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Research Article

BIOTRANSFORMATION OF CYANIDE BY A MALAYSIAN ISOLATE *RHODOCOCCUS* UKMP−5M

***Maegala N.M., Fridelina S. and Abdul Latif I.**
Institute of Bio−IT Selangor, Universiti Selangor, Jalan Zirkon A7/A, Seksyen 7, 40 000 Shah Alam, Selangor, Malaysia
**Author for Correspondence*

ABSTRACT
Rhodococcus UKMP−5M, a Malaysian isolate obtained from petroleum−contaminated soil was assessed for its potential to detoxify cyanide. The resting cells harvested at early stationary phase demonstrated the highest rate of cyanide removal corresponding to 0.34mm/hrs/mg dry weight even though the bacterium could not grow solely on potassium cyanide, KCN. An addition of 0.8%v/v glucose to the bioconversion medium witnessed an increase in the cell density of the isolate and subsequently higher cyanide−degrading activity measuring to 67% degradation was recorded. It was revealed that the enzyme(s) responsible for nitrile hydrolysis may be able to mineralize cyanide as well since they have demonstrated strong sequence homology to all available nitrilase sequences and their existence in nature were most likely to be constitutive. Hence, the role of inducer in the cultivation of resting cells of *Rhodococcus* UKMP−5M for cyanide detoxification was investigated in the present study. It was revealed that the employment of acetonitrile as an inducer did not promote higher cyanide removal efficiency resulting in 58% cyanide−degrading activity in comparison to 55% degradation when induced and uninduced resting cells obtained from Medium I was utilized, respectively ($p > 0.05$). This discovery is highly significant since it revealed the presence of low level constitutive synthesis of cyanide−degrading enzyme(s) in this bacterium. In addition, inducing the bacterium with acetonitrile during cultivation did not inhibit the cyanide removal efficiency of *Rhodococcus* UKMP−5M. Besides, the resting cells of the isolate also demonstrate satisfactory storage stability. These properties offer an attractive alternative in utilizing this actinomycete for biological treatment of wastewater containing cyanide.

Keywords: *Biotransformation, Cyanide, Inducer, Resting Cells, Rhodococcus UKMP−5M, Thermostability*

INTRODUCTION
Rhodococcus species have remarkable ability to degrade many pollutants besides producing biosurfactants or emulsifiers with beneficial applications. They are increasingly becoming more valuable in the field of bioremediation and biotechnology. This is due to their indigenous property in contaminated sites and thus making them suitable to be used as inocula for bioremediation (Bell *et al*., 1998).
The metabolic diversity of *Rhodococcus* is more versatile than the pseudomonads with great persistent in the environment even though they often demonstrate slow growth. In addition, catabolite repression is absent in *Rhododcoccus* suggesting that pollutants like hydrocarbons, phenols and nitriles (organic cyanide) would be degraded even in the presence of more easily assimilable carbon sources (Quek *et al*., 2006). Taking this into account, the exploitation of this bacterium with such a versatile competency in degrading environmental pollutants, particularly cyanide has been investigated previously. *Rhodococcus rhodochrous* above all showed remarkable potential in degrading cyanide at a concentrations as high as 260mg/L and can be considered a source for the isolation of cyanidase (Keusgen *et al*., 2001). Hong (2007) reported a rhizosphere microbial community, *Rhodococcus* species isolated from two cyanogenic plants which apparently utilized oxidative reactions for cyanide degradation. It is also a fascinating revelation to note that two facultative autotrophs, both actinomycetes, of the genus *Nocardia* and in another case a gram−positive filamentous organism, probably again an actinomycete, have been found to be capable of growing on cyanide as a carbon and nitrogen source. This indicates that *Rhodococcus*, an actinomycete, also has a prospect to degrade cyanide as it belongs to the genus *Nocardia* (Ezzi and

Lynch, 2005). In addition, there is a report suggesting the ability of *Rhodococcus* species to grow in media supplemented with cyanide by expressing alternative oxidases so permitting bacterial respiration (Dumestre *et al*., 1997).

It was understood that the utilization of resting cells may have an added advantage in detoxifying the highly toxic cyanide. This is primarily due to the ability of whole cells to accumulate factors that enable them to tolerate variety of environmental challenges (Adjei and Ohta, 1999). Therefore, in the present work, the viability of employing the whole cells of locally isolated *Rhodococcus* UKMP−5M for its potential activity in cyanide detoxification is assessed. The most desirable conditions such as the effect of inducer and the addition of glucose for enhanced cyanide−degrading activity were determined accordingly. In addition, the thermostability of the resting cells were examined for storage stability.

