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Microbial production of 2-hydroxynicotinic acid from nicotinic acid by intact cells of MCI3289

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Abstract

We investigated the microbial production of 2-hydroxynicotinic acid (2-HNA) from nicotinic acid (NA) by intact cells of MCI3289, which was isolated from soil by enrichment culture with 6-methylnicotinic acid as the sole carbon source. The strain which showed the 2-HNA-producing activity was revealed to be a member of the β-subgroup of *Proteobacteria*. The 2-HNA-producing activity was induced by the addition of 6-methylnicotinic acid and 2-hydroxy-6-methylnicotinic acid to the culture medium. Intact cells of this strain incubated at 30°C in an optimal reaction mixture containing 15 g/l of NA produced 6.40 g/l of 2-HNA for 29 h.

Keywords: Nicotinic acid; 2-Hydroxynicotinic acid; Proteobacteria; 6-Methylnicotinic acid

1. Introduction

Some pyridine-related compounds are useful intermediates for the synthesis of pharmaceuticals and agricultural chemicals. Several methods have been reported for the preparation of 3,6-disubstituted pyridine using microorganisms [1,2]. Lehky et al. [3] reported the microbial production of 6-hydroxynicotinic acid (6-HNA) from NA by *Achromobacter xylosoxydans* [4]. Nagasawa et al. [5], Hurh et al. [6,7] also prepared 6-HNA from NA using *Pseudomonas fluorescens* TN5. We have developed an industrial process for the microbial production of 6-hydroxycyanopyridine from 3-cyanopyridine by intact cells of *Comamonas testosteroni* MCI2848 [8]. While the development study was being carried out, we started searching for the microbial enzyme which catalyzes hydroxylation of nicotinic acid (NA) to 2-hydroxynicotinic acid (2-HNA) since the 2,3-disubstituted pyridine compounds are also important intermediates for industrial synthesis and little is known about its microbial production. Recently, Tinschert et al. [9] investigated the enzymatic synthesis of 2-HNA from NA using whole cells of the 6-methylnicotinic-acid-degrading bacterium, Mena 23/3-3c. This strain, which grows with 6-methylnicotinic acid as its sole source of carbon and energy, showed the activity.

This paper reports screening of microorganisms, identification of the strain, and the optimization of cultural and reaction conditions for the production of 2-HNA from NA.

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2. Materials and methods

2.1. Chemicals

6-Methylnicotinic acid and 2-hydroxy-6-methylnicotinic acid were purchased from Aldrich Chemical (Milwaukee, WI, USA). NA and 2-HNA were provided by Tokyo Kasei Kogyo (Tokyo, Japan). Other chemicals used were commercial products.

2.2. Microorganisms and cultivation

6-Methylnicotinic-acid-utilizing microorganisms were isolated from soil samples using a medium containing 1.0 g of 6-methylnicotinic acid, 1.0 g of $(NH_4)_2SO_4$, 1.0 g of KH_2PO_4, 3.0 g of K_2HPO_4 and 250 mg of $MgSO_4 \cdot 7H_2O$ in 1000 ml distilled water, pH 7.0. A basal medium for cultivation consisted of 5.0 g of yeast extract, 5.0 g of corn steep liquor, 1.0 g of 6-methylnicotinic acid, 1.0 g of $(NH_4)_2SO_4$, 1.0 g of KH_2PO_4, 3.0 g of K_2HPO_4 and 250 mg of $MgSO_4 \cdot 7H_2O$ in 1000 ml of tap water (pH 7.0). The strain was cultivated in a 1-l jar fermenter system (Biot, Tokyo, Japan) with cultivation conditions as follows: pH adjusted to 7.0 with 500 g/l DL-malic acid, temperature 30°C, agitation at 600 rpm, and aeration at 1.0 volume per volume min (vvm). The strain was cultured for 50 h.

