

## Chromobacterium Violaceum for Rapid Measurement of Biochemical Oxygen Demand

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### Article history

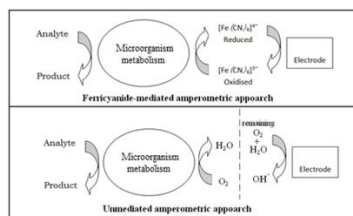
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### Graphical abstract



### Abstract

**Abstract:** Biochemical Oxygen demand (BOD) is an important parameter indicating the total biodegradable organic pollutants in waters. Fast BOD determination could be achieved using BOD biosensor. However, most of the developed BOD biosensors are dependent on dissolved oxygen concentrations. Low solubility of oxygen in water decreases the reliability of oxygen-dependent BOD biosensor. However, replacement of oxygen with a mediator solution solves this problem. In the present study, an effective ferricyanide-mediated approach was modified from ferricyanide-mediated BOD assay and used for BOD determination in a water system. Several different types of microorganisms were isolated from different organic-rich environmental sources and their ability to use ferricyanide during organic (standard glucose-glutamic acid solution) degradation were effectively assessed using ferricyanide-mediated BOD assay. Around 90% of the GGA was degraded by *Chromobacterium violaceum* after 1 hour of incubation period. Therefore, *C. violaceum* has been found to be a potential microorganism to be used as a biosensing element in the BOD biosensor. This assay is not only effective in selecting the suitable microorganisms for ferricyanide-mediated BOD detection; it also could be applied to select the suitable microorganisms for other mediated microbial biosensor and bioremediation by changing the substrate and conditions.

**Keywords:** Biochemical Oxygen Demand (BOD); Ferricyanide; Ultramicroelectrode (UME); *Chromobacterium violaceum*, redox-mediated biosensor; substrate specificity

### Abstrak

Keperluan Oksigen Biokimia (BOD) merupakan satu parameter penting yang menunjukkan jumlah bahan pencemar organik biodegradasi dalam air. Penentuan BOD yang pantas boleh dilaksanakan menggunakan biopenderiaan BOD. Walau bagaimanapun, kebanyakan biopenderiaan BOD yang dibangunkan bergantung kepada kepekatan oksigen terlarut. Kelarutan rendah oksigen dalam air mengurangkan kebolehpenerimaan oksigen yang bergantung kepada biopenderiaan BOD. Penggantian oksigen dengan satu pengantara penyelesaian dapat mengatasi masalah ini. Dalam kajian ini, pendekatan pengantara ferisianida yang berkesan telah diubah suai daripada analisis pengantara BOD ferisianida dan digunakan untuk penentuan BOD dalam sistem air. Beberapa jenis mikroorganisma telah diasingkan daripada pelbagai sumber alam sekitar yang kaya dengan bahan organik dan keupayaan mereka untuk menggunakan ferisianida semasa degradasi organik (larutan standard glukosa- asid glutamik) telah dinilai dengan berkesan menggunakan analisa pengantara BOD ferisianida. Sekitar 90% daripada GGA telah didegradasi oleh *Chromobacterium violaceum* selepas tempoh 1 jam pengeraman. Oleh itu, *C. violaceum* adalah mikroorganisma yang berpotensi untuk digunakan sebagai satu elemen dalam biopenderiaan BOD. Analisa ini tidak hanya berkesan dalam memilih mikroorganisma yang sesuai untuk mengesan pengantara BOD ferisianida, tetapi ia juga boleh digunakan untuk memilih mikroorganisma lain yang sesuai sebagai pengantara biopenderiaan yang mikrob dan biopemulihan dengan menukar substrat dan keadaan-keadaan tertentu.

