Biodegradation of Acetonitrile and Benzonitrile by a Newly Isolated \textit{Rhodococcus Pyridinivorans} Strain I-Benzo from Leather Tanning Waste

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Abstract. Nitriles are toxic organo-cyanide compounds, but extensively used in various industries as solvents, plastics, synthetic rubber, pharmaceuticals, herbicides, and starting materials for other industrially important chemicals. The wider use of these toxic compounds could lead to an environmental pollution, which have a negative impact on health. Some microbes are reported to be able to utilize both aliphatic and aromatic nitriles as growth substrates and convert them into non-toxic compounds, some of which also have economic value as well. An indigenous bacterial isolate I-benzo, capable of growing on and utilizing of a high concentration of acetonitrile (CH\(_3\)CN) and benzonitrile (C\(_6\)H\(_5\)CN), could be isolated from leather tanning waste by the enrichment-culture technique. Based on 16S rDNA sequence, the strain was identified as \textit{Rhodococcus pyridinivorans}. These bacterium was shown to able to grow on acetonitrile (0.2 - 2.0 M) and on benzonitrile (5-25 mM), as a sole source of energy, carbon and nitrogen, respectively. The best growth of \textit{R. pyridinivorans} strain I-benzo was on 500 mM acetonitrile and on 15 mM benzonitrile. During the degradation of both nitriles using whole cells of the bacterium, amide and carboxilic acid were detected in the reaction media, indicating that nitrile hydratase and amidase involved in the metabolism of the substrate. The involvement of both enzymes on the conversion of acetonitrile and benzonitrile was also proved by the ability of \textit{R. Pyridinivorans} I-benzo to grow on their intermediate degradation products, acetamide (CH\(_3\)CONH\(_2\)) and benzamide (C\(_6\)H\(_5\)ONH\(_2\)), respectively. Based on these results, \textit{R. pyridinivorans} strain I-benzo could be expected as a potential candidate for biological treatment for nitriles-containing wastes, although further research is still needed before being applied on a field scale.

1. Introduction

Nitriles (R-CN) are known as highly toxic compound and most of them mutagenic and carcinogenic in nature and possible cause severe health hazards [1, 2]. However, nitriles are manufactured on large scale by several industries and extensively used for synthesizing a variety of polymers and other important chemicals [3]. Acetonitrile (CH\(_3\)CN), for example, is commonly used in industries as organic solvent for manufacturing of pharmaceuticals, photografic film, extractants, etc. On the other hand, benzonitrile (C\(_6\)H\(_5\)CN) is widely used as an active ingredient of herbicides, such as dichlofenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), and ioxynil (4-hydroxy-3,5-diodobenzonitrile) [4, 5].
The extensive used of nitriles in various industries has sparked a great interest in finding new nitrile-degrading microorganisms as well as their involved enzymes (nitrile hydratase and amidase) to be used both as biocatalyst in commercially chemicals synthesis and also as agents for detoxification of nitrile/cyanide containing waste [6, 7, 8]. Although there are several chemical methods could be used for handling this kind of toxic waste [9, 10], these methods are expensive and hazardous chemicals are used as the reagents and some of them create additional toxic and biological persistent chemicals. In general, biological treatments are eco-friendly, cost effective and sometimes more efficient and thus considered as a feasible alternative to the chemical methods [11, 12, 13].

Many authors have reported nitriles biodegradation by bacteria. The microorganisms used include Nocardia rhodochrous LL100-21 [14], Arthobacter sp. I-9 [15], Pseudomonas [16], Klebsiella pneumoniae [17] and Rhodococcus sp. [18]. We have also reported that some indogenous bacteria were capable to degrade nitrile compounds; Corynebacterium sp. D5, Rhodococcus pyridinivorans strain TPIK [19], Rhodococcus pyridinivorans GLB5 [20], and also Micrococcus endophyticus.

