Bioremediation of Cyanide by Optimized Resting Cells of Rhodococcus Strains Isolated from Peninsular Malaysia

Maegala Nallapan Maniyam, Fridelina Sjahrir, and Abdul Latif Ibrahim

Abstract—**Five strains of locally isolated Rhodococcus species from various sources in Peninsular Malaysia were evaluated for their cyanide bioremediation prospective. The partially characterized Rhodococcus UKMP-5M which was used as reference strain failed to propagate exclusively in potassium cyanide (KCN). Therefore, the potential use of resting cells grown in nutrient broth without cyanide was tested to detoxify KCN at various concentrations ranging from 3 mM to 15 mM. The physical characteristics for the cultivation of Rhodococcus UKMP-5M namely temperature, pH and percentage of inoculum were optimized in order to be used as source for cyanide treatment. It was found that the maximum biomass was generated amounting to 8.3222 g/L when the incubation temperature was set to 30 °C at pH 6.6 with an initial inoculum loading of 2 % (v/v). Whole cells with biomass amounting to 1 g/L of the strain were able to rapidly transform almost 50 % of 12 mM cyanide over a period of 10 hours. The biotransformation was carried out at 30 °C, pH 7 and with an agitation at 160 rpm in the absence of added organic and inorganic substances. In addition, by employing the identical amount of biomass and other experimental conditions, the different strains of locally isolated Rhodococcus species were compared for their ability to metabolize cyanide. It was revealed that Rhodococcus UKMP-5M had the highest percentage of cyanide removal efficiency followed by Rhodococcus zopfii, Rhodococcus sp1, Rhodococcus NAM81 and Rhodococcus sp2 which corresponded to 47.78 %, 29.17 %, 23.61 %, 18.33% and 11.67 % respectively.**

Index Terms—**Bioremediation, Physical characteristics, Cyanide, Rhodococcus.**

I. Introduction

Cyanide is a carbon nitrogen radical, which may be found in a wide variety of life forms and their large scale presence in the environment is attributed to the manufactured sources which are used extensively in industries. Cyanide is commonly found as a contaminant in wastewaters through release from metal finishing, electroplating and coal coking industries which is the crucial factor that contributes to the bulk occurrence of cyanide in the environment [1]. Cyanide in various forms is highly toxic,carcinogenic, and mutagenic.

Hence, control and remediation of cyanide – contaminated water is desired because of the potential hazards associated with cyanide. There are several conventional methods used in treating effluents containing cyanide before discharging it into the environment. The most common ones are the alkaline chlorination, sulfur oxide/air process and hydrogen peroxide process. Although these methods of treatment can be used in detoxifying free cyanide bearing waste, they pose significant drawbacks as listed below:

- The disability to treat cyanide that is complexed with metals.
- High costs of reagents, equipment, maintenance and royalty payments.
- The generation of unfavorable by-product such as chlorinated compounds.

Thus, with rapid growth in many applications that utilize cyanide, it is necessary to use bioremediation as the most promising platform to prevent contamination of soils and wastewater. Many attempts to develop biological processes for the detoxification of cyanide have been concentrated on cyanide-degrading fungi [2] and several studies have been established on the use of bacterial strains such as *Klebsiella, Pseudomanas, Acinetobacter, Bacillus, Burkholderia* and *Alcaligenes* [3].

Meanwhile, it was also interesting to note that the utilization of *Rhodococcus* in degrading cyanide was rather promising. *Rhodococcus rhodochrous* in particular showed remarkable potential in degrading cyanide even at a concentration of 260 mg/L and can be considered as source for the isolation of cyanidase [4]. Rhizosphere microbial community, *Rhodococcus* sp. which was isolated from two cyanogenic plants apparently utilized oxidative reactions for cyanide degradation as well [5]. It was also a fascinating revelation to observe that two facultative autotrophs, both actinomycetes, of the genus *Nocardia* and in another case a gram-positive filamentous organism, probably again an actinomycete, have also been found to be capable of growing on cyanide as a carbon and nitrogen source. This indicated that *Rhodococcus*, an actinomycete, had a huge potential to degrade cyanide as it belonged to the genus *Nocardia* [6]. Hence, in the present work, the viability of using locally isolated *Rhodococcus* strains for their potential in cyanide bioremediation is assessed.

II. Materials and Methods

A. Microorganisms and growth conditions

Strains of locally isolated *Rhodococcus* species were kindly provided by the Culture Collection Unit, Institute of

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Bio-IT Selangor. To the production medium containing (g/L) nutrient broth, 8.0; glucose, 8.0; and L-proline, 10.0, 1 ml (2 % v/v) of 24 hours preculture of *Rhodococcus* UKMP-5M (optical density equivalent to 1.000-1.200) grown in 8.0 g/L nutrient broth were inoculated and left to shake at an agitation of 160 rpm.

