

Pathways of reductive degradation of crystal violet in wastewater using free-strain *Burkholderia vietnamiensis* C09V

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Abstract A new strain isolated from activated sludge and identified as *Burkholderia vietnamiensis* C09V was used to biodegrade crystal violet (CV) from aqueous solution. To understand the degradation pathways of CV, batch experiments showed that the degradation using *B. vietnamiensis* C09V significantly depended on conditions such as pH, initial dye concentration and media components, carbon and nitrogen sources. Acceleration in the biodegradation of CV was observed in presence of metal ions such as Cd and Mn. More than 98.86C of CV (30 mg l^{-1}) was degraded within 42 h at pH 5 and $30 \text{ }^{\circ}\text{C}$. The biodegradation kinetics of CV corresponded to the pseudo first-order rate model with a rate constant of 0.046 h^{-1} . UV–visible and Fourier transform infrared spectroscopy (FTIR) were used to identify degradation metabolites. Which further confirmed by LC-MS analysis, indicating that CV was biodegraded to *N,N*-dimethylaminophenol and Michler’s ketone prior to these intermediates being further degraded. Finally, the ability of *B. vietnamiensis* C09V to remove CV in wastewater was demonstrated.

Keywords Biodegradation · Crystal violet · Pathways ·
Burkholderia vietnamiensis C09V

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Introduction

Wastewater from the textile industry has become a major environmental hazard in recent years and this effluent contains toxic dyes and heavy metals (Daneshvar et al. 2007). Many efforts have been made to develop strategies that treat dyes in wastewater, including physical, chemical and biological techniques. Physical and chemical methods are useful but their limitations include high cost, secondary pollutants and versatility (Forgacs et al. 2004). An alternative is biodegradation because it is cost-effective and environmentally friendly, and furthermore does not produce large quantities of sludge despite representing a slower rate of crystal violet (CV) biodegradation (Mohan et al. 2007).

A number of microorganisms has been reported to degrade crystal violet. The biodegradation of triphenylmethane dyes using white rot fungi has been reported, where biodegradation mechanisms of dyes depended mainly on the enzyme being secreted by white rot fungus (Cha et al. 2001). The degradation of CV using either *Bacillus subtilis* IFO 13719 or *Nocardia corallina* IAM 12121 has been documented (Yatome et al. 1993), where the CV at a low concentration was degraded, and it has been reported that bacterial process is much faster than fungal decolorisation/degradation of dyes (Garg et al. 2012). However, the growth of bacterial was completely inhibited at a higher concentration. More recently, the biodegradation of CV, malachite green and methyl violet B by *Shewanella decolorationis* NTOU1 suggested that biodegradation of these dyes involved a reduction to its leuco form (Chen et al. 2008). *Staphylococcus epidermidis* has been used to biodegrade CV, phenol red, malachite green, methyl green and Fuchsin with a high decolourisation capacity (Ayed et al. 2010). These bacteria are useful in the biodegradation of CV, but the majority of these dyes are chemically stable and resistant to its decomposition by microbial organisms. Therefore, isolating new strains is one option that may

increase the capacity of biodegradation of dyes and help us to understand its mechanism.

In this study, a new bacterium—*Burkholderia vietnamiensis* C09V (*B. vietnamiensis* C09V)—was isolated from activated sludge at the Textile Printing & Dyeing Mill (Fuan, Fujian, China). *B. vietnamiensis* has been reported in the biodegradation of toluene (Kumar et al. 2008) and documented as: firstly, reducing the toxicity of nickel and uranium; and secondly, playing a role in the degradation of trichloroethylene (Nostrand et al. 2007). It has not, however, been reported as being used to biodegrade CV. Hence, this study aimed to: (1) biodegrade CV using *B. vietnamiensis* C09V under various conditions and examine the conditions affecting the biodegrading efficiency; (2) study the kinetics of biodegradation and the metabolites as confirmed by ultraviolet (UV), Fourier transform infrared spectroscopy (FTIR) and high-performance liquid chromatography-mass spectrometry (HPLC-MS), and propose the degradation of CV; and (3) degrade the CV in wastewater to determine whether or not *B. vietnamiensis* C09V has the potential to biodegrade CV.

Materials and methods

Cultivation medium and cultivation

All the chemicals used in the experiment were analytical reagent grade. CV (purity $\geq 96\%$) was purchased from Sigma-Aldrich and used without further purification.

The phosphate-buffered basal medium (BM) was composed of (gram per liter of distilled water unless otherwise indicated): K_2HPO_4 1.8, $NaH_2PO_4 \cdot 12H_2O$ 3.0, $MgCl$ 0.1, KNO_3 1.0 and glucose 3.0. Solid BM plate contains (in grams per liter of BM): agar 20. Luria–Bertani medium (LB medium) was composed of (in grams per liter): $NaCl$ 10, peptone 10 and yeast extract 5. Flasks containing the medium were sterilised by autoclaving at $121\text{ }^\circ\text{C}$ for 20 min.

Microorganism and sequence analysis of the 16S rRNA gene

B. vietnamiensis C09V, a facultative anaerobic bacterium, was originally isolated from wastewater sludge collecting from the Textile Printing & Dyeing Mill based at Fuan, Fujian in China. The isolated strain was identified using Gram staining and morphology characteristics. The strain is Gram-negative stained, short rod-shaped and pink- to orange-pigmented as well as motile without flagellum. The colonies on the BM agar plate were round, smooth and translucent with entire margins. Further identification was done by employing 16S ribosomal RNA (rRNA) gene sequencing. A fragment of 16S rRNA was amplified using a Gene Amp PCR system (PE, USA). Sequencing on both strands of polymerase chain reaction (PCR)-amplified fragments was carried out using the dideoxy

chain termination method in an ABI 3730 automated sequencer (Invitrogen, Shanghai, China). Using the basic local alignment search tool (BLAST) program (Nievas et al. 2006), 16S rRNA homology searches of the NCBI database were carried out, the aim being to find similar sequences in the 16S rRNA database. The phylogenetic tree was drawn using the bootstrap program MEGA (ver. 4.0).

