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**Analysis of the changes in microbiota associated with indigo reduction
in natural indigo fermentation**

(藍染め発酵液のインジゴ還元に関連する微生物叢の変遷の解析)

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応用生物学専攻 博士後期課程

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General introduction

Indigo has been used as a dye for thousands of years by humans around the world. Enormous quantities of indigo dye are produced for use in blue denim, conferring a characteristic discoloration and faded appearance in jeans. Due to its insolubility in water, before dyeing, indigo needs to be reduced to its water-soluble form leuco-indigo. The reduced form can be easily absorbed by the fabric. When exposed to oxygen in the air, the reduced leuco-indigo will be re-oxidized back to the insoluble form and will remain within the fabric, with a blue color. Although the bacterial indigo reduction process is environmentally friendly, it takes 4–14 days for microorganisms to start producing leuco-indigo. The fermentation process is difficult to maintain because the dyeing process causes instability for the microbiota involved in the fermentation. Further, the judgment for the correspondence for changing situation was difficult.

Traditional indigo can be obtained from several plant species and is reduced by natural fermentation or natural reducing agents in an alkaline fluid. With the development of modern industry and the popularity of indigo, chemical materials derived from petroleum have been used for synthesis. Synthetic indigo is reduced with chemical reducing agents, such as sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), in industrial dyeing. Low-cost synthetic indigo and efficient chemical reductants have quickly occupied the market of plant-derived indigo. However, during the chemical synthesis of indigo, environmentally hazardous chemicals, such as formaldehyde, hydrogen cyanide, sodamide, and strong bases, are produced. Furthermore, because the reductants are easily oxidized, excessive quantities are needed to maintain a strong reducing ability. These chemical reductants cannot be recycled from wastewater. To reduce the cost of wastewater treatment, many small factories directly discharge the wastewater into nature. The toxic and corrosive oxidation byproducts (sulfate and sulfite) cause various environmental and human

health problems.

Owing to an increasing awareness of the environment and human health, demand for safe and recyclable plant-derived indigo is gradually increasing. The market for natural indigo has gradually recovered in recent years, although synthetic indigo is still predominant. At the same time, the safe and environmentally friendly traditional fermentation reduction method, which has long been used in Europe and Asia, has been rethought and repositioned. To address the easy oxidation of leuco-indigo by exposure to the air and to ensure continuous reduction, microorganisms provide an alternative to chemical reducing agents; they only need small substrate quantities and are able to proliferate and renew continuously. However, during the dyeing process, clothes or fibers need to be immersed in fermentation fluid, and outside microbes are inevitably introduced. It is crucial to maintain stable reduction, even after contamination by external microbes. The preparation and maintenance of traditional indigo fermentation fluid require the rich experience of craftsmen, which is directly related to the quality of the reduction and dyeing conditions. This uncertainty is a key factor limiting the extended use of traditional methods.

To fully utilize the advantages of traditional natural fermentation and improve its efficiency and stability, it is particularly important to study the composition and dynamics of bacterial communities in naturally fermented fluids. Japanese traditional indigo, called *sukumo*, is composted leaves of knotweed (*Polygonum tinctorium*) and is reduced by natural fermentation. After adding hot ash extract, the *sukumo* fluid can be reduced in about a week, and the reduced state can be maintained from 6 months to nearly a year under the management of experienced crafts persons, although the reduced indigo fluid is occasionally used for dyeing under open-air conditions. However, the reduced indigo fluid always gets deteriorated eventually.

For the clarification of the mechanisms for the indigo reduction in the early stage of the

fermentation, the stability of indigo reduction state during in the stable period and the final deterioration, the analysis of successive change of the microbiota is a promising procedure. Therefore, the relationship between the fermentation process and the changes in the bacterial community was analyzed using the next-generation sequencing (NGS), including Chapter 1) the evaluation of the initial stage of fermentation concomitant with indigo reduction, Chapter 2) comparison of the long- and short-term natural indigo fermentation for clarification of the stability of the indigo-reducing state, and Chapter 3) finding of common trends in transitional change among three different batches.

Chapter 1 describes the relationship between the initiation of indigo reduction at the early fermentation period and changes in the microbiota. The origin of indigo-reducing bacteria in the indigo fermentation fluid is also discussed. The uncertainty and slow initiation of the reduction of indigo are crucial problems in the natural fermentation procedure. Therefore, the results of this analysis will contribute the development of improved methods for indigo fermentation. Chapter 2 describes differences in microbial changes among fermentation fluids for long and short periods of indigo reduction. Despite the high risk of contamination during the process, the fluid exhibits a tremendous ability to maintain the indigo-reducing state. This study provides important insight into the long-term maintenance of the indigo-reducing state. Finally, Chapter 3 describes analyses of the microbiota during fermentation using three batches with different maintenance procedures. Similarities in microbial changes during indigo fermentation as well as differences due to differences in management are revealed. This study provides a more comprehensive understanding of the changes in the microorganisms throughout the process, including factors related to the initiation of indigo reduction, succession of indigo-reducing bacteria, and the maintenance and eventual deterioration of the reducing state.

General overview

1.1 History of indigo

The earliest known use of indigo worldwide constitutes 6000-year-old cotton fabrics from the Preceramic site of Huaca Prieta on the north coast of Peru, which retain traces of a blue pigment that was analyzed and positively identified as an indigoid dye [92]. Evidence also exists of indigo being used in mummy cloths in ancient Egypt from 2500 BC [5, 25]. In China, natural indigo has been used for thousands of years as well. For example, the famous metaphor for learning: indigo blue is extracted from the indigo plant but is bluer than the original plant, is contained within the Xunzi Quanyue, an ancient Chinese collection of philosophical writings attributed to Xun Kuang (313-238 BC). In Japan, it is speculated that the indigo plant was introduced from China through the Silk Road route. Although the timing remains unclear, records of indigo plant cultivation remain from the Asuka era (AD 592-710). From the Edo era (AD 1603-1868), the production of Japanese indigo (*Polygonum tinctorium* Lour.) has flourished in various parts of the country, with that especially from Awa Province (currently a part of Tokushima Prefecture) being known as exhibiting good quality, leading to produced indigo also being called *awa-ai*. However, from the beginning of the 19th century, the production of Japanese indigo declined because of the inflow of natural indigo from India and chemical indigo from Europe.

Globally, the current consumption of indigo dye is enormous owing to the popularity of blue jeans, with that of indigo and other vat dyes reaching approximately 33 million kg annually. Compared to natural indigo, numerous kinds of synthetic dyes exist with better fastness properties, particularly against light, washing, and chlorine bleaching; however, the particular fading of color that is so characteristic of indigo has underlain its extreme popularity with the jeans-wearing public.

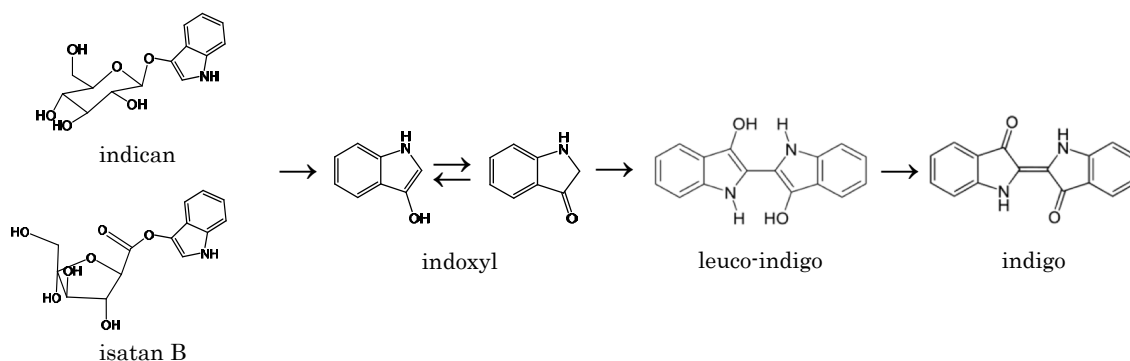
1.2 Methods of obtaining indigo

1.2.1 Transformation of indigo from plant-based precursors

Since ancient times, indigo has been derived from various indigo-containing plants including woad (*Isatis tinctoria*), a biennial species whose habitat is found in the steppe areas of south-eastern Europe, Asia, and in parts of North Africa. Another woad species, *Isatis indigotica*, is cultivated as an herb known as *banlangen* and *daqingye* in China. Knotweed (*Polygonum tinctorium*), an annual species whose habitat can be found predominantly in China, Japan, and eastern Russia, has also been used for thousands of years as a source of blue dye. Currently, the knotweed plant is commonly exploited for the extracted dye obtained by precipitation from water extract in China and from fermented leaves (*sukumo*) in Japan. *Strobilanthes cusia*, a perennial plant also termed *Ryukyu-ai* with habitats in India, southeastern Asia, south China, and Okinawa, produces a precipitate indigo dye that is still used in Okinawa, Japan. However, although this represented an important dye in ancient China, this species is rarely used as a dye but rather as an herb in modern-day China, known as *south-banlan*. Finally, *Indigofera tinctoria*, termed *indo-ai* in Japan, can be found in subtropical and tropical regions along with *Indigofera suffruticosa*, which are subjected to widespread cultivation owing to their high indigo content.

Notably, indigo itself does not exist in the leaves of indigo-producing plants. Instead, its precursor indoxyl- β -D-glucoside (indican) is present in *P. tinctorium* and *Indigofera* species [80], with isatan B in addition to indican being found in *Isatis* species [5]. The precursors are broken down to indoxyl and sugar moieties by enzymes in the plant or by alkali with aeration in the extraction method. For example, a β -glucosidase has been purified and characterized from *P. tinctorium* that preferentially catalyzes the hydrolysis of indican [62]. Free indoxyl has been suggested to form indigo through an indoxyl radical, which first forms leuco-indigo that is then oxidized to indigo [85]. As indigo is not soluble in water or other commonly used solvents, it

must be solubilized before it can be used for dyeing. In contrast, leuco-indigo, the reduced form of indigo, is soluble in water and thus can be used in the dyeing process.



1.2.1.1 European method

In Europe, a composting procedure using woad (*I. tinctoria*) was developed in the Middle Ages. Harvested woad leaves are cut into very small pieces and gathered into a ball that can fit in the palm of a hand. These prepared balls are fermented for a short period under appropriate moisture conditions and then allowed to dry for several weeks to produce dried woad balls, which can be stored. However, before they can be used in dyeing, the balls need to be couched. This entails crushing the balls into powder, which is then wetted and allowed to ferment for several more weeks. After couching, the woad is transformed into a dark clay-like material, which is dried and packed tightly prior to use [47].

1.2.1.2 Japanese method

In Japan, a method for composting indigo-containing plants has been developed in Tokushima Prefecture. The leaves of knotweed plants (*P. tinctorium*) are harvested and air-dried, then approximately three tons of dried leaves are mixed with a roughly equivalent weight of water and piled to approximately 1 m height. Microbial oxidative fermentation of the leaves is promoted

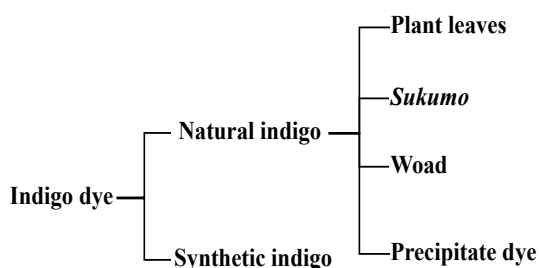
under aerobic conditions and at an appropriate temperature by controlling the water content and adjusting the turnover frequency by a trained craftsman [1]. The temperature reaches 60 °C because of microbial activity. This microbial oxidation step is carried out for up to 100 days, with the resultant brown composted product termed as *sukumo*. Due to time-consuming and labor-intensive factors, this natural indigo *sukumo*, as well as European woad, has become less and less produced.

1.2.1.3 Chinese method

In some parts of China, such as in Yunnan and Hainan Provinces, indigo is extracting from several species of indigo-yielding plants, such as *Strobilanthes cusia*, *Wrightia laevis*, *In. tinctoria*, *In. suffruticosa*, and *Persicaria tinctoria* [52, 106]. Fresh leaves and stems are cut and then soaked in tap water and held in an underwater environment for 2-3 days in a large vat. The vat contents are agitated up and down once daily with a wooden rake to ensure that all the leaves are soaked and fermented. In this process, the precursors of indigo are broken down to indoxyl and sugar moieties by enzymes in the leaves. After the fermentation, the plant residues are removed from the vat. Lime powder is then added to the liquid followed by vigorous stirring to induce leuco-indigo formation by indoxyl, which is then oxidized to insoluble indigo. The suspension with indigo is placed in a large plastic vat from several hours through one or two nights to allow precipitation. Once the indigo paste has precipitated to the bottom, the supernatant is carefully removed. The paste is then collected and its state maintained through the regular addition of water. This method of directly extracting indigo is currently the most popular method around the world for obtaining natural indigo because of the short time requirement, high indigo dye yield, and convenient storage and transportation.

1.2.2 Chemical synthesis

The structure of indigo was first suggested by von Bayer in 1869 and the first commercially successful synthesis of indigo was based on the process published by Heumann in 1890 [16, 95]. Accordingly, in 1897 the German chemical company BASF began production, which rendered indigo suddenly affordable [88]. This synthetic process converted phenylglycine-*o*-carboxylic acid by fusion with sodium hydroxide into indigo via indoxyl-2-carboxylic acid [15]. With the improvement of chemical process and efficiency, the chemically synthesized indigo makes this dye widely available worldwide. However, artificial indigo is industrially synthesized from aniline (a toxic compound derived from benzene, which is in turn derived from petroleum) via a process that involves hazardous chemicals such as formaldehyde and strong bases.



1.2.3 Biotechnological process

Fermentation has also been explored as a promising approach for indigo production. For example, the use of hydrocarbon-degrading bacteria expressing mono-oxygenases or dioxygenases was investigated as an alternative for the chemical synthesis of indigo [8, 70]. Recombinant *Escherichia coli* have been also used for indigo production. In particular, Berry et al. developed a fermentation process with *E. coli* that had been modified with *Pseudomonas putida* genes encoding naphthalene dioxygenase. However, the method also produced indirubin, which provided an undesirable red hue [7]. In turn, Hsu et al. identified the gene coding for the

glucosyltransferase PtUGT1 from the knotweed plant (*P. tinctorium*). The expression of PtUGT1 in *E. coli* supported high indican conversion by stabilizing the reactive precursor (indoxyl → indicant), with the indican being subsequently hydrolyzed to indoxyl by β -glucosidase, which spontaneously oxidizes to form indigo via a leuco-indigo intermediate [40]. Nevertheless, although various biotechnological processes have been identified as being capable of producing indigo, no large-scale industrial biotechnological process has yet been developed [19].

1.3 Reduction of indigo

As indigo is insoluble in water, it needs to be reduced to its water-soluble form, leuco-indigo, prior to its use in dyeing. The reduced form is absorbed into the fabric and remains therein when re-oxidized back to insoluble form by exposure to air. Several different methods have been invented for indigo reduction and dyeing, all initiating with the fermentation vat, which was used for centuries prior to the advent of modern technology.

1.3.1 Natural fermentation

1.3.1.1 European method

In Europe, couched woad was processed in a woad vat, in which material to be dyed was immersed. Hot water was added to the couched woad and the vat was maintained at 50 °C. During the ensuing fermentation, the pH was maintained as alkaline through the periodic addition of potash or lime. In the woad vat, the insoluble form of indigo was converted by reduction to the soluble form, leuco-indigo [77]. Within the whole preparation and fermentation process using the woad vat, the fermentation microorganisms mainly derive from the woad balls and couching process. Moreover, the addition of hot water to the couched woad and the maintenance of high (50 °C) temperature serve to screen for heat-resistant microorganism populations.

1.3.1.2 Japanese method

In Japan, traditional *sukumo* reduction is implemented by fermentation with extreme alkalinity (pH 10.3-11.0) [72]. *Sukumo* is mixed with hot wood ash extract (60-80 °C), the fluid is stirred once or twice daily, and slaked lime is added to maintain the extreme alkalinity. Wheat bran or wheat gluten is then added to the fluid as the substrate for the indigo-reducing microbes when the dyeing efficiency decreases. During this process, indigo is solubilized via bacterial indigo-reducing activity. Generally, the applicable dyeing efficiency can be maintained for approximately six months on average by skilled artisans [72]. Notably, although constituting distinct plants originating from distant areas, a large number of microorganisms are similarly accumulated after a long period of aerobic fermentation during the production processes (couching and composting) of both European woad and Japanese *sukumo*, followed by screening of the microbial populations at a higher temperature when preparing the successive liquid fermentation.

1.3.1.3 Chinese method

In Yunnan Province, China, the indigo paste is added to plant water (defined below), together with alkaline ash extract, maize (*Zea mays* L.) wine, and hot maize or chilies (*Capsicum annuum*). The plant water, which consists of the boiling water extract of leaves and stems of *Buddleja officinalis*, *Tithonia diversifolia*, *Peristrophe bivalvis*, and *Persicaria hydropiper*, is considered as a source of nutrients. To obtain darker reddish textiles, the boiling water extract of leaves and stems of *Iresine herbstii* along with its ash extract is added to the dye vat. Fermentation is allowed to continue for about a week, until the color of the dye liquid turns yellow-green. The dye liquid is then stirred using a wooden stick to reveal bright bubbles, and the dye liquor should taste both

slightly sweet and spicy. When all of the above conditions are satisfied, the liquid is considered to be suitable for dyeing traditional clothing [52]. However, the microbiota of this fermentation-based reduction has not been fully studied.

1.3.2 Chemical reductants

Since the late 19th century, natural indigo has been replaced over a relatively short period of time by synthetic dyes and, in parallel, the use of chemical reductants such as sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), also known as sodium hydrosulfite, which has been exploited as a major reducing agent in the industrial reduction of vat dyes including indigo owing to its chemical and economic properties. The reductant causes a swift reduction of indigo along with other vat dyes. However, it cannot be recycled from the wastewaters and used again in the reduction process [6]; moreover, it is unstable and very easily oxidized by atmospheric oxygen [11]. Consequently, large amounts of dithionite and NaOH are needed beyond the stoichiometric requirements of the reduction process [18]. Furthermore, the oxidation byproducts cause various problems with regard to the disposal of wastewaters. The generation of sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), and thiosulfate ions ($\text{S}_2\text{O}_3^{2-}$) exerts a harmful effect on the environment owing to their toxicity in addition to their corrosive effects. Sodium dithionite also negatively impacts the aerobic processes during water treatment and toxic hydrogen sulfide (H_2S) can form anaerobically from the sulfate deposits present in the wastewaters [10, 48].

1.3.3 Other indigo-reducing methods

To eliminate or minimize the production of harmful byproducts from chemical reducing agents during indigo production, alternatives such as organic reducing agents [14], borohydride [60], and electrochemical [84] along with biological reduction of indigo [75] have been proposed.

Moreover, pre-reduced indigo has been introduced to the dye-houses, in which the leuco-indigo is produced by reduction via a catalytic hydrogenation process [87].

1.4 Previous studies related to bio-reduction

Ancient humans had developed a number of environmental-friendly and renewable bioprocesses to reduce indigo by natural fermentation. Using modern technology to explore the scientific principles of traditional craftsmanship is expected to facilitate the development of environmentally friendly indigo-reducing procedures. In addition, these techniques can be modified for the development of highly sophisticated procedures [1]. As described in section 1.2.3, a sustainable method has been developed for producing indigo via recombinant *E. coli*, in which a glucose moiety is utilized as a biochemical protecting group to stabilize the reactive indigo precursor indoxyl to form indicant, preventing spontaneous oxidation to indigo. Notably, no reducing agent is required when dyeing with indicant in this procedure [40]. This strategy was designed to eliminate the reducing agent for dye solubilization, but the cost of this technology is too high at this stage.

1.4.1 An early study on indigo reducing bacteria

In order to identify the indigo-reducing bacteria in indigo dyeing fermentation, numerous indigo-reducing bacterial species have been isolated from various types of indigo fermentation fluid. The first indigo-reducing bacterium was discovered in 1960 by Takahara and Tanabe [94]. Based on phenotypic characterization, they identified that the isolate belonged to the genus *Bacillus*. Because of its novel characteristics, the isolate was considered as a new species, which was named *Bacillus alkaliphiles*. This species is a motile, Gram-negative facultative anaerobe with a strong ability to reduce the redox potential of indigo fermentation fluid. The optimum pH

for its growth is 10-11.5. This species grows at temperatures between 10 and 50 °C, with an optimum temperature of 30 °C. However, as the strain was not deposited in a culture collection, we cannot directly compare *B. alkaliphiles* with recently isolated indigo-reducing bacteria.

1.4.2 European method

In 1998, the indigo-reducing bacterium *C. isatidis* was isolated from a couched woad vat prepared using a traditional medieval European procedure [77, 78]. The growth pH range of this bacterium is 5.9-9.9, with an optimum pH of 7.2 (at 50 °C), which is much lower than those of the indigo-reducing isolates from Japanese fermentation procedures (Section 1.4.3). In addition, three additional strains considered to have a role in removing oxygen from the fermentation fluid were identified. Two strains, *Bacillus pallidus* and *Ureibacillus thermosphaericus*, which are related to the coaching process, and *Bacillus thermoamylovorans* likely consume oxygen through respiration [13]. Moreover, evaluation of the indigo reduction mechanism using *C. isatidis* concluded that *C. isatidis* can reduce indigo because it both produces an extracellular factor that decreases indigo particle size, and generates a sufficient reducing potential [67].

1.4.3 Japanese method

In Japan, indigo-containing media have been used to isolate and indicate indigo-reducing bacteria. In particular, two species in genus *Alkalibacterium* were isolated from polygonum indigo fluid obtained from an indigo craft center in Date City, Hokkaido, named *Alkalibacterium psychrotolerans* [104] and *Alkalibacterium iburiense* [65]. In addition, another new indigo-reducing species named *Alkalibacterium indicireducens* [105] was isolated from a fermented *sukumo* originated from a polygonum indigo sample obtained from Tokushima Prefecture. Furthermore, to screen indigo-reducing bacteria, a fermented polygonum indigo liquor aged for

4 days in our laboratory was used, leading to the isolation of an indigo-reducing species belonging to genus *Oceanobacillus*. This bacterium was then named *Oceanobacillus indicireducens* [32]. Almost concurrently, two other indigo-reducing bacteria were isolated from a 10-month-old indigo fluid from Date City, Hokkaido, named *Amphibacillus indicireducens* and *Amphibacillus iburiensis* [33, 34]. Subsequently, on the basis of phenotypic and chemotaxonomic characteristics along with phylogenetic data, three isolates were identified as representing novel species of novel genera, including *Fermentibacillus polygoni*, *Polygonibacillus indicireducens* [35, 38], and *Paralkalibacillus indicireducens* [36]. Moreover, from a fermented *sukumo* liquor also obtained from an indigo craft center in Date City, Hokkaido, the novel indigo-reducing species *Bacillus fermenti* was identified [37]. Table 1 shows the indigo-reducing bacteria isolated from Japanese indigo fermentation fluids. Indigo reducing *Alkalibacterium* spp. and *Amphibacillus* spp. are aerotolerant anaerobes, which are able to hydrolyze cellulose and xylan. In contrast, the other strains comprise facultative anaerobes, which are not able to hydrolyze cellulose and xylan, except *Polygonobacillus polygoni* In2-9^T. In addition, although the spore production rate is very low in *Amphibacillus* spp., except for *Alkalibacterium* spp., the remaining eight species can form spores, which is beneficial for their survival under extreme conditions.

Notably, Nishita et al. found that obligate anaerobes that do not metabolize oxygen are difficult to isolate using conventional media owing to their slow growth, whereas media containing *sukumo* hydrolysate facilitated the isolation of novel species of indigo-reducing bacteria [69]. Accordingly, media were developed to accelerate the isolation of indigo-reducing bacteria that are difficult to isolate using conventional media, leading to the isolation of bacterial strains such as *Poly. indicireducens*, *Para. indicireducens*, and *B. fermenti* as described above. It was also clarified that *Bacillus cohnii*, which had already been reported, constituted an indigo-reducing bacterium. Furthermore, a strain belonging to the genus *Amphibacillus*, considered as a

new species, was also isolated using wheat bran extract-containing medium.

