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

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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 (± 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 °C for 3 min of predenaturation; followed by 30 cycles of 95 °C for 30 sec of denaturation, 50 °C for 30 sec of annealing, 72 °C for 90 sec of extension, and 72 °C 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 °C 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 °C 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 °C for 72 h (120 rpm). 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_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 °C 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

adding 0.25 μL of 5 N HCl. After the enzymatic reaction stopped, samples were neutralized by adding 5 mL of 0.25 N NaOH. Samples were then centrifuged, and the resulting supernatant was taken for determination acetonitrile, acetamide and acetic acid.

Analysis of acetonitrile, acetamide, and acetic acid performed in Gas Chromatography (GC) equipped with Flame Ionization Detector (FID) using a Porapak Q column. Analysis conditions were set as follows: 1 mL sample is injected at 225, 240, and 240 °C of oven, injector, and detector temperature, respectively. N_2 was used as a carrier and H_2 was used as detectors, and these gases were pumped at 11 mL/min (Sunarko *et al*. 2000).

Ammonia Concentration Assay. Determination of ammonia was performed using the Nessler method. 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 then measured using a spectrophotometer at a wavelength of 420 nm (Oliver *et al*. 1989).

DNA Extraction and PCR Amplification for Nitrilases Gene. DNA extraction and PCR (Polymerase Chain Reaction) amplification were carried out in order to obtain genomic DNA for nitrilases gene characterization. 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 NHase gen was amplified using primer pairs of α-NH1-F (5'-GTGAACCAGATGTCAGTAACGATCG-3') and α-NH1-R (5'-CGCTCAGGCAGTCCTTGGTGACG-3'), and amidase gen was amplified using primer pairs of amd1-F (5'-GTGAAGCCGATCACATCAGGAGC-3') and amd2-R (5'-CGGGTACCAATCCCTTACCGTCG-3') (Brandão *et al*. 2003). The PCR components and PCR condition were set as previously described in the bacterial identification. 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 of the α-NHase and Amidase Genes. Nucleotides sequences of α-NHase and amidase of *R*. aff. *qingshengii* strain 100A and 100D were aligned with available α-NHase and amidase equences retrieved from GenBank. The phylogenetic analysis of nitrilases genes of selected bacteria was carried out by using the same method as in the identification of bacteria.

RESULTS

Identification of Bacteria. BLAST result showed that three *Rhodococcus* species, namely, *R. qingshengii*, *R. baikonurensis*, and *R. erythropolis* were the most homologous bacteria with bacterial isolates strain 100A and 100D, with a maximum identity of sequence homology reaches 99 and 100%, respectively. In the phylogenetic analysis, the type species of these species were included in the analyses together with other *Rhodococcus* species. The phylogenetic tree generated from the ML analyses of 16S rRNA sequence showed that the genus *Rhodococcus* is monophyletic with 92% bootstrap support (BS) (Figure 1). Strain 100A and 100D nested in the same clade with *R. qingshengii*, *R. baikonurensis*, and *R. erythropolis* with 83% BS. The clade indicated a close relationship between strain 100A and 100D to those of *R. qingshengii*, *R. baikonurensis*, and *R. erythropolis*. Multiple nucleotide sequence analyses among these isolates showed that there were eight nucleotides differences of *R. erythropolis* to each of strain 100A and 100D, and there were three nucleotides differences of *R. baikonurensis* to each of strain 100A and 100D.

The same analyses showed that *R. qingshengii* was identical to that of strain 100D and only single nucleotide difference of *R. qingshengii* to strain 100A. Based on the phylogenetic tree and multiple nucleotide sequence analyses, the bacterial strain of 100A and 100D were identified as *Rhodococcus* aff. *qingshengii*. Code 'aff.' or 'affinity to' refers to the closest only nucleotide sequences and phylogenetic affinity of strain 100A and 100D to that of *R. qingshengii*.

Bacterial Growth Assay. This assay showed that *R*. aff. *qingshengii* strain 100A and 100D were capable to grow properly after incubated for 72 h. It was indicated by the increasing of OD value in the mineral medium amended with 100 mM acetonitrile (Figure 2).

Log phase of *R*. aff. *qingshengii* strain 100D was found at 24-48 h while the log phase of *R*. aff. *qingshengii* strain 100A was found at 8-32 h. The highest enzyme activity of *R*. aff. *qingshengii* strain 100A was found at 76.04 mmol/min/mL at 52 h, and *R*. aff. *qingshengii* strain 100D was found at 61.34 mmol/min/mL at 72 h. During the bacterial growth incubated for 7 days (Figure 3), the log phase of *R*. aff. *qingshengii* strain 100A started from 2nd day to 3rd day,while the log phase of *R*. aff. *qingshengii* strain 100D started from the first day to 2nd day. During bacterial growth, a change in pH value

