Journal of Applied Biology & Biotechnology Vol. 10(Suppl 2), pp. 107-113, June, 2022
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2022.10s212

Process optimization for efficacious biodecolorization of crystal violet by Malaysian *Rhodococcus pyridinivorans* using monothetic analysis

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ARTICLE INFO

Article history:
Received on: January 08, 2022
Accepted on: March 16, 2022
Available online: June 20, 2022

Key words:
Crystal violet,
Decolorization,
Optimization;
one-factor-at-a-time,
Rhodococcus pyridinivorans.

ABSTRACT

Environmental concern over the discharge of improperly treated textile wastewater has been on the rise. The present study explored the use of the limitedly studied, tropical *Rhodococcus pyridinivorans* for the decolorization of crystal violet, a typically encountered dye in the textile industry. The effect of agitation speed, temperature, pH, nutritional source, initial dye concentration, and size of inoculum on crystal violet removal were evaluated using one-factor-at-a-time method. Decolorization of 0.6 mM crystal violet carried out at agitated mode showed 73 ± 3% crystal violet removal efficiency in comparison to static mode (43 ± 2%) after 24 h. This crystal violet removal efficiency escalated to 91 ± 2% after optimizing the culture conditions at 45°C and pH 7 using maltose as carbon source and 12% (v/v) size of inoculum. Furthermore, the optimization process reduced the incubation time to achieve almost complete decolorization by 67%. Ultraviolet–visible analysis revealed that the decolorization of crystal violet primarily occurred through biodegradation. The findings from this study validated the potential of *R. pyridinivorans* as an effective biocatalyst to remediate crystal violet. *R. pyridinivorans* will be attempted to decolorize different triphenylmethane dyes, namely, malachite green in future studies.

1. INTRODUCTION

Triphenylmethane dyes are multipurpose and serve a major role in numerous industrial applications [1]. As one of the triphenylmethane dyes, crystal violet has been expansively used as a biological stain and textile dye [2]. The aromatic structure of crystal violet is stable and thus, it endures environmental degradation and is able to persist in wastewater and soil for an extended period of time [3]. Crystal violet was reported to be recalcitrant, exhibiting the potential to cause cancer and lead to gene mutation [2]. Exposure to crystal violet can cause humans severe damages to the eyes, difficulties in inhalation, vomiting, diarrhea, and skin irritation due to its corrosive and toxic nature [4]. Improperly treated crystal violet-containing wastewater, on the other hand, remains toxic and when discharged, it can be detrimental to agricultural productivity due to death of soil microorganisms as a result from crystal violet toxicity [5]. Therefore, textile wastewater containing crystal violet needs to be competently treated to ensure safe disposal into the environment.

There are a few physical and chemical methods popularly used to treat colored wastewater, namely, adsorption and ozonation. However, their applications are restricted due to the showcase of many weaknesses such as high cost, generation of secondary pollutants, and incomplete removal of the toxic colorants [6]. On the contrary, the use of microorganisms, particularly bacteria to remediate textile dyes, has been on the rise. This is attributable to the metabolic activities of bacteria which can be exploited to transform dyes to by-products exhibiting reduced toxicity at an enhanced rate of decolorization [7,8].

Rhodococcus, in particular, plays a key role in environmental biotechnology and industrial biotechnology, demonstrating promising potential in the bioremediation of toxic pollutants and biotransformation to generate commercially beneficial compounds [9-11]. *Rhodococcus* acquires the characteristics which allow this genus to survive in severe and extreme conditions and they possess a diverse array of anabolic genes, catabolic genes, and unique cell wall structure along with numerous enzymatic capabilities [12]. In view of this, *Rhodococcus* has been attempted in the present study to bioremediate crystal violet.

Although the use of biological method is advantageous and the employment of *Rhodococcus* as the biocatalyst provides added values to the method, the application to solve real-time problems is still challenging due to fluctuating conditions such as pH values which can hamper cell growth and metabolic activities [13]. Therefore, optimization using one-factor-at-a-time (OFAT) approach (monothetic analysis) was prioritized in the present study to obtain accelerated rate of crystal violet removal. This method of optimization is still relevant due to its straightforward approach and simplicity in analyzing data to obtain the most favorable medium and physical parameters such as pH, temperature, and agitation formulation for crystal violet removal in this case [14]. In addition, although a previous study by

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Li *et al*. [6] demonstrated the use of *Rhodococcus qingshengii* JB301 in decolorizing crystal violet, the application of *Rhodococcus* isolated from a tropical environment for crystal violet removal is scarcely reported in the literature. Thus, the present study concentrated on discovering the optimum conditions required for enhanced removal of crystal violet by a Malaysian *Rhodococcus pyridinivorans*.