MATERIALS AND METHODS

Chemicals

All chemicals and media ingredients used in the present study were of analytical grade and purchased either from Sigma (USA), Fisher Scientific (Singapore) or Merck (Germany).

Microorganism

The Malaysian isolate *Rhodococcus* UKMP−5M, obtained from petroleum−contaminated soil, was kindly supplied by the Culture Collection Unit, Institute of Bio−IT Selangor, Malaysia.

Preparation of Resting Cells of Rhodococcus UKMP−5M

The production medium was prepared containing 8.0g/L nutrient broth, 0.8%w/v glucose and 1.0%w/v L−proline and the pH was left unadjusted. An inoculum concentration of 2%v/v was then transferred aseptically (Biosafety Cabinet 4ft-EN12469, ESCO Micro (M) Sdn. Bhd) into the production medium. The flask was left to shake on a rotary shaker at 160rpm and 30°C (Jeio Tech SI-600R, Korea). The resting cells of *Rhodococcus* UKMP−5M was collected by centrifuging at 419×*g* for 30min at 4°C (Eppendorf 5702R, South Asia) at 12, 24 and 48hrs corresponding to lag, exponential and early stationary phases, respectively. The washed cells in an appropriate amount of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7) were later subjected to residual cyanide analysis.

Effect of Glucose on the Biotransformation of Cyanide by Rhodococcus UKMP−5M

The effect of additional carbon source on cyanide removal efficiency was assessed by adding glucose of concentration 0.8%w/v into vessel containing resting cells harvested at early stationary phase in an adequate volume of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7) and of 12mM KCN.

Effect of Inducer on the Biotransformation of Cyanide by Rhodococcus UKMP−5M

Rhodococcus UKMP−5M was cultivated in two basal nutrient media of different composition. Acetonitrile amounting to 0.5%v/v was added as an inducer into each medium and cells were harvested at early stationary phase corresponding to 48hrs for medium I and 72hrs for medium II, respectively by centrifuging at 419×*g* for 30min at 4°C.

Medium I: Nutrient broth (8g/L), Glucose (8g/L) and L−proline (10g/L)
Medium II: Yeast extract (0.5g/L), KH_2PO_4 (0.5g/L), K_2HPO_4 (0.5g/L), $MgSO_4$ (0.5g/L) and $CaCl_2$ (0.01g/L)

The washed pooled cells were resuspended in a suitable amount of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7) and used further as resting cells for conversion of 12mM KCN. The utilization of induced suspended cells as compared to that of uninduced whole cells was investigated for both mediums.

Thermostability

Resting cells of *Rhododcoccus* UKMP−5M harvested at early stationary phase of 48hrs were pre−incubated at 4, 30, 40 and 50°C (MEMMERT 108L Incubator INB500, Germany) for 30 and 60min, respectively. The pre−incubated resting cells were subsequently added to screwcap bottles consisting of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7) and 12mM KCN for biotransformation activity.

Conversion of 12mM Cyanide by Resting Cells of Rhodococcus UKMP−5M

The biotransformation of cyanide was carried out in screwcap bottles containing 3.5mg biomass (dry cell weight). Following this, an adequate volume of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7) was added to 50mm

filter−sterilized KCN in order to attain a concentration of 12mM cyanide. The mixtures in triplicate were then incubated in a rotary shaker operated at 150rpm for 10hrs at 30°C. Control experiments were established in identical conditions without bacterial cells. The cell pellet was separated by centrifuging at 11000×*g* for 15min at 25°C to obtain transparent spent media. Aliquot of the resulting clear supernatant measuring to 100µl were withdrawn and diluted to 10ml before assaying for remaining cyanide in triplicate.

Analysis of Residual Cyanide and Glucose and Determination of Dry Cell Weight

The concentration of remaining cyanide was determined as detailed by Nagashima (1977). The changes in the concentration of glucose in the biotransformation medium were determined following the protocols detailed by Ezzi and Lynch (2005). The dry cell weight was determined through the protocol described by Tripathi *et al*., (2009).

Statistical Analysis

Experimental errors were valued and presented by error bars (standard error) typically with three determinations. An SPSS version 17.0 was used to analyse all data. 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 to be significant statistically.

RESULTS AND DISCUSSION

Biotransformation of Cyanide by Resting Cells of Rhodococcus UKMP−5M Harvested at Different Growth Phase

The resting cells were grown in nutrient broth without KCN and separately harvested during the lag, exponential and early stationary phases. Low cyanide−degrading activities were observed for cells collected at lag and exponential phase yielding specific activities of $0.12^{b} \pm 0.07$ and $0.17^{b} \pm 0.04$mM/hrs/mg dry weight, respectively. Meanwhile, cells harvested during early stationary phase exhibited significantly elevated activities amounting to $0.34^{a} \pm 0.01$mm/hrs/mg dry weight, approximately 64 and 49% higher than that of the lag and exponential phase, respectively (data with uncommon superscript was significantly different ($p < 0.05$)). Similar, findings were also reported by Adjei and Ohta (1999) and Meyers *et al*., (1991).

