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Comparison of Nitrile Hydratases in Rhodococcus Rhodochrous DAP 96253 and DAP 96622 Growing on Inducing and Non-Inducing Media

Fengkun Du
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COMPARISON OF NITRILE HYDRATASES IN RHODOCOCCUS RHODOCHROUS DAP 96253 AND DAP 96622 GROWING ON INDUCING AND NON INDUCING MEDIA

by

FENGKUN DU

Under the Direction of George E. Pierce

ABSTRACT

Nitrile hydratase activity in Rhodococcus rhodochrous DAP 96253 can be induced with multiple inducers that include urea, cobalt (Co), iron (Fe) and nickel (Ni). When induced with Co/urea, cells of R. rhodochrous DAP 96253 expressed the highest level of nitrile hydratase activity (~200 units/min·mg·cdw) when compared with the other inducers tested. Cells induced with Co had the second highest nitrile hydratase activity (~7 units/min·mg·cdw), whereas in the uninduced cells, nitrile hydratase activity was lower than 1 unit/min·mg·cdw. Similarly in R. rhodochrous DAP 96622, when induced with Co/urea, the nitrile hydratase activity of R. rhodochrous DAP 96622 cells was around 50 units/min·mg·cdw which was the highest of all inducers tested. When induced with Co only, the nitrile hydratase activity of R. rhodochrous DAP 96622 was around 20 units/min·mg·cdw, and the nitrile hydratase activity of R. rhodochrous DAP 96622 uninduced was the same as the nitrile hydratase activity of uninduced R. rhodochrous DAP 96253.

When Co/urea induced R. rhodochrous DAP 96253 cell lysate was examined on gradient SDS-PAGE and analyzed by Image Quant TL, the nitrile hydratase bands (both α and β subunits)
accounted for more than 55% of the total cytosolic proteins. Whereas in Co/urea induced *R. rhodochrous* DAP 96622, the nitrile hydratase bands accounted for around 25% of the total cytosolic proteins. According to matrix-assisted laser desorption ionization time-of-flight mass spectrometry results, amidase in *R. rhodochrous* DAP 96253 was approximately 38 kDa from the nitrilase/cyanide hydratase family and amidase in *R. rhodochrous* DAP 96622 was 55 kDa from the amidase signature family.

In addition, the nitrile hydratase regulation system in both *R. rhodochrous* DAP 96253 and DAP 96622 strains are different. Moreover, the nitrile hydratase regulation system in *R. rhodochrous* DAP 96253 is different from *R. rhodochrous* J1.

Purified nitrile hydratase from *R. rhodochrous* DAP 96253 may form a protein complex with glutamine synthetase, resulting in a nitrile hydratase activity of approximately 1500 units/mg-proteins, and nitrile hydratase from *R. rhodochrous* DAP 96622 is not a protein complex and results in a nitrile hydratase activity of 950 units/mg-proteins.

**INDEX WORDS:** *Rhodococcus*, Nitrile hydratase, Nitrilase, Amidase, Glutamine Synthetase
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FENGKUN DU

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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AEC</td>
<td>Anion Exchange Chromatography</td>
</tr>
<tr>
<td>AMD</td>
<td>Acrylamide</td>
</tr>
<tr>
<td>AN</td>
<td>Acrylonitrile</td>
</tr>
<tr>
<td>Co-NHase</td>
<td>Cobalt containing nitrile hydratase</td>
</tr>
<tr>
<td>Fe-NHase</td>
<td>Iron containing nitrile hydratase</td>
</tr>
<tr>
<td>GF</td>
<td>Gel Filtration</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine Synthetase</td>
</tr>
<tr>
<td>H-NHase</td>
<td>High molecular weight nitrile hydratase</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic Interaction</td>
</tr>
<tr>
<td>L-NHase</td>
<td>Low molecular weight nitrile hydratase</td>
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<td>NHase</td>
<td>Nitrile hydratase</td>
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1. INTRODUCTION

1.1. Background

Nitriles have been widely used as solvents, extractants, precursors for synthetic compounds, herbicides, and pesticides (Henahan and Idol, 1971; Ahmed and Farooqui, 1982; Johannsen et al., 1986; Kobayashi et al., 1992b; Kobayashi and Shimizu, 2000; Li et al., 2007). Due to their widespread use, nitriles have been reported in industrial effluents and agricultural runoffs (Wyatt and Knowles, 1995a; Kobayashi and Shimizu, 2000). Most aliphatic nitriles are potentially toxic because they commonly release cyanide (The National Institute for Occupational Safety and Health, 1978; Ahmed and Farooqui, 1982; Beasley and Glass, 1998; Kobayashi and Shimizu, 2000; Cummings, 2004).

One such aliphatic nitrile, acrylonitrile (AN) [2-propenenitrile (CH₂CHCN)], is a colorless liquid at room temperature, often appearing yellow when not pure, and is produced mainly by the Sohio process in industry. The global production of AN was estimated to be over 5 million tons in 2005 (American Chemical Society, 2007). Acrylonitrile is a very important precursor for producing a variety of industrial products, such as synthetic fibers, rubbers and plastics (Baxter et al., 2006). It also is used in the production of acrylamide, which is an important chemical precursor with a global production of over 200,000 tons per year (Alfani et al., 2001).

1.1.1. Adverse environmental and health effects of acrylonitrile

Acrylonitrile is acutely toxic to animals, and is considered to be a potential human carcinogen (Sakurai et al., 1978; U.S. Environmental Protection Agency, 1994; Hazardous Substances Data Bank, 2000; Agency for Toxic Substances and Disease Registry, 2001; National Pollutant Inventory, 2006). Acrylonitrile enters the environment mainly through waste water
effluents generated during its production (Wyatt and Knowles, 1995b). AN can cause severe soil and groundwater contaminations as a result of accidental spillage. Such an incident occurred in Gujarat, India in 2001 when an earthquake caused an AN spill, resulting in surrounding soil contamination (Deshkar et al., 2003). Due to this event, governmental agencies strived to remove and eliminate AN from the soil. However, eight months after the spill occurred, many of these efforts (including natural attenuation) failed to significantly decrease the AN concentration in the soil (Deshkar et al., 2003; Baxter et al., 2006).

1.1.2. Current methods in conversion of nitriles

Industrial waste containing AN is mainly handled by chemical methods, such as ozone treatment. However, chemical treatment is very costly and generates secondary pollutants (Adjei and Ohta, 1999; Li et al., 2007). Bioremediation is considered to be a promising alternative method to degrade AN in the environment (Ryan et al., 1991; Adjei and Ohta, 1999; Li et al., 2007).

In nature, nitriles can be produced by many plants, animal, fungi, bacteria, and algae (Blum and Woodring, 1962; Jones, 1962; Eisner et al., 1963; Dunnill and Fowden, 1965; Conn, 1969; Eyjolfssson, 1970; Conn, 1973; Knowles, 1976; Sykes, 1981; Johannsen et al., 1986; Nagasawa and Yamada, 1989; Legras et al., 1990). Since nitriles are naturally produced, nitrile degraders are also widespread in nature and have been isolated (Harper, 1977a; Legras et al., 1990; Cowan et al., 1998). These microorganisms are in some instances, able to use nitriles as a sole carbon and/or nitrogen source. *Acinetobacter* sp. strain AK226, for example, was reported to carry out biotransformation of 2-(4'-isobutylphenyl)propionitrile (Ibu-CN) to Ibuprofen by nitrilase (Yamamoto et al., 1990). In addition, *Arthrobacter, Corynebacterium, Pseudomonas, Nocardia*, and *Rhodococcus* also have been reported to metabolize nitriles (Asano et al., 1982;
Harper, 1985; Watanabe, 1987; Nawaz et al., 1989; Martínková et al., 1992; Wyatt and Knowles, 1995a; Wyatt and Knowles, 1995b). There are two major pathways for the metabolism of nitriles (Figure 1):

i). Nitriles can be converted to their corresponding acid and ammonium by nitrilase, without forming amide (AMD) (Asano et al., 1980; Kobayashi and Shimizu, 2000);

ii). Nitriles can be converted to amides by nitrile hydratase, and then converted to acid and ammonium by amidase (Asano et al., 1980; Kobayashi and Shimizu, 2000).

Figure 1. Enzymatic conversion of nitriles

1.2. Nitrile hydratase

Nitrile hydratases (NHases) have been studied since the early 1960’s and were known as “nitrilases” for over a decade (Hook and Robinson, 1964). Acetonitrile hydratase was first reported as “nitrile hydratase” in Arthrobacter sp J-1, which is responsible for converting acetonitrile to acetamide (Asano et al., 1980). Due to their ability to transform nitriles into corresponding amides, NHases have been widely used for several decades in the chemical industry as the catalyst to produce amides (Yamada et al., 1986; Nagasawa and Yamada, 1989; Yamada and Kobayashi, 1996). Three generations of NHases have been reported in industry for producing acrylamide: the first generation of catalysts was from Rhodococcus sp. N-774, Rhodococcus sp. N-771 and Rhodococcus. sp. R312; the second generation was from
*Pseudomonas chlororaphis* B23, and the third generation was from *Rhodococcus rhodochrous* J1 (Watanabe *et al.*, 1987a; Watanabe *et al.*, 1987b; Mayaux *et al.*, 1990; Kobayashi and Shimizu, 1998). NHases from *Rhodococcus* sp. R312, *Rhodococcus* sp. N-774 and *Rhodococcus* sp. N-771 are identical in amino acid sequence, with a molecular mass of approximately 85 kDa; however, the quaternary structure differs among the various species. The first generation NHases are composed of hetero-tetramers containing two α and two β subunits—α2β2 (Nagasawa *et al.*, 1986; Huang *et al.*, 1997; Kobayashi and Shimizu, 1998). The α subunit is 26 kDa, while the β subunit is 27.5 kDa, and one iron atom is contained per αβ hetero-dimer (Nagasawa *et al.*, 1986; Huang *et al.*, 1997). The NHase from *P. chlororaphis* B23, is about 100 kDa, and consists of four subunits, each with an identical molecular mass. It was also noted that for 1 mole of enzyme (NHase), 4 moles of Fe(III) are present (Nagasawa *et al.*, 1987). The first and second generations of NHases are ferric NHases containing non-heme, low-spin Fe(III) as the active center, and both enzymes have a preference for aliphatic nitriles with three to six carbons (Nagasawa *et al.*, 1987; Nagasawa and Yamada, 1989). The third generation catalyst, a cobalt-containing NHase, was first reported in *R. rhodochrous* J1 (Nagasawa *et al.*, 1988; Nagasawa *et al.*, 1991). Two types of NHases are produced by *R. rhodochrous* J1: high-molecular-mass NHase (H-NHase) and low-molecular-mass NHase (L-NHase) (Kobayashi *et al.*, 1991; Nagasawa *et al.*, 1991). H-NHase has a molecular mass of 500-530 kDa, containing 10αβ hetero-dimers (α10β10), and is induced by cobalt, amide and urea, (Kobayashi *et al.*, 1991; Kobayashi and Shimizu, 1999). Additionally, H-NHases have a preference for aliphatic nitriles as the substrate (Kobayashi *et al.*, 1991). L-NHase has a molecular mass of 100-130 kDa, containing 2 αβ hetero-dimers (α2β2), and is induced by cyclohexanecarboxamide and cobalt (Kobayashi and Shimizu, 1998; Wieser *et al.*, 1998). The α subunit and the β subunit in both H-
NHase and L-NHase are 26 kDa and 29 kDa, respectively (Kobayashi et al., 1991). Cobalt is necessary for the enzymatic activity of both H- and L-NHase in *R. rhodochrous* J1 but is not required for the expression of the NHase genes, as demonstrated when H-NHase gene was cloned and expressed in *Escherichia coli* (Yamada and Kobayashi, 1996). Both NHases from *R. rhodochrous* J1 contain non-corrin cobalt as the active center, and one Co$^{3+}$ is contained in each $\alpha\beta$ hetero-dimer (Brennan et al., 1996; Wieser et al., 1998; Sari et al., 2007). Cobalt-NHases also have been reported in *R. rhodochrous* M8 (Pogorelova et al., 1996). In the NHase produced by *R. rhodochrous* J1 and *R. rhodochrous* M8, cobalt cannot be substituted by other metals (Pogorelova et al., 1996; Yamada and Kobayashi, 1996). Aside from cobalt NHases that have been reported in *R. rhodochrous* J1 and *R. rhodochrous* M8, it also has been reported that *R. rhodochrous* DAP 96253 and DAP 96622 produce cobalt NHase when cultured in carbohydrate rich media containing cobalt, urea, and an inducer cocktail that contains acetonitrile, AN, succinonitrile, fumaronitrile and AMD (Pierce, 1999; Pierce, 2000; Ganguly, 2007; Tucker, 2008). Previous studies have shown that when other metals such as iron, magnesium, and manganese are substituted for cobalt, the NHase activity in *R. rhodochrous* DAP 96622 and DAP 96253 was significantly lower than that seen in cobalt induced NHases (Tucker, unpublished data). When induced by iron, the NHase activity is significantly higher than the NHase activity measured without metal induction. When induced by magnesium and manganese, however, the NHase activity is quite similar to that with no metal induction (Tucker, unpublished data). As both the active center and inducer for NHase, cobalt also enhances the level of NHase transcription (Pogorelova et al., 1996; Wieser et al., 1998; Pierce, 1999; Pierce, 2000). The effects of Co and Fe on NHase activity have, however, not been well established in *R. rhodochrous* DAP 96622 and DAP 96253.
1.3. Amidase

Microorganisms like *Pseudomonas* spp., utilizing amides as their sole carbon and nitrogen sources have been reported as early as 1926 by Den Dooren de Jong (Den Dooren de Jong, 1926; Kelly and Clarke, 1962). Amidases (EC 3.5.1 and 3.5.2) hydrolyze carboxylic acid amides to carboxylic acids and ammonia (Figure 2) (Chebrou *et al.*, 1996; Pertsovich *et al.*, 2005). Amidases have been categorized into four families: amidase signature family (GGSS motif in the primary structure), urease, nitrilase/cyanide hydratase and acyl transferases (Chebrou *et al.*, 1996; d’Abusco *et al.*, 2005; Pertsovich *et al.*, 2005).

$$\text{O} \quad \text{R-C-NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{Amidase}} \text{O} \quad \text{R-C-O}^- + \text{NH}_4^+$$

Figure 2. Catalytic conversion of amide by amidase

were reported as 105-120 kDa with the subunit molecular mass of approximately 55 kDa (Mayaux et al., 1990; Kobayashi et al., 1993; Ciskanik et al., 1995; Kobayashi et al., 1997; Fournand et al., 1998; d’Abusco et al., 2001; d’Abusco et al., 2005; Littlechild, 2011). Amidases from *R. erythropolis* MP50 and *S. solfataricus* MT4 were reported as homooctamers (Hirrlinger et al., 1996; d’Abusco et al., 2001; d’Abusco et al., 2005). Amidase from *R. erythropolis* MP50 was reported as a holoenzyme of 480-500 kDa and the subunit was approximately 61 kDa on an SDS-PAGE gel (Hirrlinger et al., 1996). Similarly, the native state amidase from *S. solfataricus* MT4 was proved to be approximately 400 kDa and the subunit was around 56 kDa (d’Abusco et al., 2001). This homooctomer amidase from *S. solfataricus* MT4 consisted of homodimers (~110 kDa) and the assembly of the octamer by the dimers was pH-dependent (d’Abusco et al., 2005). Amidases from this amidase signature family were reported as wide-spectrum amidases acting on both aliphatic and aromatic amidases (Mayaux et al., 1990; Kobayashi et al., 1993; Ciskanik et al., 1995; Hirrlinger et al., 1996; Kobayashi et al., 1997; Fournand et al., 1998; d’Abusco et al., 2001; d’Abusco et al., 2005; Littlechild, 2011). Some amidases, such as that from *S. solfataricus* MT4, were able to use nitriles as the substrate with extremely low activity (Cilia et al., 2005). Most amidases, as mentioned above, from this amidase signature family cannot utilize urea as their substrate (Kobayashi et al., 1993; Ciskanik et al., 1995; Fournand et al., 1998). The amidase from *R. erythropolis* MP50 can use urea as the substrate, however, the activity was reported to be very low (Hirrlinger et al., 1996).

