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Biodegradation of cyanide by acetonitrile-induced cells of *Rhodococcus* sp. UKMP-5M

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A *Rhodococcus* sp. UKMP-5M isolate was shown to detoxify cyanide successfully, suggesting the presence of an intrinsic property in the bacterium which required no prior cyanide exposure for induction of this property. However, in order to promote growth, *Rhodococcus* sp. UKMP-5M was fully acclimatized to cyanide after 7 successive subcultures in 0.1 mM KCN for 30 days. To further shorten the lag phase and simultaneously increase the tolerance towards higher cyanide concentrations, the bacterium was induced with various nitrile compounds sharing a similar degradatory pathway to cyanide. Acetonitrile emerged as the most favored inducer and the induced cells were able to degrade 0.1 mM KCN almost completely within 18 h. With the addition of subsequent aliquots of 0.1 mM KCN a shorter period for complete removal of cyanide was required, which proved to be advantageous economically. Both resting cells and crude enzyme of *Rhodococcus* sp. UKMP-5M were able to biodegrade cyanide to ammonia and formate without the formation of formamide, implying the identification of a simple hydrolytic cyanide degradation pathway involving the enzyme cyanidase. Further verification with SDS-PAGE revealed that the molecular weight of the active enzyme was estimated to be 38 kDa, which is consistent with previously reported cyanidases. Since the recent advancement in the application of biological methods in treating cyanide-bearing wastewater has been promising, the discovery of this new bacterium will add value by diversifying the existing microbial populations capable of cyanide detoxification.

Key Words—adaptation; ammonia; cyanidase; cyanide biodegradation; formate; inducer; *Rhodococcus* sp. UKMP-5M

Introduction

Despite its toxicity, there is a substantial requirement for cyanide employment in many industrial processes

such as in steel manufacture, gold mining and electroplating which contributes to the presence of anthropogenic sources of cyanide (Dash et al., 2009b). The liberation of cyanide from industries worldwide has been estimated to be more than 14 million kg/year (Ebbs, 2004). Thus, it is necessary to effectively neutralize the cyanide waste products generated through these industrial activities due to risks associated with the use of cyanide, which include accidental spills and injuries.

In order to remediate the waste products generated

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by various cyanide-utilizing industries, development and subsequent implementation of several physical and chemical processes have been established (Baxter and Cummings, 2004). However, employing this method of treatment proved to be disadvantageous even though it could be acceptable in the detoxification of cyanide in contaminated wastes. Chemical processes are burdened with high operating costs and royalty payments, are often ineffective in treating cyanide complexes due to slow reaction rates, produce sludge which requires licensed disposal, generate undesirable by-products and are generally only suitable for treatment of aqueous-based waste forms (Dash et al., 2009b). Thus, a more attractive alternative which can achieve high degradation efficiency at low costs is desired for treatment of cyanide-containing wastewater.

Biological degradation can be regarded as a potentially inexpensive and environmentally friendly approach to detoxify effluents containing cyanide in comparison to conventional cyanide removal technologies (Dash et al., 2009b). A wide range of microorganisms are known to metabolize the highly toxic cyanide and thus have developed metabolic cyanide detoxification pathways which have been utilized in industry in the last 40 years (Dumestre et al., 1997; Hong, 2007). Among other approaches, the use of bacteria emerged as the most accepted and most feasible form of biological treatment of cyanide. Bacterial strains were able to adapt to grow in the presence of cyanide either by inducing enzymes for degradation and detoxification of cyanide or through the formation of cyanide-resistant enzymes (Knowles, 1976).

Since the utilization of bacterial strains has proven to be viable for practical application in detoxifying cyanide-bearing wastewater, the need to screen for more cyanide-degrading microorganisms is great. *Rhodococcus* sp. UKMP-5M, a bacterial strain isolated from petroleum-contaminated soils, demonstrated promising potential in cyanide degradation as reported earlier (Nallapan Maniyam et al., 2011, 2012). The present work aims to further characterize the cyanide-degrading ability of growing cells of *Rhodococcus* sp. UKMP-5M besides investigating the effect of inducer on cyanide degradation by this local isolate. The identification of intermediates and endproducts of cyanide degradation along with kinetic study were also carried out to elucidate the possible metabolic pathway of cyanide degradation by this bacterium.

