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Bioelectrochemical transformation of nicotinic acid into 6-hydroxynicotinic acid on *Pseudomonas fluorescens* TN5-immobilized column electrolytic flow system

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Abstract

Pseudomonas fluorescens TN5 catalyzes the hydroxylation of nicotinic acid (NA) into 6-hydroxynicotinic acid (6HNA), an important compound as a starting material for the synthesis of a new type of pesticides. Under aerobic conditions, however, 6HNA is metabolized in the *P. fluorescens* cells. The use of $Fe(CN)_6^{3-}$ as an extracellular electron acceptor enhances the biotransformation of NA into 6HNA and completely suppresses the subsequent oxidation of 6HNA. The function of the *P. fluorescens* cell was combined with the electrode process by immobilizing the *P. fluorescens* cells on the carbon fiber electrode surface in the column, where $Fe(CN)_6^{3-}$ was used as an electron transfer mediator. Continuous-flow electrolysis of NA in the presence of $Fe(CN)_6^{3-}$ at the *P. fluorescens*-immobilized column electrode realized the accelerated and complete transformation of NA into 6HNA without any by-product.

Keywords: Bioelectrocatalysis; Nicotinic acid; 6-hydroxynicotinic acid; *Pseudomonas fluorescens*; Column electrolysis

1. Introduction

The use of whole cells as well as enzymes for transformation of organic compounds has been receiving great attention [1–4]. The advantages of the utilization of microorganisms as catalysts are low cost, moderate reaction conditions, and a wide spectrum of the functions of microorganisms [1]. One of the examples is the regioselective hydroxylation of nicotinic acid (NA) into 6-hydroxynicotinic acid (6HNA) catalyzed by *Pseudomonas fluorescens* [5]. Since 6HNA is an important compound as a starting material for the synthesis of a new type of pesticides with a 6-position substituted pyridine backbone structure [6–10], extensive efforts have been paid to chemical synthesis of 6-position substituting pyridines [11]. However, severe by-product formation is unavoidable in such organic syntheses, which subsequently requires

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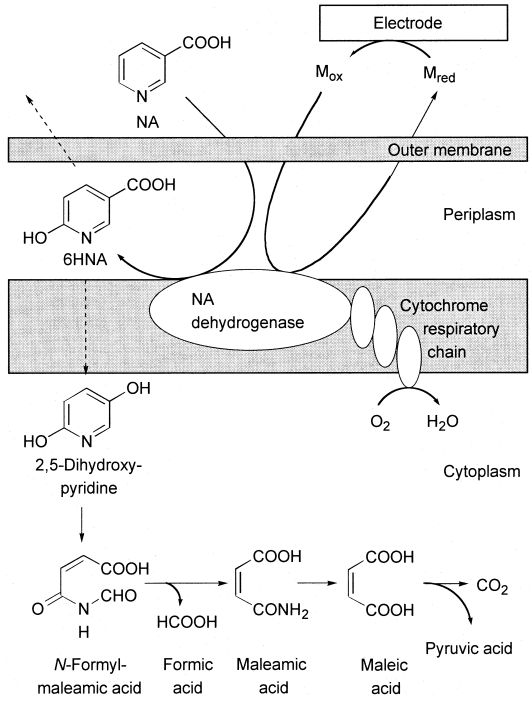
tedious isolation processes. This is the reason why the biotransformation of NA into 6HNA is interesting and important.

The biochemistry and physiology of *P. fluorescens* have been extensively studied. NA is the carbon and energy source of the *P. fluorescens* bacteria. A schematic pathway of NA metabolism is illustrated in Scheme 1. The oxidation of NA is catalyzed by NA dehydrogenase, a membrane-bound enzyme linked to the cytochrome respiratory chain [12]. Although dioxygen is the final electron acceptor in the respiratory chain [12], the oxygen atom of the hydroxyl group in 6HNA is derived from water [13]. 6HNA is metabolized finally into pyruvate in the bacterial cells [14–16]. Recently, the authors (T.N. et al.) have screened *P. fluorescens* TN5 with high catalytic activity for the hydroxylation of NA into 6HNA and utilized the strain for the 6HNA production with high yields [17].

On the other hand, the authors (T.I. et al.) have shown that some artificial electron acceptors such as $Fe(CN)_6^{3-}$ and *p*-benzoquinone can be used in place of O_2 in the *P. fluorescens*-catalyzed NA oxidation and the catalytic reaction has been coupled with the electrode process to construct NA biosensors [18–20]. The reaction scheme of the bioelectrocatalysis is also illustrated in Scheme 1.