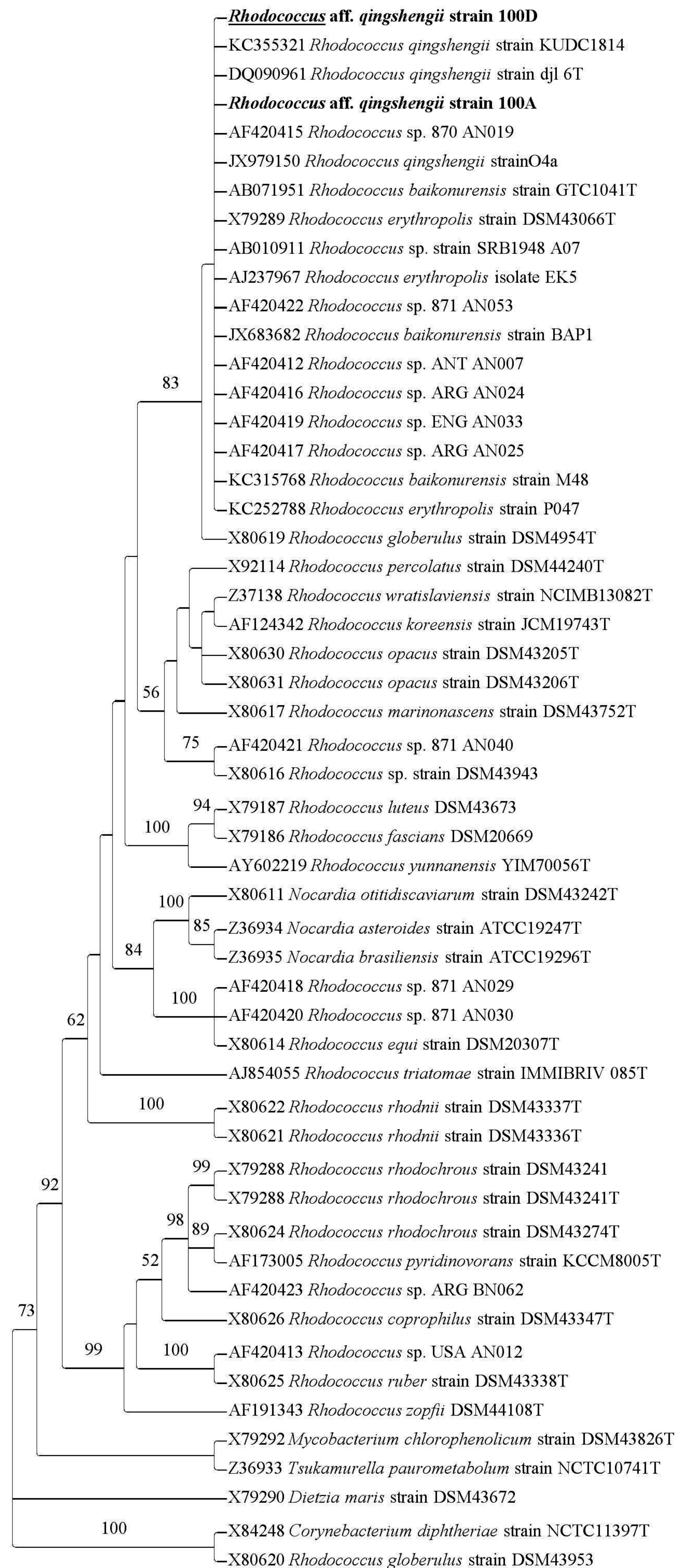

Figure 1. The phylogenetic tree from the Maximum Likelihood analyses of 16S rRNA sequence of *Rhodococcus* aff. *qingshengii* 100A and *Rhodococcus* aff. *qingshengii* 100D.

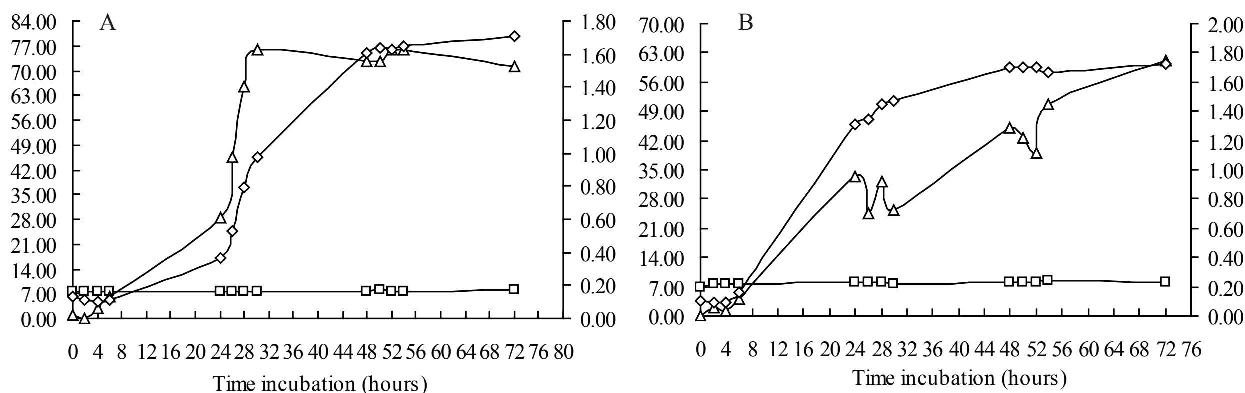

Figure 2. Bacterial growth incubated for 72 hours in the mineral medium amended with 100 mM acetonitrile. (A) Isolate *Rhodococcus* aff. *qingshengii* 100A and (B) Isolate *Rhodococcus* aff. *qingshengii* 100D. -□- pH, -△- Total enzyme activity (mmol/mnt/mL), -◇- OD.