Materials and Methods

Chemicals. All chemicals and media ingredients used were of analytical grade and purchased either from Sigma (St. Louis, MO), Fisher Scientific (Singapore) or Merck (Darmstadt, Germany).

Microorganism and preparation of inoculum. *Rhodococcus* sp. UKMP-5M, a petroleum-contaminated soil isolate from an oil refinery in Malacca, Malaysia, was kindly supplied by the Culture Collection Unit, Institute of Bio-IT, Selangor, Malaysia. The seed culture was prepared by inoculating a loop of the bacterium (which was routinely maintained on agar nutrient plate) into 50 ml medium consisting of 4.5 g glucose, 0.5 g yeast extract, 0.5 g KH_2PO_4 , 0.5 g K_2HPO_4 , 0.5 g MgSO_4 and 0.01 g CaCl_2 in 1 L deionized water. The flask was kept on an incubator shaker (Jeio Tech SI-600R, Seoul, Korea) at 30°C and agitated at 160 rpm for 24 h. This culture was used as a standard inoculum throughout this study.

Adaptation to cyanide and cyanide biodegradation experiments. A 5 ml seed culture (10%, v/v) with an optical density in the range of 0.8 to 0.9 was transferred aseptically into the production medium containing 4.5 g glucose, 0.5 g yeast extract, 0.5 g KH_2PO_4 , 0.5 g K_2HPO_4 , 0.5 g MgSO_4 and 0.01 g CaCl_2 in 1 L deionized water. Next, the production medium was amended following two different conditions as established below:

Production Medium A: supplemented with 0.1 mM KCN

Production Medium B: supplemented with 0.1 mM NH_4Cl

Cyanide was sterilized separately by filtration using 0.2 μm regenerated cellulose membrane filter (Sartorius Stedim, Goettingen, Germany) and added to the prepared production medium after being cooled to room temperature. The suspension cultures were maintained at 30°C and 160 rpm and further subcultured by transferring 10% (v/v) inoculum into 50 ml of fresh production Medium A at designated intervals. The cultures were adapted to cyanide for more than 4 weeks by successive subculturing into production Medium A. The exact protocol described above was duplicated for production Medium B which was replaced with 0.1 mM NH_4Cl instead of 0.1 mM KCN for comparison purposes.

Effect of inducer on biodegradation of cyanide. The seed culture which was prepared as described

previously was separately supplemented with different inducers, namely acetonitrile, acrylonitrile, benzonitrile, propionitrile and 1,2-dicyanobutane, at a concentration of 0.2 mM. Next, 10% (v/v) of these cultures acclimatized with the respective inducers with an optical density of 0.7 to 0.8 was transferred aseptically (Biosafety Cabinet 4ft-EN12469, Esco Micro (M) Sdn. Bhd., Kuala Lumpur, Malaysia) into production Medium A. The flasks were then maintained on a rotary shaker at 160 rpm and 30°C with pH 6.3 for 48 h. Negative controls without bacterial cells were also included to determine the loss of cyanide due to air stripping. Cell growth and remaining cyanide concentrations were determined after 24 and 48 h respectively. The specific activity of the cyanide degrading enzyme(s) was defined as the amount of cyanide being degraded by 1 mg (dry cell weight) *Rhodococcus* sp. UKMP-5M in 1 h under the conditions described above.

Following this, varying concentrations of KCN (0.1 to 1.0 mM) were added to 50 ml of production medium. The media were then inoculated with acetonitrile-induced cells and later subjected to analysis of growth and remaining cyanide concentrations at selected intervals.

Determination of cyanide degradation pathway. An inoculum concentration of 2% (v/v) was aseptically transferred into sterilized nutrient broth medium (i) without the addition of an inducer and (ii) induced with 0.2 mM acetonitrile. The flasks were then kept on a rotary shaker at 160 rpm and 30°C and the pH was left unadjusted. After the early stationary phase was attained (48 h), the content of the production vessels was spun down (Eppendorf 5702R, Kuala Lumpur, Malaysia) at $419\times g$ for 30 min at 4°C and the supernatants were decanted. The cell pellets were then washed twice with 0.1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7) and resuspended in 10 ml of the same buffer. The optical density of the cell suspensions was adjusted to 1, corresponding to 2 g/L dry cell weight. Cyanide biotransformation was initiated by the addition of KCN to a final concentration of 3 mM in a system containing 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7) and 2 g/L cell suspensions obtained from *Rhodococcus* sp. UKMP-5M grown in nutrient broth without the addition of an inducer. The screwcap bottles were incubated on the orbital shaker at 160 rpm for 24 h at 30°C. Control experiments were established under identical conditions without bacterial cells.