1.5 Indigo-reducing enzymes

An indigo-carmin-reducing enzyme has been characterized from an indigo-carmin-reducing *B. cohnii* strain isolated from an indigo fermentation fluid and identified based on its 16S rRNA gene sequence [68]. The indigo-carmin-reducing enzyme was purified from disrupted cells of the isolate and its enzymatic activity was determined using NADH and indigo carmine as the electron donor and acceptor, respectively. The enzyme is stable at the pH range of 3.5-9.5, with an optimum pH for activity of 7.5. The optimum temperature for activity is 30 °C. The reported enzyme is considered to be a kind of azoreductase [68], which have been reported in *Bacillus* spp. [74, 81]. Such azoreductases are thought to react with soluble substrates such as indigo carmine although it has not been ascertained whether these azoreductases are able to react with the water-insoluble indigo substrate. In addition, an indigo reductase was identified from the alkaliphilic *Bacillus* sp. AO1 [93]. The enzyme efficiently reduced indigo carmine in an NADH-dependent manner and exhibited strict specificity for electron acceptors. The enzyme oxidized NADH in the presence, but not the absence, of indigo. Moreover, the reaction rate was enhanced by adding organic solvents to solubilize indigo. Absorption spectrum analysis showed that indigo absorption decreased during the reaction. Together these observations suggested that the enzyme could reduce indigo *in vitro* and potentially in *Bacillus* sp. AO1. This is the first study to identify an indigo reductase, providing new insight into a traditional approach for indigo dyeing [93]. However, this bacterium does not represent a predominant member of the indigo-reducing bacterial group present in indigo fermentation fluid.

1.6 Bacterial community in fermented indigo fluids

The initiation of indigo reduction is expected to be associated with transitional changes in the microbiota in indigo fermentation vats under anaerobic alkaline conditions [1]. In order to understand the changes in the composition of the microbiota upon initiation of indigo reduction, in 2011, the bacterial community associated with indigo fermentation was investigated using PCR-DGGE and a 16S rRNA gene clone library [2]. The change in the bacterial community ascertained by DGGE analysis revealed that that dissimilarity value from the 1st to the 2nd day was 66.9%, whereas that from the 3rd to 4th day was smaller but also increased to 28.2%. From the 3rd day (not reduced) to the 4th day (well-reduced), it was found that the majority of *Halomonas* spp. (from 54% to 8%), which are considered to be aerobic and linked to indigo reduction, was substituted by *Amphibacillus* spp. (from not detected to 19%), even though to date only three species of *Alkalibacterium* had been newly found as indigo-reducing bacteria from Japanese *sukumo* fluids. In addition, strains belonging to genera *Amphibacillus* and *Oceanobacillus* were considered to play important roles in sustaining the indigo-reducing state. As demonstrated in subsequent studies, the genera *Amphibacillus* and *Oceanobacillus* were also confirmed to contain indigo-reducing bacteria, with the bacteria belonging to *Amphibacillus* exhibiting especially strong reducing ability. Analysis of microbiota in this study suggested that indigo-reducing bacteria constitute a majority in the bacterial community during indigo-reducing-state fermentation. Moreover, *Bacillus* spp. (14% at the 3rd day) were shown to be present at the start of fermentation, although their function was not clarified. In addition, the drop of redox potential was not linked to the initiation of indigo reduction as it was stated that no obvious indigo reduction occurred during the 2nd day of fermentation, although the redox potential was -640 mV.

For the fermentation of Japanese *sukumo*, the indigo-reducing state is maintained for six months on average or longer although the indigo reduction process is performed via natural

fermentation and occasionally used for dyeing under open-air conditions. In order to understand the mechanism underlying long-term indigo fermentation and develop a low-cost and long-term indigo-reducing bioprocess, the microbiota in one early-phase batch and two aged batches of Japanese indigo fermentation fluid were examined [72]. It was found that the microbiota in the aged fermentation fluid consisted mainly of the genera *Alkalibacterium*, *Amphibacillus*, *Anaerobacillus*, and *Polygonibacillus*, and the family Proteinivoraceae. This indicated that indigo-reducing bacteria constitute the majority in the bacterial community and fluctuate at different rates, with the evolving bacterial community consisting of various indigo-reducing species that replace the previous group of indigo-reducing bacteria. The transition speed in the aged fermentation fluid was slower than that in the early-stage fluid. Nevertheless, the microbiota in the aged fermentation fluid maintained for more than six months was drastically changed within three months [72]. This indicated that despite being stabilized a few months after the initiation of indigo reduction, microbiota can drastically get altered at any time.

The microbiota of woad vat fermentation liquor aged 12 months have also been examined, using a combination of culture-dependent and -independent PCR-DGGE analyses and next-generation sequencing of 16S rRNA amplicons [61]. It was found that eumycetes and coliforms were present at < 1 log CFU/mL, whereas total mesophilic aerobes and spore-forming bacteria and their spores were detected at > 5 log CFU/mL. Both facultative and obligate anaerobes existed in the woad vat and were involved in the formation and reduction of indigo in addition to its degradation. The authors inferred that indigo-reducing bacteria constituted only a small fraction of the microbial community in the natural woad dye vats. Nonetheless, it was considered that unknown reducing bacteria remain to be identified, or only promote the reduction reaction by consuming oxygen or assisting electron transfer within the unknown functional community.

Despite recent advances in research on bacterial community in fermented indigo fluids, there

are many unresolved issues, including 1) the mechanisms underlying the initiation of indigo-reduction concomitant with transitions in the microbiota; 2) the factors determining the stability of the indigo-reducing state, and 3) the mechanisms underlying the deterioration of the indigo-reducing state.

Table 1 Characteristics of indigo-reducing bacteria isolated from Japanese indigo fermentation fluid.

	<i>Alkalibacterium psychrotolerans</i>	<i>Alkalibacterium iburense</i>	<i>Alkalibacterium indicireducens</i>	<i>Amphibacillus indicireducens</i>	<i>Amphibacillus iburense</i>	<i>Oceanobacillus indicireducens</i>	<i>Fermentibacillus polygani</i>	<i>Polygonibacillus indicireducens</i>	<i>Parakalibacillus indicireducens</i>	<i>Bacillus fermenti</i>	<i>Bacillus cohnii</i>
Aerotolerant anaerobic	+	+	+	+	+	-	-	-	-	-	-
Facultatively anaerobic	-	-	-	-	-	+	+	+	+	+	+
Spore formation	-	-	-	+	+	+	+	+	+	+	+
Hydrolysis of cellulose	+	+	+	+	+	-	-	-	-	-	not determined
Hydrolysis of xylan	+	+	+	+	+	-	-	V*	-	-	not determined
Oxidase	-	-	-	-	-	+	+	+	+	+	+
Catalase	-	-	-	-	-	+	+	+	+	+	+
Growth pH range	9-12	9-12	9-12.3	9-12	8-9.1**	7-12	7.5-12	8-12	8-11	8-12	not determined
Optimum growth pH	9.5-10.5	9.5-10.5	9.5-11.5	10	8.9-9.0**	10	9-10	10	9-10	9-10	not determined
Growth temperature range (°C)	5-45	5-45	15-40	17-39	26-39	18-48	12-40	10-45	18-40	15-45	10-47
Optimum growth temperature (°C)	34	30-37	20-30	35	36	39	30-33	35-37	33	33-40	not determined
Growth NaCl concentration range (%)	0-17	0-14/16	0-14/15	0-7	0-7	0-10	0-5/10	0-10	0-10	0-7	not determined
Optimum growth NaCl concentration (%)	2-12	3-13	1-11	0-1	0-3	1-11	0	3	7	0-3	not determined
Published year	2004	2005	2008	2013	2013	2013	2016	2016	2017	2018	1993

* Variable: *Polygonibacillus indicireducens* In2-9⁺ is positive; strain D2-7 is negative.

** These pH values are based on time, while other descriptions are based on the initial pH of the medium.

Chapter I

Analysis of the microbiota involved in the early changes associated with indigo reduction in the natural fermentation of indigo

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Abstract

Constituents of the seed microbiota and initial changes in the microbiota in fermentations are important in fermentation progression. To identify the origin of indigo-reducing bacteria and understand the initial changes in the microbiota that occur concomitantly with the initiation of indigo reduction during indigo fermentation, we analysed the initial changes in the microbiota. The proportions of the reported indigo-reducing taxa *Alkalibacterium*, *Amphibacillus* and *Polygonibacillus* increased to 24.0% on the 5th day, to 15.2% on the 7th day and to 42.8% at 4.5 months, and the relative abundances of these taxa were 0.048%, 0.14% and 0.02%, respectively, in *sukumo* (composted Japanese indigo plant material used for fermentation). In the early phase of the microbiota transition, two substantial changes were observed. The first change may be attributed to the substantial environmental changes caused by the introduction of heated wood ash extract (pH $\geq$ 10.5, temperature $\geq$ 60 °C). This change increased the proportions of *Alkalibacterium* and the family *Bacillaceae*. The second change in microbiota might be initiated by the consumption of oxygen by aerobic microorganisms until the 5th day followed by an increase in the abundance of the obligate anaerobe *Anaerobranca* and the aerotolerant *Amphibacillus* and a decrease in the abundance of *Bacillaceae*. This experiment

demonstrated that the 0.048% *Alkalibacterium* in the original material was augmented to 23.6% of the microbiological community within 5 days. This means that using the appropriate material and performing appropriate pretreatment and adjustment of fermentation conditions are important to increase the abundance of the taxa that reduce indigo.

2.1 Introduction

In Japan, indigo-dyeing has traditionally been performed by natural fermentation [1, 2, 72]. The indigo remains in a reduced state (leuco-indigo) for 6 months on average, and the fermentation fluid has a greater chance of being contaminated in an open environment than in a closed environment, especially during the dyeing process. By using the ecosystem of indigo fermentation, we have been trying to understand how this system can be maintained for 6 months or even longer, what the role of each constituent bacterium is, and what triggers the changes in the microbiota that are observed during the traditional process. By answering these questions, we expect to adjust the microflora to improve the dyeing process.

Traditional Japanese indigo dyeing consists of two microbial activity steps. In the first step, the indigo plant is composted to concentrate the contained indigo and make the dye material appropriate for storage, resulting in a material called *sukumo*. *Sukumo* is similar to the couched woad that is used in traditional medieval European indigo dyeing processes [16, 77, 78]. As a result of the composting process, this step also plays a role in seeding microorganisms for the second step. In preparation for the second step, *sukumo* is treated with hot wood ash extract (pH 10.5-11, 60-80 °C). This treatment of microorganisms in the *sukumo* is the first selection for microorganisms that can adapt to thrive in indigo fermentation fluid (an anaerobic alkaline environment). These anaerobic, harsh conditions (pH 10.5-11, 60-80 °C) appropriately select the indigo-reducing microorganisms that could benefit the indigo fermentation.

Indigo-reducing bacteria play a significant role in reducing the indigo-containing *sukumo*. Eleven species of indigo-reducing bacteria from traditional Japanese fermentation have been identified. The identified indigo-reducing bacteria can be roughly divided into two types: aerotolerant bacteria, such as species of the genera *Alkalibacterium* [65, 104, 105] and *Amphibacillus* [33, 34] and facultative anaerobes, such as *Oceanobacillus indicireducens* [32], *Fermentibacillus polygoni* [35], *Polygonibacillus indicireducens* [38], *Paralkalibacillus indicireducens* [36] and *Bacillus* spp. [37, 69]. In addition, obligate anaerobes might also contribute to indigo reduction. Thus, there are many types of indigo-reducing bacteria in indigo fermentation fluid. Each type of bacterium plays a role in sustaining the indigo-reducing state over a long fermentation period. Although it is expected that the genera *Alkalibacterium* and *Amphibacillus* may play a central role in indigo reduction in the system [2, 72], the detailed mechanisms underlying indigo reduction have not been elucidated.

This natural fermentation system for dyeing exhibits changes in its microbiota not only during the initiation of fermentation but also during the long fermentation period. First, we studied the changes in the microbiota in the early phase and in ten-month-old fermentation fluid by PCR-DGGE and clone library analysis based on the 16S rRNA gene sequence. On the fourth day after the initiation of fermentation, the dyeing intensity increased dramatically. Concomitant with this change, a distinct substitution of the genus *Halomonas* by the aerotolerant anaerobic genus *Amphibacillus* was observed [2]. This phenomenon can be explained by the change in the microbiota towards bacteria with increased adaptability to alkaline anaerobic conditions.

Okamoto et al. [72] attempted to understand the mechanisms underlying the maintenance of the reduced state of indigo during fermentation over 6 months, and the microbiota of fermentation fluid that was older than 6 months was analysed. Although members of the genera *Alkalibacterium* and *Amphibacillus* were often present throughout the fermentation period, the

major indigo-reducing bacteria changed depending on the fermentation stage. In addition, the changes in the microbiota occurred faster in the early fermentation stage than in later stages. This finding indicates that a certain time after the initiation of fermentation, the microbiota became stable. During this stable phase, members of the obligate anaerobic genus *Anaerobacillus* were present.

As described above, various species of indigo-reducing bacteria have been detected in various indigo fermentation fluids. It is reasonable to consider that indigo-reducing bacteria originate from *sukumo*, which is the main material for indigo fermentation. However, it has not been confirmed that indigo-reducing bacteria do indeed originate from *sukumo*. In this study, we attempted to detect indigo-reducing bacteria in *sukumo* by next-generation sequencing (NGS). Rapid and assured initiation of indigo reduction is one of the crucial challenges associated with natural indigo fermentation. The uncertainty associated with the occurrence of indigo reduction in the early phase of indigo fermentation necessitates the employment of the chemical reductant sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). Clarification of the mechanisms underlying the initiation of indigo reduction will simplify the reduction. To understand the mechanisms underlying the changes in the initial fermentation phase including *sukumo* and the pretreatment that are concomitant with the initiation of indigo reduction, we analyzed the changes in the microbiota that occur during the initiation of fermentation with *sukumo* by NGS.

2.2 Materials and methods

2.2.1 Preparation and maintenance of the indigo fermentation system

The indigo fermentation vat was prepared using 532 g of *sukumo* (composted Japanese indigo [*Persicaria tinctoria*]) and 5 L of wood ash extract (70 °C) containing wood ash precipitate. The pH of the mixture was adjusted to 11.2 by adding $\text{Ca}(\text{OH})_2$ and K_2CO_3 . The

wood ash extract was prepared by mixing 532 g of wood ash with 7 L of water and then boiling the mixture for 10 min. The prepared fermentation vat was kept at 26 °C for 1 day and then heated to 60 °C and maintained at 26 °C. The fermentation vat was stirred with a bar once a day. The pH of the fermentation fluid was decreased by the production of metabolites by the microorganisms involved in the process. The pH of the fermentation fluid was adjusted to between 10.3 and 11.3 by adding $\text{Ca}(\text{OH})_2$, and the staining intensity was checked by occasionally dipping a small portion of cotton textile into the fluid.

2.2.2 DNA extraction and PCR

Fermentation fluid, including the precipitated debris, was collected on the 2nd, 5th, 7th, and 9th days after initiation of fermentation for analyses of the microbiota. In addition, 4.5- and 5.5-month-old samples were also collected. An aliquot of the obtained indigo fermentation fluid sample was centrifuged at $15,000 \times g$ for 10 min to obtain a cell pellet sample, and then, DNA was directly extracted from the sample pellet using ISOIL (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

Primers targeting the bacterial 16S rRNA gene (341–805 in *Escherichia coli*), namely, 5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3', were integrated with adaptors and used for PCR-based amplification of the gene from the extracted DNA. The PCR solution (total 100 μl) consisted of 20 μl of 5 $\times$ Phusion HF buffer (Thermo Fisher Scientific, Waltham, USA), 2 μl of 2.5 mM dNTP mix (TaKaRa Bio-Company, Shiga, Japan), 25 ng of isolated DNA, 2 U of Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific) and 50 pmol of each primer. The PCR was performed under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 25 cycles of 40 s at 95 °C, 40 s at 53 °C and 60 s at 72 °C. At the end of amplification, the amplicons were subjected to a

final 7-min extension at 72 °C. The size and purity of the PCR product was checked by 2% agarose gel electrophoresis. The corresponding DNA band in the agarose gel was extracted and purified with the Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions.

2.2.3 NGS analysis

The 1st PCR amplicons were subjected to PCR to attach Illumina sequencing adaptors. The PCR products were purified with AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's instructions. The integrity and concentration of the obtained DNA were checked with a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The 16S rRNA gene library was sequenced on an Illumina MiSeq platform using 300-bp reads and MiSeq v3 reagents at Hokkaido System Science (Sapporo, Japan).

The sequences identified by NGS analysis were processed using the open-source software pipeline "Quantitative Insight into Microbial Ecology" (QIIME), version 1.9.1 (Caporaso et al. 2010). Operational taxonomic units (OTUs) were selected based on 97% sequence similarity using the UCLAST algorithm (Edger 2010). The rarefaction curves, observed species, and Chao 1 index (Hill et al. 2003) were determined by the QIIME alpha diversity analysis script. From the OTU tables and phylogenetic trees, an unweighted UniFrac distance matrix was presented as PCoA (principal coordinate analysis), and the corresponding plots were prepared using the beta diversity analysis script in QIIME. EMPEROR software (Vázquez-Baeza et al. 2013) was used to visualize the 3D PCoA plots.

The NGS data from this study are publicly available in the DNA Data Bank of Japan (DDBJ) under accession no. DRA007978.

2.3 Results

2.3.1 The *sukumo* microbiota

To elucidate the changes in the microbiota that lead to indigo reduction, we analysed the microbiota in the initial fermentation period of indigo by NGS. It is reasonable that the microbiota associated with indigo reduction in the fermentation fluid originates from *sukumo*, which is the main material for fermentation. However, there is no evidence to support this hypothesis to date. First, we analysed the microbiota of the *sukumo* used in this study (Fig. I -1) by NGS analysis and obtained 10481 reads, and we examined whether indigo-reducing bacteria were present in the fluid. The *sukumo* microbiota consisted of 9 phyla, and major constituents in the *sukumo* were members of the class Clostridia (15.6%), family Bacillaceae (14.6%), class Gemma-3 (phylum Gemmatimonadetes) (12.3%) and family Alcaligenaceae (9.4%). The results indicated that the indigo-reducing taxa *Alkalibacterium*, *Amphibacillus* and *Polygonibacillus* originated in the *sukumo* and that these bacteria constituted 0.048%, 0.14% and 0.02% of the microbiota, respectively.

2.3.2 Changes in the microbiota of the fermentation fluid

To understand the changes in the microbiota during the early phase of indigo fermentation, the fermentation fluid collected from the 2nd to 9th day after the initiation of fermentation was examined by NGS. For comparison, the microbiota of 4.5- and 5.5-month-old fermentation fluid was also examined. A total of 496562 reads were obtained, with 82760 reads per sample on average. The identification of major OTUs in the dominant taxa and the fluctuation in the proportions (%) depending on the fermentation period are shown in Table I -1. After the *sukumo* was treated with the hot wood ash extract, incubated at 26 °C, heated again to 60 °C and maintained at 26 °C (2nd day), the major constituent was Bacillaceae (62.3%) (Fig. I -1).

These heat-resistant bacteria increased in proportion during this treatment (Table I -1). On the 5th day, the major constituents were Bacillaceae (33.3%) and the genus *Alkalibacterium* (23.6%). The microbiota on the 2nd day was similar to that on the 5th day, except for the proportion of the genus *Alkalibacterium*. Although the proportion of the genus *Alkalibacterium* in the *sukumo* was negligible (0.048%), during the observation period, the abundance of this genus was highest on the 5th day. This finding indicates that the bacteria in this genus exhibit high adaptability under the initial fermentation conditions.

On the 7th day, a matte membrane that may be attributed to reoxidized reduced indigo in the fermentation fluid appeared at the surface of the fermentation fluid. On the 8th day, a shiny membrane appeared at the surface of the fermentation fluid and intense staining of soaked cotton textiles was observed. Although the proportion of the genus *Amphibacillus* was 0.006% on the 2nd day, this proportion was increased on the 5th day (0.26%), and the relative abundance of this genus peaked on the 7th day (15.2%). In addition, although the proportion of the genus *Anaerobranca* was only 0.90% on the 5th day, it was suddenly increased on the 7th day (50.1%). Considering the corresponding dyeing intensity, this genus may contribute to indigo reduction. This taxon also originated from *sukumo* and had a relative abundance of 0.22%. The microbiota on the 7th day was similar to that on the 9th day, except for the presence of *Tissierella/Soehngenia*. The major constituents in the fermentation fluid on the 7th and 9th days were anaerobic metabolizers that adapt to alkaline environments. On the other hand, the abundance of Bacillaceae, which may include aerobic metabolizers, continuously decreased from the 2nd day (62.9%) to the 7th day (7.0%). In 4.5-month-old fermentation fluid, the proportion of Bacillaceae further decreased to 0.68%. On the other hand, the proportion of *Polygonibacillus*, which are indigo-reducing bacteria, increased from not being detected on the 9th day to 42.8% at 4.5 months, and these species were the most abundant taxa (Fig. I -1,

Table I -1). Although there was a long interval (1 month), the microbiota of the 4.5- month-old fermentation fluid was very similar to that of the 5.5-month-old fermentation fluid. The major constituents in the 4.5- and 5.5-month-old fermentation fluid were *Polygonibacillus*, which accounted for more than 38% of the microbiota. Other indigo-reducing genera, namely, *Amphibacillus* (7.9–8.1%) and *Alkalibacterium* (2.3–3.6%) (Table I -2), were also present during this period. In addition, the possible indigo-reducing genus *Anaerobranca* (11.2–10.1%) was also present (Table I -1). The abovementioned complexity of indigo-reducing bacteria may lead to the stability of the microbiota.

The identification of major OTUs belonging to genera *Alkalibacterium* and *Amphibacillus* and the fluctuations in the proportion (%) depending on the fermentation period are shown in Table I -2. This indicated that the major species most likely belonging to *Alkalibacterium* and *Amphibacillus* were *Alkalibacterium indicireducens* and *Amphibacillus indicireducens*, respectively. The first drastic change in the microbiota attributed to the substantial environmental changes caused by the introduction of heated wood ash extract (pH $\geq$ 10.5, temperature $\geq$ 60 °C) increased the proportion of *Alkalibacterium indicireducens* (from 0 [sukumo] to 0.06% [2nd day]). The relative abundance of the species further increased to 23.3% after 3 more days (5th day).

To observe the changes in the diversity of the microbiota, rarefaction curves were computed for the sequences and observed species based on the observed OTUs (Fig. I -2). The diversity of the microbiota decreased from the *sukumo* stage until the 2nd day of fermentation and then increased until the 5th day. The initial decrease was in accordance with the timing of when selective pressure was applied by the introduction of hot wood ash extract (pH $\geq$ 10.5, temperature $\geq$ 60 °C) to the *sukumo*. The increase from the 2nd day to the 5th day is consistent with the abundance of Bacillaceae (Fig. I -1, Table I -1). After the 5th day, the diversity

decreased for 9 days. This pattern is in accordance with the decreasing abundances of Bacillaceae in the anaerobic environment. The diversity is thought to have continuously increased from then until 4.5 months.

To observe the intensity of changes in the microbiota, an unweighted PCoA was performed (Fig. I -3). The change in the microbiota from the 2nd day to the 5th day was relatively slow, and then a considerable change that accomplish the initiation of indigo reduction occurred from the 5th day to the 7th day. The change in the microbiota from the 7th day to the 9th day was relatively small. In addition, the change in the microbiota from the 9th day to 4.5 months was relatively small considering the length of the interval, and the difference in the plots between 4.5 and 5.5 months was very small, showing that the microbiota became very stable (Fig. I -1).