Biodegradation experiments

Since the growth of this strain was much faster in LB medium (data not shown), strain C09V was first cultivated at $30\text{ }^\circ\text{C}$ overnight in LB medium and then harvested by centrifugation (7,000 rpm, 10 min). The pellet was washed twice in 15 ml BM and then re-suspended in 10 ml BM. Cell suspension was transferred to a series of 50 ml culture bottles containing fresh BM (30 ml) using a gyratory shaker (SKY-2102, Shanghai, China) operated at 150 rpm in the dark. Sterile controls were prepared using autoclaved culture before the introduction of CV. The CV concentration following degradation in various samples was determined using UV–visible spectrophotometry. The biomass produced was determined following the methodology described (Silveira et al. 2009).

The wastewater containing crystal violet was collected from an industrial sewage treatment plant at Fuzhou, where the crystal violet concentration was 40.37 mg l^{-1} and pH was 8.59. The samples were stored at $4\text{ }^\circ\text{C}$ before being used. Inoculum was added to wastewater (30 ml) in a 50 ml culture bottle at pH 5 and $30\text{ }^\circ\text{C}$ in the dark for 72 h. Sterile control was used to compare this process to that of biodegradation. During the culture period, bacterial growth was monitored by spectrophotometry at 600 nm (Chen et al. 2009). Chemical oxygen demand (COD_{cr}) was measured by a closed reflux titration method (Clesceri et al. 1999). The average values of the parallel measurements were used in all analyses and the standard deviations of these means.

Characterisations and measurements

Fourier transform infrared spectra (FTIR) concerning the ‘initial’ and ‘final’ sample of biodegradation of crystal violet were obtained with a Fourier transform infrared spectroscope (FTIR Nicolet 5700, Thermo, USA). Samples for FTIR measurement were prepared by mixing extracted metabolites with spectroscopically pure KBr in the ratio 1:100 and pressed into a sheer slice. An average of 16 scans was collected for each measurement with a resolution of 2 cm^{-1} .

Aliquots of cell cultures (50 ml) collected after decolourisation of CV (100 mg l^{-1}) were centrifuged at $10,000\times g$ for 10 min, and the collected supernatant collected was extracted with 100 ml of ethylacetate. The extracts were dried and evaporated with nitrogen gas; acetonitrile was added prior to LC/MS analyses. Metabolites were identified using

liquid chromatography-mass spectrometry (HPLC/Q-TOF-MS, Bruker, Germany). A C18 analytical column (3.0 × 50 mm, 2.2 μm particle size) was used for the separation at 30 °C with an injection volume of 10.0 μl. The mobile phase consisted of a mixture of (A) methanol and (B) 5 mmol l⁻¹ ammonium acetate in MilliQ water with a flow rate was 0.3 ml min⁻¹. A linear gradient was applied starting at 35 % of (A), to 45 % of (A) in 2 min, up to 90 % in the next 7 min and at 90 % for 2 min, then to 35 % in 0.1 min and stay at 35 % for 2 min. The mass spectrometer was operated in positive ESI mode. The source conditions were as follows: end plate offset: -500 V, 91 nA; capillary: 4,500 V, nebuliser: 0.6 Bar; dry gas: 6.0 l min⁻¹ and dry temperature: 180 °C.

CV concentration was measured using a UV-visible spectrophotometer (722 N, Shanghai, China) at 588 nm. The removal efficiency of crystal violet was calculated using the following equation (Parshetti et al. 2006):

$$\% \text{Decolorization} = \frac{(A_0 - A_e)}{A_0} \times 100 \quad (1)$$

where A_0 = the initial absorbance of the medium; and A_e = the absorbance of decolourised medium.

Results and discussion

Phylogenetic analysis of *B. vietnamiensis* C09V

The 16S rRNA gene sequence of *B. vietnamiensis* C09V was deposited at GenBank under Accession No. JF922108. The sequences of the 16S rRNA gene of the strain were determined and compared to NCBI to match the closest by BLAST. The result indicated that the 16S rDNA sequences of the strain had up to 100 % similarity with a culture of *B. vietnamiensis*. Fig. 1 illustrates a phylogenetic tree construction based on 16S rRNA. It highlights the phylogenetic position of the *B. vietnamiensis* C09V strain and other related microorganisms, which were found in the NCBI GenBank database. Sequence comparison indicated that the *B. vietnamiensis* C09V strain was in the phylogenetic branch of *B. vietnamiensis*.

Effect of operational conditions

Effect of pH

Since culture medium pH can have an impact on enzyme activity, transport process and nutrient solubility (Kulkarni and Chaudhari 2007; Margesin and Schinner 2001), the effect of solution pH on the biodegradation of crystal violet was investigated, where initial culture medium pH values ranged from 4.0 to 8.0 after 42-h incubation. As shown in Fig. 2a, the

degradation of crystal violet increased when pH rose from 4.0 to 5.0, and a further increase in pH from 5.0 to 8.0 caused a decline in the degradation of crystal violet. The highest level of removal of crystal violet occurred at pH 5.0. More than 96.4 % of crystal violet was degraded. Degradation, however, declined from 96.4 to 75.2 % when the pH ranged from 5.0 to 8.0. These results are consistent with the study that examined solubilising insoluble P using *B. vietnamiensis*, where the optimal pH ranged from 5.0 to 7.0 (Park et al. 2010). The increase in biodegradation as pH rose from 4.0 to 5.0 can be attributed to cells growing in size and hence increasing the enzyme concentration. However, the fall in degradation as pH increased can be explained by the cell growth becoming inhibited under alkaline conditions and hence reducing crystal violet degradation. In addition, the pH of the cultivation medium is likely to affect the CV due to its positive charge under alkaline conditions. This results in biodegradation by accepting electrons based on its pathway, where CV was first biotransformed into Michler's ketone and *N,N*-dimethylaminophenol (Chen et al. 2008).

