Acrylonitrile degradation by whole cells of *Corynebacterium* sp. D5, isolated from polluted industrial waste

B Sunarko and N Sulistinah

Division of Microbiology, Research Center for Biology, Indonesian Institute of Sciences Cibinong Science Center-Botanical Garden (CSC-BG), Jl. Jakarta Km 46, Cibinong, Indonesia

Email: bambang.sunarko@lipi.go.id; bsunbiol@gmail.com

**Abstract.** Acrylonitrile is a toxic organo-cyanide compound extensively used as solvents and in the manufacture of plastics, polymers, synthetic fibers, resin, dyestuffs, pharmaceuticals and vitamins. Because of its acute neurotoxicity, mutagenicity, carcinogenicity and teratogenicity, discharge of acrylonitrile contained wastewater can lead to serious environmental pollution if is not controlled. Microbial degradation has been considered as a way of removing highly toxic nitriles from industrial waste. In our studies, the biodegradation of acrylonitrile was demonstrated by using whole cells of *Corynebacterium* sp. D5 isolated from polluted industrial wastewater. Although the bacterium could not utilized the compound as a source of carbon, energy and nitrogen for its growth, *Corynebacterium* sp. D5 was capable to degrade acrylonitrile (CH$_2$=CH-CN) into acrylamide (CH$_2$=CH-CONH$_2$) and acrylic acid (CH$_2$=CH-COOH). The acrylonitrile degradation took place via a two-steps reaction involving nitrile-hydratase and amidase. During the degradation, the highest nitril hydratase activity was 4.894 nmol.(min.mg)$^{-1}$ with the optimum temperature and pH was 25°C and pH 7.0, while the highest amidase activity was 1,315 nmol.(min.mg)$^{-1}$, with the optimum temperature and pH was 50°C and pH 6.0, respectively. Besides on acetonitrile as inducer, *Corynebacterium* sp. D5 was also able to grow on various saturated low molecular weight of nitrile and amide compounds.

1. Introduction

Acrylonitrile (CH$_2$=CH-CN) is an important raw material and chemical intermediate used in the production of acrylic fibers, plastics and synthetic rubber, and is used in the preparation of solvents, pharmaceuticals and insecticides [1]. Of all the commercial nitriles, acrylonitrile is manufactured on the largest scale. Due to its acute neurotoxicity, mutagenicity, carcinogenicity and teratogenicity, discharge of acrylonitrile contained wastewater can lead to serious environmental pollution. Currently, deep well injection disposal technology and wet air oxidation are used to address acrylonitrile contaminated wastewaters [2]. However, these two technologies are costly and have the potential to generate secondary air or water pollution.

Biological treatments are considered as feasible alternatives for acrylonitrile removal, since it has been known some microorganisms degrade acrylonitrile. Many authors have reported biodegradation of acrylonitrile by bacteria, such as by *Brevibacterium imperiale* CBS 49874 [3], *Corynebacterium nitriphilus* ATCC 21419 [3], *Micrococcus luteus* BST20 [4], *Arthrobacter nitroguajacolicus* ZJUTB06-99 [5], *Rhodococcus rhodochrous* DAP 96622 [2], and by *Rhodococcus ruber* Strain AKSH-84 [6]. According to Kobayashi et al. [7], microbial degradation of nitriles proceeds by two distinct routes: (a) nitrile hydratase hydrolyzes nitriles into amides, which are subsequently hydrolyzed to acids and ammonia by an amidase, or (b) nitrilase transforms nitriles directly into acids and ammonia.
Recently, indigenous bacterial isolate *Corynebacterium sp.* D5 has been isolated from cyanide-contaminated industrial wastewater. The purpose of this study was to explore the ability of *Corynebacterium sp.* D5 in degrading acrylonitrile, hence it could be used for the biological treatment of acrylonitrile contaminated wastewater in the future. In present investigation, we report the pathway of acrylonitrile biodegradation by whole cells of *Corynebacterium sp.* D5, the biodegradation products, and the possible enzymes involved.