Amidases from the nitrilase/cyanide hydratase family were reported from *Rhodococcus* sp. R312 (multiple amidases have been reported from this strain and this particular amidase was identified as acylamide amidohydrolase, EC 3.5.1.4), *R. rhodochrous* M8, *P. aeruginosa*, *Bacillus stearothermophilus*, *Methylophilus methylotrophus* and others (Clarke, 1972; Silman et
Amidases from this nitrilase/cyanide hydratase family were reported to be in the form of homotetramer or homohexamer (Pertsovich et al., 2005). Most native state amidases that are homotetramer have a molecular mass of approximately 150 kDa (Thiéry et al., 1986; Silman et al., 1989; Silman et al., 1991; Soubrier et al., 1992; Kotlova et al., 1999; Cheong and Oriel, 2000; Pertsovich et al., 2005). The native state amidase produced in *P. aeruginosa* was observed as a homohexamer with a molecular mass of 200 kDa (Kelly and Clarke, 1962; Ambler et al., 1987; Brammar et al., 1987; Novo et al., 1995). The molecular mass of the subunit of the amidases from this nitrilase/cyanide hydratase family was reported to be approximately 38–43 kDa (Kelly and Clarke, 1962; Thíery et al., 1986; Ambler et al., 1987; Brammar et al., 1987; Silman et al., 1989; Silman et al., 1991; Soubrier et al., 1992; Novo et al., 1995; Kotlova et al., 1999; Cheong and Oriel, 2000; Pertsovich et al., 2005). Most amidases from this family prefer short chain aliphatic amides (C5 or shorter), although some amidases, such as an amidase from *B. stearothermophilus*, from this family showed very low activity against aromatic amides (benzamide) (Kelly and Clarke, 1962; Clarke, 1972; Clarke, 1984; Maestraci et al., 1984; Thíery et al., 1986; Mayaux et al., 1990; Silman et al., 1991; Soubrier et al., 1992; Novo et al., 1995; Kotlova et al., 1999; Cheong and Oriel, 2000; Pertsovich et al., 2005).

Amidase from *Mycobacterium tuberculosis* (acetamidase) was reported from the urease family (Mahenthiralingam et al., 1993; Chebrou et al., 1996). The molecular mass of this amidase was reported to be approximately 47 kDa on the SDS-PAGE (Mahenthiralingam et al., 1993).
1.4. Nitrilase


Nitrilases (EC 3.5.5.1) belong to the nitrilase/cyanide hydratase family, which also contains cyanide hydratases, cyanide dihydratases and aliphatic amidases, although the aliphatic amidases are not closely related with other members of this family (Novo *et al.*, 1995; O’Reilly and Turner, 2003). This nitrilase/cyanide hydratase family further belongs to nitrilase superfamily which contains thirteen branches (Pace and Brenner, 2001; Singh *et al.*, 2006). These thirteen branches are nitrilase, aliphatic amidase, amino-terminal amidase, biotinidase, b-ureidopropionase, carbamylase, prokaryote NAD synthetase, eukaryote NAD synthetase, apolipoprotein N-acyltransferase, Nit and NitFhit, NB11, NB12, and nonfused outliers (Pace and Brenner, 2001; Singh *et al.*, 2006). Nitrilases (EC 3.5.5.1), cyanide hydratases and cyanide dihydratases belong to the nitrilase branch (Pace and Brenner, 2001).
Native state nitrilases from different species were reported to contain 2, 6, 8, 10, 12, 14, 16 and 26 subunits depending on the species (Harper, 1977a; Harper, 1977b; Harper, 1985; Stalker et al., 1988; Kobayashi et al., 1989; Kobayashi et al., 1990; Yamamoto and Komatsu, 1991; Bhalla et al., 1992; Stevenson et al., 1992; Lévy-Schil et al., 1995; Almatawah et al., 1999; Dhillon et al., 1999; Nagasawa et al., 2000; O’Reilly and Turner, 2003). The size of nitrilase ranges from 74 to 880 kDa on non-denaturing PAGE and the molecular size for the subunits of most nitrilases ranges from 37 to 45 kDa (Harper, 1977a; Harper, 1977b; Harper, 1985; Stalker et al., 1988; Kobayashi et al., 1989; Kobayashi et al., 1990; Yamamoto and Komatsu, 1991; Bhalla et al., 1992; Stevenson et al., 1992; Lévy-Schil et al., 1995; Almatawah et al., 1999; Dhillon et al., 1999; Nagasawa et al., 2000; O’Reilly and Turner, 2003). Nitrilases from B. pallidus, F. oxysporum f.sp. melonis, F. solani IMI196840, Nocardia sp. NCIB 11216 and NCIB 11215, R. rhodochrous ATCC 39484, and R. rhodochrous J1 were reported as aromatic nitrilases (Harper, 1977a; Harper, 1977b; Harper, 1985; Goldlust and Bohak, 1989; Kobayashi et al., 1989; Stevenson et al., 1992; Almatawah et al., 1999; Nagasawa et al., 2000; O’Reilly and Turner, 2003). The aromatic nitrilases show no or very low activity against aliphatic nitriles and cyanide (Harper, 1977a; Harper, 1977b; Harper, 1985; Goldlust and Bohak, 1989; Kobayashi et al., 1989; Stevenson et al., 1992; Almatawah et al., 1999; Nagasawa et al., 2000; O’Reilly and Turner, 2003). Nitrilases from Alcaligenes faecalis JM3, Alcaligenes faecalis ATCC 8750 and P. fluorescens DSM 7155 were reported as acylacetonitrilases and these two nitrilases show very high activity against phenylacetonitrile but very low or no activity against other nitriles (both aromatic and aliphatic nitriles) (Nagasawa et al., 1990; Yamamoto et al., 1992; O’Reilly and Turner, 2003). Aliphatic nitrilases were reported in R. rhodochrous K22, Acidovorax facilis 72W, C. testosterone, Pseudomonas sp. (S1) and Acinetobacter sp. AK226 (Kobayashi et al.,
1990; Yamamoto and Komatsu, 1991; Lévy-Schil et al., 1995; Dhillon et al., 1999; Dhillon and Shivaraman, 1999; Gavagan et al., 1999). Aliphatic nitrilases have high activity against aliphatic nitriles but none against aromatic nitriles (Kobayashi et al., 1990; Yamamoto and Komatsu, 1991; Lévy-Schil et al., 1995; Dhillon et al., 1999; Dhillon and Shivaraman, 1999; Gavagan et al., 1999). Aliphatic nitrilase from Acinetobacter sp. AK226 can act on both aliphatic and aromatic nitriles but acts with a higher activity against aliphatic amides (Yamamoto and Komatsu, 1991).

1.5. Glutamine synthetase

Glutamine synthetase (GS; EC 6.3.1.2), an important enzyme in nitrogen assimilation, converts ammonia and glutamate to glutamine with the hydrolysis of ATP (Figure 3) (Kumada et al., 1993).

Figure 3. Glutamine synthetase catalyzed reaction (Merrick and Edwards, 1995).

Due to the fact that GS binding to ATP initiates the reaction from glutamate to glutamine and the structure of Blue Sepharose resin functioning group is similar with ATP, ADP and NADP, GS purification was reported using affinity chromatography (Blue Sepharose) (Brun et al., 1992).

Three types of GS have been reported: GS Type I was reported to be a homodecamer with each subunit approximately 55 kDa. GSI was widely reported in bacteria and it has not been found in eukaryotes (Kumada et al., 1993; Amaya et al., 2005). GS type II was found in both prokaryotes (symbiotic bacteria with plants) and eukaryotes. GSII was reported to be a
homooctamer with the subunit size of about 36 kDa (Amaya et al., 2005). GS type III was reported as a homohexamer in anaerobic bacteria and cyanobacteria, with a subunit of around 75 kDa (Amaya et al., 2005).

The glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway is a very important pathway among bacteria (Merrick and Edwards, 1995). After glutamate and ammonia are converted into glutamine by GS (Figure 3), the amide group from glutamine is transferred by GOGAT to α-ketoglutarate forming two molecules of glutamate (Figure 4) (Merrick and Edwards, 1995). The purpose of the GS/GOGAT pathway is to incorporate inorganic nitrogen-ammonia with α-ketoglutarate to form glutamate for form biomass (Berlicki, 2008).

![Figure 4. Glutamate synthetase catalyzed reaction (Merrick and Edwards, 1995).](image)

Six subunits of GSI form a ring structure and two rings form a face-to-face active GSI enzyme. An active bifunnel active site is formed between two monomers and 12 active sites are formed per enzyme (Figure 5) (Eisenberg et al., 2000).

GSI is inhibited by serine, alanine, glycine, AMP, CTP, tryptophan, histidine, carbamoyl phosphate and glucosamine-6-phosphate (Eisenberg et al., 2000). These nine compounds are the end products of glutamine metabolism (Eisenberg et al., 2000). Each of the nine inhibitors can partially inhibit GSI activity and the combination of all nine inhibitors can cause the loss of almost all enzyme activity (Eisenberg et al., 2000).
1.6. Understanding *R. rhodochrous* DAP 96253 and DAP 96622

Members of the genus *Rhodococcus* are non-motile, non-sporulating aerobic bacteria (van der Geize and Dijkhuizen, 2004). The genus *Rhodococcus* is closely related with *Nocardia, Corynebacterium, Mycobacterium* and some species from *Rhodococcus* were once classified under these genera (Goodfellow and Alderson, 1977). *Rhodococcus* was first used as a genus name in 1891 (Bell *et al.*, 1998). Rhodococci contain mycolic acids and belong to suprageneric mycolata taxon (Goodfellow, 1992). They are rod-shaped or cocci cells forming limited mycelium with branching side projections (Goodfellow, 1989). Most *Rhodococcus* species are able to form colonies that are pink, orange or red due to the production of carotenoids (Warhurst and Fewson, 1994). *Rhodococcus* species were reported from a variety of natural environments that contain animals, plants, animal feces, soil, rocks, groundwater and sea water (Bell *et al.*, 1998).
Microorganisms from the genus *Rhodococcus* exhibit diverse metabolic capabilities, rhodococci were reported to utilize xenobiotics such as hydrocarbons, chlorinated organic compounds, steroids, s-triazines and lignin due to the fact that rhodococci were indigenous species contaminated environments (Bell et al., 1998). In addition, rhodococci were reported to produce biosurfactants and biofloculants (Finnerty, 1992; Kurane and Tomizuka, 1992; Kurane et al., 1994; Bell et al., 1998). Cellular biosurfactants such as mycolic acids were reported to enhance the adherence of cells to hydrophobic pollutants and facilitate the cellular entry of the hydrophobic compounds (Fiechter, 1992). Moreover, the extracellular biosurfactant from rhodococci were able to increase the available surface area by dispersing the hydrophobic pollutant (Finnerty, 1992). In addition to biodegradation, *Rhodococcus* species were reported to bioaccumulate heavy metals such as radioactive metals, and desulphurize coal and crude oil (Kilbane, 1989; Tomioka et al., 1994; Gray et al., 1996; Bell et al., 1998). The metabolic diversity among rhodococci can be attributed to the presence of both a large genome and large linear plasmids (van der Geize and Dijkhuizen, 2004). The genome of *Rhodococcus* sp. strain RHA1 was reported to be approximately 10 Mb containing three linear plasmids and the largest reported was 1100 kb (van der Geize and Dijkhuizen, 2004). A number of biodegradation genes were found on the plasmids (van der Geize and Dijkhuizen, 2004).

1.7. Objectives:

The first aim of this research was to investigate the effects of Co, Fe and Ni on NHase activity of both *R. rhodochrous* DAP 96253 and DAP 96622. Both Co and Fe have been reported as the active center for different NHases, and Ni is close to Co in the Periodic Table. For this purpose, both *R. rhodochrous* DAP 96253 and DAP 96622 were induced with Co/urea, Co, Fe/urea, Fe, Ni/urea, Ni and urea, and the NHase activities were then evaluated. The potential
differences in the protein profiles when induced under various conditions were studied by an 8-20% linear gradient SDS-PAGE. The NHase/cytosolic protein ratios were also compared under these conditions. It was also hypothesized that NHase from both strains were similar, but the amount of NHase expressed in the cells affected the NHase activity.

The second aim of this research was to investigate other enzymes possibly involved in nitrile hydrolysis. To this end, cytosolic protein profiles from cells induced with Co/urea from both strains were compared. Proteins bands that were expressed differently between both cells were extracted and identified by Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry (MALDI TOF/TOF MS). It is hypothesized that other enzymes from \textit{R. rhodochrous} DAP 96253 and DAP 96622 were different and this caused the expression level of NHase to be different in both strains.
2. MATERIALS AND METHODS

2.1. Media and Organisms

1) Yeast Extract Malt Extract Agar (YEMEA) uninduced (UI)- 4g yeast extract (Becton Dickinson, Sparks, MD), 10g malt extract, 4g glucose and 20g agar (Difco laboratories, Sparks, MD) in 1L deionized water.

2) YEMEA Co/urea (Co/urea) - 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD), cobalt (CoCl$_2$·6H$_2$O, 0.201g/L, J.T. Baker Chemical Co, Phillipsburg, NJ,) iron (FeCl$_3$·6H$_2$O, 0.228g/L, Sigma Chemical Company) and urea (7.5g/l, Sigma, St Louis, MO) in 900ml deionized water.*

3) YEMEA Co (Co)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD) and cobalt (CoCl$_2$·6H$_2$O, 0.201g/L, J.T. Baker Chemical Co, Phillipsburg, NJ,) in 1L deionized water.

4) YEMEA Fe/urea (Fe/urea)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD), iron (FeCl$_3$·6H$_2$O, 0.228g/L, Sigma Chemical Company) and urea (7.5g/l, Sigma, St Louis, MO) in 900ml deionized water.*

5) YEMEA Fe (Fe)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD) and iron (FeCl$_3$·6H$_2$O, 0.228g/L, Sigma Chemical Company) in 1L deionized water.

6) YEMEA Ni/urea (Ni/urea)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD), nickel (NiCl$_3$·6H$_2$O, 0.201g/L, Sigma-Aldrich) and urea (7.5g/l, Sigma, St Louis, MO) in 900ml deionized water.*

7) YEMEA Ni (Ni)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD), nickel (NiCl$_3$·6H$_2$O, 0.201g/L, Sigma-Aldrich) in 1L deionized water.

8) YEMEA urea (Ni/urea)- 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco
laboratories, Sparks, MD) and urea (7.5g/l, Sigma, St Louis, MO) in 900ml deionized water.*

9) YEMEA Co/acrylamide: 4g yeast extract, 10g malt extract, 4g glucose, 20g agar (Difco laboratories, Sparks, MD), cobalt (CoCl₂·6H₂O, 0.201g/L, J.T. Baker Chemical Co, Phillipsburg, NJ) and acrylamide (8.89g/l, Sigma, St Louis, MO) in 900ml deionized water.*

10) YEMEA urea/isovaleronitrile (urea/isovaleronitrile): Due to the fact that isovaleronitrile is a volatile, flammable and toxic compound, a biphasic medium was selected with 300ml agar and 150ml broth. 300ml agar was autoclaved in a 500ml flask: 1.2g yeast extract, 3g malt extract, 1.2g glucose, 6g agar (Difco laboratories, Sparks, MD) and urea (7.5g/l, Sigma, St Louis, MO) in 300ml deionized water (Urea was filtered into the agar). 150ml broth was autoclaved, cooled to room temperature and added on the top of the agar: 0.6g yeast extract, 1.5g malt extract, 0.6g glucose and urea (7.5g/l, Sigma, St Louis, MO). 450mg isovaleronitrile was filtered into the medium after inoculation. Isovaleronitrile was added into the medium every 2 days with an amount of 450mg.*

* Urea or acrylamide were dissolved in 100 ml DI water first and then filter-sterilized into the corresponding YEMEA after autoclave. Isovaleronitrile was directly filtered into the biphasic media.

The organisms screened were *R. rhodochrous* DAP 96253 and DAP 96622.