The influences of temperature and pH on growth of this bacterium were tested in the range of 20 to 40 °C and 5 to 10 respectively. The impact of initial addition of inoculum varying from 1 to 5 % (v/v) was evaluated as well. The optimized physical parameters were then employed in order to cultivate *Rhodococcus* UKMP-5M. The cells were harvested at early stationary phase (48 hours) by centrifuging the culture at 419 × g for 30 minutes at 4 °C, washed twice with 100 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer to be stored at 8 °C until further analysis. The optical density of the suspended cell was adjusted to 0.500 at 600 nm which corresponded to 1.0 g/L dry cell weight.

B. Biotransformation of cyanide

An amount of 2.5 ml of the suspended cells were transferred into screwcap bottles and cyanide in the form of KCN were added to a final concentration of 3, 6, 9, 12 and 15 mM respectively and left to shake at 160 rpm and 30 °C. Hundred microlitre of samples were removed, diluted to 10 mL and assayed for remaining cyanide at 2, 4, 7, 10, 24 and 30 hours respectively by employing a modified barbituric acid method [7].

Identical culture conditions were employed to cultivate the other locally isolated Rhodococcus strains. For the purpose of strain comparison study, the percentage of cyanide degradation was determined after 10 hours of incubation with an initial cyanide concentration of 12 mM following the similar experimental method as mentioned above.

C. Statistical analysis

All chemicals were of analytical grade obtained from various commercial sources. Control experiments were established without bacterial cells and experiments were carried out in triplicate and experimental errors were estimated and depicted with error bars. All data were analyzed by using SPSS version 17.0. Comparison between groups was performed by using Duncan analysis. A one way ANOVA test (95 % confidence interval) was used to evaluate differences between groups and $p < 0.05$ was considered statistically significant.

III. Results and Discussion

A. Optimization of physical parameters of Rhodococcus UKMP-5M

The strain was unable to grow aerobically on minimal medium with KCN as the sole source of carbon and nitrogen which was in agreement with previous reports [2], [3], [8]. The addition of initial inoculums perhaps were unable to sustain a basal metabolism and needed ample of energy and time in order to produce enzymes that could metabolize the highly toxic substrate, KCN [9]. Besides, the system may also require supplementary carbon source as well as the presence of yeast extract to support the growth of *Rhodococcus* UKMP-5M which will eventually facilitate the detoxification of cyanide. The prospect of this hypothesis is currently being investigated. Meanwhile, the potential use of resting cells grown in the nutrient broth to eliminate cyanide was examined. The physical properties for the cultivation of *Rhodococcus* UKMP-5M were optimized in order to generate the utmost biomass.

At optimum temperature, all aspects of cell metabolism function at their finest whereby the cells were able to rapidly increase in size leading to efficient enzymatic systems. It was found that the ideal temperature for the cultivation of *Rhodococcus* UKMP-5M was at 30 °C as supported by previous reports [10], [11], a feature of many bacteria isolated from environmental habitats, generating an amount of 6.3222 g/L dry cell weight. Malaysia with temperature ranging from 28 °C to 33 °C could greatly benefit from these findings since most actual cyanide bioremediation sites were operated at ambient temperature which coincided with the optimal growth of *Rhodococcus* UKMP-5M. The strain proliferated reasonably well at 25 and 35 °C which corresponded to a biomass of 2.9889 and 2.5889 g/L respectively as illustrated in Fig. 1. *Rhodococcus* UKMP-5M survived better in the temperature range between 25 °C and 35 °C implying the typical characteristics of a mesophile [10]. The growth reduced rather substantially at 20 and 40 °C resulting in merely 0.5922 and 0.1033 g/L dry cell weight respectively. The movement of molecules decelerated as the temperature was lowered suggesting the inability of enzymes to mediate in chemical reactions and eventually all activities were brought to a halt due to the viscosity of the cell interior. Meanwhile, at higher temperature, enzymes started to denature and the total affect was detrimental to cellular growth [11].

In order to design an effective bioremediation strategy, identification of the pH optimal for growth would be essential. The effect of initial pH on the growth of *Rhodococcus* UKMP-5M was examined at 30 °C using stepwise addition of either 0.1 M HCl or 0.1 M NaOH. The strain proliferated rather well at a relatively wide pH range from 6 to 9 [12] with maximal growth attained at pH 6.6, the pH of the medium without any alteration resulting in 4.3222 g/L dry cell weight. Growth dramatically decreased outside this pH range although the bacterium remained viable with a biomass of 0.2556 and 0.2311 g/L respectively. This may be due to lowered stability of plasma membrane coupled with inhibited enzyme membrane as well as proteins transport [13]. It was rather apparent from Fig. 2 that the alkaline conditions appeared to encourage the cultivation of this isolate since it could tolerate higher pH considerably as compared to that of acidic conditions, a distinct characteristic of an actinomycete 10]. It was also observed that *Rhodococcus* UKMP-5M exhibited highest growth at near neutral pH which was acknowledged by the fact that the cytoplasm in most bacteria has a pH level of 7.

