Bioelectrochemical Studies on Indigo Reduction Mechanisms in Indigo Fermentation

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March 2021

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General Introduction

In Japan, *Aizome* (indigo dyeing) is a famous traditional dyeing method in Japan. Traditional indigo dyeing consists of two important steps: preparations of the dye material (called *sukumo*) and dye suspensions, both of which involve fermentation steps by microorganisms. Indigo plants (*Polygonum tinctorium Lour.*, Syn: *Persicaria tinctoria* (Aiton) H. Gross) biosynthesize indican (indoxyl-β-D-glucoside) that is the precursor of indigo (Fig. 1 A). Therefore, the first fermentation process is the transformation of indican into indigo for the preparation of indigo dye.

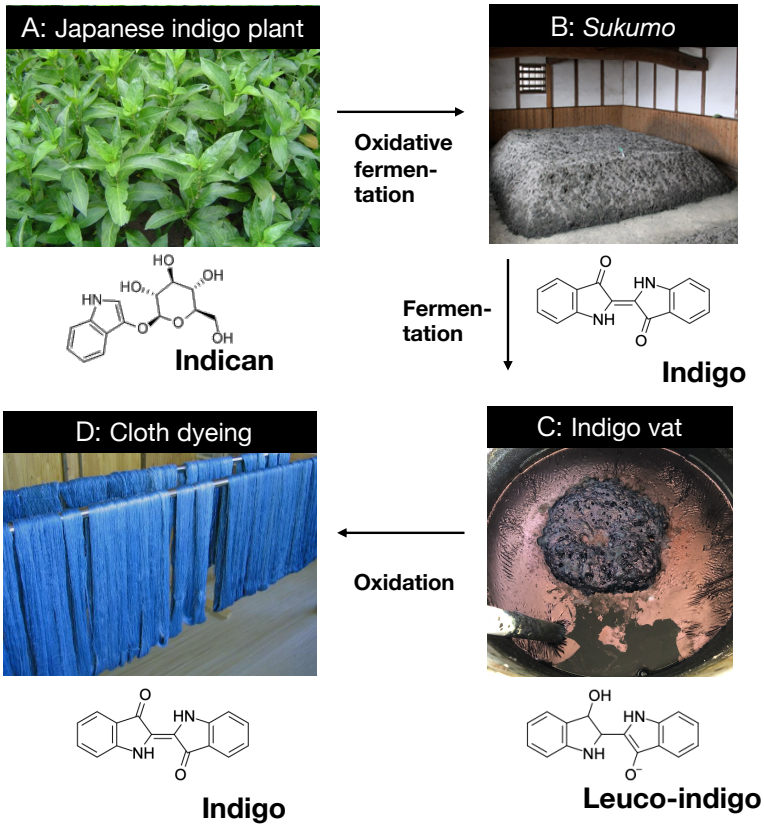


Figure 1.

Pictures of traditional indigo dyeing processes. (A) Japanese indigo plant leaves containing indican. The leaves are decomposed by aerobic microorganisms at suitable moisture contents and temperature controlled by skilled craftsmen for 100 days. (B) *Sukumo:* oxidatively fermented indigo plant leaves to generate indigo from indicant. (C) Top view of an indigo fermentation tank in which insoluble indigo is microbially reduced to leuco-indigo under anaerobic alkaline conditions (pH 10–12). (D) Blue-colored fibers that adsorbed leuco-indigo in the indigo fermentation tank and were exposed to the air.

The leaves are harvested and air-dried. Then, approximately three tons of dried leaves are mixed with the same amount of water and stacked to ca. 1 m in height (Fig. 1 B). Once every 2 to 3 days, the leaves are mixed up and down with water for sufficient aeration (oxidative fermentation). The temperature reaches about 60 ℃ due to the fermentation heat during the plant tissue decomposition. The microbial oxidative fermentation of the leaves proceeds under aerobic conditions and at an appropriate temperature with the sophisticated techniques of trained craftsmen. This operation takes up to 100 days to produce composted and fermented product called *sukumo*, and it has been developed in Tokushima Prefecture, Shikoku, Japan.

The second fermentation process of preparing the dye suspensions, insoluble indigo is microbially reduced into water-soluble leuco-indigo (Fig. 1 C). The reduction of indigo to leuco-indigo is catalyzed by microorganisms in highly alkaline suspensions (pH 10.3–10.5), which are prepared with wood ash extract, slaked lime, wheat bran, Japanese *sake*, etc. When the leuco-indigo is adsorbed by clothing fibers and is exposed to the air, it is re-oxidized into indigo and is fixed to the fibers (Fig. 1 D).

A number of indigo-reducing microorganisms were isolated from fermentation vats and several enzymes involved in indigo reduction, such as an azoreductase, were characterized. However, little is known about the mechanism of the microbial indigo reduction as well as the significance of the alkali environment of the fermenting dye suspensions and the advantage of the addition of *sake*. On the other hand, traditional indigo fermentation requires a lot of experience because proper management (e.g., stirring once a day, maintaining pH, adding nutrients) is needed to maintain the fermentation liquid in a reduced state of indigo (leuco-indigo) for a long time, but there is no method to directly monitor the amount of indigo in the dye suspension. In this research, the author aimed to bioelectrochemical characterization of indigo reduction and development of in situ indigo reduction, monitoring system in indigo fermentation.

In chapter 1, the author aimed to elucidate the mechanism of microbial indigo reduction, we directly performed cyclic voltammetry (CV) on alkali fermenting dye suspensions. Leuco-indigo was successfully detected on CV in the supernatant of a dye suspension. In addition, the cyclic voltammetric experiments on the sediment-rich (that is, microbe-rich) suspension derived form the fermenting dye suspension showed steady-

state catalytic oxidation and reduction waves that catalyzed by some redox enzymes in the microbes and the indigo/leuco-indigo redox couple as a mediator. A model bioelectrocatalytic system for the reductive fermentation of indigo was constructed by using isolated indigo-reducing bacteria strain K2-3′, methyl viologen (MV^{2+}) as a soluble mediator, and acetaldehyde. As a result, it confirmed that acetaldehyde can both donate electrons to MV^{2+} and accept electrons from $MV^{\bullet+}$; the reactions were catalyzed by redox enzymes in the K2-3′ strain.

In chapter 2, CV was carried out by floating the electrodes set up in apolyethylene vinyl alcohol (PEVA) material tube holder in the fermentation vat. The anodic and cathodic peaks in the suspensions were found to indicate electrochemical oxidation of leuco-indigo and re-reduction of indigo, respectively. To develop a method for *in situ* leuco-indigo monitoring, a traditional Japanese fermentation procedure was performed on a laboratory scale. Vat 1 contained ca. 5 L dye suspension of last year, and Vat 2 was prepared with nothing in it. In both vats, leuco-indigo levels could be measured over time. In Vat1, catalytic oxidation wave was observed as indigo/leuco-indigo-mediated bioelectrochemical catalytic oxidation of a reductant one day earlier than in Vat 2. CV clearly showed the amount of leuco-indigo, which cannot be detected by the staining intensity of the towel. In addition, the author proposed examination of revive methods for fermentation suspension by using CV. It is expected that if the amount of indigo present in dye suspension and *sukumo* extract can be determined by artificial reduction and measurement by CV, the dye suspension can be controlled more efficiently in the future.

In chapter 3, to clarify the conditions for efficient indigo reduction in dye fermentation suspension, 1) we identified indigo-reducing bacteria isolated from the indigo-fermenting suspensions and the *sukumo* by the new indigo-reduction assay method, 2) we tested some mediators that facilitate indigo reduction, and 3) we analyzed microbiota of the indigo fermentation suspension. The new indigo-reducing assay method was used not only for the measurement of the indigo-reducing activity, but also for the isolation of new indigo-reducing bacteria which were not isolated by the indigo carmine-reducing assay method. In addition, anthraquinone was found to be effective as a mediator to facilitate the indigo-reducing activity. It was demonstrated that there was no significant

difference among the microbiota of *sukumo* produced by different producers and the indigo dye suspension. Although the number of indigo-reducing bacteria decreased with the addition of nutrient sources, the staining intensity increased. These results indicate that the ratio of the numbers between indigo-reducing bacteria and the others is important to maintain the indigo-reducing activity.

Chapter 1

Mechanistic Insights into Indigo Reduction in Indigo Fermentation: A Voltammetric Study

Abstract

Indigo is one of the oldest natural blue dyes. Microorganisms and their enzymatic activities are deeply involved in the traditional indigo staining procedure. To elucidate the mechanism of the microbial indigo reduction, we directly performed cyclic voltammetry on alkaline fermenting dye suspensions. A pair of characteristic redox peaks of leuco-indigo was observed in a supernatant fluid of the fermenting dye suspension. On the other hand, it was found that the indigo/leuco-indigo redox couple mediated two-way microbially catalyzed oxidation and reduction in a sediment-rich suspension of the fermenting suspension. Acetaldehyde was supposed to be the electron donor and acceptor of the catalytic reactions. In order to verify the bioelectrocatalytic reaction, we isolated indigo-reducing bacterium K2-3′ from the fermenting suspension, and the two-way bioelectrocatalysis was successfully restaged in a model system containing K2-3′ and methyl viologen (as a soluble mediator instead of indigo) as well as acetaldehyde at pH 10.

1. Introduction

Indigo was used as a pigment until around 1600. Around that period, Dutch sailors visited India and acquired technical knowledge of the fermentation method for indigo production, and indigo spread as a blue dye all over the world[1]. Indigo has long been a widely known dye as a natural source of blue. Natural indigo production declined due to the development of artificial production in chemical industries, however, craftsmen who perform indigo dyeing are still very active in Japan. Japanese *Aizome* (indigo dyeing) is highly appreciated even in foreign countries and characterized by a deep blue color called “Japan blue”.

A number of indigo-reducing microorganisms were isolated from fermentation vats[2–14] and several enzymes involved in indigo reduction, such as an azoreductase, were characterized[15–18]. However, little is known about the mechanism and the electron donor

of the microbial indigo reduction as well as the significance of the alkali environment of the fermenting dye suspensions and the advantage of the addition of *sake*. The fermenting dye suspensions in which the indigo reduction takes place are highly complex regarding nutrients and microbial organisms, and thus the mechanism and the electron donor of the microbial indigo reduction in such environments remains unclear for long. It is considered that both indigo and leuco-indigo coexist in a fermenting dye suspension. Various analytical methods were reported for the quantitative and qualitative analyses of indigo dyes in aqueous solutions, for example, UV-Vis spectrophotometry with the addition of organic solvents such as dimethyl sulfoxide, dichloromethane[19,20], and redox titration using potassium hexacyanoferrate[21]. However, when a part of the fermenting dye suspension is exposed to oxygen, leuco-indigo is immediately oxidized and converted into indigo. Spectroscopic methods are not applicable to detect or quantify indigo and leuco-indigo in the fermenting dye suspension. Several electrochemical techniques were reported for ex-situ analyses of indigo[22–25]. Therefore, in this study, we applied cyclic voltammetry to in-situ detection of leuco-indigo in fermenting dye suspension and attempted to get mechanistic insights into the mechanisms of microbial indigo reduction.

2. Experimental

2.1 Preparation of indigo dye suspensions by fermentation

The traditional Japanese fermentation procedure was performed on a laboratory scale. Wood ash (500 g) was mixed with 5 L of boiling water, and the wood charcoal was allowed to settle in the fluid overnight. The resultant supernatant (S1) was used as lye. The sediment was mixed with 10 L of boiling water, and the supernatant (S2) was prepared in the same manner as described above. Two kg of *sukumo*, which was purchased from *Nii Seiaisyo* (Tokushima, Japan), was well mixed with 1 L of boiling water in a 45-L plastic bucket. Ten L of S2 and 100 ml of Japanese *sake* (rice wine) were added, and the total volume was made up to 20 L with boiling water. The pH of the mixture was adjusted to 10–11 with slaked lime. Two days later, 20 g of wheat bran was mixed with 1 L of boiling water, and the mixture was transferred to a fermentation vat. Two more days later, 5 L of S1 and 5 L of warm water (30 ℃) were added.

The fermenting dye suspension in the fermentation vat was mixed with a bar once a day. The pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO) were measured using a D-75 (Horiba) electrode. The pH of the fermenting dye suspension decreased with the metabolite production by the microorganisms involved in the process. The pH of the fermenting dye suspension was kept in the range from 10 and 12 by adding slaked lime, and the staining intensity was occasionally checked by dipping a small portion of cotton textile into the dye suspension (Fig. 1).

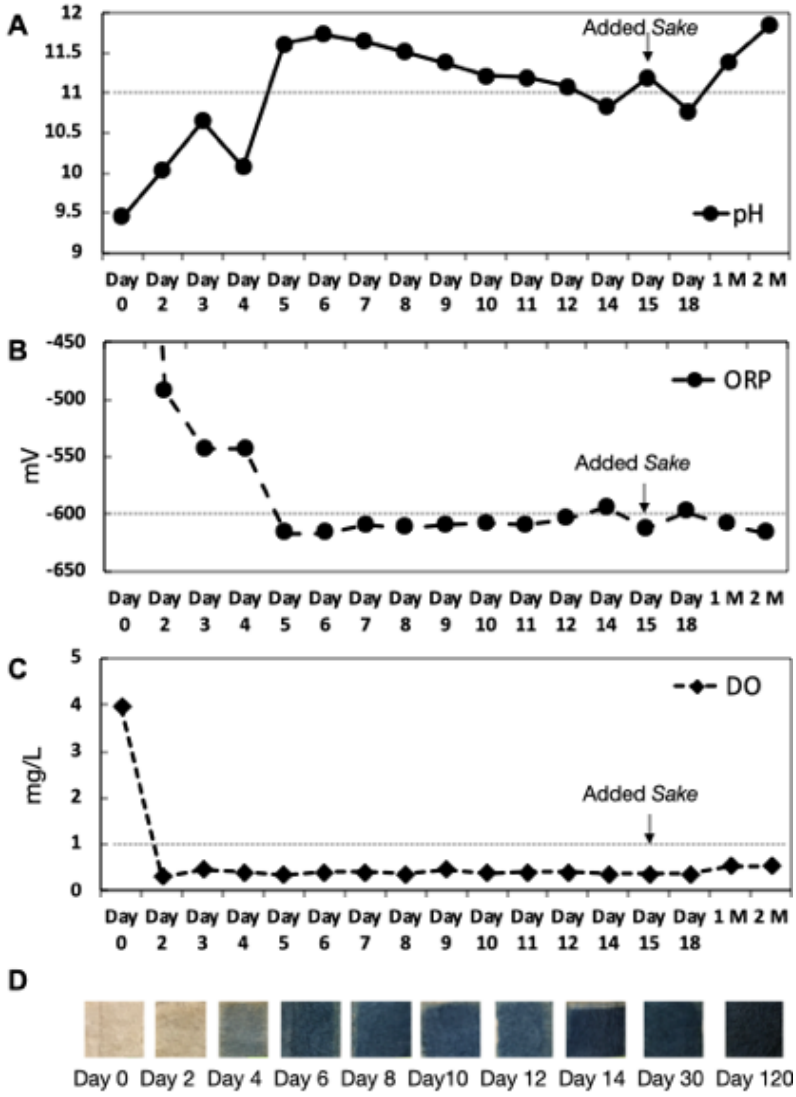


Fig. 1.

Changes in (A) pH, (B) ORP, (C) dissolved oxygen of an indigo fermentation suspension in a fermentation vat, and (D) the textile dyeing intensity. Each piece of 100 %-cotton towel was dipped into the fermentation suspension three times and then exposed to the air. Sample labels represent the number of fermentation days elapsed.

For in-situ voltammetric measurements, 50 mL of the well-mixed fermenting dye suspension was sampled and left for 30 min under anaerobic conditions. 38 mL of the supernatant was removed and submitted to voltammetric analysis under anaerobic conditions. The remained sediment suspension (12 mL) was re-mixed and directly submitted to voltammetric analysis under anaerobic conditions. The supernatant and sediment-rich suspension reflect the situations of the upper and the bottom layers of the fermentation vat, respectively.

2.2 Isolation of an indigo-reducing microorganism

A drop of the suspension in the *Aizome* tank (fermenting tank) was inoculated into Alkaline Gifu Anaerobic Medium (Nissui Pharmaceutical, Japan) with 1 % (w/v) Na_2CO_3 (alkali-GAM) and incubated again at 28 °C under anaerobic conditions with Aneropack Kenki (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The culture broth was streaked on a 1.5 % gellan gum plate of alkali-GAM and then incubated at 28 °C under anaerobic conditions with Aneropack Kenki, and isolates were obtained. Indigo-reducing activity of the isolates was measured by the following method; the isolates were cultivated in 10 mL of alkali-GAM at 28 °C for 16 h in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA), of which the atmosphere was N_2 with 1–2 % H_2. After cultivation, 10 mL of the culture broth was centrifuged at 1,500 *g* for 10 min. Ten mL of indigo suspension (the total amount per volume corresponding to ca. 1 mM (M ≡ mol L^{-1})) in 20 mM sodium carbonate buffer (pH 10.0) was added to the harvested cells, followed by incubation at 28 °C for 16 h in an anaerobic chamber; during the cultivation, microbial reduction of indigo proceeded with some electron donors contained in the harvested cell paste, possible candidates being some microbial metabolites such as acetaldehyde. After the reaction, the reaction mixture was centrifuged at 1,500 *g* for 10 min. The supernatant containing leuco-indigo was exposed to the air, and the optical density at 660 nm (OD_{660}) of the resulting indigo was measured. The increase in OD_{660} compared to that of the brank (without microorganisms) was regarded as a measure of the indigo-reducing activity. The isolate showing the highest indigo-reducing activity was obtained and named K2-3′. As the result of 16S rRNA analysis, K2-3′ was identified as *Enterococcus* sp.*,* with a similarity of 100 %. The nucleotide sequence reported in this study was deposited in the DDBJ database under

DDBJ/EMBL/GenBank accession number LC586216.

