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Characterization of α-Nitrile Hydratase and Amidase of *Rhodococcus* aff. *qingshengii* from Indonesia

AERMA HASTUTY[1*], WIBOWO MANGUNWARDOYO[2], BAMBANG SUNARKO[1]

[1]Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences-LIPI, Jalan Raya Bogor Km 46, Cibinong 16911, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia, Depok 16424, Indonesia

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A study on biotransformation of acetonitrile using Gram-positive bacteria has been conducted. Two isolates of nitrile-degrading bacteria (strain 100A and 100D) were screened from sediments of a contaminated river in Cibinong, West Java. The bacterial isolates were identified as *Rhodococcus* aff. *qingshengii* based on molecular phylogenetic analyses of 16S rRNA sequence. These bacteria were capable to grow on medium containing 100 mM acetonitrile, but unable to grow on medium amended with 25 mM benzonitrile. Analyses using Gas Chromatography (GC) indicated that *R*. aff. *qingshengii* strain 100A and 100D has the ability to produce nitrile hidratase and amidase. The highest enzyme activity on mineral medium with the addition of 100 mM acetonitrile was 73.49 mmol/min/mL by strain 100A, and 70.52 mmol/min/mL by strain 100D. In addition, the ammonia concentration produced by strain 100A and 100D were 180.20 and 54.10 mM, respectively. These results were supported by molecular characterization using specific primers, where strain 100A and 100D positively contain genes encoding α-nitrile hydratase (α-NHase) and amidase. There was a difference at the first position of amino acid composition of the gene encoding α-NHase between strain 100A (Methionine[1]) and strain 100D (Glycine[1]), but the amino acids composition of amidase of both strain were identical. This is the first report of *R*. aff. *qingshengii* as nitrile-degrading bacterium in Indonesia.

Keywords: biotransformation, nitrile, nitrilases, 16S rRNA, *Rhodococcus*

INTRODUCTION

Nitrile hydratase (NHase) (EC 4.2.1.84) and amidase (EC 3.5.1.4) are group of nitrile-converting enzymes that hydrolyze nitriles into the corresponding higher-value amides and acids (Nagasawa & Yamada 1990). NHase and amidase, including other nitrile-converting enzymes, have attracted interest not only because of their role as biocatalyst in the production of solvents, extractans, drug intermediates (chiral synthons), pesticides as well as in the synthesis organic amine, amide, ester, carboxylic acid, aldehydes, ketones, and heterocyclic compounds (Banerjee *et al*. 2002), but also because nitrilases have advantages over the chemical hydrolysis due to their milder pH and temperature conditions, and the absence of by-products (Nagasawa *et al*. 2000). For example, the application of NHase from *Rhodococcus rhodochrous* J1, which is currently used in the production of acrylamide (Nagasawa *et al*. 1993; Yamada & Kobayashi 1996) and nicotamide (Nagasawa *et al*. 1988) at industrial scale. Wyatt and Knowless (1995) reported that NHase has also practical importance as biocatalysts for environmental bioremediation in the removal of nitriles from waste streams.

The potential of NHase and amidase in the industries has encouraged extensive research and exploration in the discovery of more novel bacteria producing these enzymes from various habitats and geographical regions. Until now, the majority of bacteria producing NHase and amidase enzymes have been reported from shallow marine sediment (Langdahl *et al*. 1996), deepsea sediments (Heald *et al*. 2001), geothermal habitats (Pereira *et al*. 1998), and various soils (Blakey *et al*. 1995; Brandão *et al*. 2003). Polluted environment (Cahill 2004; Kabaivanova *et al*. 2005; Coffey *et al*. 2009) have also been explored in the discovery of novel bacteria producing NHase and amidase enzymes. In the current study, two strains of α-NHase and amidase producing bacteria isolated from polluted river in Cibinong, West Java (Indonesia) were determined using sequence analyses generated from 16S rRNA region. The genes encoding α-NHase and amidase of these bacteria were also characterized and sequenced.

*Corresponding author. Phone: +62-21-8765066/8765067, Fax: +62-21-8765062, E-mail: mima_hdy@yahoo.com

MATERIALS AND METHODS

Organisms and Growth Condition. Two nitrile-metabolizing bacteria (strain 100A and 100D) were isolated from polluted-river sediment using mineral medium (MM) (Meyer & Schlegel 1983) supplemented with 10-25 and 50-100 mM of acetonitrile. Selected bacteria were grown on nutrient agar (NA) DIFCO and mineral medium (MM) with the following composition (per 1000 mL): 0.4475 g $Na_2HPO_4 \cdot 2H_2O$; 0.1 g KH_2PO_4; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.01 g $CaCl_2 \cdot 2H_2O$; 0.001 g $FeSO_4 \cdot 7H_2O$, 0.01 g yeast extract, and 1 mL micro-elements (Meyer & Schlegel 1983). The micro-element composed of 0.1 g $ZnSO_4 \cdot 7H_2O$; 0.03 g $MnCl_2 \cdot 4H_2O$; 0.3 g H_3BO_3; 0.2 g $CoCl \cdot 6H_2O$; 0.01 g $CuCl_2 \cdot 2H_2O$; 0.02 g $NiCl_2 \cdot 2H_2O$; 0.9 g $Na_2MO_4 \cdot 2H_2O$; 0.02 g Na_2SeO_3 within 1000 mL distilled water (Pfennig 1974). Acetonitrile and benzonitrile were added in the medium at concentration of 100 and 25 mM, respectively.

