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Penerbit Akademia Baru

Journal of Advanced Research Design

Journal homepage: www.akademiabaru.com/ard.html
ISSN: 2289-7984


# Enhanced Decolourization of Congo Red Dye by Malaysian Rhodococcus UCC 0010 Immobilized in Calcium Alginate

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**ABSTRACT**

The world's once glorious environment is heavily impacted by pollution caused by many industrial activities. The textile industry in particular has its share in pollution of water, land and air. With limited water resources and fast emerging nature of the textile industry in our nation, this raises concern in regards to detrimental effect of improper textile industry waste management. This waste is toxic and noxious which negatively affect living organisms and the environment when discharged without proper treatment. Utilization of microorganism through green chemistry approach is an alternative for industrial application to be sustainable, eco-friendly, cost- and time-effective compared to conventional effluent treatment methods that have considerable drawbacks. This study is interested in subjecting Rhodococcus sp. that is sturdy and resilient to decolourize di-azo dye Congo red, a highly recalcitrant and toxic dye since no previous studies have been reported with similar objective. Screening studies showed 8 strains were capable of forming halo zone around colony on 0.1 g/L Congo red agar plate with UCC 0010 giving largest halo zone with 1 cm diameter at day 5 of incubation. Similar results were obtained for Congo red decolourization in 24 hours at static condition for resting cells and heat-killed cells with 46d ± 1.5 % and 46d ± 7 % dye removal respectively. This shows dye removal is majorly via biosorption since cells were deeply coloured. Live cells yielded higher decolourization compared to resting cells with 67c ± 1.7% decolourization, possibly due to active metabolic state. Cells immobilized in both calcium alginate and gellan gum showed significantly higher decolourization and biosorption to matrix in control set. However calcium alginate is selected as best entrapment matrix since drastic difference was seen between control set and dye solution inoculated with biobeads (beads containing UCC 0010) indicating enzymatic action for Congo red decolourization.

***Keywords:***
Congo red; Rhodococcus; dye decolourization; immobilization



## 1. Introduction

In the past few years, there has been some news reported in our neighbouring countries such as blue dogs roaming the streets of Mumbai, India [6], tons of dead fish floating ashore Valankulam tank in India [2], health problems and infertile land in Bangladesh [18]. The major common occurrence between all of these news were found to be water pollution, especially caused by companies related to textile industry which have discharged their effluents into precious water bodies without prior treatment. Similar cases of environmental issues caused by textile industry could be a common scenario in Malaysia in the future. This is because of the fast growing nature of the textile industry

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which might impose a future threat to the well-being of our nation's environment. The textile industry in Malaysia has earned a revenue total of RM428.8 million, which were invested by foreign and domestic investors in the industry in year 2017. The textile industry in Malaysia has become one of the largest earning industries in terms of export sales to USA, Japan and Turkey, grossing up to RM15.3 billion in the year 2017 ("Textiles and textile products", 2018), clearly showing that the industry has a prominent contribution to the nation's economy.

Although Malaysia's textile industry has a positive effect on the country's economy, the possibility of detrimental impact of the industry towards the nation's natural gift; its' rich environmental resources, should be taken into consideration, particularly wastewater management. It is noted from the total volume of industrial waste water generated by various industries, Malaysia's textile industry is responsible for 22% of the total volume, which may cause serious damage to the environment as well as health due to usage of organic dyes [14]. Apart from that, a total of 70% of dyes that are used in textile industry are of the class azo dye which are very toxic and possess mutagenic as well as carcinogenic properties in reductive conditions that can affect living organisms in the environment as well as human health [7]. Untreated effluents containing organic dyes cause an increase in color, increase of oxygen demand in terms of both chemical and biochemical, toxicity, pH, and turbidity of the wastewater [14]. This shows it is necessary to properly treat textile industry effluents containing organic azo dyes in order to prevent environmental and health concerns.

There are many physical (coagulation-flocculation, adsorption, filtration, reverse osmosis) and chemical treatment (advanced oxidation processes using photocatalytic oxidation, Fenton Chemistry, oxidizing agents, combination of UV light and hydrogen peroxide) methods for textile industry effluent treatment. However, there are many drawbacks when it comes to using these methods such as low decolourization efficiency, high cost, large amount of sludge production, generation of secondary waste and formation of toxic by-products [8]. Biological method for effluent treatment serves to reduce majority of the disadvantages faced when using physical and chemical method and is opted nowadays whereby microorganisms such as bacteria, fungi and algae are exploited for their metabolic pathways since it is cost effective, eco-friendly, requires less water consumption and sludge production as well as generates non-hazardous by-products by converting dyes using various types of enzymes such as laccase, NADH-DCPIP reductase and tyrosinase [11,8].