2. MATERIALS AND METHODS

2.1. Chemicals and Media Composition

The preparation of standard stock solution was carried out using crystal violet purchased from Merck (Germany). Reputable suppliers such as Merck (Germany), R&M Chemicals (UK), or Bendosen (Malaysia) were engaged to purchase analytical grade media ingredients, namely, yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4$, and $CaCl_2$. These chemicals required no further treatment or purification and can be directly used for experimentation. Minimal salt medium (MSM) consisting of 100 mL modified 10X M9 salts (50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 5 g/L NaCl), 2 mL 1 M $MgSO_4$, 0.2 mL 0.5 M $CaCl_2$, and 2 mL 100 g/L yeast extract in 900 mL deionized water was used throughout this study as the standard medium. The pH of the medium was adjusted using stock solutions of 2 M HCl and 2 M NaOH as needed.

2.2. Isolation of *R. pyridinivorans*

Sludge sample located at Kuala Lukut, Negeri Sembilan, Malaysia, was used as the sampling area to isolate *R. pyridinivorans*. This strain was routinely conserved and stored at the Culture Collection Unit of Institute of Bio-IT Selangor, Universiti Selangor. This strain was placed at the GenBank® (NCBI) and identified with an accession number MN56069 [15].

2.3. Decolorization Study

The decolorization medium was composed of sterilized MSM medium amended with 0.6 mM crystal violet and supplemented with 10% (v/v) inoculum (optical density kept in the range of 1.0–1.1 at 600 nm) grown in the same medium (without 0.6 mM crystal violet) to exponential phase (36–48 h) at 30°C and 160 rpm (SI-600R, Lab Companion). The decolorization study used 0.6 mM crystal violet since this was the minimum concentration in which *R. pyridinivorans* could grow during the initial screening stage on solid agar medium (effect of higher concentrations of crystal violet on the decolorization will be tested during the optimization study). The decolorization study was carried out in triplicate at 30°C for 24 h under agitated mode (160 rpm) and static mode (0 rpm), respectively. A control experiment was set-up without the addition of inoculum.

The initial reading of the sample absorbance was taken at 0 h and the second reading was taken after 24 h. An amount of 1.5 mL samples was removed from the decolorization medium into the Eppendorf tubes and centrifuged at 4°C and 14 000 × g for 15 min (Tomy MX-305 High-Speed Refrigerated Microcentrifuge, Japan). The absorbance was taken at multiple wavelengths ranging from 200 to 700 nm using ultraviolet–visible (UV–Vis) spectrophotometer (Biospectrophotometer BioMate 3, Thermo Scientific, USA). The maximum absorbance wavelength (λ_{max}) of 590 nm found in this protocol was used for subsequent studies. The percentage of decolorization was determined following the formula below:

$$\text{Colour removal efficiency} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Readings were taken in triplicate and reported as averages with error bars indicating the standard errors.

2.4. Optimization Study

Optimization studies were carried out using the MSM medium added with 0.6 mM crystal violet (concentrations were varied for initial dye concentration study) and inoculated with 10% (v/v) inoculum (concentrations were varied for size of inoculum study) using OFAT approach (one factor was varied whereas the others were kept constant). When the effect of carbon source on crystal violet removal was studied, yeast extract was replaced accordingly. The optimization study was carried out for 24 h when the effect of different initial pH (4–10) and temperature (25–45°C) was tested. The incubation period was reduced to 12 h when varying 0.5 g/L carbon source (glucose, sucrose, maltose, orange peel, shredded coconut shells, sugarcane bagasse, and banana peel), size of inoculum (4% [v/v]–20% [v/v]), and initial dye concentration (0.1–2.4 mM). The incubation period was reduced to determine the optimum condition for crystal violet removal using these factors since 24 h incubation period yielded almost similar results among all tested parameters. The control experiment was established in the absence of inoculum.