Effect of temperature

The effect of temperature ranging from 20 to 40 °C on the biodegradation of crystal violet was investigated (Fig. 2b), where biodegradation increased from 68.77 to 97.94 % when the temperature rose from 20 to 35 °C. When the temperature was increased further the degradation of crystal violet significantly decreased to 62.68 % at 40 °C. This is because the bacteria grew quickly within a relatively narrow temperature range and a higher temperature has a more adverse effect due to oxygen solubility falling when temperature increases; this triggers a reduction in the metabolic activity of aerobic bacteria (Bamforth and Singleton 2005). Generally, the biodegradation rate increases as temperatures also increase because this scenario favours bacterial growth, while harsh temperatures prohibit cellular enzymes, leading to a decrease in the degradation rate (Zavala et al. 2004). Nevertheless, the relatively higher degradation rates at temperatures ranging from 20 to 40, indicating that *B. vietnamiensis* C09V can be used to treat dye wastewater.

Effect of inoculum concentration

To estimate the optimum biomass of *B. vietnamiensis* C09V, the biodegradation of 30 mg l⁻¹ crystal violet was monitored with inoculum concentrations from 2 to 7 %, which presented the inoculum concentrations of 6.26, 9.39, 12.52, 15.65, 18.78, and 21.91 mg l⁻¹, respectively, as shown in Fig. 2c. It can be seen that dye removal significantly increased as the amount of biomass increased until it reached the value of 97.24 % at an inoculum concentration of 5 %. A similar observation was reported in the decolourisation of malachite green by *Sphingomonas paucimobilis* (Zavala et al. 2004).

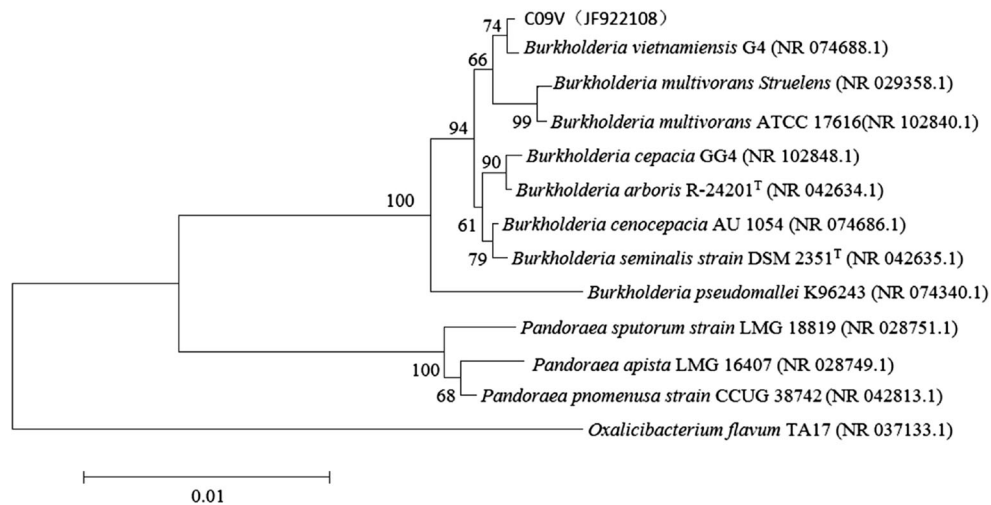


Fig. 1 Phylogenetic tree of *Burkholderia vietnamiensis* C09V based on 16S rRNA gene sequence. (GenBank sequence accession numbers are given in parentheses. Bootstrap values > 50 % are indicated in the main nodes in a bootstrap analysis of 1,000 replicates; the scale bar represents

the expected number of substitutions averaged over all sites analysed. Numbers at the nodes show the percent bootstrap value. Besides, the length of the nucleotide sequence considered is 1,422 bp, and *Oxalicibacterium flavum* strain TA17 was used as a bacterial outgroup.)