In this paper, we tried to utilize the mediated bioelectrocatalytic system as a bioelectrochemical transformation system of NA into 6HNA. [2] Since continuous-flow column electrolysis is very useful in achieving rapid and quantitative bulk electrolysis [22–26], *P. fluorescens* cells were immobilized on a column electrode. Special interest has been paid to clarify what is the benefit of the bioelectrochemical system compared with the conventional aerobic transformation. Then we characterized the *P. fluorescens*-catalyzed biotransformation process using several electron acceptors. The results



Scheme 1. Metabolic pathway of NA in *P. fluorescens* and bioelectrocatalytic mechanism of NA hydroxylation.

revealed that the use of $Fe(CN)_6^{3-}$ as an extracellular electron acceptor enhances the *P. fluorescens*-catalyzed biotransformation of NA into 6HNA and completely suppresses the subsequent oxidation of 6HNA. Based on these findings, we finally realized continuous and complete conversion of NA into 6HNA.

2. Experimental

2.1. Materials

P. fluorescens TN5 cells, cultured overnight according to a literature [17], were harvested at the late exponential phase by centrifugation at $7000 \times g$ for 10 min. The cells were washed three times with a saline solution (0.85% NaCl) and stored at -30°C. A portion of the stored cells was suspended in the saline solution before use. The cell density of the suspension was estimated spectrophotometrically at 610 nm us-

[2] Preliminary results have been published in Ref. [21].

ing an absorption coefficient of 7.7×10^{-12} cm^2 $cell^{-1}$. All chemicals used were of reagent grade without any further purification.

2.2. Column electrolytic system and coulometry of $Fe(CN)_6^{4-}$

Column electrolysis was performed using a potentiostat and a Hokuto Denko HX-110 electrolysis cell (Tokyo, Japan) with a column electrode (inner diameter 8 mm, length 50 mm) packed with carbon fiber. The current was monitored with a recorder.

Coulometric analysis of $Fe(CN)_6^{4-}$ was carried out in a flow injection analysis mode using the column electrolytic system as a detector. The electrode potential was set at 0.4 V vs. Ag/AgCl/sat. KCl, which is enough for complete electrolysis of $Fe(CN)_6^{4-}$. The charge was calculated from the peak area in current–time curves.

2.3. HPLC analysis of NA and 6HNA

HPLC analysis was performed with an HPLC system coupled with a photodiode array detector using a Develosil ODS-A-5 column (4.6×150 mm; Nomura Chemical, Aichi, Japan). A phosphate buffer of pH 2.5 containing 30% (v/v) methanol and 5 mM sodium 1-octanesulfonate was used as a mobile phase for the determination of NA and 6HNA and flowed at 1.0 ml min^{-1}. Caffeine was used as an internal standard.

2.4. Measurements of NA dehydrogenase activity in P. fluorescens cell suspension

The NA oxidation in *P. fluorescens* TN5 cell suspensions was performed in 10 mM phosphate buffer (pH 7.0, ionic strength was adjusted to 150 mM with NaCl) at room temperature in a 50-ml vial. The vials were opened to the atmosphere in the aerobic oxidation, while the suspensions were purged with argon gas to remove dissolved O_2 in anaerobic oxidation using $K_3Fe(CN)_6$ as an electron acceptor. The *P. fluorescens* cell suspensions were kept under stirring, and the NA oxidation reaction was initiated by the addition of an aliquot of *P. fluorescens* suspension with an optical density of 20. The suspension was periodically sampled and filtered with a 0.2-μm pore membrane filter to remove the *P. fluorescens* cells. The filtrate was immediately subjected to the HPLC analysis of NA and 6HNA after appropriate dilution with the mobile phase. In anaerobic experiments, the concentration of $Fe(CN)_6^{4-}$ in the filtrate was also determined by coulometry using the column electrolytic method.