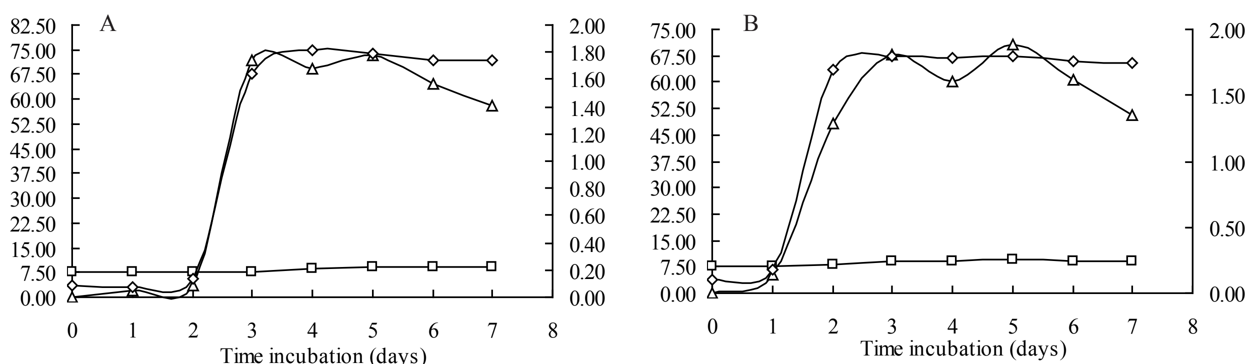

Figure 3. Bacterial growth incubated for 7 days in the mineral medium amended with 100 mM acetonitrile. (A) Isolate *Rhodococcus* aff. *qingshengii* 100A and (B) Isolate *Rhodococcus* aff. *qingshengii* 100D. -□- pH, -△- Total enzyme activity (mmol/mnt/mL), -◇- OD.

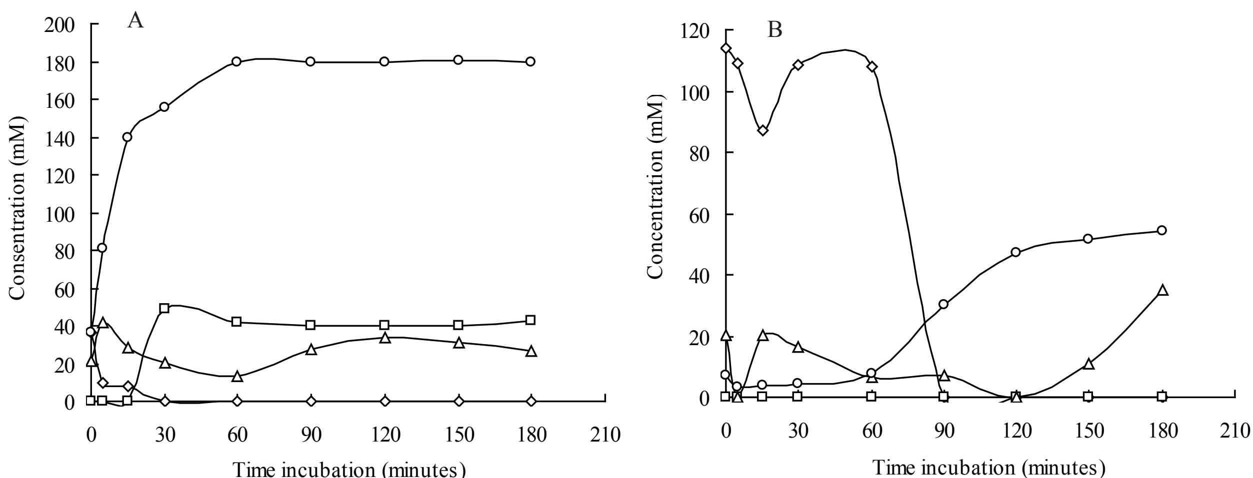

Figure 4. The nitrile biotransformation assay. (A) *Rhodococcus* aff. *qingshengii* 100A (B) *Rhodococcus* aff. *qingshengii* 100D. -◇- Acetonitrile, -□- Acetic acid, -△- Acetamide, -○- NH_3.

was found unpredictable. Range of pH was found about 7-9. During the bacterial growth, there was an increase in enzyme activity of *R*. aff. *qingshengii* strain 100A and 100D with 73.49 and 70.52 mmol/min/mL, respectively at fifth day.

Nitrile Biotransformation Assay. The nitrile biotransformation assay showed that NHase and amidase enzymes involved during the biotransformation of nitrile by *R*. aff. *qingshengii* strain 100A and 100D. In the current study, highest ammonia levels obtained was 180.20 mM produced in the acetonitrile biotransformation by *R*. aff. *qingshengii* strain 100A at 50 min incubation (Figure 4). This was supported by faster reduction

level of acetonitrile in the medium containing *R.* aff. *qingshengii* strain 100A compared to medium containing *R.* aff. *qingshengii* strain 100D.