The remaining washed cells of *Rhodococcus* sp.

UKMP-5M in 50 mM phosphate buffer was kept frozen at -80°C before being disrupted for preparation of crude enzyme. In order to determine the crude enzyme activity, the cell suspensions were disrupted by using 4.5 g 425–600 μm unwashed glass beads (Sigma) for every 3 ml of 2 g/L resting cells suspended in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer for 45 min at 275 rpm. The resulting disrupted cells were centrifuged at $13,148\times g$ for 15 min at 4°C. The disappearance of cyanide and the formation of endproducts were measured in sealed vials at 30°C containing 100 μl cell extract (~ 5 mg protein/ml), 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7) and 1 mM KCN for 20 min without the addition of a co-enzyme. Boiled crude extract was substituted for control experiments. Samples collected at selected intervals were evaluated for cyanide, ammonia, formamide and formate concentrations in triplicate for both resting cells and crude enzyme activity.

SDS-PAGE. SDS-PAGE was performed on 12.5% polyacrylamide linear gradient gel based on the method described by Laemmli (1970). Proteins were detected by staining the gel with 0.2% Coomassie brilliant blue R-250 in 50% ethanol/10% acetic acid. Comparison with the mobilities of standard proteins was carried out in order to determine the molecular mass of the enzyme polypeptide.

Analytical methods.

Analysis of residual cyanide: Appropriate amounts of samples were withdrawn after a designated time interval, diluted if necessary and clarified by centrifugation at $13,148\times g$ for 15 min at 4°C to obtain clear supernatant. Cyanide determination was then carried out spectrophotometrically (Biomate 3, Thermo Scientific, Waltham, MA) following a modified barbituric acid-pyridine method against an established standard curve in the range of 0.0 to 0.1 mM KCN (Nagashima, 1977).

Determination of cell density and dry cell weight : Growth of cultures was monitored by measuring the optical density in triplicate at a wavelength of 600 nm by a spectrophotometer. Distilled water was used as a reference. For the determination of dry cell weight, 10 ml of the culture supernatant was added in triplicate to pre-weighed (Sartorius TE214S) 15 ml Falcon tubes and centrifuged for 15 min at $13,148\times g$ and 4°C. After a subsequent washing step with 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, cell pellets were oven-dried (Carbolite, Hope Valley, UK) until constant mass was achieved at 80°C for at least 24 h (Tripathi et al., 2009).

Determination of cyanide reaction products : For-

mate was determined enzymatically using the commercial formate dehydrogenase (Sigma) following the protocol established by Kao et al. (2003). A colorimetric method was employed in order to determine the formation of formamide based on its conversion to ferric hydroxamate (Kao et al., 2003). A modified indophenol blue method was used to determine the concentration of ammonia colorimetrically (Fawcett and Scott, 1960).

Statistical analysis. All experiments were carried out in triplicate. Experimental errors were estimated and depicted by error bars (standard error), typically with three determinations. 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.

Results and Discussion

Adaptation to cyanide

The ability of *Rhodococcus* sp. UKMP-5M to adapt to the highly toxic cyanide was tested in two different production media with production Medium A in the presence of 0.1 mM KCN. The bacterium demonstrated adaptation to cyanide after seven successive subcultures for 30 days in cyanide-containing production Medium A (Fig. 1). Biomass growth in production Medium A was much poorer than that in production Medium B at first and second subculture, most probably due to the toxic effect of cyanide (Suh et al., 1994). The growth of *Rhodococcus* sp. UKMP-5M resumed gradually during the third, fourth and fifth subculture in production Medium A, indicating the development of respiratory resistance and induction of a cyanide-degrading enzyme system that facilitated the cells' detoxification of the cyanide environment (Suh et al.,

Fig. 1. Adaptation to cyanide by successive subcultures in cyanide-containing medium.

Growth of *Rhodococcus* sp. UKMP-5M was monitored in production Medium A supplemented with 0.1 mM KCN (○) and production Medium B supplemented with 0.1 mM NH_4Cl (●) for 30 days. Incubation was at 30°C and 160 rpm. Error bars represent the mean standard error for three determinations.