A 2 % v/v of inoculum supported the highest growth of this particular isolate as portrayed in Fig. 3 yielding to 8.3222 g/L dry cell weight. Increasing the concentration of seed culture beyond 2 % in the cultivation vessel found to be disadvantageous for the propagation of *Rhodococcus*

UKMP-5M. This could be possibly due to the increased viscosity in the medium because of higher inoculum concentration, which probably limited efficient mass transfer.

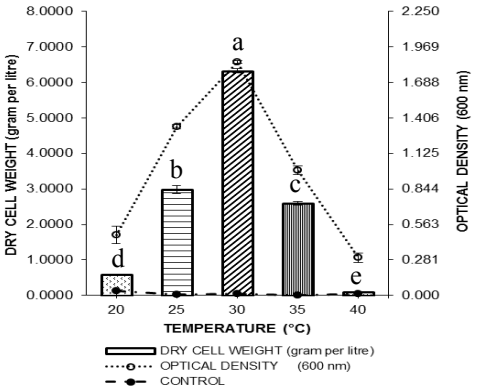

Fig. 1. Effect of temperature on the growth of Rhodococcus UKMP-5M cultivated in nutritionally optimized nutrient broth medium at an agitation of 160 rpm for 48 hours at pH 6.6 with 2 % (v/v) inoculum. Error bars represent standard error between three determinations. Statistically significant differences ($P < 0.05$) were observed among the tested range of temperature.

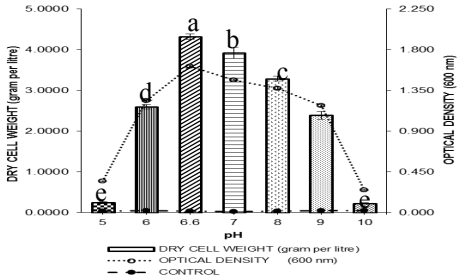

Fig. 2. Effect of pH on the growth of Rhodococcus UKMP-5M cultivated in nutritionally optimized nutrient broth medium at an agitation of 160 rpm for 48 hours at 30 °C with 2 % (v/v) inoculum. Error bars represent standard error between three determinations. Statistically significant differences ($P < 0.05$) were observed among the tested range of pH.

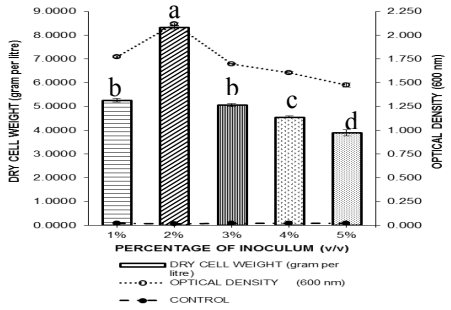

Fig. 3. Effect of initial inoculum loading on the growth of Rhodococcus UKMP-5M cultivated in nutritionally optimized nutrient broth medium at an agitation of 160 rpm for 48 hours at 30 °C and pH 6.6. Error bars represent standard error between three determinations. Statistically significant differences ($P < 0.05$) were observed among the tested range of percentage of inoculum.

B. Biotransformation of cyanide by Rhodococcus UKMP-5M

The optimized biomass was able to rapidly degrade up to 47.78 % of 12 mM cyanide after 10 hours of incubation as depicted in Fig. 4. It was clearly noticeable that 63.33 % of 3 mM KCN was degraded within 10 hours of incubation followed by 50.56 % of 6 mM KCN, 48.52 % of 9 mM KCN, 47.78 % of 12 mM KCN and 1.11 % of 15 mM KCN. The bacterium was not able to metabolize or transform 15 mM KCN as the percentage of degradation decelerated drastically which was attributed to the intolerable toxicity level of cyanide. As illustrated in Fig. 4, it was rather apparent that the percentage of degradation became constant after 10 hours of incubation. This may be due to the limited capability of the resting cells amounting to 1 g/L to express sufficient amount of cyanide degrading enzymes to support continuous degradation and eventually to completely break down cyanide. Meanwhile, cyanide concentrations in the control system remained almost unchanged throughout the experimental process indicating that the depletion of cyanide is primarily due to biodegradation activity.

