Isolation of cyanide-degrading bacteria and molecular characterization of its cyanide-degrading nitrilase

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Abstract

Hydrogen cyanide is an industrially important chemical, and its annual production is more than 1.5 million tons. Because of its toxicity, the cyanide-containing effluents from industries have caused many environmental problems. Among various methods to treat the contaminated soils or water, the biological degradation is regarded to be promising. We isolated two cyanide-degrading microorganisms, *Pedobacter* sp. EBE-1 and *Bacillus* sp. EBE-2, from soil contaminated with cyanide. Among these bacteria, *Bacillus* sp. EBE-2 exhibited significantly a high cyanide-degrading ability. *Bacillus* sp. EBE-2 might be used for the remediation of cyanide contaminated water or soil. A nitrilase gene was cloned from *Bacillus* sp. EBE-2. *Bacillus* nitrilase was expressed in *Escherichia coli* and purified. *Bacillus* nitrilase exhibited cyanide-degrading activity as a large oligomer. Since formic acid formation from cyanide was observed, *Bacillus* nitrilase is likely to be a cyanide hydrolase. Although there exist various homologous enzymes annotated as carbon–nitrogen family hydrolases, this is the first report on the cyanide degrading activity. The structure and catalytic site of *Bacillus* nitrilase were studied by homology modeling and molecular docking simulation. Volume 00, Number 0, Pages 1–7, 2021

Keywords: *cyanide, Bacillus, nitrilase, biocatalysis, biodegradation, protein modeling*

1. Introduction

Cyanide is a compound containing the C≡N group. The cyanide anion is highly toxic. It binds to iron at the catalytic center of the mitochondrial protein cytochrome *c* oxidase and prevents electron transport from cytochrome *c* to oxygen [1]. As a result, the electron transport chain is disrupted. Thus, cells can no longer aerobically produce ATP for energy. Hydrogen cyanide (HCN) is an industrially important chemical and has been used in the pharmaceutical industry, polymer manufacturing, mining and steel manufacturing, electroplating, and agrochemical production. More than 1.5 million tons of HCN are produced annually. The cyanide-containing effluents from such industries have caused many environmental problems. Therefore, various cyanide detoxification strategies have been developed. Chemical and physical treatments are effective in the detoxification process of cyanide [2]. However, the cyanide-degrading abilities affected by multiple factors, such as the chemical composition of the waste, the amount of effluent, and the area of contamination. Microbial degradation of cyanide from ore mining wastewater is regarded as the most successful method for cyanide removal [3, 4].

In the biodegradation process, free cyanide and metal cyanide complexes are transformed into ammonia and

Abbreviations: *CNO, cyanide oxygenase; CynD, cyanidase.*

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bicarbonate. Because various cyanide compounds are produced in nature, multiple microorganisms, including fungi, algae, yeasts, and bacteria, can degrade cyanide compounds. Many fungal species, including the genera *Fusarium*, *Penicillium*, *Scytalidium*, and *Trichoderma*, are known to degrade cyanide [5, 6]. The yeast *Cryptococcus cyanovorans* could utilize up to 10 mM NaCN as a sole carbon source [7]. Bacterial species belonging to the genera *Pseudomonas*, *Arthrobacter*, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Rhodococcus*, and *Serratia* have been reported to exhibit cyanide-degrading ability [8–12].