### 2.2. Chemicals and Solutions

#### 2.2.1. Sodium phenate solution

25 g phenol (Sigma- Aldrich Co., St. Louis, MO) was melted in a water bath and added to 800 ml 55 °C DDI water. 78 ml 4N sodium hydroxide (Sigma- Aldrich Co., St. Louis, MO) solution was added into the phenol solution and mixed by stirring (Fawcett and Scott, 1960). The sodium phenate solution was good for one month stored at 4 °C in the dark.
2.2.2. Sodium nitroferricyanide solution (0.01%)

Sodium nitroferricyanide stock solution was prepared by adding 1g of sodium nitroferricyanide (Sigma-Aldrich Co., St Louis, MO) into 99 ml DI water. Sodium nitroferricyanide stock solution was good for one month stored at 4 °C in the dark (Fawcett and Scott, 1960). Working solution was made by adding 1 ml of stock solution into 99ml DI water (Fawcett and Scott, 1960).

2.2.3. Sodium hypochlorite (0.02N)

Sodium hypochlorite working solution was made by adding 2.44 ml of commercial 6.15% Chlorox into DDW water to make a total volume of 100ml (Fawcett and Scott, 1960). Sodium hypochlorite was made fresh each time.

2.3. Methods:

2.3.1. R. rhodochrous DAP 96253 and DAP 96622 cultivation

Cultures were prepared by adding 1ml R. rhodochrous DAP 96253 or DAP 96622 glycerol stock to 50ml Nutrient Broth (Difco, Sparks MD). The above cultures were grown for 2 days at 30°C, 45 rpm. Nutrient agar (Difco, Sparks MD) plates were inoculated with the above nutrient broth culture and grown for 2 days at 30°C. These nutrient agar plates were used to inoculate different YEMEA plates with an approximate ratio of 1 nutrient agar to 4 YEMEA plates. YEMEA plates were incubated at 30°C for approximately 7 days.

The biphasic medium was inoculated with R. rhodochrous DAP 96253 from 1 nutrient agar plate. After inoculation, 450mg (566 µl) isovaleronitrile was filtered into the biphasic medium. The biphasic medium was cultured at room temperature on a shaker (Innova™ 2000 Platform Shaker, New Brunswick Scientific) at a speed of 60 rpm for 10 days.
2.3.2. NHase Assays for Whole Cells

NHase was quantified using 1000mg/L or 5000mg/L (v/v) standard solutions of testing nitrile (Table 1, Table 2) (Sigma-Aldrich Corporation, St. Louis, MO); individual standard solutions (9 ml) were added into 15 ml Falcon tubes. A YEMEA plate was scraped to obtain 1-300 mg of cells (wet wt.) (Table 1, Table 2) and then the cells were suspended into 1 ml of 50mM sodium phosphate buffer (pH 7) in a microcentrifuge tube (for Co/urea induced cells, approximately 40mg of cells were harvested from the plate and proper dilution was made to bring the cell suspension down to 1mg/ml). Then, 1ml of the cell suspension was combined with 9 ml nitrile standard solution. This suspension was vortexed for approximately 1.5 min to allow the cells to react with acrylonitrile, 1.3 ml suspension was taken into a new microcentrifuge tube and centrifugation was started when the reaction time was 2 min. The reaction was stopped by centrifuging at 13,200 rpm for 2 min. 1ml supernatant was immediately transferred to a 1.5 ml microcentrifuge tube pre-loaded with 10 μl of 2M sulfuric acid (Fisher Chemical, Fairlawn, NJ), this was mixed for 1 min, and then 10 μl of 4N sodium hydroxide (Fisher Scientific, Fairlawn, NJ) was added to neutralize the acid. Duplicate samples with the volume of 1.3 ml were placed in two separate 1.5 ml Eppendorf tubes and centrifuged (Beckman Microfuge Lite, Palo Alto, CA) for 2 min at 13,000 rpm (approximately 16,000g). The supernatant was transferred into clean Eppendorf tubes.

Commercial amidase (1000 units/ 440 μl, stored at -20°C) (Sigma-Aldrich Corporation, St. Louis, MO) was added into above supernatant to complete the conversion from amide to acid and ammonia. A working amidase solution with a concentration of 0.25 unit/μl was prepared by adding 1 volume of the original amidase solution to 49 volume of double deionized water. 10 μl (2.5 units) of the above working solution was added to each Eppendorf tube and vortexed for 30
sec. These Eppendorf tubes were incubated at 37°C for 30 min.

Ammonia was determined by modification of the method by Fawcett and Scott (1960). After 30 min incubation, the solution was transferred to 15 ml glass test tubes. Then, 2 ml of sodium phenate, 3 ml 0.1% aqueous sodium nitroferricyanide (Sigma-Aldrich Corporation, St. Louis, MO) and 3 ml 0.02N sodium hypochlorite (Clorox, 6.15%) were consecutively added into each test tube. The test tubes were mixed by vortexing lightly. The test tubes were incubated in the dark at 27°C for 30 min to complete color reaction. The test tubes were vortexed and color development of the above solutions were determined by OD reading at 630 nm for 10 sec using a microplate reader (Wallac Victor 1420 multilabel counter, Perkin Elmer Life Sciences, Shelton, CT). Triplicates were prepared from each sample for OD reading and the data was averaged.

One unit of NHase is defined as the conversion of 1 μM of AN per minute per mg dry weight of cells at 30 °C, pH 7.
Table 1. NHase assay of *R. rhodochrous* DAP 96253 and DAP 96622 cell weight, substrate concentration (acrylonitrile, acetonitrile) and dilution factors before adding commercial amidase.

<table>
<thead>
<tr>
<th>Cell Samples</th>
<th>Cell Wet Weight (mg)</th>
<th>Nitrile Concentration (mg/L)</th>
<th>Dilution Factor Before Adding Amidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co/urea</td>
<td>~1*</td>
<td>5000</td>
<td>1:25</td>
</tr>
<tr>
<td>Co</td>
<td>15-25</td>
<td>5000</td>
<td>1:25</td>
</tr>
<tr>
<td>Fe/urea</td>
<td>~12</td>
<td>1000</td>
<td>1:5</td>
</tr>
<tr>
<td>Fe</td>
<td>~50</td>
<td>1000</td>
<td>1:5</td>
</tr>
<tr>
<td>Ni/urea</td>
<td>~25</td>
<td>1000</td>
<td>1:5</td>
</tr>
<tr>
<td>Ni</td>
<td>~300</td>
<td>1000</td>
<td>1:5</td>
</tr>
<tr>
<td>Urea</td>
<td>~50</td>
<td>1000</td>
<td>1:5</td>
</tr>
<tr>
<td>Uninduced</td>
<td>50-100</td>
<td>1000</td>
<td>1:5</td>
</tr>
</tbody>
</table>

Note: The amount of cells, substrate concentration and dilution factors were determined from pre-tests with 10% substrate depletion.

*Note: Approximately 40mg of cells were harvested from the plate and diluted to 1mg/ml with 50mM phosphate buffer/pH 7.2. When carrying out NHase assay, 1ml of cell suspension was used.

Table 2. NHase assay of Co/urea induced *R. rhodochrous* DAP 96253 and DAP 96622 cell weight, substrate concentration and dilution factors before adding commercial amidase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate Concentration (mg/L)</th>
<th>Cell Weight (mg)</th>
<th>Dilution Factor Before Adding Amidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>5000</td>
<td>1</td>
<td>1:25</td>
</tr>
<tr>
<td>Propionitrile</td>
<td>5000</td>
<td>1</td>
<td>1:25</td>
</tr>
<tr>
<td>Acrylonitrile</td>
<td>5000</td>
<td>1</td>
<td>1:25</td>
</tr>
<tr>
<td>Allyl Cyanide</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
<tr>
<td>Isovaleronitrile</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
<tr>
<td>Trimethylacetonitrile</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
<tr>
<td>Heptyl cyanide</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
<tr>
<td>Chloroacetonitrile</td>
<td>5000</td>
<td>2</td>
<td>1:25</td>
</tr>
<tr>
<td>3-Chloropropionitrile</td>
<td>5000</td>
<td>2</td>
<td>1:25</td>
</tr>
<tr>
<td>Adiponitrile</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>1000</td>
<td>50</td>
<td>1:5</td>
</tr>
</tbody>
</table>
2.3.3. NHase Assays for Cell Lysate and Proteins During Purification

0.5mg of Co/urea cell lysate or 0.25mg of proteins during the purification process was used for NHase assay. 5000mg/L (v/v) standard solutions of acrylonitrile were prepared (Sigma-Aldrich Corporation, St. Louis, MO); individual standard solutions (9 ml) were added into 15 ml Falcon tubes. 1 ml of protein sample in 50mM sodium phosphate buffer (pH 7) was prepared in a microcentrifuge tube. Then, 1ml of the protein sample was combined with 9 ml nitrile standard solution. This solution was vortexed for approximately 2 min to allow the NHase to react with acrylonitrile. The reaction was stopped by adding 100 μl of 2M sulfuric acid (Fisher Chemical, Fairlawn, NJ), this was mixed for 1 min, and then 100 μl of 4N sodium hydroxide (Fisher Scientific, Fairlawn, NJ) was added to neutralize the acid. 40μl of the solution was taken out and added to 960 μl of 50mM phosphate buffer (pH 7.2). Amidase was added according to the method for NHase Assay for Whole Cells above and the color reaction was also performed according to the method 2.3.2. for NHase Assay for Whole Cells above.

2.3.4. Nitrilase Assay Using Acrylamide Substrate

Nitrilase was quantified using 1000mg/L (v/v) standard solution of testing acrylonitrile (Sigma-Aldrich Corporation, St. Louis, MO); 9 ml of the 1000mg/L acrylonitrile solution was added into 15 ml Falcon tubes. Approximately 50 mg (wet weight) of cells was obtained from a YEMEA agar plate and then the cells were suspended into 1 ml of 50mM sodium phosphate buffer (pH 7) in a microcentrifuge tube. Then, the 1ml cell suspension was combined with 9 ml acrylonitrile 1000mg/L standard solution. This suspension was vortexed for approximately 1.5 min to allow the cells react with acrylonitrile, 1.3 ml suspension was taken into a new microcentrifuge tube and centrifugation was started when the reaction time was 2 min. The reaction was stopped by centrifuging at 13,200 rpm for 2 min. 1ml supernatant was immediately
transferred to a 1.5 ml microcentrifuge tube pre-loaded with 20 μl of 2M sulfuric acid (Fisher Chemical, Fairlawn, NJ), this was mixed for 1 min, and then 20 μl of 4N sodium hydroxide (Fisher Scientific, Fairlawn, NJ) was added to neutralize the acid. Duplicate samples with the volume of 1.3 ml were placed in two separate 1.5 ml Eppendorf tubes and centrifuged (Beckman Microfuge Lite, Palo Alto, CA) for 2 min at 13,000 rpm (approximately 16,000g). The supernatant was transferred into clean Eppendorf tubes.

Ammonium was detected in the same manner mentioned in 2.3.2. NHase Assay for Whole Cells above.

2.3.5. Nitrilase Assay Using Benzonitrile Substrate

The reaction steps for this nitrilase assay using benzonitrile substrate were the same mentioned in 2.3.4. Nitrilase Assay Using Acrylamide Substrate but the substrate was 1000mg/L benzonitrile solution. Ammonium was also detected in the same manner mentioned in 2.3.2. NHase Assay for Whole Cells above.

2.3.6. Cell Dry Weight

Cells were harvested from YEMEA agar plates and weighed directly in microcentrifuge tubes. Approximately 300 mg of cells were harvested from different inducing condition. Cells were suspended with 1 ml DDI H$_2$O and centrifuged at 13,200 rpm for 5 min. After centrifugation, supernatant was discarded. The pellet was washed 2 more times with the DDI H$_2$O.

After washed with DDI H$_2$O, cell pellet was re-suspended in 1ml DDI H$_2$O and transferred to a 5ml crucible. The microcentrifuge tube was washed 3 times with 0.5ml DDI H$_2$O and all washes were transferred to the crucible. The crucible has been pre-dried at 105 ºC for 24 hour and the dry weight has been recorded. After all tested cell samples were added into the
crucibles, the crucibles were dried at 105 °C for 24 hour. The total weight of dry cell and crucible were determined. Cell dry weight was calculated by subtracting the empty dry crucible weight from the cell/crucible total dry weight.

2.3.7. Preparation of *R. rhodochrous* DAP 96253 and DAP 96622 cell free lysate

20 grams of *R. rhodochrous* DAP 96253 or DAP 96622 was harvested from YEMEA agar plate into a 15ml Falcon tube, and 40 ml of lysis buffer (0.1M HEPES/44mM sodium butyrate/KOH pH 7.2 (HKB buffer)) and 2g of glass beads (diameter=0.5mm) were added. The cell suspension was mixed by vortex. The cell suspension was then sonicated using an Ultrasonic Converter Horn (Model No. CL4; Serial Number F1903) on a Sonic Dismembrator Ultrasonic Homogenizer (Fisher Scientific F550). Total net sonication time was 50 minutes. An ice bath was required during the sonication process.

The above cell lysate was transferred into two centrifuge tubes and centrifuged at 13,200 rpm (~16,300g) at 4 °C for 30min (Allegra™ 64R Centrifuge, Beckman Coulter™). The supernatant was transferred to two new centrifuge tubes and centrifuged for another 30min at 13,200 rpm (~16,300g) at 4 °C. Three centrifugations were needed to remove all the cell debris from the supernatant.

2.3.8. Extraction of cell envelope proteins

1 gram of *R. rhodochrous* DAP 96253 was scraped from each type of YEMEA plates, added to 2 ml of 50mM phosphate buffer (pH 7.2), and mixed thoroughly by vortexing for 30 sec. Duplicates were made from above suspension by transferring into two microcentrifuge tubes (Eppendorf, 1.5 ml), and centrifuged at 13,200 rpm (16,100 g) for 10 min at room temperature using an Eppendorf Centrifuge 5415D. The supernatant was discarded. Cell pellets were resuspended in 50mM PBS, mixed thoroughly and sonicated for 10min (net sonication time).
The suspension was centrifuged at 13,200 rpm, the supernatant was collected and this supernatant was recorded as ‘no pre-sonication extraction (NPSE) cytosol. The pellets were collected and washed three times with 50mM PBS. The pellets were collected by centrifuging at 13,200 rpm for 10 min. The supernatant was discarded and the pellets were resuspended in cell envelope protein extraction solution (1 ml of 2% SDS (GE Healthcare Biosciences, Uppsala, Sweden) in 50 mM Tris-HCl (pH 6.8)). The suspension was treated at 100 °C for 4 min using a standard heat block (VWR Heat Block, VWR Scientific, Batavia, IL). After centrifugation (13,200 rpm for 10 min), the supernatant was collected as cell envelope protein extract.

Proteins in the cell envelope protein extracts were precipitated with acetone. One volume of the cell envelope proteins in the extraction buffer was added to four volumes of cold acetone (-20 °C) and set in the freezer at -20 °C for 1 hour. The solution was mixed every 10 min by flipping over several times. Protein pellet was collected by centrifuging the acetone/cell envelope protein extract at 13,200 rpm (~16,300 rcf) for 30 min at 4 °C. After decanting the supernatant, the protein pellet was centrifuged at 13,200 rpm (~16,300 rcf) for 5 min at 4 °C to remove more liquid. The protein pellet was then dried at 4 °C for 3~5 minutes, ensuring that the protein pellet was not over-dried. 50mM phosphate buffer/pH 7.2 was gradually added to the pellet and a glass rod was used to gently break the protein pellet until the protein pellet was totally dissolved. The dissolved cell envelope protein sample was centrifuged at 13,200 rpm (~16,300 rcf) for 15 min at 4 °C to remove un-dissolved components. The supernatant was collected and quantified by BCA protein assay (Thermo Scientific Pierce BCA Protein Assay Kit).

2.3.9. Linear Gradient SDS-PAGE

20% gel solution and 8% gel solution for separation gel were prepared according to Table
3. Linear gradient gel was casted according to Figure 6. Separation gel solutions were pumped into the plates, covered with butanol saturated with water and set for 2 hours to complete polymerization. Butanol was discarded from the top of the separation gel and the separation gel was rinsed three times with DDI water. After the separation gel was made, 4% Stacking gel was prepared and casted on the top of the separation gel according to Table 4.