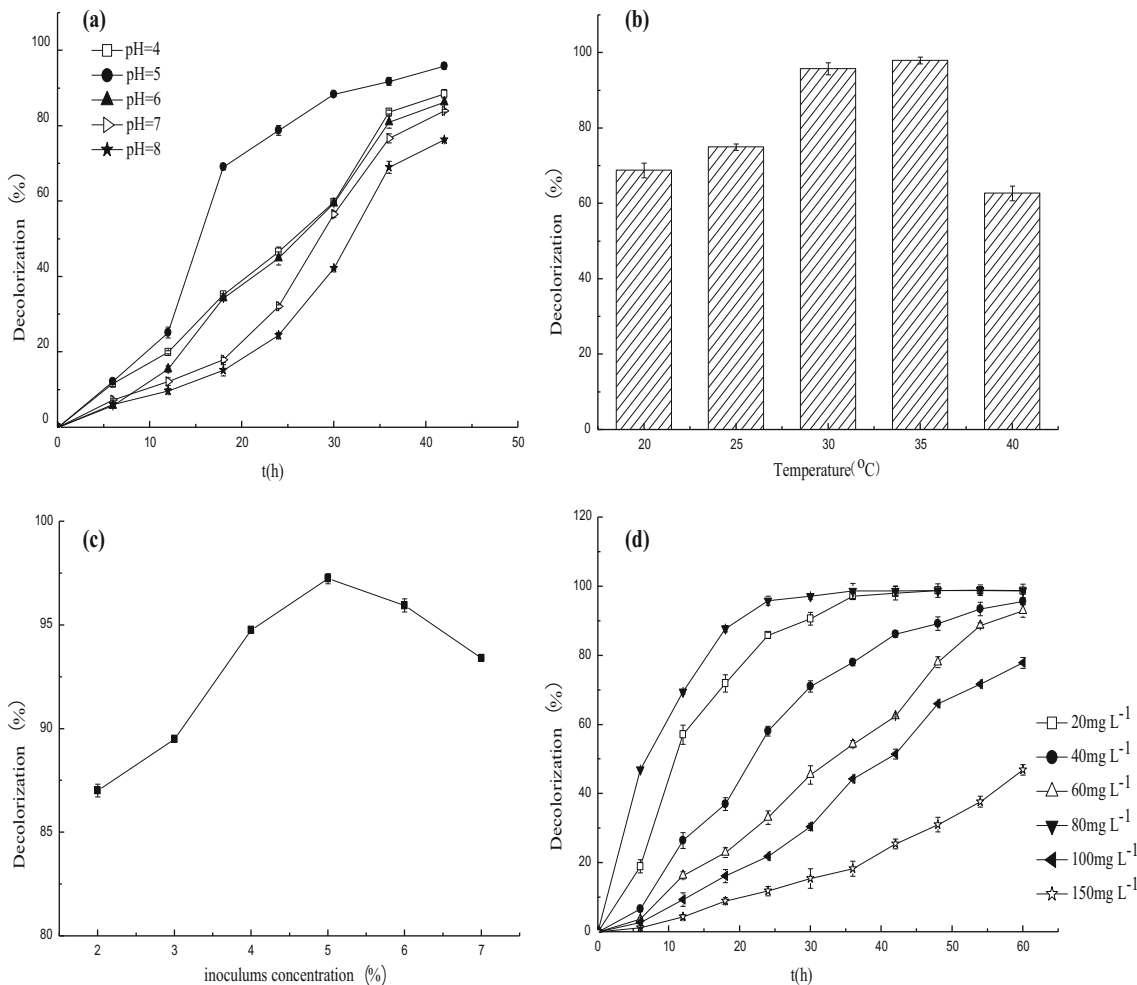


Fig. 2 Factors affecting the biodegradation of CV. **a** Initial pH (initial concentration of crystal violet: 30 mg L⁻¹; incubation time: 42 h; inoculum concentration: 0.4 % (v/v); temperature: 30 °C). **b** Temperature (pH 5.0).

c Inoculum concentration (temperature: 30 °C; pH 5.0). **d** Initial dye concentration [inoculum concentration: 0.5 % (v/v)]

Further increase in the inoculum concentration did not accelerate the biodegradation since the existence of more bacteria populations resulted in increased competition for resources (e.g., nutrients and oxygen). This resulted in prohibited bacterial growth when limited nutrient resources were depleted (Mellefont et al. 2008), and OD₆₀₀ increased from 0.037 to 1.5 when the value of dissolved oxygen decreased from 6.7 to 1.4 at an inoculum concentration of 5 %. It further indicated that the inoculum concentration of 5 % was suitable for degrading 30 mg l⁻¹ crystal violet.

Effect of initial dye concentration

The effect of initial crystal violet concentration on biodegradation was examined to assess the strain's tolerance of different concentrations of crystal violet (see Fig. 2d). This figure shows that *B. vietnamiensis* C09V could degrade crystal violet with a short lag time upon inoculation, and the adaptation period increased with higher crystal violet concentration. The degradation of 98.74 and 98.70 % was obtained after 36 h when initial concentration of crystal violet was 20 and 40 mg l⁻¹, respectively, in the culture medium. As the initial concentration of crystal violet increased from 80 to 150 mg l⁻¹, the degradation efficiency decreased from 92.88 to 46.91 % after 60 h.

It is suggested that the decrease in degradation efficiency might be due to the toxic effect of dyes on the growth of bacteria in the presence of dyes at toxic concentrations (Khehra et al. 2006). However, the amount of crystal violet removed increased when the initial concentration of crystal violet also rose, because a higher initial dye concentration provided a driving force that overcame all mass transfer resistances of the dye between the aqueous and solid phases (Parshetti et al. 2006). The tolerated high concentration (150 mg l⁻¹) of crystal violet in the culture medium confirms that the *B. vietnamiensis* C09V strain has the potential to degrade crystal violet.

Effect of media composition

Effect of carbon source

Fig. 3a shows the effect of different carbon sources on the degradation of crystal violet over time through the addition of glucose, sucrose, starch and granulated sugar (3.0 g l⁻¹) into the media containing the inoculated bacteria, and the control which did not have carbon sources. After 42-h incubation, the degradation efficiency of crystal violet was highest in the presence of glucose (98.8 %), followed by sucrose (86.7 %), granulated sugar food-sugar (78.9 %) and starch (78.9 %), while it was only 25.9 % when carbon sources were not added (control). This indicates that degradation activity of bacteria

