Nitrile Hydrolysing Enzymes-Producing Bacterium Isolated from Gandang Dewata Mountain, West Sulawesi

N Sulistinah¹ and R Riffiani¹

¹Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI)
Jl. Raya Jakarta-Bogor Km 46 Cibinong, Indonesia

E-mail: nuniksulistinah@gmail.com

Abstract. Nitrile compounds are widely used and manufactured by the chemical industry for synthesis of important compounds. They are known to be highly toxic because of the CN group on its molecular structure and most of them are hydrophobic, mutagenic and carcinogenic. Nevertheless, some microbes were reportedly able to hydrolyse nitrile by involving nitrile-hydrolysing enzymes, such as nitrilase, nitrile hydratase and amidase. Nowadays, the application of these enzymes is increasingly recognized because it may be used as a biocatalyst to produce some of important pharmaceutical compounds and/or synthetic chemical that has high economic values. These enzymes also have a significant role in environmental protection due to their capability to eliminate toxic compounds. The probability to acquire potential microbes-degrading nitriles in nature and extreme environments is extremely high. Some bacteria have been isolated from Gandang Dewata mountain areas. SB1D1 bacterial isolate was identified as Rhodococcus pyridinivorans. It showed potential activity as bacteria-hydrolysing nitrile. The bacteria was able to grow on CH₃CN (acetonitrile) until the concentration of 1000 mM and also it was demonstrated to degrade 50 mM C₆H₅-CN (benzonitrile) and produce benzamide, benzoic acid even in low concentrations. SB1D1 isolate is a gram-positive bacterium and the colonies are light orange in colour. Based on the literature review, it is known that R. pyridinivorans is widely studied detail and usually used for bioremediation agent to degrade toxic compounds, such as pyridine, PAH etc. Therefore, R. pyridinivorans strain SB1D1 is a good candidate as nitrile hydrolysing-bacteria for degrading aliphatic and aromatic nitrile.

1. Introduction
Nitriles are organic compound that contain CN with the general formula RCN. They are found everywhere in nature, produced and widely used in the manufactures for synthesizing important intermediates in the chemical industry, such as amide, carboxylic acid, amines, ester etc.[1]. Most of synthetic nitriles have been extensively used in the manufacture of extractant, solvent, pharmaceutical, drug intermediate, and pesticides [2]. The excessive use of these compounds triggers environmental problems in the future. Although nitriles are toxic in nature, but many microbes are able to hydrolysenitriles to less-toxic and valuable carboxylic acid using nitrile-hydrolysing enzymes (nitrile hydratase, amidase and/or nitrilase). These enzymes have an important role in the microbial nitriles degradation. Even based on their capability to eliminate toxic compounds, these enzymes also have a
significant role in environmental protection [3]. Nitrilase have been reported to be found in various microbes such as *Alcaligenes*, *Bacillus*, *Bulkmorderia*, *Pseudomonas*, and *Rhodococcus*.

The enzymes synthesized by potential microbes are similar to nitrile-hydrolysing enzymes that have been utilized as biocatalysts for the production of important chemical compounds as well as bioremediation agents for the polluted environment [4].

Recently, it has been reported that many industries are interested in the utilization of nitrile-hydrolysing enzymes isolated from potential microbes as biocatalysts for producing of medicinal or chemical compounds with high economic value. There has been many reports concerning the exploitation of some microbes degrading nitriles and amides to commercially synthesize important compounds, such as for the production of acrylamide, nicotinic acid, benzoic acid, R-(-)-mandelic acid, and S- (+)-Ibuprofen [5]. For example, acrylamide has been commercially produced by Mitsuhi-Rayon Chemical Co., and the application of the utilization of nitrile compounds into drug substances can be found in the process of biotransformation of mandelonitrile racemic compounds into R-(-)-mandelic acid and S-(-)-mandelic acid by involving the nitrile enzyme [5]. Its hydrolysis products (amides and carboxylic acid derivatives) have also been widely applied in pharmaceutical, chemical, cosmetic, herbicide, perfume, food [4].

In general the reaction of nitrile hydrolysis can be illustrated in the following:

![Figure 1. Nitrile-degrading enzymes system (Agarwal, 2012)](image)

**Figure 1.** Nitrile-degrading enzymes system (Agarwal, 2012)

Gandang Dewata Mountain, Mamasa Regency is the second highest mountain at Sulawesi. The location is reported to possess mega diversity level and very high uniqueness. With extreme highland conditions (> 1600 m above sea level), it has the potential microbiota for further development (i.e. bioprospecting), associated microorganisms, especially nitrile-hydrolysing bacteria which has the ability as a nitrile producing hydrolysing enzymes as biocatalysts for food production and raw materials of drugs.

