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Enhanced Biodegradation of Crystal Violet by Immobilized Cells in Rhodococcus Strain ucc 0004 as Biological Tool

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Kanchana Rajendran[1], Maegala NallapanManiya[2,*]

[1] Institute of Bio-IT Selangor, Universiti Selangor, Jalan Zirkon A7/A, Seksyen 7, 40000 Shah Alam, Selangor, Malaysia
[2] Centre for Foundation and General Studies, Universiti Selangor, Jalan Timur Tambahan, 45600 Bestari Jaya, Selangor, Malaysia

ABSTRACT

Every year million tons of dyes and toxic compounds are discharged as industrial waste which pose a threat to the environment and human health. Thus, environmental concern over discharge of coloured wastewater has been on the rise. A competent method which is environmentally acceptable and economically viable is desired to treat the textile effluent prior to discharge into the environment. In the present study, twenty-three locally isolated Rhodococcus strains were examined as biological tools for decolourization of crystal violet. The tested microorganisms which were locally isolated Rhodococcus strains demonstrated promising ability to decolourize crystal violet as evidenced by the change in the colour of the dye from dark purple to pale purple on nutrient agar plates. One of the best strains namely Rhodococcus strain UCC 0004 formed clear zone around the colonies of the bacteria after 5 days of incubation period. This strain demonstrated good growth and completely decolourized 0.6 mM crystal violets after 5 days of incubation period. Further investigation was carried out by comparing the ability between immobilized cells and whole cells of Rhodococcus strain UCC 0004 for efficient crystal violet removal. The results showed that the percentage of crystal violet decolourization was highest ability to degraded when immobilized cells in gellan gum (99 a ± 0 %) and calcium alginate (98a ± 2 %), were used compared to heat killed cells which yielded (11c ± 0 %) of crystal violet removal. These findings clearly indicated that the immobilized cell of Rhodococcus strain UCC 0004 has a huge potential as biological tool to remediate actual wastewater containing crystal violet.

Keywords:
biodegradation; crystal violet; decolourization, immobilization, Rhodococcus; textile industry; water pollution

1. Introduction

Crystal violet is used extensively in textile dyeing, manufacturing industries, and as staining agent to classify microorganisms [14]. Crystal violet is used as a mutagenic and bacteriostatic agent, it act as an antimicrobial agent, in poultry feed to avoid the growth of fungal [10]. However, crystal violet has been reported as recalcitrant dye molecule due to its long term persistence in the environment [1]. Crystal violet is viewed as a hazardous chemical and it becomes essential for the industrial wastewaters to be treated adequately before being released into the environment.

There are many method to remove textile wastewater treatment such as physical and chemical treatments such as coagulation-flocculation, adsorption, filtration advanved oxidation processes using photocatalytic oxidation, oxidizing agents, combination of UV light and hydrogen peroxide.

* *Corresponding author.*
E-mail address: maegala@unisel.edu.my (Maegala Nallapan Maniyam)

Biological treatment gain attention as wastewater treatment to reduce majority of the disadvantages. This is became, biological method are reliable, less cost, produce less sludge and requires less water consumption [3].

There are many, different type of microorganisms such as *Agrobacterium radiobacter* [3], *Bacillus cereuc Pseudomonas putida* [11] and *Aeromonas hydrophila* (Bharagava, R., 2018) they could degrade crystal violet [3]. These microorganisms have been isolated from sludge of a textile printing wastewater treatment plant, which successfully decolourized and degraded more than 90% of crystal violet dye [2], but we are looking for microorganisms to completely decolorize and degrade at higher concentration of crystal violet within short duration. *Rhodococcus* appears to be very encouraging for this type of study with its ability to degrade various organic and inorganic compounds and also tolerate huge amount toxic compounds [4]. Many studies have been carried out using locally isolated *Rhodococcus* to decolourize and degrade some textile dyes such as Methylene blue [9] and Methyl red [8].