Varieties of enzymatic pathways are involved in the processes for the biotransformation and biodegradation of cyanide compounds. Cyanide compounds are degraded by four different pathways: the oxidative pathway, hydrolytic pathway, reductive pathway, and substitution pathway [13]. In the aerobic pathway, cyanide is converted into ammonia and carbon dioxide using three different enzymes, namely, cyanase, cyanide monooxygenase, and cyanide dioxygenase [3, 14]. Among these enzymes, cyanide monooxygenase and cyanide dioxygenase can degrade free cyanide. Cyanase catalyzes the degradation of cyanate, which is produced from cyanide by oxidation in soil. Using bicarbonate as a nucleophile reactant, cyanase converts cyanate to an unstable carbamate, which further spontaneously breaks down to form ammonia and carbon dioxide by decarboxylation [15]. Cyanide oxygenase (CNO) from *Pseudomonas fluorescens* NCIMB 11764 is a pterin-dependent enzyme that catalyzes the mono-oxygenase conversion of cyanide to formic acid and ammonia [16]. CNO consists of four protein components (P1–P4). P1 is a multimeric, 230-kDa flavoprotein exhibiting the properties of a peroxide-forming NADH oxidase (oxidoreductase). P2 is a NADH peroxidase. P3 is a sizable oligomeric protein (~300 kDa) with cyanide dihydratase activity. The remaining P4 component is a carbonic anhydrase [17]. Cyanide dioxygenase is also thought to be a pterin-dependent enzyme [14]. Most microbes exploit the hydrolytic pathway for the degradation of cyanide compounds. Various hydrolases and nitrilases can degrade free as well as organic cyanide compounds. In the hydrolytic pathway, direct cleavage of the carbon–nitrogen triple bond by nitrilase family enzymes results in the formation of the corresponding amide or acid and ammonia [18]. Nitrilases are generally homo-oligomers of an $\alpha\beta\beta\alpha$–$\alpha\beta\beta\alpha$ sandwich fold protein and use the triad of cysteine, glutamic acid, and lysine as the active center [19]. Among nitrilases, cyanidase (cyanide dehydratase, CynD) and cyanide hydratase can degrade free cyanide. Cyanide hydratase exists throughout the fungal kingdom and converts cyanide into formamide as the product [20, 21]. In the cell, cellular amidases normally convert formamide into formate and ammonia. CynD exhibits high sequence homology with other bacterial nitrilases with different substrates [22]. Cyanide hydratase is also homologous to nitrilases. However, the sequence identity between CynD and cyanide hydratase is only approximately 25%–30%. CynD and cyanide hydratase are more similar to other nitrilases than to each other.

Nitrogenase works in the reductive pathway. Nitrogenase reduces cyanide into methane and ammonia [13]. In substitution pathway, cyanides are converted to thiocyanate by the transfer of a sulfur group from thiosulfate or 3-mercaptopyruvate by rhodanese or 3-mercaptopyruvate sulfurtransferase. The produced thiocyanate is further assimilated as a nitrogen source [13].

In this study, we isolated two cyanide-degrading microorganisms, *Pedobacter* sp. and *Bacillus* sp., from soil contaminated with cyanide. Among these species, *Bacillus* sp. exhibited significantly high cyanide-degrading ability. *Bacillus* sp. has the nitrilase gene. *Bacillus* nitrilase was expressed and purified. It was found to be a cyanide hydrolase. The structure and catalytic site were studied by homology modeling.

2. Materials and Methods

2.1. Isolation of cyanide-degrading bacteria

Groundwater obtained from a site contaminated with cyanide was used as the source of cyanide-degrading bacteria. The groundwater was supplemented with free cyanide in the form KCN at approximately 12 mg/L. The sample was incubated with or without the addition of 0.1% yeast extract. The number of bacteria was counted as colony forming units. The cyanide concentration was measured by the chloramine T/isonicotinic acid/1,3-dimethylbarbituric acid method using visocolor ECO cyanid (MACHEREY-NAGEL, Düren, Germany) with slight modification. The cyanide-degrading bacteria were isolated by several repeated of plating and cyanide degradation activity assays.

2.2. 16S rRNA gene cloning and sequence

The 16S rRNA gene was amplified by PCR using the primers 16SrRNA_Fw5′-AGAGTTTGATCCTGCCTCAG-3′ and 16SrRNA_Rv 5′-GGTTACCTTGTTACGACTT-3′. The amplified DNAs were cloned into the pMD20 vector by TA cloning and applied for DNA sequencing.