The concentrations of all protein samples were determined by BCA protein assay (Thermo Scientific Pierce BCA Protein Assay Kit) (See Appendix). Protein samples were diluted with double deionized H₂O (D.D.I. H₂O) to 1mg/ml. 1 volume of diluted protein sample was added to 1 volume of 2X loading buffer (0.125M Tris-HCl, pH 6.8/4% SDS/20% glycerol/3% DTT/0.002% bromophenol blue). The protein samples mixed with loading buffer were treated at 90 °C for 5 min using a standard heat block (VWR Heat Block, VWR Scientific, Batavia, IL) and cooled down at room temperature.

Table 3. 8-20% gradient gel.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>20% Gel Solution (ml)</th>
<th>8% Gel Solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.875 M Tris-HCl, pH 8.8</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Proto Gel solution</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Water</td>
<td>0.9</td>
<td>11.7</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.225</td>
<td>0.225</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.3g</td>
<td>---</td>
</tr>
</tbody>
</table>

Mix well and de-aerate for 10min

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>75 µl</th>
<th>75 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulfate (10%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>5.63 µl</td>
<td>5.63</td>
</tr>
<tr>
<td>Total Volume</td>
<td>22.5</td>
<td>22.5</td>
</tr>
</tbody>
</table>
Figure 6. Linear gradient gel casting process.

Table 4. 4% stacking gel.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>2 Gels (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/0.8% bisacrylamide (Protol Gel Solution)</td>
<td>3.33</td>
</tr>
<tr>
<td>5X resolving gel buffer (1.875M Tris, pH8.8)</td>
<td>5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.25</td>
</tr>
<tr>
<td>DDI Water</td>
<td>16.14</td>
</tr>
<tr>
<td></td>
<td>Mix well and de-aerate for 10min.</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>250 µl</td>
</tr>
<tr>
<td>100% TEMED</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>25</td>
</tr>
</tbody>
</table>
The treated protein samples were analyzed by 1-D gel electrophoresis. 8-20% gradient acrylamide gels were used and the gel size was 16*18cm. 13μg protein sample was loaded in each well. Electrophoresis was carried out using SE 600 Ruby system (Amersham Sciences, Piscataway, NJ). Voltage was set as 600v, power was set as 100w, current was set as 50mA/gel and temperature was set as 4°C. The total running time was three and half hours. Gels were fixed by a fixing solution (methanol : acetic acid : H₂O ratio 3:1:6) for 30min. Staining stock solution was made by adding one PhastGel™ Blue R tablet (Amersham Biosciences, Piscataway, NJ) into 80ml methanol, and after the solution was mixed for five minute, 80ml of DDI H₂O was added. Staining stock solution was filtered before use. Gels were stained with a volume of stock solution and 9 volume of fixing solution on a shaker (Innova™ 2000 Platform Shaker, New Brunswick Scientific) at 60 rpm for at least three hours. The gels were destained with 1 part of fixing solution and 2 parts of DDI water. Solution was changed until protein bands were clearly visible. Gels were also imaged with Typhoon Imager 9400 (GE Healthcare). The intensity of the bands was analyzed with Image Quant TL.

Bands of interest were extracted from the gel and sent to Georgia State University core facility for analysis. Gel bands were treated by trypsin digestion with the ProteoProfile™ Trypsin In-Gel Digest Kit (#PP0100, Sigma, St. Louis, MO). The samples were further cleaned with a ZipTip® (#ZTC18M096, Millipore, Billerica, MA) and analyzed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry (MALDI TOF/TOF MS). MALDI TOF/TOF MS peptide sequence results were compared to the protein database of *Rhodococcus* to identify the protein bands.
2.3.10. Purification of NHase from *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 966622

All chromatography steps during the purification of NHase, amidase (from GGSS motif family) and amidase (~38 kDa) were performed on a GE ÄKTA Explorer™ (Amersham Pharmacia, Piscataway, NJ) fast protein liquid chromatography (FPLC) system. Samples were loaded automatically by a pump P-960™ from the system. The GE ÄKTA Explorer™ (Amersham Pharmacia, Piscataway, NJ) FPLC system was controlled by UNICORN® 5.11 platform. Protein peaks were determined by UV280 and UV260, and both wavelengths were detected by a UV-900 detector from the system. Eluted samples were collected by a Frac-950 fractionator with an assigned volume by UNICORN® 5.11 platform for each fraction.

**Step 1: Anion exchange chromatography (First AEC)**

8 ml cell-free lysate of *R. rhodochrous* DAP 96253 or DAP 966622 in HKB buffer was diluted to 50ml with 20mM NaH$_2$PO$_4}$/10mM sodium butyrate/pH 7.2 (anion exchange equilibration buffer). The diluted cell-free lysate was loaded on a HiPrep™ Q FF 16/10 anion exchange column. Prior to loading the sample, the column was equilibrated with 6 column volumes of anion exchange equilibration buffer with a flow rate of 3ml/min. Sample was loaded and eluted at 3 ml/min. After the diluted cell-free lysate was loaded on the AEC column, the column was washed with 3 column volumes of anion exchange equilibration buffer to wash off unbound components. Bound proteins were eluted with 6 column volumes of a linear gradient with 1M NaCl/20mM NaH$_2$PO$_4}$/10mM sodium butyrate/pH 7.2 elution buffer. Eluted samples were collected with 3 ml for each fraction. Protein fractions of the same peak were collected as the same protein sample and a sub sample of each peak was examined by 8-20% linear gradient SDS-PAGE to determine the target peak for NHase.
Step 2: Second anion exchange chromatography (Second AEC)

Peaks containing target proteins were diluted with anion exchange equilibration buffer to 100ml and loaded back on a HiPrep™ Q Fast Flow (HiPrep™ Q FF) 16/10 anion exchange column. Sample was loaded, eluted and collected with the same method in Step 1. A sub sample (0.5 ml) of the target peak was examined by 8-20% linear gradient SDS-PAGE to determine the purity of NHase.

Step 3: Hydrophobic interaction chromatography (First HIC)

Sample after the second AEC was added with solid ammonium sulfate to achieve approximately 45% saturation before loading (ammonium sulfate was added ~5 min before the sample was started to load to prevent protein samples from aggregating). Before sample was loaded, a 5 ml HiTrap™ Butyl HP column (GE Healthcare) was equilibrated with 6 column volumes of 2M (NH₄)₂SO₄/20mM NaH₂PO₄/10mM sodium butyrate/pH 7.2. After the sample was loaded, bound proteins were eluted with 20 column volumes of a linear gradient with 20mM NaH₂PO₄/10mM sodium butyrate/pH 7.2 elution buffer. Eluted samples were collected with 2.5 ml for each fraction. During the HIC process, the flow rate was 5 ml/min constant. Protein fractions of the target peak were collected as the same protein sample and a sub sample (0.5 ml) of the target peak was examined by 8-20% linear gradient SDS-PAGE to determine the purity of NHase.

Step 4: Size exclusion chromatography-gel filtration (First GF)

The sample from HIC was then loaded on a HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR) column (GE Healthcare). 150mM NaCl/50mM NaH₂PO₄/10mM sodium butyrate/NaOH pH 7.2 (GF running buffer) was used as the running buffer for gel filtration column. Before the sample was loaded, the HiPrep™ 26/60 Sephacryl™ S-300 (HR) column
was equilibrated with 2 column volume GF running buffer at a flow rate of 1.3 ml/min. 13ml of the post HIC sample was loaded at a loading rate of 1ml/min and eluted with GF running buffer at a flow rate of 1ml/min. Eluted samples were collected with 3 ml for each fraction. Protein fractions of the same peak were collected as the same protein sample and a sub sample of each peak was examined by 8-20% linear gradient SDS-PAGE to determine the target peak for NHase.

Step 5: Second hydrophobic interaction chromatography (Second HIC).

Solid ammonium sulfate was added into the target protein sample after GF to reach 35% ammonium sulfate saturation. After ammonium sulfate was dissolved by vortexing, this protein sample was set on ice for an hour and then centrifuged at 13,200 rpm (~16,300g) at 4ºC for 30min. The supernatant was loaded on a 5 ml HiTrap™ Butyl HP column (GE Healthcare). This second HIC was performed the same as Step 3 shown above.

Step 6: Size exclusion chromatography-gel filtration (Second GF)

The sample from the second HIC was then loaded back on HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR) column (GE Healthcare). 150mM NaCl/50mM NaH₂PO₄/10mM sodium butyrate/NaOH pH 7.2 (GF running buffer) was used as the running buffer for gel filtration column. Eluted samples were collected with 3 ml for each fraction. Protein fractions of the same peak were collected as the same protein sample and a sub sample of each peak was examined by 8-20% linear gradient SDS-PAGE to determine the target peak for NHase.
3. RESULTS

3.1. NHase activity

3.1.1. NHase activity of *R. rhodochrous* DAP 96253 and DAP 96622

The dry/wet weight ratio of cells from YEMEA agar plates supplemented with Co/urea, Co, Fe/urea, Fe, Ni/urea, Ni, urea and uninduced samples were all tested to be approximately 20% (Table 5). Thus 20% cell dry weight ratio was used to calculate all the NHase activities of cells harvested from YEMEA agar plates.

The NHase activities of induced whole cells using acrylonitrile as the substrate are shown in Table 6 and Figure 7. When induced with Co/urea, *R. rhodochrous* DAP 96253 gave the highest activity of approximately 200 units/min·mg cell dry weight (cdw). Co-induced cells gave the second highest activity of around 7 units/min·mg cdw, which is approximately 4% that seen from Co/urea-induced cells. Fe/urea- and Ni/urea-induced cells showed activities of 3.1 and 2.6 units/min·mg cdw (1.3% and 1.6% of the Co/urea cells), respectively. The NHase activities of otherwise induced and uninduced cells were lower than 1% of the Co/urea-induced cells (Table 6).

Similarly, using acrylonitrile as the substrate, *R. rhodochrous* DAP 96622 cells induced with Co/urea gave the highest NHase activity, approximately 53 units/min·mg cdw using acrylonitrile as the substrate, which was about 23% of the activity of *R. rhodochrous* DAP 96253 induced with Co/urea. Co-induced *R. rhodochrous* DAP 96622 cells yielded a NHase activity of approximately 23 units/min·mg cdw, roughly 12% of the activity of *R. rhodochrous* DAP 96253 when induced with Co/urea (Table 7). When the cells were otherwise induced, the NHase activities were lower than 1 unit/min·mg cdw. When grown on YEMEA agar supplemented with Fe and urea, *R. rhodochrous* DAP 96622 showed minimum growth.
Table 5. Cell dry weight to cell wet weight growing on YEMEA media with different inducers.

<table>
<thead>
<tr>
<th>Sample Names</th>
<th>Cell Dry Weight/Cell Wet Weight (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co</td>
<td>19.7%</td>
</tr>
<tr>
<td>Co/urea</td>
<td>19.0%</td>
</tr>
<tr>
<td>Fe</td>
<td>19.6%</td>
</tr>
<tr>
<td>Fe/urea</td>
<td>20.0%</td>
</tr>
<tr>
<td>Ni</td>
<td>19.4%</td>
</tr>
<tr>
<td>Ni/urea</td>
<td>17.3%</td>
</tr>
<tr>
<td>Urea</td>
<td>18.2%</td>
</tr>
<tr>
<td>Uninduced</td>
<td>19.5%</td>
</tr>
</tbody>
</table>

Table 6. NHase(s) activity of *R. rhodochrous* DAP 96253 whole cells induced different metals.

<table>
<thead>
<tr>
<th>Samples</th>
<th>NHase activity (unit/min·mg cdw)*</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEMEA Co/urea</td>
<td>200±24</td>
<td>100</td>
</tr>
<tr>
<td>YEMEA Co</td>
<td>7.4±1.3</td>
<td>3.7</td>
</tr>
<tr>
<td>YEMEA Fe/urea</td>
<td>3.1±0.5</td>
<td>1.6</td>
</tr>
<tr>
<td>YEMEA Fe</td>
<td>0.9±0.09</td>
<td>0.5</td>
</tr>
<tr>
<td>YEMEA Ni/urea</td>
<td>2.6±1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>YEMEA Ni</td>
<td>0.13±0.037</td>
<td>0.1</td>
</tr>
<tr>
<td>YEMEA urea</td>
<td>0.93±0.29</td>
<td>0.5</td>
</tr>
<tr>
<td>YEMEA uninduced</td>
<td>0.29±0.05</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note:

CDW: Cell Dry Weight in mg.

Acrylonitrile was used as the substrate. Co/urea induced sample used 5000mg/L acrylonitrile, and all other samples used 1000mg/L acrylonitrile.
Figure 7. NHase(s) activity of *R. rhodochrous* DAP 96253 whole cells induced with different metals (acrylonitrile substrate).
Table 7. NHase(s) activities of *R. rhodochrous* DAP 96622 whole cells induced with different metals (acrylonitrile as substrates)

<table>
<thead>
<tr>
<th>Samples</th>
<th>NHase activity (unit/min·mg-cdw)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP 96253 Co/urea*</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>DAP 96622 Co/urea*</td>
<td>53</td>
<td>27</td>
</tr>
<tr>
<td>DAP 96622 Co*</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>DAP 96622 Fe/urea</td>
<td>No Growth</td>
<td>NA</td>
</tr>
<tr>
<td>DAP 96622 Fe</td>
<td>No Growth</td>
<td>NA</td>
</tr>
<tr>
<td>DAP 96622 Ni/urea</td>
<td>0.27</td>
<td>0.1</td>
</tr>
<tr>
<td>DAP 96622 Ni</td>
<td>0.04</td>
<td>0.0</td>
</tr>
<tr>
<td>DAP 96622 urea</td>
<td>0.19</td>
<td>0.1</td>
</tr>
<tr>
<td>DAP 96622 uninduced</td>
<td>0.20</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note:

*Substrate concentration was 5000mg/L. 1mg live cells were used for the assay. The rest of the conditions used 1000mg/L acrylonitrile as the substrate working solution.*
3.1.2. NHase activity of *R. rhodochrous* DAP 96253 and DAP 96622 against different nitriles

The NHase activities of *R. rhodochrous* DAP 96253 cells were also tested against acetonitrile, propionitrile and acrylonitrile, and yielded 300, 210 and 200 units/mg cdw, respectively. The NHase activities against the chlorinated aliphatic nitriles Chloroacetonitrile and 3-Chloropropionitrile were 140 and 11, respectively. For all other tested aliphatic nitriles, and against both adiponitrile and benzonitrile, the NHase activities were lower than 1 unit/ min-mg cdw (Table 8).