When 0.6 mM initial dye concentration was evaluated, repeated addition of 0.6 mM dye was carried out for 4 cycles. Once complete decolorization occurred, crystal violet stock solutions were pipetted into the decolorized dye solution to give a final concentration of 0.6 mM. The flasks were incubated and hourly absorbance reading was taken until decolorization was completed or when maximum decolorization had occurred.

2.5. Statistical Analysis

The comparisons between groups were determined using one-way analysis of variance (ANOVA) IBM SPSS version 23. Duncan test (represented by different letters) was selected for *post hoc* analysis. $P < 0.05$ was labeled as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Decolorization of Crystal Violet under Shaking and Static Condition

The control system exhibited negligible abiotic loss of crystal violet. When compared with the control system, decolorization carried out at static condition did not yield significant color changes. On the other hand, when the decolorization was established under shaking condition, substantial changes in the color were observed. The initial violet color changed to colorless (after centrifugation to remove the cell pellet) possibly indicating the conversion of crystal violet to other metabolites by *R. pyridinivorans*. The static condition showed 43 ± 2% decolorization while the shaking condition recorded 73 ± 3% decolorization after 24 h incubation at 30°C. Therefore, the employment of shaking condition was favorable for crystal violet decolorization, resulting in 70% enhanced decolorization ($P < 0.05$) compared to that of static condition. In addition, shaking condition promoted cell growth after 24 h (optical density at 600 nm is 0.983 ± 0.078) which possibly assisted the decolorization in comparison to static condition (optical density at 600 nm is 0.1923 ± 0.037).

Similar results were observed by Bharagava *et al*. [16], reporting 95% crystal violet removal at agitated mode (110 rpm) in comparison to 45% crystal violet removal at static condition by *Aeromonas hydrophila*. Another study by Gao *et al*. [17] reported identical findings when an

endophytic fungus, *Bjerkandera adusta* SWUSI4 was found to be more effective in the decolorization of crystal violet under shaking condition (72%) than static condition (27%). Shaking condition allowed seamless transfer of oxygen between the cells and medium, leading to increased cell concentration [16] which suited the function of *R. pyridinivorans* as an aerobic bacterium. Under agitated mode, primary enzymes such as lignin peroxidase and manganese through oxidative reactions may largely contribute to higher decolorization of crystal violet [16] as *Rhodococcus* strains were known to possess these enzymes [18-20]. On the contrary, the decolorization of crystal violet by *Aspergillus niger* was established at static condition which may be caused by the presence of azoreductase [2]. Therefore, it can be assumed that the discrepancy of decolorization between static and agitated modes is microorganism dependent and related to the types of enzymes generated by these microorganisms.

3.2. UV–Vis Analysis

The UV–Vis spectrophotometric scanning between 200 nm and 700 nm yielded the formation of three major peaks in the visible region of the spectrum, namely, at 260 nm, 330 nm, and 590 nm at 0 h of incubation period [Figure 1] corresponding to aromatic rings and the -C=C- bond. It was particularly interesting to observe the disappearance of one of the major peaks at 590 nm after 24 h of incubation period under shaking condition, suggesting the disrupt of conjugated structure and removal of electron-donating group. This peak decreased without any further changes or shifts in the maximum wavelength (λ_{max}) to complete the decolorization after 24 h at 160 rpm. In contrast, Chen *et al*. [21] observed that with the increase of biodegradation time for the removal of crystal violet by *Pseudomonas putida*, there was a slight shift in the characteristic wavelength from 588 nm to 580 nm suggesting that a series of N-demethylated intermediates may be generated. Static cultures of *R. pyridinivorans* did not facilitate rapid removal of crystal violet and marginal reduction in the intensity of the peak at 590 nm was detected after 24 h of incubation period.