2.3. Cloning, expression, and purification of *Bacillus* nitrilase

The *Bacillus* nitrilase gene was obtained by PCR using the primers BNit_Fw 5′-CATATGAAAATCGCCCTTGT-3′ and BNit _Rv5′-CTCGAGCTAATAGATATCCAGACGCTGA-3′ and cloned into the pMD20 vector. After sequence analysis, the gene was cloned into the *Nde*I/*Xho*I site of the pET23b vector (pET-BNit). *E. coli* BL21 (DE3) transformed with pET-BNit was cultured in LB media supplemented with 100 μg/mL ampicillin at 18 °C for 70 H. The cells were harvested by centrifugation at 5,000*g* for 10 Min at 4 °C. The harvested cells were suspended in buffer (50 mM Tris–HCl, pH 8.0) and disrupted by sonication, and the suspension of disrupted cells was centrifuged at 24,000*g* for 30 Min at 4 °C. The supernatant was applied to an anion-exchange column (TOYOPEARL DEAE-650M; Toyobo, Osaka, Japan) equilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT). Proteins were eluted with a linear gradient of NaCl in buffer A. Fractions containing *Bacillus*

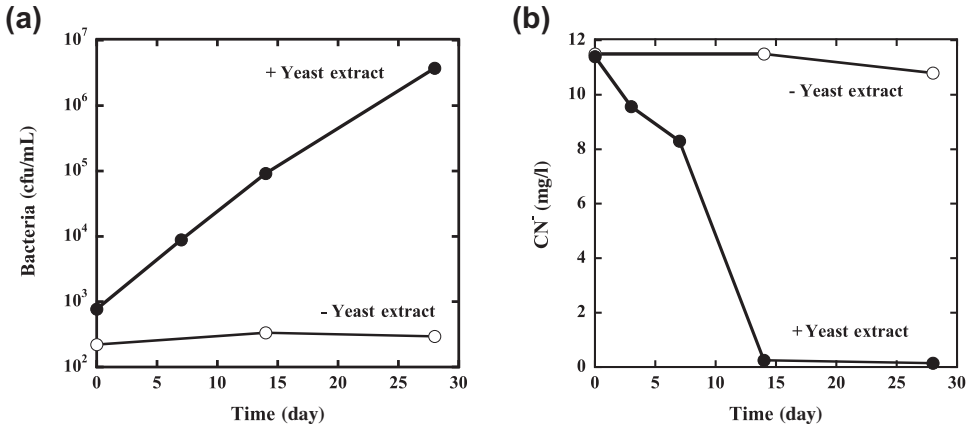

FIG. 1 *Enhancement of cyanide degradation with the growth of bacteria by the addition of yeast extract. The groundwater was supplemented with free cyanide as KCN to be approximately 12 mg/L. The water was incubated with or without the addition of 0.1% yeast extract. Growth of bacteria (a) and decrease of cyanide (b) are shown.*

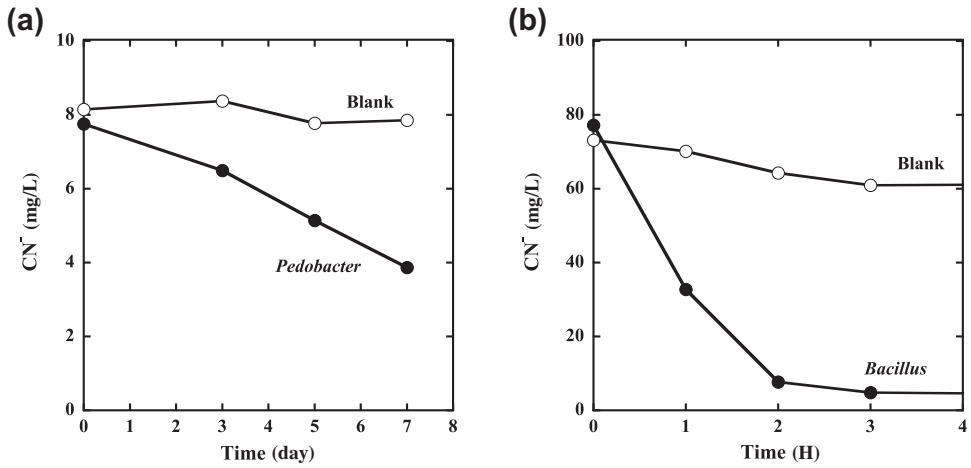

FIG. 2 *Cyanide degradation abilities of isolated bacteria. The cyanide-degrading activities of the isolated bacterial strains were investigated by incubating them in physiological salt solutions containing KCN. (a)* Pedobacter *sp. EBE-1. The initial cell density was 3 × 10⁹ CFU/mL. (b)* Bacillus *sp. EBE-2. The initial cell density was 2 × 10⁶ CFU/mL.*