Recently, an indigenous bacterial isolate I-benzo, capable of utilizing toxic nitrile as substrate, has been isolated from leather tanning waste from Garut, West Java. In this study, we report the identity of the isolate I-benzo, its growth and biodegradation capability on aliphatic nitrile (acetonitrile) and aromatic nitrile (benzonitrile), its biodegradation products, and the possible enzymes involved. We hope, these bacterial isolate could potentially be used for the biological treatment of nitrile/cyanide contaminated wastewater in the future.

2. Materials and Methods

2.1. Chemicals

Acetonitrile and acetamide were purchased from Merck. Benzonitrile was obtained from Junsey chemical, Co, Ltd Japan and benzamide from Sigma Aldrich Steinheim Germany. Media ingredients were procured from Difco.

2.2. Selective enrichment and Bacterial Isolation

1.0 gram of leather tannery waste sample was suspended aseptically in Erlenmeyer flask (100 ml) which contained 50 ml of sterile minimal medium [21] supplemented with 5 mM benzonitrile. The suspension was incubated at room temperature (28°C-30°C) on rotary shaker for one weeks. After 7 days, 5 ml of this culture was transferred to 50 ml of fresh sterile minimal medium supplemented with different concentration of benzonitrile. The process was repeated three times and step by step the concentration of benzonitrile was raised up to 25 mM. After ± 3 weeks of acclimatization, the enrichment culture was used for isolating nitrile-degrading microorganism by pouring the culture on nutrient agar or on minimal agar medium containing benzonitrile. The pure cultures were maintained on minimal medium agar or on nutrient agar plate for further test.

2.3. Microorganism and Inoculum Preparation

R. pyridinivorans strain I-benzo, which was isolated from leather tannery waste, was maintained on Nutrient Agar slant before use for further investigation. The inoculum was prepared using minimal medium supplemented with benzonitrile as a sole source of energy, carbon and nitrogen for the growth. The bacterial culture was then incubated on rotary shaker for 72 hours. R. pyridinivorans strain I-benzo was maintained at Microbiology Division, Research Center for Biology, LIPI and its purity was checked periodically by plating on agar plate.

2.4. Growth condition

The pure culture of R. pyridinivorans strain I-benzo was inoculated into Erlenmeyer flask (100 ml) contained 50 ml minimal medium. Acetonitrile, benzonitrile or their intermediate degradation products (acetamide and benzamide) was added to the culture medium as the sole source of energy, carbon and nitrogen. The bacterial culture was then incubated on rotary shaker (± 121 rpm) at room temperature (28-30 °C) for 48-72 hours. Bacterial growth was monitored by measuring the optical density at 436 nm.
2.5. Biomass Production
Biomass of *R. pyridinivorans* strain I-benzo was produced in Erlenmeyer flask (1000 ml) contained 500 ml of minimal media supplemented with benzonitrile (25 mM). 3% inoculum (v/v) was inoculated into the medium. The bacterial culture was then incubated on rotary shaker (±121 rpm) at room temperature (± 28 °C) for 72 hours (exponential phase). Cells were harvested by centrifugation at 10,000 rpm for 15 minutes at 4 °C, and washed twice with 50 mM phosphate buffer (KH₂PO₄, pH 7.2). The cell suspension was centrifuged and the pellet was stored at - 4°C before being used for subsequent testing.

2.6. Biodegradation of acetonitrile and benzonitrile by whole cells of *R. pyridinivorans* strain I-benzo
The biodegradation of acetonitrile and benzonitrile was carried out by adding 1,0 g of the cell to 75 ml of 500 mM /2% (v/v) acetonitrile in 50 mM phosphate buffer (KH₂PO₄, pH 7.2). These reaction mixture was then incubated on rotary shaker at room temperature for 180 minutes. Samples (1.0 ml) were taken periodically at interval of 15 minutes. The enzymatic reaction was stopped by adding 250µl of 4N HCl, and the samples were centrifuged. Residual of acetonitrile or benzonitrile in the supernatan and the degradation product were analyzed by GC (for acetonitrile) and HPLC (for benzonitrile), while ammonium was measured by the Nessler method.