2.3 Bioelectrochemical characterization of fermentation suspensions

Electrochemical measurements were carried out with an ALS1205C voltammetric analyzer at room temperature (ca. 25 °C) under a nitrogen atmosphere. The working electrode was a glassy carbon disk electrode (GCE, 3 mm in diameter; BAS, Tokyo, Japan). The GCE was polished with 0.05 μm alumina powder and washed with distilled water. The reference electrode was a handmade Ag|AgCl|sat.KCl electrode. The counter electrode was 5 cm Pt-wire (Nilaco, Tokyo, Japan). The electrode potential in this paper is referred to the reference electrode unless otherwise stated.

To maintain anaerobic conditions, the electrochemical cells were continuously purged with N_2 gas. Cyclic voltammetry was carried out at a scan rate of 10 mV s^{-1} under quiescent conditions.

2.4 Bioelectrocatalytic activity of K2-3′ against acetaldehyde

Strain K2-3′ was inoculated into five tubes containing 10 mL of alkali-GAM and incubated at 37 °C for 18 h under anaerobic conditions with Anaeropack Kenki (Mitsubishi Gas Chemical Co., Inc., Japan). After the cultivation, K2-3′ was harvested by centrifugation at 1,500 *g* for 10 min. The cells were suspended in 100 mM sodium carbonate buffer (pH 10.0) and then collected by centrifugation. The collected cells were washed twice with the 100 mM sodium carbonate buffer (pH 10.0). The reaction mixture contained the washed cells (ca. 0.7 g mL^{-1}), 0.24 mg mL^{-1} methyl viologen dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride) *n*-hydrate (Wako Pure Chemicals, Osaka, Japan), and 9.3 mM acetaldehyde (Wako Pure Chemicals, Osaka, Japan) in the 100 mM sodium carbonate buffer (pH 10.0). Electrochemical measurements were carried out before and after adding acetaldehyde solution.

3. Results and discussion

3.1 Voltammetric behavior of the indigo fermenting dye suspensions

In order to obtain insights into the mechanism of the indigo fermentation, cyclic voltammetric measurements were done to the supernatant and the sediment-rich suspension of the indigo fermenting dye suspension. The supernatant gave a couple of

characteristic peaks with the peak potentials of –0.51 V and –0.71 V (Fig. 2A). We expected that the anodic and cathodic peaks could be assigned to the electrochemical oxidation of leuco-indigo and re-reduction of indigo, respectively. To confirm this, cyclic voltammograms were measured for a chemically reduced indigo solution in the 100 mM sodium carbonate buffer (pH 10.0); indigo was reduced with sodium hydrosulfite under anaerobic conditions. As shown in Fig. 2B, the cyclic voltammogram of leuco-indigo was very similar to that of the supernatant of the indigo dye suspension (Fig. 2A). These results verify our expectation. The small peak height of the cathodic wave compared with the anodic one seems to be due to partial precipitation of electrochemically generated indigo;

$$\text{leuco-indigo}^- \rightleftarrows \text{indigo}\ (\downarrow) + 2e^- + H^+, \qquad (1)$$

where leuco-indigo exists as a deprotonated monoanion in an alkaline solution, because the pK_a values of the hydroxy groups are 8.0 and 12.7[26]. The formal potential can be judged to be close to or slightly more positive than –0.61 V (as the midpoint potential of the voltammogram in Fig. 2A). The anodic peak height can be utilized as a measure of the concentration of leuco-indigo in fermenting dye suspensions. The in-situ voltammetric method was also applied to direct monitoring of leuco-indigo in the fermentation vats during the fermenting process. Details will be reported elsewhere.

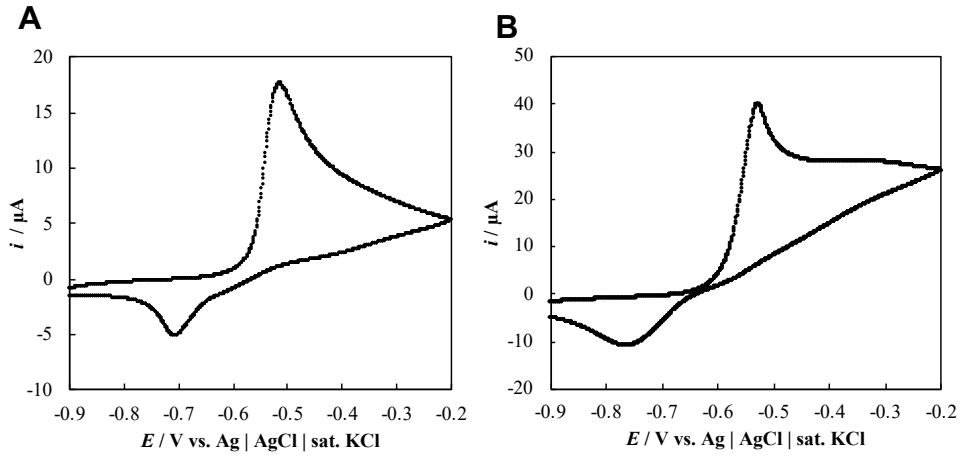


Figure 2.

Cyclic voltammograms recorded at a scan rate (v) of 0.01 V s^{-1} with a bare GC electrode under a complete nitrogen atmosphere in (A) the supernatant of the fermenting dye suspension (pH 10.65), and (B) a chemically reduced leuco-indigo solution (sodium dithionite ($Na_2S_2O_4$)) in 100 mM carbonate buffer (pH 10.0).

In contrast, the sediment-rich suspension showed steady-state catalytic waves in cyclic voltammetry, as shown in Fig. 3.

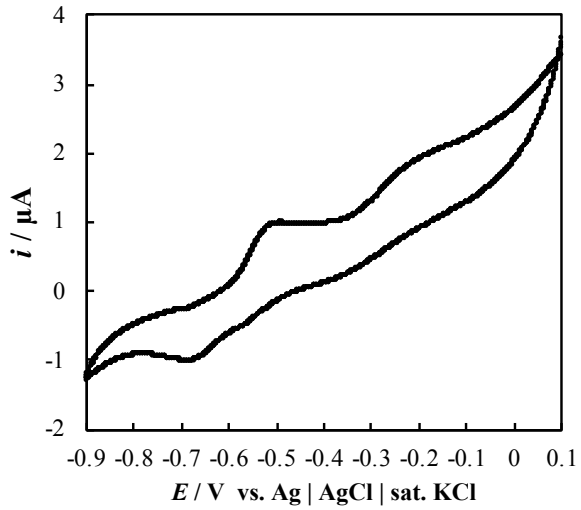


Figure 3.

Cyclic voltammograms recorded in a sediment-rich suspension of the fermenting dye suspension at $v = 0.01$ V s^{-1} with a bare GC electrode under a nitrogen atmosphere.

The concentration of any soluble electron donors and acceptors (and extracellular soluble enzymes if any) in sediment-rich suspension should be equal to that in the supernatant, but the population of microbial organisms in sediment-rich suspension is much higher than that in the suspension. Therefore, it can be concluded that the electrocatalytic reactions are catalyzed by the microbial organisms in the sediments.

Two steady-state oxidation waves were observed with half-wave potentials of –0.56 V and –0.29 V. Since the half-wave potential of the negative-side catalytic oxidation wave was close to the peak potential of leuco-indigo (Fig. 2 A), the wave is characterized as indigo/leuco-indigo-mediated bioelectrochemical catalytic oxidation of a reductant (Red_1) in the fermenting dye suspension. The catalyst is a redox enzyme(s) (redox enzyme 1) in the microorganism;

$$Red_1 + \text{indigo} \xrightarrow{\text{redox enzyme 1}} Ox_1 + \text{leuco-indigo} \qquad (2)$$

The enzymatically generated leuco-indigo is oxidized at the electrode (Eq. (1)). From the thermodynamic viewpoint (that is, to satisfy the down-hill condition in the electron transfer in Eq. (2)), the formal potential of the Ox_1/Red_1 redox couple should be

more negative than that of the indigo/leuco-indigo couple (ca. –0.6 V). Table 1[27] summarizes the formal potentials of some redox compounds considered in this research.

Table 1. Formal potentials of half-reactions of some redox compounds considered. [a]

Half-Reaction	***E*°'**			
	/ V vs. SHE	**/ V vs. Ag/AgCl/ sat. KCl**		
	(pH 7)	**(pH 7)**	**(pH 10)**	**(pH 12)**
Acetaldehyde + $2H^+ + 2e^-$ ⇌ Ethanol	–0.197	–0.394	–0.571	–0.689
FAD + $2H^+ + 2e^-$ ⇌ $FADH_2$ (*free coenzyme*)	–0.219	–0.416	–0.593	–0.711
$NAD^+ + H^+ + 2e^-$ ⇌ NADH	–0.315	–0.512	–0.601	–0.660
$2H^+ + 2e^-$ ⇌ H_2	–0.421	–0.618	–0.795	–0.913
$MV^{2+} + e^-$ ⇌ $MV^{\bullet+}$	–0.440	–0.637	–0.637	–0.637
CO_2 (g) + $H^+ + 2e^-$ ⇌ Formate	−0.443	–0.640	–0.728	–0.788
$Acetate^- + 3H^+ + 2e^-$ ⇌ Acetaldehyde + H_2O	–0.581	–0.778	–1.044	–1.221

[a] Ref. 27. The equilibrium potential of the Ag|AgCl|sat.KCl electrode is –0.197 V vs. the standard hydrogen electrode (SHE) at 25 ℃.

The most likely candidate for Red_1 is acetaldehyde in Table 1 (bottom), and then the oxidized product Ox_1 is assigned to acetate. Acetaldehyde is possibly supplied by enzymatic oxidation of ethanol, which is supplied by alcohol fermentation from glucose (and some other sugars) or often by addition of *sake* in the practical fermentation process. The sour aroma during the dye fermentation in the fermentation vat is reasonably ascribed to acetic acid generated by the reaction (Eq. (2)). Hydrogen (H_2) might be one of candidates for Red_1, but H_2 cannot exist stably in the fermenting dye suspension. Formate might not be ruled out as one of candidates for Red_2 from the thermodynamic view point (Table 1), but we will focus on acetaldehyde as Red_2 in this paper and continue our research on formate also in future. A similar reaction proceeds in the positive-side catalytic oxidation wave (Fig. 3). Unfortunately, since a (non-catalytic) oxidation peak

was not detected around –0.29 V in the supernatant of the fermenting dye suspension (Fig. 2 A), the redox compound that mediated the bioelectrocatalytic oxidations seem to exist only in the sediment-rich suspension.

On the other hand, the peak-shaped reduction wave of indigo (Fig. 2 A) changed into a steady-state catalytic reduction wave with a half-wave potential of –0.64 V in the sediment-rich suspension (Fig. 3). The wave is characterized as indigo/leuco-indigo-mediated bioelectrochemical catalytic reduction of an oxidant (Ox_2) in the suspension, but the catalytic reaction rate is not so high. The catalyst is a redox enzyme(s) (redox enzyme 2) in the microorganism;

$$Ox_2 + \text{leuco-indigo} \xrightarrow{\text{redox enzyme 2}} Red_2 + \text{indigo}. \quad (3)$$

The enzymatically generated indigo is reduced at the electrode (Eq. (1)). From the thermodynamic viewpoint, the formal potential of the Ox_2/Red_2 redox couple will be more positive than that of the indigo/leuco-indigo couple (ca. –0.6 V). The most likely candidate for Ox_2 is also acetaldehyde in Table 1 (top), and the reduced product Red_2 can be assigned to ethanol.

3.2 Two-way bioelectrocatalysis for acetaldehyde in a model system containing K2-3′ and methyl viologen

In order to verify our consideration described in Section 3.1, we constructed a model system for the indigo fermenting suspension. Since the solubility of indigo is very low, we used water-soluble methyl viologen (MV^{2+}) in place of indigo as a mediator of the bioelectrocatalysis, since the formal potential of the $MV^{2+}/MV^{\bullet+}$ couple is close to that of indigo in alkaline solutions (Table 1);

$$MV^{2+} + e^- \rightleftarrows MV^{\bullet+}. \quad (4)$$

We utilized bacterium K2-3′ as a catalysis that was isolated from the fermenting dye suspension and exhibited the reduction activity toward indigo.

Voltammetric studies were performed in 100 mM sodium carbonate buffer (pH 10.0) in the presence of 0.3 g of wet cells of K2-3′ (per 300μL) using $MV^{2+}/MV^{\bullet+}$ (0.24 mg/mL) as a mediator. MV^{2+} gave a couple of non-catalytic reversible redox waves in the absence of acetaldehyde even in the presence of the K2-3′ cells (Fig. 4A, curve red). The midpoint potential (−0.64 V) is in good agreement with that in the literature (–0.637 V,

Table 1).

When acetaldehyde (9.3 mM) was added to the model system, the peak-shaped wave changed into a steady-state sigmoidal one due to bioelectrocatalysis of the oxidation and reduction of acetaldehyde. In the catalytic reaction, the strain K2-3′ cells and the $MV^{2+}/MV^{\bullet+}$ couple acted as a catalyst and a mediator, respectively. The data clearly support our idea that acetaldehyde can act as both an electron donor for MV^{2+} and an acceptor for $MV^{\bullet+}$. MV^{2+} and $MV^{\bullet+}$ were replaced with indigo and leuco-indigo, respectively, in the indigo fermenting suspension. Therefore, we can safely conclude that the microbial reduction of indigo in fermenting dye suspensions is catalyzed by an acetaldehyde dehydrogenase (Eq. (2), in which Red_1 is acetaldehyde and Ox_1 is acetate). We suppose that the enzyme (redox enzyme 1 in Eq. (2)) may be a molybdopterin enzyme. Further study is also required for the production of acetaldehyde under such alkaline conditions of the fermenting due suspension.

An NADH-dependent indigo-reducing enzyme was reported[17]. Since the formal potential of the NAD^+/NADH couple is –0.601 V at pH 10, as shown in Table 1, NADH can barely act as an electron donor to indigo under the condition. When the pH is above 10, NADH becomes more difficult to act as an electron donor to indigo.

On the other hand, this work has also raised the possibility that acetaldehyde is reduced back to ethanol by redox enzyme 2 in Eq. (3), most probably an NAD(P)-independent molybdopterin alcohol dehydrogenase[28]. Especially when we used $MV^{\bullet+}$ as an electron donor, strong acetaldehyde-reducing activity (as a reverse reaction of alcohol dehydrogenase) was observed compared with leuco-indigo (Figs. 3 and 4A). The reason is that the formal potential of the $MV^{2+}/MV^{\bullet+}$ couple is more negative than that of indigo.

However, the enzymatic leuco-indigo oxidizing reaction with acetaldehyde (Eq. (3) in which Ox_2 and Red_2 are acetaldehyde and ethanol, respectively) is not convenient for the indigo reduction in the dye fermentation. Here, we should consider the pH dependence of the formal potential ($E^{\circ\prime}$) as given by:

$$E^{\circ\prime}{}_{\mathrm{pH}_2} = E^{\circ\prime}{}_{\mathrm{pH}_1} - 2.303\frac{mRT}{nF}(\mathrm{pH}_2 - \mathrm{pH}_1), \quad (5)$$

where m and n are the numbers of protons and electrons, respectively, R, T, and F being the gas constant, absolute temperature, and Faraday constant, respectively. The m values of the acetate/acetaldehyde, acetaldehyde/ethanol, and indigo/leuco-indigo redox couples

are 3 (Eq. (6)), 2 (Eq. (7)), and 1 (Eq. (1)), respectively, in the two-electron transfers (n =2).

$$CH_3COO^- + 2e^- + 3H^+ \rightleftarrows CH_3CHO + H_2O, \quad (6)$$

$$CH_3CHO + 2e^- + 2H^+ \rightleftarrows CH_3CH_2OH. \quad (7)$$

Therefore, under highly alkaline conditions, acetaldehyde behaves as a very strong electron donor (to indigo). On the other hand, its electron-accepting ability (from leuco-indigo) is drastically weakened (Fig. 4B), and then the anticipated re-oxidation of leuco-indigo with acetaldehyde would be minimized at strongly alkaline conditions. These redox properties of the participants appear to be the keystone to explanation of the significance of alkaline conditions in indigo dye fermentation.