Many different types of microorganisms have been studied for decolourization and biodegradation of carcinogenic azo dye Congo red, for example bacteria *Staphylococcus* sp. EY-3 [15], *Shewanella xiamenensis* BC01 [13], white rot fungi *Alternaria alternata* CMERI F6 [3] and a microbial consortium isolated from soil samples from wastewater disposal site [9]. Although these microorganisms were able to successfully decolourize and degrade Congo red dye to a certain degree, it is necessary to continue carrying out studies for Congo red decolourization using other novel microorganisms to achieve complete mineralization of the dye. The bacteria of *Rhodococcus* genus seems to be very promising candidate for azo dye decolourization with its ability to degrade various natural and recalcitrant compounds, survive and tolerate harsh and toxic environment due to possession of an assortment of catabolic genes which may correlate to its high enzymatic activity [10] and are suitable to be used for biotechnology application owing to its unique cell structure composed of mycolic acid with hydrophobic nature and ability to produce biosurfactant, allowing it to survive and tolerate harsh, toxic environment [4]. *Rhodococcus* isolated and maintained by the Unisel Culture Collection Unit can degrade range of dyes such as Methylene blue [11], Methyl red [10], Coomassie brilliant blue and Crystal violet [19] as well as Methyl orange [12].

There have been no studies carried out on Congo red decolourization using *Rhodococcus* sp. to the best of our knowledge. Therefore, in the present study, 23 locally isolated *Rhodococcus* strains maintained at Unisel Culture Collection were screened to determine most competent strain to

decolourize and grow on Congo red dye. Congo red decolourization activity by the best strain will then be studied with different modes of cells including cells entrapped in different support matrix such as calcium alginate and gellan gum. This method is used to compare Congo red decolourization activity of immobilized cells and whole cells.

## 2. Material and Methods

All chemicals used in this study were used without any purification and obtained from Merck (Germany), R&M Chemicals (UK) or Bendosen (Malaysia). All experiment protocol was carried out aseptically under sterile conditions.

*Screening*
*Revival of 23 strains of Rhodococcus sp.*

Twenty-three strains of *Rhodococcus* sp. bacteria isolated from multiple sites in Peninsular Malaysia by researchers in previous years that are maintained at Unisel Culture Collection Unit at Institute of Bio-IT Selangor, UNISEL Shah Alam were revived from stock cultures stored in cryobeads at – 80℃ by streaking the beads onto nutrient agar plates and incubated at 30℃ for 48 hours. The resulting colonies were used as inoculum for subsequent steps.

*Screening for Congo red decolourization activity*

Nutrient agar (20 g/L) was prepared and autoclaved (HVE-50 Hirayama, Japan) at 12℃ for 20 minutes. 0.1 g/L Congo red was weighed and mixed into sterilized nutrient agar and poured onto petri dishes and allowed to set followed by UV-sterilization on laminar flow hood (Biosafety Cabinet 4ft-EN12469, ESCO Micro (M) Sdn. Bhd.). Two petri dishes were labelled as control and wrapped with parafilm. Remaining plates were streaked with 23 strains of *Rhodococcus* sp. in duplicate and labelled respectively and wrapped with parafilm. All the Congo red-containing agar plates were incubated at 30℃ for 7 days (MEMMERT 108L Incubator INB500, Germany) and observed for decolourization at intervals of 24 hours. The best decolourizing strain was determined by comparison based on the diameter of decolourized zone present around colony of each *Rhodococcus* sp.

*Different modes of Rhodococcus strain UCC 0010 for Congo red decolourization*
*Preparation of seed culture*

Strain UCC 0010 was cultivated in sterilized 8 g/L nutrient broth under aseptic conditions and incubated in shaking incubator at 30℃, 160 rpm (S1600R (B3L) Lab Companion, Jeiotech, Korea) for 72 hours. After 72 hours, the growth of seed culture was determined by measuring its optical density at wavelength 600 nm ($OD_{600}$) using UV-Vis spectrophotometer (Biospectrophotometer Biomate 3, Thermo Scientific (USA)) with distilled water as blank. The $OD_{600}$ value of seed culture was maintained at 1.0 – 1.2 prior to inoculation into production medium to generate whole cells.