Gas chromatography analysis showed a reduction in the concentration of acetic acid and acetamide (Figure 4). It was probably due to the acetic acid and acetamide were utilized by *R.* aff. *qingshengii* strain 100A and 100D for the synthesis of cells during bacterial growth in biotransformation process. During the biotransformation, acetonitrile concentration in the medium inoculated with *R.* aff. *qingshengii* strain 100A started to decrease after 5 min incubation into 9.78 mM. The acetonitrile was completely degraded after 30 min incubation. However, in the medium inoculated with *R.* aff. *qingshengii* strain 100D, the acetonitrile was completely degraded after 90 min incubation.

Molecular Characterization of Nitrile Degrading Enzymes (α-NHase and Amidase). NHase consists of two sub-units, α and β, with molecular weight 23 kDa. About 400 bp bands were formed of which indicated the presence of α-NHase gene in the *R.* aff. *qingshengii* strain 100A and 100D. Several strains of *R. erythropolis* and *R. rhodochrous* showed high homology to those of α-NHase nucleotide sequence of *R.* aff. *qingshengii* strain 100A and 100D with 99-100% similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic tree generated from maximum likelihood analyses of α-NHase nucleotide sequence showed that four monophyletic clades were formed (Figure 5). The first clade contains *R. rhodochrous* and *R. erythropolis* ARG AN024 (rainforest soil, Argentine), 871 AN053 (sea sediment, Japan), ARG AN025 (rainforest soil, Argentine), ANT AN007 (lake sediment, Antarctic), DSM 13002 (garden soil, Germany), 67 BEN001 (deep-sea sediment, Japan), DSM 43.006 (soil, unknown) with 65% BS. The second clade contains *R. erythropolis* 871 AN042 (deep-sea sediment, Japan), ENG AN033 (swamp, England) and IND AN014 (mangrove forest, Indonesia). *Rhodococcus* aff. *qingshengii* strain 100A nested in the same clade with *R. erythropolis* 122 AN065 (deep-sea sediment, Japan) with 64%