**Kata kunci:** Keperluan Oksigen Biokimia (BOD); Ferisianida; Elektrodultramikro (UME); *Chromobacterium violaceum*, biopenderiaan dengan pengantara redoks; pengkhususan substrat

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## 1.0 INTRODUCTION

In the last few decades, intense industrialization has led to rapid development of urban population. As a result of urbanization, environmental pollution has become a serious problem due to manifold wastes discharged from the agriculture, domestic, and industries into the environment [1]. BOD is an important and widely used parameter to access the degree of pollution in the water system; it represents the amount of oxygen required for microbial aerobic decomposition processes of biodegradable organic pollutants in the water system. Thus, BOD indicates the quantity of biodegradable organic pollutants and the oxygen used to oxidise inorganic material in the water systems [2]. Although some portions of organic pollutants can be degraded easily by the indigenous microorganisms, the level of dissolved oxygen (DO) in the water after decomposition of the organic pollutants gives a significant impact to the health of the aquatic ecosystem. The reduction of DO levels was reported to cause lethal and sublethal effects (physiological and behavioural) in aquatic organisms, especially in fish [3].

The standard method for BOD analysis is the conventional 5-day biochemical oxygen demand (BOD<sub>5</sub>) method described by the American Public Health Association (APHA) Standard Methods Committee [4] and the Japanese Industrial Standard (JIS) Committee [5]. Although BOD<sub>5</sub> is a universal method of measuring most wastewater samples and the required equipment is inexpensive, it is time-consuming and requires complicated procedure and skilled analysts to obtain reproducible results. Therefore, the BOD<sub>5</sub> is not suitable for *in situ* determination or on-line process monitoring of wastewater treatment systems where fast feedback is required [6].

Hence, in order to overcome the weakness of BOD<sub>5</sub>, several alternative methods to BOD<sub>5</sub> have been widely developed, such as BOD assay and BOD biosensor. In the past decade, alternative BOD assays assimilated the use of ferricyanide mediator were introduced [7; 8; 9; 10]. These assays were proved to give a great benefit on the perspective of time constraint and the detection range of BOD [9]. However, the preparation and complexity of the assays were the limitation to the assays.

Fast determination of BOD could also be achieved by the biosensor-based methods. A biosensor is a self-contained

integrated device, which is capable of providing specific analytical information using a biological sensing element, which is immobilized onto a transducer [12]. Nowadays, microbial biosensor has been widely used in toxicity detection, heavy metal detection, specific carbohydrate detection, and organic pollution detection [13]. However, a large portion of the developed microbial biosensors for BOD detection has focused on measuring the remaining dissolved oxygen levels or concentration after the immobilized microbes utilized DO to degrade the organic pollutants in the sample over a certain period. Due to poor solubility of oxygen (O<sub>2</sub>) in water (8.7 mg/l at 25°C), DO rapidly becomes the limiting reactant in the biodegradation process. Subsequently, the amount of organic compounds biodegraded within the short time is small, which represents only a small fraction of the total organic content [10]. This results in narrow response ranges and poor reliability of microbial BOD biosensor.

Recently, ferricyanide-mediated amperometric approach has been developed to overcome the oxygen limitation problems [4; 5; 14; 15]. Ferricyanide can serve as a redox mediator, which can be reduced by certain microorganisms, thereby, by-passes the oxygen reduction step during microbial metabolic process. In this approach, O<sub>2</sub> is replaced by the ferricyanide ion, which serves as an alternative electron acceptor which is preferentially reduced to ferrocyanide during the metabolic oxidation of organic pollutants (Figure 1). The ferrocyanide is then reoxidized at a working electrode which is held at a sufficiently high electric potential. As a result, a current is generated and detected using the electrode system [14]. The high solubility of ferricyanide enables the use of higher microbial population without rapid depletion of ferricyanide. Since the metabolic rate of the microorganisms is not affected in the presence of the large amount of ferricyanide, the upper detection limit of the sensor is increased and the excessive dilution of samples might not be necessary as required for the oxygen-dependent sensor. Moreover, the reduced form of the mediator has an initial background concentration of zero, thereby, increases the accuracy when quantifying the amount of oxidised mediator converted to its reduced form [10]. Hence, mediated microbial sensors have received much attention for rapid BOD measurement [5; 8; 9; 10; 11; 14; 16; 17; 18].