In addition, the intensity of the peak at 330 nm reduced substantially after aerobic treatment due to decolorization which was not achieved in the static cultures, demonstrating only a slight decrease in the peak intensity. Similar finding was reported by Ayed *et al*. [22] which observed the diminishing intensity of the peaks at 592 nm and 330 nm, respectively, when the decolorization of crystal violet by *Bacillus* sp. was carried out at agitated mode (150 rpm). Interestingly, the characteristic wavelength at 260 nm at 0 h decreased and shifted to 265 nm after 24 h of incubation period under agitated mode which suggested the potential formation of intermediates during aerobic decolorization of crystal violet. This peak at 260 nm only reduced to a small extent in terms of peak strength under static condition. These alterations implied the formation of series of N-demethylated intermediates and cleavages of the whole conjugated chromophore structure. These findings, therefore, supported the role of biodegradation in addition to decolorization for crystal violet removal by *R. pyridinivorans*. Analysis of metabolites and the parent dye of crystal violet using Fourier-transform infrared spectroscopy and mass spectroscopy will be carried out as future study to identify the decolorization products and predict the biodegradation pathways.

3.3. Optimization of Study Using OFAT Approach

3.3.1. Effect of initial pH

The initial pH value of the culture medium was adjusted within a range of 4–10 to examine the effect of pH on the decolorization of crystal violet by *R. pyridinivorans*. It was fascinating to observe that the locally isolated actinomycete was able to decolorize the colorant at all tested pH values as tabulated in Table 1 although the growth was more favorable at alkaline conditions. This is due to the fact that *Rhodococcus* is an actinomycete and actinomycetes preferentially thrive under alkaline condition [23].

Near neutral and alkaline condition seemed to be favorable for crystal violet decolorizing activity. Optimum removal of crystal violet was observed at pH 7 yielding $71^{a} \pm 1\%$ whereas an average of 62% crystal violet decolorizing activity was recorded at pH values of 8 to 10. Bharagava *et al*. [16] also reported pH 7 as the optimum culture condition for the removal of crystal violet by *A. hydrophila*. Optimum pH value of 7, furthermore, was observed for the removal of crystal violet by *Nocardiopsis alba* which achieved 49% decolorization of 100 mg/L of the colorant [24]. In addition, *R. qingshengii* JB301 isolated

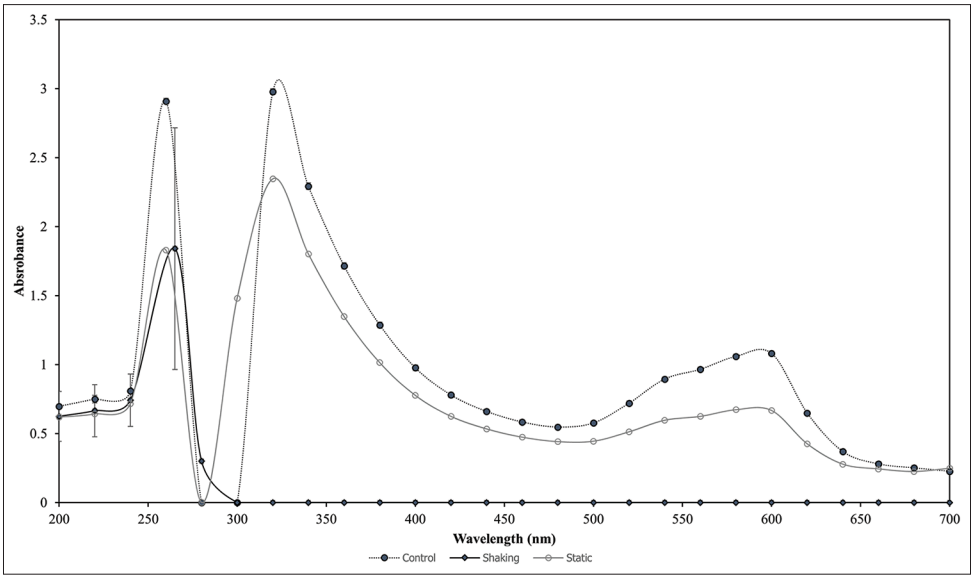

Figure 1: Ultraviolet–visible spectra of crystal red subjected to decolorization by *Rhodococcus* sp. at static and shaking conditions, respectively. Incubation was carried out 30°C and the pH was left unadjusted for 24 h with 0.6 mM crystal violet. Error bars represent standard error between three replicates.