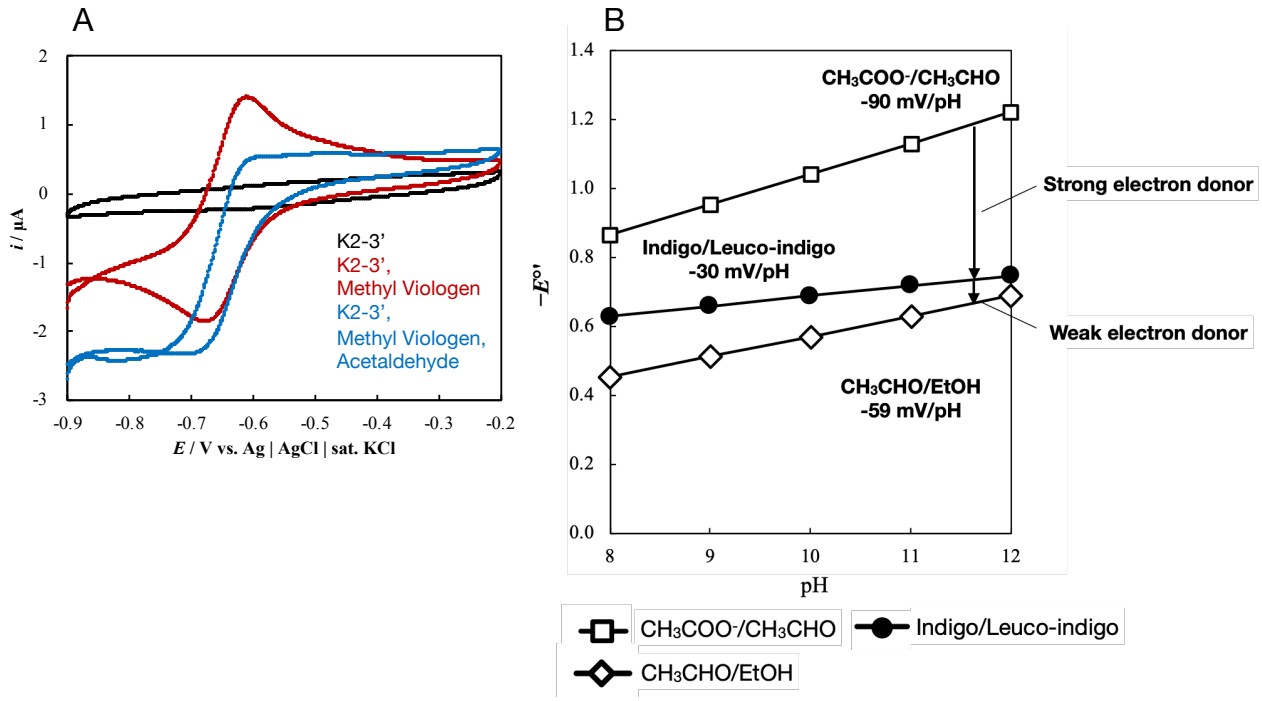
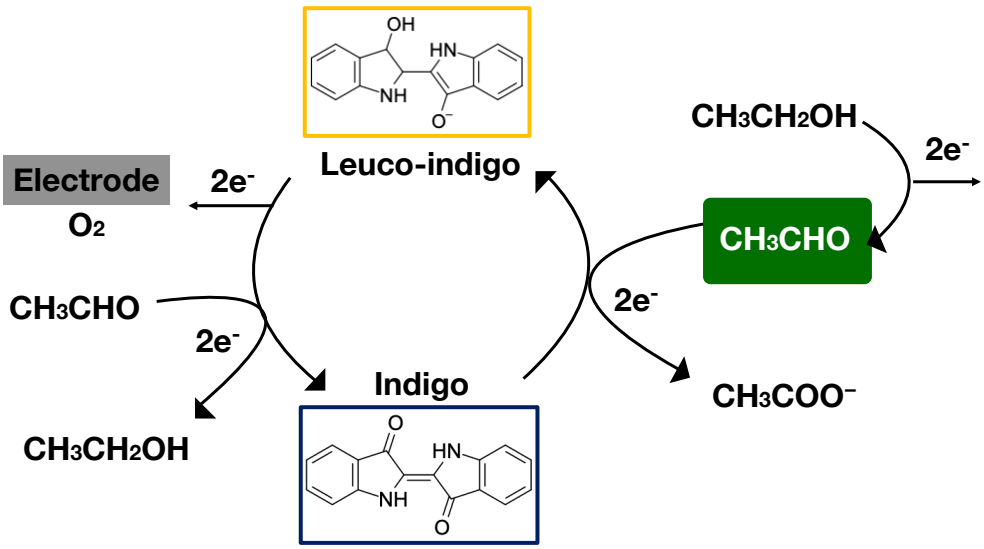


Figure 4.

Cyclic voltammograms of a model bioelectrochemical-system containing the k2-3′ strain as an electrocatalyst and MV^{2+} as a mediator in 100 mM sodium carbonate buffer (pH 10.0). (Black) The k2-3′ strain alone, (red) after the addition of 10 mM MV^{2+}, and (blue) after further addition of 100 mM acetaldehyde. All experiments were performed with a bare GC electrode at v =10 mV s^{-1} under a nitrogen atmosphere. (B) The pH-dependence of the formal potentials of (open squares) acetate/acetaldehyde, (circles) indigo/leuco-indigo, and (open diamonds) acetaldehyde/ethanol.

4. Conclusion

Leuco-indigo was directly detected by cyclic voltammetry in the supernatant of the fermenting dye suspension. The voltammetric experiments in the sediment-rich (that is, microbe-rich) suspension of the fermenting dye suspension showed steady-state catalytic oxidation and reduction waves. A model bioelectrocatalytic system involving indigo-reducing bacterium strain K2-3′ and MV^{2+} as an electrocatalyst and a mediator, respectively, confirmed that acetaldehyde can both donate electrons to MV^{2+} and accept electrons from $MV^{\bullet+}$; the reactions were catalyzed by redox enzymes in the K2-3′ strain. We can propose a mechanism for indigo fermentation as shown in Scheme 1.



Scheme 1.

Proposed redox reactions of indigo and leuco-indigo occurring in indigo dye fermenting suspensions.

Indigo in *sukumo* is reduced by acetaldehyde with an (NAD-independent) acetaldehyde dehydrogenase to generate leuco-indigo and acetate predominantly in the bottom of the fermentation vat. Acetaldehyde can be supplied by alcohol fermentation. Therefore, the sour aroma of acetic acid and the addition of *sake* in the practical fermenting process are very reasonable. Leuco-indigo is auto-oxidized by O_2 into indigo especially on the surface of the fermenting dye suspension. In addition, part of leuco-indigo may be re-oxidized into indigo by acetaldehyde as the reverse-reaction of alcohol dehydrogenase. In order to weaken the electron accepting ability (and also to strengthen

the electron donating ability) of acetaldehyde, it is very important to keep the strongly alkaline conditions during the dye fermentation. This situation is strictly practiced in the practical fermenting process. We are attempting to isolate and clone the enzyme(s) participating in the fermenting process.

5. References

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Chapter 2

Voltammetric In-situ Monitoring of Leuco-Indigo in Indigo-Fermenting Suspensions

Abstract

Cyclic voltammetry was successfully applied to in-vivo monitoring of leuco-indigo in indigo-fermenting suspensions under quiescent conditions without deoxygenation; the working and counter electrodes were kept on the surface of each suspension by a polyethylene vinyl alcohol tube holder. The anodic peak current was used as a measure of the leuco-indigo concentration. The voltammetric wave shape suggested partial solubilization of the indigo with some macromolecules in the fermenting suspensions, which lead to an in-situ method without any electrode surface pretreatment. The anodic peak current well reflected the dyeing activity of a suspensions. The results obtained for laboratory-level fermentation systems clarified the number of days required for dye fermentation, the effectiveness of addition of old suspension as an additive for preparing fresh fermenting suspensions, and the importance of addition of a nitrogen-based nutrient as well as a glucose-based one to recover the indigo-reducing activity. The method can also be applied to determine the amounts of indigo in used dye suspensions and extracts of fermented indigo leaves (*sukumo*) by adding a chemical reduction pretreatment.

1. Introduction

Indigo belongs to a group of dyes known as vat dyes. A "vat" is a vessel that was originally used to ferment indigo leaves[1]. Regarding the worldwide consumption of dyes for dyeing of cellulose fibers, vat dyes account for a relatively large portion of the dye market (about 31 %). It is estimated that at least 10 g of indigo is necessary to dye one pair of trousers, and the world production of indigo is close to 10^7 kg/a due to the vast annual sales of 10^9 pairs of blue jeans[2,3].

Vat dyes such as indigoids and anthraquinone are insoluble in water, but can be reduced with suitable reductants under alkaline conditions into a water-soluble leuco-form[1]. Sodium dithionite ($Na_2S_2O_4$) has been the main industrial reducing agent for a long time, but its consumption in a vat leads to the generation of environmentally

unfavorable products that cause practical problems in the disposal of the dye waste[3]. It was reported that 20 % of the dyes used in the dyeing process are discharged in wastewater[4]. On the other hand, traditional Japanese indigo dyeing is very eco-friendly, because it uses only natural materials[5], and the sediment of the waste dye suspensions is reused as compost for the growth of plants.

Aizome (Japanese indigo-dyeing technique) uses *sukumo*, which is made by fermenting indigo leaves. The preparation of a dye suspension by fermentation was described in our previous paper[6]. The reduction of indigo in *sukumo* is initiated by indigo-reducing bacteria in the dye vat[7]. An indigo-fermenting dye suspension including *sukumo* exhibits the dyeing activity within several days from the start of the preparation. We previously reported that acetaldehyde derived from ethanol is the most likely candidate as the reducing reagent for indigo and is oxidized into acetate, while a small amount of acetaldehyde may be re-reduced into ethanol by leuco-indigo[6]. Additionally, the traditional indigo fermentation requires strict conditions in which proper management (e.g., mixing the suspension once a day, maintaining pH, and adding nutrients for indigo-reducing bacteria) is needed to maintain the reduced state of indigo (leuco-indigo) in the fermenting suspension for a long time[8]. In order to maintain the dyeing activity, it is necessary to prevent the oxidation of leuco-indigo and to maintain the anaerobic state of the indigo dye suspension. Therefore, there is a large need for in-situ monitoring of the leuco-indigo concentration in indigo-fermenting suspensions.

Under highly alkaline and anaerobic conditions in the dye suspension, indigo is reduced to leuco-indigo by microorganisms.[7] After the soluble leuco-indigo adheres to the cloth, it is air-oxidized to insoluble indigo to dye the cloth. We are very interested in how the insoluble indigo is reduced to leuco-indigo by some microorganisms in such a harsh environment. Therefore, we first needed to develop a novel method for quantifying leuco-indigo in the dye suspension to understand the redox reaction of indigo and leuco-indigo. Various methods were reported for analyzing indigo dyes in aqueous solutions. Indigo is frequently measured by UV-Vis spectrophotometry with the addition of organic solvents such as dimethyl sulfoxide or dichloromethane[9,10]. Redox titration of leuco-indigo with potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) was also reported[4]. However, it is not easy to directly apply these methods to measure the concentration of leuco-indigo

and/or indigo in fermentation vats that contain complex and colored mixtures. Cyclic voltammetry was applied to the quantification of indigo but only in an organic solvent[11]. The low solubility of indigo disturbs voltammetric measurements in aqueous solutions. Pulse electrolysis for electrode surface refreshment was proposed for repeated use of glassy carbon electrodes in aqueous leuco-indigo solutions[12]. Rotating disk voltammetry was applied to measure the concentration of leuco-indigo[13,14]. This method is more accurate than the UV-Vis spectrophotometric method and can be used even for dark blue-colored dye suspensions. However, a rotating disk voltammetric instrument is not convenient for in-situ measurements. We reported electrochemical redox behavior of indigo and leuco-indigo in dye suspensions.[6] On the other hand, it was difficult to quantitatively measure leuco-indigo in dye vats because it is rapidly converted to indigo in the presence of even a small amount of oxygen. Here, we propose an in-situ electrochemical method for quantitative measurement of leuco-indigo in dark blue-colored dye suspensions in dye vats. The method was applied to model practical cases. Based on the measurements, we also propose some treatments to maintain and recover the dyeing activity of the dye-fermenting suspensions.

2. Experimental

2.1 Reagents

Chemical reagents such as indigo-carmine (Tokyo Chemical Industry Co., Ltd., Japan), sodium dithionite (Wako Pure Chemical Industries, Ltd., Japan), D(+)-glucose (Kanto Chemical Co., Inc., Japan), yeast extract (Oriental Yeast Co., Ltd., Japan), and hipolypepton (Fujifilm Wako Pure Chemical Corporation, Japan) were commercially obtained .

2.2 Old fermenting suspension and *sukumo* extract

The traditional Japanese fermenting procedure was performed on a laboratory scale. A fresh dye suspension (40 L) was prepared in a vat (45-L plastic bucket) in the same way as described in our previous paper[6] and used for dyeing experiments last year. Portions of the used fermenting suspension were kept in a refrigerator and used as appropriate as old dye suspension. The *sukumo* extract was prepared from 25 g of *sukumo* soaked in 110 mL of hot water (ca. 40 °C) for 3 h.

2.3 Preparation of two types of fresh fermenting suspension

A fresh dye-fermenting suspension (40 L) was prepared in two vats (Vats 1 and 2) in the same way as described in our previous paper[6]. In the traditional dye-fermenting process, the old dye suspension used for dyeing is often mixed with a fresh dye-fermenting suspension; this process corresponds to the artificial addition of bacterial flora for indigo fermentation. According to the traditional procedure, 5 L of the old dye suspension was added to the fresh fermenting suspension (40 L) in Vat 1. For comparison, Vat 2 contained only the fresh dye suspension, i.e., without any old dye suspension. Japanese *sake* (100 mL) was added to the fermentation suspensions on days 0, 30, and 37, and 200 mL of *sake* was added on days 12 and 45. On day 58, 20 g of wheat bran was mixed with 1 L of boiling water, and then the mixture was added to the fermentation vats. The suspensions in the fermentation vats were gently mixed once a day. The pH, oxidation-reduction potential (ORP), and fermenting suspension temperature were measured using a D-75 electrode (Horiba, Japan). The pH of the fermentation suspension was kept in the range of 10 to 12 by adding slaked lime. All data are given in Fig. S1. The dyeing activity was checked by dipping pieces of 100 %-cotton towel three times into the dye-fermenting suspension, followed by their exposing to the air.

2.4 Voltammetric measurements

Electrochemical measurements were carried out with an ALS1205C voltammetric analyzer (BAS Inc., Japan) at room temperature. The working electrode was a stationary glassy carbon electrode (3 mm in diameter; BAS, Tokyo, Japan). The glassy carbon electrode was polished with 0.05 μm alumina powder and washed with distilled water. The reference electrode was a handmade Ag|AgCl|sat.KCl electrode with a KCl salt bridge tube. The counter electrode was 5 cm Pt-wire (Nilaco, Tokyo, Japan). The potential in this study is referred to the reference electrode.

A leuco-indigo solution was prepared by reducing an indigo (0.02 g) suspension in 9 mL of 0.01 M NaOH with 88 mg of $Na_2S_2O_4$ for 1 h at 50 °C under anaerobic conditions (1 M = 1 mol L^{-1}). After the addition of 1 mL of 1 M Na_2CO_3/$NaHCO_3$ buffer (pH 10) to the leuco-indigo solution, voltammetric measurements were performed under a nitrogen-atmosphere. An indigo- carmine (1 mM) solution was also subjected to cyclic voltammetry under a nitrogen-atmosphere.