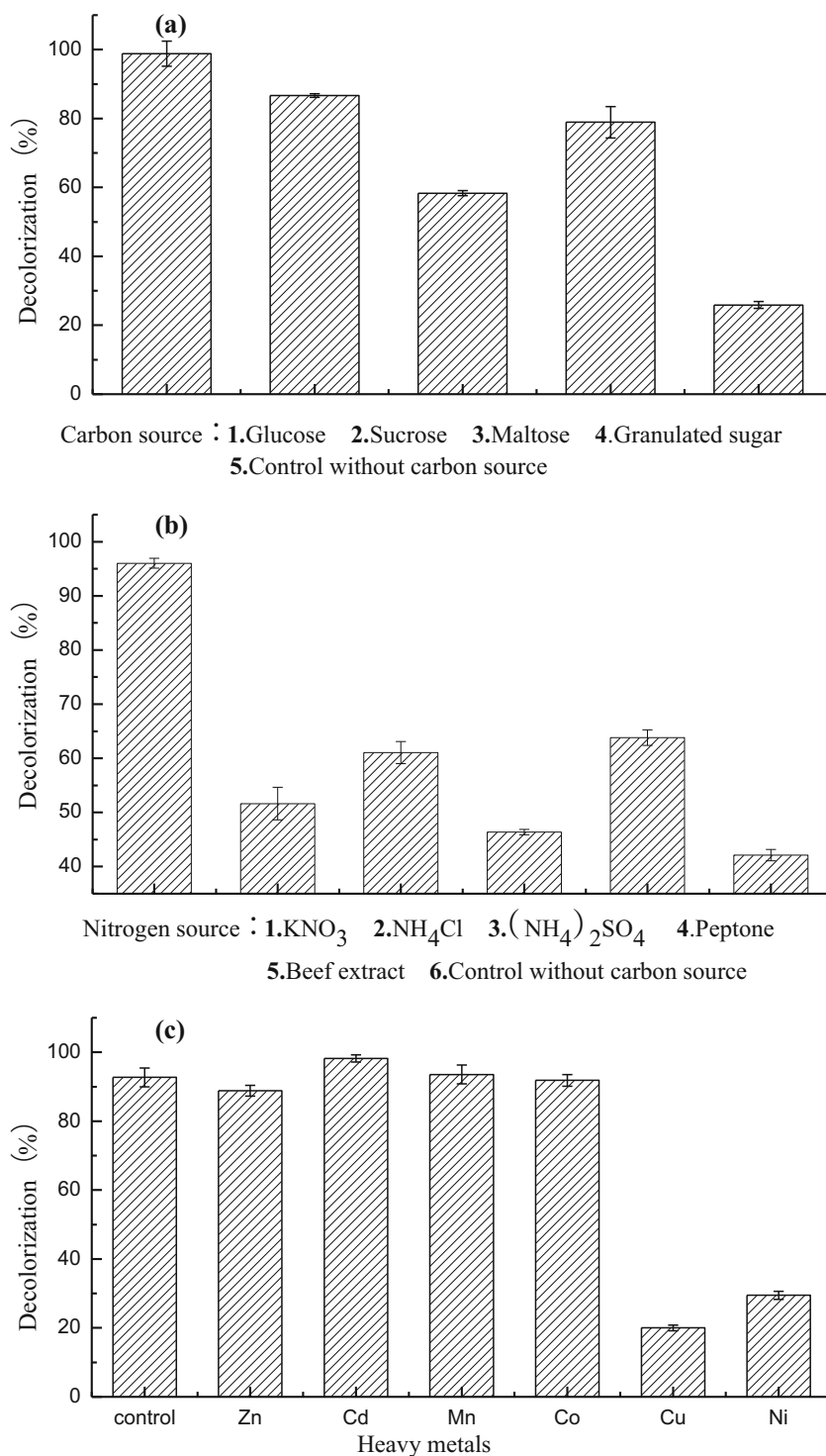
was linked to the carbon sources. However, the crystal violet in solution could also be used as a carbon source since 25.9 % crystal violet was removed in the control solution. These results correlate with the observation of the degradation of Blue G by *C. versicolour* using starch, molasses and fructose (Kapdan et al. 2000). Here, 100 % removal was achieved by glucose after 5-day incubation, followed by fructose (92 % in 9 days), starch and molasses (65 % in 9 days and 14 % in 6 days). This is probably due to the glucose being oxidised directly as an electron donor and being used by bacteria as energy material (Guo et al. 2010). However, sucrose and food-sugar were hydrolysed into either glucose or fructose, which can then be used by the *B. vietnamiensis* C09V strain. Soluble starch was hydrolysed into dextrin, then into maltose and finally into glucose, which can then be used by microorganisms (Guo et al. 2010). Hence, glucose as a carbon source was employed in this study since it is one of the co-substrates functioning as the electron donor in the biodegradation process (Telke et al. 2008).

Effect of nitrogen source

Fig. 3b shows the effect of different nitrogen sources on the degradation of crystal violet when peptone, beef extract, KNO₃, NH₄Cl and (NH₄)₂SO₄ (1.0 g l⁻¹) were added to the media inoculated with *B. vietnamiensis* C09V bacteria, while the control did not have nitrogen sources added to it. After 42-h incubation, the degradation of crystal violet was increased in the medium containing all tested nitrogen sources compared to the control. The degradation efficiency of crystal violet using different nitrogen sources occurred in the following order: KNO₃ (96.1 %) > beef extract (63.8 %) > (NH₄)₂SO₄ (61.1 %) > NH₄Cl (51.6 %) > peptone (46.4 %) > control (42.2 %). This suggests that nitrogen sources present in the media affected dye decolourisation by altering bacteria's production of enzymes (Kaushik and Malik 2009). In addition, the *B. vietnamiensis* C09V bacteria utilised either NO₃⁻ or NH₄⁺ which was formed by hydrolysis as N-source for the growth of microorganisms (Guo et al. 2010). It was reported that the complete decolourisation occurred: poly R-478 by *Lentinus edodes* at 1 mM NH₄Cl nitrogen (in 18 days); 1 mM peptone nitrogen (in 21 days); and 2 mM malt extract nitrogen (in 17 days) (Hatvani and Mecs 2002). Therefore, KNO₃ emerged as the most suitable nitrogen source in further experiments.

In summary, the best conditions for degrading 30 mg l⁻¹ crystal violet by *B. vietnamiensis* C09V include 3 g l⁻¹ glucose, 1 g l⁻¹ KNO₃, 5 % inoculum concentration, pH of 5 and a temperature of 35 °C. The results showed that 93.1 % crystal violet was removed within the first 30 h, and then 98.8 % crystal violet was degraded after 42-h incubation.