nitrilase were pooled and dialyzed with buffer A overnight. The dialyzed protein solution was applied to a RESOURCE Q column (Cytiva, Tokyo, Japan) equilibrated with buffer A. Proteins were eluted with a linear gradient of NaCl in buffer A. Fractions containing *Bacillus* nitrilase were concentrated by ultrafiltration (Amicon Ultra, Merck Millipore, Billerica, CA) and then applied to a HiLoad 26/60 Superdex 200 pg size-exclusion column (Cytiva) equilibrated with buffer B (50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and 150 mM NaCl).

2.4. Cyanide degradation assay

Cyanide-degrading activity was assayed by mixing the *Bacillus* nitrilase solution with 270 ng of KCN in 1.5 mL of buffer A. The decrease in cyanide was measured after incubation at 30 °C for 30 Min. The cyanide hydrolase activity was measured as follows. The reaction was started with 100 μL of *Bacillus* nitrilase (0.8 mg/mL in buffer A) and 1.4 mL of reaction solution [676 ng of KCN, 6.9 μM NAD^+, and 1 unit of formic acid dehydrogenase (Sigma–Aldrich Japan, Tokyo, Japan) in buffer A]. Formation of formic acid was measured by the increase in NADH absorbance at 340 nm.

2.5. Homology modeling

The three-dimensional structure of *Bacillus* nitrilase was constructed by the automated protein modeling server SWISS-MODEL [23] on the basis of homology modeling with reference