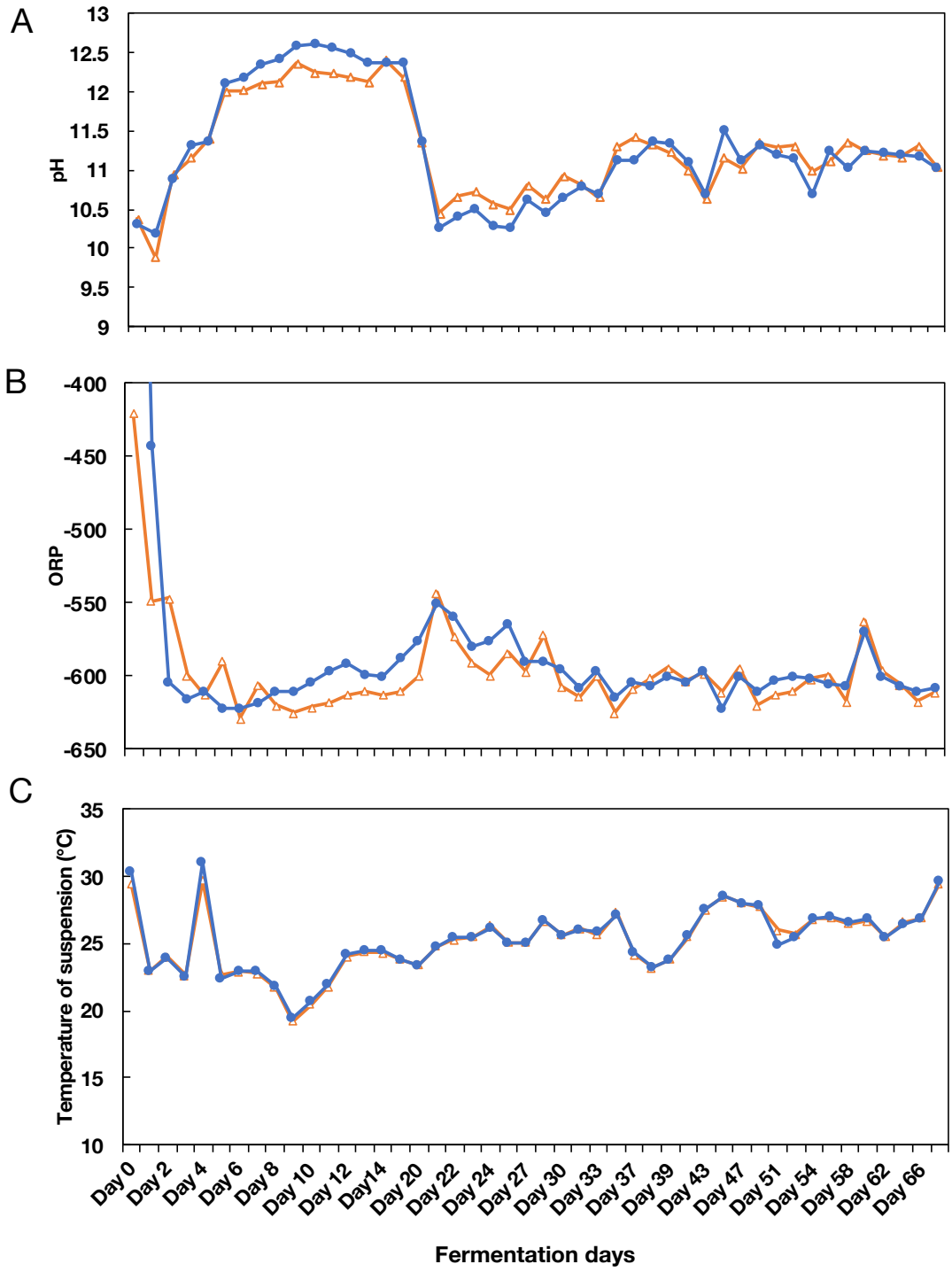

Fig. 1.

Changes in (A) pH, (B) ORP, and (C) temperature of an indigo fermentation suspension in a fermentation vat. Closed circles, Vat 1; open triangles, Vat 2.

In order to directly monitor the leuco-indigo concentration during the dye fermentation,

the working and counter electrodes were kept on the surface of the dye fermenting suspension by a PEVA tube holder, and a salt bridge was used for connection to the reference electrode, as shown in Fig. 1A. The working and counter electrodes and the salt bridge were immersed 3 cm from the suspension surface. Voltammetric measurements were performed at a scan rate (v) of 10 mV s^{-1} without deoxygenation of the dye-fermenting suspension.

In order to check the additional effect of α-cyclodextrin (CD) on the cyclic voltammograms of leuco-indigo, a leuco-indigo solution was prepared by reducing 0.02 g of indigo suspension in 9 mL of 0.01 M NaOH with 88 mg of $Na_2S_2O_4$ for 1 h at 50 °C under anaerobic conditions. One mL of 1 M Na_2CO_3/$NaHCO_3$ (pH 10) was added to the reduced solution before voltammetric measurements. The α-CD stock solution comprised 8.4 g of α-CD in 7.4 mL of 100 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10).

Indigo in the old dye suspension and in the *sukumo* extract was reduced with $Na_2S_2O_4$; to 1 mL each of the old dye suspension and the *sukumo* extract supernatant, 1 mL of a 10 M NaOH solution and 0.35 g of $Na_2S_2O_4$ were added. Electrochemical measurements were performed under nitrogen-atmosphere.

3. Results and discussion

3.1 In-situ detection of leuco-indigo in a fresh dye-fermenting suspension

We previously reported that leuco-indigo was successfully detected on cyclic voltammetry under anaerobic conditions in samples supernatants of dye-fermenting suspensions[6]. Since the concentration of dissolved oxygen was below 0.5 mg L^{-1} in the fermenting suspension[6], we expected that voltammetric measurements were directly applicable to dye-fermenting suspensions without deoxygenation pretreatment. The leuco-indigo concentration in the dye fermenting suspension was monitored as shown in Fig. 2.

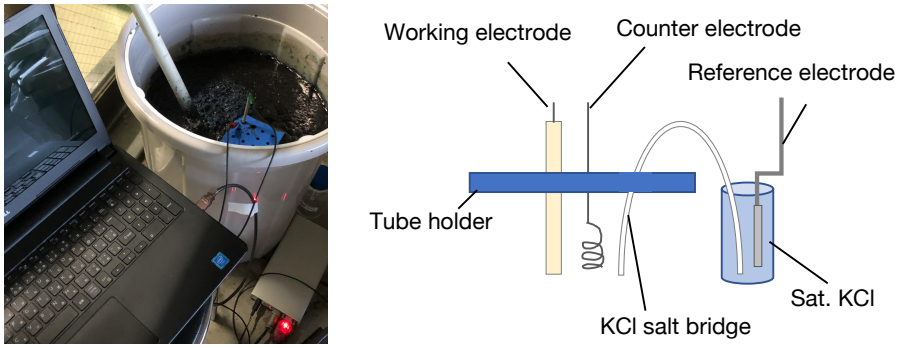

Fig. 2. The in situ indigo monitoring system and cyclic voltammograms of a dye-fermentation suspension. (A) Picture of the in situ indigo monitoring system (left) and layout of the electrodes (right).

Fig. 3A (black) shows a cyclic voltammogram of a fresh dye-fermenting suspension (7 days after the start of the fermentation); the voltammogram was taken under quiescent conditions without mixing of the dye-fermenting suspension. A characteristic sharp anodic peak and a small cathodic peak were observed. The characteristic sharp anodic peak at –0.55 V and the small cathodic peak at –0.72 V indicate the oxidation of leuco-indigo to indigo and the reduction of indigo to leuco-indigo, respectively. The shape of the cyclic voltammogram is very close to that of chemically reduced leuco-indigo (Fig. 3B). These results indicate that the anodic and cathodic peaks for the fermenting suspension can be assigned to the electrochemical oxidation of leuco-indigo and re-reduction of indigo, respectively. The low solubility of indigo seems to be due to the small cathode peak height compared with the anodic one. Compared to indigo-carmine (ca. 0.06 V at v = 10 mV s^{-1}, Fig. 3C), the relatively large peak separation (ca. 0.16 V at v = 10 mV s^{-1}) suggests some hindrance in the electrode kinetics, most probably due to the adsorption of indigo (and leuco-indigo) on the electrode surface (Fig. 3A, B)[12].

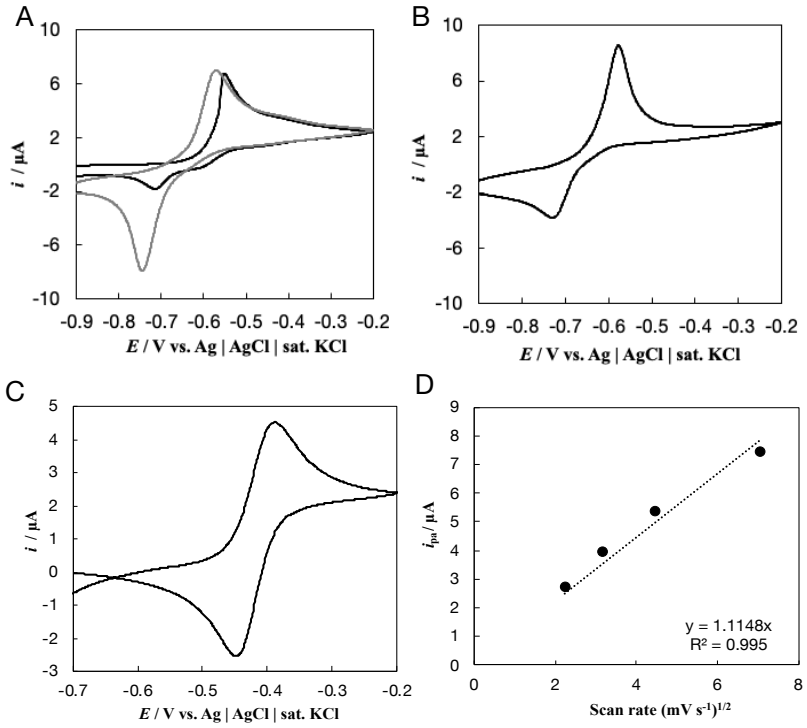

Fig. 3. (A) In-situ cyclic voltammograms of a dye-fermenting suspension (7 day after the start of the fermentation) at v = 10 mV s^{-1} before (black) and just after (gray) mixing of the suspension. (B) Cyclic voltammogram of 0.1 g L^{-1} of leuco-indigo generated by reduction of indigo with $Na_2S_2O_4$ at v = 10 mV s^{-1}. (C) Cyclic voltammogram of indigo-carmine (1 mM) at a stationary glassy carbon electrode at a scan rate of 10 mV s^{-1}; start of potential scan at −0.7 V vs Ag|AgCl|sat.KCl in 100 mM sodium carbonate buffer (pH 10). (D) Anodic peak current as a function of the square root of the scan rate for cyclic voltammograms obtained for indigo-carmine.

Interestingly, the cyclic voltammetric shape was drastically different after mixing of the dye-fermenting suspension (Fig. 3A, gray), although the voltammogram was recorded under quiescent conditions. The anodic and cathode peak potentials shifted slightly to the negative side, and the cathodic peak height became almost identical with that of the anodic peak. The height change in the cathodic peak suggests an increase in the solubility of indigo, most probably through complex formation with some water-soluble macromolecules that existed predominantly in the sediment before the mixing. The peak potential shift to the negative side suggests the stabilization of indigo compared with leuco-indigo. Actually, inclusion complex formation between indigo and α-cyclodextrin was reported[15], and the complex formation was verified in part by cyclic voltammetric measurements in preliminary experiments (Fig. 4). Although the true binding partners in the fermenting dye suspension could not be identified in this work, it is concluded that the increased solubility of indigo after mixing of the dye-fermenting suspension is responsible for the result of in-situ monitoring of indigo by the voltammetric method with repeatedly used glassy carbon electrodes under quiescent conditions.

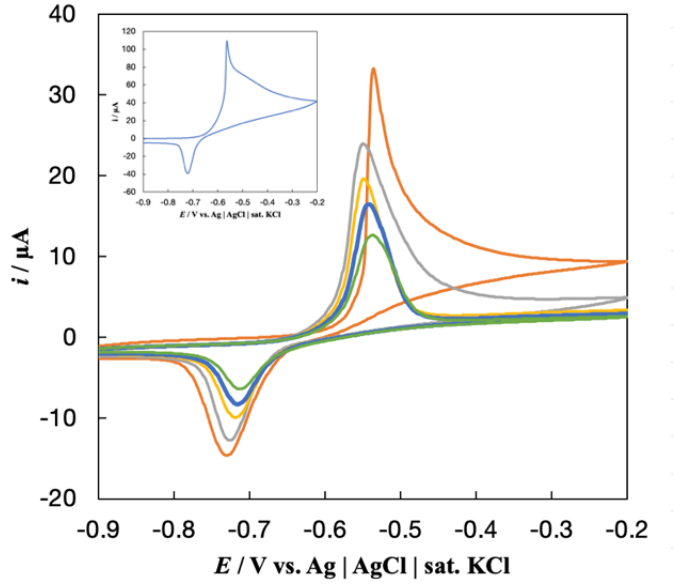

Fig. 4. Cyclic voltammograms were recorded after addition of 0.1, 0.3, 0.5, 0.7, and 1.0 mL of the α-CD stock solution at 50 °C and v = 10 mV s−1. The inset shows a cyclic voltammogram in the absence of α-CD. The peak height of the voltammograms decreased with the addition of α-CD. The results indicate a decrease in the diffusion coefficient of indigo/leuco-indigo through inclusion complex formation with α-CD.

Compared with the shapes of the cyclic voltammograms in Fig. 3A, we observed a small cathodic peak at –0.6 V in Fig. 3A (black). Unfortunately, we could not identify the redox species in the complex dye-fermenting suspension. Interestingly, the small peak almost disappeared after mixing of the dye-fermenting suspension in Fig. 3A (grey), most probably due to an increase in the solubility of indigo under the conditions in Fig. 3A.

In any event, we can conclude that the cyclic voltammetric method involving floating electrodes was successfully applied to in-situ determination of leuco-indigo in the fermenting dye suspension in dye fermentation vats. In order to determine the leuco-indigo concentration from the cyclic voltammetric peak current, we used water-soluble indigo-carmine as a tentative marker; indigo-carmine gave a reversible cyclic voltammogram (Fig. 3C). The anodic peak height (i_{pa}) increased linearly with the scan rate (Fig. 3D), indicating a diffusion-controlled process at least up to v = 50 mV s^{-1}. Therefore, we set the scan rate (v) to 10 mV s^{-1} in this work. From the data in Fig. S2A, we tentatively set the conversion factor as 0.25 mM μA^{-1} (at v = 10 mV s^{-1}) and determined the leuco-indigo concentration from the anodic peak current in the dye-fermenting suspension. In addition, in this work, in-situ voltammetry was performed under quiescent conditions after mixing of the dye-fermenting suspension. Strictly speaking, the electrode kinetic of leuco-indigo is not so good as compared with indigo-carmine, and some complex formation of indigo/leuco-indigo should decrease the peak current due to a decrease in the diffusion coefficient. Therefore, the conversion factor given above should be considered to be the minimum one, and the actual conversion factor might be few times larger than the tentative value proposed here.

Vat 1 was prepared by addition of the old dye suspension to the fresh fermenting suspension, in contrast, Vat 2 contained only the fresh dye suspension, i.e., without any old dye suspension (see Materials and Methods). As shown Fig. 5A, on the starting day of the dye fermentation (day 0), no redox peaks assignable to indigo/leuco-indigo redox couple were observed. Small catalytic oxidation waves appeared on day 1 for Vat 1. Fig. 5B shows the dependence of the anodic peak current (i_{pa}) on the number of fermenting days. The i_{pa} value increased with the number of fermenting days at the beginning of the dye fermentation. The i_{pa} seemed to reach the maximum values on day 3 or 4 and then remained almost constant for a few days. The microbial reduction of indigo and auto-oxidation of leuco-indigo seem to be in a steady state in the vats.

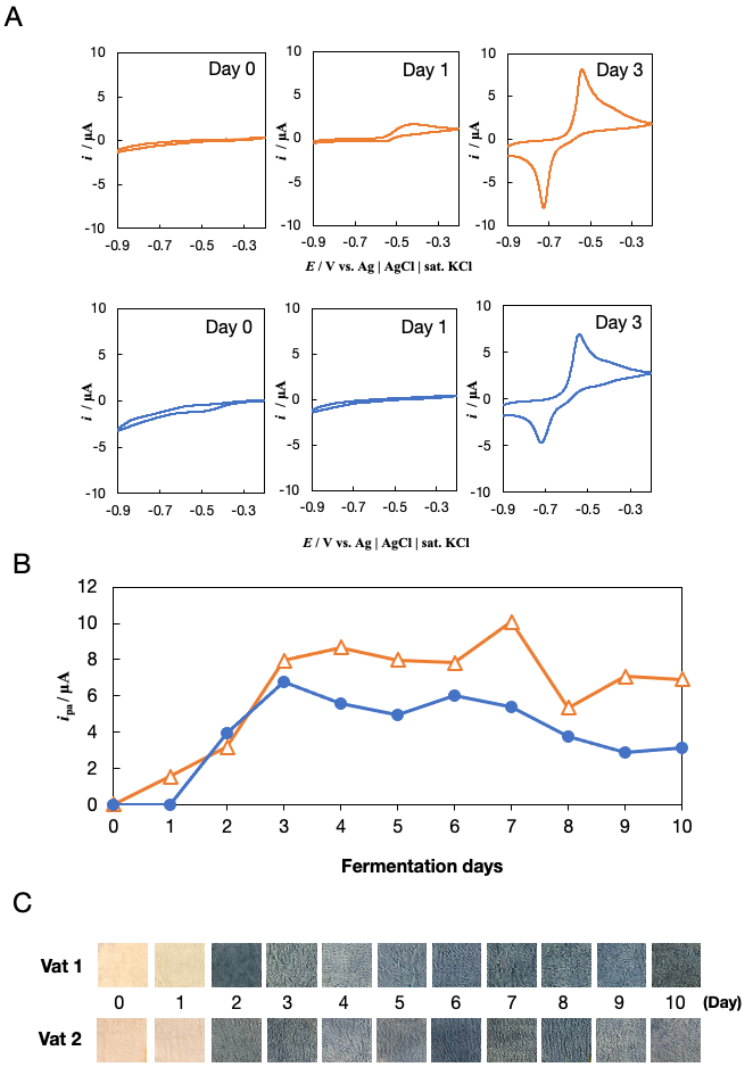

Fig. 5. (A) The changes in waveforms of *in situ* cyclic voltammograms from day 0 to day3 in Vat 1 and 2. Vat1 is shown in the top, Vat2 is shown in the bottom, and the date is shown in the upper right corner of the graph. (B) The oxidation peak current of leuco-indigo from day 0 to day 10 are plotted in black for Vat1 and in gray for Var2, and (C) the textile dyeing intensity.