The purpose of this study here were to isolate and screen a potential indigenous microbes as a nitrile-hydrolysing microorganisms, especially benzonitrile. It was expected to obtain potential indigenous bacteria from Gandang Dewata mountain ecosystem which has the high ability and activity to degrade/convert both aliphatic and aromatic nitriles. Thus, it can be used as a base for further research and provide information and contribution on microbial data of nitrile-hydrolysing bacteria in the region.

2. **Materials and Methods**

2.1. **Collection samples**

Soil samples were collected in forest, plantation, surrounding Gandang Dewata area with altitude about 1500-1600 m above sea level and soil pH 6.0-6.5.

2.2. **Bacterial growth on nitriles**

Nitriles were used as a sole source of energy, carbon, and nitrogen. The culture was inoculated in minimal medium with certain concentration of nitrile with the following composition: Na₂HPO₄ (0.357 g/L), KH₂PO₄ (0.1g/L), MgSO₄·7H₂O(0.1 g/L), CaCl₂·2H₂O (0.01 g/L), FeSO₄·7H₂O (0.001
2.7. Assay for nitrile-hydrolysing activities
Nitrile-hydrolysing activity was assayed by determining the ammonia released from the nitrile reaction mixtures that contained the cells of bacteria based on Nessler’s reagents and measuring the decrease of benzonitrile (substrate) and the increase of hydrolysis product concentration. The substrate used was 50 mM benzonitrile (dissolved in 10 ml phosphate buffer pH 7.2). About 1.0 grams cell (w/v) was added to the substrate and incubated in rotary shaker at room temperature for 60 min. Sampling of 1.0 ml was done at minutes 0, 30 and 60. Activity of the enzyme was stopped by adding with 250 µl 4N HCL. Then, the samples were centrifuged for 10 min, the supernatant obtained was injected into the chromatography column. The ammonium concentration was measured using spectrophotometer at 420 nm wavelength.

2.8 Analysis of benzonitrile hydrolysis product
Hydrolysis product of benzonitrile were measured by using HPLC Agilent 1100 with column C18 (Supelco, 5 µm: 15 cm x 4.6 mm) and the mobile phase used Ortho phosphoric acid (0.2%): acetonitrile (25:75), flow rate 1mL/minute; λ 254 nm, column temperature was 25°C.

3. Results and Discussion
3.1 Selection, identification and morphological characteristics of a nitrile-hydrolysing bacteria.
By using enrichment nitrile medium culture technique, sixty eight isolates were obtained from soil isolated at Gandang Dewata. The screening of 68 isolate showed that 17 isolates demonstrated positive reaction. It was characterized by discoloration after the addition of INT (Iodonitrotetrazolium) and the activity was tested based on the formation of ammonium as a release product of nitrile hydrolysis (Fig 3). Identification of 17 isolates was shown in Table 1 as followed:

| No | Isolates Code | Identification nitrile-hydrolysing bacteria based on 16S RNA | Homology (%) |
|----|---------------|-------------------------------------------------------------|--------------|
| 1  | SB3-E6        | Luteipulveratus mongoliensis strain MN07-A0370 genome       | 99           |
| 2  | SB1-B2        | Bacillus mycoides strain ATCC 6462, complete genome         | 99           |
| 3  | SB2-D1        | Roseovariustolerans strain EL-16                             | 99           |
| 4  | SB1-C2        | Bacillus cytotoxicus NVH 391-98, complete genome             | 100          |
| 5  | SB1-D1        | Rhodococcus pyridinivorans SB3094                            | 100          |
| 6  | SB-E2         | Cupriavidus basilensis strain 4G11                            | 99           |
| 7  | SB-MC         | Bacillus pumilus strain NJ-M2                                | 99           |
| 8  | SB2-E4        | Chryseobacterium vrystaentense strain LMG 22846 c             | 100          |
| 9  | SB5-D1        | Paracoccus versutus strain DSM 582                            | 100          |
| 10 | SB2-A4        | Micrococcus luteus NCTC 2665                                 | 99           |
| 11 | SB2-E3B       | Cupriavidus basilensis strain 4G11                            | 99           |
| 12 | SB2-A8        | Aeromicrobium marinum DSM                                     | 98           |
| 13 | SB2-D2        | Arthrobacterarilaitensis RE117 c                              | 97           |
| 14 | SB3-A1        | Bacillus horikoshii strain FJAT                               | 100          |
| 15 | SB8-E9        | Pseudomonas protegens                                        | 99           |
| 16 | SB3-A6        | Arthrobacter phenanthrenivorans                              | 99           |
| 17 | SB2-D2.1      | Cecembialonarensis LW9                                       | 94           |

Compared to other isolates, SB1D1 was capable of growing well and had the highest activity. The strain grew on several nitriles both aliphatic and aromatic nitrile compound (Table 2). The growth of
the isolate on acetonitrile and benzonitrile was much better than the growth on lactonitrile or isobutyronitrile (data not shown).