1994; White et al., 1998).

During the third passage in cyanide, which commenced on day 5 (Fig. 1), it was observed that growth began after a lag period of 48 h (day 7) and an optical density of 0.334 was achieved on day 9 after further incubation at 30°C. Medium containing cyanide was able to support the adaptation of *Rhodococcus* sp. UKMP-5M more actively during the fourth batch, which started on day 9. Similar to the third passage, a lag phase of 48 h (day 11) was noticed during the fourth batch (Fig. 1), after which the growth of the bacterium escalated, reaching an optical density of 0.387 on day 13. Further reduction of lag periods to 36 h were observed during the fifth and sixth subculture in the presence of 0.1 mM KCN, which corresponded to day 14.5 and 20.5 respectively. Interestingly, at the sixth subculture, the optical density of the adapted cells attained an astounding value of 0.704 after 5 days of incubation (day 24), suggesting a substantial elevation in the growth rate of this bacterium in the presence of cyanide in comparison to the previous subcultures as shown in Fig. 1. *Rhodococcus* sp. UKMP-5M was fully adapted to cyanide at the seventh subculture, yielding a final optical density of 0.905 on day 30 compared to that of 1.032 with cells that were not acclimatized with 0.1 mM KCN (Fig. 1). The lag period was also shortened to 24 h (day 25) during the seventh passage, after which the growth recommenced at approximately the same rate as before cyanide addition and the yields were almost equal to those of cyanide-free cultures.

Effect of inducers on cyanide biodegradation

Introducing an inducer at the initial stage of inoculum preparation indeed expedited the growth of *Rhodococcus* sp. UKMP-5M and cyanide biodegradation by this local isolate. The utilization of all tested inducers except potassium cyanide on average managed to shorten the lag phase to less than 12 h and almost complete removal of 0.1 mM KCN occurred after 48 h of incubation, a substantial reduction in the time required to degrade the highly toxic substrate by 4 days in comparison to uninduced, adapted cells. Interestingly, the utilization of potassium cyanide as an inducer to assist and accelerate the cyanide-degrading activity of *Rhodococcus* sp. UKMP-5M was found to be unfavorable (Table 1). This observation can be attributed to the fact that high level of toxicity of cyanide in comparison to nitrile compounds was detrimental to growth of the bacterium, which subsequently halted the secretion of cyanide-degrading enzymes. No cyanide-degrading activity was recorded in the absence of an inducer after 24 and 48 h of reaction (Table 1), indicating that the uninduced cells of *Rhodococcus* sp. UKMP-5M exhibited a longer lag phase to instigate growth and the bacterium therefore required extended time for complete removal of cyanide.

Of the six compounds selected for induction, acetonitrile surfaced as the most favored inducer, exhibiting significantly higher specific activities of $0.012^a \pm 0.001$ and $0.026^a \pm 0.001$ mM/h/mg dry cell weight after 24 and 48 h of incubation (Table 1) respectively ($p < 0.05$). The inducers are ranked from the most favored to the

Table 1. Effect of cells induced with various nitrile compounds on cyanide biodegradation by *Rhodococcus* sp. UKMP-5M. Specific activity represents means of triplicate samples $\pm$ standard deviations.

Types of inducer	Specific activity (mM/h/mg dry cell weight)	
	24 (h)	48 (h)
No inducer	Not detected	Not detected
Acetonitrile	$0.012^a \pm 0.001$	$0.026^a \pm 0.001$
Acrylonitrile	$0.007^b \pm 0.001$	$0.018^b \pm 0.001$
Benzonitrile	$0.006^b \pm 0.001$	$0.017^{b,c} \pm 0.000$
Propionitrile	$0.004^c \pm 0.003$	$0.017^{b,c} \pm 0.000$
1,2-Dicyanobutane	$0.003^c \pm 0.000$	$0.016^c \pm 0.000$
KCN	Not detected	$0.003^d \pm 0.000$

Post-hoc Duncan analysis was carried out to determine the best inducer to enhance cyanide detoxification by *Rhodococcus* sp. UKMP-5M statistically. The inducers are ranked from the most favored to the least favored following the alphabetical orders in superscript format (most favored = ^a; least favored = ^d).