*Decolourization of Congo red dye solution*

Decolourization study was carried out using resting, immobilized and heat-killed cells. Production medium was prepared using sterile distilled water and 0.04 g/L Congo red dye. The culture conditions of production medium with different modes of cells studied are as follows:

**Table 1**
Preparation of different modes of cells from seed culture for production medium

| Mode of cells | Culture condition |
|---|---|
| Live cells | Approximately 10% v/v seed culture inoculated into production medium. |
| Resting cells | Seed culture centrifuged at 4 400 rpm, double-washed and re-suspended to final volume of 15 mL in potassium phosphate buffer (pH 7) followed by 10% v/v inoculation into production medium. |
| Heat-killed cells | 10% v/v seed culture inoculated into distilled water and autoclaved. |
| Immobilized cells (Calcium alginate & gellan gum) | 1:2 ratio of resting cells to 2 % support matrix mixed and immobilized in 0.2 M calcium chloride solution, left to harden overnight and double-washed with sterile distilled water. Approximately 100 beads added into production medium. |

The flasks were prepared in duplicates and incubated in static condition at 30°C (MEMMERT 108L Incubator INB500, Germany). Absorbance reading at 0 hour and 24 hour were taken (Biospectrophotometer Biomate 3, Thermo Scientific (USA)) with distilled water as blank.

*Percentage of Decolourization*

After the 0 hour and 24 hour absorbance reading was obtained, the percentage of decolourization was calculated using the formula:

$$\text{Percentage of decolourization, \%} = \frac{\text{0 hour absorbance} - \text{24 hour absorbance}}{\text{0 hour absorbance}} \times 100$$

*Statistical Analysis*

The calculated percentage of decolourization for each variable in each optimization parameter was tabulated into MS Excel spread sheet and standard error was determined. The data were used for statistical analysis using SPSS version 16.0 by performing one-way ANOVA with post hoc analysis by Duncan test. Value of $p < 0.05$ was considered to be statistically significant.

## 3.Result and Discussion

*Screening for Congo red decolourization activity*

The decolourization of 0.1 g/L Congo red dye by 23 strains of *Rhodococcus sp.* over 7 days is as shown in Table 2 below.

It was interesting to observe growth of *Rhodococcus sp.* colonies on all the 23 plates in the presence of Congo red dye, indicating that the tested strains of *Rhodococcus sp.* have the ability to tolerate toxic and xenobiotic properties of Congo red dye. This conforms with the statement that bacteria of *Rhodococcus* genus can tolerate and degrade many recalcitrant compounds [4]. With the results obtained through this study, textile dye Congo red can be included as one of the many recalcitrant compounds since this is the first study to have utilized bacteria of *Rhodococcus* genus to the best of our knowledge.

**Table 2**
Congo red decolourization by local *Rhodococcus sp.*

| Strain | Colour changes and presence of halo zone (diameter, cm) | Growth | Incubation days |
|---|---|---|---|
| **UCC 0001** | No changes | Moderate | 7 |
| **UCC 0002** | Paler with halo zone (0.3) | Good | 3 |
| **UCC 0003** | No changes | Moderate | 7 |
| **UCC 0004** | No changes | Good | 7 |
| **UCC 0005** | Paler with halo zone (0.2) | Good | 3 |
| **UCC 0006** | Paler with halo zone (0.3) | Good | 3 |
| **UCC 0007** | Paler with halo zone (0.1) | Good | 3 |
| **UCC 0008** | No changes | Moderate | 7 |
| **UCC 0009** | Paler with halo zone (0.28) | Good | 3 |
| **UCC 0010** | Paler with halo zone (0.5) | Good | 3 |
| **UCC 0011** | Paler with halo zone (0.25) | Good | 3 |
| **UCC 0012** | No changes | Good | 7 |
| **UCC 0013** | No changes | Good | 7 |
| **UCC 0014** | No changes | Good | 7 |
| **UCC 0015** | No changes | Good | 7 |
| **UCC 0016** | No changes | Good | 7 |
| **UCC 0017** | No changes | Good | 7 |
| **UCC 0018** | No changes | Good | 7 |
| **UCC 0019** | No changes | Good | 7 |
| **UCC 0020** | No changes | Good | 7 |
| **UCC 0021** | No changes | Good | 7 |
| **UCC 0022** | Paler with halo zone (0.15) | Good | 3 |
| **UCC 0023** | No changes | Good | 7 |