2.4 Discussion

The initiation of indigo reduction in indigo fermentation using *sukumo* occurs via natural fermentation. Ensuing rapid and reliable indigo reduction during the initiation of indigo fermentation is a crucial challenge associated with natural fermentation. In addition, the induction of favourable microbiota development during the initiation step is fundamentally important for the biological process and depends on the naturally occurring microbiota in natural fermentation. This study indicated that the utilization of material that contains favourable bacteria (*sukumo*) and an appropriate treatment in the initiation of fermentation are important for producing appropriate microbial communities. The induction of favourable microbiota development or the elimination of unfavourable microorganisms by alkaline or salt in the initiation step is fundamentally important in fermented foods, such as Spanish-style green table-olive [56], Japanese *nukadoko* [66, 73], Korean saeu-jeot [41, 50] and myeolichi-aekjeot [42]. There are reports on the positive effects of alkaline [44, 83] or heat [64] pretreatments for

the enhancement of biogas production. In this study, the analysis of microbiota at the initiation of indigo fermentation demonstrated that a very small portion of the necessary bacteria in the original material was augmented within 5 days by using appropriate material and performing appropriate pretreatment to amplify important taxa.

We analysed the initial changes in the microbiota in the early stage of indigo fermentation and observed two stages. The first rapid change occurred from the *sukumo* stage (the origin of the developing microbiota) to the 2nd day after the initiation of fermentation. This change may be attributed to the following substantial environmental changes: the change from the solid state (*sukumo*) to the liquid state (fermentation liquor) or the drastic change in pH caused by introduction of heated wood ash extract (pH ≥ 10.5 , temperature ≥ 60 °C) [1, 2]. This substantial change was reflected in not only the microbiota (Fig. I -1) but also the rarefaction analysis of the 16S rRNA gene sequence reads (Fig. I -2). Although an initial increase in taxonomic diversity was observed in Korean saeu-jeot [41, 50] and myeolichi-aekjeot [42] and Belgian lambic beer [91], an initial decrease in diversity followed by an increase was also observed in thermophilic compost [3]. The initial introduction of heat leads to the selection of microorganisms, and then, the diversity of adaptable thermophiles increases over time. The initial wood ash extract treatment (pH 10.5–11, 60–80 °C) during indigo fermentation plays a role in the selection of microorganisms in the *sukumo*, and the surviving microorganisms act as seeds for fermentation. The presence of indigo-reducing bacteria after the wood ash treatment indicates that the initial preparation is very important for the production of an appropriate microbiota.

In addition, another trigger for changes in the microbial community in this fermentation is the decrease in redox potential that occurs upon consumption of oxygen by aerobic microorganisms [2]. This environmental pressure is not as harsh as the introduction of heated

wood ash extract. However, this decrease in redox potential may induce a steady change from microorganisms that favour oxygen metabolism to strictly anaerobic microorganisms. It took approximately 7 days for the genus *Anaerobranca* to become the predominant genus in the microbiota, concomitant with the disappearance of Bacillaceae. On the other hand, the microbial diversity decreased from the 5th day to the 9th day. This finding suggested that the microbial diversity decreased from the *sukumo* stage to the 2nd day but then increased due to the increased abundance of bacteria that metabolize oxygen and that the bacterial diversity then decreased again due to a lack of oxygen. In this period, the relative abundance of the genus *Anaerobranca* increased from 0.02% on the 2nd day to 0.9% on the 5th day. Although there is no evidence that the genus *Anaerobranca* can reduce indigo, strains belonging to this genus might reduce indigo because *Anaerobranca californiensis* exhibits Fe(III) reduction [28]. Indigo reduction may occur via multiple mechanisms such as azoreductase activity [93] and extracellular electron transfer (EET), the latter of which includes operating by direct attachment of cell wall cytochromes *c* [4, 55, 89, 101], through electron shuttles (i.e., quinones and flavin) [4, 26, 71, 98] and through electron-conducting pilus-like “nanowires” [27]. An azoreductase that utilizes NADH as an electron donor was shown to reduce indigo by Suzuki et al. [93]. On the other hand, Light et al. [51] identified an eight-gene locus that encodes a specialized NADH dehydrogenase that is responsible for EET and segregates EET from the aerobic respiratory chain by channelling electrons to a discrete membrane-localized quinone pool. The orthologues of these genes are distributed in many genera in Firmicutes, including *Alkalibacterium*, *Amphibacillus* and *Oceanobacillus* [51]. In addition, metal reduction by bacterial communities under alkaline conditions and by alkaliphiles, that use flavins as electron shuttles has previously been reported [23, 24, 39, 59]. The redox potentials of electron shuttles and electron acceptors differ based on the pH values of their corresponding environments. It is desirable to clarify the

differences in EET under neutral and alkaline conditions in future studies.

Although initial changes in the microbiota have been analysed by PCR-DGGE [2], the precise fluctuations in the abundance of indigo-reducing bacteria have not been reported. In the original population, the proportion of indigo-reducing bacteria was too small to detect by PCR-DGGE and clone analyses. In this study, it was proven that the indigo-reducing taxa *Alkalibacterium*, *Amphibacillus* and *Polygonibacillus* originated in the *sukumo*, and the relative abundance of these taxa was determined to be 0.048%, 0.14% and 0.02%, respectively, by NGS analysis. The appearance of *Alkalibacterium*, which was derived from the *sukumo* in the initial phase (5th day), is important in this fermentation process. The appearance of *Alkalibacterium* at the initial phase and the constant presence of this genus during a long fermentation were also observed in Spanish-style green table-olive fermentation [56, 57]. This finding indicates that the appearance of the genus *Alkalibacterium* during the initiation of fermentation and the constant presence of this genus during fermentation are common phenomena in different kinds of alkaline fermentation processes and that the taxon may have an important role not only for the decreasing the amount of insoluble substrates but also for electron transfer among the microbial communities.

2.5 Conclusions

To observe the initial changes in the microbiota that occur concomitantly with the initiation of the indigo reduction during indigo fermentation, we analysed the microbiota by 16S rRNA NGS. The reported indigo-reducing taxa *Alkalibacterium*, *Amphibacillus* and *Polygonibacillus* originated in the *sukumo* (composted Japanese indigo plant material used for fermentation), and these bacteria were detected at relative abundances of 0.048%, 0.14% and 0.02%, respectively. Therefore, the constituent microorganisms in *sukumo* are important for indigo reduction. To

induce the dominance of alkaliphilic anaerobes that stabilize the microbiota in the fermentation fluid, two events occurred. First, the introduction of hot alkaline solution allowed indigo-reducing bacteria and *Bacillaceae* originating from *sukumo* to survive. *Bacillaceae* may contribute to strict anaerobic conditions by consuming oxygen. Second, the production of alkaline anaerobic conditions allowed the increase in slow growth once diminished by wood ash treatment anaerobes (*Anaerobranca*) and aerotolerants (*Amphibacillus*). This study demonstrated that a selection of appropriate material, adequate pretreatment of the original material and appropriate fermentation conditions are very important for constructing desirable microbiota in biological processes using natural fermentation.

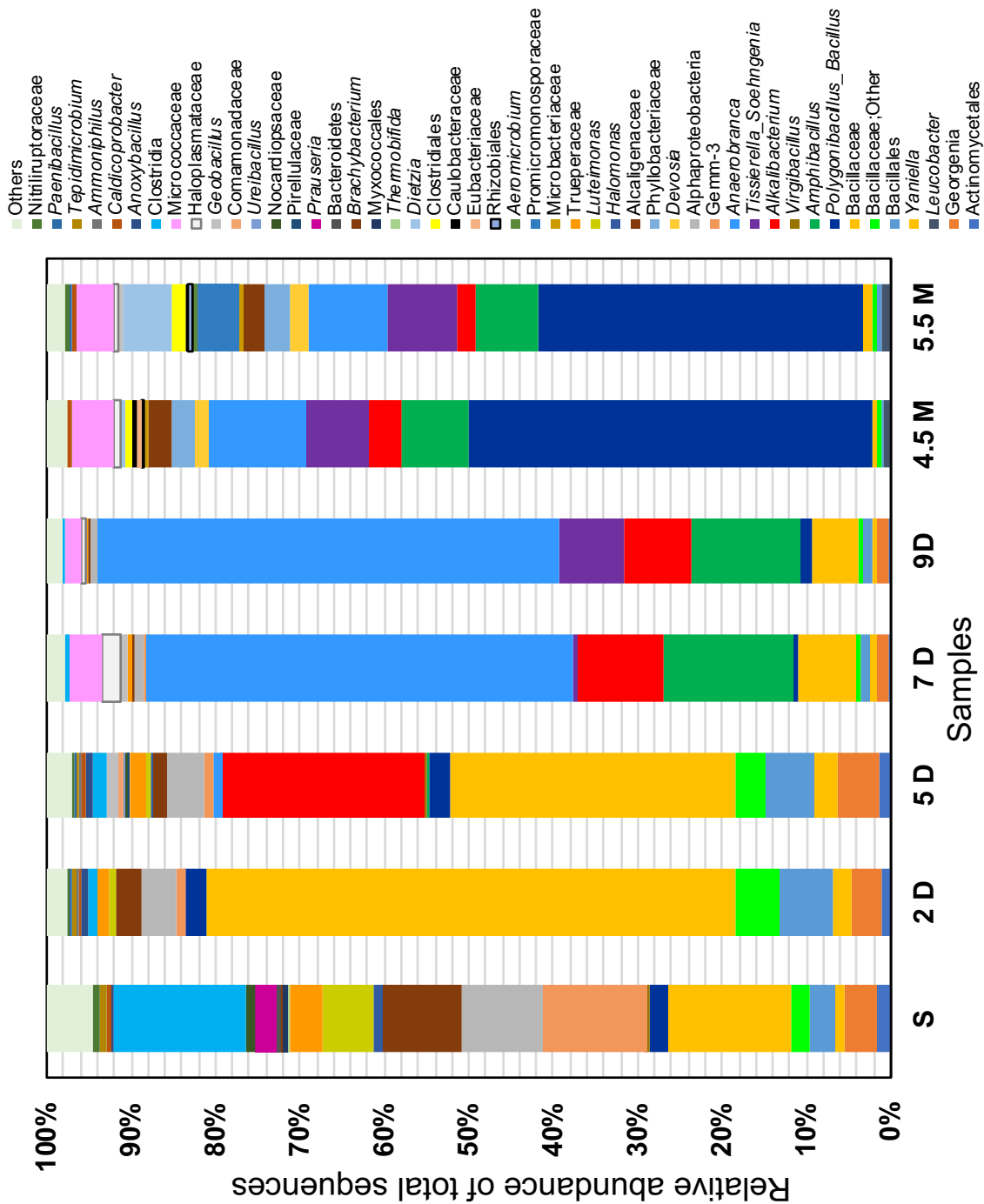


Fig. I -1 Relative abundances of total reads ($\geq 0.3\%$) in indigo fermentation fluids. *S*, *sukumo*; 2D, 2nd day sample; 5D, 5th day sample; 7D, 7th day sample; 9D, 9th day sample; 4.5M, 4.5-month-old sample; 5.5 M, 5.5-month-old sample.

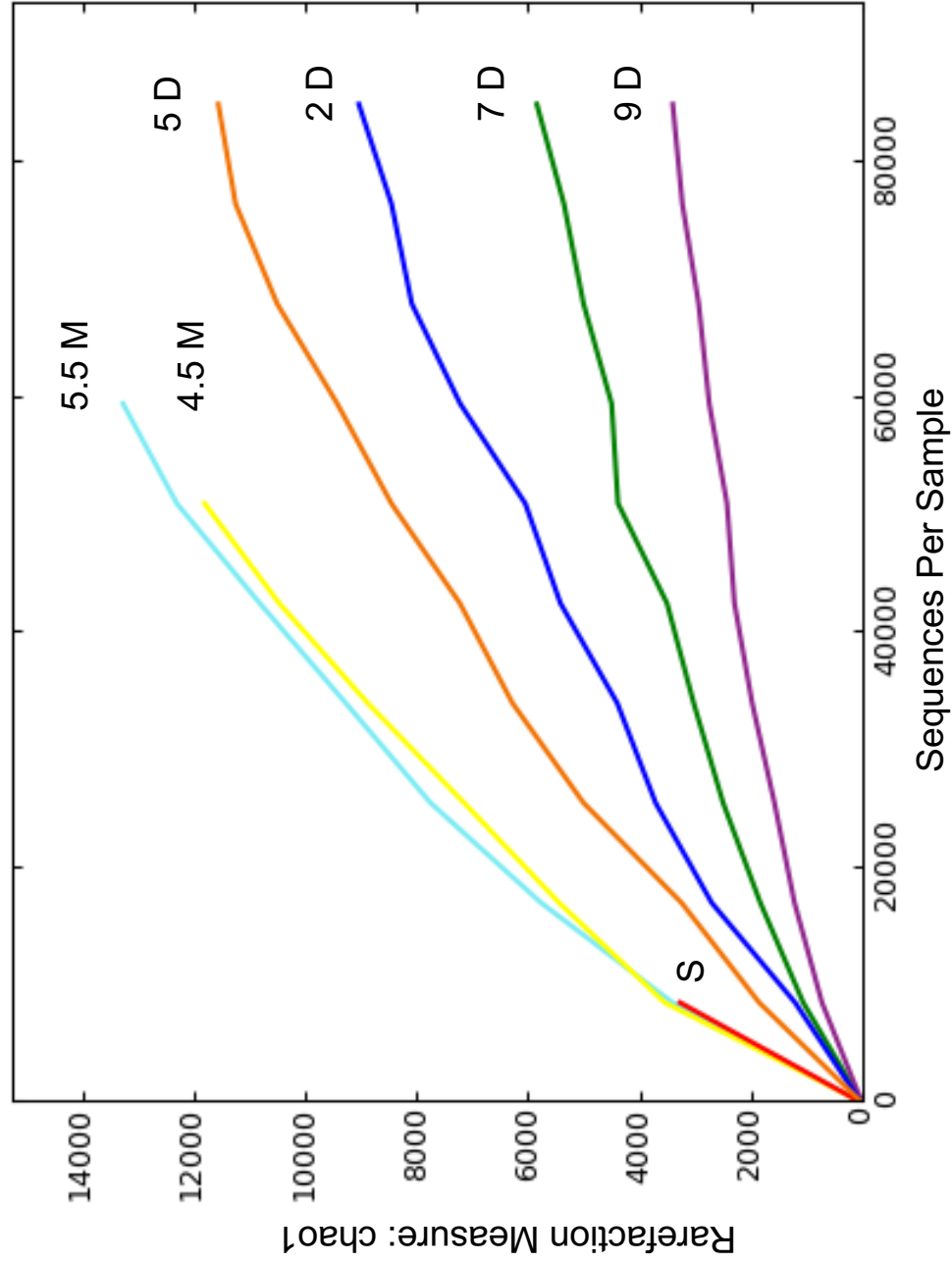


Fig. I -2 Rarefaction curve for operational taxonomic units (OTUs) defined by 3% sequence variation in 16S rRNA sequencing reads in samples from the indigo fermentation system. S, *sukumo*; 2D, 2nd day sample; 5D, 5th day sample; 7D, 7th day sample; 9D, 9th day sample; 4.5M, 4.5-month-old sample; 5.5M, 5.5-month-old sample.

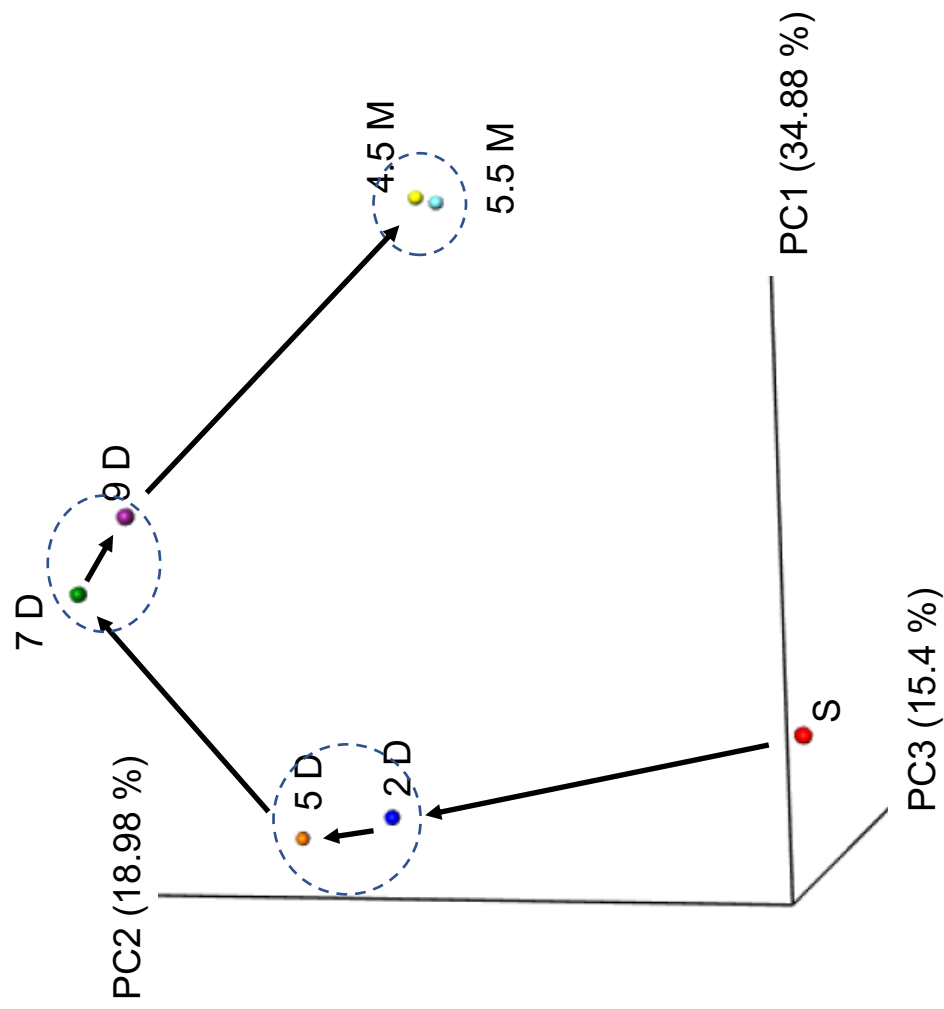


Fig. I -3 Unweighted PCoA plot for the bacterial community in the indigo fermentation system. S, *sukumo*; 2D, 2nd day sample; 5D, 5th day sample; 7D, 7th day sample; 9D, 9th day sample; 4.5M, 4.5-month-old sample; 5.5M, 5.5-month-old sample.

Table I -1 Identification of major OTUs in dominant taxa and the fluctuation of the relative abundance (%) depend on the fermentation period. Bold numbers indicate the high-abundance periods.

OTU	Highest similarity in database	<i>Sukumo</i>	2D	5D	7D	9D	4.5M	5.5M
<i>Anaerobranca</i>								
denovo4245	<i>Anaerobranca gottschalkii</i> LBS3 (NR_025050.1) 375/403 (93.1%)	0.22	0.02	0.90	50.1	54.7	11.2	10.1
<i>Bacillaceae</i>								
<i>Pseudogracilibacillus marinus</i>								
denovo9384	NIOT.bflm.S4 (NR_151980.1) 409/429 (95.3%)	1.23	8.88	2.99	0.58	0.37	0.005	0.01
<i>Oceanobacillus luteolus</i> WM-1								
denovo9384	(NR_126270.1) 415/427 (97.2%)	5.79	31.5	13.2	2.51	1.92	0.11	0.14
<i>Sinibacillus soli</i> GD05 (NR_133856.1)								
denovo7014	417/428 (97.4%)	1.62	13.3	8.95	1.68	1.40	0.10	0.11
<i>Polygonobacillus_Bacillus</i>								
<i>Polygonibacillus indicireducens</i> In2-9								
denovo8024	(NR_152690.1) 419/428 (97.9%)	0.02	0.001	0	0	0	42.8	38.3

Table I -2 Identification of major OTUs belonging to the genera *Alkalibacterium* and *Amphibacillus* and the fluctuations of the relative abundances (%) depend on the fermentation period. Bold numbers indicate high-abundance periods.

OTU	Rate of identified OTUs	<i>Sukumo</i>	2D	5D	7D	9D	4.5M	5.5M
denovo837	<i>Alkalibacterium iburiense</i> M3 (NR_041242.1) 427/427 (100%/all OTUs	0.029	0	0.017	1.3	2.2	0.24	0.25
denovo9383	<i>Alkalibacterium indicireducens</i> A11 (NR_041510.1) 427/427 (100%/all OTUs	0	0.060	23.3	8.7	5.7	3.1	1.9
	denovo837+denovo9383/all OTUs	0.029	0.060	23.3	10.1	7.9	3.3	2.2
	denovo837+denovo9383/all <i>Alkalibacterium</i> OTUs	60.0	100.0	98.7	99.5	98.9	91.5	92.7
	All <i>Alkalibacterium</i> OTUs/all OTUs	0.048	0.060	23.6	10.2	8.00	3.6	2.3
denovo8667	<i>Amphibacillus indicireducens</i> C40 (NR_1113557.1) 428/428(100%/all OTUs	0.048	0.0024	0.21	13.2	11.3	4.5	4.7
denovo11571-1	<i>Amphibacillus iburiensis</i> N314 (NR_114343.1) 426-427/428 (99.5-99.8%/all OTUs	0.0095	0	0.012	0	0	0.28	0.30
denovo11571-2	<i>Amphibacillus indicireducens</i> C40 (NR_1113557.1) 424-425/428 (99.1-99.3%)	0	0	0	0.81	0.56	0	0
	denovo8667+all denovo11571 / all OTUs	0.057	0.0024	0.22	14.0	11.9	4.8	5.0
	denovo8667+all denovo11571/all <i>Amphibacillus</i> OTUs	42.9	33.3	85.8	92.0	93.0	61.2	61.6
	All <i>Amphibacillus</i> OTUs/all OTUs	0.14	0.0060	0.26	15.2	12.8	7.9	8.1

Chapter II

Characterization of the microbiota in long- and short-term natural indigo fermentation

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Abstract

The duration for which the indigo-reducing state is maintained in indigo natural fermentation is batch dependent. The microbiota was analyzed in two batches of *sukumo* fermentation fluids that lasted for different duration (Batch 1: less than 2 months; Batch 2: nearly 1 year) to understand the mechanisms underlying the sustainability and deterioration of this natural fermentation process. The transformation of the microbiota suggested that the deterioration of the fermentation fluid is associated with the relative abundance of Alcaligenaceae. Principal coordinates analysis (PCoA) showed that the microbial community maintained a very stable state in only the long-term Batch 2. Therefore, entry of the microbiota into a stable state under alkaline anaerobic condition is an important factor for maintenance of indigo fermentation for long duration. This is the first report on the total transformation of the microbiota for investigation of long-term maintenance mechanisms and to address the problem of deterioration in indigo fermentation.

3.1 Introduction

Indigo is one of the oldest dyes used by humankind. Before synthetic dyes were developed in the 19th century, indigo was traditionally derived from various blue dye plants, including woad (*Isatis tinctoria*), native to Europe and southeastern Russia; knotweed (*Polygonum tinctorium*), used in China, Korea, and Japan; and *Indigofera* (*Indigofera tinctoria* and *Indigofera suffruticosa*), used in India [1, 9, 99]. Plant leaf processing methods for dyeing have been developed in different regions, such as woad balls in Europe, *niram* in Korea and *sukumo* in Japan. However, precipitated indigo extracted from plants has been used in India, China and Southeast Asia [13, 52].

Indigo itself does not exist in the leaves of indigo-producing plants. The original state of indigo dye is always indican (indoxyl- β -D-glucoside) [80]. Therefore, transformation of indican to indigo is necessary for the production of indigo dye. The most popular method is the extraction of indigo dye by short-term fermentation. Fresh leaves are soaked in a large vat for 2-3 days until soft. Then, lime hydrate (Ca(OH)_2) is added to the liquid, and the mixture is thoroughly stirred. The indigo then precipitates and accumulates at the bottom of the vat. Alternatively, indigo-containing plants are decomposed by microorganisms, as performed for woad in Europe and *sukumo* in Tokushima Prefecture, Japan. [1, 72]

In Tokushima Prefecture, the leaves of knotweed plants are harvested and air-dried, and then, approximately 3 tons of dried leaves are mixed with a roughly equivalent weight of water and piled to approximately 1 m in height. Microbial oxidative fermentation of the leaves is promoted under aerobic conditions and at an appropriate temperature by controlling the water content and adjusting the turnover frequency through the skillful techniques of a trained craftsman [1]. The temperature reaches 60 °C because of microbial activity, and the mixture becomes alkaline. This microbial oxidation step is carried out for up to 100 days, and the brown

composted product is called *sukumo*. During this process, the precursor is broken down to indoxyl and sugar moieties by plant enzymes, and free indoxyl has been suggested to form indigo via indoxyl radicals, which first form leuco-indigo and are then oxidized to indigo [85].