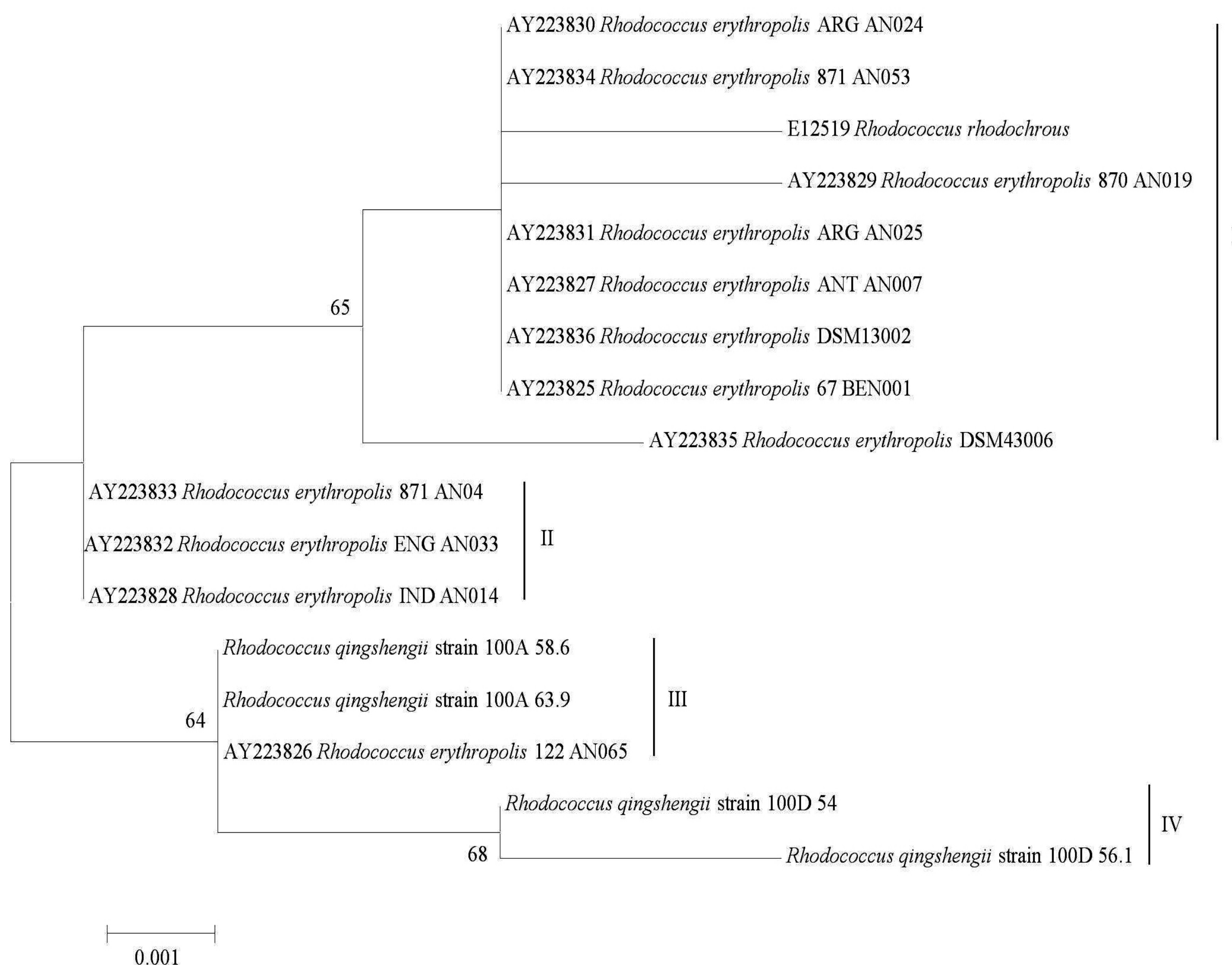

Figure 5. Phylogenetic tree generated from maximum likelihood analyses of α-NHase nucleotide sequence for *R.* aff. *qingshengii* 100A and *R.* aff. *qingshengii* 100D.

BS, while two sequences of *R*. aff. *qingshengii* strain 100D formed independent clade with 68% BS.

In the electrophoresis analyses of amidase gene of *R*. aff. *qingshengii* strain 100A and 100D, about 1900 bp bands were formed. BLAST analyses of amidase nucleotide sequences showed highest similarity with amidase nucleotide sequence belonging to several strains of *R. erythropolis* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree generated from ML analyses based on amidase nucleotide sequences showed that *R*. aff. *qingshengii* strain 100A and 100D nested in the same clade with *Rhodococcus* sp. N774, *Microbacterium* sp. AJ115, *Rhodococcus* sp. N771, *Brevibacterium* sp. strain R312, *R. erythropolis* strain AJ270, and *R. erythropolis* strain AJ300 with 100% BS (Figure 6).

DISCUSSION

Bacterial Growth Assay. Inability of these bacteria in degrading benzonitrile was probably due

