = 5; t = 20, A = 50, B = 50; t = 35–40, A = 10, B = 90; t = 45, A = 95, B = 5. The column effluent was introduced into the ESI source of the MS.

3. Results and discussion

3.1. Characterization of VBR-degrading bacteria

The PCR amplification and sequencing procedures were according to Weisburg et al. [11] and resulted in the isolate being identified as *A. calcoaceticus* YC210 with 99.2% similarity (GenBank accession no. GU339280). The phylogenetic tree analysis of the isolate indicates that the isolate YC210 has a high similarity with *A. calcoaceticus* (GU339280) (Fig. 1): both isolate YC210 and *A. calcoaceticus* (GU339280) are in the same cluster. *A. calcoaceticus* was isolated in BMM containing VBR as the sole carbon source. This microorganism comprised non-fermentative aerobic gram-negative rods that are widely dispersed in nature [12]. *A. calcoaceticus* YC210 was observed to enter the late logarithmic growth phase after 18 h of culture in BMM containing 50 mg/l VBR. The optimal pH range for the growth of *A. calcoaceticus* was 5–7, as estimated by cell numbers (data not shown), and the optimal temperature range was 20–35 °C (data not shown). These results are similar to those reported by Zhan et al. [13] who used *A. calcoaceticus* to degrade phenol. Consequently, in accordance with our results and their data, we performed all subsequent experiments at pH 7.0 and 30 °C to optimize the activity of *A. calcoaceticus* YC210.

3.2. Effect of reaction time and initial VBR concentration on VBR removal

To gain an understanding of the adsorption and biodegradation of VBR by *A. calcoaceticus* in terms of VBR removal, a batch removal study using autoclaved cells and living cells in BMM containing 10 mg/l MG was carried out. During the first 30 min, the efficiency of autoclaved cells and living cells for decoloring VBR was 7.5% and 85.0%, respectively (data not shown). The maximum decolorization efficiency of VBR by autoclaved cells only attained 12.6% (30–150 min), while that with living *A. calcoaceticus* increased with increasing reaction time up to 150 min, at which time the removal equilibrium was reached at a decolorization efficiency of 82.3 ± 0.5%. This indicated that autoclaved cells could remove dye by an adsorption mechanism but the major dye removal is probably biological oxidation by living *A. calcoaceticus* cells in the decolorization processes [14,15]. Because the decolorization efficiency of VBR by *A. calcoaceticus* exhibited an arc increasing curve between 30 and 150 min of the reaction time, we conducted a kinetic analysis. The results indicated that the biodegradation behavior of VBR by *A. calcoaceticus* YC210 fitted first order kinetics well: $\ln C = -0.0102t + 0.9243$, $R^2 = 0.983$.

The decolorization efficiency of *A. calcoaceticus* YC210 was studied in media containing different concentrations of VBR. Fig. 2 shows the dependence of VBR concentration on the biological decolorization efficiency after 150 min of reaction time. When the initial dye concentration was increased from 8 to 450 mg/l, the decolorization efficiency of VBR increased as well – from 77.4 ± 1.5%