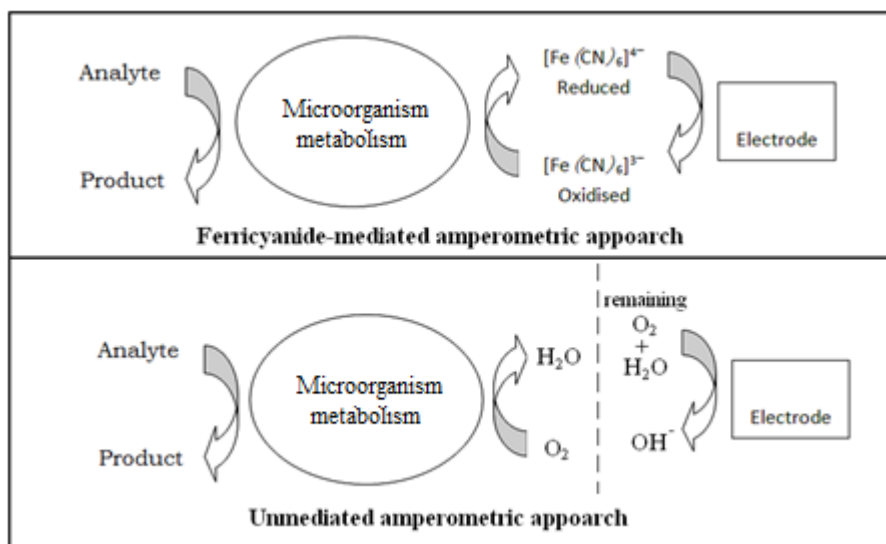


Figure 1 Scheme of ferricyanide-mediated amperometric approach and unmediated amperometric approach (modified from Yoshida et al., 2000).

Furthermore, most of the reported BOD biosensors focus on the use of conventional electrode as working electrode in the BOD detection. However, the use of ultramicroelectrode (UME) is rather limited in BOD biosensor. UME is defined as an electrode that has at least one dimension (the critical dimension) smaller than 25  $\mu\text{m}$ . The small size of UME gives them relatively large diffusion layers and small overall currents. These features allow the UME to achieve useful steady-state conditions rapidly and very high scan rates (V/s) with limited distortion. Therefore, highly accurate measurements are possible to be made with two-electrode system even in non-polar solvent or resistive solution without deliberately adding supporting electrolytes. Thus, it allows electrochemical measurements to be carried out with or without low amount of supporting electrolyte. Hence, UME permits *in situ* characterization and avoids disturbance of the equilibria which can be the source of impurities and can limit the electro activity range [10]. Therefore, it can be used as a chip-based microelectronic device for natural *in situ* water monitoring.

In the present study, the possibility of FM-BOD assay is explored to screen for potential microorganisms, which could be used for FM-BOD detection.

## 2.0 MATERIALS AND METHODS

### 2.1 Chemicals

All chemicals used were of analytical reagent grade, and all solutions were prepared in deionised water. The standard GGA solution (150 mg glucose/L and 150 mg glutamic acid/L) was prepared according to the APHA standard (1992) methods [19]. The BOD<sub>5</sub> values of the standard GGA solution were  $198 \pm 30$  mg O<sub>2</sub>/L. Potassium ferricyanide solutions were prepared in phosphate buffered saline solution (PBS, 10.8872 g/L K<sub>2</sub>HPO<sub>4</sub>/20.9016 g/L K<sub>2</sub>HPO<sub>4</sub>/ 7.456 g/L KCl, pH 7).