2.5. Immobilization of P. fluorescens cells on column electrode surface

P. fluorescens TN5 cells were immobilized on the carbon fiber by circulating a *P. fluorescens* suspension with an optical density of 20 through the column electrode with a peristaltic pump for 60 min. The carbon fiber in the column has high affinity to microbial cells, thus *P. fluorescens* cells were easily adsorbed on the fiber electrode surface without any chemical treatment or physical entrapment. In order to get uniform distribution of the cell population in the column electrode, the cell suspension was alternatively introduced from each side of the column. After the immobilization treatments, the buffer solution was passed through the column. The number of cells immobilized on the carbon fiber electrode surface was roughly estimated from a decrease in the optical density of the cell suspension at 610 nm after the immobilization treatment.

2.6. Evaluation of electrochemical biotransformation system

NA solutions containing $K_3Fe(CN)_6$ were continuously flowed through the *P. fluorescens*-immobilized column electrode with an

HPLC pump. The electrode potential was fixed at 0.4 V vs. Ag/AgCl/sat. KCl. An electrolyzed solution eluted from a *P. fluorescens*-immobilized column electrode was subject to HPLC analysis for the determination of NA and 6HNA.

3. Results and discussion

3.1. Accelerated biotransformation of NA into 6HNA in the presence of $Fe(CN)_6^{3-}$

Fig. 1(A) shows the time course of biotransformation of NA into 6HNA in a suspension of *P. fluorescens* cells under aerobic conditions. In the presence of *P. fluorescens* cells, NA was oxidized by NA dehydrogenase in *P. fluorescens* cells with an unknown electron acceptor to yield 6HNA. The electron is transferred to dissolved dioxygen (O_2) as the final electron acceptor in the respiratory chain (Scheme 1) [12].

The hydroxylation rate, represented by the initial slope of the profile in Fig. 1(A), was not affected by an increase in the O_2 concentration.

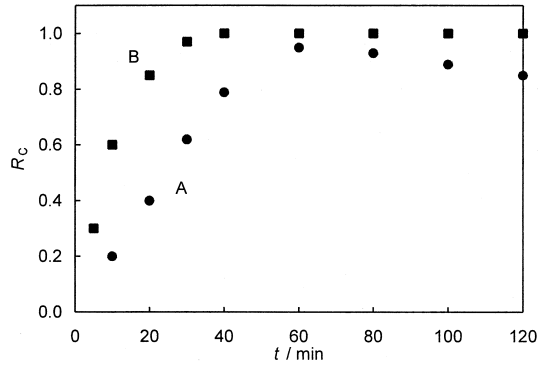


Fig. 1. Time dependence of the 6HNA production from NA (1 mM) in *P. fluorescens* suspension of 8.32×10^{12} cells ml^{-1} at pH 7.0 phosphate buffer in the presence of (A) 0.25 mM [O_2] and (B) 5 mM $K_3Fe(CN)_6$ as an electron acceptor. R_c is the conversion ratio defined by the relative concentration of 6HNA against the initial concentration of NA in the suspension.

This is due to a low apparent Michaelis constant for O_2 (<20 μM) in the *P. fluorescens*-catalyzed NA oxidation [20].

The conversion of NA into 6HNA became almost complete at ca. 60 min and then decreased gradually with the time. The decrease in the 6HNA concentration is due to the subsequent metabolism of 6HNA [14–16]. The oxidative degradation of 6HNA is induced by a decrease in the concentration of NA [17]. To suppress the subsequent metabolism of 6HNA, a successive addition of NA in the cell suspension was proposed for an aerobic production system [17]. Even in such cases, the control of the reaction time is essential to prevent the subsequent oxidation of 6HNA. Therefore, it is very difficult to construct a continuous biotransformation system to achieve the 100% conversion under aerobic conditions. It would be considered to utilize isolated NA dehydrogenase in place of whole cells. However, the isolated enzyme was too unstable to be used as a biocatalyst for the biotransformation of NA into 6HNA [12], most probably due to the membrane-bound characteristics. Whole cells can be used as a bag of enzymes to prevent the degradation of unstable enzymes. This is one of the advantages of whole cell-based biotransformation.