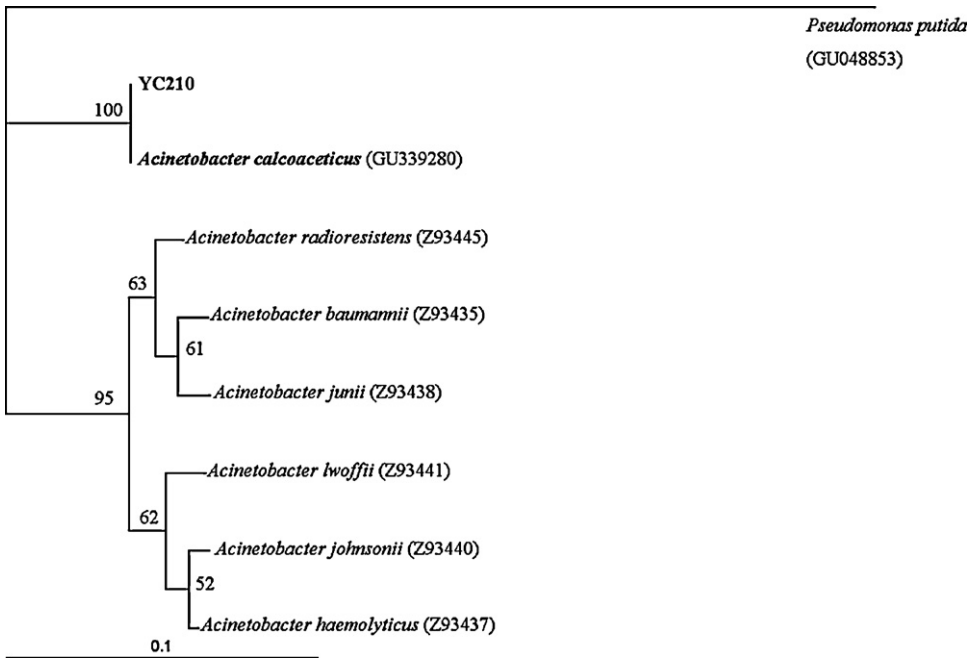

Fig. 1. Bootstrap N-J tree of isolate YC210 (in this study) illustrating its high similarity to *A. calcoaceticus* (GU339280) based on 16S rDNA sequence comparisons. *P. putida* (GU048853) was used as an outgroup in this analysis. The scale bar indicates 1% sequence dissimilarity.

up to 94.5 ± 1.4%. The results indicated that the initial concentration of VBR possesses a strong driving force to overcome the mass transfer resistances between VBR and the *A. calcoaceticus* cells [16]. However, the decolorization efficiency of malachite green (a triphenylmethane dye, but whose chemical structure is simpler) by *P. pulmonicola* only achieved 92.7% after 210 min of reaction time [15]. Hence, we suggest that both the structure of the dye and the bacteria species have a significant influence on decolorization [17].

3.3. Effect of bacterial cell concentration and pH on VBR removal

The decolorization efficiency of VBR by *A. calcoaceticus* YC210 increased with increasing cell concentration of *A. calcoaceticus* from 10^4 to 10^8 cfu/ml (Fig. 3a). The optimal decolorization efficiency, 90.7 ± 0.6%, was found at 10^8 cfu/ml of *A. calcoaceticus*. However, an overabundance of bacterial cells (i.e. 10^9 cfu/ml) had no appreciable effect on the decolorization efficiency. Prior to degrade dye by microbes, the adsorptive efficiency of *A. calcoaceticus* was significantly increased in the concentration of 10^7 cfu/ml in which the higher VBR adsorption was developed and has led to higher decolorization efficiency. That is be regarded the characteristics of *A. calcoaceticus*. Similar relationships between decolorization efficiency and cell concentration have been reported by Shahvali et al. [14], who treated textile wastewater with *Phanerochaete chrysosporium*, and by Sani and Banerjee [18], who treated textile wastewater with *Kurthia* sp. However, Moosvi et al. [6] reported that there was no proportionate increase of decolorization with increase initial concentration of bacteria. Thus, the relationship between decolorization efficiency and cell concentration in a biological system seems to alter from case by case. In this study, 10^8 cfu/ml of *A. calcoaceticus* was the optimal cell concentration to decolorize VBR.

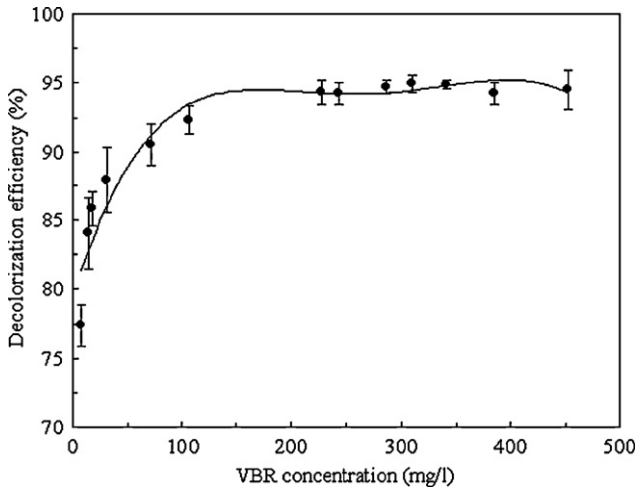

Fig. 2. Effect of VBR concentration on the decolorization efficiency of VBR by culture medium with *A. calcoaceticus* YC210. pH: 7.0; bacterial cells: 10^8 cfu/ml; 30 °C. Results are means of triplicate experiments (SD is indicated with error bars).