### 2.2 Isolation of Microorganisms

Microorganisms were isolated from different organic-rich environmental sources, such as petroleum refinery liquid effluent (P), sludge from textile industry (TS), raw pineapple liquid waste (PA), river water (R), and raw palm oil mill effluent wastewater (POME). Samples from different environmental sources were streaked onto Nutrient Agar (20 g/L Nutrient Agar, MERCK) and Potato Dextrose Agar (39 g/L Potato Dextrose Agar, Difco™/10 g/L tartaric acid) plates using dilution streak plate technique, and were incubated at 37°C for 24 hours. Pure cultures were obtained by restreaking single colonies with different morphologies onto new respective agar plates.

### 2.3 Screening of Potential Microorganisms

Ferricyanide-mediated BOD assay [8] was slightly modified and was used to screen the potential microorganisms using ferricyanide as an electron acceptor during the degradation according to the standard GGA.

### 2.3.1 Preparation of Inoculum

Overnight culture (10% v/v) was aseptically inoculated into Tryptic Soy Broth (TSB) and incubated aerobically at 37°C with shaking at 200 rpm. Cultures were grown until the end of the exponential phase (as determined by growth curves) and harvested by centrifugation at 4000 rpm for 15 min at room temperature. Cell pellets were washed twice with PBS and subsequently were re-suspended in PBS. The optical density of the suspension at 600 nm wavelength of light (OD<sub>600</sub>) was adjusted to 10 using 100 VIS Spectrophotometer (Jenway).

### 2.3.2 Preparation of the Samples

Three solutions were prepared. The sample solution consisted of potassium ferricyanide (1.5 mL, 131.696 g/L), standard GGA solution (3.5 mL, BOD<sub>5</sub>: 198 mg O<sub>2</sub>/L), and cells suspension (5.0 mL, OD<sub>600</sub> 10). Endogenous solutions were prepared by replacing the standard GGA solution with deionized water. Blank solutions were prepared by replacing ferricyanide solution with PBS. All the solutions were prepared in a closed serum bottle, and the solutions were sparged with oxygen free nitrogen gas for 15 minutes.

### 2.3.3 Incubation of the Samples

The samples were incubated at 37°C with shaking at 100 rpm for 5 hours in a shaking water bath. After one hour interval, 1.0 mL aliquots were withdrawn and centrifuged at 4000 rpm for 15 minutes. Subsequently, 1.0 mL of supernatant was diluted to 30x dilution using PBS solution (pH 7.0) and sparged with oxygen free nitrogen gas for 10 minutes. The production of ferrocyanide in supernatant solution was then analysed using voltammetry.

### 2.3.4 Voltammetric Detection

The concentration of ferrocyanide was determined chronoamperometrically. Chronoamperometric measurements were conducted in a single potential time base mode at +450 mV. eDAQ e-corder 410 (Model ED410) and eDAQ picostat (Model EA162) were used in the measurement, together with a three electrode system which consisted of a 10  $\mu\text{m}$  Platinum (Pt) microelectrode (working electrode), 1.0 mm diameter Pt gauze auxiliary electrode, and Ag/AgCl reference electrode. The amperometric current value, obtained one minute after the imposition of the applied potential, was taken as the limiting current value and used as the analytical signal.

### 2.3.5 Calculation of the FM-BOD<sub>5</sub> Equivalent Values:

The FM-BOD<sub>5</sub> equivalent value (Eq 1) was obtained by dividing the limiting current values obtained from the sample with the limiting current values obtained from the GGA standard solution and multiplied by BOD<sub>5</sub> value of the standard GGA solution (BOD<sub>5</sub>: 198 mg O<sub>2</sub>/L). The ratio of the limiting current values obtained from the sample to the limiting current values obtained from the GGA standard solution indicates the amount of biodegradation that can occur in the standard GGA solution for a given batch culture of microorganisms [9].

$$\text{FM - BOD}_5 \text{ equivalent value} = \left\{ \frac{i_{\text{lim}}(\text{sample}) - i_{\text{lim}}(\text{endogenous})}{i_{\text{lim}}(\text{GGA}) - i_{\text{lim}}(\text{endogenous})} \right\} \times 198 \text{ mg / mg O}_2/\text{L} \quad (\text{Equation 1})$$

