A New Indigenous Cyanomethane-Degrading Bacterium Isolated From Gold Mining Waste Water
(Bakteri Indigeneous Pendegradasi Sianometana yang Diisolasi dari Limbah Cair Tambang Emas)

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ABSTRACT

The gold mining wastewater effluent is potential source for isolating cyanides-degrading bacteria, since cyanide is commonly used in gold extraction process in the mining industry. An indigenous bacterial strain LP3, capable of growing on and utilizing of a high concentration of cyanomethane (up to 1.0 Molar), could be isolated from Cikotok gold mine effluent. Based on 16S rDNA sequence, the strain was identified as *Rhodococcus pyridinivorans*. During the growth on cyanomethane (CH_3CN), ethanamide (CH_3ONH_2) and ethanoic acid (CH_3COOH) were detected in the growth media, indicating that nitrile hydratase and amidase involved in the metabolism of the substrate. The involvement of both enzymes on the conversion of cyanomethane was also proved by our study on cyanomethane biodegradation using whole cells of *R. Pyridinivorans* LP3. Besides cyanomethane, the *R. pyridinivorans* LP3 could also utilize various aliphatic, aromatic, heterocyclic nitriles and amides as growth substrates. Base on these results, *R. pyridinivorans* LP3 is expected to be used as a potential candidate for biological treatment for cyanide-containing wastes, although further research is still needed, before being applied on a field scale.

Keywords: biocatalyst, cyanide degrading bacteria, gold mining, *Rhodococcus pyridinivorans* LP3

ABSTRAK

Limbah cair tambang emas merupakan sumber yang potensial untuk mendapatkan isolat bakteri yang mampu mendegradasi senyawa sianida, karena senyawa tersebut sering digunakan untuk proses ekstraksi bijih emas dari matriks pembawanya. Dari air buangan tambang emas Cikotok dapat diisolasi satu isolat bakteri LP3 yang mampu tumbuh dalam siano-metana (CH_3CN) dalam konsentrasi yang tinggi (1 Molar). Berdasarkan analisis sekuen 16S rDNA, isolat bakteri tersebut dapat diidentifikasi sebagai *Rhodococcus pyridinivorans*. Selama pertumbuhannya dalam siano-metana, terdeteksi adanya etana-amide (CH_3ONH_2), dan asam etanoat (CH_3COOH) di dalam media pertumbuhan. Hal ini mengindikasikan bahwa enzim nitril-hidratase dan amidase terlibat dalam metabolisma siano-metana. Keterlibatan kedua enzim tersebut juga diperkuat oleh hasil pengujian biodegradasi siano-metana dengan menggunakan sel utuh *R. pyridinivorans* LP3. Selain pada siano-metana, *R. pyridinivorans* LP3 juga mampu tumbuh pada beberapa senyawa sianida organik alifatik, aromatik, dan heterosiklik, serta beberapa senyawa amida. Ini berarti bahwa *R. pyridinivorans* LP3 mampu memanfaatkan senyawa-senyawa tersebut sebagai sumber karbon, energi, maupun nitrogen untuk pertumbuhannya. Dari hasil penelitian ini, *R. pyridinivorans* LP3 diharapkan dapat dimanfaatkan sebagai kandidat potensial untuk pengolahan limbah yang mengandung sianida, walaupun masih perlu penelitian lanjutan, sebelum diaplikasikan dalam skala lapangan.

Kata Kunci: bakteri pendegradasi sianida, biokatalis, *Rhodococcus pyridinivorans* LP3, tambang emas

INTRODUCTION

Organic cyanide compounds are widely manufactured by several industries, such as cosmetics, herbicides, pesticides, pharmaceuticals, drug intermediates and food additives (Agarwal *et al*., 2012; Banerjee *et al*. 2002). Cyanomethane, one of the simple aliphatic cyanide compounds, is commonly used in industries as organic solvent for manufacturing of pharmaceuticals, photografic film, extractants, *etc*. It is also produced mainly as byproduct of vinyl cyanide manufacture. However, most of organic cyanide compounds are known highly toxic, carcinogenic and mutagenic in nature and possibly cause severe health hazards.