2.3. Assay methods for 2-HNA-producing activity

Cells harvested from the cultured broth were incubated with 15 mg of NA in 25 mM of potassium phosphate buffer (pH 7.0, total volume of 1.0 ml). The reaction was allowed to proceed at 30°C for 30 min with vigorous shaking in a test tube, then the reaction mixture was diluted and the cells were removed by centrifugation ($12\,000 \times g$ for 3 min). The amounts of 2-HNA produced and NA consumed were determined by high-pressure liquid chromatography (HPLC) with a UV detector under the following conditions: column, Wakosil-II $5C_{18}$ (5 by 30 mm; Wako Pure Chemicals, Osaka, Japan); mobile phase, 0.1% phosphoric acid: CH_3CN = 90:10 (v/v); flow rate, 0.5 ml/min.

2.4. Identification of the microorganism

Cell morphology, Gram reaction, motility and 16S rDNA gene sequencing were determined by using cells grown on the cultivation medium agar. Anaerobic growth was observed in GasPak Anaerobic System (BBL Microbiology Systems, Cockeysville, MD, USA).

Cells used for ubiquinone analysis were harvested in liquid medium for isolation. Quinone compositions were determined by HPLC method of Tamaoka et al. [10]. The eluent for HPLC was a mixture of methanol and isopropanol (2:1, v/v). Ubiquinone was detected by spectrophotometer at 275 nm and identified by the retention time comparison with standard coenzyme Q. Cells used for 16S rDNA sequencing were lysated by incubating with Proteinase K at 60°C for 20 min. The enzymatic reaction was stopped by incubation in boiling water for 5 min. Supernatant of this preparation was used for PCR amplification. The 16S rDNA gene of the strain was amplified by PCR method with two primers, 5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8 to 27 in *Escherichia coli* numbering [11]), and 5′-AAGGAGGTGATCCAGCCGC-3′ (complementary to positions 1522 to 1540). The PCR products were ligated to the pT7blue vector (Novagen, WI, USA) and transformed into competent JM109 *E. coli* cells. Plasmid DNA was sequenced using the ABI Dye Terminator Cycle Sequencing FS Kit (Applied Biosystems, CA, USA) and a model ABI 377 automatic sequencer (Applied Biosystems). The sequences were aligned using CLUSTAL W program which is the improved version of the CLUSTAL V [12]. Pairwise evolutionary distances were calculated by the method of Kimura [13]. Phylogenetic dendrograms were reconstructed by the neighbor-joining method [14], and the topology of the phylogenetic tree

was evaluated by the bootstrap resampling method with 1000 replicates [15]. The published sequences which we used in this paper have been deposited in the DDBJ/GenBank/EMBL nucleotide sequence data bases under the following accession numbers: *Alcaligenes feacalis*, M22508; *Alcaligenes xylosoxidans* subsp. *denitrificans*, M22509; *Bordetella bronchiseptica*, X57026; *Burkholderia andropogonis*, X67037; *Burkholderia caryophylli*, X67039; *Burkholderia capacia*, M22518; *Burkholderia gladioli*, X67038; *C. testosteroni*, M11224; *Nitrosolobus multiformis*, L35509; *Pseudomonas aeruginosa*, X06684; *Pseudomonas lemignei*, X92554; *Ralstonia eutropha*, M32021; *Ralstonia pickettii*, X67042; *Ralstonia solanacearum*, X67036; *Telluria chitinolytica*, X65590; *Telluria mixta*, X65589; and *Zoogloea ramigera* ATCC25935, X74914.

2.5. 2-HNA production

Cells harvested from 0.4 l of cultured medium (1-l jar fermenter system) were incubated with 6.0 g of NA in 25 mM of potassium phosphate buffer (pH 7.0, total volume of 0.4 l). The reaction was allowed to proceed in a 1-l jar fermenter system agitated at 600 rpm at 30°C, with aeration of 0.5 vvm.

3. Results and discussion

3.1. Screening of 2-hydroxynicotinic-acid-producing strains

A total of 0.1 g of soil was incubated in a test tube containing 6 ml of screening medium at 30°C for 7 days on a shaker. From 200 soil samples, we were able to isolate only two strains (MCI3288 and MCI3289) which produced 2-HNA from NA. In these two strains, MCI3289 was selected as the strain with the highest 2-HNA-producing activity and was used in further experiments.