## 2.4 Identification of Isolate

Promega DNA Purification kit was used to extract the genomic DNA. After the extraction, the bacterial 16s rRNA (1354 bp) was amplified by polymerase chain reaction (PCR) with 2 universal primer (pH and pA). The amplified PCR products were sent to First BASE Laboratories Sdn Bhd for purification and sequencing.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Isolation of Microorganisms

A total of 14 types of bacteria (coded P1, P2, P3, PA1, PA2, PA3, TS1, TS2, TS3, R1, R5, MC5, POME22, and S2) and 3 types of

yeasts (Y1, Y2 and Y3) were isolated from different environmental sources in Malaysia. The colonies of the isolates were characterized on the basis of colour, shape, texture, elevation, and margin (Table 1). The purpose of this isolation was to select the microbes that were capable of degrading a wide range of organic compounds. Thus, all the microbes were isolated from the sources that were rich in organic compounds. For example, raw pineapple liquid waste consists of sugars and lignocellulosic components [20], while textile wastewater contains various complex organic compounds made from dyes, detergents, solvents, grease, and oils [21]. POME consists of a spectrum of carbohydrates, nitrogenous compounds, free organic acids, lipids, and mineral constituents [22], and petroleum refinery liquid effluent mainly consists of oil products such as saturated hydrocarbon and polycyclic aromatic hydrocarbons [23].

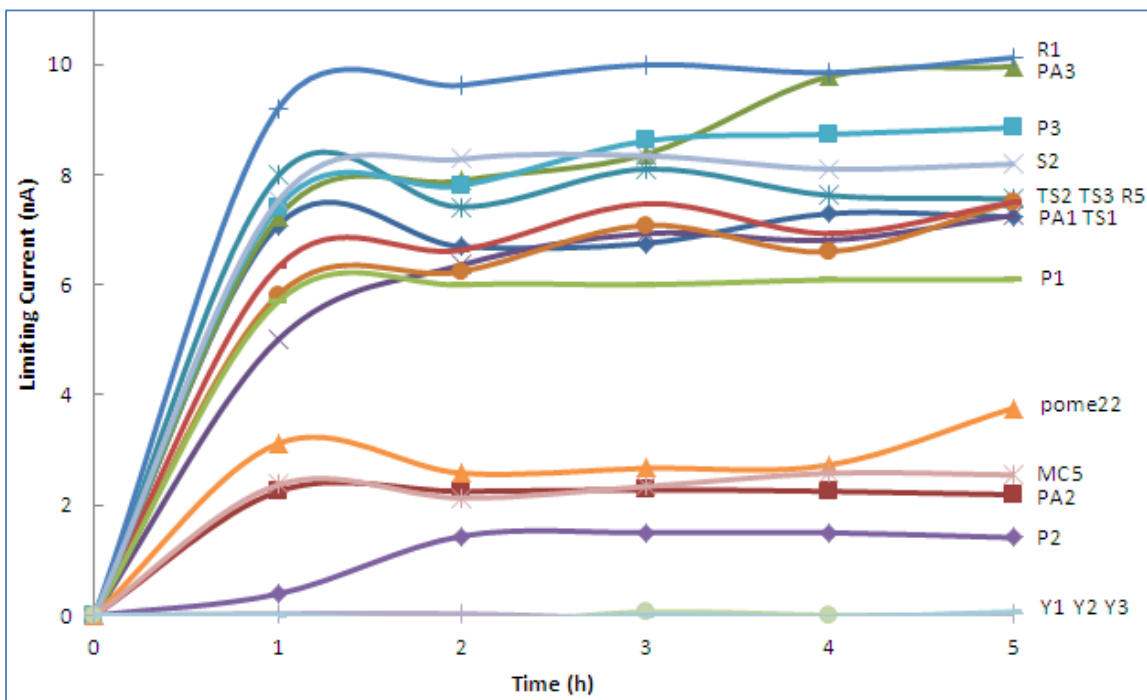
**Table 1** Colonies of microorganisms isolated from different environmental sources with their morphologies