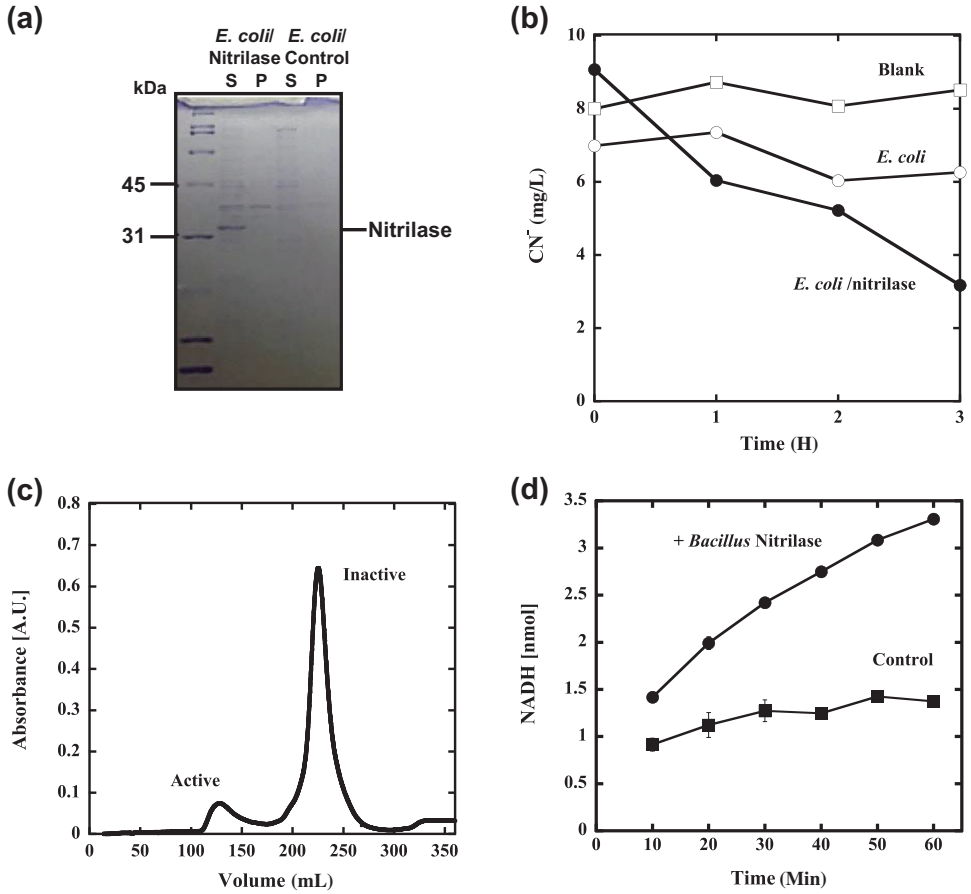

FIG. 3

Expression, cyanide-degrading activity, and purification of Bacillus *nitrilase. (a) SDS-PAGE analysis of the expression of* Bacillus *nitrilase. S, supernatant of crude extract; P, precipitate of crude extract. (b) Cyanide-degrading activity of recombinant* E. coli *expressing* Bacillus *nitrilase. (c) Size-exclusion chromatography of the purified* Bacillus *nitrilase. (d) Cyanide hydrolase activity of* Bacillus *nitrilase. Formation of formic acid from cyanide was observed by the production of NADH.*

to the crystal structure of the putative carbon–nitrogen family hydrolase from *Staphylococcus aureus* (PDB ID: 3P8K), which shared the highest sequence identity to that of *Bacillus* nitrilase.

2.6. Molecular docking

Molecular docking was performed using AutoDock Vina [24] with preprocessing of structures using AutoDock Tools. Cyanide ion was docked to the modeled structure of *Bacillus* nitrilase within a box (with dimensions of 40 Å × 40 Å × 40 Å).

3. Results and Discussion

3.1. Isolation of cyanide-degrading bacteria

To observe microbial degradation of cyanide in the groundwater obtained from the site contaminated with cyanide, the effect of the addition of yeast extract was investigated. The groundwater was supplemented with free cyanide as KCN to be approximately 12 mg/L. The water was incubated with or without the addition of 0.1% yeast extract. An increase in bacteria occurred when the groundwater was supplemented with yeast extract (Fig. 1a). As bacteria grew, cyanide concentration decreased in the condition with yeast extract (Fig. 1b). On the contrary, no bacterial growth and a decrease of cyanide were observed without yeast extract (Figs. 1A and 1b). We then tried to isolate cyanide-degrading bacteria from the culture by repeated plating and cyanide degradation activity assays. Finally, we isolated two different cyanide-degrading bacteria.

The 16S rRNA sequences showed that one strain belongs to the genus *Pedobacter*, and the other strain is *Bacillus* sp. *Pedobacter* sp., named *Pedobacter* sp. EBE-1, was highly homologous to *P. heparinus* strain DSM 2366 [25]. The *Bacillus* sp., named *Bacillus* sp. EBE-2, was similar to *B. pumilus* strain PDSLzg-1 [26].

The cyanide-degrading activities of these bacterial strains were investigated by incubating *Pedobacter* sp. EBE-1 (Fig. 2a) and *Bacillus* sp. EBE-2 (Fig. 2b) in physiological salt solutions containing KCN. Among them, *Bacillus* sp. EBE-2 showed relatively higher activity than that of the other strain. The *Bacillus* sp. EBE-2 could degrade 80 mg CN^-/L in 2 H by 2×10^6 CFU/mL. Previously, 13 bacterial species were isolated from electroplating wastewater to assess their ability to biodegrade free cyanide [27]. A mixed culture mainly dominated