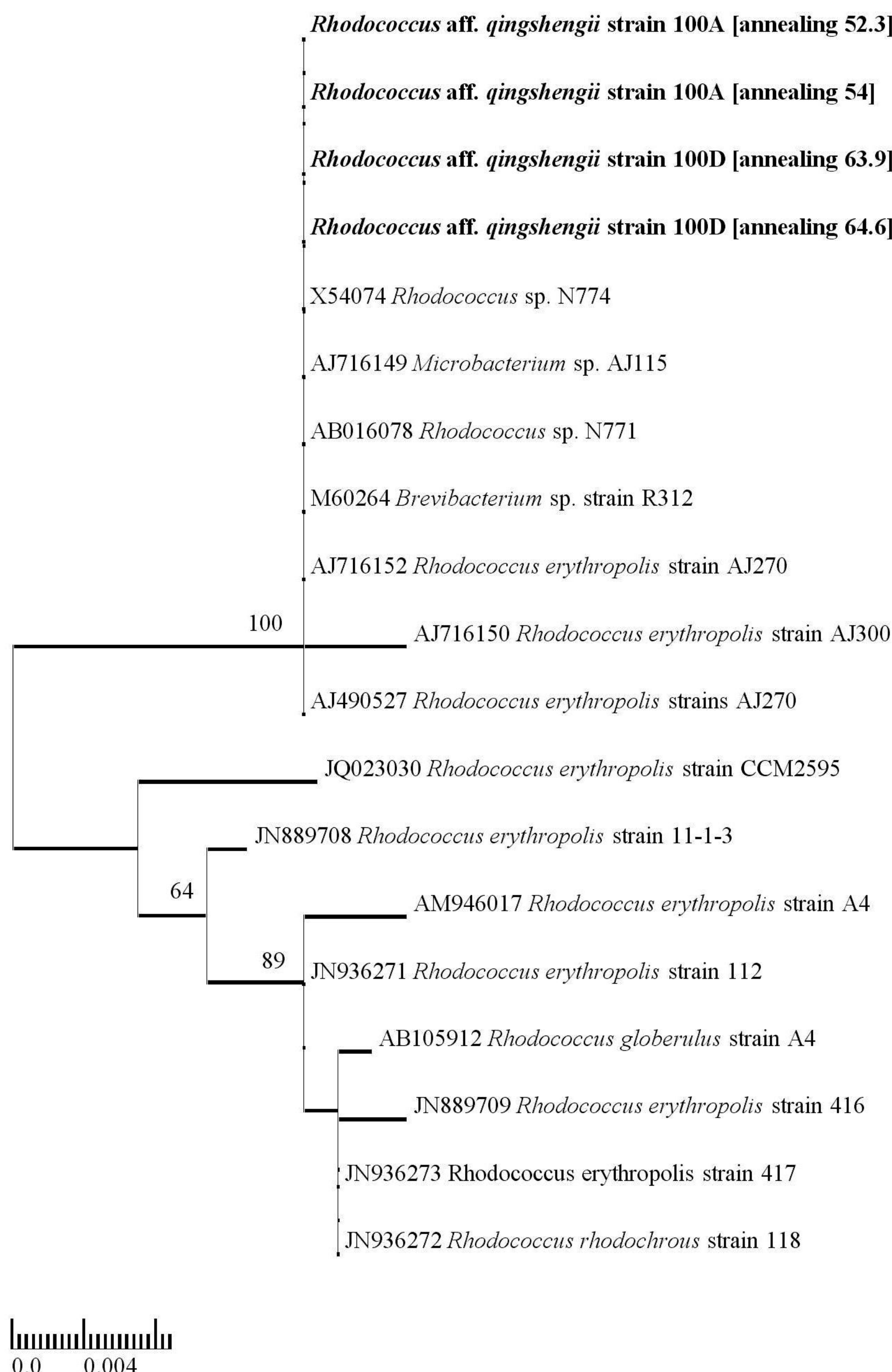

Figure 6. Phylogenetic tree generated from maximum likelihood analyses of amidase nucleotide sequence for *Rhodococcus* aff. *qingshengii* 100A and *Rhodococcus* aff. *qingshengii* 100D.

to the complexity in benzonitrile chemical bond of which contains cyclic structure (Sulistinah & Riffiani 2011). In general, screening of microbial producing NHase is difficult because of gene regulation of NHase is heavily influenced by amide compounds and few species of bacteria are able to produce NHase using selective media (Kobayashi & Shimizu 1998).

During the growth phase, the pH value of medium was changed to alkaline. This was due to the accumulation of ammonia in the medium during bacterial growth. Suhartono (1989) noted that the presence of extracellular enzyme activities and microbial growth in fermentation process often yields by-products that change the pH of the medium. It is interesting to know that *R*. aff. *qingshengii* strain 100A and 100D to the alkaline pH. It is possible that both strains are alkali tolerant, therefore, an increase in the pH value in the medium during the growth phase did not inhibit the growth of *R*. aff. *qingshengii* strain 100A and 100D, and did not inhibit the activity of nitrile degrading enzyme. Until now, there is no evidence that the degradation of nitrile compounds by *Rhodococcus* spp. can be carried out in alkaline conditions. Other than *Rhodococcus* spp., Sorokin *et al*. (2007) reported that *Natronocella acetinitrilica* isolated from soda lakes capable of producing the enzyme NHase, and can grow at pH 10 in medium amended with 3M acetonitrile. Duthaler (1994) stated that the enzymatic hydrolysis reaction of nitrile compounds in alkaline conditions has its own advantages, especially when cyanide compounds contribute in the enzymatic reactions, e.g. Strecker reaction when combined with enzymatic hydrolysis reaction of α-aminonitrile (enantio-selective) will produce α-aminoamida and α-amino acids.

Nitrile Biotransformation by *R*. aff. *qingshengii* Strain 100A and 100D. During the assay of biotransformation of nitrile compounds, acetonitrile was the only carbon and nitrogen source for the growth of *R*. aff. *qingshengii* strain 100A and 100D. This situation caused a decrease in the acetonitrile concentration in the medium because the bacteria will produce enzymes to metabolize the substrate through the process of degradation (Nawaz *et al*. 1989). During the biotransformation process of acetonitrile by *R*. aff. *qingshengii* strain 100A and 100D, amide compounds was produced in the form of acetamide, and carboxylic acid in the form of acetic acid and ammonia. These process involving NHase and amidase enzymes.

In general, the first product formed during the biotransformation process was acetamide. This product was the result of the acetonitriles biotransformation by NHase into amide compound in the form of acetamide, followed by the formation of acetic acid and ammonia as a result of biotransformation of acetamide by amidase enzyme. The biotransformation of nitrile compounds by bacteria usually takes place in two steps, of which the first reaction involves NHase and amidase, whereas the second reaction involves only nitrilase (Kim *et al*. 2001). It was supported by the formation of acetic acid, acetamide and ammonia during the biotransformation process as previously also reported by Asano *et al*. (1982) and Kim *et al*. (2001). Basically, the activity of nitrile compounds biotransformation characterized by the formation of ammonia (Heald *et al*. 2001).

Molecular Characterization of Nitrile Degrading Enzymes (α-NHase and Amidase). Kobayashi *et al*. (1992) reported that the amino acid sequences of the two sub-units are not interconnected and the structural genes of NHase are usually adjacent in the same operon, although the coding sequence of sub-units of α and β are variable.

Multiple alignment analyses of amino acid sequences of *R*. aff. *qingshengii* strain 100A and 100D with several α-NHase nucleotide sequences belonging to *R. erythropolis* obtained from the GenBank database showed a closest similarity between *R*. aff. *qingshengii* strain 100A with *R. erythropolis* 122-AN065. The latter species isolated from deep-sea sediments (Brandão *et al*. 2003). This was supported by the phylogenetic analysis where *R*. aff. *qingshengii* strain 100A nested in the same clade with 64% BS (Figure 5). Although *R*. aff. *qingshengii* strain 100D was isolated from the same source to that of strain 100A, however, the amino acid composition of their α-NHase gene was different. The differences found in the amino acid position 1 where *R*. aff. *qingshengii* strain 100D encodes Glycine[1] (G), while strain 100A encodes Methionine[1] (M) (Figure 7). *Rhodococcus erythropolis* and *R. rhodochrous* encode Methionine[1] (M) at this position. This result showed that there were variations in the amino acid composition of the gene encoding α-NHase on intra- and inter-species. Nevertheless, the significance of amino acid substitutions on the characteristics of α-nitrile hidratase activity is unknown. Pratush *et al*. (2011) reported that the Methionine (M) and Isoleucine (I) characterized hydrophobic properties, and are grouped into aliphatic amino acids. Methionine (M) allegedly has a correlation with functional protein stability at high temperature (55 ^{o}C) and acidic conditions (pH 5.5). Variability in the gene