Various immobilization methods were used to the degradation rate of bacteria by stabilizing the enzyme involved in decolourization textile dyes. Therefore, it would be helpful and meaningful to study the decolourization and degradation of crystal violet dye in the presence of various immobilization methods. Thus, the objectives of the present studies are to investigate decolourization of crystal violet by, twenty three locally isolated *Rhodococcus* strains and to compare colour removal of crystal violet immobilized in (gellan gum and calcium alginate) and heat killed cells.

2. Methodology Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), Fisher Scientific (Singapore) and Merck (Darmstadt, Germany), respectively and was used without further purification. In this study, all experiment protocols were conducted using aseptic technique under a sterile environment.

Revival of Rhodococcus Strain on Nutrient Agar

Twenty-three *Rhodococcus* strain were used for this study, which were isolated from various locations in Peninsular Malaysia. These strains were maintained and conserved as growing culture in the Culture Collection Unit of Institute of Bio-IT Selangor, Universiti Selangor, Malaysia at -80 °C. A single bead was removed and utilized to directly inoculate on nutrient agar plate.The strains grown on nutrient agar were used for subsequent preparation of inoculums.

Growth of Rhodococcus Strain 0004 on Crystal Violet Dye

Nutrient agar solution was prepared using 20 g/L of nutrient agar powder and it was added with distilled water. Then, the solution was autoclaved at 121 ℃ for 20 minutes and added with 0.6 mM of crystal violet dye and mixed well.

The crystal violet agar solution was poured into petri dishes before solidifying it under sterile condition. The available 23 locally isolated *Rhodococcus* strain were streaked on the centre of the plate. The growth of the bacteria was observed by the formation of colourless zone around the colonies of *Rhodococcus* strain which signify the decolourization activities.These observations were madefor 14 days. The fastest growing strain with the largest formation of clear zone was selected for secondary screening.

Preparation of Starter Culture

Nutrient broth (8.0 g/ L) solution was prepared and autoclaved at 121℃ for 20 minutes, prior to inoculation of loop full *Rhodococcus* strain UCC 0004. Then, the culture was incubated in an incubator shaker (SI – 600R, Lab Companion) at temperature 30 ℃ and agitation for 160 rpm for 72 hours (Tokiran et al., 2016).

The growth of starter culture was observed by measuring its optical density reading at the wavelength of 600 nm (OD600)by using ultraviolet visual (UV-Vis) spectrophotometer [Biospectrophotometer Biomate 3, Thermo Scientific (USA)] model with distilled water as blank. The OD600 value of starter culture was maintained at 1.2 - 1.3 before inoculating into the production medium.

Preparation of Resting Cell

Seed culture of 5 % (v/v) was inoculated into 8.0 g/ L of nutrient broth. The medium was left to shake for 72 hours at 30 ℃ and 160 rpm. The cells were collected by centrifugation at 4 ℃, 16 000 × *g* for 30 minutes (Eppendorf 5702R, South Asia). After centrifuge, the cells were wash twice with phosphate buffer (pH 7). Lastly, the collected *Rhodococcus* strain UCC 0004 cells were stored in phosphate buffer at 4 ℃ until further use for preparation of immobilized cells.

Preparation of Immobilization Cells of Rhodococcus Strain UCC 0004

Nutrient broth (8.0 g/ L) was prepared and autoclaved at 121℃ for 20 minutes. Nutrient broth solution were inculcated with 5 mL (10 % v/v) starte culture and amended with 0.6 mM crystal violet. The flasks were incubated at 45°C for 24 hours. The cells were concentrated by centrifuging at 14 000 × *g* for 30 minutes at 4°C. The supernatant was decanted while the cell pellet was washed twice using a phosphate buffer solution (pH 7.0) under sterilized condition.

Immobilization using gellan gum and calcium alginate was adopted from Nallapan Maniyam *et al*. ,[9]. The beads were prepared by using 0.2 % (w/v) of gellan gum and sodium alginate under sterilized condition respectively, with 2:1 ratio of *Rhodococcus* strain UCC 0004. The mixture was added into 0.3 M of calcium chloride ($CaCl_2$) solution and the beads were formed into 0.4 cm diameter size.