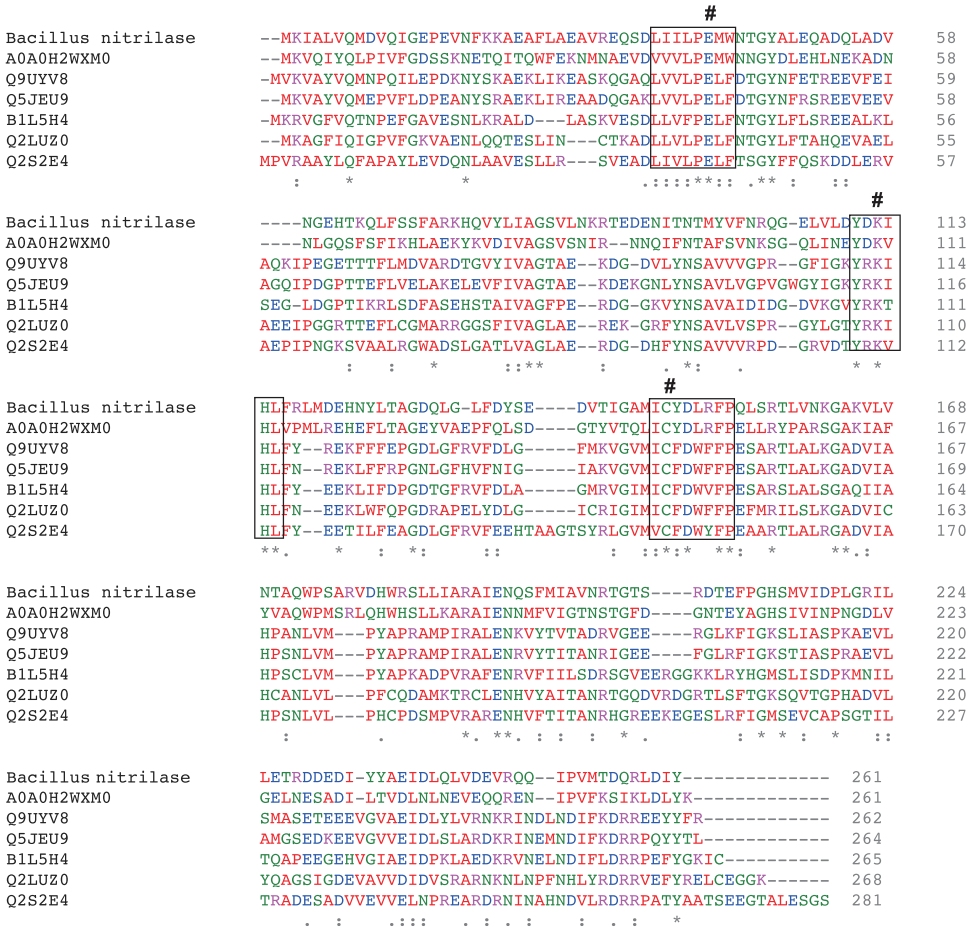

FIG. 4 *Sequence alignment of* Bacillus *nitrilase with other nitrilases. Amino acid sequence of* Bacillus *nitrilase (Bacillus_Nitrilase) was aligned with other nitrilase family proteins (A0A0H2WXM0,* Staphylococcus aureus *hydrolase, carbon–nitrogen family; Q9UYV8,* Pyrococcus abyssi *nitrilase; Q5JEU9,* Thermococcus kodakarensis *carbon–nitrogen hydrolase; B1L5H4,* Korarchaeum cryptofilum *nitrilase/cyanide hydratase; Q2LUZ0,* Syntrophus aciditrophicus *carbon–nitrogen hydrolase family protein; Q2S2E4,* Salinibacter ruber *hydrolase, carbon–nitrogen family). The putative catalytic regions are boxed. The residues constituting the catalytic triad are marked with #.*

by *Bacillus* sp. (*B. safensis*, *B. lichenformis*, and *B. tequilensis*) could degrade 131 (65.5%) and 177 (44.3%) mg CN^-/L in cultures containing 200 and 400 mg CN^-/L over a period of 8 days, respectively. Therefore, *Bacillus* sp. EBE-2 seems to have significantly high cyanide-degrading activity.