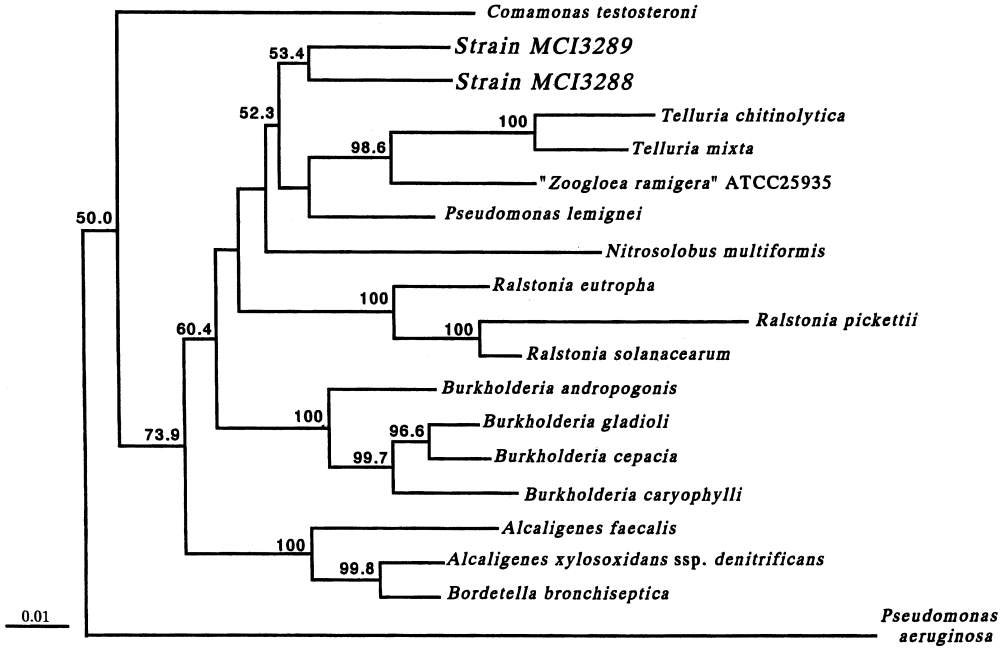

Fig. 1. Phylogenetic relationships between MCI3288, MCI3289 and related genera based on 16S rDNA sequences. Unrooted phylogenetic tree showing the relationship of strain MCI3288 and MCI3289 to reference organisms. The numbers indicate the percentage of bootstrap samples, derived from 1000 samples. The numbers over 50% are written. Bar signifies one base change per 100 nucleotide positions.

3.2. Identification of microorganisms

Strain MCI3289 was isolated from soil by enrichment culture with 6-methylnicotinic acid as sole carbon source. This strain does not grow on a nutrient agar. It formed white and round colonies on the medium agar for growth, after 1 or 2 weeks incubation at 30°C. Cells of this strain were short Gram-negative rods, non-sporing and nonmotile. It did not grow under anaerobic conditions. Catalase and oxidase were positive. Predominant quinone of MCI3289 was ubiquinone-8 while menaquinone could not be detected. The 16S rDNA sequence analysis showed phylogenetic relationships with some genera in the β-subgroup of the *Proteobacteria*, but no strong homologies to any recognized bacteria were found (Fig. 1). Strain MCI3289 constructed the same phylogenetic branch with the other isolate, MCI3288 (taxonomic data not shown). The highest degree of sequence homology was 95.3% with strain MCI3288 and *P. lemignei*, which is not an authentic member of the genus *Pseudomonas*. Strain MCI3289 was found to have 94.1% homology with *Z. ramigera* ATCC25935, 90.8–92.4% with *Ralsonia* species and 91.1–92.3% with *Burkholderia* species.

As described above, strain MCI3289 was found to be a member of the β-subgroup of the *Proteobacteria*. Furthermore it is probable that this strain is new at the genus or at least species level. The complete 16S rRNA gene sequence is deposited in the DDBJ under accession number AB006750.