2.7. Substrates consumption and product degradation
Substrate consumption (acetonitrile) and formation of the degradation product (acetamide and acetic acid) were determined by gas chromatography (Shimadzu GC-14B, Japan) using a GC Shimadzu equipped with a flame ionization detector. The column used in this study was an Porapaq Q of 80-100 mesh with the operational conditions were as follows: column temperature was 225°C, injector and detector temperature were 240°C, hydrogen as carrier gas, and injection volume was 1.0 µl. Benzonitrile and formation of its degradation products (benzamide and benzoic acid) were detected and quantified by HPLC (Agilent 1100) equiped with C18 column (Supelco, 5 µm : 15 cm x 4.6 mm). Ortho phosphoric acid (0,2%) : acetonitrile (25:75) was used as the mobile phase pumped with the flow rate of 1 ml/minute. The column temperature was 25°C and the wave length used for detection was 254 nm.

3. Results and Discussion
3.1. Identification of bacterial Isolate I-benzo
Bacterial isolate I-benzo, which was isolated from the leather tannery waste, was a gram positive bacteria, non-motile, non-spore former and a small rod or coccioid shaped cells. The isolate grew well at pH 6-7 under room temperature (±30°C). Colonies on the solid agar medium were generally white opaque, circular in nature with smooth ridges and gradually become orange. Physical appearance of the colonies of isolate I-benzo was shown in figure 1.

Based on 16S rDNA sequence analysis, bacterial isolate I-benzo has 100% homology with the type strain *Rhodococcus pyridinivorans*. Thus confirming its identity as *Rhodococcus pyridinivorans* and there after designated the bacterial isolate I-benzo as *Rhodococcus pyridinivorans strain I-benzo*. The phylogenetic relationship of the strain was shown in figure 2. Rhodococcus are a diverse group of microorganisms commonly found in many environmental niches from soils to seawaters and as plant and animal pathogens. Some species belong to this genus have been isolated from polluted soil, water, and sewage purification units [22]. They exhibit a remarkable ability to degrade many organic compounds and their economic importance is becoming increasingly apparent.
3.2. Growth of *R. Pyridinivorans* strain I-benzo on various concentration of acetonitrile and benzonitrile

As shown in figure 3 & 4, *R. pyridinivorans* strain I-benzo was able to utilize both aliphatic nitrile (acetonitrile) and aromatic nitrile (benzonitrile), as a sole source of energy, carbon, and nitrogen for its growth. Although the isolate was able to grow on high concentration of acetonitrile (2M) and of benzonitrile (25 mM), the growth was maximum when the strain was grown on 0.5 M acetonitrile and on 15 mM benzonitrile. Up to these concentration, the growth of the isolate was lower, but still higher than their growth on controls (minimal medium without addition of acetonitrile or benzonitrile). It seems that at certain level of concentration, the growth of *R. Pyridinivorans* strain I-benzo could be inhibited by the substrate. The ability of *R. Pyridinivorans* strain I-benzo to grow well at high concentration of acetonitrile and benzonitrile could be an early indication that the isolate could also degrade both compounds, as shown also by our previous study on *R. pyridinivorans* strain TPIK, and *R. pyridinivorans* strain LP3 [19, 20].