3.2. Cloning, sequencing, expression, and purification of *Bacillus* nitrilase

Then, we tried to find the enzyme responsible for cyanide degradation from *Bacillus* sp. EBE-2. Although some *B. pumilus* strains are known to have the CynD gene and exhibit cyanide-degrading activity, the CynD gene does not exist in the genome of the *B. pumilus* strain PDSLzg-1. A nitrilase gene (WP_047202096.1) was observed in the genome of *B. pumilus* strain PDSLzg-1. Considering that nitrilase is responsible for cyanide degradation, we tried to clone the nitrilase gene from *Bacillus* sp. EBE-2. Expecting high sequence identity with the nitrilase from *B. pumilus* strain PDSLzg-1, we designed the appropriate primers. The PCR-amplified gene was highly homologous to that of the nitrilase of *B. pumilus* (WP_047202096.1). These genes were highly homologous, and there were only three amino acid replacements. Then, we tried to express and purify *Bacillus* nitrilase. The gene was cloned into the pET23b vector (pET-BNit). *E. coli* BL21(DE3) cells harboring pET-BNit expressed a protein with a molecular mass of approximately 30 kDa and could degrade cyanide (Figs. 3a and 3b). *Bacillus* nitrilase was purified by chromatography. Interestingly, the purified *Bacillus* nitrilase appeared as two different peaks in the size-exclusion chromatograph (Fig. 3c). Their molecular masses were estimated to be approximately 24 and 509 kDa. Although a large portion of *Bacillus* nitrilase appeared in the low-molecular-weight fraction, probably as monomers, it did not exhibit cyanide-degrading activity. A previous study on *Rhodococcus* nitrilase has shown that inactive dimers assemble into a large, functional oligomer,