3.3. Identification of the product from nicotinic acid

The reaction product of MCI3289 and authentic 2-HNA had the same retention time (8.7 min) in HPLC analysis (Fig. 2). The product was identified by ^{1}H nuclear magnetic resonance (NMR) and mass spectroscopy. ^{1}H NMR (270 MHz, DMSO): δ 14.8 (1H, s), 13.3 (1H, s), 8.39 (1H, d), 7.96 (1H, d), 6.69 (1H, t). MS: m/z 139 (M). Many microorganisms are known to produce 6-HNA from NA catalyzed by nicotinic acid dehydrogenase (EC 1.5.1.13), however, 6-HNA was not detected in the reaction mixture of this strain by HPLC analysis. 2-HNA was the only product of enzymatic reaction from NA found in this strain.

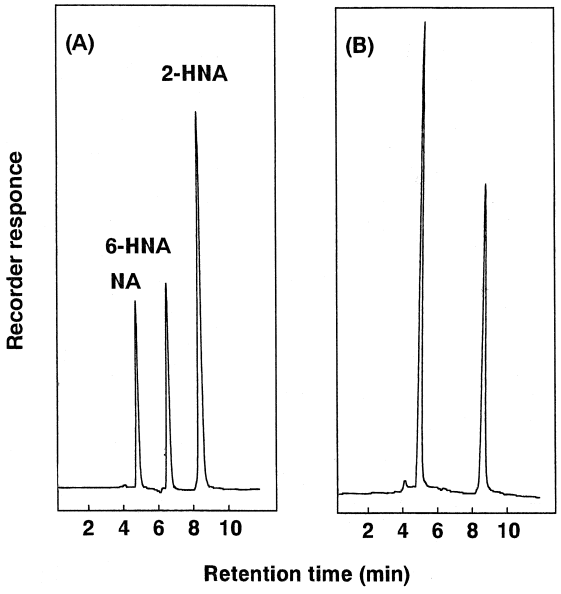

Fig. 2. HPLC analysis of authentic samples (A) and reaction mixture of MCI3289 (B). The eluate was monitored at 230 nm with an UV detector.

3.4. Effect of inducers on the 2-HNA-producing activity

Various pyridine compounds were tested as inducers. As shown in Table 1, the addition of 6-methylnicotinic acid and 2-hydroxy-6-methylnicotinic acid to the medium increased the 2-HNA-producing activity. When 6-methylnicotinic acid was used as an inducer, accumulation of 2-hydroxy-6-methylnicotinic acid along with degradation of 6-methylnicotinic acid were observed by HPLC-analysis of the culture medium. This result suggests that MCI3289 metabolized 6-methylnicotinic acid to 2-hydroxy-6-methylnicotinic acid by the enzyme which catalyzes the hydroxylation at the C2 position of 6-methyl-

Table 1
Effect of inducers on the 2-hydroxynicotinic acid-producing activity

Inducers (0.5 g/l)	Growth ($O.D._{660}$)	Relative activity (%)
6-Methyl nicotinic acid	1.59	76.7
2-Hydroxy-6-methylnicotinic acid	1.62	100
Nicotinic acid	1.09	4.2
2-Chloronicotinic acid	1.22	1.3
6-Chloronicotinic acid	0.89	1.1
2-Hydroxy nicotinic acid	1.13	2.2
6-Hydroxy nicotinic acid	1.63	1.5
2-Chloro-6-methyl nicotinic acid	1.25	1.5

MCI2848 was cultivated in 500-ml shaking flasks containing 50 ml of culture medium at 30°C for 2 days. The reaction time was 30 min.

nicotinic acid. We estimated that the 6-methylnicotinic-acid-degrading enzyme might be responsible for the hydroxylation of NA to 2-HNA in this strain (Fig. 3).

3.5. Reaction conditions for 2-HNA production with MCI3289

The effect of pH on the activity was investigated. For a 30-min reaction, pH 6.0 to 7.5 seemed to be preferable. The optimal pH for 2-HNA production was at about 7.0 with 50 mM potassium-phosphate buffer. The optimal temperature was 40°C for the 1-h reaction. However, for the 24-h reaction, the highest accumulation of 2-HNA was attained at 30°C. The optimum concentration of NA was studied. The optimum concentration of NA for 2-HNA production was 10 to 15 g/l. Substrate inhibition was found above 20 g/l of NA.