Since the key catalyst in the conversion of NA into 6HNA is not oxygenase but dehydrogenase, some artificial electron acceptors such as $Fe(CN)_6^{3-}$ and *p*-benzoquinone can be used in place of O_2 in the whole cell level [20]. Fig. 1(B) shows the time course of the biotransformation of NA into 6HNA in the *P. fluorescens* cell suspension in the presence of $Fe(CN)_6^{3-}$ under anaerobic conditions. The overall reaction may be written by:

$$\mathrm{NA} + 2\mathrm{Fe(CN)_6^{3-}} + \mathrm{H_2O} \rightarrow 6\mathrm{HNA} + 2\mathrm{Fe(CN)_6^{4-}} + 2\mathrm{H^+} \quad (1)$$

It is noteworthy that the rate of the anaerobic transformation using $Fe(CN)_6^{3-}$ as an extracel-

lular electron acceptor was larger than that of the aerobic transformation. This phenomenon might be described as follows. It would be considered that the aerobic oxidation of NA into 6HNA is regulated by some reduced intermediate accumulated in the normal metabolic pathway. The use of $Fe(CN)_6^{3-}$ as an extracellular electron acceptor, which means the electron leak without accumulation of the reduced intermediates within the cell, makes NA dehydrogenase free from such a redox regulation. This situation would be responsible for the enhanced activity of NA dehydrogenase.

In addition, in the presence of $Fe(CN)_6^{3-}$ under anaerobic conditions, the succeeding oxidation of 6HNA was completely suppressed, as shown in Fig. 1(B). This means that $Fe(CN)_6^{3-}$ cannot serve as an electron acceptor for enzymes concerning the metabolism of 6HNA. This conclusion is clearly evidenced by a stoichiometric generation of 6HNA and $Fe(CN)_6^{4-}$. The molar ratio of $Fe(CN)_6^{4-}$ and 6HNA during the anaerobic batch experiments was 2.00–2.08, which is in good agreement with that expected from Eq. (1). Most probably, the specificity of $Fe(CN)_6^{3-}$ as an electron acceptor would be ascribed to very low membrane permeability due to the large negative charge. The 6HNA-metabolizing enzymes would locate in the cytoplasm and hardly contacts with $Fe(CN)_6^{3-}$. In contrast, NA dehydrogenase is a membrane-bound enzyme and then it can utilize $Fe(CN)_6^{3-}$ as an electron acceptor.

Judging from the above results, $Fe(CN)_6^{3-}$ is superior to O_2 as an electron acceptor from the standpoint of the 6HNA production. $Fe(CN)_6^{3-}$ enhances the biotransformation of NA into 6HNA and suppresses the subsequent oxidative metabolism of 6HNA. Benzoquinone and related compounds can work as better electron acceptors than $Fe(CN)_6^{3-}$ [18–20]. However, the quinones were not suitable in this work because non-enzymatic unknown reaction occurs between NA and the quinones, although the side reaction was slow. $Fe(CN)_6^{3-}$ is also advantageous from the view point of the specificity as an electron acceptor, as described above.

3.2. Construction and characterization of P. fluorescens-immobilized column electrolytic systems

The $Fe(CN)_6^{3-/4-}$ redox couple can work as a mediator between the NA dehydrogenase reaction and the electrode process. In addition, the column electrolytic method allows rapid and complete bulk electrolysis. Therefore, we expected that the combination of the NA dehydrogenase reaction using $Fe(CN)_6^{3-}$ as an electron acceptor and continuous-flow column electrolysis of $Fe(CN)_6^{4-}$ allows an accelerated and complete transformation of NA into 6HNA, where small amounts of $Fe(CN)_6^{3-}$ will be sufficient compared with NA. Thus, *P. fluorescens* TN5 cells were immobilized on the carbon fiber electrode surface in the column to construct a bioelectrochemical transformation system of high performance.

The *P. fluorescens* cells were simply adsorbed on the carbon fiber electrode surface by circulating *P. fluorescens* cell suspension. The number of the *P. fluorescens* cells immobilized on the column electrode (N_{Cell}) was estimated as 1.1×10^{13} cells. The cell distribution in the *P. fluorescens*-immobilized column was evaluated as follows. First, 5 mM NA in 10 mM deaerated phosphate buffer (pH 7.0) was passed through the *P. fluorescens*-immobilized column electrode at 0.1 ml min^{-1} with the HPLC pump to reduce all of NA dehydrogenase in the *P. fluorescens* cells. After washing the column thoroughly with deaerated buffer without NA, 5 mM $K_3Fe(CN)_6$-containing deaerated phosphate buffer (pH 7.0) was flowed at 0.1 ml min^{-1} through the column electrode, where the reduced NA dehydrogenase in *P. fluorescens* cells is reoxidized with $Fe(CN)_6^{3-}$. The generated amount of $Fe(CN)_6^{4-}$ was coulometrically determined on the column electrolytic system. All experiments here were carried out under completely anaerobic conditions to prevent aer-

obic oxidation of the reduced NA dehydrogenase in the *P. fluorescens* cells.