**Figure 1.** *Rhodococcus pyridinivorans* strain I-benzo on nutrient agar

**Figure 2.** The phylogenetic tree of I-benzo isolates based on 16S rDNA sequences using the neighboring-Tamoin method of 3-parameter Tamura and Gamma distributed with 1000 replication bootstrap values
3.3. Biodegradation acetonitrile dan benzonitrile using whole cells of *R. pyridinivorans* strain I-benzo. As shown in figure 4a, acetonitrile was completely degraded by whole cells of *R. pyridinivorans* strain I-benzo into acetamide (CH$_3$CONH$_2$) and acetic acid (CH$_3$COOH). At the end of the degradation process, however, all acetamide formed was then totally hydrolyzed into acetic acid. Similarly, as shown in figure 4b, benzonitrile was also degraded by the cells of the isolate into benzamide (C$_6$H$_5$CONH$_2$) and benzoic acid (C$_6$H$_5$COOH), although the concentration of benzamide formed relatively small compared to benzoic acid. The formation of amide and carboxylic acid during the degradation of both nitrile compounds was indicating that nitrile hydratase and amidase were involved in the metabolism of the substrate by *R. Pyridinivorans* I-benzo, as formulated by Yamada *et al.* [23], which is presented in figure 4c.
The involvement of both enzymes in the biodegradation of acetonitrile and benzonitrile was also proved by the appearance of the degradation products (ammonia), as indirect indication of the presence of both enzyme activities, when cells of *R. Pyridinivorans* I-benzo was incubated in the phosphat buffer supplemented with acetonitril/acetamide and with benzonitrile/benzamide (table 1). Generally, simple aliphatic nitriles, such as acetonitrile, are metabolized in a two-steps reaction by nitrile hydratase and amidase [8], while aromatic nitriles, such as benzonitrile, are metabolism in one step reaction, involves the nitrilase, which catalyzes the hydrolysis of benzonitrile directly into benzoic acid and ammonium without forming amide compounds [8].

**Table 1. Activity of *R. pyridinivorans* strain I-benzo based on Nessler test**

| Substrates (nitrile) | *Rhodococcus pyridinivorans* strain I-benzo | Substrates (amides) | *Rhodococcus pyridinivorans* strain I-benzo |
|---------------------|--------------------------------------------|--------------------|--------------------------------------------|
| (1)                 | (2)                                        | (3)                | (4)                                        |
| acetonitrile        | +++                                       | acetamide          | +++                                       |
| benzamide           | +++                                       | benzamide          | +                                          |

As reported by many authors, nitrile-degrading bacteria are important biocatalysts for synthetic processes as well as for the efficient treatment of toxic organo-cyanide-contaminated industrial effluents. Their application is expected to reduce the energy expenses and their employment could decrease the distribution of various pollutants in the environment [24]. *Rhodococcus* represents a genus of considerable industrial interest. This genus has been proven to have a great ability to degrade nitriles, both aliphatic and aromatic, as well as other toxic compounds, such as *Rhodococcus* sp. [18], *Rhodococcus rhodochrous* J1 [25], *Rhodococcus* sp. UKMP-5M [26], and *Rhodococcus pyridinivorans* strain TPIK [20].

**4. Conclusion**

*Rhodococcus pyridinivorans* strain I-benzo, isolated from leather tannery waste, was able to grow on a high concentration of acetonitrile (CH₃CN) (up to 2000 mM) and benzonitrile (C₆H₅CN) (up to 25 mM), respectively and could utilize both substrates as a sole of energy, carbon, and nitrogen for its growth. With the acetonitrile as substrate, the optimal growth of *R. pyridinivorans* strain I-benzo was at 500-1000 mM, while with benzonitrile at 15-25 mM. Using whole cells of *R. pyridinivorans* strain I-benzo, acetamide (CH₃CONH₂) and acetic acid (CH₃COOH) were identified as degradation product of acetonitrile, while benzamide (C₆H₅CONH₂) and benzoic acid (C₆H₅COOH) of benzonitrile. Based on these products, the biodegradation of both acetonitrile and benzonitrile by *R. pyridinivorans* strain I-benzo most likely took place via a two-steps reaction involving nitrile-hydratase and amidase. Lastly, although further research is still needed, *R. pyridinivorans* strain I-benzo could be expected as a potential candidate for biological treatment for nitrile/cyanide-containing wastes.
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