least favored following the alphabetical orders in superscript format (most favored = ^a; least favored = ^d). Considerably lower specific activities of $0.007^b \pm 0.001$, $0.006^b \pm 0.001$, $0.004^c \pm 0.003$, $0.003^c \pm 0.000$ and $0.000^d \pm 0.000$ mm/h/mg dry cell weight were observed for biodegradation of cyanide by acrylonitrile-, benzonitrile-, propionitrile-, 1,2-dicyanobutane- and potassium cyanide-induced cells respectively after 24 h of incubation (Table 1). A similar trend was also noticed with the respective induced cells after 48 h of incubation (Table 1). In all the six cases of induced cells, complete mineralization of cyanide was achieved solely due to the biodegradation process, as evident from increase in growth of the bacterium and negligible abiotic loss of cyanide in the control system established without bacterial cells. Acetonitrile was found to be the best inducer to augment the synthesis of nitrile hydratase/nitrilase in many previous reports (Kaplan et al., 2006; Prasad et al., 2005; Ramakrishna and Desai, 1992). In addition, the presence of yeast extract in the medium may have further stimulated the synthesis of iron-containing enzymes when acetonitrile was used as an inducer (Ramakrishna and Desai, 1992).

Moreover, it was found that *Rhodococcus* sp. UKMP-5M, the bacterium used in the present study, was able to grow well in nitrile-containing medium and the whole cells were competent to hydrolyze nitrile to its endproducts (Nallapan Maniyam et al., 2011). 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 capable of converting KCN as well. Besides, the presence of a close relationship between the enzyme cyanide dihydratase (cyanidase) from *Pseudomonas stutzeri* AK61 with approximately 60% amino acid identity to the nitrilases of *Comamonas testorenoni* and *Bacillus* sp. OxB-1 was observed (Nolan et al., 2003). Site-directed mutagenesis of the *Fusarium lateritium* cyanide hydratase gene further indicated that there was a strong correlation between the loss in the cyanide hydratase activity and nitrilase activity. This observation suggested that the active site of cyanide-degrading enzymes and nitrilase in the protein was the same (Nolan et al., 2003). Thus, the utilization of different nitriles having a similar degradatory pathway to that of cyanide as inducers was advantageous in shortening the lag period and subsequently enhanced the rate of cyanide biodegradation by *Rhodococcus* sp. UKMP-5M.

Cyanide biodegradation by acetonitrile-induced cells of Rhodococcus sp. UKMP-5M

Growth of *Rhodococcus* sp. UKMP-5M in the production medium supplied with 0.45% glucose was monitored at different concentrations of KCN. It was observed that an increase in KCN concentration in the culture medium extended the lag phase of cell growth periods. Interestingly, cyanide decomposition continued to occur during the lag periods at all tested cyanide concentrations even though measurable growths of the bacterium did not take place (Fig. 2A, B). This preliminary phase may be essential to induce various phenomena leading to cyanide degradation such as cyanide-insensitive respiration, a siderophore-based mechanism for iron acquisition in the presence of cyanide and specific enzymes or metabolic intermediates involved in the biodegradation pathway (Huertas et al., 2010).

Figure 2, A and B demonstrate typical cell growth with corresponding cyanide consumption by the locally isolated *Rhodococcus* sp. UKMP-5M strain. *Rhodococcus* sp. UKMP-5M managed to reduce the concentration of cyanide from 0.1 to 0.054 mM with 56% removal efficiency within 6 h of study and the optical density (growth) increased from 0.129 to 0.162. After 18 h, the cyanide removal efficiency escalated remarkably to 96% and the cells of the bacterium increased to 0.761.

Rhodococcus sp. UKMP-5M required 48 h to successfully reduce the concentration of cyanide to 0.02 mM KCN when the medium was supplemented initially with 0.1 mM KCN (Fig. 3). When almost entire consumption of 0.1 mM KCN occurred after 48 h of incubation, addition of a second aliquot of 0.1 mM KCN was made to the culture. Interestingly, complete removal of 0.1 mM KCN was observed within 8 h of incubation (which corresponds to 56 h in Fig. 3), a substantial reduction in the time acquired to fully detoxify cyanide by 83% in comparison to the first cycle, which needed 48 h (Fig. 3). Upon subsequent pulses, the rates of cyanide consumption were distinctly faster than that observed upon initial inoculation, indicating that adaptation to cyanide had occurred. Similar observations were made by Barclay et al. (1998), Kim and Kang (1993) and Kunz et al. (1992). In contrast, resting cells of *Bacillus pumilus* C1 needed 2 h to remove 100 mg/L KCN completely and further addition of 100 mg/L cyanide witnessed continued degradation albeit at a slower rate (Meyers et al., 1991).