Fig. 2 shows the results. The current began to increase at ca. 2.5 min after introduction of the $K_3Fe(CN)_6$ solution and then reached the maximum at ca. 8 min. The time lag in the current increase corresponds the time for the $K_3Fe(CN)_6$ solution to reach the column head from the HPLC pump. After reaching the maximum, the current remained almost constant up to ca. 25 min. The retention time of the $K_3Fe(CN)_6$ solution was assessed to be ca. 20 min based on the column volume (2 ml) and the flow rate (0.1 ml min^{-1}). This value is close to the time of the plateau region between 8 to 25 min. This means that the $K_3Fe(CN)_6$ flow begins to elute from the column at ca. 25 min. The tailing characteristics of the *I–t* curve would be ascribed to irregular flow in the column due to large difference in the inner diameter between the column (8 mm) and the line tubing (0.8 mm).

Since $Fe(CN)_6^{4-}$, generated in the oxidation of the reduced NA dehydrogenase in the *P. fluorescens* cells, is rapidly and completely reoxidized at the column electrode, the current (I) represents the oxidation rate of the reduced NA dehydrogenase, as expressed by:

$$I = n_{Eox} F \frac{dm_{Enz,Ox}}{dt} \quad (2)$$

where n_{Enz} is the number of the electrons per NA dehydrogenase molecule, F is the Faraday constant, $m_{Enz,Ox}$ is the amount of the oxidized NA dehydrogenase, and t is the time. Since the electron transfer from the reduced NA dehydrogenase to $Fe(CN)_6^{3-}$ is acceptably fast, the oxidation of the reduced NA dehydrogenase undergoes in the front of the $K_3Fe(CN)_6$ flow in the column. Therefore, the appearance of the plateau region in the *I–t* curve revealed that the *P. fluorescens* cells were distributed almost homogeneously in the column. Alternative introduction of the cells from both sides of the column (see Section 2) was essential to make such homogeneous immobilization of the cells. Reproducible results were obtained for a given

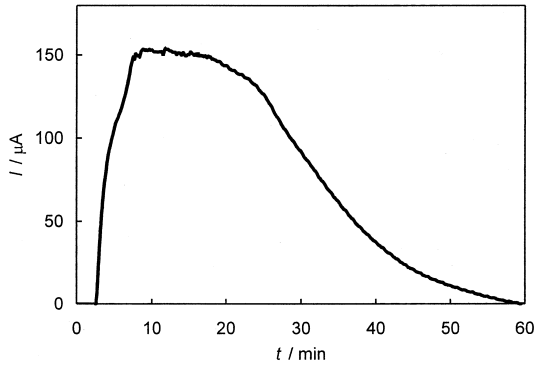


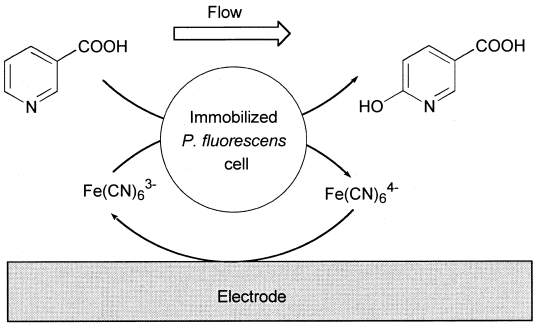
Fig. 2. Current–time curve observed at 0.4 V for the oxidation of reduced NA dehydrogenase in *P. fluorescens* cells immobilized on a column electrode with 5 mM $K_3Fe(CN)_6$. NA dehydrogenase in the cell was completely reduced with NA. All experiments were performed under anaerobic conditions. See text for details of the procedure.

column within 5% error. This means that the *P. fluorescens* cells were well immobilized (or adsorbed) on the carbon fiber electrode surface.

The total charge under the *I–t* curve was 268 mC, which represents the total amount of NA dehydrogenase (m_{Enz}) in the column, as expected from Eq. (2). Assuming that $n_{Enz} = 2$, m_{Enz} was calculated as 8.3×10^{17} molecules. The result means that a single *P. fluorescens* cell contains 7.9×10^4 molecules of NA dehydrogenase ($= m_{Enz}/N_{Cell}$). Interestingly, the evaluated number of NA dehydrogenase per cell is comparable to that of glucose dehydrogenase in *Escherichia coli* cell (3.5×10^4 molecules per cell) [27].