Due to its insolubility in water, indigo in *sukumo* needs to be reduced to the water-soluble form (leuco-indigo) before dyeing. The reduced form can be easily absorbed into fibers, and when exposed to the air, the soluble leuco-indigo is oxidized back to the insoluble indigo and stays within the fibers. Traditional *sukumo* reduction is implemented by fermentation under extreme alkalinity (pH 10.3-11.0) [72]. *Sukumo* is mixed with hot wood ash extract; the fluid is stirred once or twice daily; and wheat bran or wheat gluten is added as a substrate for the indigo-reducing microbes when the dyeing efficiency decreases. In this step, oxidized indigo is solubilized via bacterial reduction. Generally, the required dyeing efficiency can be maintained for approximately 6 months on average by skilled artisans [72].

Since the late 19th century, natural indigo has been replaced over a relatively short period by synthetic dyes, and in parallel, the use of chemical reductants, such as hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), has been established in the industry for modern vat-based dyeing with indigo. Fermentation vats continue to be used on a small scale and even in domestic dyeing processes across Europe, Africa, Asia and America [30]. In addition to maintaining the cultural heritage, traditional techniques for indigo fermentation have significant advantages in terms of recyclability and human health and environmental safety [61, 75].

In the past decade, attempts have been made to develop and optimize environmentally friendly alternatives for the formation and reduction of indigo. To elucidate the mechanism underlying indigo fermentation, many indigo-reducing bacterial species have been isolated from various types of indigo fermentation fluids. In the genus *Alkalibacterium*, there are 10 named species, 3 of which are indigo-reducing bacteria: *Alkalibacterium iburiense* [65],

Alkalibacterium psychrotolerans [104] and *Alkalibacterium indicireducens* [105]. There are ten named species within the genus *Amphibacillus*, and 2 of these species are indigo-reducing bacteria: *Amphibacillus indicireducens* [33] and *Amphibacillus iburiensis* [34]. In addition, some other species belonging to the class Bacilli, such as *Oceanobacillus indicireducens* [32], *Fermentibacillus polygoni* [35], *Polygonibacillus indicireducens* [38], *Paralkalibacillus indicireducens* [36], and *Bacillus fermenti* [37], have been shown to be indigo-reducing bacteria in recent years.

In addition, Okamoto et al. [72] explored the microbiota in aged fermented fluids with culture-dependent and clone library-based methods. They found that the microbiota mainly consists of the genera *Alkalibacterium*, *Amphibacillus*, *Anaerobacillus* and *Polygonibacillus* and the family Proteinivoraceae. Aino et al. [2] investigated the bacterial community structure associated with indigo fermentation using denaturing gradient gel electrophoresis (DGGE) and clone library analyses of a PCR-amplified 16S rRNA gene and found a marked substitution of *Halomonas* spp. by *Amphibacillus* spp., corresponding to a marked change in the state of indigo reduction. *Alkalibacterium* spp. were not predominant in the early phase of fermentation, but were abundant in aged fluid (10 months) obtained from Date City, Japan. Milanović et al. [61] explored the microbiota of woad vat fermentation fluid aged 12 months with PCR-DGGE and pyrosequencing and found that eumycetes and coliforms were present at levels below the detection limit, whereas total mesophilic aerobes and spore-forming bacteria and their spores were detected at great at high levels. Both facultative and obligate anaerobes were present in the woad vat and were involved in the formation, reduction, and degradation of indigo. They inferred that indigo-reducing bacteria constitute only a small fraction of the microcosm.

During long periods of fermentation, there are many chances of contamination by environmental microorganisms. Subtle changes in nutrients, pH, and agitation can affect on the

state of reduction. Therefore, the maintenance time differs greatly among fermentation broths, and the differences and trends for microbial communities are remain unknown. To investigate factors associated with defining the timing of the reducing state of fermentation fluid and the changes in bacterial dynamics during the whole fermentation process, we used two batches of *sukumo* fermentation broth. The reduction of one of the broths lasted for only approximately 7 weeks, and that of other lasted for nearly 1 year. Next-generation sequencing (NGS) was used to monitor the total transformation of bacteria during the whole fermentation process of Japanese *sukumo*.

3.2 Materials and methods

3.2.1 Indigo fermentation samples

Two batches of *sukumo* fermentation fluids were used: Batch 1 was fermented for approximately 7 weeks (51 days), and Batch 2 was fermented for nearly 1 year (345 days). Batch 1 contained 380 g of *sukumo* (produced in Tokushima Prefecture, Japan) and the same weight of wood ash (fine powder of burnt charcoal made from *Quercus phillyraeoides*) in 5 L of water. The wood ash was first added to 5 L of water and boiled by simmering on a stove for 10 min. When the liquid cooled to 60 °C at room temperature, the *sukumo* was added and mixed well, and then, the fluid was placed at 26 °C in a thermostatic room protected from light. On the next day, the fluid was heated again to 60 °C with constant stirring and then returned to the thermostatic room. Batch 2 was made from 532 g of *sukumo* (produced in Tokushima Prefecture, Japan) and 7 L of wood ash supernatant by heating to 70 °C, and then placed in the same thermostatic room. Batch 2 was also heated again to 60 °C on the next day and then maintained at 26 °C. During the subsequent long-term fermentation, the broths were stirred every day, and the pH of the fermentation fluid was maintained between 10.3 and 11 with $\text{Ca}(\text{OH})_2$ in addition

to small amount of Na_2CO_3 , NaOH and lactic acid for Batch 1 and with $\text{Ca}(\text{OH})_2$ and a small amount of K_2CO_3 for Batch 2. For precise adjustment of pH in preparation for the fermentation, NaOH , Na_2CO_3 and K_2CO_3 were used. On the other hand, for maintenance of pH during fermentation, $\text{Ca}(\text{OH})_2$ was used for the precipitation of acid. The oxidation-reduction (ORP) [53] was measured using a 9300-10D (Horiba, Kyoto, Japan) electrode.

According to the method used by traditional craftsmen, the reduction state was roughly evaluated by cotton cloth dyeing. In this method, a piece of white cotton cloth of approximately $2\text{ cm} \times 3\text{ cm}$ is soaked in the fermentation fluid for 30 sec and then removed and exposed to air for oxidation, and then, the impurities are washed away in flowing water. Reduced fermentation broth will stain the white cotton cloth blue. From Batch 1, samples fermented for 1 day, 7 days (1 week), 14 days (2 weeks), 21 days (3 weeks), 28 days (4 weeks), 35 days (5 weeks), 42 days (6 weeks), 49 days (7 weeks) and 51 days (7 weeks + 2 days) were examined. From Batch 2, samples fermented for 1 day, 9 days, 30 days (1 month), 135 days (4.5 months), 165 days (5.5 months), 240 days (8 months), and 345 days (11.5 months) were used for the subsequent analysis.

3.2.2 DNA extraction, PCR amplification, and PCR product purification

The obtained samples were centrifuged at $15,000 \times g$ for 10 min to obtain the sample pellet. Total bacterial DNA was directly extracted from the sample pellets using ISOIL (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions: 950 μl of Lysis Solution HE and 50 μl of Lysis Solution 20S were added to the samples and, after mixing well by inversion, incubated at $65\text{ }^\circ\text{C}$ for 1 h. The samples were then centrifuged ($12,000 \times g$, 1 min, room temperature). Then, 600 μl of each supernatant to a new tube, and 400 μl of Purification Solution was added, and mixed well. Then, 600 μl of chloroform was added, and each sample

was vortexed for 15 sec and then centrifuged ($12,000 \times g$, 15 min, room temperature). Then, 800 μ l of the aqueous layer was transferred to a new tube while taking care not to transfer the intermediate layer; 800 μ l of Precipitation Solution was added, and the samples were mixed well and then centrifuged ($20,000 \times g$, 15 min, 4 °C). The supernatant was discarded; 1 ml of Wash Solution was added and mixed by inverting a few times; and the mixture was then centrifuged ($20,000 \times g$, 10 min, 4 °C). The supernatant was discarded; 1 ml of 70% ethanol and 2 μ l of Ethachinmate was added and the mixture was vortexed and then centrifuged ($20,000 \times g$, 5 min, 4 °C). The supernatant was discarded; the precipitates were air-dried and then dissolved in 50 μ l of TE (pH 8.0).

The DNA extracts were purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. The V4 region of the bacterial 16S rRNA genes was PCR-amplified using a composite pair of primers containing unique 35- or 34-base adapters, which were used to tag the PCR products from the corresponding samples. The forward primer was 515F (5'-GTGBCAGCMGCCGCGGTAA-3') with an adaptor (GCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and 10 bp of sample-specific barcode, and the reverse primer was 805R (5'-GACTACHVGGGTATCTAATCC-3') with an adaptor (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). The primer pair 515F-805R amplified approximately 290-bp fragments of the bacterial 16S rRNA genes. PCR was performed in a 100 μ l solution containing 20 μ l of 5 $\times$ Phusion HF buffer (Thermo), 2 ml of a 2.5 mM dNTP mixture (TaKaRa, Ohtsu, Japan), 25 ng of isolated DNA, 2 U of Phusion Hot Start II DNA polymerase (Thermo) and 50 pmol of each primer. The amplification reactions were performed as follows: initial thermal denaturation at 98 °C for 30 sec followed by 25 cycles of heat denaturation at 98 °C for 10 sec, annealing at 55 °C for 20 sec and extension at 72 °C for 30 sec.

The fragment lengths of the PCR products were confirmed by agarose gel electrophoresis, and the products were then purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. The purified PCR products with the expected bands were extracted after agarose gel electrophoresis with the Wizard SV Gel and PCR Clean-up System (Promega) following the manufacturer's instructions.

3.2.3 Next-generation sequencing (NGS)

The extracted V4 region of the 1st PCR products was submitted to the Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). A 2nd PCR was performed with an index-adapted primer to generate paired-end (2× 301 base pair) libraries for Illumina shotgun sequencing, and the products were purified. NGS was carried out on the Illumina MiSeq platform (Illumina, San Diego, USA). The obtained raw reads were preprocessed with cutadapt version 1.1, Trimmomatic version 0.32 and fastq-join version 1.1.2-537. All failed sequence reads and low-quality, tag and primer sequences were removed. Clustering analysis based on operational taxonomic units (OTUs) with 97% identity and taxonomic classification annotated by each representative sequence were performed by using QIIME software version 1.9.1 [12]. Taxonomic analysis and annotation of the output data were also carried out by QIIME software, which can perform OTU picking, taxonomic assignment, diversity analysis and graphical visualization [12, 82]. Nucleotide sequence identity analysis was also carried out with bacterial 16S rRNA reference sequences in the BLAST database [43].

The sequence data obtained in this study have been deposited in DDBJ under the accession number DRA007978.

3.2.4 Diversity analysis

Alpha diversity is usually used to represent species diversity in a sample. In this study, rarefaction curves were computed with sequences and observed species based on OTUs at a 97% similarity level with respect to the total number of reads for each sample. Observed species curves were obtained to show the trend in bacterial species detected in the samples. In addition to alpha diversity, beta diversity within all samples was computed in this study. Beta diversity analysis involves the explicit comparison of microbial communities based on composition. Beta diversity metrics thus assess the differences between microbial communities. PCoA based on Bray-Curtis distances was used to evaluate the similarity of the microbiota in every sample. Both rarefaction curves and PCoA were carried out with QIIME software version 1.9.1.

3.3 Results

3.3.1 Indigo-reducing fermentation

The 2 batches of *sukumo* fermentation fluid started reduction at approximately 1 week, which is characterized by the formation of a film with a metallic luster on the surface of the fluid. That is reduced leuco-indigo in the liquid was oxidized back to insoluble indigo by oxygen in the air at the portion of the liquid surface that was in contact with the air (Fig. II-1).

Batch 1 did not receive any nutrients until the staining became weak on the day 46, on the next day (day 47) the batch received two pieces of wheat-gluten bread (approximately 1 g). Batch 2 received wheat bran when the dyeing became weak (from week 3), and the reducing state continued until approximately month 11, when the final deterioration occurred and dyeing could no longer occur. However, the staining intensity became weak at 4.5 months (135 days) (Fig. II-2).

Although the redox potential of Batch 2 was not estimated, the changes in pH and redox

potential in Batch 1 and the change in pH in Batch 2 were measured (Fig. II-3). The initial redox potential decrease in redox potential occurred due to the survival of aerobic bacteria after the initial heat treatment. In addition, further reduction in the redox potential may occur via external transmission of electrons from anaerobic metabolic pathways associated with lactic acid production. It has been reported that the magnitude of the reduction of redox potential is depend on the bacteria present in the fluid [67]. To maintain the fermentation fluid at pH of approximately 10.3, $\text{Ca}(\text{OH})_2$ was added. The changes in pH are depicted in Fig. II-3.

When the fermentation broth deteriorated, the film disappeared, and the liquid turned brown (Fig. II-1). Batch 1 and Batch 2 exhibited deterioration on day 51 and month 11.5 (day 345), respectively. Although the basic preparation and maintenance procedures were the same for both batches, the characteristics of the fermentation fluids were different. This difference was because of the accumulation of inhomogeneity in the *sukumo* and the subtle differences in the temperature and pH of the wood ash extract at the initiation of fermentation, which was reflected in the differences in the microbiota on day 1 (Fig. II-2). Furthermore, the subtle differences in maintenance procedures also induced differences in the characteristics of the fermentation fluid.

3.3.2 Whole bacterial community analysis by NGS

The whole community structure of the *sukumo* fermentation fluids were analyzed based on OTUs identified by NGS. NGS analysis indicated that the fluids contained a wealth of different bacterial species. The bacterial communities comprised a total of 22 phyla in Batch 1 and 24 phyla in Batch 2, including Archaea, which were detected in samples of Batch 1 from day 1, 7 and 51 and samples of Batch 2 from day 9, although the relative levels of Archaea were consistently less than 0.1%. The top 3 relative levels were exhibited by the phyla Proteobacteria,

Firmicutes and Actinobacteria, which accounted for more than 90% of the phyla in every sample. In the beginning, the abundance of the phylum Firmicutes increased substantially in the middle and late stages of fermentation, the abundance of the phylum Proteobacteria increased substantially, and the abundance of the phylum Tenericutes (0.2-0.3%) also increased, especially in Batch 1 (data not shown).

At the genus level, 208 and 279 different genera were distinctly detected in Batch 1 and Batch 2, respectively. During the whole fermentation of the two batches, most of the significant bacterial populations were similar, and the batches also showed significant changes in the relative content of various genera or families (Fig. II -2). The abundance of the genera *Anaerobranca* and *Tissierella* increased substantially in the beginning and decreased slightly thereafter. The family Alcaligenaceae was detected in small amounts, but the abundance increased greatly on day 21 in Batch 1 and on day 240 in Batch 2. There were also significant differences between the two batches. In the beginning, the abundance of the genus *Prauseria* decreased substantially in Batch 1, and the abundances of the families Rhodospirillaceae and Trueperaceae decreased substantially in Batch 2. The abundances of the genera *Clostridium* and *Coprococcus* increased from the middle period in Batch 1, while the the abundances of genus *Devosia* and family Phyllobacteriaceae increased from the middle period in Batch 2, and the genera *Acholeplasma* and *Azoarcus* were detected in only deteriorated samples from Batch 2. In addition, the genus *Caldicoprobacter* had a relative abundance of 2.1% on day 28, which increased to 28.7% on day 35 and this genus was present at more than 20% after day 35 in Batch 1. *Pseudomonas* was present at 0% at week 2 but at 36.9% on day 21 in Batch 1 maintaining a relatively high level thereafter. However, *Pseudomonas* had a relative abundance of 16.9% in only the deteriorated sample and an abundance of 0% in the other samples of Batch 2.

Based on the reported indigo-reducing bacteria, the most likely indigo-reducing population includes *Amphibacillus*, *Alkalibacterium* and *Oceanobacillus*. Comparisons of sequences of *Marinilactibacillus*, which were identified by NGS and search against the BLAST database showed that these sequences share 100% similarity with sequences of *Amphibacillus* spp. and *Alkalibacterium* spp. in the target 16S rRNA V4 region fragments. This result indicates that *Marinilactibacillus* may also contain indigo-reducing bacterial species. The relative abundance of *Amphibacillus* remained steady in both batches and the abundances of *Alkalibacterium* and *Marinilactibacillus* first increased and then decreased, while that of *Oceanobacillus* continued to decrease with fermentation. The total relative abundances of these genera showed a general trend consisting of an initial increase followed by a decrease. In all samples, the total amount of reducing bacteria peaked in the early stage of fermentation, on day 14 for Batch 1 and day 9 for Batch 2. In addition, the total amount in the long-term broth was more than that detected in short-term broth, which was more than the 8% detected before deterioration in Batch 2; however, the value in Batch 1 was 5.6% on day 28, decreasing to 1.5% by day 42.

3.3.3 Statistical analysis with OTUs

3.3.3.1 Observed species from rarefaction curves

Alpha diversity is usually used to represent species diversity in a specific environment. In this study, rarefaction curves were computed with sequences and observed species based on OTUs; these curves show the trends in bacterial species detected in the samples. Because the sequences of each sample were different, the observed species were obtained from the intersection nodes of 3818 sequences of Batch 1 and 3730 sequences of Batch 2 (Fig. II -4). The number of bacterial species decreased in the beginning of the fermentation, going from 242 species on day 1 to 145 species on day 7 and 138 species on day 14 in Batch 1 and from 175

species on day 1 to 133 species on day 9 in Batch 2. Then, this number increased with fermentation progression until deterioration, resulting in 323 species on day 51 in Batch 1 and 281 species on day 345 in Batch 2. In both the short- and long-term fermentation broths, the number of observed bacterial species showed a U-shaped trend. In the present sequence analysis, the number of bacterial species in the deterioration period was approximately 3 times that at the beginning of reduction.

The bacterial populations in the *sukumo* fermentation broths changed during the whole fermentation process. The bacterial diversity of both batches decreased from the beginning to the reducing stage, and then increased until the final deterioration in the fermentation broths. That is, in the initial reduction state, the bacterial diversity was relatively low, and in the subsequent state, the bacterial diversity increased. Low bacterial diversity is conducive to fermentative reduction, and high diversity increases the risk of deterioration of the fermentation broth. However, comparison of Batch 1 and Batch 2 showed that the short-term Batch 1 maintained low bacterial diversity for a very short time (within 28 days), and the bacterial diversity of Batch 1 reached that of Batch 2 on day 165, which represents a six-fold difference in time. Based on this result, it is speculated that prolonging the period of low bacterial diversity in the stationary phase would play a positive role in maintaining a long period of reduction.

3.3.3.2 Principal coordinates analysis

The Bray-Curtis index was used to evaluate the similarity of the microbiota between all samples (Fig. II -5). The bacterial composition changed rapidly at the beginning (day 1 to 7 of Batch 1, day 1 to 9 of Batch 2), and then remained steady in the freshly reduced samples (day 7 to 14 of Batch 1, day 9 to 30 of Batch 2). Then, the bacterial population rapidly changed after day 14 in Batch 1. However, in Batch 2, there was little transformation during the three weeks

from day 9 to 30, and in the next few months from the days 135 to 240. However, the bacterial flora transformed slowly from the beginning to the reduction stage to the final deterioration stage. This result also indicated that the bacterial taxa replaced the previous group at all times. The bacterial population in Batch 1 transformed faster than that in Batch 2. The OTUs identified as *Bacillus* clarified that it contained *P. indicireducens* and *Bacillus cohnii*. The change in abundance of the major constituents and genera during indigo reduction was estimated. These total abundances of these bacterial community transformations are produced major members of each fermentation batch (Fig. II -6). The slow transition in Batch 2 after day 9 was attributed to the slow transition of the genus *Anaerobranca* and *P. indicireducens* (Fig. II -6B). On the other hand, the change velocities of reported indigo-reducing bacteria in the genera *Amphibacillus*, *Alkalibacterium*, and *Oceanobacillus* and the *Bacillus cohnii* were not as high as those of the dominant taxa (Fig. II -6A B). Fig. II -6 shows that the deterioration of the fermentation fluid is associated with the relative abundance of Alcaligenaceae in both batches.

3.4 Discussion

This study pioneered the use of indigo fermentation broths that maintained short-term and long-term reduction states and examined the bacterial communities and differences between these two batches. Although transformation in the microbiota occurred in both short-term and long-term batches, the change velocity decreased after day 9 in Batch 2. On the other hand, the change velocity of the microbiota increased after day 14 in the short-term Batch 1 compared that observed in the previous 7 days (days 7 to 14).

The presented results suggest entry into a stable state at approximately 10 days in the long-term fluid is important for long-lasting indigo fermentation. The microbiota generated within 10 days of fermentation initiation exhibited a retention ability. This period may

correspond to the period in which obligate anaerobes were dominant in the fluids. The most predominant taxa transformed successively as follows: seeds (mostly oxygen metabolizing aerobes) → obligate anaerobes (i.e., *Anaerobranca*) → facultative anaerobes (i.e., *Bacillus* in Fig. II-2; *P. indicireducens* in Fig. II-6B). In addition to the most predominant organisms, reported aerotolerant indigo-reducing genera were present at relative abundance of 7.4-16.3% during the indigo reduction period. Comparison with the short-term fermentation fluid showed that the successive transitional change from predominance of the obligate anaerobe *Anaerobranca* to *P. indicireducens* was associated with the stable state of the microbiota. This stability was demonstrated by the endurance of the indigo-reducing state, although a substantial decrease in pH occurred in the Batch 1 approximately 95-102th day. Although the taxa were different from those in the present study, the predominance of obligate anaerobes in long-term indigo fermentation fluid was reported previously [72]. This retention is attributed to the environmental pressure of alkaline anaerobic conditions [1] and the persistence of the bacteria adapted to the environment (i.e., obligately anaerobic alkaliphiles). Natural fermentation processes that utilize spontaneously occurring microbiota have been used to produce several fermented foods, beverages and condiments [41, 42, 86, 91]. In most of these cases, acidic and/or salty environments define the adapted microbiota. In the case of traditional Korean fish sauce, fermentation (myeolchi-aekjeot) samples exhibit stable states of the microbiota under the appropriate fermentation conditions [42].

On the other hand, according to the microbiota, the long-term stable state was maintained appropriately until day 14. On day 21, *Pseudomonas* was the predominant taxon, and its dominance lasted until day 51. This change may have resulted from the introduction of lactic acid on day 13. When lactic acid was introduced, the redox potential of the fermentation fluid increased to -530 mV (Fig. II-3A). In the nondyeing deteriorated stage, Gram-negative

bacteria were predominant in both batches. In addition to the high redox potential, a localized low-pH niche may eventually have been formed in the fermentation fluid and this event may have increased the levels of Gram-negative bacteria such as *Pseudomonas* and *Alcaligenaceae* (Fig. II -2, Fig. II -6). This difference may have been due to Batch 1 having a small fermentation volume, which readily induces high redox potential, and a localized low pH niche may have been produced due to stirring with a thinner glass bar than that used for Batch 2. Normally, the abundances of these taxa hardly increase under highly anaerobic and high-pH conditions, which are dominated by obligately anaerobic alkaliphiles. Detection of unfavorable taxa in the initial stage of propagation could prevent further propagation. Therefore, it is considered that there may be a period of susceptibility in which propagation of unfavorable taxa could occur. For example, during the period of entry into the obligate anaerobe-dominated phase, may be susceptible to unfavorable environmental changes. When the high redox potential occurred on day 13, the proportion of *Anaerobacillus* began to decrease (Fig. II -6A). Bacteria belonging to *Alcaligenaceae* (genus *Alcaligenes*) are often observed in reduced indigo fermentation fluid [2, 61]. Therefore, a taxonomic study to identify *Alcaligenaceae* species observed in this study is desirable. The relative abundance of the *Alcaligenaceae* and the time periods at which these species are present are important for determination of the staining intensity of textiles.