The decolorization efficiency of VBR in culture medium with/without *A. calcoaceticus* (control) was determined to understand the decolorization behaviors at different pH values (pH 5–9). The results showed that culture media without *A. calcoaceticus* had an insignificant decolorization efficiency within the pH range 5–9 (data not shown). In the *A. calcoaceticus* YC210 system, the decolorization efficiency increased from pH 5 to 6, leveled off between pH 6 and 7 (91.1 ± 0.5%), and sharply decreased to 80.1 ± 1.3% at pH 8 (Fig. 3b). For the genus of *Acinetobacter*, the isoelectric point was at a pH of 5.8 [19]. At a lower pH (pH < 5.8), the H^+ ions compete effectively with dye cations to adsorb on the cell surface, causing a decrease in color removal efficiency. At a higher pH (pH > 5.8), the surface of *A. calcoaceticus* changes to being negatively charged, which results in an increased electrostatic attractive force between the bacterial cell and the positively charged VBR molecule. This increased electrostatic force accounts for the significant increase in the decolorization efficiency of VBR at pH 6–7. As for the decrease in decolorization efficiency of VBR at pH 8–9 conditions, we suggested the optimal pH range for the growth of *A. calcoaceticus* YC210 is pH 5–7 that gives an additional explanation. Similar trends were found in the study of the decolorization of triphenylmethane and azo dyes by *Pseudomonas* sp. [20].

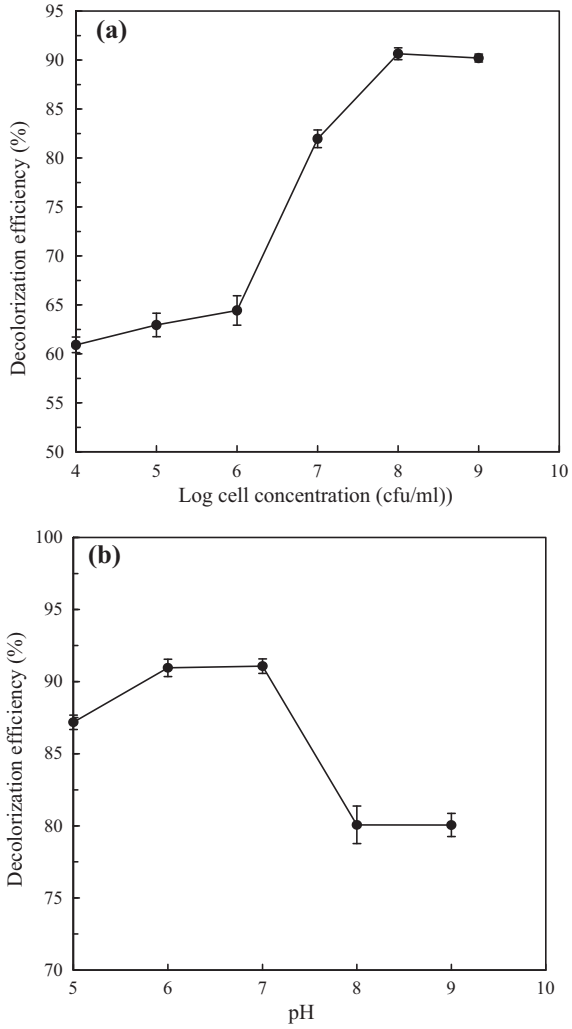

Fig. 3. (a) Effect of bacterial cell concentration on the decolorization efficiency of VBR by *A. calcoaceticus* YC210. VBR: 60 mg/l; pH: 7.0; 30 °C. (b) Effect of pH value on the decolorization efficiency of VBR by *A. calcoaceticus* YC210. VBR: 60 mg/l; pH: 5–9; bacterial cells: 10^8 cfu/ml; 30 °C. Results are means of triplicate experiments (SD is indicated with error bars).