Fig. 2. Growth profile of acetonitrile-induced *Rhodococcus* sp. UKMP-5M cells (A) and time course of cyanide consumption by *Rhodococcus* sp. UKMP-5M (B).

The control was established in the absence of inoculum. Cyanide removal was initiated by the addition of various concentrations of KCN (0.1–1 mM) into production medium. Incubation was at 30°C for 624 h at 160 rpm. Error bars represent the mean standard error for three determinations.

Fig. 3. Removal of 0.1 mM KCN during biodegradation of cyanide by acetonitrile-induced *Rhodococcus* sp. UKMP-5M cells.

A lag period was observed during the addition of the initial aliquot of cyanide. Subsequent aliquots of cyanide added after a designated time interval shown as (*) in the graph were rapidly removed without any lag phase. Incubation was carried out at 30°C for 66 h at 160 rpm. Error bars represent the mean standard error for three determinations.

When the production medium was supplemented with 0.25 mM initial cyanide concentration, 98% cyanide degradation was recorded after 9 days of incubation (Fig. 2B). Interestingly, the early stationary phase was achieved at an optical density of 0.879 on day 9 (Fig. 2A) even though cyanide was completely degraded after 96 h of incubation (Fig. 2B). It was found that increasing the cyanide concentration in the growth medium resulted in deteriorated optical density, which indicated that the reduction of cell density occurred with the increase in cyanide concentration (Dash et al., 2009a). Moreover, as the bacterial population continues to grow, depletion of one or more essential nutrients in the growth medium may occur. This will result in the accumulation of waste materials, toxic metabolites and inhibitory compounds which will shift the conditions of the medium such as pH and temperature, thereby creating an unfavorable environment for the bacterial growth and thus, the growth rate will stabi-

lize. Increasing the concentration of cyanide to 0.5 mM witnessed 99% degradation after 11 days; however, only moderate growth with an optical density of 0.600 was recorded (Fig. 2A, B).

It was interesting to observe that the local isolate, *Rhodococcus* sp. UKMP-5M, possessed appreciably higher cyanide-degrading ability in comparison to previously published reports (Das and Santra, 2011; Potivichayanon and Kitleartpornpairat, 2010). In addition, it was fascinating to note that both the microorganisms were isolated from cyanide-acclimatized environment and yet their performance in degrading cyanide was comparable to *Rhodococcus* sp. UKMP-5M, which was isolated from petroleum-contaminated soils. This observation coincided with findings reported by Patil and Paknikar (2000) stating that it was not a prerequisite for any microorganism to be isolated from cyanide-contaminated sources in order to carry out efficient cyanide-degrading activity.

Increasing the concentrations of cyanide witnessed deteriorated growth rates of the bacterium which could be attributed to the toxicity of cyanide at higher concentration (Adjei and Ohta, 2000). A prolonged lag phase was observed as the concentrations of cyanide were increased gradually, which is consistent with this assumption. It took 18 days for the isolate to degrade 0.75 mM completely and an extra 8 days to remove 99% of 1 mM KCN (Fig. 2B). The optical density of the bacterium reached 0.621 after 18 days of incubation upon addition of 0.75 mM KCN and the growth fell to 0.464 after 26 days when the production medium was amended with 1 mM KCN (Fig. 2A). Cyanide removal due to air stripping was also observed in an uninoculated control but at significantly slower rates than in inoculated cultures, revealing that the reduction of cyanide concentration in the medium containing active cells of *Rhodococcus* sp. UKMP-5M was mainly due to the biodegradation process.

Determination of cyanide degradation pathway

In the present study, a new bacterium species identified as *Rhodococcus* sp. UKMP-5M, isolated from petroleum-contaminated soil, demonstrated promising ability in degrading cyanide to non-toxic endproducts, namely ammonia and formate via the hydrolytic reaction catalyzed by cyanidase. To the best of our knowledge, this is the first evidence reporting an elaborate cyanide degradation pathway of a *Rhodococcus* strain, since the possible route of KCN biodegradation by this bacterium was not well defined in any of the literature except for a publication by Hong (2007). Thus, different experiments were attempted in this study to elucidate the metabolic pathway of KCN utilization by *Rhodococcus* sp. UKMP-5M.