In this study, *Amphibacillus*, *Alkalibacterium*, and *Oceanobacillus* were detected in the initial sample (day 1) of Batch 1, and *Amphibacillus* and *Oceanobacillus* were detected in the initial sample (day 1) of Batch 2. These genera have been isolated from microbial indigo fermentation using *sukumo* as the source of the dye and have been indicated to include several indigo-reducing bacteria [32-34, 65, 104, 105]. Although the correlation between the relative proportions of *Amphibacillus*, *Alkalibacterium*, and *Oceanobacillus* and dyeing intensity

exhibited a 7-14-day lags, there was a correlation observed in short-term fermentation. On the other hand, a direct correlation between the relative ratio of *Amphibacillus* and *Oceanobacillus* and the dyeing intensity was observed in long-term fermentation (supplementary Fig. II-1). Changes in the relative abundances of dominant taxa other than *Amphibacillus*, *Alkalibacterium* and *Oceanobacillus* were examined (Supplementary Fig. II-1). In both batches, the abundance of *Anaerobranca* decreased as the fluids exhibited a shift toward deterioration (Fig. II-6). Therefore, *Anaerobranca* is predicted to be a favorable bacterium for maintenance of a reducing state in indigo fermentation. On the other hand, Alcaligenaceae species were also predominant in both deteriorated fermentation fluids (Fig. II-6). Although the mechanisms of inhibition of indigo-reduction by Alcaligenaceae are unknown, it is possible that these organisms degrade indigo via the activity of enzymes such as laccase [90, 102].

There remains still a great possibility that a large number of potential indigo-reducing bacteria were not detected by NGS with the primer pair 515F-805R in this study [46]. In addition, the determined gene sequences were not always long enough to identify the corresponding species that were reported as indigo-reducing bacteria. Furthermore, although we detected their presence in this study, several taxa have not been identified as indigo-reducing bacteria. For example, the genus *Anaerobranca* was abundant in both Batch 1 and Batch 2. It was assumed that *Anaerobranca* spp. include indigo-reducing bacteria because of their phylogenetic position adjacent to indigo-reducing bacteria and their Fe^{3+} -reducing characteristics [28].

Many studies have examined the microbiota in natural fermentation systems, such as fermented fish, fermented soybean condiments, lambic beer and fermented rice bran [41, 42, 49, 86, 91, 97, 100]. In most of these cases, fermentation occurs in salty and/or acidic environments. However, the largest difference is that indigo needs to be continuously used for dyeing during

the fermentation process and that the reduction reaction needs to continue after dyeing. Most natural fermentation processes can be transferred to seed cultures of single microbial species because there is little chance of intensive contamination from external environments. However, maintenance of a stable microbiota under alkaline anaerobic conditions is much more important in indigo fermentation than in other natural fermentation processes due to the requirement for long-term maintenance. Determination of the reducing period of the fermentation fluid and the changes in bacterial dynamics during the whole fermentation process will be helpful for identification of a stable and efficient bacterial community for indigo reduction, and the findings will be applicable for the stabilization of other alkaline anaerobic bioprocesses [23, 24, 39, 57].

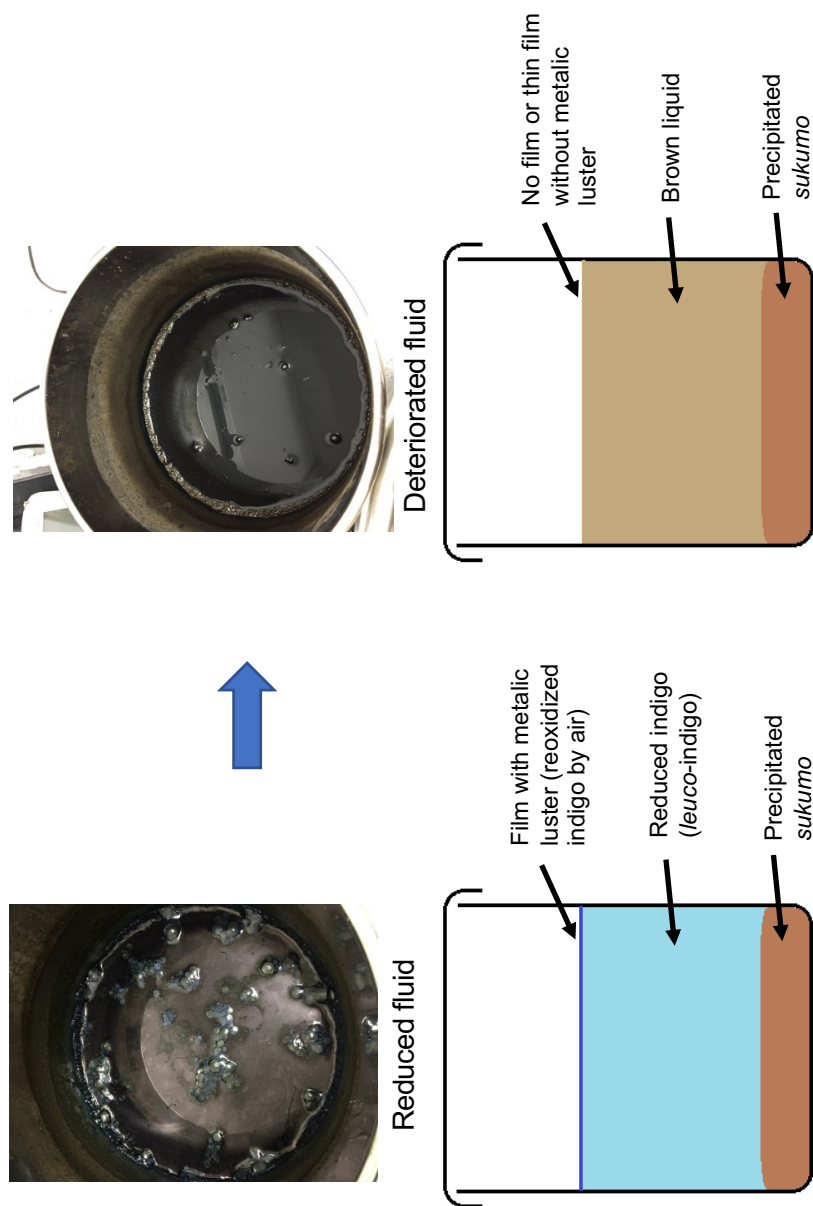


Fig. II-1 Change in appearance of indigo fermentation fluids from the reducing state to deterioration. Reducing fluid (soluble indigo) is characterized by the formation of a film with a metallic luster that is the result of indigo reoxidized upon exposure to air at the surface of the fluid (A). Deteriorated fluid is brown and lacks the film or has a thin film with no metallic luster (B).

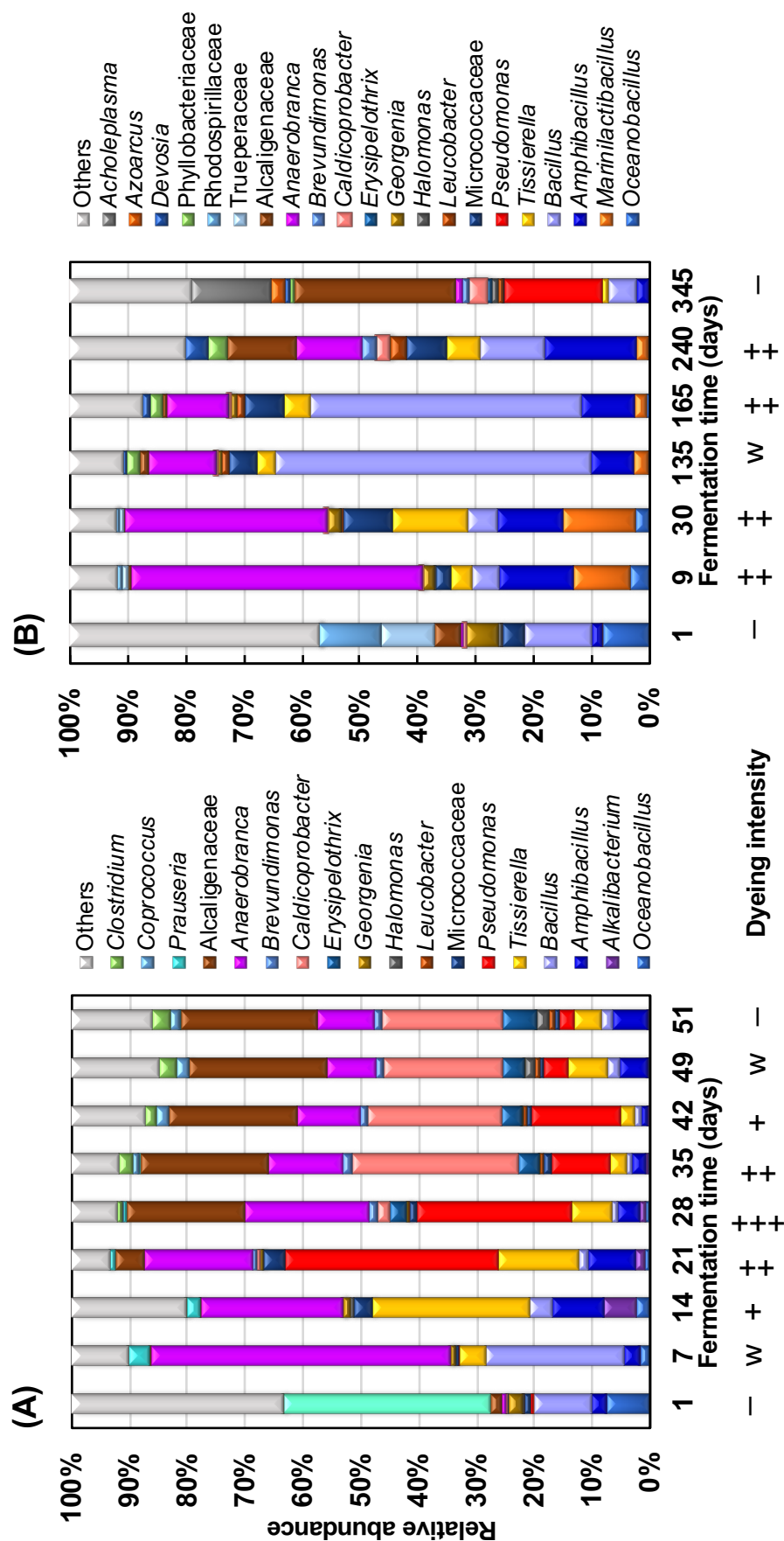


Fig. II-2 Relative abundance of the bacterial constituents of short-term (Batch 1 [A]) and long-term (Batch 2 [B]) batches. Batch 1 (A) was fermented from day 1 to 51 and the long-term Batch 2 (B) was fermented from day 1 to 345 (11.5 months) for indigo fermentation (26 °C). The corresponding staining intensity is described below. ++: very strong, +: strong, w: weak and -: no staining.

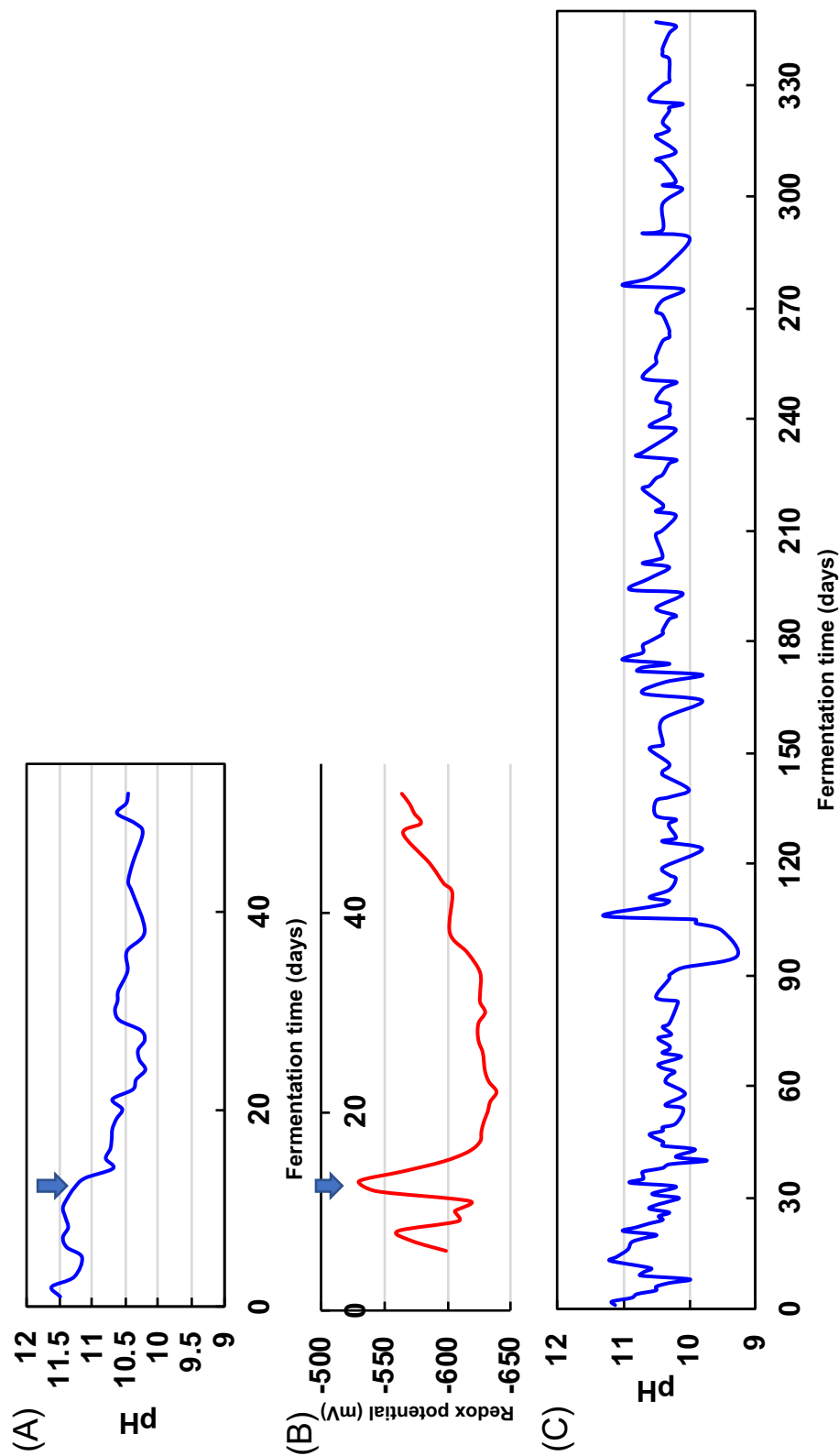
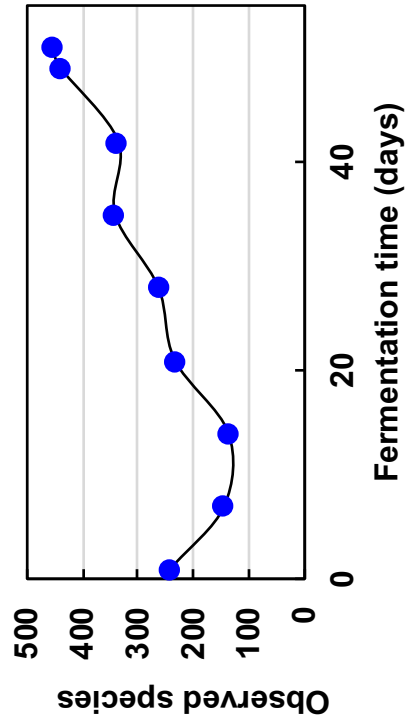


Fig. II-3 Fluctuation in abiotic parameters in indigo fermentation batches. Fluctuation in pH (A) and redox potential (B) in Batch 1 and pH (C) in Batch 2. The batches were stirred every day, and the pH of the fermentation fluid was maintained between 10.3 and 11 in Batch 1 with the addition of Na_2CO_3 , NaOH , $\text{Ca}(\text{OH})_2$, and lactic acid and in Batch 2 with $\text{Ca}(\text{OH})_2$ and K_2CO_3 .

(A)



(B)

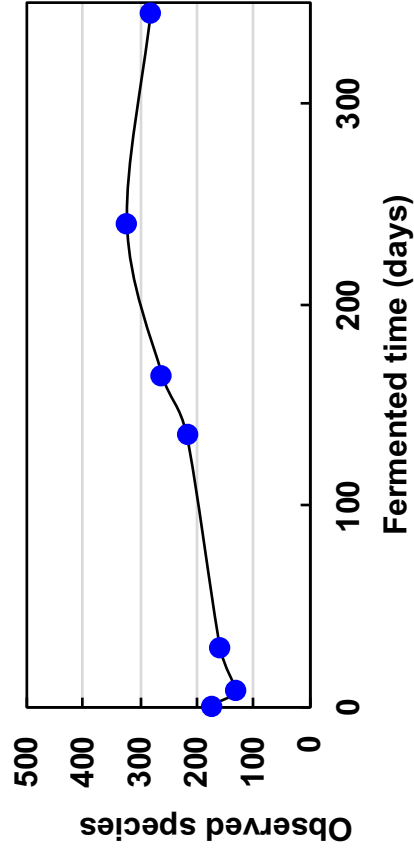


Fig. II-4 Changes in microbial diversity fluctuation in indigo fermentation. Fluctuation based on the number taxonomic units for each sequence read in the samples of Batch 1 (3818 sequences read) (A) and Batch 2 (3730 sequences read) (B). These data were based on the rarefaction curves showing the bacterial diversity of indigo fermentation samples from Batch 1 and Batch 2.

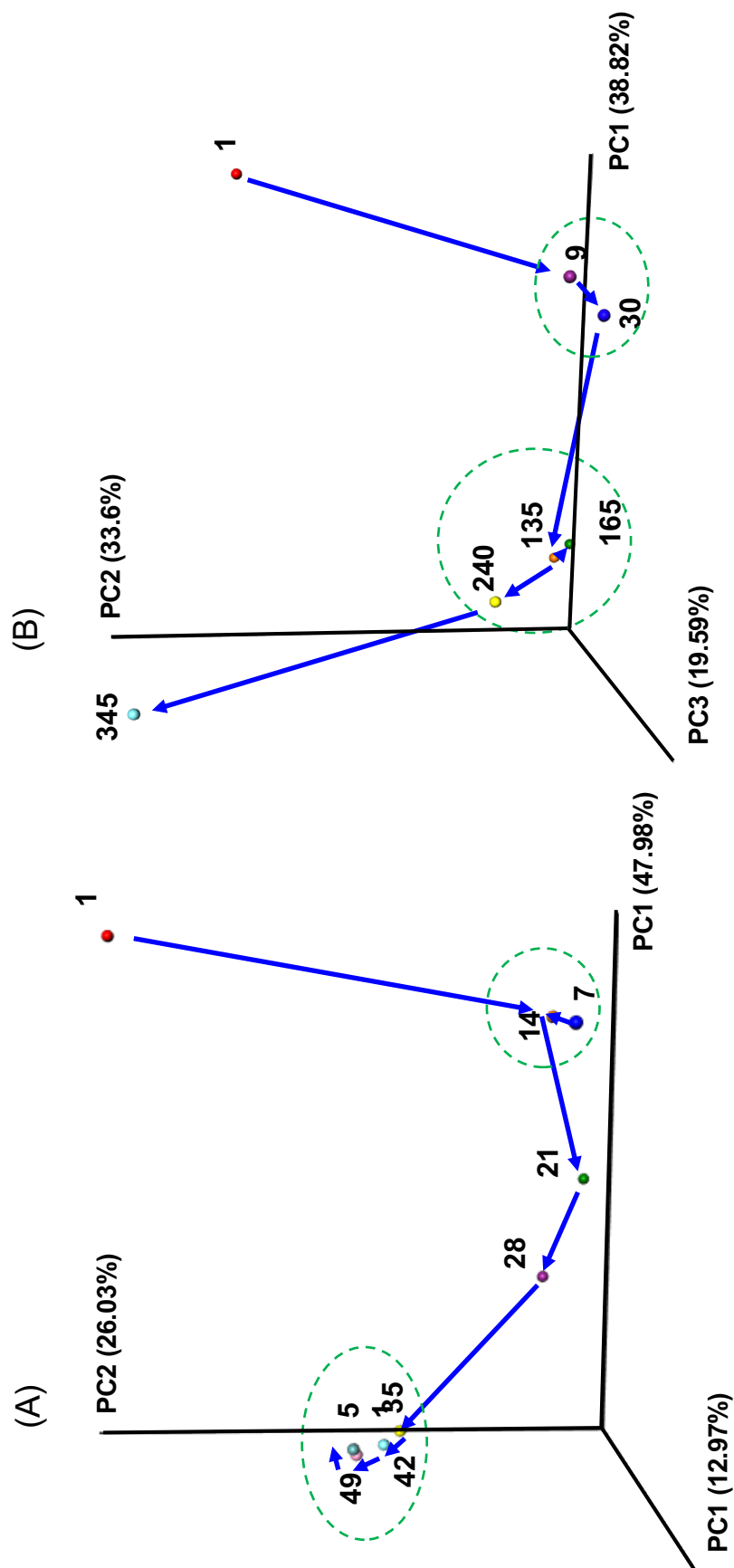


Fig. II -5 PCoA plot showing the changes in bacterial community during indigo fermentation. The plot shows Batch 1 (A) and Batch 2 (B). The numbers beside the symbols represent the fermentation time (days). The arrows indicate the routes of the changes in bacterial community during the fermentation period in Batch 1 and Batch 2. The dotted circles indicate that the microbiota remained stable.

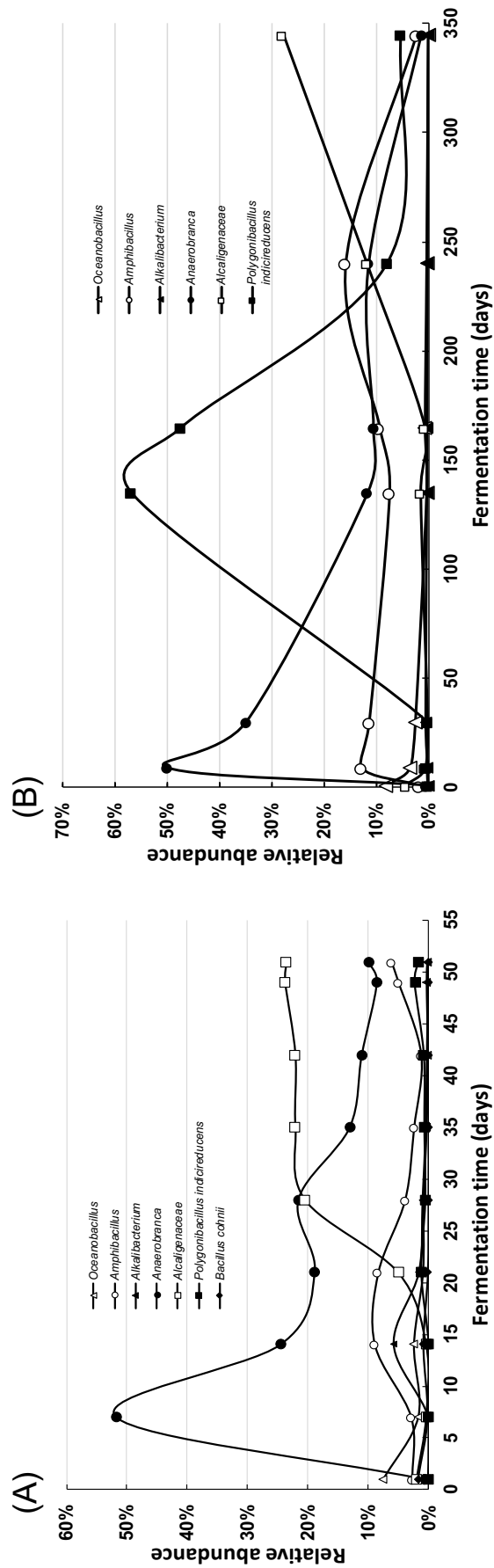


Fig. II-6 Fluctuation in the relative abundance of the indigo-reducing genera and dominant taxa. Short-term Batch 1 (A) and long-term Batch 2 (B) during each fermentation period in different fermentation periods. The presented results are based on next-generation sequence (NGS) analyses.