3.4. Apparent kinetic analysis

The VBR degradation rate in this system was calculated using the following equation derived from the Lineweaver–Burk plot:

$$\frac{1}{R_{VBR}} = \frac{K_m}{R_{VBR,\max}} \times \frac{1}{C_{VBR}} + \frac{1}{R_{VBR,\max}}$$

where R_{VBR} (mg-VBR/g-cell/h) = VBR degradation rate; C_{VBR} (mg/l) = VBR concentration; $R_{VBR,\max}$ (mg-VBR/g-cell/h) = maximum apparent VBR degradation rate; K_m (mg/l) = apparent half-saturation constant. From the linear relationship between $1/C_{VBR}$ (x) and $1/R_{VBR}$ (y), $R_{VBR,\max}$ and K_m was obtained by calculating from the slope and intercept; the relationship is shown in Fig. 4. When the initial VBR concentration was increased from 8 to 498 mg/l, the VBR degradation rate increased significantly between 8 and 450 mg/l and then leveled off at 450–500 mg/l (data not shown). The regression equation in Fig. 4 is expressed as $y = 25.373\,x + 0.1443$ ($R^2 = 0.9962$) for the system inoculated with *A. calcoaceticus* YC210. The apparent kinetic parameters $R_{VBR,\max}$ and K_m are calculated to be 6.93 mg-VBR/g-cell/h and 175.8 mg/l, respectively. The variations in the K_m value for different substrates are mainly due to the affinity between cells and substrates. The decrease in K_m suggests an enhancement

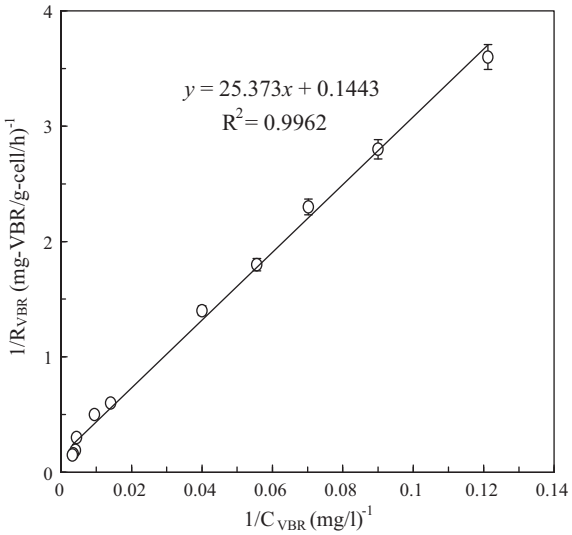

Fig. 4. Relationship between VBR degradation $1/R$ and $1/C$ in the medium inoculated with *A. calcoaceticus* YC210. pH: 7.0; bacterial cells: 10^8 cfu/ml; 30 °C. Results are means of triplicate experiments (SD is indicated with error bars).

in biomass affinity for the target substrate [15]. These important parameters ($R_{VBR,\max}$ and K_m) can be used for comparison if other biological systems are to be utilized in the removal of VBR.

3.5. Separation and identification of VBR metabolism intermediates

We isolated the intermediates of the VBR decolorization process using HPLC and ESI-MS and identified these by comparison with commercially available standards. Fig. 5 shows the VBR biodegradation intermediates formed by *A. calcoaceticus* YC210 after a 7-day culture period (sampling analysis from culture day-1 (upper), day-3, day-4, day-6, and day-7 (down), respectively). Peak A is the parent VBR dye (retention time: 54.6 min), and peaks B–I (retention time: 37.2–52.4 min) are degradation intermediates. Nine components were successfully detected, all with the retention times less than 60 min. The maximum absorption band of each intermediate in the visible spectral region in Table 1 was measured. The intermediates were further identified using the HPLC–ESI mass spectrometric method; the relevant mass spectra are illustrated in Table 1. The intermediates appeared to be in their acidic forms based on their molecular peaks. Other intermediates may have been under the detection limit. From these results, intermediates can be classified into several groups.