Whole cells grown in nutrient broth in the absence of cyanide were able to remove KCN rather effectively. It is reasonable to suggest that the direct transformation of KCN by means of resting cells occurred even though the strain did not exhibit growth on KCN as sole source of carbon and nitrogen. Similar findings were reported by Dhillon and Shivaraman (1999). Although *Pseudomonas* species (S1) failed to propagate on KCN, the bacterium was capable of direct conversion of KCN to formic acid (Dhillon and Shivaraman, 1999). This biotransformation was possible due to constitutive enzyme(s) that may be present in the culture and were active in transforming nitriles into carboxylic acids and ammonia and may be competent to convert KCN as well. *Rhodococcus* UKMP−5M was noticed to proliferate well in nitrile−containing medium and the whole cells were able to hydrolyse nitrile to its corresponding carboxylic acid and ammonia (Nallapan Maniyam *et al.*, 2011), suggesting the existence of a similar pattern here.

Moreover, there have been numerous reported observations stating the ability of *Stemphlium loti*, *Fusarium lateritium* and *Gloeocerocops sorghi* to detoxify cyanide and yet were not able to grow on KCN (Barclay *et al.*, 1998). On top of that, strong sequence homology was found between the genes encoding the enzyme cyanide hydratase, isolated and sequenced from *Fusarium lateritium*, *Fusarium solani*, *Gloeocercospora sorghi* and *Leptosphaeria maculans* to the predicted protein sequences of all available nitrilases and cyanide dihydratases sequences (Nolan *et al.*, 2003), further strengthening the current findings.

Effect of Glucose on the Biotransformation of Cyanide by Rhodococcus UKMP−5M

Supplementing the biotransformation system with 0.8%w/v glucose increased the rate of cyanide removal efficiency to 67% degradation as compared to that of 58% in the biotransformation medium without the addition of 0.8%w/v glucose as shown in Table 1. In addition, significant rise in the growth of

Rhodococcus UKMP−5M ($p < 0.05$) corresponding to an optical density of 0.74 was observed in the system with 0.8%w/v glucose whereas the cell density remained unchanged in the absence of 0.8%w/v glucose after 24hrs of incubation. Interesting, the elevated growth of the bacterium in glucose−containing biotransformation medium was apparently linked to the reduction levels of glucose in the medium. This indicated the addition of glucose supported the presence of higher metabolic activity and energy production due to increased biomass which linearly enhanced cyanide−degrading activity of the strain *Rhodococcus* UKMP−5M. Similar observation was reported by Ezzi and Lynch (2005) whereby the fungal strains of *Trichoderma* and *Fusarium* managed to consume 2000ppm of cyanide within 32 days with the addition of 25mm glucose whereas it took 90 days for the same strains to degrade cyanide without the supplement of glucose. Glucose served as a source of energy in sustaining the biomass which eventually maintained the cyanide−degrading capability of the fungal strains. Meanwhile, the degradation of $K_2Ni(CN)_4$ by resting cells of Klebsiella *oxytoca* cultivated on 50mm phosphate buffer was enhanced remarkably when 0.8%w/v of glucose was added into the buffer (Kao *et al.*, 2003). The white−rot fungi, *Phanerochaete chrysosporium* utilized glucose to promote growth and rapid establishment of the fungus within the cyanide substrate and it assisted cyanide degradation by providing a more easily metabolizable carbon source (Hossain *et al.*, 2005). It was also found that 80% of 65mm $K_2Ni(CN)_4$ was degraded by *Cryptococcus humicolus* MCN2 with 3g/l glucose whereas supplementing the growth medium with 8g/l glucose observed complete degradation of cyanide (Kwon *et al.*, 2002). Hence, the presence of glucose is rather vital in cyanide degradation as it appears to act as a co−metabolite as it provides energy for growth and maintenance.