Chapter III

Transition of the bacterial communities associated with indigo reduction throughout the entire fermentation process

Abstract

The factors contributing to a sustained state of indigo reduction during natural fermentation are not clearly established. In this study, changes in the microbiota throughout the process of *sukumo* reduction were evaluated in three batches with different operating procedures. Batch 1 was fermented for 51 days, Batch 2 was fermented for 345 days, and Batch 3 was fermented for 280 days. All three batches were characterized by an initial increase in Bacillaceae, which survived heat treatment and probably contributed to reductions in the redox potential of the fermentation fluid at initiation, followed by increases in the obligate anaerobe *Anaerobranca* and the indigo-reducing species *Amphibacillus indicireducens*. The early reversal in the ratio of Bacillaceae to *Anaerobranca* was likely important for the initiation of indigo reduction. The maintenance of a high pH and low redox potential (anaerobic environment) as well as the occupation of niches by obligate anaerobes and aerotolerant or facultative anaerobic indigo-reducing bacteria are impotent to maintain a stable indigo-reducing environment. An increase in the rate of neutralophilic *Parapusillimonas granuli* was commonly observed in the deterioration state. This suggests that the ratio of *P. granuli* to indigo-reducing bacteria is important for the deterioration from the indigo-reduced state. From the indigo-reducing state to the deteriorated state, bacterial diversity increased. This can probably be explained by microenvironmental variation at the bottom of the batch. These findings provide a basis for the optimization and expanded use of traditional fermentation for safe, environmentally friendly indigo production.

4.1 Introduction

Indigo is one of the oldest dyes used by humans, with evidence of its use dating back 6,000 years [92]. Currently, indigo dye is used extensively for the production of blue jeans. As a vat dye, insoluble indigo needs to be reduced to its water-soluble form, leuco-indigo. In the traditional process, indigo reduction occurs by natural fermentation, a process that is still used to this day for the production of European woad [16, 30, 61, 75, 77], Japanese *sukumo* [1, 2, 72], and Chinese Landian Yao [52], among other dyes. In the current industrial process, the chemical reductant sodium dithionite is the preferred reducing agent owing to its high efficiency and low cost, but it is not environmentally friendly. Owing to interest in reliable and environmentally friendly alternatives for indigo reduction, the traditional fermentation reduction method has attracted substantial attention.

To improve traditional natural fermentation, it is important to understand the mechanisms underlying the process. A major limitation in the fermentation process is the uncertainty in the occurrence of the reducing state. Additionally, the importance of various factors, such as daily treatments, stirring the fermentation fluid, pH adjustment, and wheat bran feeding, is unclear. In the indigo-reducing state, the fermentation fluid is particularly robust, enabling intense utilization. The repeat immersion of fabrics provides an opportunity for many microbial contaminants and the introduction of oxygen.

The indigo-reducing *Clostridium isatidis* has been isolated from a couched woad (*Isatis tinctoria*) vat prepared by a traditional medieval European procedure [77] and has been detected from a variety of woad vats in the UK and Nigeria [77, 78]. In an analysis of bacterial diversity in fermenting dye vats with woad prepared and maintained in a functional state for approximately 12 months, indigo-reducing bacteria constituted only a small fraction of the unique microcosm [61].

Japanese indigo, called *sukumo*, is composted leaves of knotweed. Traditional *sukumo* reduction is achieved by natural fermentation in an extremely alkaline fluid. Different from European woad, the indigo-reducing species *C. isatidis* has not been found. However, at least 11 indigo-reducing species have been isolated from Japanese indigo fermentation fluids. In 2011, Aino et al. investigated the bacterial community associated with indigo fermentation. The main aerobic bacteria *Halomonas* spp. was replaced with *Amphibacillus* spp. at the beginning of fermentation. These results suggested that indigo-reducing bacteria represent a majority of the bacterial community in the indigo-reducing state [2]. In 2017, Okamoto et al. examined the microbiota in one early-phase batch and two aged batches of Japanese indigo fermentation fluid. They also reported that indigo-reducing bacteria are major components of the bacterial community. These indigo-reducing bacteria fluctuate in abundance and are replaced. The transition in the aged fermentation fluid was slower than that in the early-stage fluid [72].

Changes in the bacterial community in an early stage of *sukumo* fermentation fluid have been evaluated [publication 1]. This previous study also indicated that the appropriate treatment in the initiation of fermentation is important for producing appropriate bacterial communities. Indigo-reduction initiated by the consumption of oxygen by aerobic bacteria (until day 5) is followed by an increase in the abundance of the obligate anaerobe *Anaerobranca* and the aerotolerant *Amphibacillus*.

In another study, bacterial communities in natural indigo fermentation fluids after short-term and long-term reduction were characterized [publication 2]. The stabilization of the microbiota (within the first 10 days) with retention ability is an important factor for the long-term maintenance of indigo reduction. This study showed the microbial community transformed from oxygen-metabolizing aerobes to obligate anaerobes and facultative anaerobes, with gram-negative bacteria as the dominant taxa in the deterioration stage.

The wide range of indigo materials (e.g., *sukumo*, wood ash extract, and feeding substances), preparation conditions (e.g., temperature and pH), and operating methods (e.g., frequency of feeding and stirring efficiency) in indigo fermentation are expected to be key determinants of changes in the microbiota. In this study, three batches of *sukumo* fluids with different reducing terms due to different operation procedures were evaluated by the same analytical methods. The study aims were to explain the similarities and differences in the formation of the indigo-reducing state among batches, the determinants of stable indigo reduction, and the factors that hinder the reduction of indigo with respect to the operation procedure.

4.2 Materials and methods

4.2.1 Indigo fermentation fluids

Three batches of *sukumo* fermentation fluids were used (Table III). Batch 1 was fermented for 51 days without feeding, Batch 2 was fermented for 345 days with low-frequency feeding (every 2-6 weeks and the first feeding on the 22nd day), and Batch 3 was fermented for 280 days with higher-frequency feeding (every 1-2 weeks and the first feeding on the 35th day). In all three batches, the *sukumo* was produced by a craftsman (Mr. Sato) in Tokushima Prefecture, Japan. Both Batch 1 and 3 contained 380 g of *sukumo* and the same weight of wood ash (fine powder of burnt charcoal made from *Quercus phillyraeoides*) in 5 L of water. The wood ash was first added to 5 L of water and boiled by simmering on a stove for 10 min. When the liquid cooled to 60 °C at room temperature, *sukumo* was added and mixed well. The fluid was placed at 26 °C in a thermostatic room protected from light. On the next day, Batch 1 was heated again to 60 °C with constant stirring and then returned to the thermostatic room. Batch 3 was not heated on the next day. Batch 2 was made from 532 g of *sukumo* (produced in Tokushima Prefecture, Japan) and 7 L of wood ash supernatant by heating to 70 °C. It was placed in the same thermostatic room,

heated again to 60 °C on the next day, and then maintained at 26 °C. During the subsequent long-term fermentation, the fluids were stirred every day, and the pH of the fermentation fluid was maintained between 10.3 and 11 with $\text{Ca}(\text{OH})_2$ in addition to a small amounts of Na_2CO_3 , NaOH , and lactic acid for Batch 1; with $\text{Ca}(\text{OH})_2$ and a small amount of K_2CO_3 for Batch 2; and with only $\text{Ca}(\text{OH})_2$ for Batch 3. $\text{Ca}(\text{OH})_2$ was also used for acid precipitation. The redox potential was measured using a 9300-10D (Horiba, Kyoto, Japan) electrode.

The reduction state of indigo was roughly evaluated by cotton cloth dyeing. In this method, a piece of white cotton cloth of approximately 2 cm × 3 cm was soaked in the fermentation fluid for 30 sec, removed, and exposed to air for oxidation for 5 min. Impurities are rinsed in flowing tap water.

4.2.2 DNA extraction and library preparation

The samples were centrifuged at $15,000 \times g$ for 10 min to obtain the sample pellet. Total bacterial DNA was directly extracted from the sample pellets using ISOIL (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The V3-V4 region of the bacterial 16S rRNA gene was PCR-amplified using a composite pair of primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). The PCR for sample of Batch 1 and 2 was performed in a solution of 100 μL containing 20 μL of 5× Phusion HF buffer (Thermo, Waltham, MA, USA), 2 mL of a 2.5 mM dNTP mixture (TaKaRa, Otsu, Japan), 25 ng of isolated DNA, 2 U of Phusion Hot Start II DNA polymerase (Thermo), and 50 pmol of each primer. The amplification reactions were performed under the following conditions: initial thermal denaturation at 98 °C for 30 sec, followed by 25 cycles of heat denaturation at 98 °C for 10 sec, annealing at 55 °C for 20 sec, and extension at 72 °C for 30 sec.

The PCR for sample of Batch 3 was performed in a solution of 20 μL containing 2 μL of 10×

Ex buffer, 1.6 μ L of a 2.5 mM dNTP mixture, 1 units of Ex-taq (above TaKaRa, Otsu, Japan), 1 ng of isolated DNA, and 1 μ L of each 10 μ M primer. The amplification reactions were performed under the following conditions: initial thermal denaturation at 94 °C for 2 min, followed by 20 cycles of heat denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec.

4.2.3 Next-generation sequencing

The 1st PCR products of samples from Batch 1 and 2 were submitted to Hokkaido System Science Co., Ltd. (Sapporo, Japan) for sequencing. The PCR products for samples from Batch 3 were submitted to the Bioengineering Lab. Co., Ltd. (Sagamihara, Japan). The 2nd PCR was performed with an index-adapted primer to generate paired-end (2×301 bp) libraries for Illumina shotgun sequencing, and the products were purified. Next-generation sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA). The raw reads were preprocessed, and all failed sequence reads and low-quality reads, tags, and primer sequences were removed. A clustering analysis based on operational taxonomic units (OTUs) with a 97% identity threshold and taxonomic classification were performed using QIIME version 1.9.1 [12]. A taxonomic analysis and annotation of the output data were also performed using QIIME, including OTU picking, taxonomic assignment, diversity analyses, and graphical visualization [12, 82]. Nucleotide sequence identity was estimated with bacterial 16S rRNA reference sequences in the BLAST database [43].

4.2.4 Diversity analysis

Alpha diversity is a common indicator of species diversity. In this study, rarefaction curves were computed for sequences and observed OTUs at a 97% similarity level with respect to the

total number of reads for each sample. OTU curves were obtained to evaluate trends in bacterial species detection. In addition, beta diversity was computed; beta diversity analyses involve the explicit comparison of microbial communities based on the composition and assessments of the differences between microbial communities. A principal coordinate analysis (PCoA) based on Bray-Curtis distances was used to evaluate the similarity of the microbiota in every sample. Both rarefaction curves and PCoA were implemented in QIIME version 1.9.1.

4.3 Results

4.3.1 Reduction state

The reducing state of the fermentation fluids can be qualitatively evaluated by dyeing. Batch 1 showed staining on day 7 of fermentation and the staining became substantially lighter on day 49, with no staining on day 51. Batch 2 showed staining on day 6, and staining was lost on day 345. Batch 3 showed slight staining on day 4 and deep staining on day 8. On days 31 and 63, the staining intensity decreased. During this period, the pH was adjusted to around 10.6 by the addition of wheat bran and wheat gluten, and the staining intensity gradually recovered. After 280 days, the staining gradually decreased and was finally lost around day 350. The duration of the reducing state in Batch 1 was significantly shorter than those of Batch 2 and 3. This can be explained by the lack of nutrients for indigo-reducing bacteria or the early growth of miscellaneous bacteria due to insufficient stirring.

4.3.2 Bacterial community analysis by NGS

The bacterial community structure of the three batches of *sukumo* fermentation fluid was analyzed based on OTUs identified by NGS (Fig. III -1, III -2 and III -3). There were 26 taxonomies in Batches 1 and 3, and 28 taxonomies in Batch 2. At the beginning of the

fermentation, the *sukumo*-derived bacterial populations (including *Georgenia*, *Prauseria*, and members of the Alphaproteobacteria and Trueperaceae) were quickly replaced by newly emerging populations, first of Bacillaceae, and then of Anaerobrancaceae and the indigo-reducing taxa *Amphibacillus* and *Alkalibacterium*. In the late stages of fermentation, Alcaligenaceae tended to increase. In Batch 3, *Pseudomonas* was present consistently throughout the fermentation period. This is probably due to the difference in heat treatment at the start of fermentation. The abundance of Alcaligenaceae and *Pseudomonas* was higher after day 21 in Batch 1. We consider this to be related to the higher microbial diversity and deterioration of the fluid.

The minor OTUs ($\leq 2\%$ in each sample) were combined; their total percentages are shown above each figure. The relative content of the communities comprising these minor OTUs showed a U-shaped curve throughout the fermentation process, during which the initial reduction (day 7 in Batch 1, day 6 in Batch 2 and day 8 in Batch 3) represented the minimum value. At this time, the presence of the dominant bacterial population (e.g., Anaerobrancaceae) inhibited the growth of minor bacteria.

4.3.3 Bacterial diversity

The prominent taxa with a relative content of at least 1% were selected for analyses of total counts and percentages (Fig. III-4). Five taxa in Batch 1 on day 7 accounted for 91.9% of all bacteria. Seven taxa in Batch 2 on day 6 accounted for 91.9% of bacteria. On days 4 and 8 in Batch 3, 7 and 9 taxa accounted for 95.6% and 92.4% of bacteria, respectively. Combined with the reduction status of the fermentation broth, our results indicated that at the initial stage of reduction, a small number of taxa were dominant.

The rarefaction curves based on the observed OTUs are shown in Fig. III-5. In all 3 batches, the observed OTUs decreased gradually after the beginning of fermentation, reached a minimum

at the beginning of reduction, and gradually increased until weakening and deterioration. These findings show that during the reduction process in *sukumo* fermentation, the general trend in bacterial diversity can be described by a slight increase at the beginning, followed by a reduction, and then a relatively rapid increase followed by a gradual increase. This pattern reflects the increase in Bacillaceae, followed by its reduction, and the increasing diversity of alkaline anaerobic microorganisms, followed by an increasing trend towards a neutrophilic microenvironment.

Fig. III-6 summarizes the PCoA results for the similarity among bacterial communities based on distances between samples. A few days before reduction was observed (e.g., S to day 7 in Batch 1, days 0-6 of Batch 2, and days 0-8 of Batch 3), the bacterial communities changed rapidly. After reduction began (e.g., days 7-14 of Batch 1, days 6-26 of Batch 2, and days 8-14 of Batch 3), there was a short period of stability. It should be noted that the short-lived Batch 1 had a drastic change in the community within the 7-day period from days 14 to 21. This drastic change was not observed in the long-lived Batch 3 after day 2. In the long-lived Batch 2, a change of an equivalent magnitude was detected over the 115-day period from days 26 to 141. These results show that the long-term stable maintenance of indigo reduction is related to the stability of the bacterial community.

4.3.4 Aerobes and anaerobes

The frequencies of Bacillaceae and Anaerobrancaceae were very high in all bacterial communities in the early stage of fermentation. Representative sequences of the dominant OTUs were identified by BLAST searches against the bacterial 16S rRNA sequence database. The indigo-reducing bacteria (e.g., *Oceanobacillus indicireducens*) were eliminated and the remaining spore-forming aerobes, mainly *Bacillus* and small numbers of *Virgibacillus* (97-99%),

Sinibacillus (97-99%), and *Pseudogracilibacillus* (96-99%), were the major taxa belonging to Bacillaceae, as shown in Fig. III-7. Representative sequences of the dominant OTUs in the family Anaerobrancaceae were identified as *Anaerobranca* spp., an obligate anaerobe. Spore-forming aerobes in Bacillaceae from *sukumo* had relative contents of at least 14.3%. These spore-forming aerobes increased on day 1 (Batch 1 and 2) or day 2 (Batch 3), but then decreased rapidly, corresponding to a surge in the obligate anaerobic *Anaerobranca*, which only accounted for 0.2% of bacteria at the beginning (in *sukumo*) but increased to 56.1% (day 7 of Batch 1), 62.8% (day 6 of Batch 2), and 39.6% (day 2 of Batch 3). Considering the redox potential, we found that the consumption of oxygen by aerobes promotes the rapid decrease in redox potential, which is beneficial for the reduction of indigo. At the beginning of fermentation, when the relative content of Bacillaceae was greater than that of Anaerobrancaceae, the redox potential dropped sharply, and indigo reduction did not yet occur. When Bacillaceae exceeded Anaerobrancaceae, reduction started. Thereafter, these taxa were gradually replaced by other bacterial populations.

4.3.5 Neutralophilic bacteria

The family Alcaligenaceae (Fig. III-8) was common in fermented *sukumo* fluids of different origins, especially in the late stage of fermentation and deteriorating fluids (e.g., days 28-51 of Batch 1, day 345 of Batch 2, and days 28 and 280 of Batch 3). Alcaligenaceae was derived from *sukumo* (9.4%), and its relative content was suppressed to the lowest level at the beginning of the reduction period, reaching 0.2% on day 7 of Batch 1, 0.5% on day 6 of Batch 2, and 0.1% and 1.1% on days 4 and 8 of Batch 2. It is important to note that the short-lived Batch 1 consistently contained a large number of Alcaligenaceae since day 28, while the long-lived Batch 2 and 3 had no great increases in Alcaligenaceae in the middle of the fermentation period. The increase in the abundance of Alcaligenaceae tended to coincide with an increase in bacterial diversity when the

pH decreased and redox potential increased. Syrup is conventionally added as a nutrient source, but in our analysis, syrup had a negative effect on indigo reduction. Furthermore, when the content of Alcaligenaceae exceeded 10%, the risk of deterioration of the fermentation fluid increased. In addition, representative sequences of the dominant OTUs assigned to the family Alcaligenaceae were *Parapusillimonas granuli* (427/427 bp, 100%), a neutrophilic bacterium [45]. In addition, 5 strains of *P. granuli* were isolated from Batch 3. Growth was optimal at pH 7-8 in liquid clostridial nutrient medium, and proliferation was not detected above pH 10. Although the pH of *sukumo* fermentation fluids was maintained at above 10, a large number of neutrophilic bacteria appeared in the late stage of fermentation, especially in the short-lived Batch 1 and the deteriorated Batch 2.

4.3.6 Indigo-reducing bacteria

Based on the research of indigo-reducing species shown in Table 1, there are 7 species of indigo-reducing bacteria in Batch 1 and 3, and 8 species in Batch 2 were detected, although there may be other unknown indigo-reducing species, such as *Anaerobranca* spp. as discussed in Chapter II. Changes in the percentage of each indigo-reducing bacterium and in total indigo-reducing bacteria over time are shown in Fig. III-9. With respect to the total reducing bacteria, the maximum in batch 1 was only 14.5% (day 14) and the frequency was typically less than 10%. The maximum frequencies in Batch 2 and 3 were 66.5% (day 135) and 40.9% (day 224) and they were typically above 20%. The total content of indigo-reducing bacteria in long-lived Batch 2 and 3 was significantly higher than that in short-lived Batch 1. In particular, it should be noted that all 3 batches of indigo-reducing bacteria showed an increase and subsequent decrease at the beginning of fermentation, but the increases in Batch 2 and 3 were higher after feeding, while the unfed Batch 1 exhibited an increase to a limit of about 15%. Due to the difference in pretreatment

methods, although they were all derived from the same *sukumo*, the species of indigo-reducing bacteria at the initial stage differ among the 3 batches of fluids, including *Oceanobacillus indicireducens* and *Bacillus cohnii* in Batch 1, *Alkalibacterium indicireducens* and *Amphibacillus indicireducens* in Batch 2, and *Bacillus cohnii* and *Am. indicireducens* in Batch 3. In addition, *Polygonibacillus indicireducens* emerged in the middle of the fermentation period and coexisted with *Am. indicireducens* until the end. Among all detected indigo-reducing species, only *Am. indicireducens* showed an increase in the initial reduction in Batch 1, 2, and 3 (from 0.17% to 1.54%, 0.22% to 7.60%, and 0.42% to 1.51% and 3.15%), and these changes coincided with the change in dominance from Bacillaceae to Anaerobrancaceae. Although the percentages are constantly changing, only *Am. indicireducens* was abundant throughout the whole process. In all 3 batches of fermentation fluids, the relative content of indigo-reducing bacteria changed, forming a relay-like relationship throughout the whole fermentation process.

4.4 Discussion

We evaluated similarities and differences in microbial changes in 3 batches of *sukumo* fermentation fluid. A common feature was the initial reduction of indigo, accompanied by the reversal of the dominance of obligate anaerobes, i.e., Anaerobrancaceae, relative to spore-forming aerobes, i.e., Bacillaceae. This indicates that pretreatment (60 °C) is important for the selection of necessary microorganisms. With respect to differences among the 3 batches, the proliferation of indigo-reducing bacteria was lower in the short-lived Batch 1 than in other long-lived batches. This can probably be explained by the high pH (around pH 11.5) at the early fermentation stage (i.e., within 10 days) [publication 2]. This indicates that pH regulation at the induction period is very important to induce a favorable microbiota.

Sukumo is pretreated by the addition of hot (60-80 °C) ash extract. This sudden

environmental change in *sukumo*, resulting in a strong alkali state and transient high temperature, selected for a heat-resistant and strong alkali-resistant population, including spore-forming aerobes in Bacillaceae (Fig. III-7). Bacillaceae proliferated rapidly during the early stages of fermentation. As a result, the aerobic state in the fermentation fluid was instantly changed to a micro-anaerobic (dissolved oxygen in the fluid) or anaerobic condition. The consumption of oxygen concomitant with the high pH background led to a rapid drop in the redox potential of the fermentation fluid. The decrease in the redox potential accelerates the construction of a strictly anaerobic environment, which causes a sudden reversal of the dominant position between obligate anaerobic Anaerobrancaceae and aerobic Bacillaceae. Previous reports have shown that an initial drop in redox potential is not directly linked to indigo reduction [2]. Another study has shown a substantial change in the microbiota occurs in the early stage of fermentation, characterized by appearance of Bacillaceae and thereafter by *Anaerobanca* sp. [publication 1]. In this study, data from 3 different batches indicated that indigo reduction occurs when the ratio is reversed from Bacillaceae as the dominant group to Anaerobrancaceae in *sukumo* fermentation fluids.

The maintenance of the indigo-reducing state during long-term indigo fermentation is an important goal. Among the three batches, only Batch 1 was short-lived. This was presumably related to the low ratio of indigo-reducing bacteria. For example, the maximum frequency of *Am. indicireducens* was around 6% in Batch 1, whereas the maximum frequencies were around 15% and 24% in Batch 2 and Batch 3, respectively. The total ratio of reducing bacteria in batch 1 was only 14.5% (day 14) and was often less than 10%, whereas those for Batch 2 and 3 reached 66.5% (day 135) and 40.9% (day 224). In addition, the ratio of indigo-reducing bacteria was mostly above 20% in both Batch 2 and Batch 3. This low ratio of indigo-reducing bacteria in Batch 1 is probably due to the high pH at the early fermentation stage. The pH of the fermentation fluid remained at around 11.5 for the first 10 days after the initiation of fermentation. On the other hand,

the pH during the early fermentation period in first 10 days were always kept at below pH 11.3 in both Batch 2 and Batch 3. Although a high pH is thought to effectively eliminate unfavorable species for indigo fermentation, it also damages bacteria that are beneficial for this process. Therefore, the regulation of the initial pH is very important for the generation of a favorable, long-lasting microbiota.

A key issue in indigo fermentation is the maintenance of the indigo-reducing state. During the practical dyeing process, fabric is continuously immersed in the fermentation fluid. Therefore, the dyeing process itself provides many opportunities for contamination with microorganisms from outside of the fermentation fluid. Therefore, retaining the capacity of the fermentation fluid is central to maintaining the fermentation process. Comparing the 3 batches, only Batch 1 exhibited rapid changes in the microbiota, especially on days 1-7 and days 14-21. Although rapid changes were also observed in both Batch 2 and Batch 3, the rates of change decreased after day 8 day in these cases. The decreased rate of change in the microbiota indicates that both Batch 2 and Batch 3 entered a stable state. In this state, indigo-reducing bacteria and obligate anaerobic bacteria accounted for the majority of the microbial community. Therefore, we presume that this reflects a stable state in the fermentation process, during which changes in the microbiota slowly result from the low redox potential and high pH, and niches are occupied by indigo-reducing bacteria and obligately anaerobic bacteria.