2. Materials and Methods

2.1. Chemicals
Acrylonitrile, acetonitrile, acrylamide, acrylic acid were procured from from Merck, Germany. Acetonitrile, Ethaneamide, phenylcarboxamide, niacinamide, propionamide were obtained from Sigma Aldrich, Steinheim, Germany. Media ingredients were purchased from Difco. All other chemicals used in this study were of analytical grade.

2.2. Microorganism
Microorganism used in this study was *Corynebacterium sp.* D5. The bacterial isolate was isolated from industrial wastewater using nitrile enrichment culture technique [8]. The isolate was kept at Biochemistry laboratory, Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI). The purity of the culture was checked periodically by plating on agar plates.

2.3. Culture media
The medium used for growing the *Corynebacterium sp.* D5 was mineral medium which was supplemented with microelements. Acetonitrile was added to the medium as a sole source of carbon, energy and nitrogen. The composition of the mineral medium was of the following (in g.l$^{-1}$): Na$_2$HPO$_4$, 2H$_2$O, 0.4475 g; KH$_2$PO$_4$, 0.1 g; MgSO$_4$.7H$_2$O, 0.1 g; CaCl$_2$.2H$_2$O, 0.01 g; FeSO$_4$.7H$_2$O, 0.001 g; Yeast extract, 0.01 g and microelements, 1.0 ml [9] and the microelements was (in g.l$^{-1}$): ZnSO$_4$.7H$_2$O, 0.1 g; MnCl$_2$.4H$_2$O, 0.03 g; H$_2$BO$_3$, 0.3 g; CoCl$_2$.6H$_2$O, 0.2 g; CuCl$_2$.2H$_2$O, 0.01 g; NiCl$_2$.2H$_2$O, 0.02 g; Na$_2$MoO$_4$.2H$_2$O, 0.9 g; Na$_2$SeO$_3$, 0.02 g [10]. The pH of the medium was adjusted to 7.2 using 4 N NaOH.

2.4. Growth condition
Cells of *Corynebacterium sp.* D5 (ca. 3 % w/v) was inoculated into Erlenmeyer flasks (100 ml) contained 50 ml minimal medium. Acetonitrile or other nitrile/amide compounds (100 mM) was added to the culture medium as a sole source of carbon, nitrogen and energy. The bacterial culture then incubated on orbital shaker (120 rpm) at room temperature (ca. 28 °C) for 72 hours. Bacterial growth was observed by measuring the optical density at a wavelength of 436 nm. Large scale cultivation for cell biomass was performed in Erlenmeyer flasks (1000 ml) contained 500 ml mineral medium supplemented with 200 mM acetonitrile. Bacterial cells were harvested by centrifugation at 10,000 rpm, 4 °C for 10 min. The pellet was washed twice with phosphate buffer (50 mM KH$_2$PO$_4$ and 50 mM K$_2$HPO$_4$), pH 7.2. Cell suspension was centrifuged before weighed and stored in a freezer for the biotransformation assay.

2.5. Biodegradation of acrylonitrile by whole cells
The acrylonitrile degradation was carried out by adding 1 g of the cell-pellet to 75 ml of 1% (v/v) acrylonitrile in 50 mM phosphate buffer (KH$_2$PO$_4$), pH 7.2. The reaction was incubated on the shaker incubator (120 rpm) at 30°C for 3 h. Samples (2 ml) were taken at time interval of 15 minutes, the enzymatic reaction was stopped by the adding 0.20 ml of 4N HCl, and the samples were then centrifuged. The residual acrylonitrile in the supernatant and the degradation products (acrylamide and acrylic acid) were analyzed by gas chromatography (GC), while ammonium was determined by the Nessler method.
2.6. Analytical methods
Acrylonitrile, acrylamide and acrylic acid were quantitatively determined using gas chromatography (Shimadzu GC-14B, Japan) equipped with a flame ionization detector. Columns were packed with Porapack Q of 80-100 mesh. The carrier gas was \( \text{N}_2 \) at a flow rate 160 kPa. Oven, injection and detector were operated at 240°C. Injection volumes was 1.0 µl. The acrylonitrile and degradation products were identified by their retention time and quantified by comparing peak areas with external standards. Dissolved \( \text{NH}_3 \) was determined colorimetrically by Nessler’s procedure (Oliver et al. 1989). A total of 10 mL of enzyme solution was added into 0.99 mL of 0.1 N NaOH and 20 mL Nessler reagent. The solution was incubated at 30°C for 20 min, and measured using a spectrophotometer at a wavelength of 420 nm. Enzyme activity was determined in Units which were equivalent to 1 umol of degraded substrate (acrylonitrile or acrylamide) in 1 minute per mg of dry weight cells at 25°C and pH 7.2.