No	Isolate	Colony Morphologies	Cellular Shape
1	PA1	White, slightly gummy, translucent, circular, convex with an entire margin	cocci
2	PA2	White, translucent, circular, convex with an entire margin	bacillus
3	PA3	White, irregular, umbonate with undulate margin	bacillus
4	P1	White, translucent, circular, convex and curled margin	bacillus
5	P2	White, circular, convex with an entire margin	bacillus
6	P3	White, slightly gummy, translucent, circular, convex with an entire margin	cocci
7	TS1	White, irregular, slightly convex and curled margin	bacillus
8	TS2	White, slightly gummy, translucent, circular, convex with an entire margin	cocci
9	TS3	White, slightly gummy, translucent, circular, convex with an entire margin	coccibacillus
10	R1	Purple, slightly gummy, circular, convex with an entire margin	cocci
11	R5	White, slightly gummy, translucent, circular, convex with an entire margin	coccibacillus
12	POME22	Large, white, irregular and flat with an undulate margin	bacillus
13	MC5	Large, white, circular, flat with undulate margin	cocci
14	S2	White, slightly gummy, translucent, circular, convex with an entire margin	cocci
15	Y1	White, waxy, circular, umbonate with filamentous margin	Oval with budding
16	Y2	White, waxy, circular, umbonate with entire margin	Elongated with budding
17	Y3	White, waxy, circular, umbonate with curled margin	Oval with budding

### 3.2 Screening of Potential Microorganisms

Figure 2 shows the limiting current values generated over 5 hours of incubation period in the GGA for each of the isolated microbes after the limiting current values of the endogenous control solution were subtracted from the sample. Based on the figure, R1 attained the highest limiting current after 60 minutes of incubation and this was followed by P3, TS2, PA1, PA3, R5, TS3, P1, TS1, POME22, MC5, PA2, and P2. Zero limiting current was attained by yeasts (Y1, Y2, Y3). A rapid linear increase in the limiting current value was observed for the sample solution after 60 minutes of incubation (Figure 2). This was because the GGA substrates were degraded by the microorganisms using ferricyanide as an alternative electron acceptor to oxygen. As a result, ferricyanide was reduced to ferrocyanide by the electron

transport chain systems of the microorganisms. When the potential of working electrode was shifted to +450 mV, the potential of the working electrode was high enough and sufficient to reoxidize the reduced ferrocyanide to ferricyanide. Therefore, the reduced ferrocyanide was reoxidized to ferricyanide by donating an electron to the working electrode. As a result, the limiting current obtained indicated the amount of ferrocyanide produced by the bacterial cells during GGA degradation, which is directly proportional to the amount of the degraded substrates. As shown in Figure 2, the limiting current of the sample solution reached an equilibrium after 60 minutes of incubation. This can be explained by the high rate of microbial metabolism when a large amount of substrate was present in the initial stage, and subsequently, the metabolic rate decreased with the unavailability of the substrates in the solution.