3.3. Continuous bioelectrochemical transformation of NA into 6HNA

The *P. fluorescens*-immobilized column was used as a bioelectrochemical device for continuous production of 6HNA, where the reaction solution containing NA and $K_3Fe(CN)_6$ (pH 7.0 phosphate buffer) was continuously passed through the column and the biocatalytically generated $Fe(CN)_6^{4-}$ was electrochemically reoxidized within the column, as shown in Scheme 2. By considering the practical application, the



Scheme 2. Schematic view of continuous-flow bioelectrocatalytic hydroxylation of NA into 6HNA at *P. fluorescens*-immobilized column.

bioelectrocatalytic transformation system was operated under aerobic conditions, where it becomes important to control the reaction time to suppress the subsequent oxidation of 6HNA, as described above. In this sense, the flow system is very convenient to regulate the reaction time.

The initial concentration of NA (C_{NA}) in the reaction solution was fixed at 5 mM, by considering the solubility of 6HNA in the present buffer (ca. 8 mM). Fig. 3(A) shows effects of the $K_3Fe(CN)_6$ concentration (C_{Fe}) on the conversion ratio (R_C) of NA into 6HNA, R_C being a ratio of the generated 6HNA concentration against C_{NA}. The concentrations of 6HNA as well as NA in the electrolyzed solution were determined by HPLC. The R_C value increased with an increase in C_{Fe} and reached unity at $C_{Fe} > 0.5$ mM at a flow rate (v) of 0.1 ml min^{-1}, where NA was completely converted into 6HNA without any by-product. Flow rate dependence of R_C is given in Fig. 3(B). The conversion was complete up to $v = 0.1$ ml min^{-1} at least at $C_{Fe} = 1$ mM. With further increase in v, the R_c value decreased gradually. In order to achieve the complete conversion at increased v, it is required to increase C_{Fe}. The conditions to achieve the 100% conversion ($R_C = 1$) are optimized as: $C_{Fe} = 1$ mM and $v = 0.1$ ml min^{-1} at $C_{NA} = 5$ mM.

During the bioelectrochemical oxidation of NA in the continuous-flow system, the steady-state current (I_s) was observed. The I_s value is expressed by:

$$I_s = R_C f_B n_{NA} F C_{NA} v \quad (3)$$

where $n_{NA}(= 2)$ is the number of electrons per NA molecule and f_B is the bioelectrochemical efficiency of the NA oxidation: that is, a ratio of the bioelectrochemical oxidation of NA against the total oxidation of NA. Under the optimized conditions for the complete conversion ($C_{Fe} = 1$ mM, $C_{NA} = 5$ mM and $v = 0.1$ ml min^{-1}), the