As shown in Fig. 5B, the staining activity seemed to increase earlier in Vat 1 than in Vat 2. In addition, the maximum value of the oxidation peak for Vat 1 was 10 μA, which corresponds to 2.5 mM leuco-indigo based on the tentative conversion factor set in this work. In contrast, the maximum concentration of leuco-indigo in Vat 2 was 1.4 mM. These results support the effectiveness of the traditional fermenting procedure that involves old dye suspension as an additive at the start of the dye fermentation. However, the effect is not so drastic, and fresh *sukumo* seems to contain enough microorganisms to start the dye fermentation.

The i_{pa} gradually decreased with the number of fermenting days (Fig. 5B). This

seems to be due to the consumption of leuco-indigo on cloth dyeing and auto-oxidation during the mixing treatment of the dye suspension. Some deviation might be ascribed to an unexpected difference in the auto-oxidation on the mixing of the dye-fermenting suspension and the change in the temperature of the suspension (Fig. 1C). However, the i_{pa} obtained on in-situ cyclic voltammetry seems to well reflect the dyeing activity of the dye-fermenting suspension (Fig. 5C).

3.2 Examination of reactivation methods for dye-fermenting suspensions

In order to gain some insights as to re-activation of the dyeing ability of dye-fermenting suspensions with weakened dyeing activity, three types of nutrients: Japanese *sake*, glucose, and a mixture of glucose, yeast extract and hipolypepton were examined as additives for the fermenting suspensions with weakened activity. In this experiment, the fermenting suspension from Vat 2 on day 15 was mixed and then used as a model suspension with weakened activity. To 250 mL aliquots of the fermenting suspension in Vat 2, *sake* (4.4 mM as final ethanol concentration), glucose (11.2 mM; 0.2 % (w/v)), and a mixture of glucose (11.2 mM; 0.2 % (w/v)), yeast extract (0.25 % (w/v)), and hipolypepton (0.5 %(w/v)) were separately added. The fermentation bottles were placed in an incubator at 28 ° C for 2 days. No characteristic peak was observed on in-situ cyclic voltammogram for the control bottle without nutrient addition (Fig. 6A). For the bottle with *sake* addition, a small but clear redox peak of the indigo/leuco-indigo redox couple re-appeared (Fig. 6B); the anodic peak current was 0.31 μA, which corresponds to ca. 0.08 mM generation of leuco-indigo. The direct electron donor for indigo reduction is acetaldehyde that is generated through microbial ethanol oxidation[6]. The addition of *sake* simply gives reducing power. Similarly, increased generation of leuco-indigo (0.75 μA corresponding to 0.19 mM) was observed for the bottle with glucose addition (Fig. 6C). Glucose may be transformed into ethanol on alcohol fermentation under anaerobic conditions. Therefore, the maximum ethanol concentration generated from glucose added can be taken to be 11 mM, which is 2.5 times greater than that in the *sake*-added bottle. This seems to be the reason why a large redox peak was observed for the glucose-added bottle.

Interestingly, the highest peak current was obtained for the bottle containing a nitrogen nutrient as well as a carbon one (such as glucose as reducing activity); the peak current was 2.08 μA (Fig. 6D), corresponding 0.54 mM generation of leuco-indigo. These results indicate that not only a carbon source but also a nitrogen source is effective to increase the indigo-reducing activity of microorganisms in a dye-fermenting suspension.

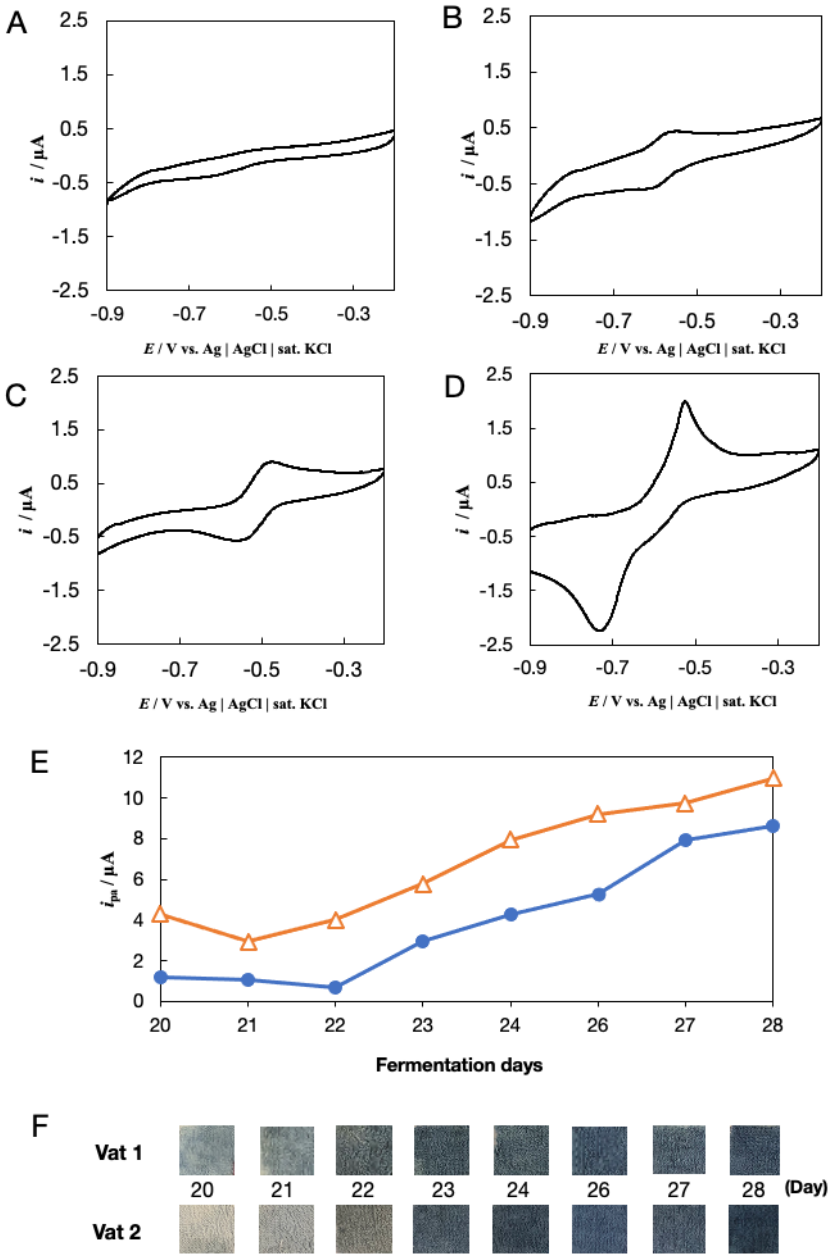

Fig. 6. Effects of nutrition sources as to re-activation of the dyeing ability of dye-fermenting suspensions. (A)−(D) Cyclic voltammograms of nutrient-supplemented dye suspension sampled from the dye-fermenting suspension (250 mL) in Vat 2 under anaerobic conditions. The nutrients added were (A) control, (B) *sake* (4.4 mM as final ethanol concentration), (C) glucose (11.2 mM; 0.2 %(w/v)), (D) glucose (11.2 mM; 0.2 %(w/v)), yeast extract (0.25 %(w/v)) and hipolypepton (0.5 %(w/v)). The voltammograms were recorded at 2 days after the nutrient addition in electrochemical cells. (E) The anodic peak current (i_{pa}) for leuco-indigo in in-situ voltammetric measurements on days 20 to 28. Closed circles, Vat 1; open triangles, Vat 2. (F) the textile dyeing intensity. The addition of the nutrients to Vats 1 and 2 was performed on day 20.

In order to verify this consideration, glucose (20 g, 0.05 %(w/v)), yeast extract (25 g, 0.0625 %(w/v)), and hipolypeptone (50 g, 0.125 %(w/v)) were added to the fermenting suspension in Vats 1 and 2 on day 20. On day 21, the pH and ORP had changed dramatically from 12 to 10 and from –600 to –550 mV, respectively (Fig. 1A, B). These substantial changes in the pH and ORP may be attributed to increases in the concentrations of acidic substances (e.g. formate, lactate, etc.) in the dye-fermenting suspension caused by microbial metabolism. As shown in Fig. 6E, the i_{pa} value of leuco-indigo increased from 2 or 3 days after the addition of the nutrient sources. In addition, the dyeing strength for the towel (Fig. 6F) was consistent with the voltammetric assessments (Fig. 6E).

3.3 Application of in-situ monitoring to dye-suspension management

The concentration of leuco-indigo in the dye-fermenting suspension decreases during cloth dyeing. However, indigo should exist in the dye-fermenting suspension. Therefore, we checked the recovery of the indigo-reducing activity by in-situ monitoring of leuco-indigo. We attempted to sufficiently remove leuco-indigo from the dye-fermenting suspension as much as possible by repeatedly dyeing much cloth on day 61. On the day after sufficient dyeing (day 62), no pH change was observed, but ORP changed from ca. –600 to –550 mV (Fig. 1A, B). The difference in the ORP could be due to a decrease in the concentration of leuco-indigo. As shown in Fig. 7A, the i_{pa} returned to the value before the sufficient dyeing (day 61) within 3 days (Fig. 7A, B). In response to the increase in i_{pa}, the color of the dyed towel became deep (Fig. 7B).

On the other hand, it is also important to know the levels of amounts indigo in the *sukumo* extract and the dye-fermenting suspension used for the dying. Thus, samples of them were chemically reduced with $Na_2S_2O_4$ and then subjected to cyclic voltammetry. Although no cathodic peak was observed for the dye-fermenting suspension sufficiently used for cloth dyeing (data not shown), a large anodic peak was observed after artificial reduction of the suspension with a i_{pa} of 60.8 μA, which corresponds to ca. 15 mM leuco-indigo (Fig. 7C). For the *sukumo* extract also, cyclic voltammetry clearly indicated the presence of chemically-reduced indigo with i_{pa} of 26.5 μA, which corresponds to ca. 6.7 mM leuco-indigo (Fig. 7D). Therefore, the voltammetric method is also useful to determine the amount of indigo in the used dye suspension and *sukumo* extract after complete chemical reduction with $Na_2S_2O_4$.

In conclusion, the cyclic voltammetric method is very useful for in-situ monitoring of leuco-indigo in dye-fermenting suspensions without deoxygenation or any

electrode surface treatment. The method can be applied to determine the amount of indigo in used dye suspensions and *sukumo* extract by adding a chemical reduction pretreatment. This work also indicated the number of days required for the dye fermentation after the start of the fermentation, the effectiveness of the addition of old suspension as an additive for preparing a fresh fermenting suspension, and the importance of the addition of a nitrogen-based nutrient as well as a glucose-based one to recover the reducing activity of indigo.

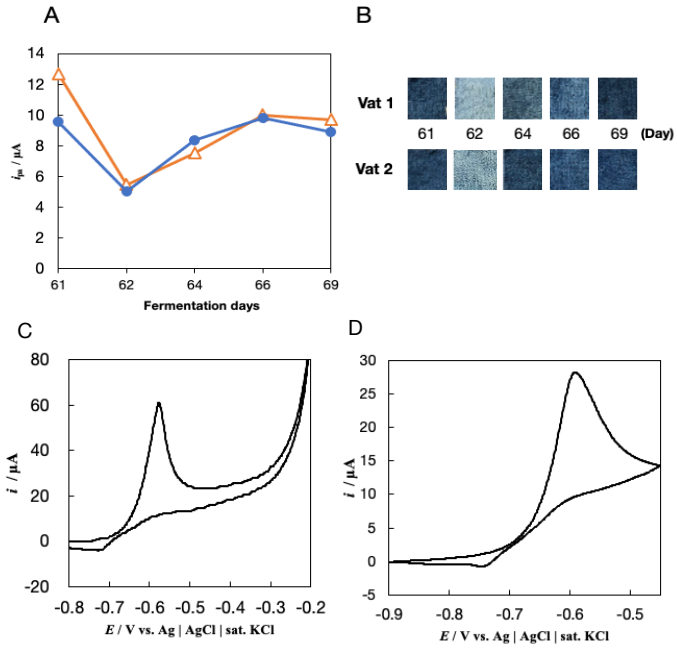

Fig. 7. Changes of the anodic peak current (i_{pa}) of leuco-indigo in dye-suspensions. (A) The anodic peak current (i_{pa}) of leuco-indigo was obtained by in-situ voltammetric measurements after removing the leuco-indigo present in the dye suspension at that time as much as possible by cloth dyeing on day 61. Closed circles, Vat 1; open triangles, Vat 2. (B) Pictures of the textile dyeing. Cyclic voltammograms of leuco-indigo artificially reduced with $Na_2S_2O_4$. (A) Cyclic voltammogram of an artificially reduced dye-fermenting suspension that was sufficiently used for cloth dyeing. (B) Cyclic voltammogram of the artificially reduced *sukumo* extract.

4. Conclusion

In order to in-situ monitoring of leuco-indigo, the working and counter electrodes were kept on the surface of the suspension with a PEVA tube holder. The anodic peak current was used as a measure of the leuco-indigo concentration, and this method is very useful for in-situ monitoring of leuco-indigo in dye-fermenting suspension without deoxygenation and any electrode surface treatments. The addition of

nitrogen-based nutrient as well as glucose-based nutrient was indicated to be of import to recover the indigo-reducing activity. The method can also be applied to evaluate the amount of indigo in the dye suspensions and the extracts of fermented indigo leaves (*sukumo*) by combining a chemical reduction pretreatment. These methods are expected to be useful for the management of indigo fermentation suspension in the future.

5. References

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Chapter 3

A Novel Assay Method for Indigo Reduction Activity and an Analysis of Microorganisms Involved in Indigo Reduction

Abstract

In natural indigo dyeing, the non-water-soluble indigo in *sukumo* is converted to the water-soluble leuco-indigo by activity of microorganisms. However, the problem was that its reduction required a lot of time. To clarify the conditions for efficient indigo reduction in dye fermentation suspension, we investigated the following three points: 1) The new indigo-reducing assay method was used not only for the measurement of the indigo-reducing activity, but also for the isolation of new indigo-reducing bacteria which were not isolated by the indigo carmine-reducing assay method, 2) anthraquinone was found to be effective as a mediator to facilitate the indigo-reducing activity, and 3) the ratio of the numbers between indigo-reducing bacteria and the others is important to maintain the indigo-reducing activity.

1. Introduction

Indigo dye was used as a pigment with a distinct blue color. Synthetic indigo is used for staining denim textures and natural indigo from the leaves of some plants of the Indigofera genus for staining traditional clothes in arts and crafts. Such products made with natural indigo are generally considered to be more comfortable and eco-friendly due to their deep blue. It has been also quantitatively reported that there is a subtle difference in color between natural indigo and synthetic indigo[1]. The traditional indigo dyeing in Japan has carried out in the indigo fermenting solutions using sukumo which is prepared by fermenting indigo leaves for approximately 100 days. Tokushima prefecture is the most famous in sukumo manufacture and natural indigo dying in Japan. The indigo fermentation solutions are prepared in an open environment, in which various microorganisms from sukumo, water, and air are contaminated. The dyeing power of the indigo fermentation solutions decreases due to continuous dyeing. It is difficult to recover the dyeing power in the indigo fermentation solutions once the dyeing power is weakened. It is important in the maintenance of the indigo fermentation solutions to analysis the symbiotic microbial flora in the indigo fermentation solutions. Such researches provides hints in long-term maintenance and recover of dying power.