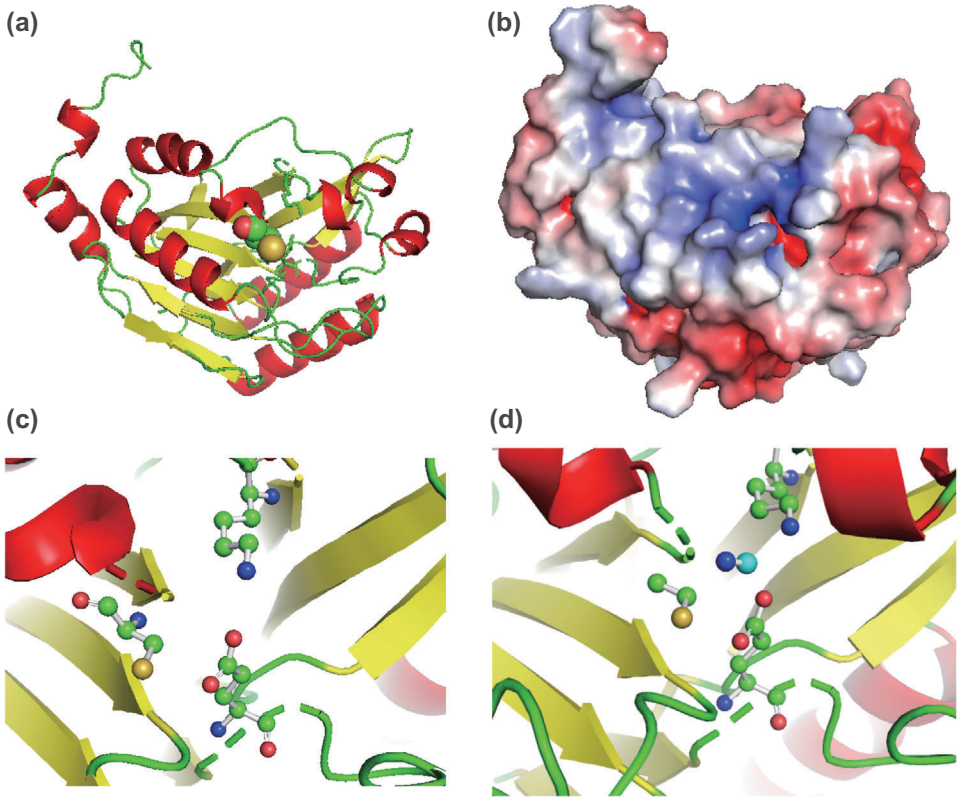

FIG. 5 *Predicted structure of* Bacillus *nitrilase. (a) Homology-based structure of* Bacillus *nitrilase. The catalytic cysteine residue is shown as a sphere model. (b) Electrostatic surface potential shown with positive charge colored blue and negative charge colored red. (c) Catalytic triad of* Bacillus *nitrilase. Cys, Glu, and Lys residues that compose the catalytic triad residues are shown as ball-and-stick models. (d) The docking model. Cyanide is also shown as a ball-and-stick model.*

a decamer, in the presence of a substrate benzonitrile [28]. Although we tried to examine the activity of the oligomers, it was difficult that the oligomer seems to disassemble spontaneously. The relatively low CN degrading activity of *E. coli* expressing *Bacillus* nitrilase compared with *B.* sp. strain EBE-2 might due to the poor active oligomer formation in *E. coli* or existence of other cyanide degrading enzymes in *B.* sp. strain EBE-2.

We tried to determine the reaction product of cyanide. As *Bacillus* nitrilase did not convert formamide to cyanide (data not shown), it was not a cyanide hydratase. Then, we examined the formation of formic acid by using formic acid dehydrogenase. As shown in Fig. 3d, conversion of cyanide to formic acid was observed. Thus, *Bacillus* nitrilase was determined to be a cyanide hydrolase.

There exist various homologous nitrilases, which are annotated as carbon–nitrogen family hydrolases (Fig. 4). However, there have been no reports on their cyanide degrading activity.

3.3. Homology modeling

Finally, we tried to make a 3D structural model by homology modeling. By automated swiss modeling, *Bacillus* nitrilase exhibited high sequence identity with the putative carbon–nitrogen family hydrolase from *S. aureus* (Fig. 4). Based on its crystal structure, a homology model was created (Fig. 5a). The putative catalytic Cys is shown as a spherical model. The thiol of cysteine attacks the cyano-carbon of nitriles (R–C≡N) to form a covalent thioimidate complex. The ammonia is released by the addition of one water molecule. Then the planar thioimidate is converted to a planar thiol acylenzyme through a tetrahedral intermediate. The acid product is released by the addition of a second water molecule and the enzyme is regenerated [29]. The surface charges are shown in Fig. 5b. The positive surface charge around the catalytic center seemed to be adapted for the negatively charged cyanide ion. Cys147, Glu41, and Lys112 form the catalytic triad in the catalytic center (Fig. 5c). Glu acts as the general base for activating the nucleophile of Cys, and Lys is the catalytic acid responsible for proton transfer to the substrate. We performed molecular docking simulations of *Bacillus* nitrilase and the cyanide ion using the modeled structure. Among 20 trials, cyanide entered at the putative catalytic site as one of the most preferred complex structures (Fig. 5d).

4. Conclusions

We have obtained two cyanide-degrading bacteria from groundwater contaminated with cyanide. Among these bacteria, *Bacillus* sp. EBE-2 was equipped with a nitrilase gene. Because of high cyanide-degrading activity, *Bacillus* sp. EBE-2 might be used for the treatment of cyanide contaminated water or soil. The catalytic site of *Bacillus* nitrilase contained a typical catalytic triad composed of Cys, Glu, and Lys with a positive surface charge. Similar to other nitrilases, *Bacillus* nitrilase also exhibited the activity in the oligomeric configuration. However, the activation mechanism by oligomerization is unknown. It is possible that some factors, small molecules or proteins, are required for the assembly of active oligomers. We found that the reaction product was not formamide but formic acid. Thus, the enzyme was determined to be a cyanide hydrolase.

5. Acknowledgement

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6. Accession Numbers

The nucleotide sequences of genes for 16S rRNAs and *Bacillus* nitrilase are deposited in the DNA data bank. The accession numbers are LC584259, *Bacillus* 16S rRNA; LC584260, *Pedobacter* 16S rRNA; LC584261, *Bacillus* nitrilase.

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