Washed cells of *Rhodococcus* sp. UKMP-5M were able to catalyze the rapid time-dependent disappearance of higher concentrations of cyanide following

growth on nutrient broth (Nallapan Maniyam et al., 2011). The bacterium was able to convert 64% of 3 mM KCN after 10 h of incubation, which was followed by stoichiometric accumulation of both ammonia and formate (Table 2). Formamide was not detected in the culture broth during the whole period of KCN degradation.

The identification of formate and ammonia as cyanide degradation products and the absence of formamide throughout experimentation led to the proposition that this bacterium may degrade cyanide via a hydrolysis pathway involving the enzyme cyanidase. Moreover, the quantification of ammonia and formate in the control system were not successful, further supporting the proposed degradation pathway of the present study. Similar observations were made by Kunz et al. (1992), Adjei and Ohta (2000), Watanabe et al. (1998), White et al. (1998) and Ingvorsen et al. (1991) demonstrating the capability of the bacteria *Burkholderia cepacia* strain C-3, *Pseudomonas stutzeri* AK61, *Alcaligenes xylosooxidans* subsp. *denitrificans*, *Pseudomonas fluorescens* NCIMB 11764 and *Pseudomonas* species to convert free cyanide directly to ammonia and formate by cyanidase (cyanide dihydratase) without producing formamide as a transitional product following the equation below:

To further verify the possibility of cyanide degradation through the enzymatic activity of cyanidase, separate incubation of 1 mM KCN as a substrate was performed with cell-free extract of induced and uninduced *Rhodococcus* sp. UKMP-5M. Reactions were allowed to proceed nearly to completion for 20 min after which the concentration of remaining cyanide and formation of possible by-products such as ammonia, formamide and formate were determined. The concentration of cyanide dropped from 1 mM to 0.28 mM, which corresponded to 72% degradation when crude extract ob-

Table 2. Formation of endproducts during cyanide consumption by resting cells and crude enzyme of *Rhodococcus* sp. UKMP-5M. Tabulated results represent means of triplicate samples $\pm$ standard deviations.

Cell condition	KCN supplied (mM)	Incubation time (min)	KCN consumed (mM)	Product recovery		
				Ammonia (mM)	Formate (mM)	Formamide (mM)
Resting cells	3.00	600	1.93 $\pm$ 0.29	1.57 $\pm$ 0.36	1.53 $\pm$ 0.46	Not detected
Crude enzyme ^a	1.00	20	0.72 $\pm$ 0.17	0.57 $\pm$ 0.06	0.53 $\pm$ 0.05	Not detected
Crude enzyme ^b	1.00	20	0.09 $\pm$ 0.02	0.04 $\pm$ 0.00	Not detected	Not detected

Crude enzyme^a: Obtained from acetonitrile-induced cells of *Rhodococcus* sp. UKMP-5M.

Crude enzyme^b: Obtained from uninduced cells of *Rhodococcus* sp. UKMP-5M.

tained from induced cells of the bacterium was used (Table 2). The breakdown of cyanide resulted in instantaneous formation of ammonia and formate with the ratio 1.08 : 1.00. No detection of formamide was recorded, further confirming the role of cyanidase in cyanide degradation by this isolate. Cell-free extracts of *Rhodococcus* sp. UKMP-5M were able to accomplish cyanide detoxification, signifying that the enzyme nitrogenase was not involved in transforming KCN into non-toxic endproducts. The crude enzyme (nitrogenase) of *Klebsiella oxytoca* failed to detoxify cyanide due to its vulnerability towards oxygen exposure as reported by Koa et al. (2003), which was not the case in the present study. Very low cyanide removal efficiency amounting to 9% degradation was observed when crude enzyme obtained from uninduced bacterial cells was employed for cyanide detoxification (Table 2). The formation of formate was not detected and a concentration of 0.04 ± 0.00 mM of ammonia was merely recorded as a result of very low cyanide consumption by these crude extracts (Table 2).