Rhodococcus Strain UCC 0004

Number of beads (50-55) were transferred into Erlenmeyer flask containing dye solution. The samples were prepared in triplicate for each type of inoculum. Heat-killed cells were prepared using 30 ml of resting cells autoclaved at 121℃ for 20 minutes. Heat killed cells were used to find out the ability of *Rhodococcus* strain UCC 0004 to perform biosorption. All the samples were then placed in an incubator at 45℃ and agitated for 160 rpm. The sample solutions were left for 24 hours until complete decolourization.

Finally, the absorbance readings were obtained by pipetting 1 ml of sample and centrifuged at 4 ℃, 16 000 × *g* for 30 minutes for clarification and for UV-Vis spectrophotometer analysis at 590 nm using [(A-B)/A] x 100%, where A = initial absorbance and B = observed absorbance.

Statistical Analysis

All the experiments were conducted in triplicate with 1 control and the values obtained reported as means ± standard error of duplicates. The comparisons between groups were performed using one-way analysis of variance (ANOVA) IBM SPSS version 23 with post hoc analysis by Duncan test. $p < 0.05$ was conceded statistically significant. The results were as ranked from a (most preferable) to c (least preferable). The standard error (SE) was calculated by using Microsoft Excel version 2013 and results were presented as means ± SE values.

3. Result and Discussion

Determination of the Most Competent Rhodococcus Strain for the Decolourization of Crystal Violet

Twenty-three locally isolated *Rhodococcus* strains were screened on 0.6 mM crystal violet dye for14 days and the result shown in Table 1.

Table 1
Decolorization of Crystal violet by *Rhodococcus* Strain

Rhodococcus strain	Colour changes	Growth	Incubation period (Day)
UCC 0001	No colour change	*	14
UCC 0002	Slightly turn light purple	**	10
UCC 0003	Slightly turn light purple	**	10
UCC 0004	Purple to colourless	****	05
UCC 0005	No colour change	*	14
UCC 0006	No colour change	*	14
UCC 0007	No colour change	*	14
UCC 0008	Purple to light purple	***	07
UCC 0009	No colour change	*	14
UCC 0010	No colour change	*	14
UCC 0011	Purple to light purple	***	09
UCC 0012	No colour change	*	14
UCC 0013	No colour change	*	14
UCC 0014	No colour change	*	14
UCC 0015	Slightly turn light purple	**	10
UCC 0016	Slightly turn light purple	**	11
UCC 0017	No colour change	*	14
UCC 0018	No colour change	*	14
UCC 0019	No colour change	*	14
UCC 0020	Slightly turn light purple	**	10
UCC 0021	No colour change	*	14
UCC 0022	Slightly turn light purple	**	13
UCC 0023	No colour change	*	09

*Growth Indicator: *Poor, **Moderate/Fair, ***Good*

Screening of twenty three locally isolated Rhodococcus strain on 0.6 mM crystal violet dye. Incubation was carried out for 14 days at 30℃. Control set was prepared with the absence of Rhodococcus strains on the plate.

The aim of this investigation was observed by growth of Rhodococcus sp. in the presence of crystal violet. Out of twenty-three strains, fourteen strains showed no colour changes and growth, namely Rhodococcus strains UCC 0001, UCC 0005, UCC 0006, UCC 0007, UCC 0009, UCC 0010, UCC 00012, UCC 0013, UCC 0014, UCC 0017, UCC 0018, UCC 0019, UCC 0021 and UCC 0023 after an incubation period of 14 days at 30 ℃. These strains failed to change the colour of crystal violet and were not able to form colourless zones around the colonies of the respective strains. This observation clearly showed that Rhodococcus strains were unsuccessful to decolourize the crystal violet dye through high toxicity level which inhibits the growth of strains on the media [13]. Plates streaked with strain UCC 0002, UCC 0003, UCC 0015, UCC 016, UCC 0020, and UCC 0022 were able to decolourize crystal violet dye before 14 days of cultivation time. These strains turned crystal violet from dark purple to light purple.