Table 1: Optimization of different culture conditions for the decolorization of crystal violet by *Rhodococcus* sp. using one-factor-at-a-time (OFAT) approach

Factor	Decolorization of crystal violet (%)	Incubation period (h)	Optical density (600 nm)
Initial pH value			
4	59^{d}±1	24	0.586^{c}±0.011
5	63^{c}±1	24	0.601^{c}±0.057
6	67^{b}±1	24	0.908^{a}±0.023
7	71^{a}±1	24	0.928^{a}±0.016
8	63^{c}±1	24	0.923^{a}±0.013
9	62^{c}±1	24	0.918^{a}±0.015
10	63^{c}±1	24	0.752^{b}±0.012
Temperature (°C)			
25	65^{d}±1	24	0.458^{b}±0.013
30	68^{d}±2	24	0.912^{a}±0.027
35	71^{c}±1	24	0.898^{a}±0.013
40	83^{b}±1	24	0.918^{b}±0.013
45	99^{a}±1	24	0.903^{b}±0.013
50	29^{e}±1	24	0.158^{c}±0.042
Carbon source			
Glucose	29^{c}±1	12	0.772^{a}±0.017
Sucrose	74^{a}±1	12	0.780^{a}±0.049
Maltose	75^{a}±1	12	0.779^{a}±0.034
Orange peel	52^{b}±2	12	0.512^{b}±0.011
Banana peel	51^{b}±1	12	0.518^{b}±0.026
Shredded coconut shells	50^{b}±2	12	0.522^{b}±0.055
Sugarcane bagasse	52^{b}±2	12	0.517^{b}±0.020
Dye concentration (mM)			
0.3	94^{a}±1	12	0.819^{a}±0.013
0.6	95^{a}±1	12	0.822^{a}±0.027
0.9	59^{b}±1	12	0.639^{b}±0.031
1.2	58^{b}±1	12	0.642^{b}±0.029
1.5	37^{c}±1	12	0.347^{c}±0.014
1.8	23^{d}±1	12	0.261^{d}±0.032
2.1	11^{e}±1	12	0.202^{e}±0.052
2.4	8^{f}±1	12	0.148^{f}±0.065
Inoculum concentration (% (v/v))			
4	17^{d}±2	12	0.381^{c}±0.049
8	50^{c}±2	12	0.576^{b}±0.034
12	91^{a}±2	8	0.733^{a}±0.033
16	67^{b}±2	12	0.572^{b}±0.038
20	49^{c}±1	12	0.392^{c}±0.124

The control system without the addition of *Rhodococcus* sp. recorded insignificant loss of crystal violet (<1 removal). The removal of crystal violet (%) was averaged between three readings and reported with ±standard errors. Different letters show significant differences among the evaluated factors (P<0.05). ^{a}Reports the highest value and ^{f}reports the lowest value when comparison was made between the parameters in each factor.

from saw dust was able to optimally decolorize and degrade crystal violet at broader pH values of 6–9, supporting the findings obtained in the present study [6]. However, the crystal violet decolorization was inhibited at pH 4 and 10 as reported by Li *et al*. [6], unlike the ability of *R. pyridinivorans* in this study, which could decolorize crystal violet at an even broader pH range between 4 and 10, making the local isolate as an ideal biocatalyst for the decolorization of actual wastewater containing crystal violet from textile finishing plants and useful for other industrial applications [6]. The control system for each pH did not show any changes in their respective values, indicating that the occurrence of decolorization was indeed mediated by *R. pyridinivorans* and was not due to shift in the pH values. In addition, no loss of crystal violet was detected in the control systems.

3.3.2. Effect of temperature

After 24 h incubation at 45°C, 99^{a} ± 1% decolorization was observed, as shown in Table 1, recording the highest removal of the colorant (P < 0.05). Interestingly, there was no significant difference (P > 0.05) in terms of growth at the temperature range between 30°C and 45°C implying that the activity of enzymes for crystal violet decolorization was temperature specific. The removal of crystal violet was much slower at 25°C and 30°C documenting 65^{d} ± 1% and 68^{d} ± 3% decolorization, respectively. Based on the results from Table 1, we could conclude that the removal of crystal violet increased with increasing temperature. However, the capability of the local isolate to decolorize crystal violet at 50°C was hampered which may be due to inactivation of enzyme at higher temperatures.