The NHase activities of *R. rhodochrous* DAP 96622 cells were also tested. When using acetonitrile, propionitrile and acrylonitrile as the substrates, the NHase activities were 66, 55 and 52 units/mg cdw, respectively. The NHase activities against the chlorinated aliphatic nitriles Chloroacetonitrile and 3-Chloropropionitrile were 42 and 2.8, respectively. For all other aliphatic nitriles, adiponitrile, and benzonitrile tested, the NHase activities were lower than 1 unit/ min-mg cdw (Table 9).
Table 8. NHase(s) activity of *R. rhodochrous* DAP 96253 whole cells against different nitriles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Formula</th>
<th>NHase activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile*</td>
<td>CH$_3$-C≡N</td>
<td>330</td>
<td>100%</td>
</tr>
<tr>
<td>Propionitrile*</td>
<td>CH$_3$CH$_2$-C≡N</td>
<td>210</td>
<td>67%</td>
</tr>
<tr>
<td>Acrylonitrile*</td>
<td>CH$_2$=CH-C≡N</td>
<td>200</td>
<td>61%</td>
</tr>
<tr>
<td>Allyl Cyanide</td>
<td>CH$_2$=CH-CH$_2$-C≡N</td>
<td>0.76</td>
<td>0.2%</td>
</tr>
<tr>
<td>Isovaleronitrile</td>
<td>(CH$_3$)$_2$CH-CH$_2$-C≡N</td>
<td>0.23</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Trimethylacetonitrile</td>
<td>(CH$_3$)$_3$C-C≡N</td>
<td>0.19</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Heptyl cyanide</td>
<td>CH$_3$-(CH$_2$)$_6$-C≡N</td>
<td>0.23</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Chloroacetonitrile*</td>
<td>ClCH$_2$-C≡N</td>
<td>140</td>
<td>42%</td>
</tr>
<tr>
<td>3-Chloropropionitrile*</td>
<td>ClCH$_2$CH$_2$-C≡N</td>
<td>11</td>
<td>3.3%</td>
</tr>
<tr>
<td>Adiponitrile</td>
<td>N≡C-(CH$_2$)$_4$-C≡N</td>
<td>0.29</td>
<td>0.1%</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>C$_6$H$_5$-C≡N</td>
<td>0.20</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

Note:*5000mg/L nitriles were used as the substrate working solution and 1mg live *R. rhodochrous* DAP 96253 cells were used. The rest of the nitriles were used with a concentration of 1000mg/L and 50mg live cells.
Table 9. NHase(s) activity of *R. rhodochrous* DAP 96622 whole cells against different nitriles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Formula</th>
<th>NHase activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile*</td>
<td>CH$_3$-C≡N</td>
<td>66</td>
<td>100%</td>
</tr>
<tr>
<td>Propionitrile*</td>
<td>CH$_3$CH$_2$-C≡N</td>
<td>55</td>
<td>83%</td>
</tr>
<tr>
<td>Acrylonitrile*</td>
<td>CH$_2$=CH-C≡N</td>
<td>52</td>
<td>79%</td>
</tr>
<tr>
<td>Allyl Cyanide</td>
<td>CH$_2$=CH-CH$_2$-C≡N</td>
<td>0.23</td>
<td>0.3%</td>
</tr>
<tr>
<td>Isovaleronitrile</td>
<td>(CH$_3$)$_2$CH-CH$_2$-C≡N</td>
<td>0.51</td>
<td>0.7%</td>
</tr>
<tr>
<td>Trimethylacetonitrile</td>
<td>(CH$_3$)$_3$C-C≡N</td>
<td>0.00</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Heptyl cyanide</td>
<td>CH$_3$-(CH$_2$)$_6$-C≡N</td>
<td>0.00</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Chloroacetonitrile*</td>
<td>ClCH$_2$-C≡N</td>
<td>42</td>
<td>64%</td>
</tr>
<tr>
<td>3-Chloropropionitrile*</td>
<td>ClCH$_2$CH$_2$-C≡N</td>
<td>2.8</td>
<td>4.2%</td>
</tr>
<tr>
<td>Adiponitrile</td>
<td>N≡C-(CH$_2$)$_4$-C≡N</td>
<td>0.66</td>
<td>1%</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>C$_6$H$_5$-C≡N</td>
<td>0.36</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Note:*5000mg/L nitriles were used as the substrate working solution and 1mg live *R. rhodochrous* DAP 96622 cells were used. The rest of the nitriles were used with a concentration of 1000mg/L and 50mg live cells.
3.1.3. SDS-PAGE analysis of proteins of *R. rhodochrous* DAP 96253 and DAP 96622

Both NHase and amidase from *R. rhodochrous* DAP 96253 were identified via SDS-PAGE and MALDI TOF/TOF MS. The α subunit of NHase is approximately 22.8 kDa and the β subunit is approximately 26.3 kDa (Figure 8), and their amino acid sequences are similar to the H-type NHases from *R. rhodochrous* J1 and *Rhodococcus* sp. M8. Amidase is about 38.2 kDa and its amino acid sequence is similar to the putative aliphatic amidase gene from *Nocardia farcinica*, aliphatic amidases of *P. aeruginosa* PAO1 (38.4 kDa) and *Helicobacter pylori* 26695 (37.7 kDa).

Using ImageQuant TL software, the ratio of NHase and amidase (~38 kDa) to recovered cytosolic proteins from Co/urea-induced cells were determined to be approximately 55% and 7%, respectively. For Co-induced cells, the NHase accounted for roughly 7% of the recovered cytosolic proteins while amidase (~38 kDa) accounted for approximately 1.5% of the recovered cytosolic proteins (Table 10). For Fe/urea, Ni/urea and urea samples, the NHase accounted for about 9.0% of the recovered cytosolic proteins, but amidase (~38 kDa) accounted for approximately 15% of the recovered cytosolic proteins (Table 10). For Co, Fe, Ni and uninduced samples, bands with similar, if not the same, molecular weight as NHase were used to calculate the volume of NHase bands, due to the fact that NHase was not over-expressed in these samples (Figure 8), and the volume of amidase (~38 kDa) bands in these samples were calculated in the same manner. The NHase in Fe-, Ni-induced and uninduced samples accounted for around 5% of the recovered cytosolic proteins, and amidase accounted for less than 1% of the total recovered cytosolic proteins. The β/α subunit ratios of NHase in Co/urea-, Ni-induced and uninduced samples were approximately 1.2, around 0.6~0.7 in Fe/urea-, Ni/urea- and urea-induced samples, 0.9 in Co- and Fe-induced samples.
Recovered cell envelope proteins were also analyzed by SDS-PAGE. A protein band with the same molecular weight with NHase β subunit was abundant. However, protein band(s) with the same molecular weight of NHase α subunit was not as abundant (Figure 10). The protein profiles of the recovered membrane proteins were different between Co/urea- and Co-induced cells (Figure 10, Lane 1 and 2).

Similarly, when *R. rhodochrous* DAP 96622 was induced with Co/urea, both NHase and amidase (~55 kDa) were identified by MALDI TOF/TOF MS (Figure 11, Lane 3). NHase α and β subunits were also 22.8 and 26.3 kDa, respectively. This NHase showed similarities to the H-type NHase from *Rhodococcus* sp. M8. NHase accounted for approximately 20% of total recovered cytosolic proteins, and this was less than that of Co/urea-induced *R. rhodochrous* DAP 96253 (Figure 11 and Table 12). In addition, in *R. rhodochrous* DAP 96253, amidase was 38.2 kDa (Figure 11, Lane 2), but in *R. rhodochrous* DAP 96622, amidase was determined to be 54.6 kDa (Figure 11, Lane 3). In *R. rhodochrous* DAP 96622, amidase accounted for approximately 12% of the total recovered cytosolic proteins (Table 12). For Fe/urea, Fe, Ni/urea, Ni, urea and uninduced samples, the NHase accounted for about 7-11% of the recovered cytosolic proteins, and amidase (~55 kDa) accounted for approximately 11% of the recovered cytosolic proteins for urea-supplemented samples (Table 10). The β/α subunit ratios of NHase for all eight samples were approximately 1.
Figure 8. SDS-PAGE comparison of cytosolic proteins of *R. rhodochrous* DAP 96253 when induced with different inducer(s)

Lane 1 & 10: Molecular markers; Lane 2: Co/urea-induced cytosolic proteins; Lane 3: Co-induced cytosolic proteins; Lane 4: Fe/urea-induced cytosolic proteins; Lane 5: Fe-induced cytosolic proteins; Lane 6: Ni/urea-induced cytosolic proteins; Lane 7: Ni-induced cytosolic proteins; Lane 8: Urea-induced cytosolic proteins; Lane 9: uninduced cytosolic proteins.

Loading amount: 13µg/well.
Table 10. ImageQuant TL analysis of cytosolic proteins of *R. rhodochrous* DAP 96253 when induced with different inducer(s).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Co/urea</th>
<th>Co*</th>
<th>Fe/urea</th>
<th>Fe*</th>
<th>Ni/urea</th>
<th>Ni*</th>
<th>Urea</th>
<th>Uninduced*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase/Total proteins (%)</td>
<td>54.8</td>
<td>6.8</td>
<td>11.2</td>
<td>5.1</td>
<td>9.0</td>
<td>4.6</td>
<td>7.8</td>
<td>4.6</td>
</tr>
<tr>
<td>NHase β/α subunit ratio</td>
<td>1.15</td>
<td>0.91</td>
<td>0.70</td>
<td>0.92</td>
<td>0.63</td>
<td>1.22</td>
<td>0.57</td>
<td>1.23</td>
</tr>
<tr>
<td>Amidase/Total proteins (%)</td>
<td>6.8</td>
<td>1.5</td>
<td>13.5</td>
<td>0.8</td>
<td>16.2</td>
<td>0.9</td>
<td>16.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Note: *For Co, Fe, Ni and uninduced samples, because NHase was not over-expressed, NHase bands were determined by band with similar molecular weight on the gel (Figure 8, Lane 5, 7 & 9).

*Amidase means [amidase]/[nitrilase] here (See Discussion, [Amidase]/[nitrilase] and NHase expression).

*Total proteins mean total recovered cytosolic proteins here.*
Figure 9. SDS-PAGE comparison of cytosolic proteins of *R. rhodochrous* DAP 96622 when induced with different inducer(s)

Lane 1 & 10: Molecular markers; Lane 2: Co/urea-induced cytosolic proteins; Lane 3: Co-induced cytosolic proteins; Lane 4: Fe/urea-induced cytosolic proteins; Lane 5: Fe-induced cytosolic proteins; Lane 6: Ni/urea-induced cytosolic proteins; Lane 7: Ni-induced cytosolic proteins; Lane 8: Urea-induced cytosolic proteins; Lane 9: uninduced cytosolic proteins.

Loading amount: 13µg/well.
Table 11. ImageQuant TL analysis of cytosolic proteins of *R. rhodochrous* DAP 96622 when induced with different inducer(s)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Co/urea</th>
<th>Co*</th>
<th>Fe/urea</th>
<th>Fe*</th>
<th>Ni/urea</th>
<th>Ni*</th>
<th>Urea</th>
<th>Uninduced*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase/Total proteins (%)</td>
<td>19.6</td>
<td>8.4</td>
<td>10.8</td>
<td>7.9</td>
<td>9.9</td>
<td>7.6</td>
<td>9.3</td>
<td>7.2</td>
</tr>
<tr>
<td>NHase β/α subunit ratio</td>
<td>0.97</td>
<td>0.94</td>
<td>1.05</td>
<td>0.89</td>
<td>1.02</td>
<td>0.96</td>
<td>0.89</td>
<td>0.93</td>
</tr>
<tr>
<td>Amidase/Total proteins (%)</td>
<td>12.6</td>
<td>2.6</td>
<td>11.8</td>
<td>1.8</td>
<td>11.0</td>
<td>2.4</td>
<td>10.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Note: *For Co, Fe, Ni and uninduced samples, because NHase was not over-expressed, NHase bands were determined by band with similar molecular weight on the gel (Figure 8, Lane 5, 7 & 9).

Amidase means [amidase]/[nitrilase] here (See Discussion, [Amidase]/[nitrilase] and NHase expression).

Total proteins mean total recovered cytosolic proteins here.
In summary, based on five different gels analyzed by ImageQuant TL, NHase in Co/urea induced *R. rhodochrous* DAP 96253 accounted for approximately 59% of the total recovered cytosolic proteins and the NHase β/α ratio was approximately 1.1. Amidase (~55 kDa) accounted for about 5% of the total recovered cytosolic proteins in Co/urea induced *R. rhodochrous* DAP 96253 cells. In Co/urea induced *R. rhodochrous* DAP 96622 cells, NHase account for 21% of the total recovered cytosolic proteins and the NHase β/α ratio was approximately 0.9. Amidase (~55 kDa) accounted for about 12% of the total recovered cytosolic proteins in Co/urea-induced *R. rhodochrous* DAP 96622 cells.

3.1.4. NHase expression and membrane in *R. rhodochrous* DAP 96253

The membrane proteins of both Co/urea-induced and uninduced *R. rhodochrous* DAP 96253 were extracted and examined on SDS PAGE. A major band NHase subunit β was seen under both conditions, but a limited amount of NHase subunit α was seen under both conditions (Figure 10, Lane 1 and Lane 2).
Figure 10. 13% SDS-PAGE comparison of cytosolic proteins when induced with different inducer(s)

Lane 1: Co/urea-induced cell envelope proteins; Lane 2: Co-induced cell envelope proteins; Lane 3: Co/urea-induced cytosolic proteins; Lane 4: Co-induced cytosolic proteins; Lane 5: Molecular markers.

Loading amount: 13 μg
Figure 11. SDS-PAGE comparison of cytosolic proteins between *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 96622 when induced with Co/urea.

Lane 1: Molecular markers; Lane 2: Co/urea-induced *R. rhodochrous* DAP 96253 cytosolic proteins; Co/urea-induced *R. rhodochrous* DAP 96622 cytosolic proteins.

Loading amount: 13µg/well.
Table 12. Comparison of NHase, amidase and amidase (GGSS family) between *R. rhodochrous* DAP 96253 and 96622.

<table>
<thead>
<tr>
<th>Strains</th>
<th><em>R. rhodochrous</em> DAP 96253</th>
<th><em>R. rhodochrous</em> DAP 96622</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase/Total proteins ratio (%)</td>
<td>59.0±3.0</td>
<td>20.7±0.6</td>
</tr>
<tr>
<td>NHase subunit β/α ratio</td>
<td>1.08±0.09</td>
<td>0.92±0.15</td>
</tr>
<tr>
<td>Amidase*/Total proteins ratio (%)</td>
<td>5.4±1.1</td>
<td>NA</td>
</tr>
<tr>
<td>Amidase (GGSS)/Total proteins ratio (%)</td>
<td>NA</td>
<td>12.3±2.2</td>
</tr>
</tbody>
</table>

Note: *Amidase means [amidase]/[nitrilase] here (See Discussion, [Amidase]/[nitrilase] and NHase expression).

Amidase (GGSS) means amidase from GGSS motif family here.

Total proteins means total recovered cytosolic proteins.

Data presented in current table were collected with five different gels analyzed by ImageQuant TL with a 95% confidence interval.

Both strains were induced with Co/urea on YEMEA agar.
3.1.5. Overexpression of nitrilase in *R. rhodochrous* DAP 96253

When *R. rhodochrous* DAP 96253 was induced with urea/isovaleronitrile, as used by Kobayashi *et al.* (1992a) to induce nitrilase in *R. rhodochrous* J1, a major band around 38 kDa was overexpressed in the cytosolic proteins (Figure 12). This protein band was identified to be putative aliphatic amidase from *N. farcinica*, and the whole cells showed 7 units/min-mg cdw nitrilase activity against benzonitrile. Thus, the protein band that was identified as amidase might actually be nitrilase, or a dynamic combination of nitrilase and amidase (see Discussion, [Amidase]/[nitrilase] and NHase expression). The ratio of amidase to total recovered cytosolic proteins of urea-induced cells was approximately 17%, while the ratio for urea/isovaleronitrile-induced cells were near 38% (Table 13). As shown in Figure 12, NHase was also induced in urea/isovaleronitrile *R. rhodochrous* DAP 96253 cells, and constituted about 10% of the total recovered cytosolic proteins, higher than that of uninduced cells (~5%) (Table 10 and Table 13).
Figure 12. *R. rhodochrous* DAP 96253 induced with urea and urea/isovaleronitrile.

Lane 1: Molecular markers; Lane 2: Urea induced cells; Lane 3: Urea/isovaleronitrile induced cells.

Loading amount: 13 µg/lane.
Table 13. Comparison of urea- and urea/isovaleronitrile-induced *R. rhodochrous* DAP 96253.

<table>
<thead>
<tr>
<th>Sample</th>
<th>urea</th>
<th>urea/isovaleronitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amidase/Total proteins (%)</td>
<td>16.8</td>
<td>37.5</td>
</tr>
<tr>
<td>NH/Total proteins (%)</td>
<td>12.6</td>
<td>10.0</td>
</tr>
<tr>
<td>NHase subunit β/α ratio</td>
<td>0.84</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Note: Amidase means [amidase]/[nitrilase] here (See Discussion, [Amidase]/[nitrilase] and NHase expression).

Total proteins mean total recovered cytosolic proteins.

Data in current table was generated by ImageQuant TL with gel image of Figure 12.
3.2. NHase Purification Development

3.2.1. Ammonium sulfate precipitation

Ammonium sulfate precipitation was carried out on Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell free-lysate. As shown in Figure 13, most NHase precipitated at 45-70% ammonium sulfate saturation. The 35-45% ammonium sulfate precipitation cut gave a NHase activity of 100 units/min·mg cdw. The NHase activity increased with increasing ammonium sulfate with the 400 units seen for 45-70%. Thus, further purification of NHase used portions of 35-70% ammonium sulfate precipitate.