The strain SB1D1 was identified as *Rhodococcus pyridinivorans* and was capable of growing on and utilizing acetonitrile and benzonitrile as the sole source of energy, carbon and nitrogen. The morphological characteristics of the strain are gram positive, non-spore forming, non-motile and cocci. The colonies colour appeared light in the early stage on the nutrient agar medium and then became orange on the late development. The strain grew well at room temperature (30 °C).

**Figure 2.** Rapid screening based on the determination of growth using INT and activity based on NH$_4$ formation

GandangDewata is conservation area reported to have mega diversity and uniqueness, therefore the opportunity to obtain potential microbes as a toxic compound degrader in this area was high. The strain SB1D1 found in this area was expected to have good ability to grow on nitrile. It has been reported that *R. pyridinivorans* was often found in soil, especially in contaminated soil. *R. pyridinivorans* strain TPIK that was isolated from tailing pond of Antam gold mine industry was also reported to have great ability to grow on acetonitrile up to 1000 mM. The isolate was capable of reducing acetonitrile as much as 90% and produced high amount of metabolite product [8]. Members of the genus *Rhodococcus* were often found in many types of environment, especially in extremely contaminated soil. Therefore, a large number of them are known well as toxic degrader strains [9]. It has been reported that *Rhodococci* have great significance in the pharmaceutical industry, environmental and waste management.

**Figure 3.** Bacteria isolates obtained from screening
3.2 Growth of *R. pyridinivorans* strain SB1D1 on nitriles

The strain growth on various nitriles was showed in Table 2. The table showed that *R. pyridinivorans* strain SB1D1 was capable of growing well and had the highest activity on acetonitrile and benzonitrile than others nitriles.

**Table 2. Growth and activity test of *R. pyridinivorans* strain SB1D1 on various nitrile**

| Substrates          | Growth | Enzyme activity |
|---------------------|--------|-----------------|
| C₂H₃N (acetonitrile)-200 mM | +++    | +++             |
| C₃H₃N (akrilonitrile)-10 mM    | -      | -               |
| C₅H₅NO (lactonitrile)-25 mM   | ++     | +               |
| C₇H₅N (isobutyronitrile)-10 mM | +      | +               |
| C₇H₅N (benzonitrile)-15 mM    | ++++   | ++              |
| C₈H₇NO (mandelonitrile)-10 mM | -      | -               |

Note: ++++/++++ = grow well ; +++ = good; ++ = fair; + = less; - = not growing

![Figure 4](image)

Figure 4. Growth of *Rhodococcus pyridinivorans* strain SB1D1 on acetonitrile in various concentration (A) and ammonium release during growth (B)

The effect of different concentration of acetonitrile and benzonitrile on *R. pyridinivorans* strain SB1D1 was shown in Figure 4 and Figure 5. The highest growth of the strain on CH₃CN was observed at 200-500mM and C₇H₅N at 25 mM. The growth of the strain gradually decreased C₇H₅N on the concentration mentioned above (Figure 4 and Figure 5). *R. pyridinivorans* strain SB1D1 has maximum tolerance ability of up to 40 mM of benzonitrile. Nevertheless, exceeding this concentration, the growth of the strain was completely inhibited due to the toxic effect of benzonitrile.
Benzonitrile is one of the aromatic nitrile compounds usually used as an active or a major component of herbicide like bromoxynil, dichlobenil. Many reports are available on the degradation of aliphatic nitriles and a few on aromatic nitriles. The toxicity level of aromatic nitriles is usually higher than aliphatic nitriles, therefore few microorganisms were able to degrade the compound [10].