In order to enhance the indigo-reducing activity, first, it is necessary to obtain

microorganisms that are highly capable of reducing indigo. As for indigo-reducing bacteria, three species belonging to the genus *Alkalibacterium* have been reported: *A. psychrotolerans*[2], *A. iburiense*[3], *and A. indicireducens*[4], and two species of the genus *Amphibacillus*, *A. indicireducens*[5] and *A. iburiensis*[6]. In addition, *Oceanobacillus indicireducens*[7], *Fermentibacillus polygoni*[8], *and Polygonibacillus indicireducens*[9] also reported as indigo-reducing bacteria. However, these bacteria were isolated by indigo carmine, a soluble blue dye similar to indigo. Park et al. tested the reducing activity of indigo in broth containing indigo, and isolated *Alkalibacterium* sp. and *Pseudomonas* sp., as indigo reducing bacteria.[10] According to medieval recipes for woad vats, madder was always to be added[11]. Second, substances that promote indigo reduction by bacteria might be involved. Madder is a dried powder obtained by grinding the roots of *Rubia tinctorum*, and which contains up to 23 anthraquinones[12]. Previous reports have shown that the addition of madder, anthraquinone-2,6-disulfonic acid (AQDA) or humic acid facilitated the reduction of indigo by indigo reducing bacteria *Clostridium isatidis*[13]. Finally, it is important to optimize the microbiota in the indigo dye vat. The indigo-reducing bacteria *Alkalibacterium*, *Amphibacillus*, and *Polygonibacillu* were reported to be derived from *sukumo* by Next-Generation Sequencing (NGS) analysis[14]. In the traditional dye fermentation process, the old dye suspension used for dyeing is often mixed with the fresh dye fermentation suspension, because adding the microbiota for indigo reduction. In addition, it is said that the indigo reducing activity of the indigo dye vats depends on the production area or the year of the *sukumo*. However, the differences of these microbiota are not clear. It also reported that the addition of heated wood ash extract ($pH \geq 10.5$, temperature ≥ 60 °C) drastically changed the environment, resulting in an increase in the proportion of *Alkalibacterium* and Bacilaceae in the indigo fermentation vat during initiate fermentation. Additionally, it was reported that the next change in microbiota was initiated by the consumption of oxygen by aerobic microorganisms up to day 5, followed by an increase in the obligate anaerobes *Anerobranca* and the aerotolerant *Amphibacillus* and a decrease in Bacillaceae[15]. Another study, the long-term maintenance and degradation of the dye through NGS analysis suggested that the degradation of the fermentation solution was associated with the relative abundance of Alkaligenaceae. On the other hand, although our previous report showed that it was important to add not only glucose-based nutrients but also nitrogen-based nutrients to recover the reducing activity of indigo[16], we could not analysis of microbiota in this condition.

The purpose of the present study was to further activate the reduction of indigo in the natural indigo dye vat. In this study, we proposed a new method for measuring indigo reduction activity using indigo. As a result, we isolated new indigo reducing bacteria from

indigo plants, *sukumo*, and indigo fermentation vat. It was also found that anthraquinone was effective as a mediator to promote indigo dyeing activity. By NGS analysis, it was shown that there was no significant difference between the microbial population of *sukumo* produced by different producers and the indigo dye suspension. Although, the addition of nutrient sources decreased the number of indigo-reducing bacteria, the staining intensity increased. We conclude that the ratio of the numbers between indigo-reducing bacteria and the others is important to maintain the indigo-reducing activity.

2. Experimental

2.1 Isolation and cultivation of bacteria

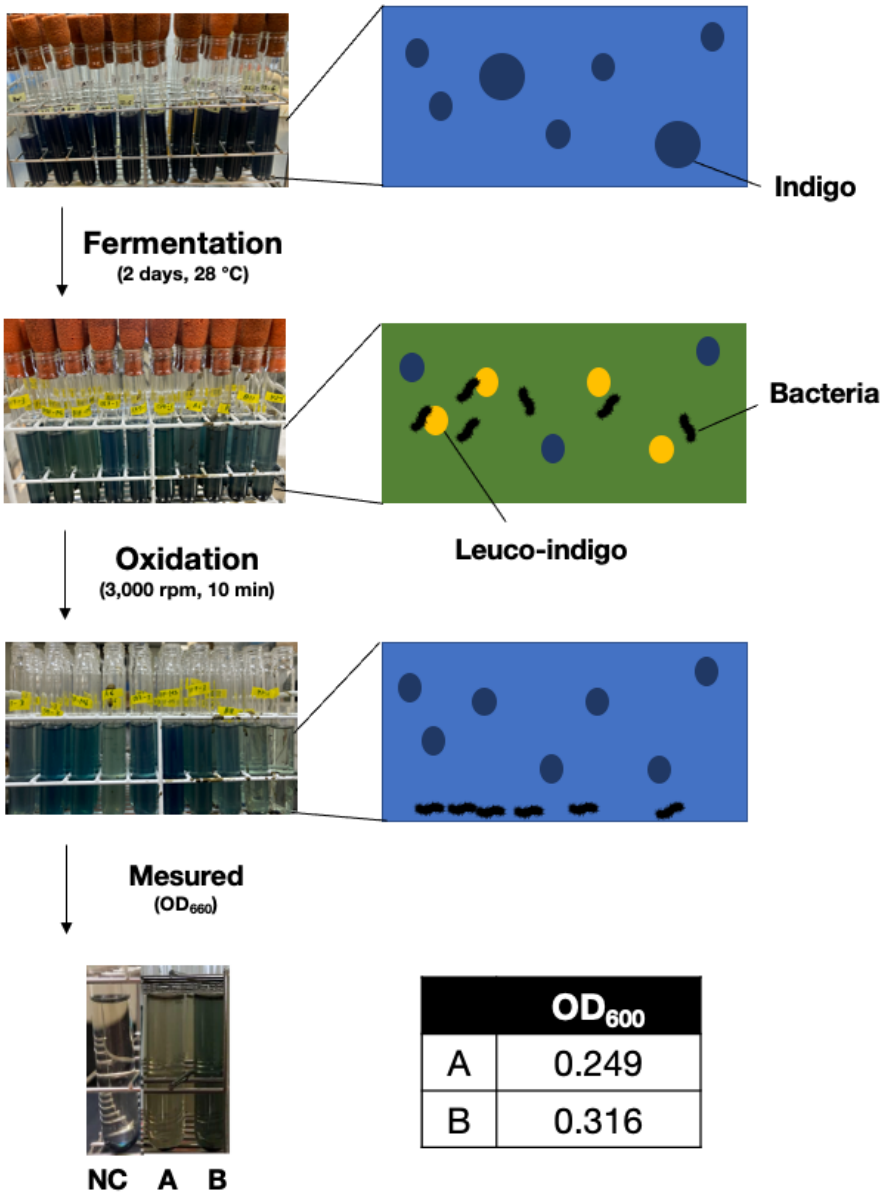
The isolation of bacteria was followed by the protocols described in the previous paper[17]. Strain 127-3 and 159-3 were isolated from an indigo plant and sukumo produced at the Japanese company of Nii Seiaisyo in Tokushima prefecture, respectively. The other bacteria was isolated indigo-dye vats managed at Nii Seiaisyo and Shikoku University in Japan. The bacteria were cultivated on 1.1% lactobacilli MRS (BD Difco, USA) agar medium with 1 % (w/v) Na2CO3 (called alkali-MRS) at 28 °C under anaerobic conditions using Aneropack Kenki (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The strain K2-3′ isolated was reported in the previous paper[17].

2.2 Evaluation of indigo-reducing activity

The bacteria were precultivated in 4 mL of alkali-MRS at 28 °C for 24 h with shaking at 300 rpm or with no shaking under anaerobic conditions. In order to evaluate of indigo-reducing assay, 100 µl of the precultured suspension was inoculated to 10 ml of indigo-containing medium (0.2 % (w/v) glucose, 0.25 % (w/v) yeast extract, 0.5 % (w/v) hipolypepton, 0.03 % (w/v) indigo, and 1 % (w/v) Na_2CO_3), followed by incubation at 28 °C for 48 h under static condition. Anthraquinone (0.2 mM) (Kishida Chemical Co., Ltd., Japan), flavin mononucleotide sodium salt (0.2 mM) (FMN, Fujifilm Wako Pure Chemical Corporation, Japan), and humic Acid (0.015 %) (Fujifilm Wako Pure Chemical Corporation, Japan) were used as mediators in the indigo-reducing assay. After the reaction, the reaction mixture was centrifuged at 3,000 rpm for 10 min (Fig. 1). The supernatant containing leuco-indigo was exposed to the air and those leuco-indigo were converted to indigo by oxidation. The optical density at 660 nm (OD_{660}) of the supernatant that turned blue was measured. The indigo-reducing activity was assessed with the value of OD_{660}. In order to evaluate the reduction of indigo carmine, the strains

isolated were inoculated on the indigo-carmine medium containing 0.2 % (w/v) glucose, 0.25 % (w/v) yeast extract, 0.5 %(w/v) hipolypepton, 0.03 % (w/v) indigo carmine (Tokyo Chemical Industry Co., Ltd., Japan), and 1 % (w/v) Na_2CO_3) and cultivated at 28 °C for 24 h. regarded as a measure of the indigo-reducing activity.

A



	OD_{600}
A	0.249
B	0.316

Fig.1 New indigo reduction assay method; indigo reduction medium (upper), 2 days after seeding (middle), after centrifuged (down). Each illustration shows a situation of medium. The absorbance in suspension of test tubes shown in the table.

2.3 16S rDNA sequence analysis for phylogenetic analysis.

The 16S rDNA gene was amplified by PCR using the following universal primers: 10F (5'-GTTTGATCCTGGCTCA-3') and 800R (5'-TACCGGGTATCTAATCC-3'). The PCR product was sequenced using ABI PRISM 3730XL Analyzer with a BigDye™ Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA). The assignment of determined sequence was performed by alignments and database identification of consensus sequences using BLAST. Similarities between the sequences were calculated using the MEGA X software program[18]. A phylogenetic tree was constructed with the neighbor-joining method using the CLUSTAL W program[19].

2.4 Indigo reducing activity by electrochemistry

Strain B3F-M5 and S5-5 were inoculated into 4 mL of alkali-MRS and incubated at 28 °C for 24 h with shaking at 300 rpm. After the cultivation, 3 ml of the culture medium was transferred to indigo reducing assay medium (300 ml) and cultured at 28 °C for 48 h at static culture condition. Two ml of the supernatant was transferred to an electrochemical cell. To maintain anaerobic conditions, it was continuously purged with N_2 gas. Cyclic voltammetry was carried out at a scan rate of 10 mV s^{-1} under quiescent conditions.

2.5 DNA extraction and PCR for NGS analysis NGS

The indigo fermentation solutions (45 L) of Vat A and Vat B were prepared with the *sukumo* derived from facilities related to traditional crafts (*Wazanoyakata* in Tokushima prefecture of Japan) and made at *Nii Seiaisyo* in 2018, respectively. The indigo fermentation solutions (45 L) of Vat 1, and Vat 2 were prepared with sukumo made at *Nii Seiaisyo* in 2019. The preparation and maintenance of the indigo fermentation solutions were described in our previous paper[16,17]. The day on the preparation started was defined as day 0.

The indigo solution (500 μl) from each Vat was centrifuged at 15,000×g for 10 min to obtain a cell pellet sample and DNA was directly extracted from the sample pellet using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The extracted DNA was submitted to Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The V4 region of the bacterial 16S rRNA genes was PCR-amplified using a composite pair of primers. The forward primer was 1st-515f_MIX (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-GTGCCAGCMGCCGCGGTAA-3′), and the reverse primer was 1st-806r_MIX (5′-

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GGACTACHVGGGTWTCTAAT -3′) with an adaptor (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). In order to improve the quality during sequence analysis, mixed primers with different lengths of 0-5 bases of random sequences were used. A 1st PCR was performed in a 10-μl solution containing 1 μl of 10 × Ex buffer, 0.8 μl of dNTPs (each 2.5 mM), 0.5 U of Ex Taq HS (TaKaRa) 25 ng of isolated DNA, and 0.5 μl of 10 μM each primer, 2 μl of Template DNA (Max 0.5 ng/μl). The amplification reactions were performed as follows: initial thermal denaturation at 94 °C for 2 min, followed by 30 cycles of heat denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s, final extension at 72 °C for 5 min. The PCR products were purified with AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions. A 2nd PCR was performed with an index-adapted primer to generate paired-end (2 × 300 base pair) libraries for Illumina shotgun sequencing, and the products were purified. To attach the index sequence to the products of the first PCR, the second PCR products were processed with the second primer set, 2nd F (5′- AATGATACGGCGACCACCGAGATCTACAC-Index2 (8 bp)-ACACTCTTTCCCTACACGACGC-3′) and the reverse primer was 2nd R (5′-CAAGCAGAAGACGGCATACGAGAT-Index1 (8 bp)-GTGACTGGAGTTCAGACGTGTG-3′) (Table 1). The second PCR denaturation at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s, final extension at 72 °C for 5 min, and purification (AMPure XP).

Table 1 Index barcodes for the second PCR with Illumina MiSeq.

	Index1	Index2
Vat A-Day 0	CATAGCCG	CAGGACGT
Vat B-Day 0	CATAGCCG	GTACTGAC
Vat 1-Day 0	CATAGCCG	TAATCTTA
Vat 2-Day 0	CATAGCCG	TATAGCCT
Vat 2-Day 4	CATAGCCG	ATAGAGGC
Vat 2-Day 8	CATAGCCG	CCTATCCT
Vat 2-Day 20	CATAGCCG	GGCTCTGA
Vat 2-Day 28	CATAGCCG	AGGCGAAG

2.6 NGS analysis

NGS was carried out on the Illumina MiSeq platform (Illumina, San Diego, USA). Raw data obtained from the Illumina MiSeq sequencing platform were defined as raw tags after removing barcode sequences and PCR primer sequences using the fastq_barcode_splitter from the Fastx Toolkit software (ver. 0.0.14). Clustering analysis based on operational taxonomic units (OTUs) with 97% identity and taxonomic classification annotated by each representative sequence were performed using Qiime2 (ver.2020.6) [5]. Primers, barcodes, 50 bp at 3' end and chimeras were removed using DADA2. Taxonomy was assigned based on Greengenes (version 13_8) at 97% OUT, trained using a Naïve Bayes classifier. Principal component analysis, calculation of cos2 values and PCA graphics was done using R software (ver. 3.6.2), factoextra (ver. 1.0.7) and ggplot 2 (ver. 3.3.2).

3. Results and discussion

3.1 Isolation of alkaliphiles bacteria

Isolated bacteria were evaluated by our indigo reduction assay. Fig. 2A (black) showed indigo reduction activity of isolation bacteria. In our assay, indigo-reducing bacteria were defined as those with an absorbance values (OD_{660}) higher than 0.2. Among these 12 strains, strain B11 and strain S5-5 did not have an indigo-reducing ability. In addition, these results were compared to the results using indigo carmine. The results for strain S5-5 were consistent with our indigo assay, but not for strains B11 and B3F-M5. We previously determined the leuco-indigo concentration from the anodic peak current in the dye-fermenting suspension using cyclic voltammetry (CV). Therefore, the anodic peak current of B3F-M5 and S5-5 strains were measured using CV (Fig. 3A, B). The average current value of the anodic peak for strain B3F-M5 was 2.3 μA, which corresponds to about 0.6 mM leuco-indigo based on the tentative conversion factor set in previous work. On the other hand, the anodic peak for strain S5-5 was not found. This result verified that our indigo reduction assay shows the indigo reduction activity by microorganisms. One of the reasons why the results did not agree with indigo carmine is that the structures of indigo and indigo carmine are different (Fig. 2B). The side chains of indigo carmine may affect the microbial reduction. Besides, indigo carmine is an indicator at pH 11.5-14, changing from blue to yellow, therefore the indigo carmine assay may just be looking at the pH change caused by the bacteria, not the reduction of indigo. Our indigo-reduction assay will help to isolate indigo reduction bacteria that could not be isolated by indigo carmine plate. Additionally, this assay also contributes to a determination of the strength of indigo reducing activity.

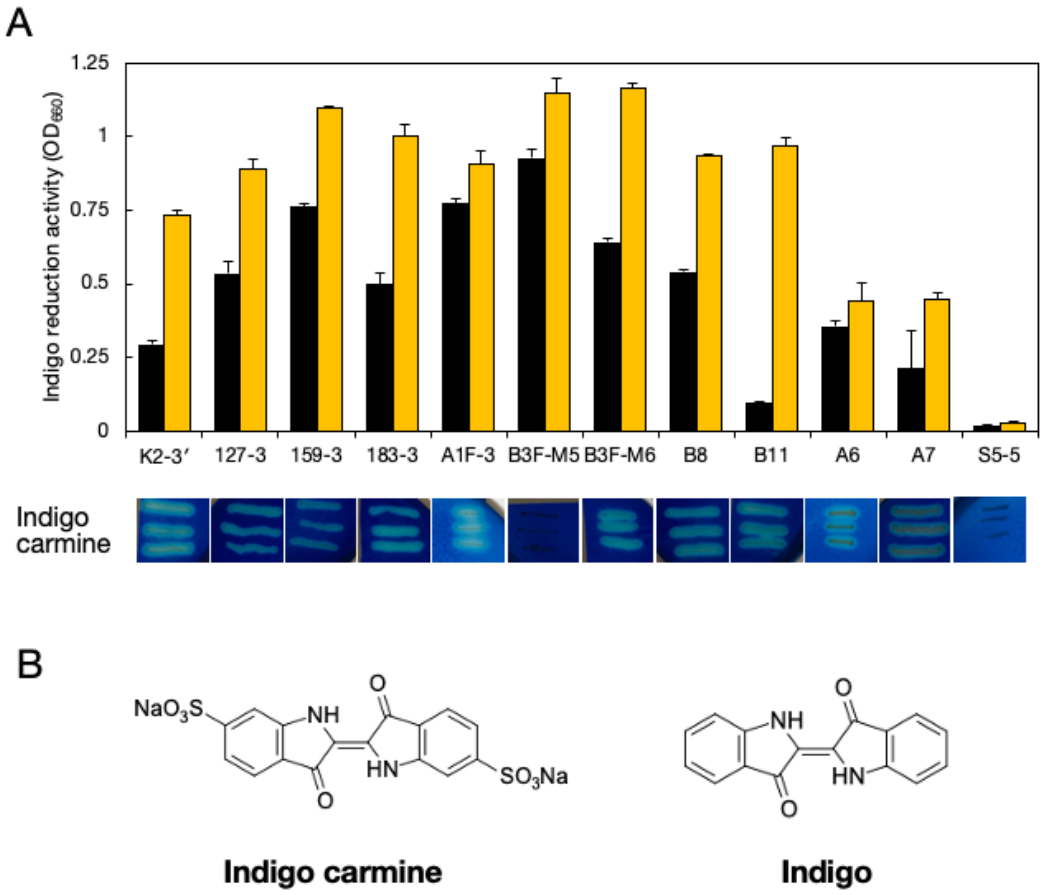


Fig.2 (A) Indigo reduction activity (OD_{660}) of indigo bacteria. Black, Control (no mediator); Yellow, Anthraquinone. Indigo carmine reduction assay of each bacterium showed under the graph. (B) the structure of indigo carmine (left) and indigo (right).