R. pyridinivorans is a thermophile, and therefore, the growth of the local isolate was still active at 45°C which led to optimum crystal violet decolorization. This finding is well supported by Bharagava *et al*. [16] which reported a temperature range between 30°C and 45°C for optimum removal of crystal violet in the range of 80% and 97% with temperature 45°C documenting substantial crystal violet decolorizing activity of 83%. In contrast, Cao *et al*. [25] reported a strain of *Cedecea davisae* which was able to perform optimum crystal violet removal at 25–35°C. Similarly, laccase from *Kabatiella bupleuri* G3 was able to carry out 40% decolorization of 250 mg/L crystal violet at an optimum temperature of 30°C [26].

R. pyridinivorans demonstrated appreciable crystal violet decolorization at a broad temperature range of 25–45°C with more than 50% decolorization of 0.6 mM crystal violet. This finding reinforced the use of the local isolate as biocatalyst for actual textile wastewater bioremediation since the strain was able to tolerate wide range of temperatures.

3.3.3. Effect of carbon source

An attempt to increase the ability of *R. pyridinivorans* to decolorize crystal violet was carried out by supplementing auxiliary carbon sources to the culture medium. Different carbon sources, namely, maltose, sucrose, and glucose along with fruit wastes such as banana peel, shredded coconut shells, sugarcane bagasse, and orange peel were added separately into the medium containing the inoculated bacteria. Crystal violet removal was also measured in the control systems devoid of carbon sources and crystal violet, respectively, to evaluate the effect of carbon sources on the decolorization of crystal violet and to monitor the abiotic loss of crystal violet, respectively.

Almost complete decolorization of crystal violet was observed after 24 h of incubation period when all tested carbon sources were used (P > 0.05). Therefore, to determine the best carbon source for the decolorization of crystal violet by *R. pyridinivorans*, the incubation period was reduced to 12 h and the results are shown in Table 1. Crystal violet decolorization was the highest in the presence of maltose and sucrose (75^{a} ± 1% and 74^{a} ± 1%), respectively, followed by fruits wastes with an average crystal violet removal of 51% and glucose (29^{e} ± 1%) while the control system without the addition of carbon source exhibited only 21 ± 1% crystal violet decolorization. This finding suggested that the presence of auxiliary carbon sources indeed assisted the growth of the isolate which simultaneously supported enhanced crystal violet decolorization. However, the strain was also able to use the dye as nutritional source for growth as evidenced by the removal of crystal violet in the control system. These results correlated with observation made by Gan *et al*. [27] indicating the presence of additional carbon source facilitated the decolorization of crystal violet by *Burkholderia vietnamiensis* C09V.

A few reports found the presence of glucose as the most favorable external carbon source for the decolorization of crystal violet [16,27]. Glucose can be directly oxidized as an electron donor which can then be used as energy material by bacteria. However, in the present study, glucose seemed to be ineffective to promote decolorization of crystal violet even though good growth of *R. pyridinivorans* was observed which may probably due to the cells assimilating the added glucose over the dye compound as the carbon source. Interestingly, the utilization of fruit wastes such as banana peel, shredded coconut shells, sugarcane bagasse, and orange peel supported the growth and crystal violet decolorization by *R. pyridinivorans* even though the extent of decolorization did not vary significantly between these wastes ($P > 0.05$). Therefore, easily available and inexpensive nutrient sources can be employed to grow the local isolate capable to perform removal of crystal violet quite substantially. This research could pave way on exploiting fruit wastes which are found in abundance in Malaysia as external carbon source for growth of microorganisms as biocatalyst for various industrial applications. This could provide an alternative for the disposal issues of agricultural residues promoting environmentally friendly wastewater treatment process [28].

3.3.4. Effect of initial concentration

The textile industry wastewater typically carries dye concentrations in the range of 60 mg/L–250 mg/L [16]. Therefore, the concentrations of crystal violet were varied in the present study from 0.3 mM to 2.4 mM whereby the concentration of 0.6 mM corresponded to 245 mg/L crystal violet. The decolorization experiment was performed with increasing concentrations of crystal violet from 0.3 mM to 2.4 mM and *R. pyridinivorans* was able to decolorize the colorant at all tested concentrations. When the concentrations of crystal violet were lower at 0.3 mM and 0.6 mM, higher crystal violet removal was observed resulting in 94 ^{a} ± 1% and 95 ^{a} ± 1% decolorization, respectively. This finding is interesting since the highest concentration of dye in the textile wastewater falls at 250 mg/L and *R. pyridinivorans* could almost decolorize 245 mg/L crystal violet (0.6 mM) completely. Therefore, this local isolate has a huge potential to be applied as the biocatalyst for bioremediation of actual textile wastewater containing crystal violet.