3.2.2. Gel filtration

Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell free-lysate was applied on a HiPrep™ Sephacryl S-300 gel filtration (GF) column to determine the target protein peak. Four identical peaks eluted at about 0.25 column volume, 0.38 column volume, 0.45 column volume and 0.53 column volume, and were analyzed by SDS-PAGE (Figures 13 and 14). The majority of NHase was eluted in the second peak. Glutamine synthetase co-eluted in the 0.38 column volume peak.

3.2.3. DEAE anion exchange chromatography

The cell-free lysate from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 affected protein binding to a HiTrap™ DEAE FF 5ml column. As a result, samples for NHase were pre-treated with a combination of ammonium sulfate precipitation followed by GF (Cell-free lysate--> Ammonium sulfate precipitation--> GF--> DEAE). As shown in Figure 16, these cleanup and purification steps yielded a single major peak with one minor shoulder peak. Figure 17 demonstrates an increase in purity from cell-free lysate, to ammonium sulfate precipitation, then gel filtration and finally anion exchange.
Figure 13. Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate with different percentage saturation of ammonium sulfate precipitation.

Lane 1: Molecular markers; Lane 2: Cell-free lysate; Lane 3: 0-35% saturation ammonium sulfate precipitation; Lane 4: 35-45% saturation ammonium sulfate precipitation; Lane 5: 45-70% saturation ammonium sulfate precipitation; Lane 6: 70-100% saturation ammonium sulfate precipitation.

Loading amount: 13 μg/lane.
Figure 14. Gel filtration on 45-70% ammonium sulfate precipitate.
Figure 15. Gel filtration analysis of 45-70% ammonium sulfate precipitate on Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.

Lane 1: Molecular markers; Lane 2: Peak 1 of gel filtration samples (Figure 6); Lane 3: Peak 2 of gel filtration samples (Figure 6); Lane 4: Peak 3 of gel filtration samples (Figure 6); Lane 5: Peak 4 of gel filtration samples (Figure 6).

Loading amount: 13 μg/lane.
Figure 16. DEAE Sepharose anion exchange of Peak 2 from gel filtration
Figure 17. Purification process: 45-70% ammonium sulfate precipitate, gel filtration and DEAE anion exchange on Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate. Lane 1: Molecular markers (SeeBlue® Plus2 Pre-Stained Standard; Invitrogen™); Lane 2: Cell-free lysate; Lane 3: 45-70% ammonium sulfate precipitate; Lane 4: Peak 2 of gel filtration samples; Lane 5: DEAE of samples from gel filtration.

Loading amount: 13 μg/lane.
3.2.4. HiTrap HIC selection kit

To improve protein binding to the HIC columns from a HiTrap HIC selection kit (GE Healthcare Life Science, Pittsburgh, PA) *R. rhodochrous* DAP 96253 cell-free lysate was pre-treated with ammonium sulfate precipitation followed by GF to remove most of the unwanted components (Cell-free lysate--> Ammonium sulfate precipitation--> GF--> HIC). The GE HiTrap™ selection kit includes seven 1ml columns: Phenyl Sepharose High Performance (Phenyl HP), Phenyl Sepharose 6 Fast Flow (low sub) (Phenyl FF (low sub)), Phenyl Sepharose 6 Fast Flow (high sub) (Phenyl FF (high sub)), Butyl Sepharose High Performance (Butyl HP), Butyl Sepharose Fast Flow (Butyl FF), Butyl-S Sepharose Fast Flow (Butyl-S FF) and Octyl Sepharose Fast Flow (Octyl FF).

As shown in Figure 18, the combination of ammonium sulfate precipitation, GF, and HIC was not sufficient to separate the glutamine synthetase (~55 kDa). Glutamine synthetase was identified by MALDI TOF mass spectrometry. Butyl HP removed the most unwanted proteins (Lane 4). Thus, a combination of ammonium sulfate precipitation, GF and 2 runs with HiTrap Butyl HP was used to purify NHase, however, glutamine synthetase was not removed (Lane 5).

3.2.5. Affinity chromatography: adenosine 5′-triphosphate–agarose

The first step in the synthesis of glutamine from glutamate and ammonium requires glutamine synthetase type I to bind to ATP. It has been previously reported that GS can be purified using affinity chromatography (see 1.5. Glutamine synthetase) (Brun *et al.*, 1992). Thus, a self-packed 1ml adenosine 5′-triphosphate–agarose column (ATP agarose A9264, Sigma-Aldrich) was used after ammonium sulfate precipitation and GF (Cell-free lysate--> Ammonium sulfate precipitation--> GF--> ATP agarose). As shown in Figure 19 Lane 2, these three in combination also did not remove glutamine synthetase from the NHase protein sample.
Figure 18. Post-GF NHase purification from Co/urea supplemented YEMEA R. rhodochrous DAP 96253 cell-free lysate.

Lane 1 & 10: Molecular markers; Lane 2: HiTrap™ Butyl-S FF; Lane 3: HIC HiTrap™ Butyl FF; Lane 4: HiTrap™ Butyl HP; Lane 5: 2X HiTrap™ Butyl HP; Lane 6: HiTrap™ Phenyl HP; Lane 7: HiTrap™ Phenyl FF (Low Sub); Lane 8: HiTrap™ Phenyl FF (High Sub); Lane 9: HiTrap™ Octyl FF.

Loading amount: 13 μg/lane.
Figure 19. Post-GF NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.

Lane 1: Molecular markers; Lane 2: Self-packed 1ml ATP agarose;

Loading amount: 13 µg/lane.
3.3. NHase purification from *R. rhodochrous* DAP 96253

By analyzing all peaks collected from the First AEC (See 2.3.8. Step 1 first anion exchange chromatography using HiPrep™ Q FF 16/10 column) on an 8-20% gradient SDS-PAGE, the NHase target peak eluted at around 53% of elution buffer and the salt concentration was around 0.53M NaCl (Figure 20). However, the appearance of a peak before the target peak required a second AEC run with the HiPrep™ Q FF 16/10 column (second Q FF). After the first AEC, 90mg of protein were collected from the peak of interest. This is approximately 45% of the total cell-free lysate and the NHase ratio increased from approximately 56% to 65% (Table 14).

During the second AEC (Q FF), a small shoulder peak was eluted before the target protein peak (Figure 21). As shown in Figure 25 Lane 3, after the first AEC (Q FF), four major bands were shown on the gel: ~50 kDa, ~38 kDa, ~27 kDa (NHase β subunit) and ~23 kDa (NHase α subunit). In addition, the second AEC (Q FF) removed more unwanted proteins compared with first AEC (Q FF) (Figure 21, Lane 3 vs. Lane 4). The second AEC only recovered around 84% of proteins from the first AEC, and this was near 38% proteins of the original total cell-free lysate. The NHase ratio decreased from approximately 65% to 63% as compared to the first AEC sample and this was because of the truncation of NHase subunit β by ammonium sulfate (Table 14) (See Discussion 4.6. Yield of NHases from both *R. rhodochrous* DAP 96253 and DAP 96622).

After the second Q FF, the protein sample showed two major peaks on the HIC (HiTrap™ Butyl HP) chromatogram. The NHase target protein was eluted at a concentration of approximately 17.5% of the elution buffer (Figure 22). The 8-20% gradient SDS-PAGE analysis indicated the removal of more unwanted proteins, including a major band around 50 kDa (Figure
25, Lane 5), however, the protein band at approximately 38 kDa was not removed. The band approximately 20 kDa was identified as NHase β subunit and the band around 55 kDa was identified as glutamine synthetase by MALDI TOF/TOF MS. This HIC process (first HIC) recovered approximately 50% of the protein from the second AEC (Q FF), and the proteins recovered from this first HIC step constitute about 19% of the original total cell-free lysate. At the same time, the NHase ratio after this first HIC increased to approximately 81% based on ImageQuant analysis of the SDS-PAGE (excluding the NHase β subunit fragment around 20 kDa) (Table 14).

The protein sample of interest from the first HIC was loaded on a GF column (HiPrep™ 26/60 Sephacryl™ S-300) and resulted in two peaks (Figure 23). The protein of interest eluted at 160ml, approximately 1/2 column volume, but a second GF was necessary to remove more unwanted protein(s). As shown in Figure 25, the majority of unwanted proteins were removed, including the protein band at approximately 38 kDa (Lane 6) and the ~20 kDa NHase β subunit fragment, but the glutamine synthetase (~55 kDa) and the 38 kDa band were not totally removed. This step recovered approximately 63% proteins from the first HIC step, accounting for about 12% of the original cell-free lysate (Table 14). The NHase ratio increased to more than 96% (Table 14).

After the first GF, the sample was concentrated and further purified by the second HIC. As shown in Figure 25, a higher percentage of the NHase β subunit fragment (~20 kDa) appeared after precipitation (Lane 7), possibly caused by the ammonium sulfate (See Discussion). After the second HIC, 75% of the proteins from the first GF were recovered, accounting for approximately 9% of the original total cell-free lysate (Table 14). The NHase ratio was
approximately 88%, not including the NHase β subunit fragment (~20 kDa) (Figure 25, Lane 7; Table 14).

As seen in the chromatogram of the second GF, only one peak eluted approximately 1/2 column volume (Figure 24). Subsequent gradient SDS-PAGE analysis revealed a trace amount of proteins around 55 kDa, identified as glutamine synthetase, and NHase α and NHase β subunits (See Discussion), and a band at 20 kDa (Figure 25, Lane 8). The second GF recovered approximately 89% of the proteins from second HIC step, and the final yield was around 8% of the original total cell-free lysate (Table 14). The NHase ratio was determined to be 98.5% after the second GF, but this ratio should be higher because the 55 kDa was a combination of GS, NHase α and β subunits (See discussion) (Figure 25, Lane 9; Table 14).

According to ImageQuant TL analysis (Figure 25), the NHase β/α ratio was around 1.2:1.0 during the whole purification process of NHase from R. rhodochrous DAP 96253 (Table 14).
Figure 20. Chromatogram of Step 1 (HiPrep™ Q FF 16/10) for NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.
Figure 21. Chromatogram of Step 2 (HiPrep™ Q FF 16/10) for NHase purification from Co/urea supplemented YEMEA R. *rhodochrous* DAP 96253 cell-free lysate.
Figure 22. Chromatogram of Step 4 (HiTrap™ Butyl HP) for NHase purification from Co/urea supplemented YEMEA R. *rhodochrous* DAP 96253 cell-free lysate.
Figure 23. Chromatogram of Step 5 (HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR)) for NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.
Figure 24. Chromatogram of Step 5 (HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR)) for NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.
Figure 25. Purification steps of NHase on Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 cell-free lysate.

Lane 1 & 8: Molecular markers; Lane 2: Cell-free lysate; Lane 3: first HiPrep™ Q FF AEC;
Lane 4: second HiPrep™ Q FF AEC; Lane 5: first HIC HiTrap™ Butyl HP; Lane 6: first Gel
filtration HiPrep™ 26/60 Sephacryl™ S-300; Lane 7: second HIC HiTrap™ Butyl HP; Lane 8:
second Gel filtration HiPrep™ 26/60 Sephacryl™ S-300.

Loading amount: 13 μg/lane.
Table 14. Purification of NHase from *R. rhodochrous* DAP 96253.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cell-free lysate</th>
<th>First AEC</th>
<th>Second AEC</th>
<th>First HIC</th>
<th>First GF</th>
<th>Second HIC</th>
<th>Second GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase ratio (% total protein)</td>
<td>56.2</td>
<td>65.1</td>
<td>62.9</td>
<td>81.4</td>
<td>96.5</td>
<td>88.1</td>
<td>98.5</td>
</tr>
<tr>
<td>NHase subunit β/α ratio</td>
<td>1.18</td>
<td>1.19</td>
<td>1.20</td>
<td>1.19</td>
<td>1.26</td>
<td>1.14</td>
<td>1.26</td>
</tr>
<tr>
<td>Protein amount (mg)</td>
<td>200.00</td>
<td>90.32</td>
<td>75.9</td>
<td>38.54</td>
<td>24.42</td>
<td>18.24</td>
<td>16.16</td>
</tr>
<tr>
<td>Yield against prior step (%)</td>
<td>NA</td>
<td>45.16</td>
<td>84.03</td>
<td>50.78</td>
<td>63.36</td>
<td>74.69</td>
<td>88.60</td>
</tr>
<tr>
<td>Yield against cell-free lysate (%)</td>
<td>NA</td>
<td>45.16</td>
<td>37.95</td>
<td>19.27</td>
<td>12.21</td>
<td>9.12</td>
<td>8.08</td>
</tr>
</tbody>
</table>

Note: All data were collected by ImageQuant TL. The NHase ratio excluded the fragmented NHase β (~20 kDa, beneath NHase α subunit shown on Figure 25).

NHase ratio means the percentage of NHase in corresponding sample of each step of purification.

Cell-free lysate means total recovered cytosolic proteins used for NHase purification.

Protein amount was determined by BCA protein assay.

Yield against prior step referred to the percentage of total protein of the current step against the prior step.

Yield against cell-free lysate referred to the percentage of total protein of the current step against the total recovered cytosolic proteins used for NHase purification.
3.4. NHase purification from *R. rhodochrous* DAP 96622

By analyzing all peaks collected from the First AEC (See 2.3.8. Step 1 first anion exchange chromatography using HiPrep™ Q FF 16/10 column) on an 8-20% gradient SDS-PAGE, similarly to the NHase from *R. rhodochrous* DAP 96253, the target peak also eluted at around 53% of elution buffer (Figure 26). This is approximately 15% protein was recovered from the original total cell-free lysate and the NHase ratio increased from approximately 22% to approximately 40% after the first AEC (Q FF) (Table 15).

A second AEC (Q FF) was thought necessary to remove unwanted proteins from the target peak (Figure 26), but was unsuccessful (Figure 27). According to the ImageQuant TL data, approximately 73% proteins were recovered from the first AEC (Q FF), constituting 11% of the original cell-free lysate (Table 15). The second AEC (Q FF) removed some of the unwanted proteins (Figure 32, Lane 3 vs. Lane 4), however, a significant amount remained in the sample (Figure 32, Lane 3 vs. Lane 4), and the NHase ratio was approximately 50% of the total recovered proteins (Table 15).

After the second AEC (Q FF), the protein sample showed several peaks on the HIC (HiTrap™ Butyl HP) chromatogram (Figure 28). The target protein for this NHase also eluted at a concentration of approximately 17.5% of the elution buffer, which was 0.35 M ammonium sulfate (Figure 28), the same as NHase from *R. rhodochrous* DAP 96253. After this first HIC step, approximately 37% proteins were recovered from the prior step, constituting approximately 4% of the original cell-free lysate (Table 15). When examined on an 8-20% gradient SDS-PAGE, two prominent bands indicated the NHase α and β subunits (Figure 32, Lane 5), and NHase accounted for 89% of the protein sample after this first HIC.
The first GF after the first HIC sample removed more bands (Figure 32, Lane 6), purifying NHase to more than 98% (Table 15). However, the total recovered protein was about 96% of the prior step (first HIC) and this was approximately 4% of the original total cell-free lysate (Table 15). Similarly with NHase from *R. rhodochrous* DAP 96253, the peak for NHase was eluted approximately 1/2 column volume (Figure 29). However, two protein bands around 50 kDa and two protein bands around 100 kDa were not removed (Figure 29).

The second HIC removed neither the two protein bands around 50 kDa nor those around 100 kDa (Figure 32, Lane 6 and Lane 7). NHase purity after the second HIC was approximately 98% (NHase β subunit fragment ~20 kDa was not included during the analysis). Approximately 70% proteins were recovered from the prior step (first GF) and this was about 30% of the original total cytosolic protein (Table 15). The major purpose for this step was to concentrate the sample for the second GF.