Table 1: Effect of Glucose on Biotransformation of Cyanide by Resting Cells of *Rhodococcus* UKMP−5M Grown in Uninduced Nutrient Broth

	Without Glucose		***With 0.8%w/v Glucose**	
	0hrs	**24hrs**	**0hrs**	**24hrs**
Growth at 600nm	0.66 ± 0.01	0.66^{b} ± 0.01	0.67 ± 0.01	0.74^{a} ± 0.03
Cyanide Removal (%)	3 ± 2.68	58^{b} ± 2.10	2 ± 1.73	67^{a} ± 2.10
Glucose Consumption (%)			3 ± 1.44	14 ± 4.28

*Glucose (0.8%w/v) was added to 3.5mg dry cell weight resting cells. Growth, cyanide removal and glucose consumption represent means of triplicate samples ± standard deviations. Data with uncommon superscript was significantly different ($p < 0.05$)

In the present study, negligible loss of glucose was observed in the uninoculated control system suggesting that glucose was indeed utilized for growth which was supported by the proportional increase in the biomass along with cyanide removal activity which was in agreement with Ezzi and Lynch (2005). This finding is in contrast to the reports by Dumestre *et al*., (1997) which observed rapid loss of cyanide in the glucose minimal medium as well as uninoculated sterile medium. They attributed this phenomenon to the well documented Kiliani reaction illustrating the reaction between cyanide and sugars catalysed at a highly alkaline pH (Dumestre *et al.*, 1997).

Effect of Inducer on the Biotransformation of Cyanide by Rhodococcus UKMP−5M

A degradation profile of cyanide by the washed cells of *Rhodococcus* UKMP−5M assayed in phosphate buffer containing 12mm KCN is as presented in Figure 1. Cells grown in induced Medium I exhibited the highest percentage of cyanide−degrading activity totalling to 58% degradation. Meanwhile, the utilization of cells harvested from uninduced nutrient broth cultivation medium showed 55% degradation, an insignificant 3% fall ($p > 0.05$) in the activity of cyanide−degrading enzyme(s). The application of cells grown in induced and uninduced Medium II displayed similar pattern of degradation, however with substantially reduced percentage of cyanide removal efficiency ($p < 0.05$) as compared to that of cells

propagated in induced and uninduced Medium I. Resting cells propagated in nutrient broth yielded substantial amount of cell density of 1.2g/l, a three−fold higher in comparison with suspended cells collected from Medium II. It may then be concluded that the overall cyanide−degrading activity seems to increase with cell density in the cultivation medium even without the presence of an inducer.

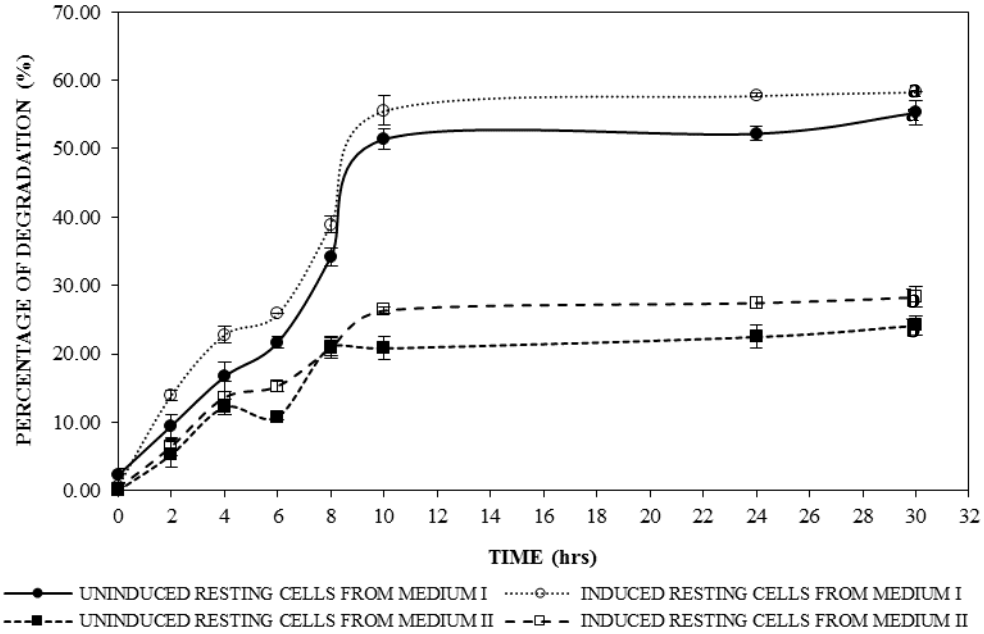

Figure 1: Effect of Induced and Uninduced Biomass on Cyanide Removal Efficiency by *Rhodococcus* UKMP−5M. Error Bars Represent Standard Error (3 Determinations). Data with Uncommon Letters was Significantly Different ($p < 0.05$)

These findings indicated that the employment of acetonitrile as an inducer did not promote higher cyanide removal efficiency suggesting the enzymes responsible for cyanide detoxification were partly inducible and partly constitutive.