As the initial concentration of crystal violet increased from 0.9 mM to 2.4 mM, the decolorization of crystal violet decreased from to 59 b ± 1% to 8 f ± 1% after 12 h of incubation period. The steady decreasing trend of crystal violet removal and increasing incubation period at higher concentrations of the colorant were observed in many previous reports [6,16,27,29]. The toxic effect of crystal violet at higher concentrations hampered the growth of *R. pyridinivorans* which simultaneously decreased the crystal removal efficiency, as shown in Table 1. In addition, metabolites from the decolorization process could saturate the bacterial cells and the active sites of the crystal violet decolorizing enzymes could be blocked by the presence of higher quantities of dye molecules, leading to decreased crystal violet removal efficiency at higher concentrations of the colorant [16].

Although the decolorization of crystal violet was inhibited at higher dye concentrations, the findings from this study exhibited more promising outcomes than reported previously. For instance, Gao *et al*. [17] reported a strain of *B. adusta* SWUSI4 which could only decolorize 34% of 250 mg/L crystal violet after an incubation period of 14 days. In addition, the incubation period taken to remove 47% of 150 mg/L crystal violet was 60 h when *B. vietnamiensis* C09V was used [27] which required 400% longer time than reported in the present study. Furthermore, all isolates obtained in the study by Jakhrani *et al.* [30] were unable to decolorize crystal violet at concentrations above 0.01 mM during the screening assay further confirming the superior ability of our local strain for crystal violet removal.

3.3.5. Repeated dye addition

Consecutive crystal violet decolorization cycles were evaluated by repeatedly adding 0.6 mM of the colorant to the optimized culture medium containing *R. pyridinivorans*. Results indicated that the local isolate was capable to decolorize crystal violet almost completely up to 4 cycles and the crystal violet removal efficiency reduced to 39 ± 1% after 10 cycles [Figure 2]. The almost complete decolorization of 0.6 mM crystal violet was observed in the first cycle after 12 h of incubation period. The successive addition of the colorant (0.6 mM) resulted in an increased rate of crystal violet removal which was achieved within 2 h of incubation period up to cycle 4. The crystal violet removal efficiency, however, declined after the 4th cycle and required extended period of incubation to achieve higher crystal violet decolorization. For example, the addition of 0.6 mM crystal violet at the 7th cycle resulted in 44 ± 1% crystal violet removal after 24 h of incubation period. The availability of nutrients to support the growth of *R. pyridinivorans* may be reduced over time which

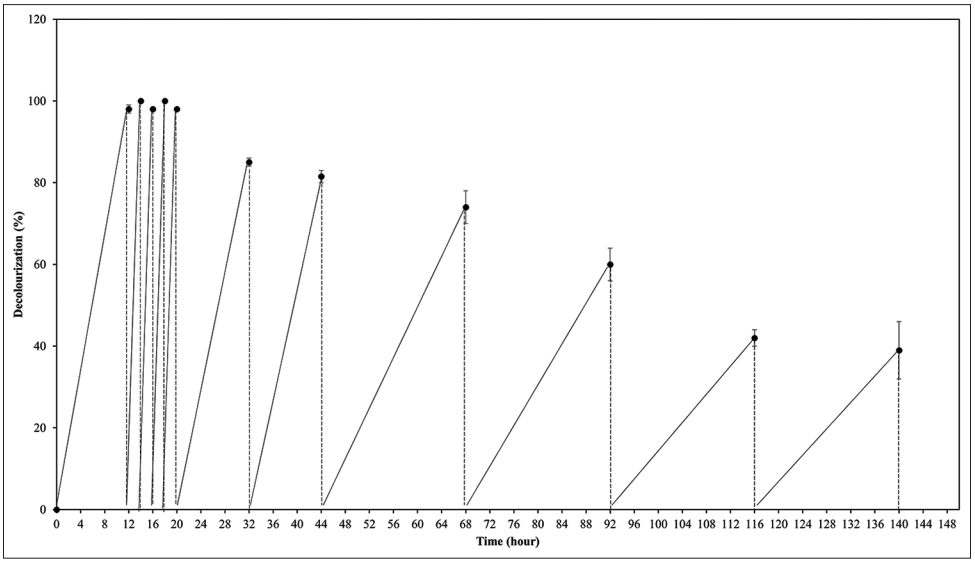

Figure 2: Repeated decolorization of 0.6 mM crystal by *Rhodococcus* sp. at optimal culture conditions. Error bars represent standard error between three replicates.