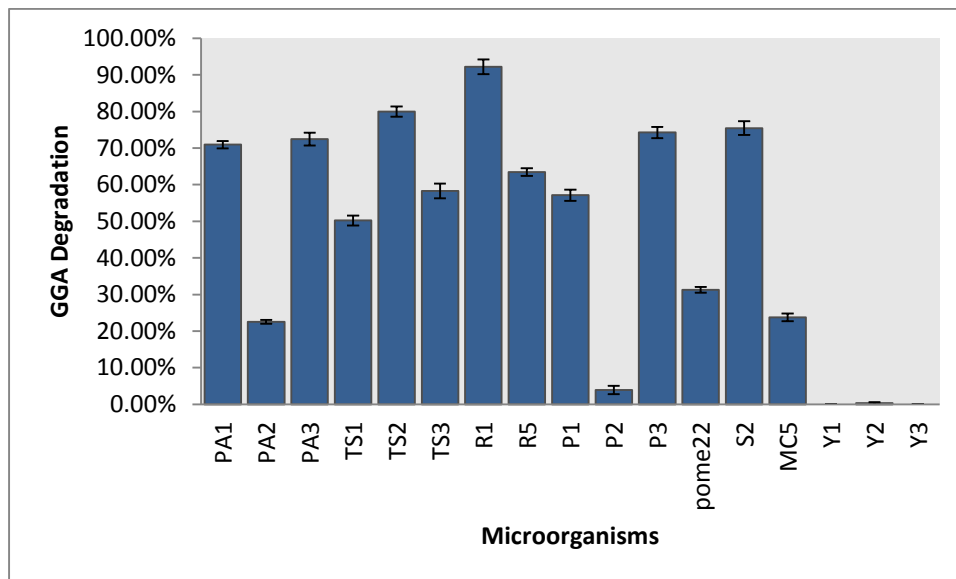


**Figure 2** Limiting current values were obtained at various incubation times for single cultures in GGA standard solution and endogenous control values have been subtracted from the sample. Microbial consortium final absorbance = 5. Ferricyanide final concentration = 60 mM. Limiting currents determined by voltammetry at  $E_{app} = +450$  mV (vs Ag/AgCl)

To give measurable analytical signal, the limiting current of the endogenous control was subtracted from the sample. The difference in the values indicates the amount of degraded substrates in the sample solution. The limiting current values of endogenous control arise from the metabolism of carbon storage polymer present within the cell during limitation of nutrients [7]. Thus, it is necessary to subtract the limiting current values of the endogenous control solution from the sample.

By referring to the generated limiting current (Figure 2) and the known amount of substrate in the sample solution, the percentage of GGA that was degraded could be calculated (Figure

3). R1 was able to degrade around 90% of the GGA in the first hour of the incubation period. In addition, PA1, PA3, TS2, R1, P3, and S2 were capable of degrading more than 60% of the GGA after 60 minutes of incubation. Similar work was reported by the Morris' group, whereby only 40% of the GGA was degraded after 60 minutes of incubation [11]. The results obtained in the present study were also compared with the more favourable conventional aerobic BOD<sub>5</sub> assay, in which approximately 60% conversion of the standard GGA solution was achieved in 5 days [7; 8]. Therefore, from the present study, the R1 can be used as a biocatalyst for further studies.