3. Results and Discussion
3.1. Effect of various nitriles and amides on the growth of Corynebacterium sp. D5
As shown in table 1, Corynebacterium sp. D5 was able to utilize five from nine tested nitrile compounds as a sole carbon, energy and nitrogen source for its growth. Acetonitrile, propionitrile, and butyronitrile served as the best substrates. However, acrylonitrile, \( \beta \)-aminopropionitrile sianopyridine and KSCN did not support the growth of Corynebacterium sp. D5. Table 1 also shows that Corynebacterium D5 could utilize three of five tested amide compounds as a sole carbon and nitrogen source. Acrylamide and nicotinamide could not serve as growth substrate for Corynebacterium sp. D5.

| Nitriles          | Growth | Amides      | Growth |
|-------------------|--------|-------------|--------|
| Acetonitrile      | +++    | Acetamide   | +++    |
| Acrylonitrile     | -      | Acrylamide  | -      |
| Propionitrile     | +++    | Propionamide| +++    |
| Butyronitrile     | +++    | -           | -      |
| Lactonitrile      | ++     | -           | -      |
| \( \beta \)-aminopropionitrile | -     | -           | -      |
| Benzonitrile      | +      | Benzamidine | +      |
| Cyanopyridine     | -      | Nicotinamide| -      |
| KSCN              | -      | -           | -      |

- \( \beta \)-aminopropionitrile: not tested

3.2. Biodegradation of acrylonitrile by whole cells of Corynebacterium sp. D5
As shown in figure 1, the cells of Corynebacterium sp. D5 were able to degrade acrylonitrile, although it could not utilize this substrate as carbon and energy source for its growth. Acrylonitrile was first converted into acrylamide and then the amide was slowly transformed into acrylic acid and amonia. The formation of acrylamide and acrylic acid during the biodegradation was an indication that the biodegradation of acrylonitrile by Corynebacterium sp. D5 took place via a two-steps reaction involving nitrile-hydratase and amidase as proposed by Kobayashi et al. [7].

In general, the success of nitrile biodegradation by whole cells depends on the activity of involved enzymes. Temperature is one of important parameter for enzyme activity. As indicated in figure 2, the optimum temperature for nitrile hydratase activity was at 25°C. At 5°C and at 50°C, the nitrile
hydratase activity of the cells was 30.14% and 69.32%, respectively, as compared to the enzyme activity at 25°C. This is suggesting that Corynebacterium sp. D5 was mesophilic in nature. Most nitrile-degrading organisms exhibited nitrile hydratase activity at around 30°C. Interestingly, as it was observed in Figure 2, the amidase of Corynebacterium sp. D5 cells was still active at high temperature (50°C) and could be classified as thermophilic enzyme. Adityarini [11] reported similar results, where the best nitrile hydratase activity was at 25°C and amidase continued to increase to 60°C with the acetonitrile substrate.

pH is another critical factor for nitrile degrading enzyme system. As shown in figure 3, nitrile hydratase activity of whole cells of Corynebacterium sp. D5 was detected at a pH range of 4.0-9.0. Maximum nitrile hydratase (2.53 x 10^{-3} Units) was observed at pH 7.0. At pH 4 and pH 9, the enzyme activity was 73.97% and 65.88% as compared to with pH 7.0, respectively. Amidase activity of Corynebacterium sp. D5 cells was also detected at a pH range of 4.0-9.0. the maximum amidase (1.65 x 10^{-3} Units) was observed at pH 6.0. At pH 4.0 and pH 9.0, the enzyme activity was 20.39% and 31.72% as compared to with optimum pH (pH 6.0).