The cyanide degrading activity was present even in the cells grown in the nutrient broth without KCN. Cells cultivated to their stationary phase were better equipped by accumulating factors that enable them to handle variety of environmental stress than exponential phase [8]. For that reason, the strains of *Rhodococcus* were harvested at their early stationary phase (48 hours) and later used as resting cells for cyanide degradation.

Although the strain was not capable of growing exclusively by utilizing KCN as the sole source of carbon and nitrogen, it was able to directly transform KCN by means of resting cells [2]. This may be due to the presence of a low level of constitutive synthesis of the cyanide degrading enzymes [8]. Moreover, *Rhodococcus* UKMP-5M was also observed to grow in medium containing nitriles and the whole cells were able to hydrolyze nitriles to its corresponding carboxylic acid and ammonia [14].

It was fascinating to notice that the predicted protein sequence from the gene encoding the enzyme cyanide hydratase that has been isolated and sequenced from *Fusarium lateritium*, *Fusarium solani*, *Gloeocercospora sorghi* and *Leptosphaeria maculans* demonstrated strong homology to all available nitrilase and cyanide dihydratase sequences. On top of that, it was also a refreshing revelation to discern the fact that the enzyme cyanide dihydratase that has been characterized from *Psedomonas stutzeri* AK61 was more closely related to nitrilase compared to that of cyanide hydratase. Furthermore, the active site for cyanide hydratase enzyme and nitrilase activity in the protein was the same for *Fusarium lateritium* [15]. Hence, the enzyme present in the culture of *Rhodococcus* UKMP-5M that transformed nitriles to carboxylic acid and ammonia may be capable of converting KCN as well.

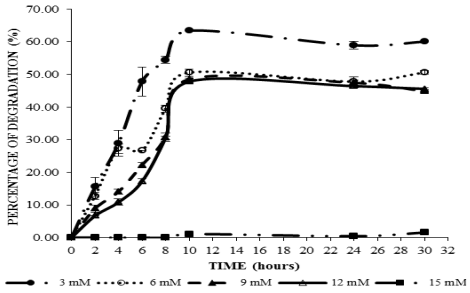

Fig. 4. Cyanide depletion patterns by resting cells of Rhodococcus UKMP-5M harvested at early stationary phase (48 Hours). Biomass (0.0025 g) was incubated with cyanide at different concentrations (3-15 mM) at 30 °C for 30 hours with shaking at 150 rpm. Error bars represent standard error between three determinations.

C. Biotransformation of cyanide by different strains of *Rhodococcus*

Within the *Rhodococcus* strains, *Rhodococcus* UKMP-5M exhibited the highest percentage of cyanide degradation which was 47.78 % whereas *Rhodococcus* sp2 displayed the least percentage of degradation, merely 11.67 %as portrayed in Fig. 5. Appreciable percentages of degradation were observed for *Rhodococcus zopfii*, *Rhodococcus* sp1 and *Rhodococcus* NAM81 which corresponded to 29.17 %, 23.61 % and 18.33 % respectively.

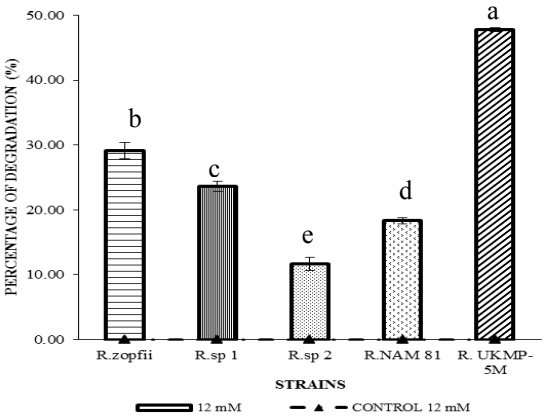

Fig. 5. Cyanide depletion patterns by resting cells of Rhodococcus strains harvested at early stationary phase (48 Hours). Biomass (0.0025 g) was incubated with 12 mM cyanide at 30 °C for 30 hours with shaking at 150 rpm. Error bars represent standard error between three determinations. Statistically significant differences ($P < 0.05$) were observed among the tested strains of Rhodococcus species

IV. Conclusions

In conclusion, we report for the first time to the best of our knowledge, an efficient cyanide-degrading bacterium, *Rhodococcus* UKMP-5M which was isolated from Peninsular Malaysia. The ability of the strain to metabolize 312 mg CN^-/L proved to be of great interest since 260 mg CN^-/L was found to be inhibitory for the growth of many microorganisms.

Meanwhile, since the utilization of resting cells played crucial role in determining the competency of cyanide removal, various physical parameters affecting the growth of the bacterium were optimized.

Rhodococcus UKMP-5M was then selected for further experiments since the strain evidently possesses huge potential to be useful in cyanide bioremediation.

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