From the results of mass spectral analysis, we confirmed that the component A, m/z = 422.18, in the liquid chromatogram is VBR; the other components are B, m/z = 408.35; C, m/z = 394.76; D, m/z = 394.76; E, m/z = 394.25; F, m/z = 380.44; G, m/z = 380.16; H, m/z = 366.77; I, m/z = 366.04. The intermediates have the wavelength position of their major absorption bands moved toward the blue region, $\lambda_{\max}$, A, 613.0 nm; B, 606.9 nm; C, 603.2 nm; D, 609.4 nm; E, 619.2 nm; F, 598.3 nm; G, 608.1 nm; H, 587.3 nm; I, 545.8 nm. The intermediates may represent the *N*-de-alkylation of the VBR.

3.6. Product identification and proposed biodegradation pathways

A sequential identification of primary and secondary metabolites enabled us to elucidate the metabolic pathway of VBR degradation by *A. calcoaceticus* YC210. The MS analysis was a powerful tool and enabled us to confirm that the components A–I,

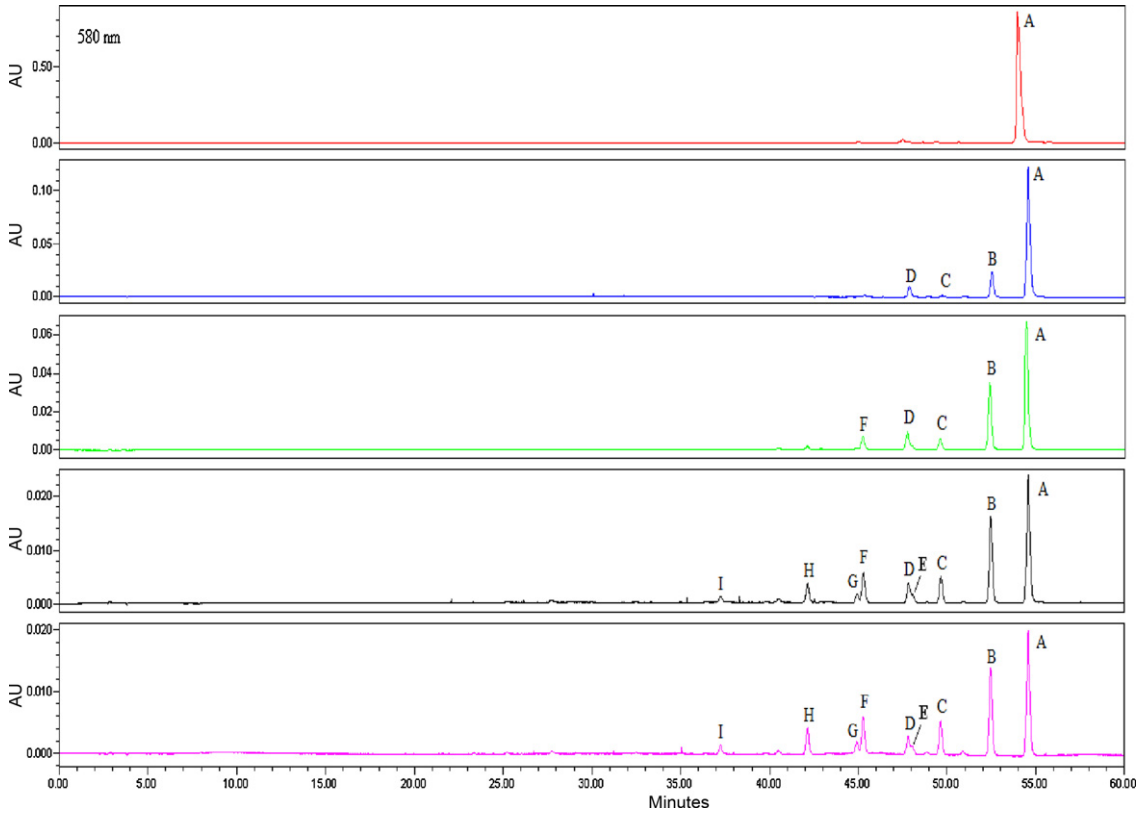

Fig. 5. Absorption spectra of the intermediates formed during the biodegradation process (7 days) of the VBR dye corresponding to the peaks in the HPLC chromatograph. Spectra were recorded using the photodiode array detector. The sampling analysis was from culture day-1 (upper), day-3, day-4, day-6, and day-7 (down).