**Figure 3** Percentages of GGA degradation by the microorganism after 60 minutes incubation period

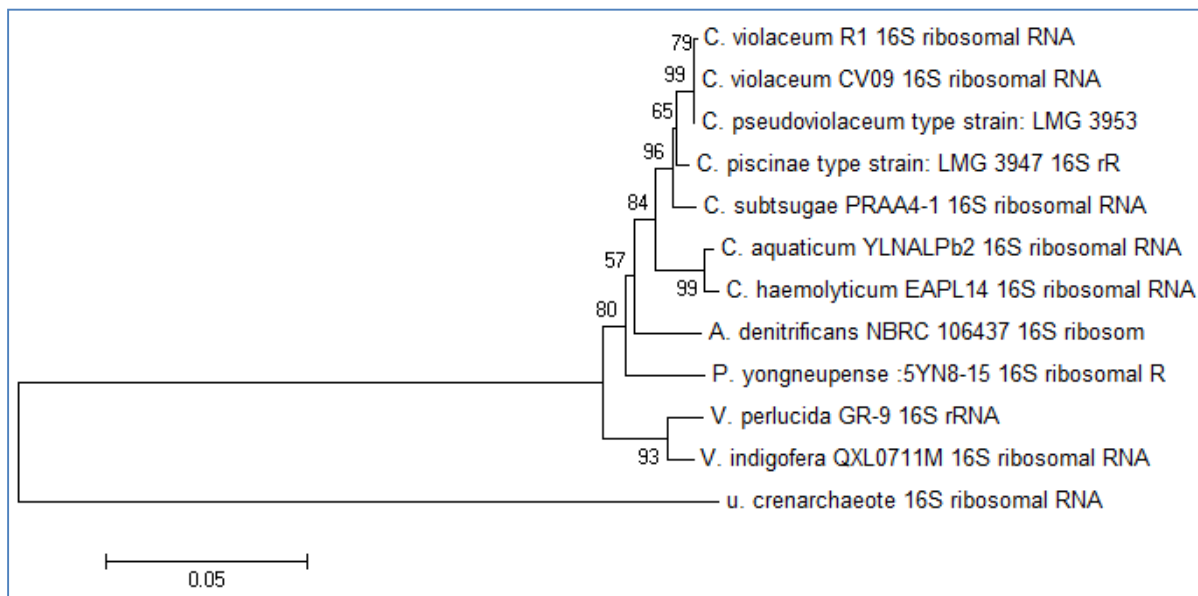


In contrast, GGA degradation was not found in yeasts. Approximately, zero current output was detected from yeasts over the 5-hour incubation. Ferrocyanide was not produced during the incubation. Yeast is classified as a eukaryotic microorganism, where the electron transport chain system is located in the mitochondria. The hydrophilic characteristic of ferricyanide prevents it from penetrating the yeast's outer cell wall [15]. Thus, during the biodegradation, ferricyanide was unable to facilitate the electron transfer from electron transport chain system of yeast to the working electrode. Therefore, double mediator system is required for eukaryotic microorganisms where a lipophilic mediator is required to transfer electron from inner mitochondria to the outer cell, and a hydrophilic mediator is

required to transfer electrons from the outer cell to the working electrode [15].

### 3.3 Identification of Isolates

R1 was identified using 16s rRNA molecular analysis. The nucleotide sequences in R1 were analysed using the BLASTn (Basic Local Alignment Search Tool) online analysis tool. The sequences were compared with GenBank using the BLASTn Programme. Subsequently, phylogenetic trees for R1 (Figure 4) were constructed. It shows that R1 is most closely related to *Chromobacterium violaceum* CV09 (accession number: FJ753567).



**Figure 4** Phylogenetic tree of R1 with other closely related members was constructed using Neighbor-Joining method with 500 replicates bootstrap test

By referring to Bergey's Manual of Systematic Bacteriology, *Chromobacterium violaceum* is a common facultative anaerobic, gram-negative coccobacillus usually found as a saprophyte in the soil and water samples in tropical and subtropical climates. Normally, it is considered to be non-pathogenic for humans [24]. An important characteristic of this microorganism is the production of a purple pigment called violacein, which is responsible for many antimicrobial activities and very potent bactericidal activity [26]. Furthermore, it has anti-viral and antitumoral properties [26]. Therefore, the colonies of *C. violaceum* appear to be purple in colour. *C. violaceum* is a capable microorganism applied in many other industries. It has been reported that chitinases produced from *C. violaceum* could be used in agriculture to fight against insects, fungi, and nematodes [27, 28], while the hydrolases action of *C. violaceum* could be used to hydrolyze plastic films of cellulose [29]. Besides, *C. violaceum* has also been reported to be used in the processes of denitrification [30] and solubilisation of gold [31].

### 4.0 CONCLUSION

In this study, an effective assay was applied to select the suitable microorganisms for ferricyanide-mediated BOD detection. By using the FM-BOD assay, the capability of various microorganisms using ferricyanide during organic degradation

was investigated. However, this assay was not only effective in selecting the suitable microorganisms for ferricyanide-mediated BOD detection; it also could be applied to select the suitable microorganisms for other mediated microbial biosensor and bioremediation by changing the substrate and conditions.

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