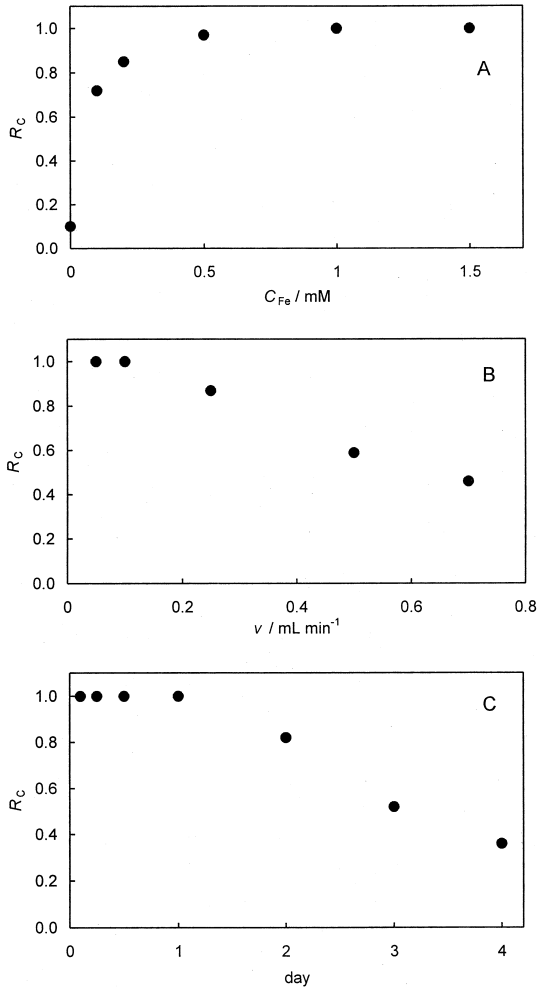


Fig. 3. Ratios of NA conversion into 6HNA (R_C) in continuous-flow bioelectrocatalytic system at $C_{NA} = 5$ mM as functions of (A) the concentration of $K_3Fe(CN)_6$ at $v = 0.1$ ml min^{-1}, (B) the flow rate of the solution at $C_{Fe} = 1$ mM and (C) the days passed after the construction of the *P. fluorescens*-immobilized column electrode at $C_{Fe} = 1$ mM and $v = 0.1$ ml min^{-1}.

observed value of I_s was 1.46 mA. According to Eq. (3), f_B was evaluated as 0.91. The electron of the residual 10% of NA would be transferred to dissolved O_2 as the natural (final) electron acceptor. The f_B value increased with C_{Fe} and became unity at $C_{Fe} = 10$ mM, where all of the electrons from NA were transferred to the electrode via the mediator and the aerobic oxidation is completely suppressed even under aerobic conditions.

Eq. (3) can be rewritten as follows using C_{Fe} and the number of the electron of $K_3Fe(CN)_6$ ($n_{Fe} = 1$):

$$I_s = R_C f_B \frac{n_{NA} C_{NA}}{n_{Fe} C_{Fe}} n_{Fe} F C_{Fe} v \quad (4)$$

The term $R_C f_B((n_{NA}C_{NA})/(n_{Fe}C_{Fe}))$ represents the turnover number during the mediating process in the column. From the above results, the turnover number was evaluated as 9.1 under the optimized conditions for $R_C = 1$. The C_{NA}/C_{Fe} ratio and then the turnover number, can be increased by a decrease of v.

As a comparison, the *P. fluorescens*-immobilized column was operated as a normal reactor at open circuit in the absence of $K_3Fe(CN)_6$. The reactor produced at most 0.5 mM of 6HNA from 5 mM NA at $v = 0.1$ ml min^{-1}. The low conversion efficiency ($R_C = 0.1$) would be attributed to the low oxidation rate of the aerobic NA oxidation and in part the depression of the dissolved O_2 in the column. In order to attain the 100% conversion ($R_C = 1$) in the normal reactor mode, C_{NA} had to decrease down to 0.4 mM. Therefore, the bioelectrochemical system proposed here has about 10-fold improved performance than that of the simple aerobic system. The result clearly indicates the bioelectrochemical acceleration of the biotransformation of NA.

The absence of by-product in the bioelectrochemical transformation system means that the reaction time regulated by v was short enough to prevent the subsequent aerobic oxidation of 6HNA. This system was relatively stable for continuous operation, but the R_C value was gradually decreased with day, as depicted in Fig. 3(C).

There are a few reports dealing with the combination of whole cell-based catalytic system and electrochemistry for biotransformation, especially for the reduction of substrates [28–30]. However, there were several problems in the reported electrolysis system. The utilization of the column electrolytic method and functional *P. fluorescens* whole cells allow the complete and rapid hydroxylation system of NA. Significance of bioelectrochemical transformation processes was also emphasized in this work.

4. Conclusion

P. fluorescens TN5-immobilized column electrolytic method allows the continuous and complete transformation of NA into 6HNA without any by-product. The use of $Fe(CN)_6^{3-}$ as an extracellular electron acceptor enhances the biotransformation of NA into 6HNA and completely suppresses the subsequent oxidation of 6HNA. Since the 100% conversion of NA can be achieved, 6HNA was easily extracted from the electrolyzed solution with butyl alcohol. Extracted 6HNA can be re-crystallized from ether to give pure white crystal powder [31]. For practical use, some improvement might be required in the long-term stabilization of the *P. fluorescens* cells immobilized on the column electrodes. Selection of better mediator and its immobilization on the column would be also important in future. On the other hand, it is easy to make the term $R_C f_B$ in Eq. (3) unity by decreasing the C_{NA}/C_{Fe} value. Under such conditions, this system can be used as an absolute determination of NA. [2] Further study for analytical application is in progress. In conclusion, the whole cell-based bioelectrocatalytic method coupled with column electrolysis will

be utilized for the production and analysis of a variety of compounds.

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