Fig. 3 Effect of media composition on the biodegradation of CV. Initial concentration of crystal violet: 30 mg l⁻¹; incubation period: 42 h; inoculum concentration: 0.5 % (v/v); temperature: 30 °C; pH 5.0. **a** Carbon source; **b** nitrogen source; **c** heavy metals (control: no metal ions)



Effect of heavy metals

In addition to dyes, heavy metals are constantly present in textile effluent and they often inhibit or cease microbial growth (Sani et al. 2001). Hence, the influence of heavy metal ions on the degradation of CV by *B. vietnamiensis* C09V was examined by adding a number of heavy metals [Zn(II), Cu(II), Ni(II), Cd(II),

Mn(II), Co(II)] to the solution. As shown in Fig. 3c, degradation of CV was enhanced only when 5 mM Cd(II) (98.3 %) was present and Mn(II) (93.6 %), compared to that in the control (92.7 %). Adding Mn(II) to the solution to enhance the degradation of CV was probably because Cd(II) and Mn(II) not only act as trace elements to promote the growth of the bacteria in media. They also play an essential role in the composition of

enzymes and enzyme activation (Murugesan et al. 2009). This was observed by the presence of Mn(II) which enhanced the decolourisation of dyes using fungal strains since Mn(II) in solution promoted the production of enzymes.

However, the degradation of CV in the presence of Zn(II) (88.8 %) and Co(II) (91.8 %) was slightly lower than that of the control (92.7 %). This may be explained by the minor inhibitory effects of Zn(II) and Co(II) on the growth of bacteria and consequently reduced the degradation rate (Murugesan et al. 2009). In contrast to Cd(II) and Mn(II), the degradation of crystal violet was significantly reduced in the presence of Cu(II) (20.0 %) and Ni(II) (29.5 %) compared to the control (92.7 %), indicating that microbial growth was inhibited in the presence of Cu(II) and Ni(II) in the solution. This scenario was observed in the inhibitory effect of heavy metals on the growth of bacteria and hence inhibited dye decolourisation (Hatvani and Mecs 2002).

Kinetics of the biodegradation of CV by *B. vietnamiensis* C09V

Biodegradation kinetics was examined in order to understand the mechanism of microbial degradation and the prospect of application. In order to analyse the biodegradation kinetics of CV by *B. vietnamiensis* C09V, a number of kinetic models have been used to fit to the data. Of these, the pseudo first-order equation is well fitted to the experimental data. The pseudo first-order kinetic equation may be represented as follows (Lin et al. 2010):

$$\ln c = kt + A \tag{2}$$

where *c* is the concentration of CV (milligram per liter) at time *t* (hour), *k* is the first-order rate constant, and *A* is a constant.

It follows that the half-life of the CV biodegradation by *B. vietnamiensis* C09V can be expressed as:

$$t_{1/2} = \frac{\ln 2}{k} \tag{3}$$

Pseudo first-order parameters for the biodegradation of CV are listed in Table 1. The biodegradation rate constant

Table 1 Kinetic equations of CV degradation by *Burkholderia vietnamiensis* C09V

Temperature (Celsius)	Kinetic equations	Rate constant (per hour)	<i>t</i> _{1/2}	Correlation coefficient
20	lnC=3.48–0.019 t	0.019	35.9	0.996
25	lnC=3.48–0.021 t	0.021	31.5	0.989
30	lnC=3.55–0.033 t	0.033	20.8	0.997
35	lnC=3.67–0.046 t	0.046	15.2	0.988
40	lnC=3.60–0.017 t	0.017	40.3	0.990

increased from 0.019 to 0.046 with *R*² being more than 0.99 when the temperature increased from 20 to 35 °C, whereas it decreased to 0.017 as the temperature reached 40 °C, suggesting that high temperatures prohibited enzyme activity and enzymes become denatured when submitted to high temperatures. This results in a decrease in degradation rate, which was confirmed in the previous section. A similar trend for half-life was obtained and is listed in Table 1, where the half-life for CV biodegradation increased as initial temperature rose. This indicates that: firstly, the first-order rate model fitted well to the biodegradation of CV (30 mg l⁻¹); and secondly, the specific biodegradation rate increases with a corresponding increase in initial temperature and then it slowed down (Zavala et al. 2004).

Characterisation of the biodegradation of CV by *B. vietnamiensis* C09V

UV–visible analysis

Fig. 4 illustrates the UV–vis spectra of crystal violet degraded by *B. vietnamiensis* C09V at 0, 24, 30 and 36 h, where UV–visible wavelength scanned from 200 to 800 nm and the initial concentration of CV was 30 mg l⁻¹. It is evident that the spectra of crystal violet exhibit large peaks with a maximum at 588 and 300 nm, corresponding to the -C=C- bond and aromatic rings (Noubactep 2009). The peaks at 588 and 300 nm significantly declined after 24-h biodegradation. However, a new peak at 360 nm was observed after 30-h biodegradation, resulting from the formation of the metabolites by the degradation of CV (Asad et al. 2007). This suggests that the removal of CV by *B. vietnamiensis* C09V includes both bioadsorption and biodegradation, where peaks at 588