The extensive industrial use of the cyanide

compounds might lead to an environmental problem and thus wastewater containing cyanides must be treated before discharging into the environment. There are several conventional methods used in treating effluents containing cyanide before discharging it into the environment. The common ones are alkaline chlorination, sulfur oxide/air process and hydrogen peroxide process (Akcil & Mudder 2003; Latkowska & Figa 2007). However, these methods are expensive and hazardous chemicals are used as the reagents (chlorine and sodium hypochlorite) and some of them create additional toxic and biological persistent chemicals. Despite cyanide's toxicity to living organisms, biological treatments are feasible alternatives to chemical methods without creating or adding new toxic and biologically persistent chemicals (Akcil 2003, Dash *et al*. 2009; Gurbuz *et al*. 2004) and thus considered as more efficient, eco-friendly and cost effective.

Many authors have reported degradation of organic cyanide compounds by bacteria. The microorganisms used include *Nocardia rhodochrous* LL100-21 (DeGeronimo & Antoine 1976), *Arthobacter* sp. I-9 (Yamada *et al*. 1979), *Pseudomonas* (Shankar *et al*. 1990), *Klebsiella pneumoniae* (Nawaz *et al*. 1991) and *Rhodococcus* sp. (Nawaz *et al*. 1994). We have also reported that some indegenous bacteria were capable to degrade organic cyanide compounds; *Corynebacterium* sp. D5 (Sunarko *et al*. 2000), *Rhodococcus pyridinivorans* strain TPIK *(*Sulistinah *et al.* 2015), *Rhodococcus pyridinivorans* GLB5 (Sulistinah *et al*. 2016), and also *Micrococcus endophyticus* (Riffiani & Sulistinah 2017).

According to Kobayashi *et al*. (1990), microbial degradation of organic cyanides proceeds by two distinct routes: (a) nitrile hydratase hydrolyzes organo cyanides into amides, which are subsequently hydrolyzed to acids plus ammonia by an amidase, or (b) nitralase transforms organo cyanides directly into acids plus ammonia.

Recently, indiginous baterial isolate LP3 has been isolated from cyanide-contaminated wastewater of Cikotok gold mining plant. LP3 was the one of six newly bacterial isolates which showed the highest growth and enzyme activity to the nitriles (Sulistinah & Sunarko 2010) The purposes of this study were to identify the new isolated bacterial LP3 and to explore its ability in degrading cyanomethane in the hope it could potentially be used for the biological treatment of cyanide contaminated wastewater in the future.

MATERIALS AND METHODS

Cyanomethane, ethanamide were obtained from Merck, Germany. Phenylcyanide, hexane-dinitrile, acetaldehyde cyanohydrin, 1,5-dimethyl-2-pyrole-carbonitrile, phenylacetonitrile, benzaldehyde cyanohidrin, phenylcarboxamide, niacinamide, propionamide, hexanediamide were procured from Sigma Aldrich, Steinheim, Germany. Media ingredients were purchased from Difco. All other chemicals used in this study were of analytical grade.

Isolate LP3 was isolated from gold mine wastewater of PT. Antam, Cikotok by cyanomethane enrichment culture technique (Sunarko *et al.*, 2000). Circa 10 ml of waste sample was added to 50 ml mineral medium (MM) supplemented with 200 mM 0.5% (v/v) cyanomethane. Further enrichment was carried out by transferring 5% (v/v) inoculum to fresh mineral medium during which cyanomethane concentration was gradually increased to 1.5 % (v/v). The purity of the culture was checked periodically by plating on agar plates.

Bacteria were identified using phylogenetic analyses. Genomic DNA was extracted from cells of LP3 by using the DNA Extraction Kit (Biorad)/the guanidium thiocyanate/EDTA/Sarkosyl (GES) method as described by Pitcher *et al*. (1989), following the manufacturer's instructions. 16S rRNA genes were amplified using general bacterial primers 20F (5'-GATTTTGATCCTGGCT CAG-3') and 520 R (5'-ACCGCGGCTGCTGGC-3'). The PCR products were purified from low-melting-point agarose using the Wizard PCR Prep kit (Promega), according to the manufacturer's instructions. DNA nucleotides were sequenced by First BASE (Malaysia) using the same primer pairs used in the PCR reaction. The sequences were first compared with those stored in NCBI GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm and aligned using Clustalw. Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis) v5.1 software (Tamura *et al*. 2011).