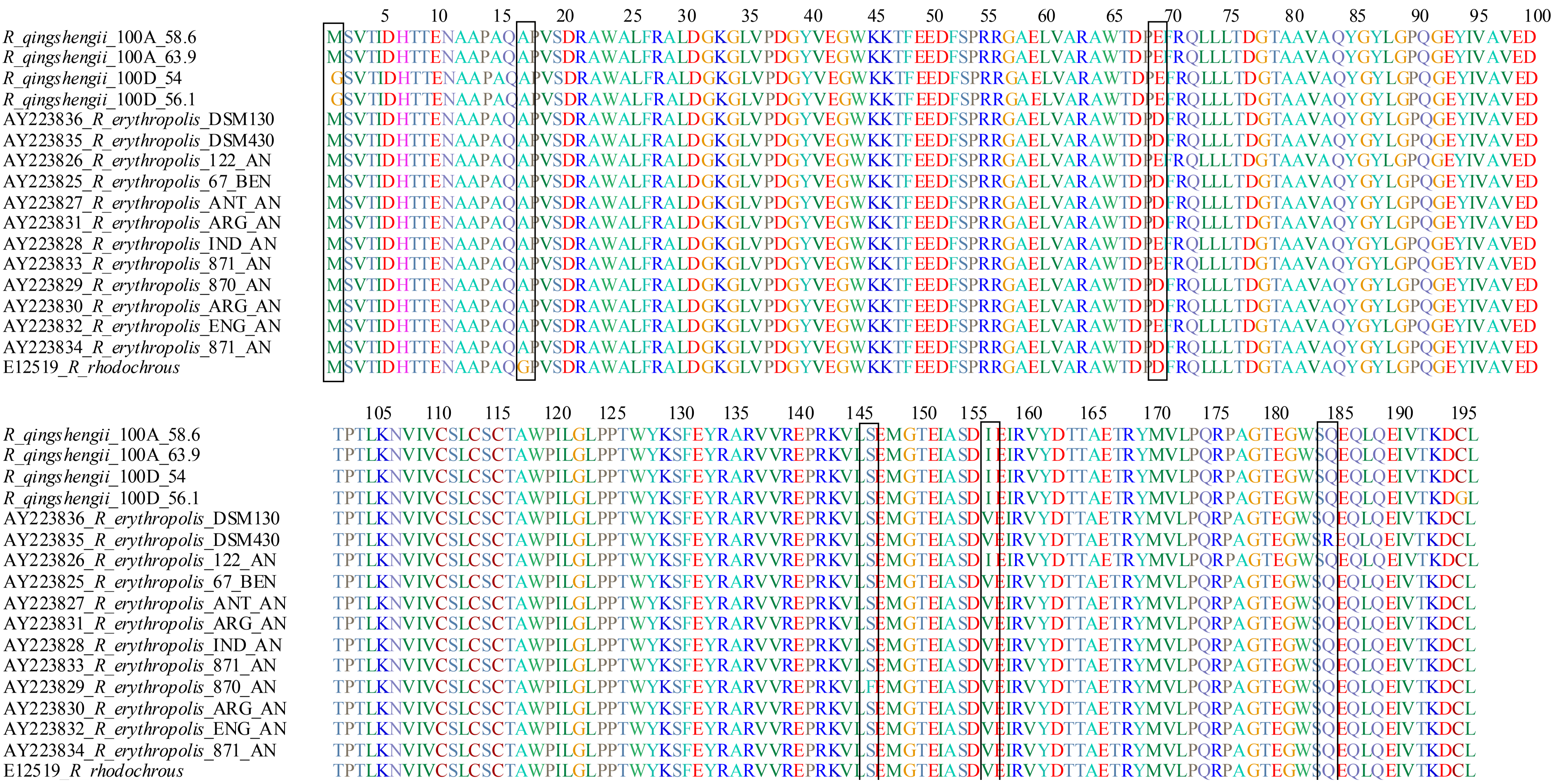

Figure 7. The differences amino acid sequence encoding α-nitrile hidratase of *Rhodococcus* aff. *qingshengii* 100A and *Rhodococcus* aff. *qingshengii* 100D.