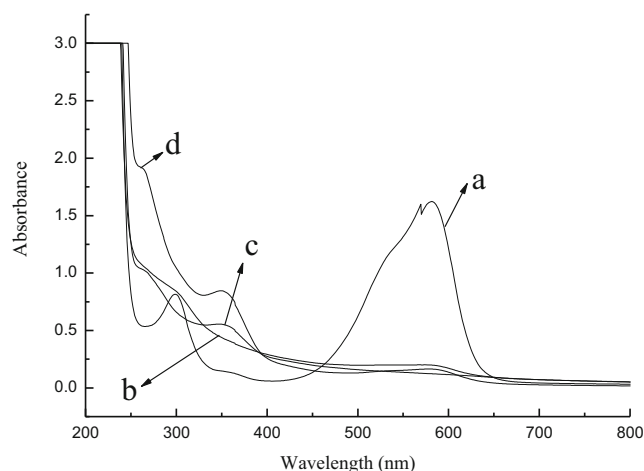


Fig. 4 UV–vis spectra of CV. **a** Before biodegradation; **b** after 24-h biodegradation; **c** after 30-h biodegradation; **d** after 36-h biodegradation

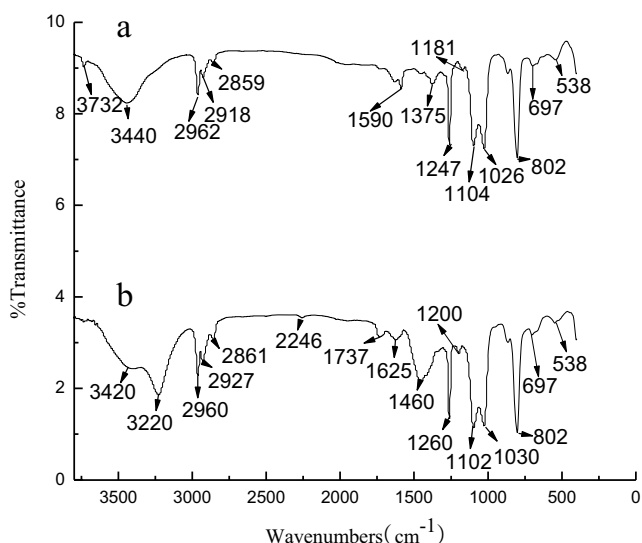


Fig. 5 FTIR analysis of the biodegraded products of CV by *Burkholderia vietnamiensis* C09V. **a** FTIR analysis of CV before biodegradation; **b** FTIR analysis the biodegraded products of CV after 36-h biodegradation. Numbers in the figure are absorption wave numbers for the stretching vibration of different functional groups

and 300 nm decrease approximately in proportion to each other in the adsorption of CV onto the biomass. In contrast the major peaks disappeared completely and a new peak appeared which resulted from the biodegradation of CV.

FTIR analysis

Initial and final biodegradation of the CV by *B. vietnamiensis* C09V scans were done in the range of 4,000–400 cm^{-1} wave

numbers using an FTIR as shown in Fig. 5, where they show the differences in changes in bands before and after the biodegradation process. Before biodegradation (Fig. 5a), the bands at 3,440 and 2,918 cm^{-1} corresponded to N-H stretching vibration, the band at 1,590 cm^{-1} corresponded to C=C stretching vibrations, the band at 1,375 cm^{-1} corresponded to CH_3 asymmetric bend, and the band at 802 cm^{-1} corresponded to benzene ring vibrations. However, a change in bands was observed following biodegradation due to the formation of metabolites as shown in Fig. 5b. Here, strong bands appeared at 1,460, 1,625, 1,242 and 884 cm^{-1} for C-H, C=O and C-O stretch vibrations and =C-H out of plane deformation vibration. In addition, two new bands at 1,737 and 3,220 cm^{-1} were also observed, corresponding to for=C-H, C=O, and C-O stretch vibrations. The change in bands clearly indicates that the formation of metabolites differs from the original substrate (Ayed et al. 2010).

LC-MS analysis

Liquid chromatography-mass spectrometry was used for the analysis of degradation products formed during the biodegradation of CV by *B. vietnamiensis* C09V. The chromatograms from LC-MS obtained from samples before and after biodegradation are presented in Fig. 6a and Fig. 6b, respectively. The peak of CV was observed at a retention time of 9.0 min before biodegradation as shown in Fig. 6a. However, four new peaks (their retention time at 8.9, 6.7, 6.1 and 5.2 min) were observed in Fig. 6b after degradation, indicating the formation of four biodegradation products. Based on fragmentation pattern and m/z values, metabolites were identified as of leuco crystal

Fig. 6 LC-MS analysis of the cell culture filtrate containing the biodegradation products of CV degraded by *Burkholderia vietnamiensis* C09V. **a** LC-MS spectra obtained before degradation, and **b** LC-MS spectra obtained after degradation for 24 h