The composition of a minimal mineral salt medium (MM) used in this study was of the following (in $g.l^{-1}$): $Na_2HPO_4.2H_2O$, 0.4475 g; KH_2PO_4, 0.1 g; $MgSO_4.7H_2O$, 0.1 g; $CaCl_2.2H_2O$, 0.01g; $FeSO_4.7H_2O$, 0.001g; Yeast extract, 0.01 g; Microelements1,0 ml, and added distilled water to 1000 ml (Sulistinah *et al.* 2015). The microelements composed of $ZnSO_4.7H_2O$, 0.1g; $MnCl_2.4H_2O$, 0.03 g; H_3BO_3, 0.3 g; $CoCl.6H_2O$, 0.2 g; $CuCl_2.2H_2O$, 0.01 g; $NiCl_2.2H_2O$, 0.02 g; $Na_2MO_4.2H_2O$, 0.9 g; Na_2SeO_3, 0.02 g within 1000 ml distilled water (Pfennig, 1974). The pH of the medium was adjusted to 7.2 using 1 N NaOH. Cyanomethane or other organo cyanide compounds was added to the above medium as a sole source of carbon, nitrogen and energy. The agar plates were prepared by adding 2% (w/v) agar to the MM medium, autoclaved and cyanomethane was added to this medium after cooling the medium to 45°C.

Cells of bacterial isolate LP3 (3 % w/v) was inoculated into Erlenmeyer flasks (500 ml) contained 250 ml minimal medium supplemented with cyanomethane at the concentration between 0.2 M – 2.0 M. The bacterial culture then incubated on orbitary shaker (120 rpm) at room temperature (± 28 °C) for 72 hours. Large scale cultivation for cell biomass was performed in 5 L fermentor (Braun, Melsungen, Germany) contained 2 L mineral medium suplemented with 200 mM cyanomethane. The growth of the LP3 isolate was monitored by spectrophotometer at 436 nm. Cells were harvested by centrifugation and washed twice with 50 mM phosphate buffer (KH_2PO_4, pH 7,2).

Approximately 2% (b/v) cell of the *R. pyridinivorans* strain LP3 and 500 mM cyanomethane were added to Erlenmeyer containing 50 mM phosphate buffer (KH_2PO_4-NaOH), pH 7.2. The reaction solution was then incubated on an orbital shaker at a temperature of ± 28 °C and at a certain time interval the sample was taken, the enzyme activity was stopped by adding 0.20 ml of 4N HCL and centrifuged to determine the concentration of cyanomethane degradation product (carboxylic acid, amide and ammonium). The concentration of carboxylic acid and amide in the filtrate were determined by gas chromatography (GC) and ammonium by the Nessler method.

The concentration of cyanomethane, acidamide, and acetic acid were measured by Gas Chromatography (GC) (Shimadzu 14B, Japan) using FID detector and Porapak Q column (Sigma Aldrich). Nitrogen gas was supplied as a carrier gas and the flow rate was 1.0 ml min^{-1}. Operational condition was as follows: column temperature 225°C, injector 240°C, detector 240°C and injection volumes 1,0 µL. The substrate and products were identified by their retention time and quantified by comparing peak areas with external standards. The concentration of ammonia in the medium was measured by Nessler method (Gerhardt *et al* 1994). The activity of intracellular cyanide degrading enzyme of the cells of *R. pyridinivorans* LP3 was calculated as the amount of degraded substrate (mmol) per milligram (dry weight) of cell biomass per minute.

The growth of bacterial isolate LP3 in various organic nitriles and amides were carried out qualitatively using a sterile macrotitter plate (24 holes) which containing 1000 µl of minimal media supplemented with a certain concentration of nitrile or amide depend on the toxicities of these compounds (Sunarko *et al.* 2000). After that, the macrotiter plate was incubated on the orbitary shaker at room temperature for 72 hours. The isolate growth was determined by the addition of an INT (Iodonitrotetrazolium) solution (Oliver *et al.* 1989). The change in color to pink indicates microbial growth. The activity of isolate was determined based on ammonia release using a Nessler reagent. High activity is characterized by the formation of yellow to brownish yellow colors.