DNA Extraction and PCR Amplification of 16S rRNA Gene. DNA extraction and Polymerase Chain Reaction (PCR) amplification were carried out in order to obtain genomic DNA for bacterial identification using sequence of 16S rRNA. The genomic DNA from bacteria were obtained from 48 h colonies using the guanidium thiocyanate/EDTA/Sarkosyl (GES) method as described by Pitcher *et al*. (1989). The 16S rRNA gene was amplified using the universal primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500R (5'-GTTACCTTGTTACGACTT–3'). The PCR components were set as follows: 2 µL DNA template ($\pm$ 100 ng), 25 µL Go Taq® PCR master mix (Promega), and 20.5 µL PCR-grade water (Sigma). The amplification was carried out using a PCR thermocycler (TaKaRa Shuzo Co., Ltd., Shiga, Japan) with the following condition: 95 ^oC for 3 min of predenaturation; followed by 30 cycles of 95 ^oC for 30 sec of denaturation, 50 ^oC for 30 sec of annealing, 72 ^oC for 90 sec of extension, and 72 ^oC for 10 min of final extension. The quality of the PCR products were determined using electrophoresis in 1.5% agarose gel. DNA nucleotides were sequenced by FirstBASE (Malaysia) using the same primer pairs used in the PCR reaction.

Phylogenetic Analysis. Bacteria were identified using phylogenetic analyses. Newly sequences of bacteria generated from 16S rRNA region were aligned with the homologous sequences obtained from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) available at the GenBank website. Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis) v5.1 software (Tamura *et al*. 2011). Maximum Likelihood (ML) was used in the analyses involving the Tamura Nei model as nucleotide substitution parameter and gap was treated as missing data. Searching of the best phylogenetic trees was done by using a heuristic method with Nearest Neighbor approach Interchange (NNI), an initial tree was generated based on Neighbor Joining analysis (NJ). Phylogenetic tree was evaluated by bootstrap method using 1000 replication.

Bacteria Growth Assay. Bacterial isolates were grown in mineral medium containing nitrile, as the sole source of carbon, energy and nitrogen. A loopful bacterial was inoculated into 50 mL mineral medium in 100 mL Erlenmeyer flask. 262 µL of 100 mM acetonitrile or 130.2 µL of 25 mM benzonitrile was added into the medium and incubated at 30 ^oC for 72 h on the shaker incubator at 120 rpm. Bacterial growth pattern was observed every 2 h by measuring the optical density (OD). Measurement of microbial growth was carried out every 24 h for 7 days at 436 nm wavelength. pH, acetonitrile concentration, and the formation of transformation products (acetamide, acetic acid, and ammonia) during bacterial growth were measured.

Bacterial Cells Production for Nitrile Biotransformation Assay. A loopful bacteria was inoculated into 50 mL mineral medium in 100 mL Erlenmeyer falsk. 262 µL of 100 mM acetonitrile was added into the medium and incubated at 30 ^oC for 72 h on the shaker incubator at 120 rpm. 2% (v/v) bacterial cells grown in 500 mL mineral medium in 1000 mL Erlenmeyer flask, and 2.626 mL of 100 mM acetonitrile was added. The flask was incubated in a shaker incubator at 30 ^oC for 72 h (120 rpm). Bacterial cells were harvested by centrifugation at 10,000 rpm, 4 ^oC for 10 min. The pellet was washed twice with phosphate buffer (50 mM KH_2PO_4 and 50 mM K_2HPO_4), pH 7.2. Cell suspension was centrifuged before weighed and stored in a freezer for the biotransformation assay.

Nitrile Biotransformation Assay. Biotransformation of nitrile was carried out by adding 2 g pellets (wet weight) into 500 mL of phosphate buffer (50 mM KH_2PO_4 and 50 mM K_2HPO_4), pH 7.2, in 1000 mL Erlenmeyer flask. Approximately 2.626 mL of 100 mM acetonitrile was added into the medium. The flask was incubated on the shaker incubator at 30 ^oC for 3 h (120 rpm). At regular intervals (0, 5, 15, 30, 60, 90, 120, 150, and 180 min), 2 mL of samples were taken for pH, OD growth and biotransformation process measurement. The reaction was stopped by