3.6. Production of 2-HNA by MCI3289

MCI3289 was cultivated in a 1-l micro jar fermenter system containing 0.4 l of basal medium. After 30 h of cultivation, 1 g/l of 6-methylnicotinic acid was fed to the culture medium. The hydroxylation activity was maximum at 50 h. The cells harvested from the culture medium were suspended in 400 ml of the reaction mixture containing 15 g of NA per l of buffer in a micro jar fermenter. The reaction was allowed to proceed at 30°C, pH 7.0 for 29 h. Fig. 4 illustrates a typical time-course of 2-HNA production from NA by intact cells of the strain. A total of 6.40 g of 2-HNA per l was produced after 29 h of reaction and the residual NA was 8.82 g/l. The molar yield of 2-HNA based on NA consumed was 91.6% and no other by-product was accumulated by MCI3289. The other strain, MCI3288, produced a blue pigment during the conversion (data not shown),

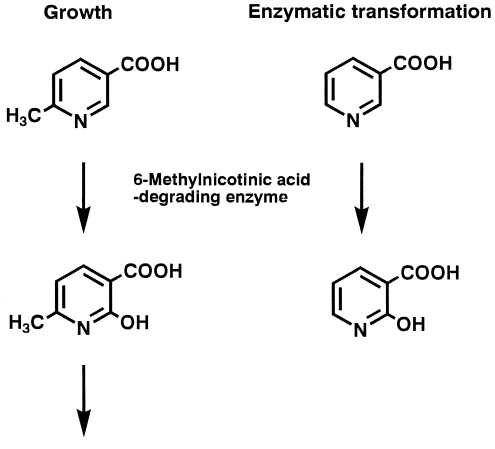

Fig. 3. Proposed mechanism of 6-methylnicotinic acid degradation and nicotinic acid enzymatic transformation by MCI3289.

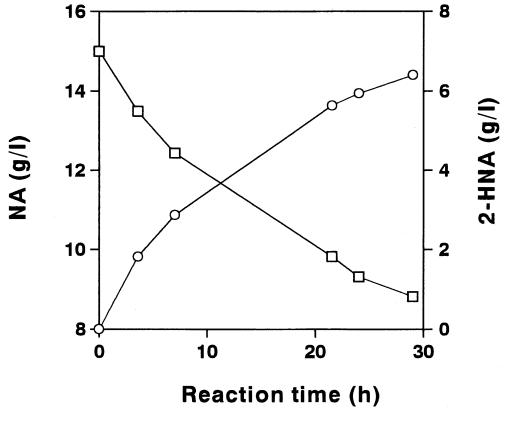

Fig. 4. Time-course of conversion of nicotinic acid (□) to 2-hydroxy nicotinic acid (○). The reaction was conducted at 30°C.

we supposed that it has the enzyme activity of 2-HNA degradation [16].

Recently, Tinschert et al. [9] detected the 2-HNA-producing activity in the resting cells of 6-methylnicotinic-acid-degrading bacterium, Mena 23/3-3c. The presence of 6-methylnicotinic acid in the culture medium was a essential to exhibit the 2-HNA-producing activity on both strains, MCI3289 and Mena 23/3-3c. Mena 23/3-3c do not grow on complex media without the addition of 6-methylnicotinic acid, however, MCI3289 could assimilate yeast extract and corn steep liquor as the carbon and energy sources. Both strains of MCI3289 and Mena 23/3-3c showed no strong similarities in characteristics to any known species. Compared with 16S rRNA-encoding DNA sequence, MCI3289 had 98.5% homology with Mena 23/3-3c. This high homology between both strains suggests a possibility that they belong to the same genus. However, MCI3289 and Mena 23/3-3c poorly show physiological characteristics, and comparative data to identify them are scanty. Therefore, a strong similarity on the species level is not concluded.

2-Chloronicotinic acid is easily prepared from 2-HNA by $SOCl_2$. Recently, 2-chloronicotinic acid has been in commercial demand as a starting material for agrochemicals and pharmaceuticals [17,18]. The present enzymatic reaction provides a new process, applicable in an industrial scale, for the production of pyridine derivatives.

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