RESULTS

Identification of Bacterial Isolate LP3

Bacterial Isolate LP3, which was capable of utilizing cyanomethane as the sole source of carbon, nitrogen and energy was isolated from the waste water of mining industry using enrichment culture techniques developed by Sunarko (2012) . The isolate was a gram positive bacteria, non-motile, non-spore former and a small rod or cocciod shaped cells. Colonies on the solid agar medium were generally white opaque, circular in nature with smooth ridges and gradually become orange. Physical appearance of isolate LP3 and their

colonies were shown in Figure 1.

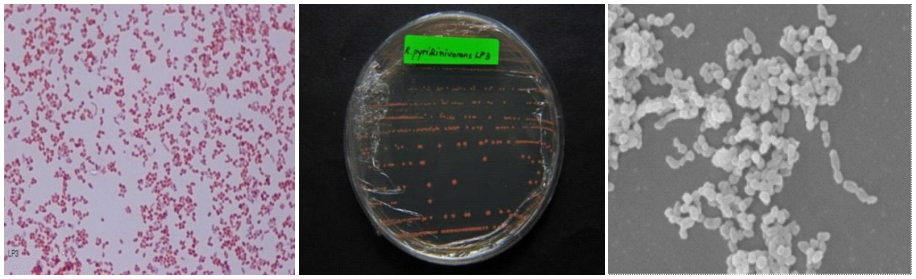
Based on 1400 bp of 16S rDNA, the isolate LP3 has 100% homology with the type strain *Rhodococcus pyridinivorans*. Thus confirming its identity as *Rhodococcus pyridinivorans* and there after designated the bacterial isolate LP3 as *Rhodococcus pyridinivorans* LP3. The phylogenetic relationship of the strain was shown in Figure. 2.

Growth of *Rhodococcus pyridinivorans* LP3 on various concentrations of cyanomethane

Growth of *Rhodococcus pyridinivorans* LP3 was investigated at different cyanomethane concentrations (Figure 3). *R. pyridinivorans* LP3 was able to grow and utilize cyanomethane as a sole source of carbon and nitrogen. The highest growth was observed at 200 mM cyanomethane. Above these concentration, the growth of *R. pyridinivorans* LP3 decreased gradually and beyond 1.5M cyanomethane, the growth was completely inhibited.

Biodegradation of cyanomethane by *Rhodococcus pyridinivorans* LP3

Based on the ability of *R. pyridinivorans* LP3 to grow on cyanomethane, further studies on the degradation of cyanomethane were conducted. As shown in Figure. 4, the cells of *R. pyridinivorans* LP3 was capable to degrade cyanomethane. During the reaction, the concentration of cyanomethane was continously decreased



A **B** **C**

Figure 1. Images of *Rhodococcus pyridinivorans* LP3. (A) cells microscopic staining; (B) on nutrient agar growing colonies, (C). cells scanning electron micrograph (SEM)