In addition, the crude extract obtained from induced cells of the bacterium demonstrated only one band with high intensity at approximately 38 kDa when examined by SDS-PAGE (Fig. 4). This finding was consistent with previous reports which revealed that the active cyanidase/cyanide dihydratase from *Pseudomonas stutzeri* AK61, *Alcaligenes xylosoxidans* subsp. *denitrificans* DF3 and *Bacillus pumilus* C1 had similar molecular masses of approximately 38 kDa (Ingvorsen et al., 1991; Meyers et al., 1991; Watanabe et al., 1998). Besides, the purified cyanide hydratase enzyme from *Fusarium solani*, *Fusarium lateritium* and *Gloeocercospora sorghi* comprised a polypeptide subunit of approximately 45 kDa (Barclay et al., 1998). No major band appeared in this region when the crude extract from *Rhodococcus* sp. UKMP-5M was subjected to analysis with SDS-PAGE, ruling out the possibility of cyanide hydratase enzyme in detoxifying cyanide in the present study. On top of that, it was interesting to observe a mild band at 38 kDa (Fig. 4) when crude extract derived from uninduced cells of *Rhodococcus* sp. UKMP-5M was subjected to SDS-PAGE analysis. This indicated that the cyanide-degrading cyanidase enzyme was partly constitutive and partly inducible. The addition of an inducer in the present study therefore enhanced the expression of cyanidase, as evidenced by the presence of a more pronounced band at 38 kDa (Fig. 4), which in turn substantially aided

Fig. 4. SDS-PAGE of the cyanide-degrading enzyme from *Rhodococcus* sp. UKMP-5M.

The cell-free extract of 10 μ l derived from the glass bead cell disruption method was loaded onto the polyacrylamide gel. Lane 1: Molecular mass standards in Daltons (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor), Lane 2: crude extract of *Rhodococcus* sp. UKMP-5M induced with 0.2 mM acetonitrile and Lane 3: crude extract of uninduced *Rhodococcus* sp. UKMP-5M.

both the growth and cyanide detoxification by this local isolate in comparison to uninduced cells.

A *Rhodococcus* strain isolated from cyanogenic plants was reported to possess an oxygen-dependent cyanide degradative pathway which resulted in the formation of ammonia and carbon dioxide as endproducts (Hong, 2007). However, there were no records of either formate or formamide generation, which was in contrast to the current findings, suggesting the presence of another possible route for cyanide degradation by *Rhodococcus* strains. The data published so far indicated that the enzyme produced by *Rhodococcus* sp. UKMP-5M was entirely different from that of the *Rhodococcus* strain discovered by Hong (2007). It would therefore be interesting to compare the amino acid sequences and other characteristics of these two enzymes. However, the possibility that formamide might be a membrane-bound intermediate product which was not released during the catalytic cycle has not been ruled out in this case (Adjei and Ohta, 2000).

Conclusion

It is still very necessary to screen for more cyanide-degrading microorganisms owing to the high toxicity of cyanide and its recalcitrant property towards biodegradation. The genus *Rhodococcus* possesses many commercially interesting characteristics that actually or potentially make them useful in environmental and industrial biotechnology. The utilization of the bacterium *Rhodococcus* sp. UKMP-5M in the current study showed promising performance in degrading cyanide and thus would make an excellent addition to the existing library of microorganisms capable of cyanide detoxification. An interesting finding was observed in the present study concerning the expedited rate of cyanide degradation when subsequent aliquots of cyanide were added to the cells fully acclimatized with cyanide, which proved to be advantageous from an economic point of view. In addition, this study was able to provide distinct benefits such as the formation of non-toxic endproducts which can be further metabolized to enhance growth properties of the bacterium, no addition of expensive or harmful chemical substances to aid the biodegradation process, easier operation and maintenance of the system and no production of unnecessary chemical sludges. These properties actually make this process a viable alternative to the available conventional technologies employed for the treatment of industrial effluents containing cyanide on a commercial scale. The simple hydrolytic pathway leading to the breakdown of cyanide proceeded via the activity of cyanidase, which was further confirmed by the absence of the intermediate formamide throughout the biodegradation process. Nevertheless, future studies on the specific activity the cyanide-degrading enzymes are obligatory to further characterize and verify metabolite assimilation by *Rhodococcus* sp. UKMP-5M. Collectively, results from this study provide us insight into the characteristics and mechanisms of KCN conversion by *Rhodococcus* sp. UKMP-5M which will be helpful in designing a practical system for the treatment of cyanide-containing wastewater.

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