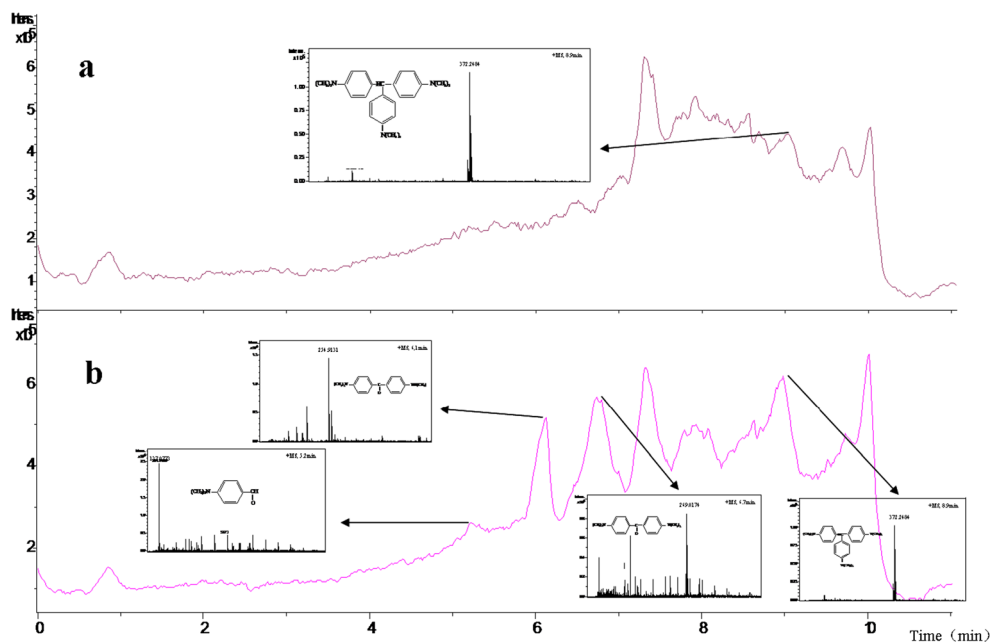
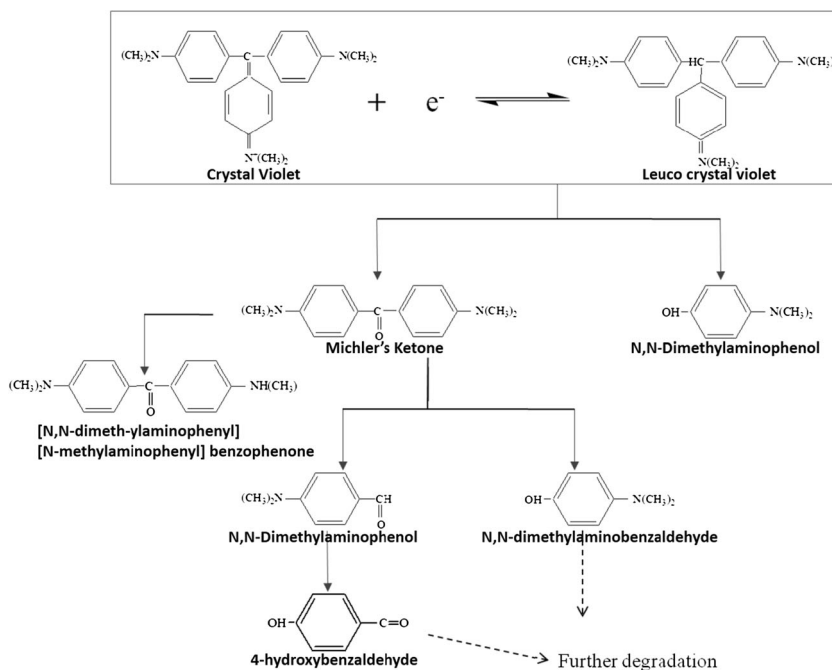


Fig. 7 Proposed biodegradation pathways of CV by *Burkholderia vietnamiensis* C09V



violet, Michler's ketone, [N,N-dimethylaminophenyl] [N-methylaminophenyl]benzophenone and *N,N*-dimethylaminobenzaldehyde. As shown in Fig. 7, crystal violet was first bio-transformed into leuco crystal violet, followed by the asymmetric cleavage by dioxygenase action between carbon of aromatic ring and alkyl carbon resulted in *N,N*-dimethylaminophenol and Michler's ketone. Then Michler's ketone was demethylated to [N,N-dimethylaminophenyl] [N-methylaminophenyl] benzophenone or reductively split into *N,N*-dimethylaminobenzaldehyde and *N,N*-dimethylaminophenol. The results were observed by UV-vis and FTIR spectra as previously mentioned. It is reported that the filamentous fungus *Cunninghamella elegans* ATCC 36112 could degrade triphenylmethane dyes through sequential *N*-demethylation to *N,N,N'*-trimethylparosaniline (Cha et al. 2001). Similar reports indicate that the intermediates in the biodegradation of triphenylmethane dyes by *Micrococcus* sp. include 4-(dimethylamino)benzophenone, Michler's ketone and 4-(methylamino)benzophenone (Du et al. 2013). Besides, the mechanism of crystal violet biodegradation was the reduction reaction, reductive splitting reaction and demethylation reaction (Chen et al. 2009).

Biodegradation of crystal violet in wastewater

To demonstrate whether *B. vietnamiensis* C09V can biodegrade crystal violet in wastewater, the crystal violet (40.37 mg l^{-1}) in wastewater was removed gradually with the value of $\text{OD}_{600\text{nm}}$ increase from 0.038 to 0.793. In this scenario, 91.1 % crystal violet was degraded, accompanied by a large COD_{Cr} decrease from 1,094.1 to 72.9 mg l^{-1} after 72 h

of incubation. Sterile controls indicated that there was no efficiency in the degradation process. The results further suggest that *B. vietnamiensis* C09V has the potential to effectively degrade crystal violet in wastewaters.

Conclusions

The results show that a new strain called *B. vietnamiensis* C09V can be used for the effective biodegradation of crystal violet. More than 98.86 % was degraded within 42 h at pH 5 and $30 \text{ }^\circ\text{C}$, and the strain could degrade CV with high concentration (150 mg l^{-1}) in the culture medium. The efficiency of CV biodegradation was increased with the presence of metal ions such as Cd and Mn, while other metal ions in the solution, such as Cu, could delay the process of CV degradation. The biodegradation kinetics of crystal violet corresponded to the pseudo first-order rate, with a rate constant of 0.046 h^{-1} . UV-vis and FTIR reveal that crystal violet was adsorbed onto the *B. vietnamiensis* C09V biomass, and then it was biodegraded. The LC-MS analysis shows that crystal violet was biodegraded to *N,N*-dimethylaminophenol and Michler's ketone prior to these intermediates being further degraded, and a possible biodegradation pathway of CV by *B. vietnamiensis* C09V was proposed. Finally, *B. vietnamiensis* C09V used to remove crystal violet from wastewater demonstrates this new strain has the potential to treat wastewater.

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