Although 23 strains *Rhodococcus sp.* subjected to Congo red dye on agar plate grew well, not all the strains were capable of showing decolourization activity. Out of the 23 tested strains, 15 strains, namely UCC 0001, UCC 0003, UCC 0004, UCC 0008, UCC 0012, strains UCC 0013, UCC 0014, UCC 0015, UCC 0016, UCC 0017, UCC 0018, UCC 0019, UCC 0020, UCC 0021 and UCC 0023 showed no changes in colour of agar plates containing Congo red dye when compared with the control plate. Formation of halo zone was also not observed. These strains did not show any decolourization with extended incubation time of 14 days. The colonies were observed to retain their pinkish orange colour indicating no adsorption of dye. The remaining 8 strains were observed to have paler plates as well as halo zones surrounding the colonies. It was observed that the colonies of the 8 strains not only turned red indicating adsorption of the dye to the cell membrane, formation of halo zone can be seen around the colonies with the strain UCC 0010 yielding 0.5 cm halo zone at 3 days incubation which increased to 1 cm halo zone at end of 5 days of incubation. Strains UCC 0007 and UCC 0022 showed the smallest halo zone diameter of 0.1 and 0.15 cm respectively whereas the remaining strains namely UCC 0002, UCC 0005, UCC 0009, UCC 0011, UCC 0006 showed comparable results with halo zone diameter in range of 0.2 – 0.3 cm. Thus, it can be said that biosorption is one of the modes for decolourization in the *Rhodococcus sp*. that were tested apart from degradation by enzymatic action on the dye molecule resulting in clear zone around the colonies. UCC 0010 was able to yield a largest halo zone of 0.5 cm with incubation time of 3 days compared to the 8 strains showing paler plates with halo zone as well as increase in halo zone diameter to 1 cm at end of 5 days of incubation as shown in Figure 1(A) and (B). Therefore, this strain was chosen for further optimization studies to enhance Congo red decolourization.

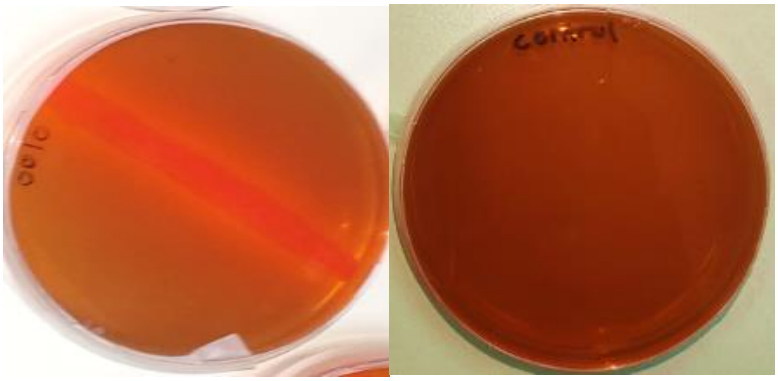

**Fig. 1(A).** Decolourization of Congo red by UCC 0010 at 3 day of incubation compared to control on the right

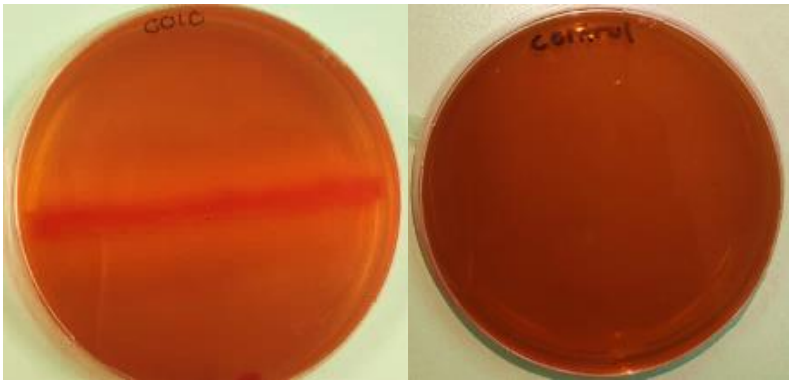

**Fig.1(B).** Decolourization of Congo red by UCC 0010 at 5 days of incubation compared to control on the right

*Comparison of different modes of Rhodococcus strain UCC 0010 for Congo red decolourization*

Comparison of dye decolourization by free cells was done using cells at different state to determine mechanisms involved in dye decolourization by UCC 0010 such as biosorption and enzymatic action. Dye decolourization by immobilized cells was studied to determine suitable matrix support for enhancement of dye decolourization. Table 3 shows the decolourization efficiency of strain UCC 0010 of different modes.