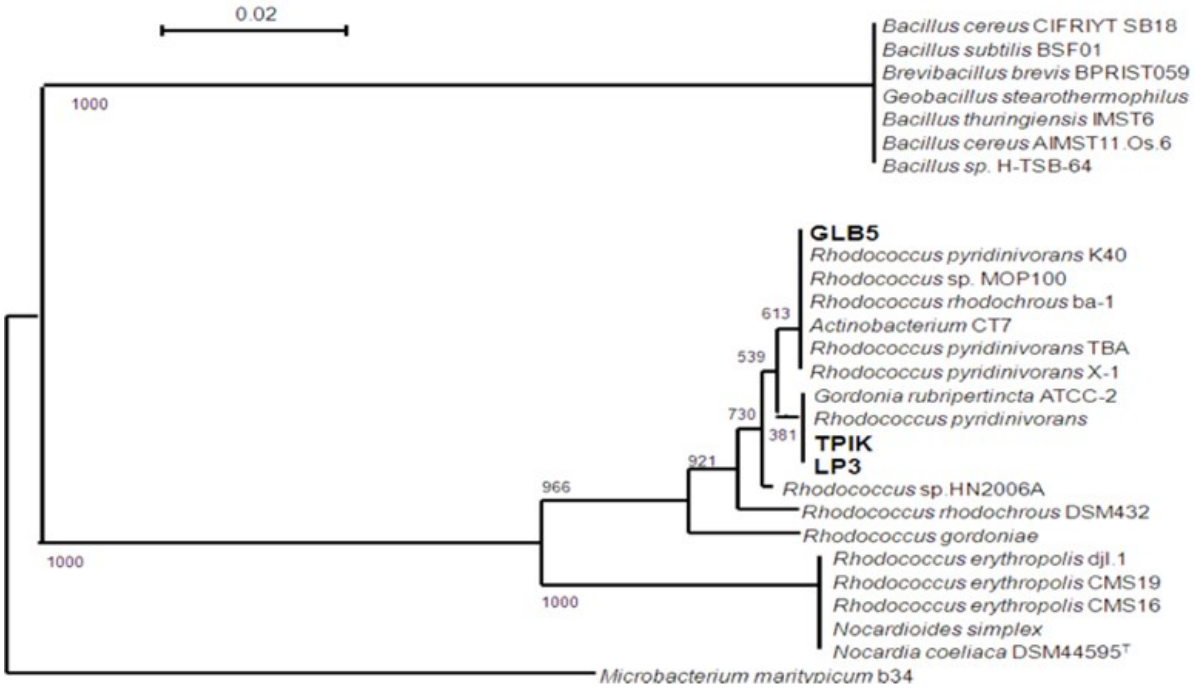


Figure 2. The phylogenetic tree from the Maximum Likehood analyses of 16S rRNA sequence of Bacterial Isolate TP3

followed by the increasing of of acetamide, acetic acid, and ammonia concentrations. The formation of these metabolites was an indication that the degradation of cyanomethane by *Rhodococcus pyridinivorans* LP3 was through a bi-enzymatic nitrile hydratase/amidase pathway.

The involvement of nitrile hydratase/amidase pathway in the biodegradation of cyanomethane by *R. pyridinivorans* LP3 was also supported by the hydrolitic activities of the whole cells of the strain on cyanomethane and on corresponding acidamide. As shown in Figure. 5, the cyanomethane grown cells of *R. pyridinivorans* LP3 were able to degrade both cyanomethane and acetamide. The degradation products of cyanomethane were acetamide and acetic acid, whereas the acetamide degradation products was acetic acid. Formation of these degradation products was also a clear indication that cyanomethane degradation in *Rhodococcus pyridinivorans* LP3 follows nitrile hydratase and amidase pathway.

Effect of various nitriles and amides on the growth and the enzyme activities of the cells of *R. pyridinivorans* LP3

Table 1 shows that *Rhodococcus pyridinivorans* LP3 was able to utilize almost all tested cyanide/nitrile when supplied them as a sole carbon and nitrogen source. Among the tested nitrile compounds, cyanomethane and phenylcyanide served as the best carbon and nitrogen source. However, phenylacetonitrile and mandelonitrile (benzaldehyde cyanohydrin) did not support the growth of *Rhodococcus pyridinivorans* LP3. Table 1 also shows that *R. pyridinivorans* LP3 could utilize all tested amide compounds as a sole carbon and nitrogen source for its growth.

Effect of growth substrates on the cyanomethane degrading activities of *R. pyridinivorans* LP3

In an attempt to test the inductivability of the cyanomethane degrading activities of *R. pyridinivorans* LP3, we have grown the strain

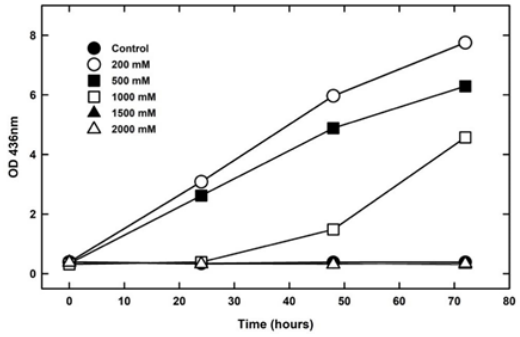


Figure 3. The effect of cyanomethane concentrations on the growth of *R. pyridinivorans* LP3 during 80 hours incubation at 28°C. Experiment were conducted in replicates.