The results of this study also indicated that the risk of the deterioration of the indigo-reducing state increases as the rate of neutrophilic bacteria, i.e., *P. granuli*, reaches 10% or more. This increase was associated with an increase in microbial diversity. As plant residues (e.g., cellulose) in *sukumo* are gradually decomposed, the fermentation liquid becomes thick and uneven stirring is likely. In this case, the microenvironment exhibits a lower pH, leading to a gradual increase in diversity and ultimately to deterioration. It is speculated that a large number of

microenvironments are created in the liquid due to the production of biofilms, especially when metabolites accumulate in the late fermentation stage. The maintenance of a stable and high pH is necessary for a long-term indigo-reducing state. A large number of neutrophilic (*P. granuli*) appear in the fermentation broth above pH 10, indicating that there are a large number of microenvironments with a pH lower than 10 in the fermentation fluid, which is not conducive to indigo reduction. *P. granuli* was abundant in short-lived Batch 1 and in deteriorating Batch 2 and 3, showing that a high rate of neutrophilic *P. granuli*, along with increases in redox potential and bacterial diversity lead to deterioration. This raises the possibility that *P. granuli* is related to the deterioration of the fermentation fluid. We have attempted to add the isolated *P. granuli* to the reduced *sukumo* fermentation fluid, but a correlation between *P. granuli* and the deterioration of reduction or to a rise in redox potential has not been demonstrated previously. In the traditional process, it is very difficult to predict the deterioration of the fermentation fluid. Generally, practitioners rely on experienced craftsmen to monitor subtle changes in color and odor. Our findings support the use of *P. granuli* as an indicator of the deterioration of indigo reduction, enabling the early implementation of steps to avoid deterioration, such as raising the pH and stirring more thoroughly.

In anaerobic conditions, the initiation of indigo reduction is associated with an increase in indigo-reducing bacteria, especially *Am. indicireducens*. The high rates of *Am. indicireducens* in Batch 2 and 3 may reflect the addition of wheat bran to these two batches of fluid. *Am. indicireducens* has the ability to degrade cellulose and xylan, components of wheat bran. This species plays a particularly important role in the whole indigo-reducing process. Two previous studies of the bacterial community during *sukumo* fermentation have shown that indigo-reducing bacteria are dominant in the fermentation broth [2, 72]. Consistent with these findings, the analysis of 3 batches of *sukumo* fluid in this study showed that long-term indigo reduction requires

relatively high frequencies of several species of indigo-reducing bacteria.

Despite those previous studies, this study is the first to clarify the relationships between aerobic bacteria, anaerobic bacteria, and indigo reduction in *sukumo* fermentation fluid; in particular, aerobes consume oxygen to promote anaerobic growth, decrease redox potential, and increase indigo reduction. It is essential to consume dissolved oxygen in the fermentation fluid in the early stages of fermentation. However, stirring and irregular dyeing inevitably introduce oxygen. The fermentation of *sukumo* itself occurs in the open air, making it impossible to avoid the dissolution of oxygen. Therefore, to maintain the reduced state, it is critical to continuously consume the dissolved oxygen in the fermentation broth. The *sukumo* fermentation fluid always form a film with a copper luster on the surface even after stirring, which is re-oxidized indigo by air from leuco-indigo [publication 2]. Besides, in addition to the re-oxidized indigo, the composition of this film can also contain a variety of bacteria, especially aerobic bacteria, which need to be further confirmed in future research. In the fermentation fluid made by the precipitation indigo instead of *sukumo*, leuco-indigo re-oxidized by air can only form a blue suspension layer on the upper part of the liquid and cannot produce this kind of film with a copper luster. Although the film is believed to help insulate oxygen from the open air, further studies are needed to explore whether the film and its specific composition contribute to the long-term maintenance of *sukumo* fermentation.

Overall, the results of this study provide insight into critical factors for indigo fermentation. In particular, they emphasize the importance of appropriate pretreatment and conditioning in the early fermentation phase to provide the necessary conditions for the appropriate microbiota. In addition, daily maintenance is important, including stirring the fluid to prevent the formation of a localized neutral pH environment and the balance of anaerobes and aerobes. The continuous feeding of the appropriate microbiota is effective to maintain a high ratio of indigo-reducing

bacteria (i.e., *Am. indicireducens* and *Al. indicireducens*).

Table III Characteristics of three batches of Japanese indigo fermentation fluid.

Batch No.	<i>Sukumo</i> source	Contained <i>sukumo</i> (g)/ Ash extract (L)	Pre-heating	Staining-beginning/ -ending	First feeding/ Frequency	Feeding substance	Start pH/ Main pH range (average level)	pH-maintaining substance	Stirring tool	Sampling day
1	Tokushima	380/ 5 (76 g/L)	1st day 60 °C, 2nd day 60 °C	7th day/ 51st day	no	-	11.5/ 10.2-11.5 (10.5)	Ca(OH) ₂ , Na ₂ CO ₃ , NaOH	glass rod	1, 7, 14, 21, 28, 35, 42, 49, 51
2	Tokushima	532/ 7 (76 g/L)	1st day 70 °C, 2nd day 60 °C	6th day/ 345th day	22nd day/ every 2-6 weeks	wheat bran, wheat gluten (little syrup)	11.1/ 9.8-11.1 (10.5)	Ca(OH) ₂ , CaCO ₃	lab spoon	0, 1, 4, 6, 8, 26, 141, 178, 269, 345
3	Tokushima	380/ 5 (76 g/L)	1st day 60 °C	8th day/ 350th day	35th day/ every 1-2 weeks	wheat bran (little wheat gluten and syrup)	10.8/ 9.9-11.1 (10.5)	Ca(OH) ₂	lab spoon	0, 2, 4, 8, 14, 28, 56, 84, 112, 140, 168, 196, 224, 252, 280

[illegible]

Fig. III-1 Relative abundance of the bacterial constituents ($>2\%$ in any sample and total minor OTUs ($\leq 2\%$ in each sample)) of Batch 1. g, genus; f, family; o, order; c, class; p, phylum.

Batch 2											
Minor OTUs (≤ 2% in each sample)											
	0	1	4	6	8	26	141	178	269	345	
o_Actinomycetales;Other	8.9%	9.0%	6.0%	2.6%	2.4%	2.4%	4.5%	6.5%	16.7%	18.3%	
Georgiella	3.8%	2.9%	1.9%	0.8%	0.7%	0.8%	0.4%	0.7%	0.0%		
Leucobacter	6.9%	11.2%	6.7%	1.2%	2.5%	3.2%	0.6%	0.9%	1.0%		
f_Micrococcaceae;g_				3.5%	3.1%	7.4%	5.1%	7.6%	11.6%	0.8%	
Yaniella	4.9%	5.2%	3.2%	0.5%	1.0%	1.4%	0.5%	0.4%	0.3%		0.5%
f_Flavobacteriaceae;g_											1.0%
o_Bacillales;f_	3.3%	6.6%	4.2%	0.8%	1.1%	1.3%	0.8%	0.0%	0.7%	2.7%	1.5%
f_Bacillaceae;Other	7.4%	2.8%	2.3%	0.7%	0.8%	1.0%	0.9%	1.1%	1.1%	0.5%	2.0%
f_Bacillaceae;g_	21.3%	30.0%	19.1%	4.7%	7.5%	8.2%	3.4%	4.3%	2.7%	0.9%	2.5%
Bacillus	4.0%	5.6%	4.5%	0.9%	2.8%	3.2%	61.4%	51.5%	10.1%	6.7%	3.0%
Amphibacillus	0.5%			8.6%	10.8%	10.6%	7.1%	9.5%	18.4%	6.1%	3.5%
Alkalibacterium			38.2%	10.1%	9.6%	12.8%	2.4%	2.6%	2.3%		4.0%
c_Clostridia;o_	1.9%	3.4%	1.4%	0.5%	0.5%	1.2%	0.5%	0.5%	0.5%		4.5%
Caldicoproductor	0.5%	0.6%	0.4%								5.0%
Tissierella				0.3%	3.9%	12.7%	2.7%	3.3%	7.8%	4.2%	5.5%
c_Clostridia;o_MBA08;f_											6.0%
f_Anaerobranchaceae;g_	0.7%	0.4%	0.8%	62.8%	50.4%	31.5%	5.9%	5.1%	4.6%	1.6%	6.5%
f_Erysipelotrichaceae;g_PSB-M-3											7.0%
p_Gemmatimonadetes;c_Gemm-3;o_	5.4%	2.3%	1.0%	0.3%	0.4%	0.0%					7.5%
c_Alphaproteobacteria;o_	12.7%	8.2%	3.8%	0.7%	0.9%	0.7%					8.0%
Devosia											8.5%
f_Phyllobacteriaceae;g_							0.7%	1.3%	5.4%	2.6%	
f_Alcaligenaceae;g_							1.8%	2.2%	6.6%	1.1%	
f_Pseudomonadaceae;g_	5.9%	4.3%	2.5%	0.5%	0.4%	0.3%	1.0%	0.5%	6.3%	25.3%	9.0%
Pseudomonas										7.8%	9.5%
Luteimonas											10.0%
Acholeplasma	2.3%	1.8%	0.7%							2.7%	>10.0%
f_Trueperaceae;g_B-42	9.4%	4.9%	2.7%	1.0%	1.1%	1.2%					

Fig. III-2 Relative abundance of the bacterial constituents (>2% in any sample and total minor OTUs (≤ 2% in each sample)) of Batch 2. g, genus; f, family; o, order; c, class; p, phylum.

Batch 3																
Minor OTUs (≤ 2% in each sample)																
	0	2	4	8	14	28	56	84	112	140	168	196	224	252	280	
<i>Georgenia</i>	11.4%	1.1%	2.2%	3.1%	8.8%	8.1%	9.7%	7.7%	6.8%	6.2%	6.5%	6.7%	6.7%	8.4%	8.0%	0.5%
<i>Brachybacterium</i>	3.4%	0.3%	0.3%	0.3%	0.6%	0.4%	0.4%	0.4%	0.5%	0.5%	0.4%	0.7%	0.8%	0.7%	0.5%	1.0%
<i>Prauseria</i>	2.1%															1.5%
<i>Yaniella</i>	9.8%	0.7%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.4%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	2.0%
<i>o_Bacillales;f_</i>	2.5%	0.5%	0.5%	0.4%	0.3%	0.3%	0.3%	0.4%	0.4%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	2.5%
<i>f_Bacillaceae;g_</i>	4.6%	0.3%	0.3%	0.4%	0.5%	0.3%	0.3%	0.4%	0.4%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	3.0%
<i>Anaerobacillus</i>	28.3%	12.3%	10.5%	4.1%	3.7%	1.9%	1.5%	1.4%	2.2%	1.9%	3.2%	2.7%	2.7%	2.3%	1.2%	3.5%
		2.4%	2.9%	0.8%												4.0%
<i>Bacillus</i>	2.2%	35.1%	11.2%	0.6%	1.8%	1.7%	0.8%	1.8%	7.3%	8.0%	15.0%	14.6%	21.6%	23.5%	12.5%	4.5%
<i>Amphibacillus</i>					4.9%	5.5%	3.9%	28.5%	16.9%	21.7%	7.4%	6.0%	27.4%	16.7%	18.8%	5.0%
<i>Alkalibacterium</i>		0.6%	2.0%					0.7%	6.7%	10.4%	8.5%	6.9%	3.0%	2.5%	2.2%	5.5%
<i>c_Clostridia;o_</i>	2.8%	0.3%														6.0%
<i>o_Clostridiales;f_</i>																6.5%
<i>Caldicoprobacter</i>	0.6%				5.8%	24.2%	9.0%	5.0%	2.8%	1.7%	2.0%	0.8%	0.7%	0.7%	1.4%	7.0%
<i>Alkaliphilus</i>			14.9%	11.5%	2.4%	0.5%										7.5%
<i>Clostridium</i>					2.7%	3.2%	3.9%	5.1%	3.4%	1.9%	1.4%	0.5%	0.3%	1.8%	2.7%	8.0%
<i>f_Tissierellaceae;g_</i>				2.7%	2.8%	1.2%	1.5%	0.7%	0.5%	0.5%	0.6%	0.5%	0.5%			8.5%
<i>Tissierella</i>			0.5%													9.0%
<i>f_Anaerobrancaceae;g_</i>				10.5%	13.8%	7.4%	6.1%	6.6%	5.8%	4.6%	6.2%	3.8%	6.3%	5.0%	5.3%	9.5%
<i>o_Thermoanaerobacterales;f_</i>			39.6%	40.0%	33.9%	24.9%	10.8%	6.1%	4.1%	5.4%	7.3%	16.1%	7.3%	5.3%	3.3%	10.0%
<i>f_Erysipelotrichaceae;g_PSB-M-3</i>								1.9%	5.3%	3.3%	2.5%	0.4%	0.4%	0.4%	2.8%	8.5%
<i>p_Gemmatimonadetes;c_Gemm-3;o_</i>																9.0%
<i>c_Alphaproteobacteria;o_</i>	8.9%	0.6%														9.5%
<i>f_Alcaligenaceae;g_</i>	14.6%	0.6%														10.0%
<i>Rheinheimera</i>	3.9%	0.3%			1.1%	2.1%	9.7%	3.3%	1.9%	2.7%	5.6%	6.2%	1.3%	2.0%	8.8%	>10.0%
<i>Pseudomonas</i>					0.5%	0.5%	0.4%	0.8%	1.4%	2.8%	2.6%	2.3%	4.1%	8.6%	6.7%	
<i>f_Trueperaceae;g_B-42</i>																
	4.9%	5.1%	14.1%	22.7%	15.7%	18.3%	12.2%	22.3%	12.2%	17.0%	17.6%	23.4%	12.2%	16.3%	21.5%	

Fig. III-3 Relative abundance of the bacterial constituents (>2% in any sample and total minor OTUs (≤ 2% in each sample)) of Batch 3. g, genus; f, family; o, order; c, class; p, phylum.

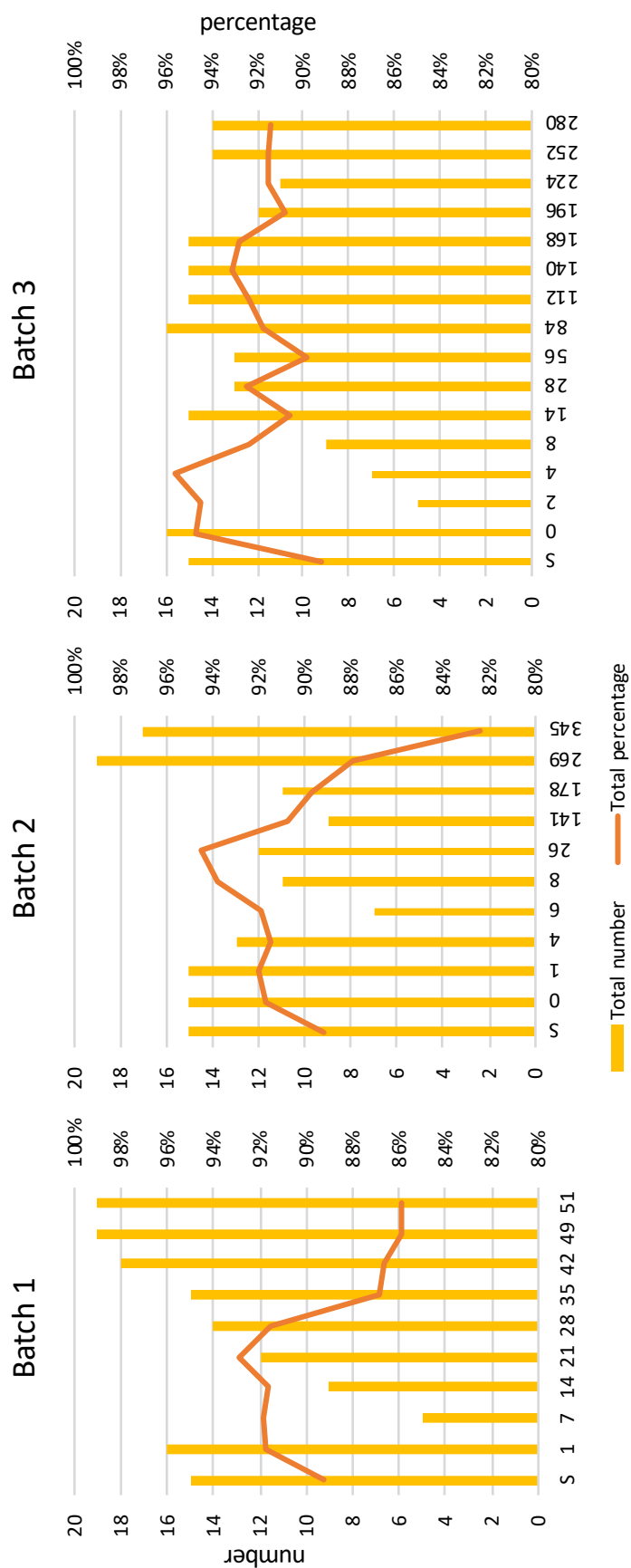


Fig. III-4 Total count and total percentage of prominent taxa (relative content $\geq 1\%$). *S*, *sukamo*; The other numbers on the horizontal axis indicate the samples of fermented days in each batch.

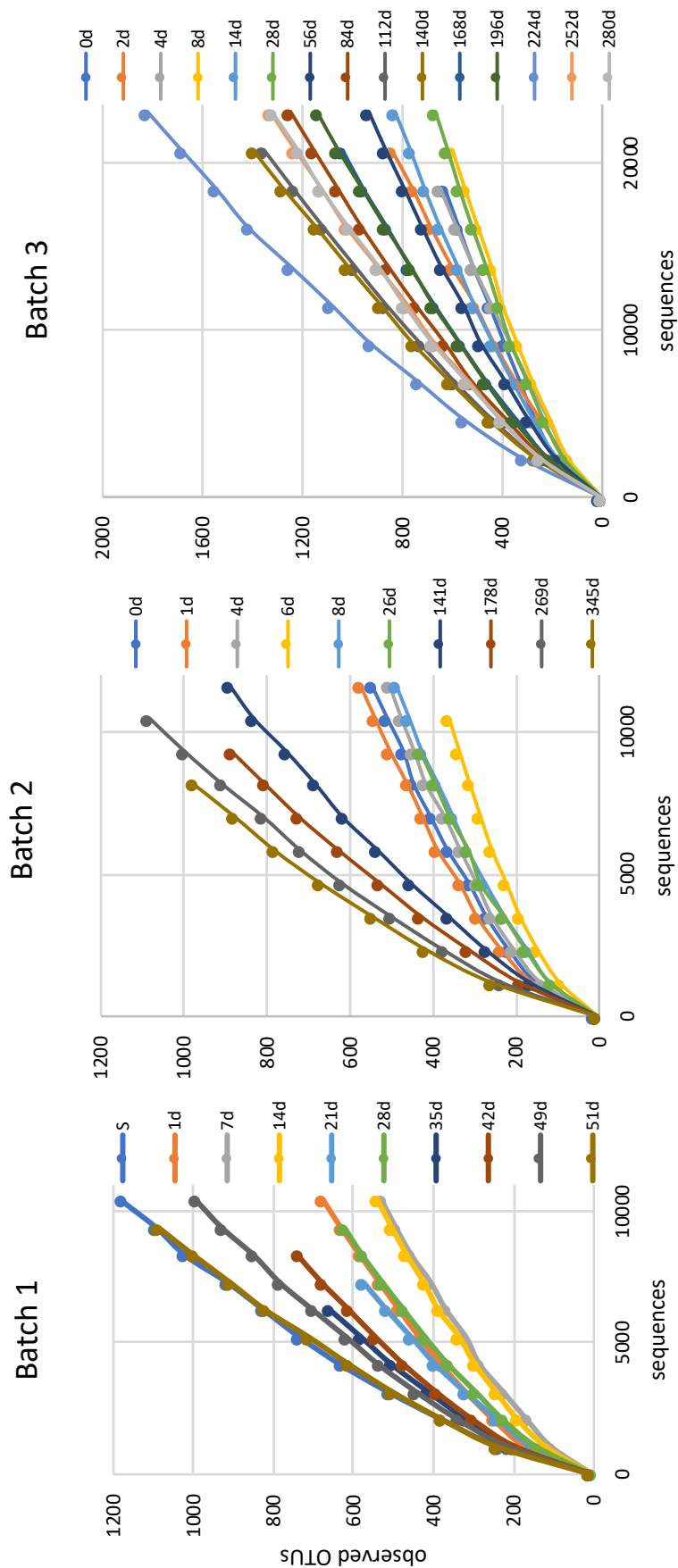


Fig. III-5 Rarefaction curve based on the observed operational taxonomic units (OTUs) defined by 3% sequence variation in 16S rRNA sequencing reads in samples from the indigo fermentation batches.

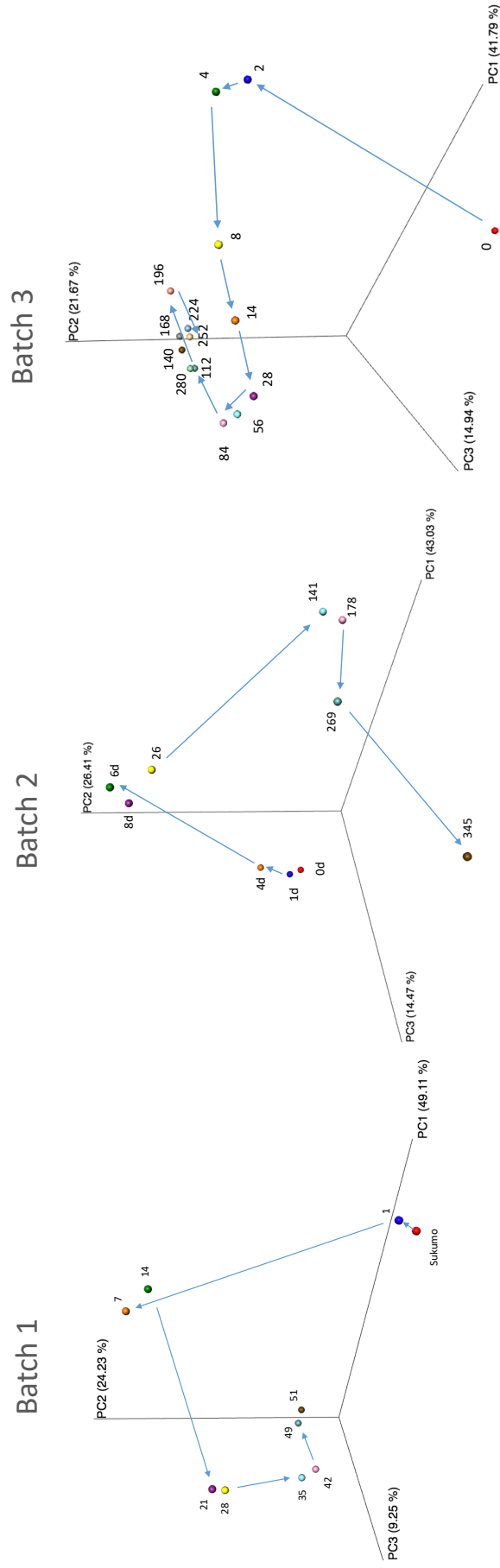


Fig. III-6 Bray Curtis PCoA plot for the bacterial community in the indigo fermentation batches. The numbers indicate the samples of fermented days.