After the second GF, the two 100 kDa bands were removed but not the two at 50 kDa (Figure 32, Lane 8). According to MALDI TOF/TOF MS results, both bands were found to contain H type NHase α and β subunits (each band contained both subunits), possibly caused by the original organization and structure of NHase, and/or the incomplete denaturation of the loaded protein sample (See Discussion). According to ImageQuant TL data, after the second GF, the purity for NHase was more than 99%. The total purified NHase accounted for approximately 2% of the original total cell-free lysate (Table 15).

The final yield of purified NHase from *R. rhodochrous* DAP 96253 (~8%) was higher than from *R. rhodochrous* DAP 96622 (~2%) (Table 15). The majority of proteins from both strains were lost during the first AEC step and the first HIC step (See discussion). The yield for first AEC step was 45% and 15% for *R. rhodochrous* DAP 96253 and DAP 96622, respectively.
(Table 14 and Table 15). The yield for the first HIC compared with the prior step (second AEC) was approximately 51% and 37% for R. rhodochrous DAP 96253 and DAP 96622, respectively (Table 14 and Table 15).

The enzyme activities of both NHases were tested and found to be around 1300 units/mg-proteins in R. rhodochrous DAP 96253 and approximately 950 units/mg-proteins in R. rhodochrous DAP 96622.
Figure 26. Chromatogram of Step 1 (HiPrep™ Q FF 16/10) for NHase purification from Co/urea supplemented YEMEA R. *rhodochrous* DAP 96622 cell-free lysate.
Figure 27. Chromatogram of Step 2 (HiPrep™ Q FF 16/10) for NHase purification from Co/urea supplemented YEMEA R. *rhodothrous* DAP 96622 cell-free lysate.
Figure 28. Chromatogram of Step 3 (HiTrap\textsuperscript{TM} Butyl HP) for NHase purification from Co/urea supplemented YEMEA \textit{R. rhodochrous} DAP 96622 cell-free lysate.
Figure 29. Chromatogram of Step 4 (HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR)) for NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 96622 cell-free lysate.
Figure 30. Chromatogram of Step 5 (HiTrap™ Butyl HP) for NHase purification from Co/urea supplemented YEMEA R. *rhodochrous* DAP 96622 cell-free lysate.
Figure 31. Chromatogram of Step 4 (HiPrep™ 26/60 Sephacryl™ S-300 High Resolution (HR)) for NHase purification from Co/urea supplemented YEMEA *R. rhodochrous* DAP 9622 cell-free lysate.
Figure 32. Purification steps of NHase on Co/urea supplemented YEMEA *R. rhodochrous* DAP 96622 cell-free lysate.

Lane 1 & 9: Molecular markers; Lane 2: Cell-free lysate; Lane 3: first HiPrep™ Q FF AEC; Lane 4: second HiPrep™ Q FF AEC; Lane 5: first HIC HiTrap™ Butyl HP; Lane 6: first Gel filtration HiPrep™ 26/60 Sephacryl™ S-300; Lane 7: second HIC HiTrap™ Butyl HP; Lane 8: second Gel filtration HiPrep™ 26/60 Sephacryl™ S-300.

Loading amount: 13 μg/lane.
Table 15. Purification of NHase from *R. rhodochrous* DAP 96622.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cell-free lysate</th>
<th>First AEC</th>
<th>Second AEC</th>
<th>First HIC</th>
<th>First GF</th>
<th>Second HIC</th>
<th>Second GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase ratio (% total protein)</td>
<td>21.9</td>
<td>40.0</td>
<td>49.5</td>
<td>88.6</td>
<td>98.2</td>
<td>98.3</td>
<td>&gt;99.9</td>
</tr>
<tr>
<td>NHase subunit β/α ratio</td>
<td>1.19</td>
<td>1.30</td>
<td>1.20</td>
<td>1.27</td>
<td>1.17</td>
<td>1.13</td>
<td>1.19</td>
</tr>
<tr>
<td>Protein amount (mg)</td>
<td>362.06</td>
<td>55.02</td>
<td>39.98</td>
<td>14.75</td>
<td>14.1</td>
<td>9.818</td>
<td>8.398</td>
</tr>
<tr>
<td>Yield against prior step (%)</td>
<td>NA</td>
<td>15.20</td>
<td>72.67</td>
<td>36.88</td>
<td>95.63</td>
<td>69.63</td>
<td>85.54</td>
</tr>
<tr>
<td>Yield against cell-free lysate (%)</td>
<td>NA</td>
<td>15.20</td>
<td>11.04</td>
<td>4.07</td>
<td>3.89</td>
<td>2.71</td>
<td>2.32</td>
</tr>
</tbody>
</table>

Note: All data were collected by ImageQuant TL. The NHase ratio excluded the fragmented NHase β (~20 kDa, beneath NHase α subunit shown on Figure 25)

NHase ratio means the percentage of NHase in corresponding sample of each step of purification.

Cell-free lysate means total recovered cytosolic proteins used for NHase purification.

Protein amount was determined by BCA protein assay.

Yield against prior step referred to the percentage of total protein of the current step against the prior step.

Yield against cell-free lysate referred to the percentage of total protein of the current step against the total recovered cytosolic proteins used for NHase purification.
4. DISCUSSION

4.1. NHase activity and NHase expression against different inducers

Among the inducers tested in *R. rhodochrous* DAP 96253, the combination of Co and urea gave the highest level of NHase activity. Corresponding to the NHase activity, only the combination of Co and urea can overexpress NHase in the cytosolic proteins (Table 6, Figure 7 and 8). In the absence of Co or urea, NHase activity was not induced (less than 1 unit/mg-cdw) (Table 6 and Figure 7). Additionally, without urea, NHase protein was not highly expressed (~ 5% of the total cytosolic proteins). This was observed by gradient SDS-PAGE (Figure 8). Urea was also reported to upregulate the expression of a microbial nickel transporter and this nickel transporter increases the uptake of Co into the cells (Kobayashi and Shimizu, 1998). Thus, even if Co upregulates NHase expression there may not be enough Co inside the cells to over-express NHase expression when compared to the Co/urea induced condition. Current analyzed data is not sufficient to determine whether Co is involved in upregulation of NHase expression. However, as long as Co was present, the NHase activity was significantly higher than the cells that were induced under Co-free conditions, even though the NHase protein was not highly expressed (~7%) (Table 6, Figure 7). An additional possibility is that the trace amount urea or other amide(s) present in the media or additional factors inside the cells enable the minimum expression of NHase. Low levels of constitutively expressed NHase may lack activity due to the absence of Co, a required cofactor for NHase activity. Fe and Ni substitution for Co resulted in significantly lower NHase activity (2%) compared to Co/urea induction. As shown by Figures 7 and 8, urea is required for the induction of NHase expression, and Co is required for the activity and overexpression of NHase.
Similarly in *R. rhodochrous* DAP 96622 cells, Co/urea induction gave the highest level of NHase activity, compared to Fe or Ni induced cells. It should be noticed that NHase in *R. rhodochrous* DAP 96622 can be induced in the presence of Co. However, the NHase activity of these Co induced cells was approximately 40% of Co/urea induced cells. In addition, Co induced *R. rhodochrous* DAP 96622 had a higher activity than Co induced *R. rhodochrous* DAP 96253 cells. This suggests that the expression systems of these strains may be different.

### 4.2. [Amidase]/[nitrilase] and NHase expression

When *R. rhodochrous* DAP 96253 was induced with either urea, urea/isovaleronitrile or Co/urea, a significant band at approximately 38 kDa was observed, and found to be homologous to putative aliphatic amidase from *N. farcinica*. Cells induced with urea or urea/isovaleronitrile showed both amidase and nitrilase activity (amidase activity used acrylamide as the substrate and nitrilase activity used acrylonitrile as the substrate). In addition, in this urea/isovaleronitrile supplemented YEMEA, *R. rhodochrous* DAP 96253 showed 7 units/mg-cdw against benzonitrile conversion which is comparable to 8 units/mg-cells reported in *R. rhodochrous* J1 (Kobayashi *et al.*, 1992a). Combined, these data suggest the alleged amidase is actually a dynamic [amidase]/[nitrilase] enzyme that may be attributed to structural organization or post-translational modifications.

According to the model of Kobayashi and Shimizu (1998) for the *R. rhodochrous* J1 H-NHase, amide positively regulates the expression of the *nhhC* gene. Its product, NhhC, in turn up-regulates the *nhhD* gene, whose product, NhhD, positively regulates *nhhB* and *nhhA* genes expressing H-type β and α subunits (Figure 33). In the L-NHase, amide is also a positive regulator for both α and β subunits for the L-type NHase (Figure 33). However, in *R. rhodochrous* DAP 96253, when induced with acrylamide and Co, the NHase activity was not
significantly induced (~15 units/mg-cell dry weight). In addition, based on the SDS-PAGE, neither [amidase]/[nitrilase] nor NHase was significantly produced (NHase accounted for approximately 22% of the total recovered cytosolic proteins, and no significant band was shown around the same molecular weight with [amidase]/[nitrilase]) (Figure 34, Lane 3). In \textit{R. rhodochrous} DAP 96253, NHase was overexpressed only when Co and urea were present and acrylamide cannot substitute for urea as an inducer, as shown in Figure 12.

According to previous results, urea was an inducer for [amidase]/[nitrilase] (Figure 8, Lane 8; Figure 12, Lane 2), therefore it is possible that [amidase]/[nitrilase] production is necessary for NHase overexpression under a Co/urea induced condition. In order to explore the relation between [amidase]/[nitrilase] production and NHase overexpression, Co/urea/isovaleronitrile was used to induce \textit{R. rhodochrous} DAP 96253 due to the fact that urea/isovaleronitrile was also able to induce [amidase]/[nitrilase] (See Results 3.1.5. Overexpression of nitrilase in \textit{R. rhodochrous} DAP 96253). Because urea/isovaleronitrile can induce [amidase]/[nitrilase], Co/urea/isovaleronitrile supplemented YEMEA should lead to NHase overexpression in \textit{R. rhodochrous} DAP 96253. According to the SDS-PAGE (Figure 35), Co/urea/isovaleronitrile induced cytosolic protein showed only three major bands on the gel: [amidase]/[nitrilase] around 38 kDa, NHase β around 27kDa and NHase α around 22 kDa. NHase accounted for approximately 53% total recovered cytosolic proteins and amidase accounted for nearly 9% total recovered cytosolic proteins. The percentage of NHase from Co/urea/isovaleronitrile was comparable with Co/urea supplemented YEMEA cells, but the [amidase]/[nitrilase] content was higher. In short, as long as this [amidase]/[nitrilase] was induced and Co was present, NHase was overexpressed. This suggests that [amidase]/[nitrilase] production is possibly related with the overexpression of NHase, and the correlation of
[amidase]/[nitrilase] with NHase may be different from the regulation system of *R. rhodochrous* J1 (Kobayashi and Shimizu, 1998)

Similarly, in *R. rhodochrous* DAP 96622, although Co/urea supplemented YEMEA cells gave the highest activity among all tested inducing conditions, NHase activity accounts for only approximately 27% of Co/urea supplemented YEMEA *R. rhodochrous* DAP 96253 (Table 7). In addition, the gradient SDS-PAGE gel showed that only approximately 21% of the total recovered cytosolic proteins were NHase, but among all screened proteins, amidase (from the nitrilase/cyanide hydratase family) was not detected around 38 kDa (Figure 11 and Table 12). According to previous data from S. Ganguly (2007), an aliphatic amidase from nitrilase/cyanide hydratase family (~38 kDa) was purified from *R. rhodochrous* DAP 96622. The identified sequence of this aliphatic amidase from *R. rhodochrous* DAP 96622 was very similar with the [amidase]/[nitrilase] from *R. rhodochrous* DAP 96253 (according to MALDI TOF/TOF MS data). Thus, the existence of an aliphatic amidase (~38 kDa) was confirmed but this amidase was not overexpressed according to S. Ganguly. One possibility is that the aliphatic amidase (reported by S. Ganguly) or [amidase]/[nitrilase] (~38 kDa) was also involved in the overexpression of NHase in *R. rhodochrous* DAP 96622. This may be due to the aliphatic amidase or [amidase]/[nitrilase] was not overexpressed in *R. rhodochrous* DAP 96622, and the low level of [amidase]/[nitrilase] caused the lower level of NHase compared with *R. rhodochrous* DAP 96253 (Figure 11). Another possibility is that amidase (GGSS family) is able to enhance the expression of NHase to some extent (Co/urea vs. Co induced) but not as efficiently as [amidase]/[nitrilase].

In conclusion, as long as urea was present in the media, [amidase]/[nitrilase] was induced; in addition, as long as Co and urea were present or Co and [amidase]/[nitrilase] were present,
NHase was over-expressed in *R. rhodochrous* DAP 96253. Thus, it is possible that, beside Co, [amidase]/[nitrilase] may also be very important in regulation of the expression of NHase in *R. rhodochrous* DAP 96253. However, the NHase expression system in *R. rhodochrous* DAP 96622 is probably different from that of *R. rhodochrous* DAP 96253. Urea and Co may also affect other proteins, such as metal transporters on the membrane as reported by Kobayashi and Shimizu (1998), and these effects from urea and Co can be studied through genetic tools for future research.

Additionally, the NHase overexpression or assembly system in *R. rhodochrous* DAP 96253 is possibly related with NHase β subunit and the cell membrane (Figure 10, Lane 1 and Lane 2). This was not reported in the self-swapping system of *R. rhodochrous* J1 (Zhou *et al.*, 2009).
Figure 33. Genetic organization and regulation of *R. rhodochrous* J1 (Kobayashi and Shimizu, 1998).
Figure 34. *R. rhodochrous* DAP 96253 Co/urea supplemented and Co/acrylamide supplemented YEMEA cell cytosolic proteins.

Lane 1: Molecular makers; Lane 2: Co/urea induced cytosolic proteins; Lane 3: Co/acrylamide induced cytosolic proteins.

Loading amount: 13 µg/well.
Figure 35. *R. rhodochrous* DAP 96253 Co/urea supplemented and Co/urea/isovaleronitrile supplemented cell cytosolic proteins.

Lane 1: Co/urea induced cytosolic proteins; Lane 2: Co/urea/isovaleronitrile induced cytosolic proteins; Lane 3: Molecular makers.

Loading amount: 13 µg/well.
4.3. NHase substrates preference

According to all the substrates tested in Table 8, NHase induced with Co/urea in *R. rhodochrous* DAP 96253 was an aliphatic NHase. The best substrate was C3 or less. This NHase can hydrolyze chlorinated short chain aliphatic nitriles (Table 8). With the same length of carbon chain, chlorinated nitriles showed lower activity than non-chlorinated nitriles as the substrates. For example, NHase activity with chloroacetonitrile as the substrate was less than acetonitrile as the substrates, and the NHase activity with 3-chloropropionitrile was less than 10% of the activity with propionitrile. In addition, with the same length of carbon chains, NHase activity with double bonded nitriles were slightly lower than nitriles without a double bond: propionitrile vs. acrylonitrile.

*R. rhodochrous* DAP 96253 showed almost no activity against nitriles with a C4 or longer chain. *R. rhodochrous* DAP 96253 showed no activity against branched nitriles (isovaleronitrile and trimethylacetonitrile) and aromatic nitriles (benzonitrile). With the only dinitrile (adiponitrile) tested, the NHase activity was very limited, and this may be caused by the long carbon chain (C6 for adiponitrile).