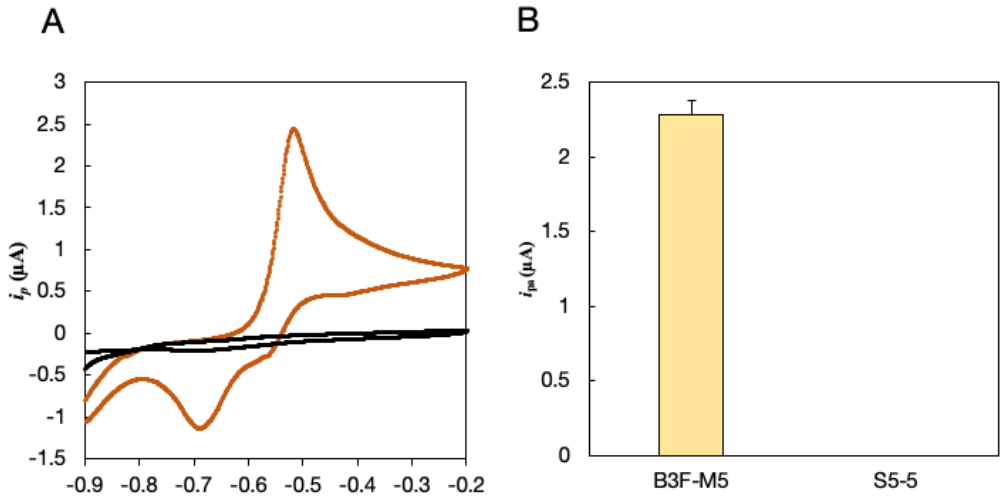


Fig.3 (A) In-situ cyclic voltammograms of a dye-fermenting suspension (7 day after the start of the fermentation) at v = 10 mV s^{-1} before (black) and just after (gray) mixing of the suspension. (B) Average of peak current in B3F-M5 strain and S5-5 strain (n=3).

3.2 Effect of mediator on bacterial indigo reduction

To examine the effect of mediator on bacterial indigo reduction, anthraquinone (AQ), humic acid, or FMN were used. Figure 4 shows the indigo reduction activity of strains 127-3, 159-3, or 183-3 when the mediator was added to the indigo reduction assay medium. All strains showed the highest indigo-reducing activity when AQ was added. Although the anthraquinone-2,6-disulfonic acid (AQDA), a water-soluble anthraquinone, was added, it had no effect on the indigo reducing activity (data not shown). In non-microbial studies, it has been reported that anthraquinone exerts a strong catalytic effect by causing a molecular "wedge effect" during the co-intercalation of Na^+ and anthraquinone into the layered indigo crystal structure[20]. The temperature in that study was 65°C, while this study was 28°C. Although there is a difference in temperature, it was considered that the anthraquinone makes the indigo more accessible to microorganism. With FMN, the indigo-reducing activity was lower than that of the control. In iron reduction studies by *Clostridium acetobutylicum*, in medium with electron mediator, hydrous ferric oxide (HFO) reduction increased to 70%–90% (with resazurin, resorufin, AQDS, or riboflavin). However, no increase in HFO reduction occurred with FMN and flavin adenine dinucleotide (FAD). The authors proposed that FMN-based metabolism in microorganisms is different in the presence and absence of HFO[21]. Therefore, we hypothesized that in the absence of indigo, FMN plays a role as a means to deliver electrons to the extracellular environment, while in the presence of indigo, FMN plays a different role in microbial metabolism.

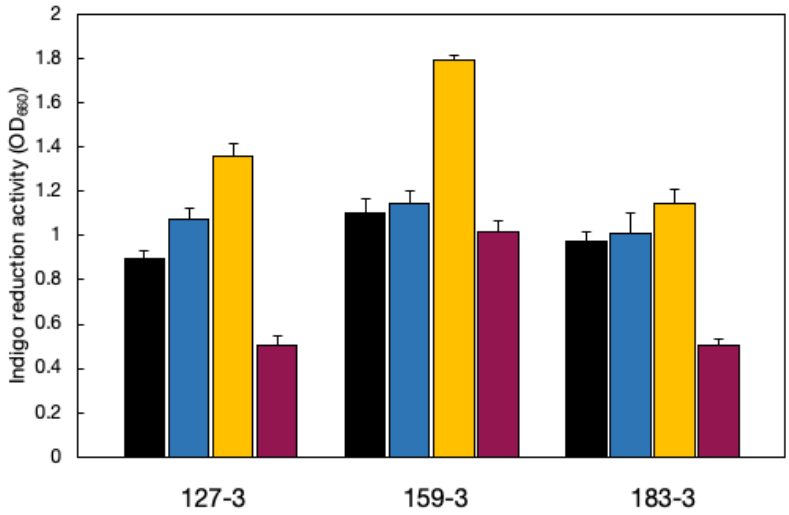


Fig.4 Indigo reduction activity (OD_{660}) of indigo bacteria under the different mediator. Black, Control (no mediator); Blue, FMN; Yellow, Anthraquinone; Red, humic acid.

Fig. 2A (yellow) showed indigo reduction activity of isolation bacteria in the presence of AQ. Indigo-reducing activity was increased by AQ in all strains. In particular, the indigo-reducing activity of strain B11 was markedly increased by the addition of AQ. This result suggests that AQ affected not only on indigo but also on the extracellular electron transfer of the strain B11. Moreover, the addition of AQ to the lab-scale indigo dye vat increased the indigo reducing activity in a preliminary study. The use of AQ might be helpful to improve indigo-reducing activity in traditional Japanese indigo dyeing, in the future.

3.3 Identification of isolation strains

Based on the sequence analysis of 16S rRNA of isolated strain, strain A1F-M3 was *Alkalibacterium* sp. with similarity 99.86 % and strain B3F-M6 was *Alkalibacterium pelagium* with similarity 100 %. To clarify the phylogenetic po sitions of the isolated strains, a phylogenetic tree was constructed that included related species using representative strains located in each clade and indigo reduction bacteria that have already been reported (Fig. 5). It was indicated that A1F-M3 and B3F-M6 were close relatives of *Alkalibacterium indicireducens.* Indigo-reducing bacteria belonging to the genus *Alkalibacterium* have been reported *A. psychrotolerans*, *A. iburiense*, and *A. indicireducens* (Fig. 5, violet)[2-4], isolation strains in this study were consistent with previous studies. Strain A6 belonged to *Exiguobacterium* sp. with similarity 100 %, strain A7 belonged to *Bacillus* sp. with similarity 100 %. Tokiwa et al., reported *Exiguobacterium* as a halophilic bacterium carried by wind and flying salt[22]. Although strain A6 were closely related to *Bacillus cohnii*, an indigo reducing bacteria, and strain A7 were closely related to *Bacillus wakoensis* AO1[23], it has indigo reductase, these were not completely consistent with previous reports. Because the majority of the *Bacillus* genus has been known to form spores in harsh environments, these two strains showed low indigo reducing activity.

The most of the isolates belonged to the genus *Enterococcus*, and strain 127-3 was *Enterococcus gallinarum* or *Enterococcus casseliflavus* with similarity 99.64 %, strains 159-3, 183-3 and B11 were *Enterococcus gallinarum* or *Enterococcus casseliflavus* with similarity 100 %, respectively. The genus *Enterococcus* has never been reported as an indigo reducing bacteria. However, *Enterococcus faecalis* strain ATCC

19433 grew in the presence of azo dyes, which are characterized by one or more R_1–NN–R_2 bonds, and metabolized them to colorless products, and characterization of FMN-dependent azoreductase[24].

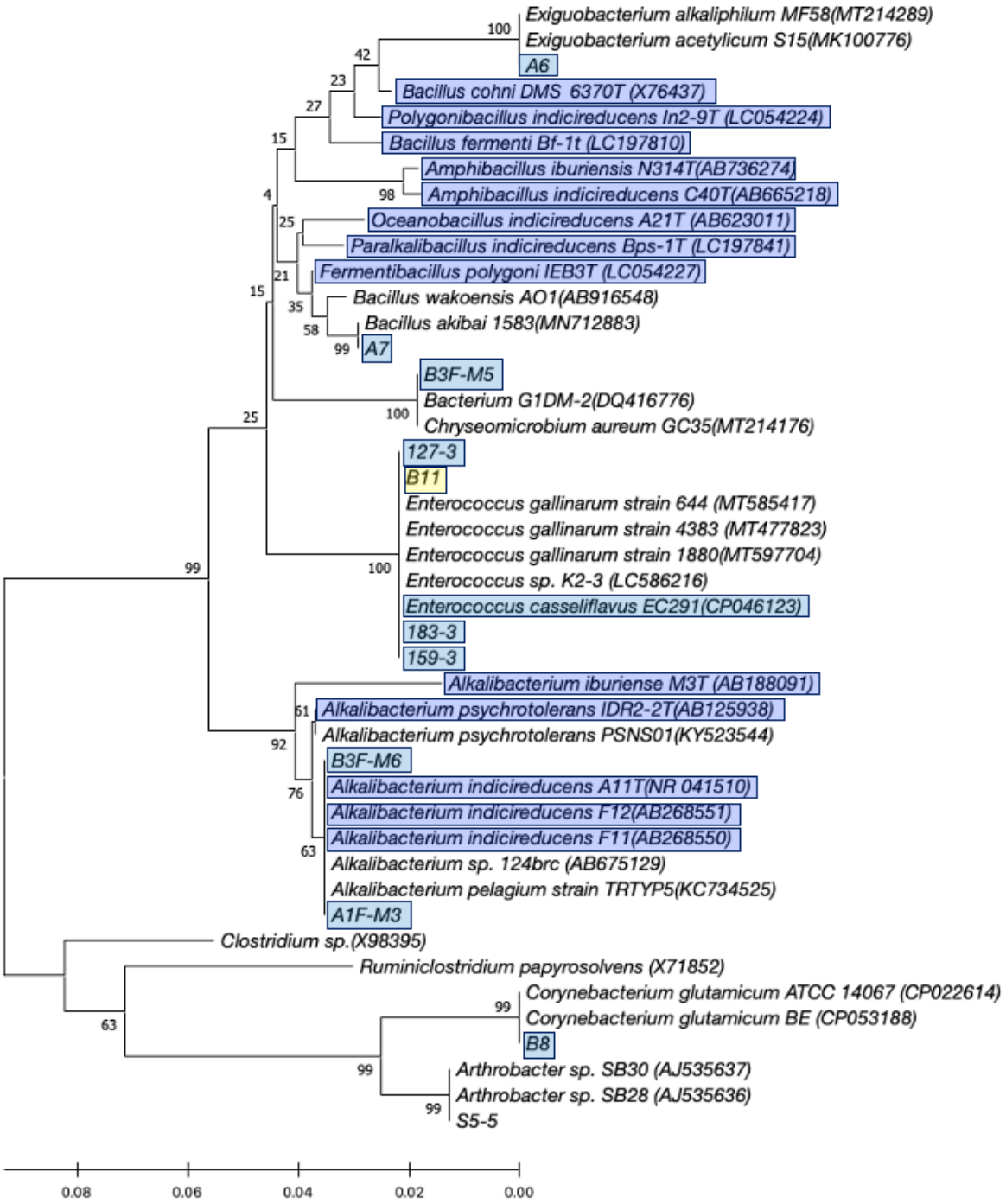


Fig.5 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.44606802 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 44 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

Therefore, Enterococcus sp. may have azoreductase for indigo reduction. These indigo reducing bacteria could have been isolated due to the use of less than the recommended volume of MRS medium for isolating lactic acid bacteria[25]. In addition, strain B8 was *Corynebacterium glutamicum* with similarity 99.27 %, and it had been reported that *Corynebacterium* sp. was obtained from fermentation liquor[26]. However, in that paper, *Corynebacterium* sp. was not reported as an indigo reducing bacteria, and this study is the first report of it. The strain B3F-M5 with the highest indigo reducing activity was *Chryseomicrobium* sp. with similarity 99%, and the strain S5-5 that did not indicate the indigo reduction activity was *Arthrobacter* sp. with similarity 99.45 %.

3.4 Comparative analysis of microbiota on Day 0

To clarify the difference in the microbiota on Day 0, each vat samples were analyzed by NGS (Fig. 6). In Vat A, *Oceanobacillus* [49] (7.1 %) and *Paenibacillaceae* [52] (7.0%) were observed, the same genus as indigo-reducing bacteria. These bacteria showed in all vats, however Vat A was the highest percentage among all Vats-Day0. *Methanoculleus* [56] (0.006%) and *Methanomassiliicoccus* [55] (0.019%) belonging to Archaea, were only showed in Vat B. In the previous study, Archaea was detected in indigo fermentation vat, the relative levels of Archaea were consistently less than 0.1%, our results support previous reports[15]. The role of Archaea in the indigo fermentation vat is unknown and will be analyzed in the future. Comparison of Vat 1 and Vat 2 showed that Vat 1 was found *Alkalibacterium* [42] (1.3%), which was not found in Vat 2. In addition, *Arthrobacter* [22], non-indigo reducing bacteria, was detected higher relative levels in Vat1. The same *sukumo* were used in Vat 1 and Vat 2, however Vat 1 was included 5 L of the old dye suspension. Therefore, these bacteria observed in Vat 1. It was found that fresh dye suspensions with old dye fermentation suspensions were effective in increasing indigo-dyeing bacteria such as *Alkalibacterium* [42], but at the same time increased non-indigo-dyeing bacteria such as *Arthrobacter* [22].