In this experiment, particularly two strains exhibited good growth on crystal violet dye which were Rhodococcus strain UCC 0004 and 0008 respectively. Rhodococcus strains UCC 0004 and UCC 0008 demonstrated the presence colourless zone within short duration of 7 days. Rhodococcus strain UCC 0008 showed there was 0.5 cm halo zone around the colonies at the end of 7 days of incubation and the agar turned from dark purple to paler purple. Rhodococcus strain UCC 0004 yielding 1 cm halo zone after 5 days of incubation and the agar become clear yellow after going through the decolourization process. From this result, biodegradation is one of the ways for decolourization of crystal violet by Rhodococcus sp. possible indication for the production of metabolites from crystal violet Rhodococcus strain UCC 0004 completely decolourized 0.6 mM of crystal violet dye after an incubation period of 5 days as shown in figure 1.

Rhodococcus strain UCC 0004 became apparent as the most competent strain for the decolourization of crystal violet after incubation period of day 5.

Decolourization of Crystal Violet Dye by Whole Cells and Immobilized Cells of Rhodococcus Strain UCC 0004

Based on screening of culture conditions to enhance crystal violet decolourization and degradation, it was observed that *Rhodococcus* strain UCC 0004 was highly significant in decolourizing the toxic dye. Cells entrapment is commonly employed for dye and effluent decolourization process [5]. This required speed of agitation 160 rpm, temperature at 45℃ and time reaction from 0 to 24 hours were used for complete decolourization.

Table 2
Decolourization of Crystal Violet Resting Cells and Immobilization Cells of Rhodococcus Strain UCC 0004

Matrices	Crystal violet removal (%)
Resting cells	$99^{a} \pm 1$
Calcium Alginate	$98^{b} \pm 2$
Gellan gum	$99^{a} \pm 0$
Heat killed cell	$11^{c} \pm 0$

Values are expressed Mean ± SE *= Significant at P < 0.05%

The immobilized cells of *Rhodococcus* strain UCC 0004 cells were exposed to 0.6 mM of crystal violet dye at 45℃ and 160 rpm. Ranked from the most favoured to the least favoured following alphabetical orders in subscript format (most favoured = a, least favoured = c).

Based on the result (Table 2) immobilization cells of *Rhodococcus* strain UCC 0004 showed the highest percentage of decolourization in Gellan gum (99^{a}± 0 %) and calcium alginate (98^{b} ± 2 %) within 24 hours duration at 45℃. Temperature is one of the factors required by live bacterial cells to produce maximum decolourization tends to correspond with the optimum cell culture growth temperature of 35-45℃ [12]. The lowest result was obtained when using heat-killed cells 11^{c} ± 0 when treated with *Rhodococcus* strain UCC 0004 even after 24 hours incubation. In this case, it might be indicating occurrence due to the adsorption by dead bacterial cells was very minimal percentage of crystal violet decolourization was the highest ability in immobilized cells Gellan gum was chosen as the matrix for entrapment due to its advantageous properties such as high binding capacity, resistance to temperature and extreme acidic condition [6]. Calcium alginate cells are the most common use bacterial gel entrapment for many bioremediation applications. Calcium alginate offer the bacterial cells protection against environmental condition compared to whole cells.

It can be concluded that the decolourization and biodegradation of crystal violet can be carried out by using immobilization cells as suitable support. Immobilization cells not only simplify separation and recovery of the bacteria and the binding agent, but it also makes the application reusable, which can reduce the overall cost. Thus *Rhodococcus* strain UCC 0004 showed good percentage of decolourization and biodegradation by immobilized cells compared with heat-killed cells.

4. Conclusion

Rhodococcus strains are a better option among microorganisms as biocatalytic for the decolourization of crystal violet due to their rapid production of biomass and high biodegradation rate. In the present studies, twenty-three locally isolated *Rhodococcus* strain were used to decolourize crystal violet. *Rhodococcus* strain UCC 0004 completely decolorized and degraded 0.6 mM crystal violet after incubation 5 days. Decolourization in immobilized cell showed 99 % colour removed compared to heat-killed cells. Furthermore, the immobilization cells allowed higher tolerance toward 45 ℃ within 24 hours in comparison heat-killed cells. In future studies, a practical large scale application for treating crystal violet containing wastewater will be developed with *Rhodococcus* strain UCC 0004 whole cells embedded in immobilize cells.

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