![Figure 5](image)

**Figure 5.** Growth profile of *Rhodococcus pyridinivorans* strain SB1D1 on various concentration benzonitrile

3.3. *Catabolic pathway of benzonitrile*

The results of growth and hydrolysis product identification by HPLC and enzyme assay in *R. pyridinivorans* strain SB1D1 were shown in **Figure 6**. Benzonitrile was converted to benzamide and then to benzoic acid and ammonia. The metabolite of benzonitrile hydrolysis was detected in 30 minutes reaction and benzoic acid concentration increased gradually in 60 minutes reaction. In this experiment, NHase and amidase was involved in the hydrolysis of benzonitrile by *R. pyridinivorans* strain SB1D1. Most of the reports stated that the metabolism of aromatic nitrile is predominantly catalysed by nitrilase and the hydrolysis of aliphatic nitrile mainly involves NH-ase and amidase. Nitrilase are responsible in the metabolism of benzonitrile to benzoic acid directly [10]. It has reported that NH-ase and amidase pathway was involved for benzonitrile degradation in *Klebsiella* sp. [11]. Microbial degradation of nitriles occurs through hydrolysis either by nitrilase, which convert them to their corresponding carboxylic acid and ammonia, or by bienzymatic pathway (NH-ase and amidase). NH-ase catalysis the formation of amides, and then amides was converted to their corresponding acids and ammonia.
Figure 6. Chromatogram standart of benzonitrile 50 mM (A), benzamide (B), and benzoic acid (C)
4. Conclusions

One potential bacterium that was obtained in the research has the ability to hydrolyse of nitrile compound both of aliphatic and aromatic. The bacteria were isolated from soil at Gandang Dewata West Sulawesi and it was identified as *Rhodococcus pyridinivorans* strain SB1D1. The strain was capable of growing and utilizing acetonitrile and benzonitrile as the sole source of energy, carbon and nitrogen. This strain was able to hydrolyse of benzonitrile to benzamide and benzoic acid, although the concentration was relatively low. Therefore, *R. pyridinivorans* strain SB1D1 is a suitable candidate of indigenous bacteria from Gandang Dewata West Sulawesi as a potential nitrile degrader which can be developed as a biocatalyst in the future for producing important compounds such as food preservative, pharmaceuticaletc. and also may be promising for the remediation of sites contaminated with nitriles. Enzymes involved in the hydrolysis reaction were nitrile hydratase, amidase and not nitrilase enzymes.

Figure 7. Chromatogram of benzonitrile hydrolysis by *R. pyridinivorans* SB1D1, in the beginning reaction (A), 30 minutes (B), and 60 minutes (C) reaction.
Acknowledgments
Authors would like to thank The Expedition Project West Sulawesi - LIPI for financial support in this experiment. We also thank to RestiSitiMuthmainah and IsmuPurnaningsih for their assistance in data analysis.

5. References
[1] Yamada H and Kobayashi M 1996 Nitrile hydratase and its application to industrial production of acrylamide Bioscience Biotechnology and Biochemistry 60 1391-1400.
[2] Banerjee A, Kaul P, and Banerjee U C 2006 Enhancing the catalytic potential of nitrilase from Pseudomonas putida for stereoselective nitrile hydrolysis App Microbiol. Biotechnol. 7277-87.
[3] Santoshkumar M, Nayak A S, Anjaneya O, and Karegoudar, TB 2010 A plate method for screening of bacteria capable of degrading aliphatic nitriles Journal Microbiology and Biotechnology 37 111-115.
[4] Agarwal A, Nigam VK, and Vidyarthi AS 2012 Nitrilases—an attractive nitrile degrading biocatalyst International Journal of Pharma and Bio Science 3232-246.
[5] Yamamoto K, Oishi K, Fujimatsu I, and Komatsu K I 1991 Production of R-(−)-mandelic acid from mandelonitrile by Alcaligenes faecalis ATCC 8750 Applied and Environmental Microbiology 59 3028-3032.
[6] Sunarko B and Atmosukarto I 2011 Screening of nitrile-degrading endophytic bacteria from biodiversity of Indonesia Teknologi Indonesia 34 108-115.
[7] Sulistinah N and Sunarko B 2010 Penapisan mikroba potensial penghasil enzim untuk biotransformasi senyawa nitril Berkala Penelitian Hayati 4F 13-18.
[8] Sulistinah N, Riffiani R, and Sunarko B 2015 Rhodococcus pyridinivorans strain TPIK, a nitrile-degrading bacterium isolated from Tailing Pond of Antam Gold Mining Industry, Pongkor Journal Teknologi Indonesia 38 61-67.
[9] Larkin M J, Kulakov L A and Allen C C 2006 Biodegradation by members of the genus Rhodococcus: biochemistry, physiology, and genetic adaptation Adv. Appl. Microbiol. 59 1-29.
[10] Harper D B 1977 Microbial metabolism of aromatic nitrile Biochemistry Journal 167 309-319.
[11] Nawaz M S, Heinze T M and Cerniglia C A 1992 Metabolism of benzonitrile and butyronitrile by Klebsiella pneumoniae Applied Environmental Microbiology 58 27-31.