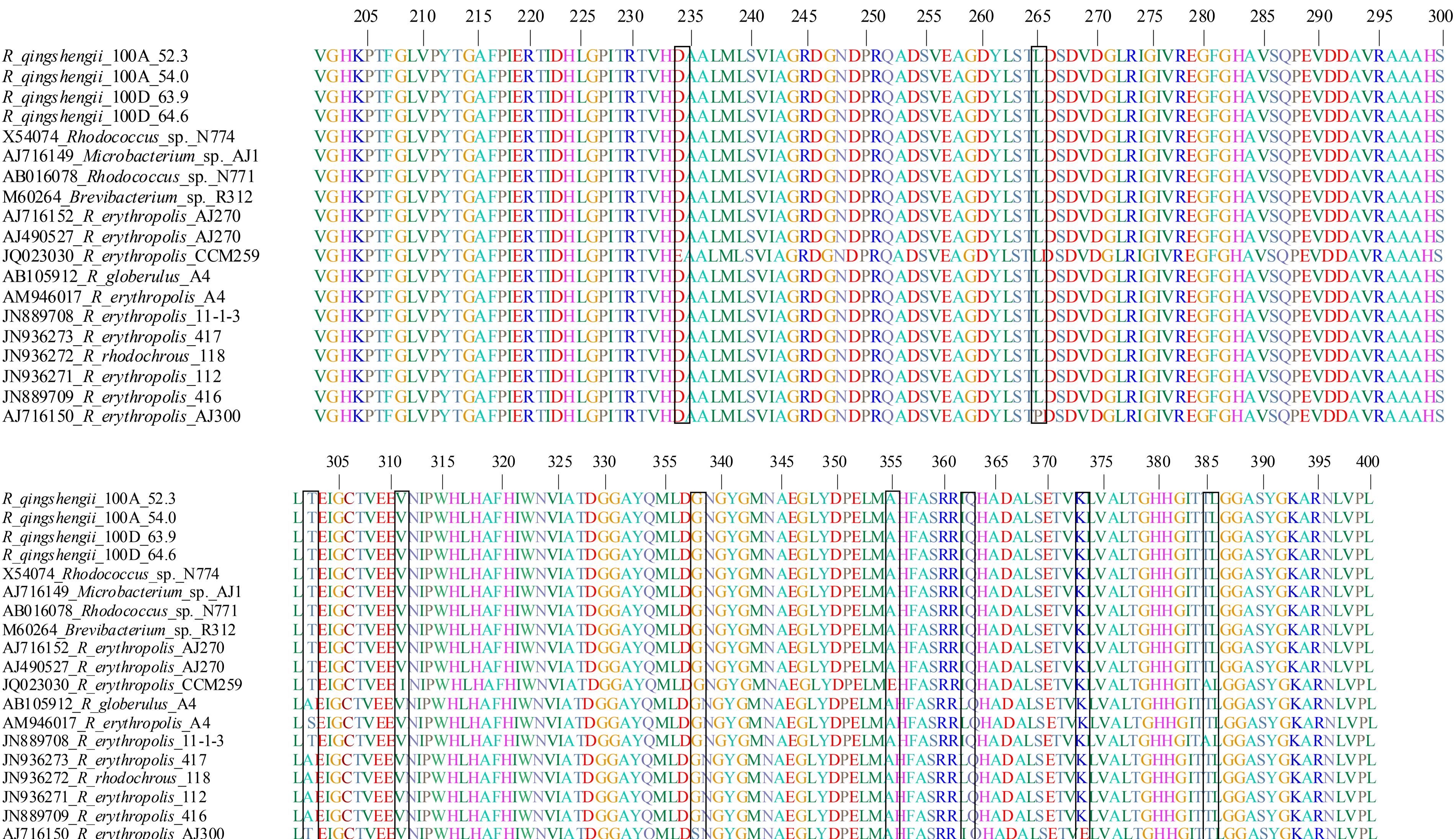

Figure 8. The differences amino acid sequence encoding amidase of *Rhodococcus* aff. *qingshengii* 100A and *Rhodococcus* aff. *qingshengii* 100D.

encoding the amino acid α-NHase also found in *R. erythropolis* (Brandão *et al*. 2003). Nakasako *et al*. (1999) reported that the variability of genes encoding amino acid α-NHase may cause variability in enzymatic activity.

In the multiple alignment analysis of the amino acid sequence encoding amidase (Figure 8) belonging to *R*. aff. *qingshengii* strain 100A and 100D as well as *R. erythropolis* AJ300 obtained from the GenBank database, it was known that the *R*. aff. *qingshengii* strain have three amino acid differences with *R. erythropolis* AJ300. The differences was found in the amino acid position 265 where *R*. aff. *qingshengii* strain 100A and 100D encodes Leucine^{265} (L), but *R. erythropolis* AJ300 encodes Proline^{265} (P). At amino acid position 338 and 373, *R*. aff. *qingshengii* strain 100A and 100D encodes Glycine^{338} (G) and Serine^{338} (S), respectively. However, *R. erythropolis* AJ300 encodes Serine^{338} (S) and Glutamic acid^{338} (E). Molecular characterization of genes encoding nitrile-degrading enzymes is important, because this method is powerful and more sensitive for further discovery and screening of nitrilases-producing bacteria. With the increasing amount of sequence information obtained from the new gene cloning and sequence genome, it allows the prediction of 'conserved' regions to develop specific primer for molecular screening of microorganisms capable of producing α-NHase and amidase, either cultivated or uncultivated. The sequencing results is fundamental for the development of molecular-based screening methods, which is faster and more sensitive than the previous molecular methods using DNA hybridization techniques (Duran *et al*. 1993).

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