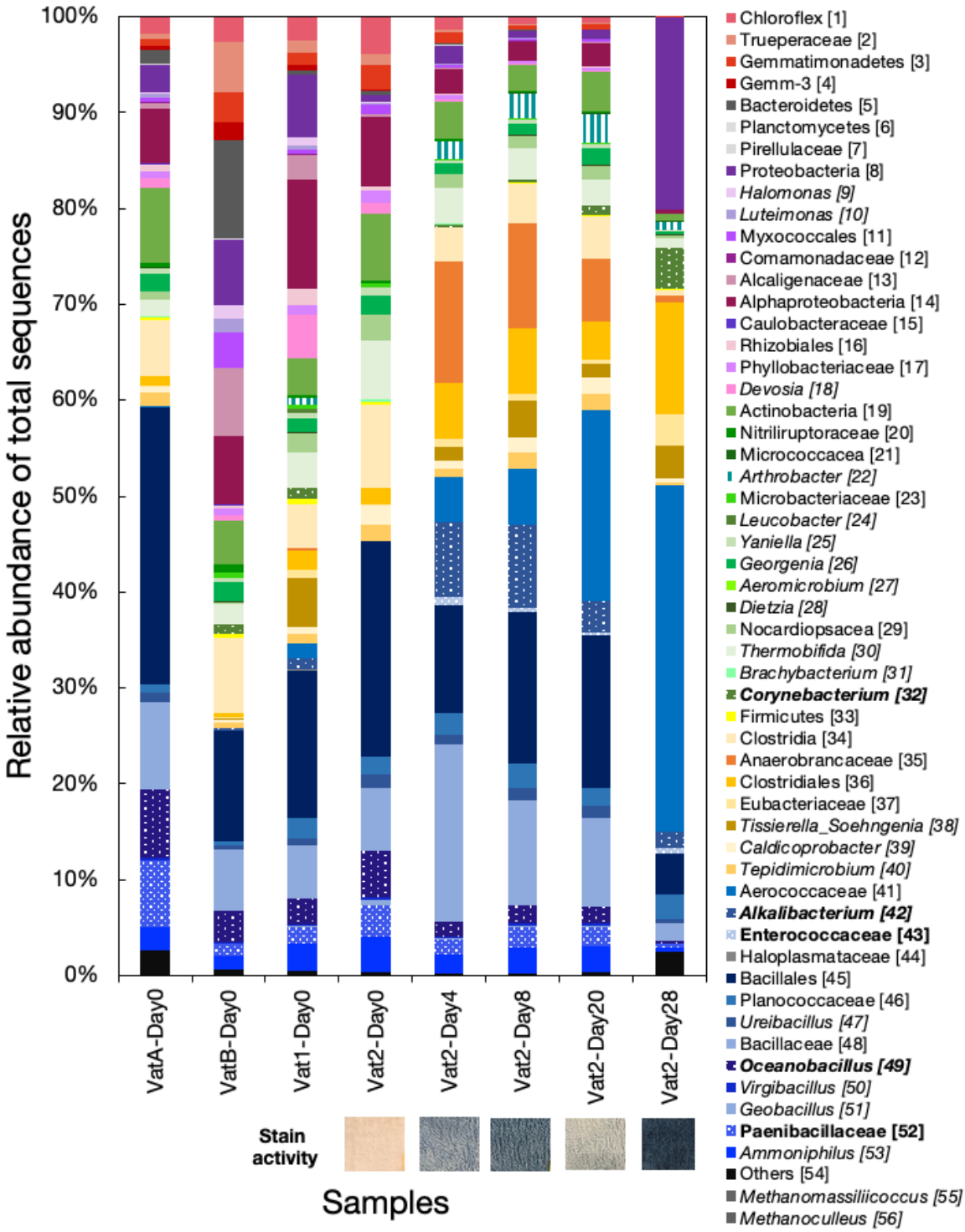


Fig.6 Relative abundances of total reads in indigo fermentation vat. Vat A, Vat B, Vat 1, Vat 2 on day 0 samples, and day 4, day 8, day 20, day 28 in Vat 2 samples, and stain activity (dipping pieces of 100 %-cotton towel three times into the dye-fermenting suspension).

Principal Coordinate Analysis (PCA) was performed to observe the differences of these samples (Fig. 7). Individual projections on the first dimension (Dim1) also suggested Vat B have different characteristics from the others. This result might be attributed to the presence of Archaea in Vat B. By contrast, Vat A showed different position on the second dimension (Dim 2). This result is suggested to be due to the characteristic that it used a different *sukumo* from the others, and which the relative level of Bacilli class [41-53] accounted for 56.79%. Finally, Vat 1 and Vat 2 showed the same direction. The PCA revealed the characteristics of each indigo fermentation vat. Interestingly, the color of the towels dyed in Vat1 and Vat2 became blue two days earlier than that of Vat A. Thus, it was suggested that not only the presence of indigo-reducing bacteria in the *sukumo* but also biomass of the bacteria and their balance with other bacteria were important.

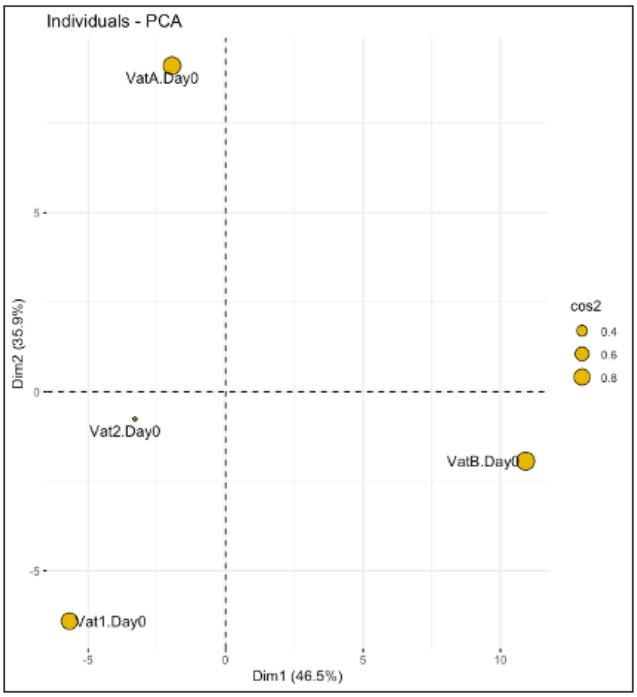


Fig.7 PCA plot for the bacterial community in the indigo fermentation on day 0.

3.5 Changes in microbiota caused by added nutrients

In order to understand the changes in microbiota affected by the added nutrients, the fermentation suspension collected from Vat 2 was examined using NGS (Fig. 6). As a nutrient, Vat 2 was added with 100 ml of Japanese *sake* on day 0, and 20 g of wheat bran on Day 2, 200 mL of *sake* on days 12, and glucose (20 g, 0.05 %(w/v)), yeast extract (25 g, 0.0625 %(w/v)), and hipolypeptone (50 g, 0.125 %(w/v)) on day 20. The staining activity was increased until day8. The relative proportions of the indigo-reducing bacteria

oceanobacillus sp. [49] and penibacillaceae [52] on day 4 were decreased than day 0, but were kept from day 4 to day 20. The reason for changes of staining activity was that indigo-reducing population includes *Alkalibacterium* [42], *Corynebacterium* [32], and Enterococcaceae [43], which were 0 % on day 0, increased to 7.8 %, 0.3 %, 0.8 %, respectively, on day 4. *Alkalibacterium* [42] has been reported increased during initial fermentation in indigo fermentation suspension, and *A.psychrotolerans* and *A. iburiense* produce acid (e.g., lactate, formate and acetate) from a number of carbohydrates[27]. Therefore, it is inferred that role of *Alkalibacterium* sp.in indigo fermentation vat is to produce acid from *sake* and wheat bran. In agreement with previous report, Anaerobrancaceae [35] was 0 % on day 0, however it was suddenly increased on day 4 (12.7 %)[14]. Another study reported that the presence of *Anaerobranca* decreased as the indigo fermentation vat moved toward deterioration[15]. Thus, it is predicted that Anaerobrancaceae contributes to the maintenance of reducing conditions in indigo fermentation vat.

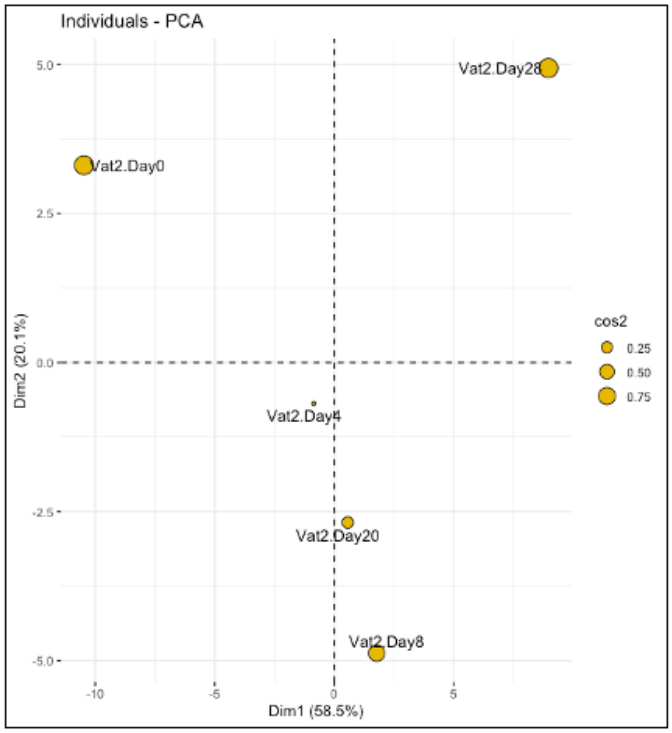


Fig.8 PCA plot for the bacterial community in vat 2 on day 0, 4, 8, 20, 28.

Although, the PCA results showed same direction on day 4, 8 and 20, i.e., similar microbiota, staining activity was decreased on day 20 (Fig. 6, 8). On day 12, *sake* was

added, but the staining activity did not recover. One reason for this result could be that the bacterial activity decreased because the pH became higher than 12 during the period from day 5 to day 20. It was reported that the growth of *A. psychrotolerans* and *A. iburiense* was equally good at all pH values in the range 9-12, with optimum growth at pH 9.5-10.5, and *A. indicireducens* optimum growth was at pH 9.5–11.5[27]. For this reason, it is suggested that although the indigo-reducing bacteria existed in the indigo dye suspension, the indigo-reducing activity was reduced, resulting in a decrease in dyeing activity. In our previous study, we proposed that the addition of a nitrogen-based nutrient as well as a glucose-based one is important to recover the indigo-reducing activity. On day 20, the pH was 11.36, but it was 10.27 on day 21 after the nutrient source. This is suggested to be due to the increase of acidic substances in the indigo dye suspension by microbial metabolism. Comparing the microbiota at day 20 and 28, there was a significant changed at day 28, and dyeing activity was recovered (Fig. 6, 8). The addition of *sake* and wheat bran caused a gradual change for microbiota, whereas the addition of carbon and nitrogen sources affected the growth of some bacteria. In particular, Proteobacteria [8], Clostridiales [36] and Aerococcaceae [41] showed a dramatic increase of relative ratio 20 %, 11.7 %, and 36.1 %, respectively. There were several reasons for the recovery of dyeing activity. The genus *Pseudomonas* is included in the phylum Proteobacteria [8], its relative ratio on day 20 was 0.1%, which increased to 2.46% on day 28. In addition, it was reported as indigo reducing bacteria, and *P. putida* MET94 has azoreductase[28]. Moreover, the indigo-reducing *Clostridium isatidis* is included in order Clostridiales [36][29-31], *Corynebacterium* sp. [32] was also increased on day 28 than day 20. On the other hand, *Arthrobacter* (non-indigo reducing bacteria) [22] was decreased. These results suggest that the increase of indigo reducing bacteria and reduce of non-indigo bacteria contributes to the staining activity. Aerococcaceae [41] is facultatively anaerobic organism, a family of lactic acid bacteria found in fish and fermented foods, and reported to have increased during alkaline fermentation[32]. However, the role of this bacterium during indigo fermentation has not been clarified. It is desirable to isolated of Aerococcuceae, and clarify the indigo reducing activity of its in future studies. Recently, it was reported that microorganisms have the ability to transfer electrons onto insoluble electron acceptors in high pH, it called extracellular electron transfer (EET)[33,34]. The isolation method using EET may be useful to obtain bacteria that could not be isolated in this study.

4. Conclusion

To clarify the conditions for efficient indigo reduction in dye fermentation suspension, we proposed the new indigo reducing assay method. It was not only for the measurement of the indigo-reducing activity, but also for the isolation of new indigo-reducing bacteria which were not isolated by the indigo carmine-reducing assay method. Next, we tested some mediators that facilitate indigo reduction, and anthraquinone was found to be effective as a mediator to facilitate the indigo-reducing activity. Finally, we analyzed microbiota of the indigo fermentation suspension by NGS analysis. It was demonstrated that there was no significant difference among the microbiota of *sukumo* produced by different producers and the indigo dye suspension. Although the number of indigo-reducing bacteria decreased with the addition of nutrient sources, the staining intensity increased. These results indicate that the ratio of the numbers between indigo-reducing bacteria and the others is important to maintain the indigo-reducing activity.

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Conclusions

In this work, the author succeeded that clarified the detailed indigo reduction mechanism by electrochemistry. We also proposed a method to measure the amount of leuco-indigo in the indigo dye vat. In addition, we have isolated new indigo-reducing bacteria and analyzed their microbiota to activate the production of leuco-indigo in the dye solution.

In chapter 1, the author succeeded that leuco-indigo was directly detected by CV in the supernatant of indigo dye suspensions. In addition, the voltammetric experiments in sediment-rich (i.e., microbe-rich) suspensions of indigo fermented dye vat showed steady catalytic oxidation and reduction waves. In a model bioelectrocatalytic system using indigo-reducing bacteria strain K2-3′ and MV^{2+} as electrocatalysts and a mediator, respectively, suggested that acetaldehyde can donates electrons to MV^{2+}, accepts electrons from $MV^{\bullet+}$, and this reaction was catalyzed by redox ebzymes of strain K2-3′.

In the traditional indigo fermentation vat, indigo in *sukumo* is reduced by acetaldehyde with an (NAD-independent) acetaldehyde dehydrogenase to generate leuco-indigo and acetate predominantly. Acetaldehyde can be supplied by alcohol fermentation. Therefore, the sour aroma of acetic acid and the addition of *sake* in the practical fermenting process are very reasonable. Leuco-indigo is auto-oxidized by O_2 into indigo especially on the surface of the fermenting dye suspension. In addition, part of leuco-indigo may be re-oxidized into indigo by acetaldehyde as the reverse-reaction of alcohol dehydrogenase. In order to weaken the electron accepting ability (and also to strengthen the electron donating ability) of acetaldehyde, it is very important to keep the strongly alkaline conditions during the dye fermentation. This situation is strictly practiced in the practical fermenting process.

In chapter 2, we proposed the in-situ monitoring system of indigo concentration in indigo dye vat. To in-situ monitoring of leuco-indigo, the author kept the working and counter electrodes on the surface of the suspension by a PEVA tube holder. The anodic peak current was used as a measure of the leuco-indigo concentration, and this method is very useful for in-situ monitoring of leuco-indigo in dye-fermenting suspension without deoxygenation and any electrode surface treatments. Next, we indicate that the addition of nitrogen-based nutrient as well as glucose-based nutrient was important to recover the indigo-reducing activity. Moreover, we showed that the method can also be applied to evaluate the amount of indigo in the dye suspensions and the extracts of fermented indigo leaves (*sukumo*) by combining a chemical reduction pretreatment. These methods are expected to be useful for the management of indigo fermentation suspension in the future.

In chapter 3, we proposed the new indigo reducing assay method using indigo. It was not only for the measurement of the indigo-reducing activity, but also for the isolation of new indigo-reducing bacteria which were not isolated by the indigo carmine-reducing assay method. Next, we tested some mediators that facilitate indigo reduction, and anthraquinone was found to be effective as a mediator to facilitate the indigo-reducing activity. Finally, we analyzed microbiota of the indigo fermentation suspension by NGS analysis. It was demonstrated that there was no significant difference among the microbiota of sukumo produced by different producers and the indigo dye suspension. Although the number of indigo-reducing bacteria decreased with the addition of nutrient sources, the staining intensity increased. These results indicate that the ratio of the numbers between indigo-reducing bacteria and the others is important to maintain the indigo-reducing activity.

Acknowledgments

The author wishes to express his sincere and heartfelt gratitude to Dr. Eiji Sakuradani, Professor of Tokushima University for providing him with his continuous guidance, valuable discussion, and encouragement throughout this study, and the author is very grateful to Dr. Kenji Kano, Professor of Kyoto University for his continuous support and advice concerning to the research of electrochemistry analysis. The author would like to thank Dr. Michiki Takeuchi, Assistant Professor of Kyoto University, for his kind supports and a number of critical comments.

The author is very grateful to Dr. Yoshitoshi Nakamura, Professor of Tokushima University, Dr. Yoshihiro Uto, Professor of Tokushima University, Dr. Chikako Asada, Associate Professor of Tokushima University, Dr. Mikito Yasuzawa, Professor of Tokushima University, Dr. Masashi Kurashina, Assistant Professor of Tokushima University,Dr. Jun Ogawa, Professor of Kyoto University, Dr. Akinori Ando, Assistant Professor of Kyoto University, Dr. Hiroko Watanabe, Assistant Professor of Kyoto University, for their critical comments and kind supports.

The author thanks Associate Professor Noriko Ariuchi of Shikoku University, Mr. Hitoshi Yoshihara and Mr. Kouji Murai of Tokushima Prefectural Industrial Technology Center, Mr. Osamu Nii of *Nii Seiaisho*, Mr. Itaro Nagao, and Mr. Ransyu Yano for teaching us the preparation procedure for indigo dye fermentation.

The author gratefully thanks to the members of laboratory, Dr. Takaiku Sakamoto, Assistant Professor of Tokushima University, Ms. Ayae Matsuka, Ms. Manami Tada, Mr. Yusuke Ikeda, Mr. Yuji Nakamura, Mr. Kazuhiro Nakamura, Mr. Takahisa Yamashita, Ms. Mayu Kikuchi, Ms. Yuka Sakaguchi, Mr. Takuya Watari, Ms. Aya Higashihara, Ms. Yui Hattori, Ms. Nanoha Nishibayashi, Momoka Matsunaga, for their kind support in the author's laboratory life.

The author thanks friends, Dr. Hirotatsu Imai, research fellow (PD) from the Japan Society for the Promotion of Science, Dr. Yudai Highchi, Assistant Professor of Hirosaki University for their kind advices and encouragement.

Finally, the author would like to express her greatest thanks to her husband Mr. Ryosuke Nishimura, for his helpful discussion, encouragement and kind supports, and her family for their continuous encouragement.

Kasumi Nakagawa
Tokushima
March 2021