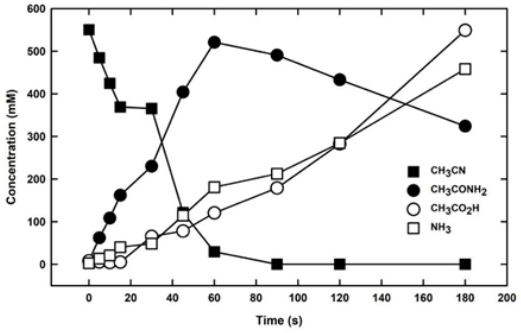


Figure 4. Biodegradation of cyanomethane by whole cells of *R. pyridinivorans* LP3

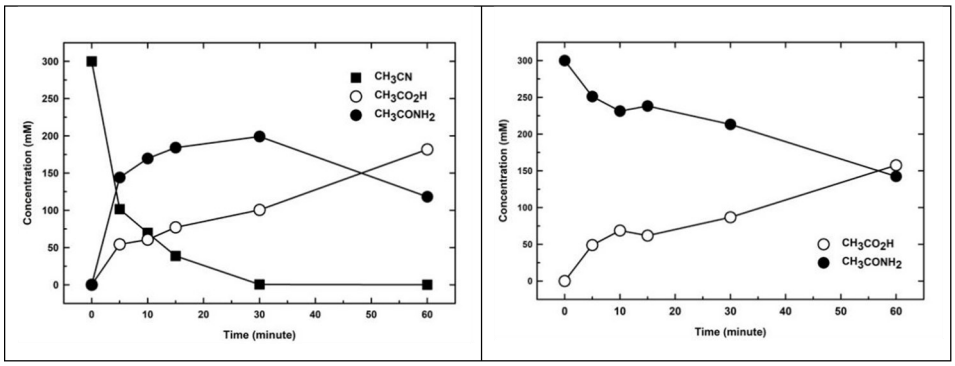
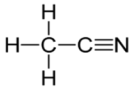
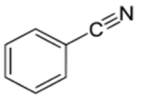
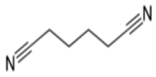
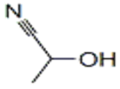
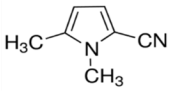
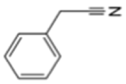
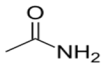
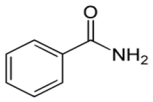
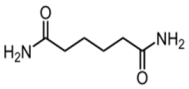
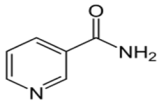
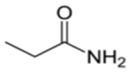
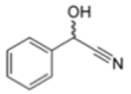


Figure 5. Degradation of cyanomethane (A) and acetamide (B) by whole cells of *R. pyridinivorans* LP3

on cyanomethane with addition of other carbon and nitrogen. As presented in Table 2, the highest cyanomethane degrading activity was shown by cells of *R. pyridinivorans* LP3, which was grown on cyanomethane as the sole of carbon and nitrogen. The addition of other carbon and/or nitrogen sources to the growth medium significantly reduced the degrading activities of the cells of *R. pyridinivorans* LP3.

Table 1. Qualitative growth of *R. pyridinivorans* strain LP3 and its degrading activities on various nitriles and amides

Substrates	Structure	Concentrations (mM)	Growth	Activity
Control *)			-	-
Cyanomethane		200	++++	++++
Phenylcyanide		25	++++	+++
Hexanedinitrile		10	+++	++
Acetaldehide cyanohydrin		10	+++	++
1,5-Dimethyl-2-pyrole-carbonitrile		10	++	+
Phenylacetonitrile		10	-	-
Ethanamide		200	++++	+++++
Phenylcarboxamide		25	++++	+++
Hexanediamide		10	++++	+++
Niacinamide		10	+++	++
Propionamide		100	++++	+++
Benzaldehyde Cyanohydrin		10	-	-

Notes : ++++ (grow well) ; +++ (good) ; ++ (fair) ; + (less) ; - (no grow), *) mineral medium without carbon, energy and nitrogen sources

DISCUSSION

Cyanomethane is widely used for the manufacture of pharmaceuticals, pesticides and synthetic rubbers, and as a solvent in laboratory research (Banerjee *et al.* 2002). As a consequence, cyanomethane is found as a pollutant in industrial wastewater streams associated with its formulation, distribution, and application. Due to its toxic effects on human health, slow rate of chemical degradation and formation of toxic intermediates, much attention has been focused on bacterial degradation of cyanomethane as a method of treating these effluents (Santoshkumar *et al*. 2011).