respectively (data not shown). Fig. 6 presents a scheme of this degradation pathway. Each of the three side chains of the VBR dye molecules terminates in two dimethyl groups (compound A). B(or E) (compound B(E)) is obtained by the removal of one methyl(or ethyl) group from the VBR molecule; C–E, F–G and H–I (compounds C–I) correspond to three pairs of isomeric molecules with two to three (or one) fewer methyl (or ethyl) groups than the VBR dye molecule. C (compound C) is formed by removal of a methyl group from two different sides of the VBR molecule, whereas the other corresponding isomer in this pair, D (compound D), is produced by the removal of two methyl groups from the same side of the VBR structure. Another corresponding isomer in this pair, E (compound E), is produced by the removal of one ethyl group from the side of the VBR structure. In the second pair of isomers, F–G (compound F–G) is formed by the removal of three methyl groups from each side of the VBR molecule, whereas G (compound G) is produced by the removal of one methyl group from one side of the VBR structure while one ethyl group is removed from the other side of the VBR structure. In the third pair of isomers, H (compound H) is produced by removal of four methyl groups from the VBR structure, whereas I (compound I) is formed by removal of two methyl and one ethyl groups from the VBR molecule. The results of the HPLC chromatograms, UV-visible spectra and HPLC–ESI mass spectra are summarized in Table 1. All of these analyses clearly reveal that VBR degradation by *A. calcoaceticus* YC210 is a stepwise dealkylation process in which the dominating mechanism of the initial step of VBR biodegradation is demethylation. A similar degradation mechanism has also been reported for crystal violet degradation by *Pseudomonas putida* [7]. These reductions may involve different enzymes and chemical reactions. Further studies will focus on the enzyme analyses to determine whether *A. calcoaceticus* YC210 has potential applications in the industrial degradation of VBR in wastewater systems. Furthermore, the stepwise demethylation process to yield mono-, di-, tri-, tetra-, and penta-dealkylated VBR species is similar to that reported by Mai et al. [21], in which VBR was degraded by TiO_2 photocatalysis.

Table 1
Identification of the *N*-de-alkylated intermediates from the biodegradation of VBR by HPLC–ESI-MS.

HPLC peaks	De-alkylation intermediates	$[M+H^+]$	Absorption maximum (nm)
A	Bis (4-dimethylaminophenyl) (4-ethylaminonaphthenyl) methylium	422.18	613.0
B	(4-Dimethylaminophenyl) (4-methylaminophenyl) (4-ethylaminonaphthyl) methylium	408.35	606.9
C	(4-Dimethylaminophenyl) (4-aminophenyl) (4-ethylaminonaphthyl) methylium	394.76	603.2
D	(4-Methylaminophenyl) (4-methylaminophenyl) (4-ethylaminonaphthyl) methylium	394.76	609.4
E	(4-Methylaminophenyl) (4-dimethylaminophenyl) (4-aminonaphthyl) methylium	394.25	619.2
F	(4-Methylaminophenyl) (4-aminophenyl) (4-ethylaminonaphthyl) methylium	380.44	598.3
G	(4-Dimethylaminophenyl) (4-methylaminophenyl) (4-aminonaphthyl) methylium	380.16	608.1
H	(4-Aminophenyl) (4-aminophenyl) (4-ethylaminonaphthyl) methylium	366.77	587.3
I	(4-Dimethylaminophenyl) (4-aminophenyl) (4-aminonaphthyl) methylium	366.04	545.8