4.4. NHase and GS in *R. rhodochrous* DAP 96253

During the development of NHase purification, ammonium sulfate precipitation, PEG precipitation (data not shown here), AEC such as Toyopearl SuperQ 650-M (Tosoh Bioscience LLC, PA, US) (data not shown here) and HiTrap™ DEAE FF 5ml, HIC with all seven different columns from the HIC selection kit, and GF with HiPrep Sephacryl 26/60 S-300 HR were not able to totally remove GS from NHase (Figure 13, 14, 16 and 24). In addition, amino acids such as glycine, alanine, serine and histidine were reported as native feedback inhibitors of GS I, and the binding of GS to these inhibitors may introduce charge or conformational changes of GS.
Single amino acids or a combination of all four amino acids were added in the loading buffers and the elution buffers of AEC and HIC columns. However, even with the addition of all four amino acids, the AEC and HIC processes did not remove GS from NHase (Data not shown here). Besides all the columns mentioned above, a self-pack ATP agarose (Sigma-Aldrich, Product number: A9264) column was also tested (affinity chromatography). This ATP is linked with agarose at the adenine N-6 position (Figure 36) of this ATP agarose. According to Eisenberg et al. (2000), during the initial binding step, ATP binds to the bifunnel structure of the GS through the γ phosphate from ATP (Figure 37 and Figure 5). The binding of ATP to GS does not require other ions or compounds (Eisenberg et al., 2000). ATP agarose should be able to bind and remove GS from NHase sample after GF. However, after GF and affinity chromatography with ATP agarose, GS was still shown on the gel of the protein sample (Figure 19, Lane 2). Thus, GS and NHase may form a protein complex and this hypothetical GS-NHase protein complex has not been reported in other strains such as R. rhodochrous J1 and M8 (Watanabe et al., 1987a; Watanabe et al., 1987b; Mayaux et al., 1990; Pogorelova et al., 1996; Kobayashi and Shimizu, 1998). In this hypothetical GS-NHase protein complex, both proteins are related with the nitrogen metabolism.

In addition to GS and NHase, when R. rhodochrous DAP 96253 was induced with Co/urea, the band at approximately 47 kDa (the band beneath the major band with a molecular weight of ~50 kDa) was identified as isocitrate lyase from R. equi (according to MALDI TOF/TOF MS data) (Figure 11). Isocitrate lyase is also related to nitrogen metabolism because of α-ketoglutarate from TCA cycle.

According to the hypothetical pathway for nitriles and amides shown in Figure 35, NHase is able to hydrolyze nitriles to amides, and [amidase]/[nitrilase] then hydrolyzes amides to
corresponding acids and ammonium. Ammonium can be incorporated with α-ketoglutarate to form glutamate by glutamate dehydrogenase or glutamate synthetase in the GS/GOGAT pathway. Due to the consumption of α-ketoglutarate from the TCA cycle, isocitrate lyase bypasses α-ketoglutarate and succinyl-CoA and directly converts isocitrate to succinate and glyoxylate. Malate synthase then converts glyoxylate and acetyl-CoA to malate. Both malate and succinate can then ensure the flow of the TCA cycle with continuous consumption of α-ketoglutarate to form glutamate from the TCA cycle (Figure 38). Especially, when induced with Co/urea, urea provided a large amount of ammonium, and this whole pathway may facilitate incorporating nitrogen into biomass (Figure 38).

Among the limited amount of protein bands that have been screened, GS and isocitrate lyase have been identified. NHase, [amidase]/[nitrilase] and GS are the essential enzymes for nitrile and amide metabolism.
Figure 36. ATP agarose structure.

Figure 37. Initial binding step of ATP to glutamine synthetase (Eisenberg et al., 2000).
Figure 38. Hypothetical pathway for nitriles and amides in *R. rhodochrous* DAP 96253 Co/urea induced cells.
4.5. NHase from *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 96622

During protein purification, the purity of NHase did not reach more than 95% before the first GF for both *R. rhodochrous* DAP 96253 and DAP 96622 (Figure 25, Figure 32, Table 14, and Table 15). In *R. rhodochrous* DAP 96253, neither GS nor amidase was totally removed after the first GF. However, in *R. rhodochrous* DAP 96622, four protein bands were not removed from the NHase, but the MALDI TOF/TOF MS suggested the two bands around 50 kDa were NHase αβ dimers. The band with a lower molecular weight (~49 kDa) could potentially be an α subunit and truncated β subunit formed during the purification process (Figure 32, Lane 6, 7 and 8). Thus, the two bands around 100 kDa may be (αβ)_2 tetramers and the band with lower molecular weight (~98 kDa) is possible (αβ)_2 tetramer with truncated β subunits (Figure 32, Lane 6 and 7). The dimers and tetramers may be formed during the denaturing (incomplete denaturation) and running process of SDS-PAGE. Thus, NHase may have been purified after the 1st NHase and the yield at this step is around 14% of the total cytosolic proteins. Dissimilar from *R. rhodochrous* DAP 96622, the band around 55 kDa in *R. rhodochrous* DAP 96253 after the first GF was identified as a combination of NHase α subunit, β subunit and GS. In addition, the [amidase]/[nitri]ase] band (~38 kDa) was not completely removed and this band was not removed until the second GF. Hence, NHase from *R. rhodochrous* DAP 96253 may be different from that in *R. rhodochrous* DAP 96622. NHase from *R. rhodochrous* DAP 96253 may form a protein complex with GS, but no other proteins were detected from NHase in *R. rhodochrous* DAP 96622 after the second GF (the purity of NHase is more than 99.9% after the second GF). Another difference between the two NHases in both strains is the purified NHase activity. Purified NHase from *R. rhodochrous* DAP 96253 showed higher activity than purified NHase from *R. rhodochrous* DAP
96622 (As shown in Results, the activity of purified NHase from *R. rhodochrous* DAP 96253 was approximately 1500 units/mg-proteins and the activity of purified NHase from *R. rhodochrous* DAP 96622 was around 950 units/mg-protein).

Despite the differences between the ways NHases organized in the cytosol, NHases from both strains showed several similarities. First, both NHases have similar if not the same charges. According to Figure 20 and 25, both NHases were eluted from HiPrep™ Q FF 16/10 column at approximately 53% elution buffer, and this was approximately 0.53M NaCl. Due to the fact that AEC separates proteins by charges, the AEC suggests that both NHases have similar charges. Second, both NHases have similar if not the same hydrophobicity. As shown in Figure 22 and Figure 28, both NHases were eluted from HiTrap™ Butyl HP 5ml column at 17.5% elution buffer, and this was approximately 0.35M ammonium sulfate. Because HIC separates proteins by the hydrophobicities, the data from HIC suggest that both NHases have similar hydrophobicity. Third, both NHases may have similar sizes. When purifying NHase by a HiPrep™ 26/60 Sephacryl™ S-300 HR column, both NHase eluted at approximately 1/2 column volume. Since GF separates proteins by sizes, the GF data suggest the sizes of both NHases are very similar if not the same.

### 4.6. Yield of NHases from both *R. rhodochrous* DAP 96253 and DAP 96622

During the purification of NHase from both *R. rhodochrous* DAP 96253 and DAP 96622 strains, a major amount of proteins were lost during the first AEC and the first HIC step. Approximately 55% cytosolic proteins from *R. rhodochrous* DAP 96253 and approximately 85% cytosolic proteins of DAP 96622 were lost during the first AEC (Table 14 and 13). In addition, around 50% of the proteins from the second AEC were lost during the first HIC for *R.
*rhodochrous* DAP 96253 and more than 60% of the proteins from the second AEC were lost during the first HIC for *R. rhodochrous* DAP 96622 (Table 14 and 13).

One reason for low yield during the first AEC was the lipids and/or pigments in the total recovered cytosolic proteins. A considerable amount of proteins were detected (data not shown here) by the BCA protein assay on the flow through fraction of the 1st AEC. In addition, some proteins were not eluted by the elution buffer with 1M NaCl, but only eluted with 1M NaCl/0.5 M NaOH (Stripping buffer) followed with 70% ethanol. This was discovered during cleaning the HiPrep™ Q FF 16/10 column (Several protein peaks were washed out during the cleaning of the column). An ammonium sulfate precipitation step could be added before the first AEC and the ammonium sulfate precipitation for this proposed step should be lower than 35% saturation of ammonium sulfate to prevent NHase lost (Figure 13). However, ammonium sulfate may affect protein binding to anion exchange columns because the sulfate ion is a bivalent ion. The yield should be tested during this proposed ammonium sulfate step for the optimization of the purification steps.

The reasons for protein lost during HIC were similar to AEC, some protein did not bind to the column or washed off the column during the early stage of elution (when the salt concentration was still significantly higher than the target elution concentration), and a significant amount of protein was lost during the cleaning of the column with 20% ethanol. Thus, some proteins were not eluted with elution buffer without salt (20 mM NaH$_2$PO$_4$/10 mM Na-butyrate/pH 7.2).

In addition to protein lost, HIC can cause protein fragmentations (Figure 25, Lane 5 and Lane 7; Figure 32, Lane 5 and Lane 7). A band around 20 kDa was identified as NHase β subunit (~27 kDa), and this means that some of the NHase β subunit was fragmented during the HIC.
process. When using ammonium sulfate precipitation to concentrate purified NHase from both *R. rhodochrous* DAP 96253 and DAP 96622, the fragmented NHase β subunit showed on the gel (Figure 39). Thus, ammonium sulfate was added to the purified NHase without precipitating or concentrating the NHase and the fragmented NHase β subunit showed up on the gel (Data not shown here). In addition to fragmented NHase β subunit on the gel, many other protein bands with 16 kDa or lower appeared on the gel (Figure 25, Lane 5 and Lane 7; Figure 32, Lane 5 and Lane 7; Figure 39, Lane 2). Hence, ammonium sulfate may truncate NHase β subunit during the purification process.
Figure 39. Purified NHase and purified NHase concentrated with ammonium sulfate from *R. rhodochrous* DAP 96253.

Lane 1: Purified NHase; Lane 2: Ammonium sulfate precipitation concentrated NHase; Lane 3: Molecular makers.

Loading amount: 13 µg/well.
5. CONCLUSION

Among tested conditions, only Co/urea can overexpress NHase in both *R. rhodochrous* DAP 96253 and DAP 96622. When induced with Co/urea, cells of *R. rhodochrous* DAP 96253 expressed higher NHase activity than cells of *R. rhodochrous* DAP 96622. The results suggested that when induced with Co/urea, the protein profiles of *R. rhodochrous* DAP 96253 and DAP 96622 were different. NHase accounted for more than 55% of the total cytosolic proteins in *R. rhodochrous* DAP 96253, and it accounted for approximately 25% of the total cytosolic proteins in *R. rhodochrous* DAP 96253. In addition, amidase in *R. rhodochrous* DAP 96253 was around 38 kDa from the nitrilase/cyanide hydratase family and amidase in *R. rhodochrous* DAP 96622 was about 55 kDa from the amidase signature family. The results also suggest that nitrile hydratase from *R. rhodochrous* DAP 96253 may form a protein complex with glutamine synthetase, and this novel protein complex was not reported in the past.
6. FUTURE DIRECTIONS

Although Co/acrylamide induced condition has been tested for the NHase overexpression, yet the acrylamide in different molarities were not tested. In order to exclude the possibility that Co/acrylamide may also overexpress NHase, different molarity concentrations of acrylamide should be tested.

In Co/urea supplemented media with half amount of urea (3.75g/L), the NHase bands still accounted for more than 55% total recovered cytosolic proteins and the protein profile from gradient SDS-PAGE did not shown significant differences from Co/urea (7.5g urea/L). The minimum concentration of urea needed to overexpress NHase should be examined.

In R. rhodochrous DAP 96253, the identity of [amidase]/[nitrilase] was not totally defined, although according to MALDI TOF/TOF MS it was defined as amidase, and based on the substrates preference and induction method it was considered as [amidase]/[nitrilase]. In order to identify what exactly this enzyme is, a genome sequence is necessary. Amidases from nitrilase/cyanide hydratase family and nitrilases from nitrilase/cyanide hydratase family share similar sequences, and the genome sequences only may not be sufficient to distinguish the posttranslational modification and/or organization differences. Thus, a crystal structure for this [amidase]/[nitrilase] is going to be necessary. Hence, crystallization is needed for the identification of this enzyme.

Based on the charge, hydrophobicity and size, both NHases from R. rhodochrous DAP 96253 and DAP 96622 are very similar, even though the activities of both purified NHases are not the same. The mechanisms behind this difference or similarities need to be studied. This will need crystallization of both NHases and genome sequence of both enzyme genes. In addition, literature on how NHase α and β subunits were organized in the holoenzyme of NHases from
other strains is not available. Thus, the crystal structure can also be compared between *R. rhodochrous* DAP 96253 and DAP 96622.

In order to understand whether [amidase]/[nitrilase] is important in NHase overexpression in both strains, gene knock-out for [amidase]/[nitrilase] should be studied in *R. rhodochrous* DAP 96253. For *R. rhodochrous* DAP 96622, an inducer for [amidase]/[nitrilase] overexpression will be necessary for studying the relationship between [amidase]/[nitrilase] and NHase overexpression. A candidate for the overexpression of [amidase]/[nitrilase] (for *R. rhodochrous* DAP 96622) will be urea/isovaleronitrile because this has been reported to induced nitrilase in *R. rhodochrous J1* and this method was also proved to be effective with *R. rhodochrous* DAP 96253 in current work (Results, Overexpression of [amidase]/[nitrilase] in *R. rhodochrous* DAP 96253)(Kobayashi et al., 1992a). Since Co/urea were not able to overexpress similar level of NHase in *R. rhodochrous* DAP 96622 compared with *R. rhodochrous* DAP 96253, if other inducers will overexpress both [amidase]/[nitrilase] and NHase in *R. rhodochrous* DAP 96622, a stronger correlation between [amidase]/[nitrilase] and NHase expression will be established.
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mechanism also carries out the oxidation of the metal ligand cysteine residues and insertion of
APPENDICES

Appendix A. BCA protein assay

Table 16. Making working standards from bovine serum albumin standard (BSA standard), 2mg/ml (Thermo Scientific, IL United States).

<table>
<thead>
<tr>
<th>Standard</th>
<th>Final Concentration to Make (µg/ml)</th>
<th>Standards to Use</th>
<th>DDI water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2000 (original BSA standard)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1600</td>
<td>200 µl 2000µg/ml (A)</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>1200</td>
<td>150 µl 2000µg/ml (A)</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>1000</td>
<td>100 µl 2000µg/ml (A)</td>
<td>100</td>
</tr>
<tr>
<td>E</td>
<td>800</td>
<td>100 µl 1600µg/ml (B)</td>
<td>100</td>
</tr>
<tr>
<td>F</td>
<td>600</td>
<td>100 µl 1200µg/ml (C)</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>400</td>
<td>100 µl 800µg/ml (E)</td>
<td>100</td>
</tr>
<tr>
<td>H</td>
<td>300</td>
<td>100 µl 600µg/ml (F)</td>
<td>100</td>
</tr>
<tr>
<td>I</td>
<td>200</td>
<td>100 µl 400µg/ml (G)</td>
<td>100</td>
</tr>
<tr>
<td>J</td>
<td>100</td>
<td>100 µl 200µg/ml (I)</td>
<td>100</td>
</tr>
<tr>
<td>K</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Proteins samples were properly diluted (1/5, 1/10, 1/20 and 1/40) with DDI H₂O. 25 µl protein sample were loaded in each well on a 96-well plate. BCA working solution was prepared by adding 1 volumes of BCA reagent B into 50 volumes of BCA reagent A. Each well with
protein sample was added with 200 µl working solution. Proteins and working reagent were mix
by shaking gently for 30 seconds, and then the 96-well plate was incubated at 37°C for 30 min.
After incubation, the color changed was determined by reading OD 600nm with Wallac Victor2.

A standard curve was made by the concentration of BSA standards and OD 600, and the
protein concentration was calculated by the OD 600 reading and the standard curve.

Appendix B. T-test for NHase assay under different conditions for \textit{R. rhodochrous} DAP 96253

Table 17. T-test for all conditions tested with acrylonitrile as the substrate (whole cell, \textit{R. rhodochrous} DAP 96253).

<table>
<thead>
<tr>
<th>T-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co vs. Co/urea</td>
<td>0.0005</td>
</tr>
<tr>
<td>Co vs. Fe/urea</td>
<td>0.0033</td>
</tr>
<tr>
<td>Fe/urea vs. urea</td>
<td>0.0087</td>
</tr>
<tr>
<td>Fe/urea vs. Fe</td>
<td>0.0040</td>
</tr>
<tr>
<td>Co/urea vs. Fe/urea</td>
<td>0.0102</td>
</tr>
<tr>
<td>Co vs. Fe</td>
<td>0.0007</td>
</tr>
<tr>
<td>Ni/urea vs. urea</td>
<td>0.1349</td>
</tr>
<tr>
<td>Fe vs. uninduced</td>
<td>0.0008</td>
</tr>
</tbody>
</table>