Substrate concentration is also playing important role in the microbial biodegradation of nitrile compounds, in general. As indicated in figure 4, nitrile hydratase of cells of Corynebacterium sp. D5 was still active at 10% (v/v) acrylonitrile with enzyme activity of 12 x 10^{-3} Units, whereas the amidase activity was only slightly affected by increased of acrylonitrile.

![Figure 1. Time course of acrylonitrile biodegradation by whole cells of Corynebacterium sp. D5.](image)

Substrate concentration is also playing important role in the microbial biodegradation of nitrile compounds. As indicated in figure 4, nitrile hydratase of Corynebacterium sp. D5 was still active at 10% (v/v) acrylonitrile with enzyme activity of 12 x 10^{-3} Units, whereas the amidase activity was only slightly affected by increased of acrylonitrile.

Nitrile degrading enzymes are an enzyme that have two types of cofactors namely Co^{2+} and Fe^{2+} [12]. Figure 5 shows the effect of Co^{2+} on the activity of nitrile hydratase and amidase of Corynebacterium sp. D5. Ion Co^{2+} was observed to be effective as cofactor of nitrile hydratase. The highest nitrile hydratase activity (1.0 x 10^{-3} Units) was observed at the concentration of 3 mM Co^{2+}. However, up to 3 mM Co^{2+}, the enzyme activity was inhibited. Meanwhile, the highest amidase
activity of *Corynebacterium sp.* D5 was observed at the concentration of 5 mM Co\(^{2+}\) with enzyme activity of \(0.24 \times 10^{-3}\) Units.

**Figure 2.** Effect of temperature on acrylonitrile degrading enzymes activity.

**Figure 3.** Effect of pH on acrylonitrile degrading enzymes activity.

**Figure 4.** Effect of acrylonitrile concentration on acrylonitrile-degrading enzymes activity.

**Figure 5.** Effect of ion Cobalt on acrylonitrile degrading enzymes activity.

4. **Conclusion**

*Corynebacterium sp.* D5 was capable to degrade acrylonitrile (CH\(_2=\)CH-CN) into acrylamide (CH\(_2=\)CH-CONH\(_2\)) and acrylic acid (CH\(_2=\)CH-COOH). The degradation took place via a two-steps reaction involving nitrile-hydratase and amidase. The highest nitrile hydratase activity was 4.894 nmol.(min.mg\(^{-1}\)) with the optimum temperature and pH were 25°C and pH 7.0, while the highest amidase activity was 1,315 nmol.(min.mg\(^{-1}\)) with the optimum temperature and pH were 50°C and pH 6.0, respectively. *Corynebacterium sp.* D5 grew on acetonitrile as inducer, but was also able to grow on various saturated low molecular weight of nitrile and amide compounds.
5. References

[1] U.S. EPA 1994 IRIS Database
[2] Zhang J and Pierce G E 2009 J Ind Microbiol Biotechnol 36 971-979
[3] Battistel E., Bernardi A and Maestri P 1997 Biotechnol Letter 19 2 131-134
[4] Fischer-Colbrie G, Matama T, Heumann S, Martinkova L, Paulo A C, and Guebitz G 2007 J Biotechnol 129 62-68
[5] Shen M, Zheng Y-G and Shen Y-C 2009 Process Biochem 44 781-785
[6] Kamal A, Kumar M S, Kumar C G and Shaik T 2011 J Microbiol Biotechnol 21 1 37-42
[7] Kobayashi M, Yanaka N, Nagasawa T and Yamada H 1990 J Bacteriol 172 9 4807-4815
[8] Sunarko B, Adityarini, Tambunan U S F, dan Sulistinah N 2000 Berita Biologi 5 2 177-185
[9] Meyer O and Schlegel H G 1983 Ann Rev Microbiol 37 277-310
[10] Pfennig N 1974 Arch Microbiol 100 197-206
[11] Adityarini 1999 Thesis (Universitas Indonesia)
[12] Yamada, H and Kobayashi M 1996 Biosci Biotech Biochem 60 9 1391-1400

Acknowledgments

The authors acknowledge the financial support from Thematic Research Program of DIPA, Indonesian Institute of Sciences (LIPI). We would like to thank Dwi Purnomo and Suri Handayani for their contribution in data collecting.