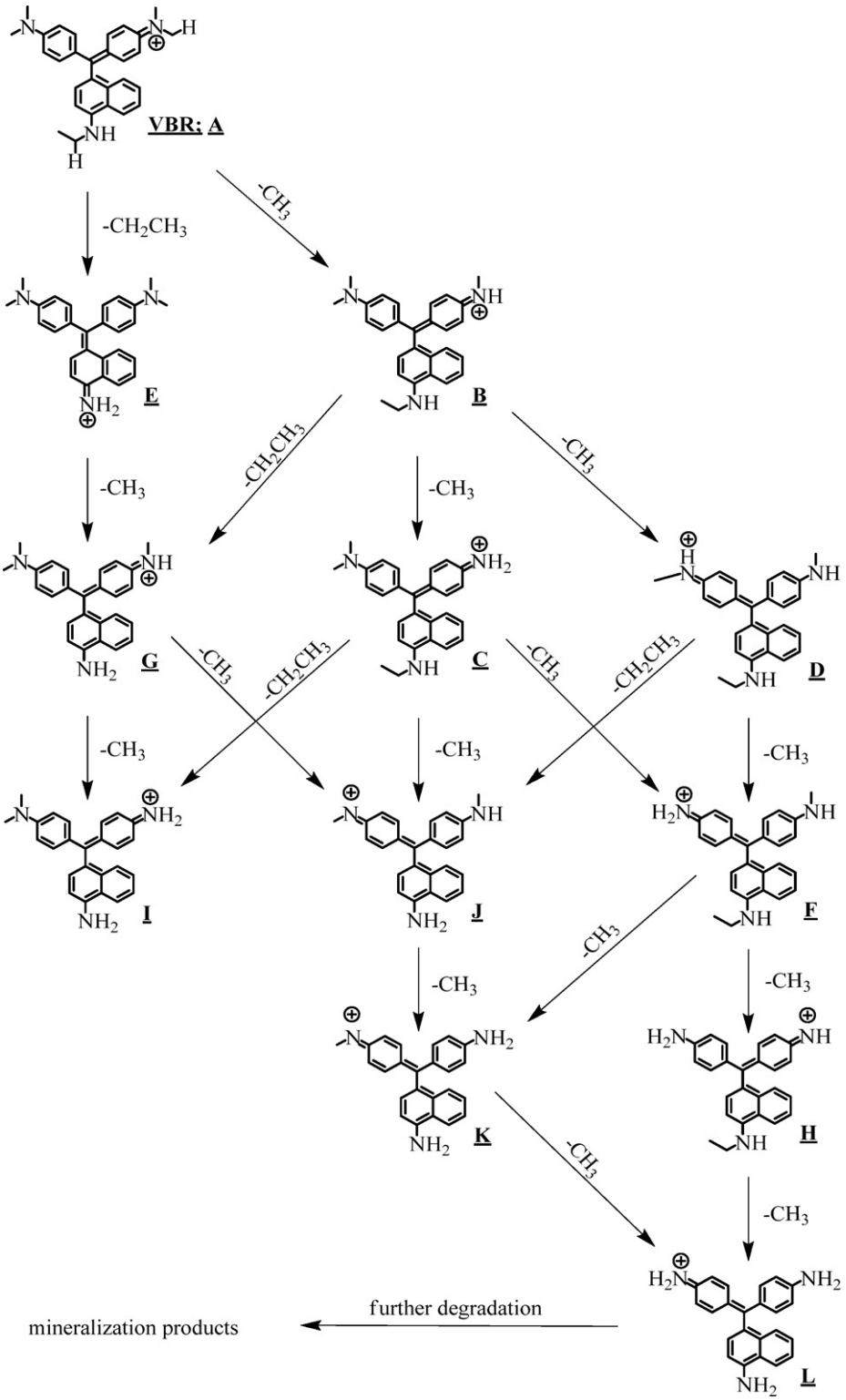

Fig. 6. The proposed degradation pathway of VBR by *A. calcoaceticus* YC210. The intermediates were identified by HPLC–ESI-MS. See Table 1 for the definition of the letters.

4. Conclusions

Based on its decolorization efficiency and characteristics *A. calcoaceticus* YC210 is an efficient degrader of VBR. The decolorization efficiency is dependent on its initial concentration, cell concentration of *A. calcoaceticus* YC210 and pH. *A. calcoaceticus* YC210 can rapidly remove high concentrations of VBR and decolorize it under slightly acid and neutral conditions. The dependence of the VBR degradation rate on VBR concentration is described by the Lineweaver–Burk equation, while HPLC–ESI-MS analysis indicates that the biodegradation of VBR by *A. calcoaceticus* YC210 is initiated via a demethylation process. The ability of *A. calcoaceticus* YC210 to decolorize VBR could have biotechnological applications.

Acknowledgment

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