This is because the growing cells of *Rhodococcus* UKMP−5M required the addition of acetonitrile as an inducer to support growth and degradation of cyanide at higher concentrations. In addition, it was interesting to note that the resting cells grown in the cultivation medium even in the absence of an inducer expressed substantial cyanide−degrading activity.

The presence of low level constitutive synthesis of cyanide−degrading enzyme(s) coupled with the robust nature of the cells harvested at stationary phase was the evidence that suggested these activities (Adjei and Ohta, 1999). The behaviour of *Rhodococcus* UKMP−5M was attributed to the factors that enable the stationary phase bacterial cells to cope with diverse environmental stress as compared to that of exponential phase. Thus, it was clear that this bacterium naturally adopted similar properties at stationary phase which facilitated cyanide detoxification. In line with our findings, Watanabe *et al.*, (1998) reported comparable observations with the degradation of 1mM KCN by whole cells of *Pseudomonas stutzeri* AK61 previously grown in the isolation medium with and without 1mm KCN as an inducer. It was revealed that both systems displayed similar cyanide disappearance pattern whereby rapid removal of cyanide occurred in the reaction mixtures (Watanabe *et al.*, 1998). In addition, it was also found that inducing 20mg CN^-/L to the Oxoid medium even during early stationary phase did not enhance the cyanide−degrading activity of resting cells of *Bacillus pumilus* C1 and the cyanide removal efficiency was completely inhibited when suspended cells cultivated in Difco medium was used (Meyers *et al.*, 1991). Furthermore, Adjei and Ohta (1999) described a cyanide-utilizing *Burkholderia cepacia* strain C3 which was able to exhibit high activities of 0.39mm/h/mg dry cell weight in detoxifying cyanide when

washed stationary phase cells grown in nutrient broth devoid of an inducer were incubated in the cyanide−containing medium.

Thermostability of Cyanide−Degrading Activity of Rhodococcus UKMP−5M

Whole cells of *Rhodococcus* UKMP−5M were preincubated at 4, 30, 40 and 50°C for 30 and 60min, respectively in order to investigate the thermostability of cyanide−degrading activity is as shown in Table 2.

It was found that the cyanide−degrading activity was completely inactivated at 50°C and very low percentage of cyanide removal efficiency was observed with cells preincubated at 40°C. Heat−treated cells at 40°C for 30min exhibited only 32% degradation and the cyanide−degrading activity fell dramatically to 11% after being subjected to slight prolongation in the incubation time of 60min at 40°C. Thus, it is rather obvious from these findings that strain *Rhodococcus* UKMP−5M exhibited mesophilic characteristics.

The utilization of pre−incubated resting cells at 4°C for 30 and 60min resulted in 51 and 46 % of cyanide removal efficiency, respectively suggesting that the storage stability of the whole cells of this particular bacterium was satisfactory. It was fascinating to note that the employment of previously incubated suspended cells at 30°C for 30 and 60min yielded in 54 and 53% degradation, respectively indicating no marked loss of activity. This coincided with the earlier finding which revealed 30°C as the optimum temperature for cyanide−degrading activity.

Table 2: Thermostability of Resting Cells of *Rhodococcus* UKMP−5M

Temperature (°C)	***Percentage of Cyanide Removal**	
	30min	**60min**
4	$51^{a,b} \pm 4.64$	$46^{b} \pm 2.93$
30	$54^{a} \pm 0.96$	$53^{a} \pm 6.25$
40	$32^{c} \pm 2.55$	$11^{d} \pm 4.33$
50	$1^{e} \pm 0.00$	$1^{e} \pm 0.00$

*Biomass 3.5mg dry cell weight resting cells were pre−incubated at 4, 30, 40 and 50°C for 30 and 60min, respectively. The pre−incubated cells were then added to vessel and incubation was carried out with 12mm KCN at 30°C, pH 7 and shaking at 150rpm for 10hrs. Specific activity represent means of triplicate samples ± standard deviations. Data with uncommon superscript was significantly different ($p < 0.05$).

Conclusion

Collectively, the results obtained from this study indicated that the actinomycete *Rhodococcus* UKMP−5M had a promising aptitude in removing higher concentration of free cyanide at an accelerated rate in comparison to growing cells.

The data presented here on the effect of the different experimental conditions revealed new insights in to designing the best approach to maximize cyanide removal efficiency whereby the excellent characteristics of *Rhodococcus* UKMP−5M made it a highly attractive candidate for industrial use. Hence, a practical system could be established by capitalizing on this bacterium for the treatment of cyanide−containing wastewater.

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