**Table 3**
Decolourization of 0.04 g/L Congo red dye by different modes of strain UCC 0010

| Modes of cell | Congo red removal (%) |
|---|---|
| Resting cells | $46^{d} \pm 1.5$ |
| Calcium alginate | $75^{b} \pm 2.5$ |
| Gellan gum | $88^{a} \pm 3$ |
| Live cells | $67^{c} \pm 1.7$ |
| Heat-killed cells | $46^{d} \pm 7$ |

The results show there is not much difference in decolourization activity of resting cells and heat-killed cells with $46^{d} \pm 1.5\%$ and $46^{d} \pm 7\%$ dye removal respectively after 24 hours incubation. When cells settled at bottom of conical flask were observed, they were reddish-brown in colour. This indicates that dye biosorption has occurred. In general, a number of mechanisms are utilized by bacteria for decolourization of dye, one of which is biosorption, indicated by deeply coloured cell colonies due to adsorption of dye molecules to different functional groups of heteropolysaccharide and lipid constituents of cell wall [20]. Live cells were interestingly able to show higher Congo red removal with $67^{c} \pm 1.7$ % via biosorption mechanism compared to resting cells. This may be attributed to active metabolic state of cells, enabling quick adaptation to the toxic environment and regulate necessary gene expression for production of enzymes responsible for decolourization and/or degradation of Congo red dye. Furthermore, it is stated that successful biosorption is influenced by physicochemical factors such as pH [16]. It is possible that neutral pH 7 of phosphate buffer in which resting cells was resuspended is not the optimum pH for decolourization activity of Congo red by UCC 0010, thus explaining the reduced decolourization efficiency of resting cells compared to live cells.

The decolourization activity was highest for immobilized cells entrapped in gellan gum followed by calcium alginate with $88^{a} \pm 3\%$ and $75^{b} \pm 2.5\%$ dye removal respectively. Both the control sets of gellan gum and calcium alginate also showed decolourization indicating adsorption of dye to matrices caused by interactions between dye molecule and matrix component [20]. Although adsorption is accountable for major dye removal, enzymatic dye decolourization was detectable in calcium alginate-entrapped cells which is explained by higher decolourization percentage observed for dye solution inoculated with biobeads (beads containing UCC 0010 strain) with average decolourization of $75^{b} \pm 2.5\%$ compared to its control set with $33.50 \pm 0.03\%$ decolourization. As for gellan gum, the difference between control set and biobeads was less than 7%, thus indicating lesser enzyme activity possibly due to reduced mass transfer from cell to external environment and vice versa which is one of the disadvantages of cell immobilization [5].

Although live cells were capable of carrying out decolourization at a reasonable efficiency, application of free cell system in effluent treatment has a major drawback of cell washout concerns [11]. This study portrays ability of calcium alginate-immobilized *Rhodococcus* strain UCC 0010 for significantly enhanced Congo red decolourization ($p < 0.05$) compared to other matrices and hence is chosen as the best matrix for Congo red decolourization as supported by previous studies. Sarim, *et al*., [17] reported bacterial isolate TS-1 removed Congo red dye by biosorption as cell pellet was coloured and culture supernatant showed no decolourization against Congo red. Bacterial isolate TS-1 immobilized in 3% calcium alginate decolourized 84.5% of 100 mg/L Congo red in 10 hours showing drastic decrease in time required for dye removal compared to free cell which gave maximum 94.8% dye removal in 48 hours, however under mild shaking conditions of 50 rpm. Similarly, bacterial consortium immobilized in calcium alginate decolourized 90% 100 mg/L Congo red in 24 hours whereas free cell consortium required 36 hours to achieve comparable result. Furthermore, immobilized bacterial consortium was able to decolourize 6-fold increase from initial dye concentration in 4 days with 88% dye removal [1]. Immobilization increases decolourization efficiency as entrapment of whole cells makes them tolerant to environmental changes and is advantageous for industrial application as the beads can be retrieved and reused for a number of cycles, thus reducing operation cost [17].

## 4. Conclusion

It is seen that bacteria are commonly used to decolourize textile dye to remove colour and reduce toxicity of dye properties. In this study, locally isolated bacteria of *Rhodococcus* genus were subjected to Congo red dye for decolourization. UCC 0010 strain was capable of adsorbing dye to its cell wall as well as give halo zone of 1 cm diameter in 5 days of incubation. Immobilized UCC 0010 strain showed significantly higher decolourization compared to free cell system with 75% decolourization of 0.04 g/L Congo red dye in 24 hours for cells entrapped in calcium alginate. This is because entrapment of cell allowed whole cells to tolerate changes in external environment. Results obtained in this study are preliminary findings for possible application of calcium-alginate immobilized *Rhodococcus* strain UCC 0010 for textile wastewater treatment. Further study will be carried out to increase dye removal percentage by optimization of physicochemical parameters such as pH, incubation temperature and additional carbon and nitrogen source. Apart from that, interaction of significant factors will be studied using response surface methodology to understand its effect on Congo red decolourization by UCC 0010.

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