In this study, a bacterial strain with a high potential for cyanomethane is reported. Compare to bacterial Isolate GLB5 & TPIK (Sulistinah *et al*. 2016), Isolate TP3 could degrade higher cocentration of cyanometane. The partial 16S rDNA gene sequence shows that this strain belongs to the *Rhodococcus* genus and highest identity with *Rhodococcus pyridinivorans* strain R-24650 with 100 % similarity. *Rhodococcus* represents a genus of considerable industrial interest. The biotechnological importance of Rhodococci derives from their ability to catabolize a wide range of compounds. Their assimilatory abilities have been attributed to their diversity of enzymatic activities. Some species belong to this genus have been isolated from polluted soil, water, and sewage purification units (Kim *et al*. 2018). Many Rhodococci have been used commercially for the biodegradation of environmental pollutants and for the biocatalytic production of high-value chemicals from low-value materials (Kim *et al*. 2018). In addition to their industrial importance, *Rhodococcus* is also an experimentally advantageous system owing to a relatively fast growth rate and simple developmental cycle (McLeod *et al*. 2006).

In the present investigation, it was observed that *Rhodococcus pyridinivorans* TPIK utilizes cyanomethane (CH_3CN) as a sole source of carbon, nitrogen and energy. During the growth, ethanamide (CH_3ONH_2) and ethanoic acid (CH_3COOH) were detected in the growth media, indicating that nitrile hydratase and amidase involved in the metabolism of the substrate (Figure 6). The involvement of both enzymes on the conversion of cyanomethane was also proved by our study on cyanomethane biodegradation using whole cells of *R. Pyridinivorans* LP3.

In general, microbial degradation of organo cyanide compounds occurs through hydrolysis either by nitralase, which converts organo cyanides to their corresponding carboxylic acids and ammonia, or by nitrile hydratase that catalyzes the formation of amide, which are subsequently

Table 2. Effect of carbon and nitrogen source on cyanomethane degrading activities of *R. pyridinivorans* LP3

Substrates	Spec. Activity	Relative Activity (%)
Cyanomethane	2.57	100
Cyanomethane + Glucose	1.58	61.48
Cyanomethane + NH_4Cl	0.93	36.19
Cyanomethane + Glucose + NH_4Cl	0.90	32.02

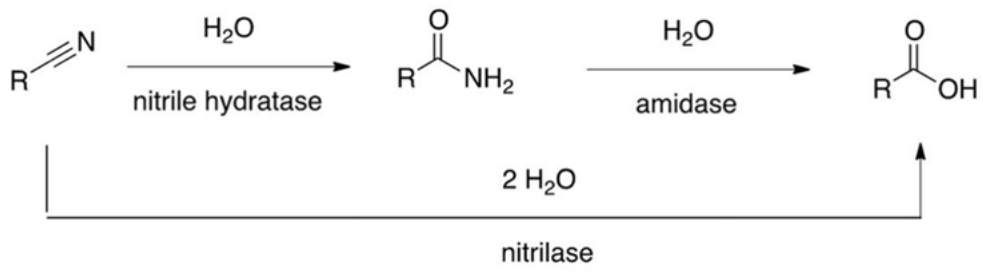


Figure 5. Enzymatic pathway of nitrile hydrolysis (Martinkova *et al*. 2008)

converted by amidase to their corresponding acids and ammonia (Banerjee *et al*. 2002). Many authors have reported degradation of organic cyanide compounds by bacteria, such as *Nocardia rhodochrous* LL100-21 (DeGeronimo & Antoine, 1976), *Arthobacter* sp. I-9 (Yamada *et al*. 1979), *Pseudomonas* (Shankar *et al*. 1990), *Klebsiella pneumoniae* (Nawaz *et al*. 1991) and *Rhodococcus* sp. (Nawaz *et al*. 1994).

CONCLUSION

Rhodococcus pyridinivorans LP3, isolated from Cikotok gold mine effluent, was capable to grow on and to utilize of high concentration of cyanomethane (CH_3CN). During the growth, ethanamide (CH_3ONH_2) and ethanoic acid (CH_3COOH) were formed. Both compounds were also released as products of cyanomethane degradation using whole cells of *R. Pyridinivorans* LP3, which indicated that nitrile hydratase and amidase were involved in the metabolism of cyanomethane. *R. pyridinivorans* LP3 could also utilize various aliphatic, aromatic, heterocyclic nitriles and amides as growth substrates. Although further research is still needed, *R. pyridinivorans* LP3 could be expected as a potential candidate for biological treatment for cyanide-containing wastes.

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