may lead to poorer secretion of crystal violet decolorizing enzymes [31] such as lignin peroxidase and manganese. Several studies have reported the importance of repeated use of microorganism as biological tool for dye decolorization for economic purposes and commercial applications [6,16]. In the present case, *R. pyridinivorans* demonstrated excellent ability to remove 0.6 mM crystal violet for ten successive cycles although prolonged incubation period of 24 h was needed. These findings emphasized the advantages of using the local biocatalyst in terms of cost saving due to repeated use and environmentally friendly approach using biological method for bioremediation.

3.3.5. Effect of inoculum concentration

Crystal violet decolorization increased with an increase in the inoculum concentration of *R. pyridinivorans* from 4% (v/v) to 12% (v/v). It was also interesting to observe that the period of incubation for efficient crystal violet removal reduced by 33% (from 12 h to 8 h; readings were taken every 4 h for 12 h) when the concentration of inoculum was varied from 4% (v/v) to 12% (v/v). The utilization of 12% (v/v) inoculum concentration resulted in the highest crystal violet removal of $91^{a} \pm 2\%$ ($P < 0.05$) within a short incubation period of 8 h. However, increasing the concentration of inoculum beyond 12% (v/v) did not support enhanced decolorization of crystal violet, yielding decreased crystal violet removal of $67^{b} \pm 2\%$ and $49^{c} \pm 1\%$ using 16% (v/v) and 20% (v/v) inoculum concentrations, respectively, after 12 h of incubation period. This may be due to the fact that the presence of higher bacteria populations limits the resources such as nutrients and oxygen for growth and crystal violet decolorization [6] as supported by the decrease in the cell growth at these inoculum concentrations [Table 1]. Similar observations were noticed when 97% decolorization of 30 mg/l crystal violet was achieved within 42 h at 30°C and pH 5 under aerobic shaking condition by 5% (v/v) *B. vietnamiensis* C09V. Increasing the concentration of inoculum to 6% (v/v) and 7% (v/v), respectively, inhibited the decolorization of crystal violet [6].

4. CONCLUSION

The present study reports effective removal of crystal violet by a novel tropical *R. pyridinivorans*. Almost complete decolorization of 0.6 mM crystal violet (95%) was achieved after 8 h when the experiment was carried out at pH 7 and 45°C using 12% (v/v) inoculum concentration and maltose as the carbon source. This finding is interesting because the biocatalyst could efficiently decolorize crystal violet within the common dye concentration range in the textile wastewater. In addition, the performance of this strain was quite consistent over a broad scale of pH (4–10) and temperature (25–45°C), which would be favorable in treating textile wastewater (mostly alkaline) and reduce the costs for heating since efficient decolorization could be achieved at normal temperatures. The decolorization of crystal violet by *R. pyridinivorans* was mostly accomplished through biodegradation as evidenced by the vanishing of major peaks and the shifting to new peaks from the UV–Vis analysis. Hence, this local strain which picturizes the richness of Malaysia's biodiversity can be considered as a capable biocatalyst for textile wastewater treatment since it can be reused for 5 cycles at reduced incubation period without significant loss in the decolorization ability. Further study to explore the ability of *R. pyridinivorans* to decolorize different triphenylmethane dyes, namely, malachite green will be endeavored to elucidate the versatility of the biocatalyst.

5. ACKNOWLEDGMENT

The authors thank Institute of Bio-IT Selangor, Universiti Selangor (Unisel), for providing the facility to execute this research work.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data; took part in drafting the article and revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest regarding the publication of this work.

8. ETHICAL APPROVALS

No animals or human subjects were involved in this study.

9. FUNDING

There is no funding to report.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Maniyam MN, Azman HH, Abdullah H, Yaacob NS. Process optimization for efficacious biodecolorization of crystal violet by Malaysian *Rhodococcus pyridinivorans* using monothetic analysis. J App Biol Biotech. 2022;10(Suppl 2):107-113. DOI: 10.7324/JABB.2022.10s212