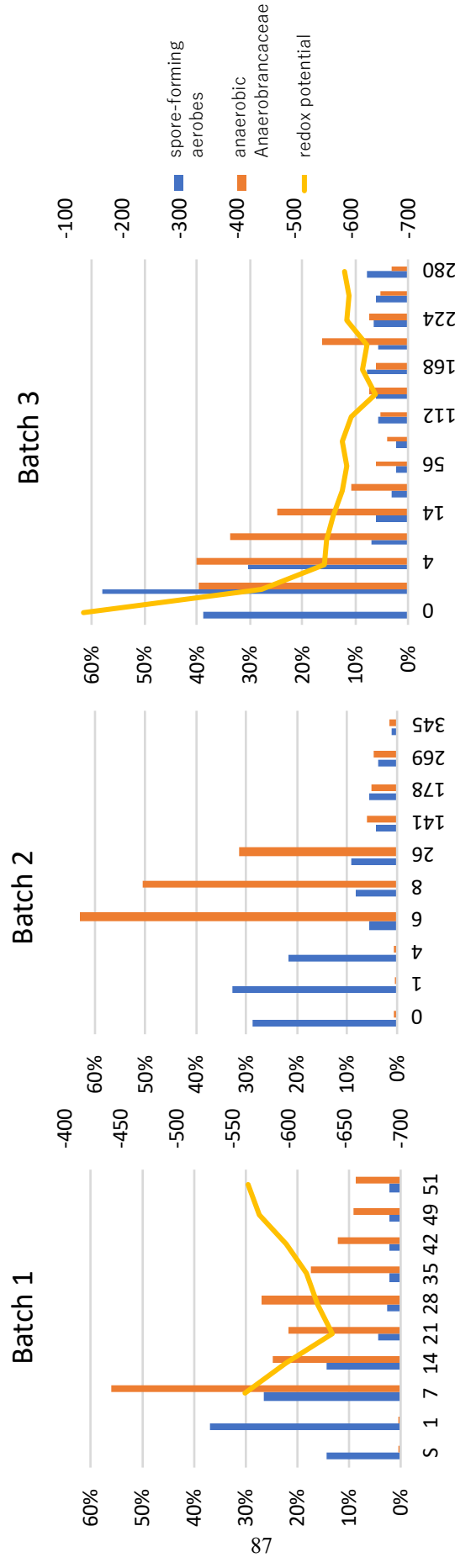


Fig. III-7 The spore-forming aerobes including *Bacillus* spp., *Lentibacillus* spp., *Oceanobacillus* spp., *Pseudogracilibacillus* spp., *Salipaludibacillus* spp., etc. The family Anaerobrancaceae is anaerobes. The yellow lines indicate redox potential of the indigo fermentation fluids. S, *sukumo*; The other numbers on the horizontal axis indicate the samples of fermented days in each batch.

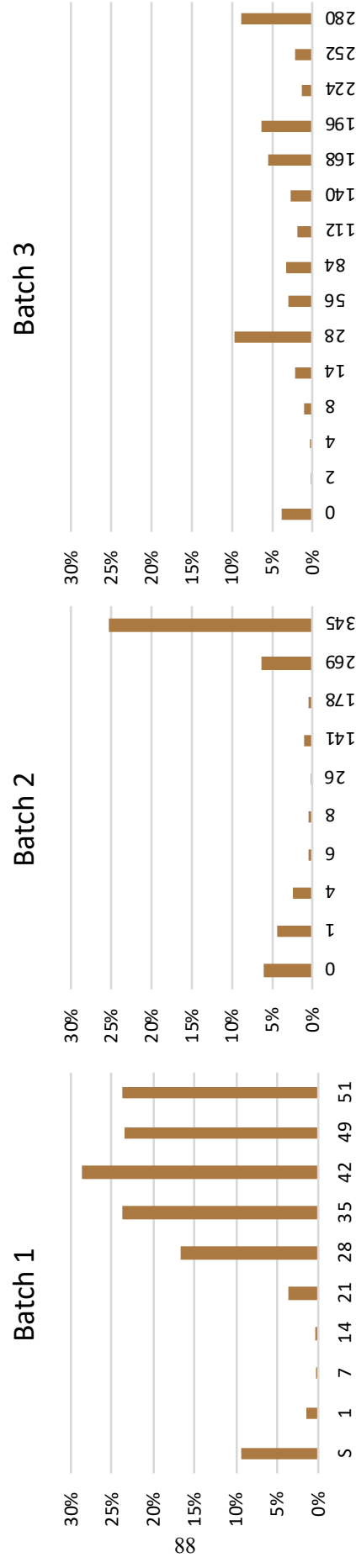


Fig. III-8 Family Alcaligenaceae detected by next-generation sequence (NGS) analyses. *S. sukumo*; The other numbers on the horizontal axis indicate the samples of fermented days in each batch.

Batch 1		S	1	7	14	21	28	35	42	49	51	
Alkalibacterium iburienne					6.04%	1.75%	1.08%	0.59%		0.16%	0.39%	0.5%
Alkalibacterium indicireducens												1.0%
Amphibacillus iburiensis				1.54%	5.47%	6.14%	2.57%	1.52%	0.87%	0.52%	0.78%	1.5%
Amphibacillus indicireducens												2.0%
Bacillus cohnii			1.72%		0.84%							2.5%
Oceanobacillus indicireducens		5.30%	11.17%	1.72%	1.88%	1.49%	0.62%	0.29%	0.33%	0.43%	0.27%	3.0%
Polygonibacillus indicireducens						1.16%	0.56%	0.89%	0.76%	2.21%	1.68%	3.5%
Total		5.41%	13.25%	3.65%	14.50%	11.26%	5.22%	3.59%	2.40%	8.46%	9.20%	4.0%
												4.5%
												5.0%
												5.5%
												6.0%
												6.5%
												7.0%
												7.5%
												8.0%
												8.5%
												9.0%
												9.5%
												10.0%
												>10.0%

Fig. III-9 Fluctuation in the relative abundance of the indigo-reducing species and total contents. The presented results are based on next-generation sequence (NGS) analyses and bacterial 16S rRNA reference sequences in the BLAST database. S, *sukumo*; The other numbers on the horizontal axis indicate the samples of fermented days in each batch.

General discussion

As interest in environmental problems has recently increased, traditional indigo fermentation using *sukumo* combined with indigo reduction by fermentation has been reconsidered. To utilize traditional methods on a wide scale, several limitations must be overcome, including the cost, initiation period of indigo reduction, and difficulty in maintaining fermentation. To improve the traditional procedure, it is necessary to analyze changes in the microbiota throughout the fermentation period. It is also important to determine the mechanisms of the initiation of indigo reduction. The initiation period fluctuates between 4 and 14 days after the beginning of fermentation. In some cases, more than 3 weeks are required. The factors necessary for indigo reduction during fermentation are unclear. Therefore, I evaluated the factors involved in this process after fermentation begins. Second, indigo fermentation fluid exhibits extraordinary resilience. The fluid is repeatedly utilized during fermentation for dyeing processes. Fabric can be immersed in the fluid many times to enhance the dyeing intensity. This process, which introduces air and contaminant microorganisms, is thought to damage the fermentation fluid. However, the indigo-reducing state persists for at least 4–6 months, including for more than 1 year in some cases. This feature is unique and intrinsic to indigo fermentation fluid but the mechanisms are unclear. Thus, I also evaluated the causes of this resiliency of fermentation. Both initial and mature indigo fermentation fluid exhibited strong resilience; however, the indigo reduction ability gradually decreased and deteriorated. To maintain the reducing ability of indigo fermentation fluid to maintain its dyeing intensity, it is necessary to determine the factors that deteriorate the indigo reducing ability of the microbiota during indigo fermentation.

In this general discussion, to clarify the three above-mentioned important issues, I will discuss the 3 different stages of indigo fermentation: 1) initiation of indigo reduction concomitant

with transitions in the microbiota; 2) stability of the indigo-reducing state; and 3) final stage underlying the deterioration of the indigo-reducing state based on accumulated data including short-lived and long-lived indigo fermentation fluids according to previous studies.

First stage

The first stage is *sukumo* composting and fluid preparation (Fig. 1A and B). The composting process requires approximately 3 months, during which microbial enrichment and selection occur. Spore-forming, oxygen-consuming aerobic and facultative anaerobic bacteria (such as Bacillaceae) are present at higher percentages within 10 days after preparing the fermentation fluid. Simultaneously, indigo-reducing bacteria (such as *Alkalibacterium*, *Amphibacillus*, *Oceanobacillus*, and *Polygonibacillus*) and lower contents of obligate anaerobes (such as *Anaerobranca*) were also detected. This study showed that the bacterial community derived from *sukumo* is fundamentally important for indigo reduction. The indigo-reducing taxa *Alkalibacterium*, *Amphibacillus*, and *Polygonibacillus* were detected in ratios of 0.048%, 0.14%, and 0.02%, respectively. To prepare the fermentation fluid, *sukumo* is treated with hot wood ash extract (pH 10.5–11, 60–80 °C). This is the first step in selecting microorganisms that can adapt to thrive in indigo fermentation fluid, which is an anaerobic alkaline environment. These conditions can be used to select indigo-reducing microorganisms that promote indigo fermentation, such as spore-forming species which metabolize oxygen such as *Bacillus* spp., *Oceanobacillus* spp., *Sinibacillus* spp., *Pseudigracilibacillus* spp., *Bacillus cohnii*, and *Oceanobacillus indicireducens*. A similar event was reported for woad dye fermentation. Additionally, *Bacillus thermoalylovorans* may consume oxygen in the fluid. After pretreatment of hot wood ash extract, spore-forming, oxygen-metabolizing bacteria showed a sharp increased for 4–7 days and then rapidly declined, decreasing the redox potential. During this short period,

among the spore-forming indigo-reducing bacteria, only *B. cohnii* shows a large increase; however, this bacteria rapidly declined before indigo reduction, indicating that *B. cohnii* participates in the initial reduction preparation but may not contribute indigo reduction because it was not detected in all batches. Until this period, the bacterial community showed the fastest migration rate and lowest diversity compared to in other stages of fermentation. In later stages of preparation (Fig. 1B), indigo-reducing aerotolerant anaerobic (*Alk. indicireducens* and *Amp. indicireducens*) and facultatively anaerobic (*Oce. indicireducens*) indigo-reducing bacteria multiply and initiate indigo reduction. Pre-treatment by heating is effective for inhibiting the proliferation of unfavorable bacteria. This may positively affect early indigo-reduction. Batches 1 and 2, which were heated twice, began to dye fabrics on days 7 and 6, respectively, whereas Batch 3 began to dye fabrics on day 8. In contrast, after heating, Batch 3 showed a higher initial aerobe content and earlier multiplication of obligate anaerobes compared to the twice-heated Batches 1 and 2, which may be beneficial for indigo reduction in the long-term. Further studies are needed to avoid the pre-heating step by maintaining a stable and high pH, which can decrease the cost of industrial application by elongating the fermentation period.

It has been reported that a drastic decrease in redox potential does not directly reflect the beginning of indigo reduction. For the batches employed in this study, indigo reduction was initiated when the ratio of obligate anaerobic Anaerobazncaseae was two-fold higher than that of Bacillaceae. Thus, for the initiation of indigo reduction, two events occurred. The first event was the consumption of oxygen in the fluid concomitant with a rapid increase in oxygen metabolizable spore-forming bacteria, which survived by pretreatment with hot wood ash extract. The second event was a reversal in the ratio of the rapidly increased Bacillaceae to obligate anaerobic Anaerobazncaseae concomitant with decreased redox potential. This early phase may be very important for the succession of microbiota in indigo fermentation. A short-lived batch did not

show a low ratio of both Bacillaceae and indigo-reducing species. This is likely because of the very high pH in first 10 days (approximately pH 11.4).

Second stage

The second stage is comprised of the stable period (Fig. 1C) and migration period (Fig. 1D). In the stable period, bacteria migrate slowly and exhibit a stable state for 10–20 days, during which time bacterial diversity is low and obligate anaerobes replace aerobes as the dominant population. In fact, before this stage (before indigo reduction), obligate anaerobes began to proliferate, and the dominant positions of aerobes and anaerobes were reversed. Thus, this stage was considered as the stable period, as the oxygen content in the fluid was very low (redox potential was lower than -550 mV), pH was high and stable ($\text{pH} \geq 10.5$), and anaerobes (such as *Anaerobranca*) were dominant, increasing the strength of the fermentation fluid and its ability to resist extraneous contamination. During this period, indigo reduction and dyeing reached an optimal state. Additionally, the percentage of indigo-reducing bacteria gradually increased. Adding nutrients (boiled wheat bran) to the fermentation fluid and maintaining the pH at approximately 10.5 can extend the reduction state. At this stage, the microbiota enters a stable state under alkaline anaerobic conditions, which is important for maintaining indigo fermentation for a long period following the migration period. During continuous and closed-circulating ethanol fermentation, the accumulation of secondary metabolites in the broth is the main inhibitor of cell growth and fermentation [20, 21]. Over long-term fermentation, large amounts of metabolites may accumulate in the fluid, which may be detrimental to maintaining the original bacterial community and promoting gradual migration of the bacterial population [20, 21]. During these months, bacterial diversity gradually increased, and the bacterial community gradually migrated. Indigo-reducing bacteria in the fermentation fluid were also replaced by new species

[72], forming a relay-like relationship throughout the indigo reduction process (Fig. 1). Among the bacteria present, *Amp. indicireducens* is important, as it is prominent during all stages of fermentation. In addition, *P. indicireducens* increased during the middle stage, suggesting that it is important in the middle and late stages of indigo reduction. Additionally, during this period, irregular staining and other operations introduce other microorganisms, which may promote gradual migration of the bacterial community.

Experimental indigo fermentation batches are not widely used for staining. In addition to anaerobic high pH conditions, occupation of niches by indigo-reducing bacteria and bacteria favorable for indigo reduction may be important for maintaining the indigo-reducing ability of the fluid. In addition to the factors mentioned above, metabolites of microorganisms and debris of deteriorated microorganisms may be involved in sustaining the ecosystem. Furthermore, appropriate feeding of the microorganisms in the fermentation fluid and continuous stirring to prevent the formation of localized neutral microenvironments are important for maintaining the indigo-reducing ability in the indigo fermentation fluid.

Third stage

The third stage is the deterioration period (Fig. 1E). After months of fermentation and irregular dyeing operations, the fermentation broth inevitably and gradually deteriorates. During this period, bacterial diversity gradually increases, and the community shows large migration from the stable period. We found that the appearance of neutrophilic bacteria (*Parapusillimonas granuli*) was related to the management of the fermentation fluid. The *P. granuli* originate from *sukumo*. Under strong alkaline conditions (pH 10.5) at the beginning of fermentation, the content of neutrophilic bacteria was suppressed to 5% or even less than 1%. However, in later stages of fermentation, their contents gradually increased, which were difficult to reduce after reaching

approximately 10% until the fermentation fluid had deteriorated. In the traditional operation method, constant stirring maintains the pH of the fermentation fluid to avoid the local pH from decreasing because of the influence of lactic acid bacteria (such as *Alkalibacterium*). In traditional indigo fermentation reduction, it is important to predict the state of the fermentation fluid in advance. Indigo-dyeing studios typically require experienced craftsman to judge the state of fermentation based on subtle changes such as liquid color, odor, and foam formed by stirring. Although we did not observe a direct relationship between the ratio *P. granuli* to indigo-reducing bacteria and deterioration of the fermentation fluid, it can be used as an indicator to monitor the internal state of the indigo fluid, particularly the neutral pH in the microenvironment, after which changes in the fermentation fluid can be predicted and timely measures can be taken to avoid deterioration and prolong the reduction period.

The ratio of *P. granuli* to indigo-reducing bacteria may be a factor in deterioration of the indigo-reducing state. Direct introduction of *P. granuli* cells prepared in the laboratory did not induce deterioration of the indigo-reducing state. Therefore, adaptation of *P. granuli* in indigo fermentation or the use of physiological conditions may be necessary to deteriorate bacteria into an indigo-reduction state. Additionally, the condition of indigo fermentation fluid may be reduced by unfavorable microorganisms.

Concluding remarks and future perspectives for natural indigo fermentation

Methods for preparing and maintaining traditional indigo fermentation fluid have been established through long-term experience. However, the mechanisms of these procedures are unclear. By analyzing the microbiota during the entire fermentation period in several batches, I found that traditional procedures such as pretreatment, feeding of wheat bran, and stirring once per day have important influences not only on the initiation of indigo reduction but also on the

maintenance of the indigo-reducing state.

Many studies have evaluated the microbiota present in natural fermentation systems, such as naturally fermented foods (vegetables, meats, fishes, and dairy products) [22, 63, 76, 79] and alcoholic beverages (wine, koumiss) [29, 54]. In most cases, fermentation occurs under salty, acidic, or alcoholic conditions, which are typically used to select an appropriate microbial community. The microorganisms involved in these natural fermentation processes exhibit high diversity in environments with pressure normally unfavorable for general microorganisms. In indigo fermentation, these factors include a high pH and anaerobic conditions. Although many natural fermentation processes are used worldwide, few of these processes occur under alkaline and anaerobic conditions. However, constructing a favorable microbial community from among numerous initial situations, successive changes in the microbiota concomitant with interactions among the microorganisms, and environmental factors defining the presence of microorganisms are common features of all fermentation processes.

This study was conducted to explore the relationships between the bacterial community and indigo reduction in a high pH and anaerobic environment. This nearly one-year natural fermentation process will not only help to develop a safer and more efficient indigo-reducing method, but also provides a reference model for microbial fuel batteries [58] and the biodegradation of mines and industrial wastewater pollution [103].

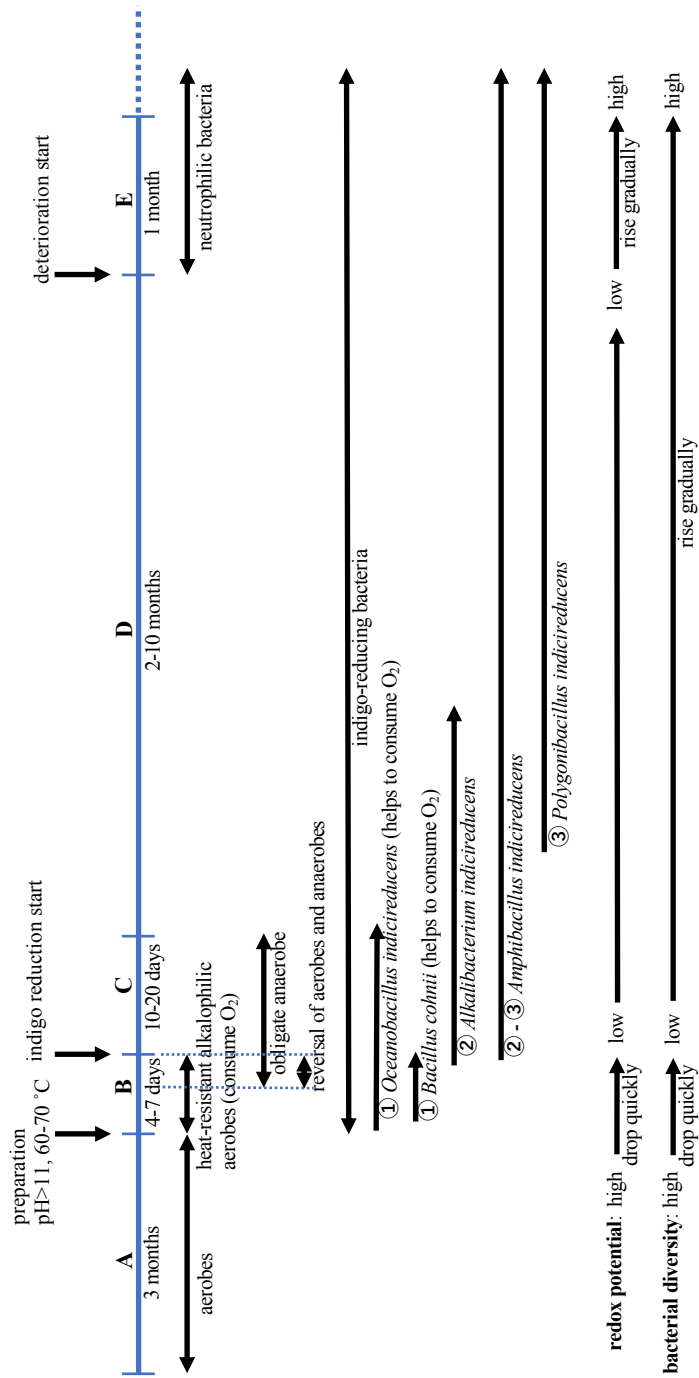


Fig. 1 The entire process of *sukumo* fermentation (shown as blue bar). A, *sukumo* composting; B, preparation; C, stable period; D, migration period; E, deterioration period

Summary

Indigo is one of the oldest dyes used by humans. Currently the rate of consumption of the indigo dye for popular blue jeans is enormous. Due to its insolubility in water, indigo dye needs to be reduced to its water-soluble form (leuco-indigo) before it can be used for dyeing. Leuco-indigo can be easily absorbed by fabrics, and when exposed to air the soluble leuco-indigo is re-oxidized to insoluble indigo and remains within the fabrics as a blue color. The Japanese traditional indigo dyeing material known as *sukumo* contains composted leaves of knotweed (*Polygonum tinctorium*) and is reduced by fermentation under anaerobic conditions and extreme alkalinity (pH 10.3-11.0). Traditional fermentation has significant advantages in safety and recyclability compared to the chemical agents (e.g., $\text{Na}_2\text{S}_2\text{O}_4$) used in industries. However, traditional fermentation is difficult to manage by ordinary people. To understand the mechanisms of initiating of indigo reduction and the maintenance of its stability in the reduced indigo state, I analyzed the changes in microbiota in differently prepared batches of indigo fermentation fluid.

1. Analysis of the microbiota involved in the early changes associated with indigo reduction in the natural indigo fermentation

The constituents of the microbiota of the seed microbiota (i.e., *sukumo*) and the initial changes in the microbiota during fermentations are important in natural fermentation progression. The origin of indigo-reducing bacteria and the initial changes in the microbiota that occurring concomitantly with the initiation of indigo reduction during indigo fermentation were analyzed. The reported indigo-reducing taxa *Alkalibacterium*, *Amphibacillus*, and *Polygonibacillus* were confirmed to originate from *sukumo*. The introduction of hot alkaline solution (pH $\geq$ 10.5, temperature $\geq$ 60 °C) during the pretreatment of *sukumo* at the initiation of

the fermentation allowed the growth of heat resistant spore-forming Bacillaceae, which originated from *sukumo*. The rapid growth of Bacillaceae at the initiation of the fermentation may contribute to lowering the redox potential by consuming oxygen in the fluid. The resulting alkaline anaerobic conditions allowed the increase in slow growth once diminished by hot wood ash treatment anaerobes (*Anaerobranca*) and aerotolerants (*Amphibacillus*). This study demonstrated that using the appropriate material (i.e., existing of indigo reducing bacteria), and performing appropriate pretreatment (i.e., hot alkaline wood ash extract), and adjusting of fermentation conditions (i.e., maintaining anaerobic high pH conditions) are important factors that benefit indigo reduction.

2. Characterization of the microbiota in long- and short-term natural indigo fermentation

The duration of indigo reduced state depends on different batches of natural indigo fermentation fluids. Two batches of *sukumo* fermentation fluids that lasted for different durations (Batch 1: less than 2 months; Batch 2: nearly 1 year) were used for microbiota analysis to understand the mechanisms underlying the sustainability and deterioration of natural fermentation process. The microbial community maintained a very stable state (i.e., changing the velocity of the microbiota was slow in PCoA analysis) in only long-term Batch 2. This is probably due to high pH, low redox potential, and dominance of favorable microorganisms for indigo-reduction (e.g., obligate anaerobe [*Anaerobranca* sp.] and indigo reducing species). This study suggested that the important factor for the maintenance of indigo fermentation for a long duration of indigo reducing state was the entry of the microbiota into a stable state under alkaline anaerobic conditions.

3. Transition of the bacterial communities associated with indigo reduction throughout the entire fermentation process

Changes in the microbiota of the three different batches (Batch 1: lasted less than 2 months;

Batch 2: lasted nearly 12 months without frequent feeding; Batch 3: lasted nearly 10 months with frequent feeding) of indigo fermentation were analyzed using the same analytical procedure. Successive change of induction of transient dominance of Bacillaceae at initiation followed by appearance of obligate anaerobe *Anaerobranca* sp. and indigo-reducing species *Amphibacillus indicireducens* was observed in all three batches. It is considered that the time for the beginning of initiating indigo reduction, the reversal change in the ratio of Bacillaceae to *Anaerobranca* sp. is important for initiation of indigo reduction. Increase in the ratio of *Parapusillimonas granui* (ca. 20%) was also commonly observed in the deterioration state. This suggests that the ratio *P. granui* to indigo reducing bacteria is important for inducing of the deterioration state. From indigo reducing state to deteriorated state, bacterial diversity was increased. This is probably because the micro acidic conditions were induced.

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