Indigofera tinctoria leaf powder as a promising additive to improve indigo fermentation prepared with sukumo (composted Polygonum tinctorium leaves)

Helena de Fátima Silva Lopes1,2 · Zhihao Tu1 · Hisako Sumi3 · Hiromitsu Furukawa4 · Isao Yumoto1,2

Received: 19 April 2021 / Accepted: 9 August 2021 / Published online: 25 September 2021
© The Author(s), under exclusive licence to Springer Nature B.V. 2021

Abstract
Being insoluble in the oxidize form, indigo dye must be solubilized by reduction for it to penetrate textile. One of the procedures is the reduction by natural bacterial fermentation. Sukumo, composted leaves of Polygonum tinctorium, is a natural source of indigo in Japan. Although sukumo has an intrinsic bacterial seed, the onset of indigo reduction with this material may vary greatly. Certain additives improve indigo fermentation. Here, we studied the effects of Indigofera tinctoria leaf powder (LP) on the initiation of indigo reduction, bacterial community, redox potential (ORP), and dyeing intensity in the initial stages and in aged fermentation fluids prepared with sukumo. I. tinctoria LP markedly decreased ORP at day 1 and stabilised it during early fermentation. These effects could be explained by the phytochemicals present in I. tinctoria LP that act as oxygen scavengers and electron mediators. Using next generation sequencing results, we observed differences in the bacterial community in sukumo fermentation treated with I. tinctoria LP, which was not influenced by the bacterial community in I. tinctoria LP per se. The concomitant decrease in Bacillaceae and increase in Proteinivoraceae at the onset of fermentation, increase in the ratio of facultative to obligate anaerobes (F/O ratio), or the total abundance of facultative anaerobes (F) or obligate anaerobes (O) (designated F + O) are vital for the initiation and maintenance of indigo reduction. Hence, I. tinctoria LP improved early indigo reduction by decreasing the ORP and hasten the appropriate transitions in the bacterial community in sukumo fermentation.

Keywords Alkaliphilic bacteria · Indigofera tinctoria · Indigo reduction ecosystem · Obligate anaerobes

Introduction
Indigo is one of the oldest dyes in human history. It was used in many ancient civilisations around the world (Chavan 2015). Natural indigo is extracted from various plants. While Indigofera tinctoria was the main source in India, Polygonum tinctorium was found in China and Japan, and woad (Isatis tinctoria) was the major source of indigo in Europe (Cardon 2007). The requirement for natural indigo declined after the first commercially available synthetic indigo was developed and commercialised in 1890. Synthetic indigo has almost entirely replaced natural indigo (Głowacki et al. 2012; Chavan 2015). Today, the global indigo production is in the thousands of tonnes. More indigo is manufactured than any other dye worldwide and it is used mainly to dye denim for blue jean fabrication (Głowacki et al. 2012). However, synthetic indigo production generates environmentally harmful chemicals (Hsu et al. 2018).

Oxidized indigo dye is insoluble in water and must be solubilised by reduced in alkali solution. The reduced product is known as leuco-indigo and readily penetrates fabrics. On contact with atmospheric oxygen, it oxidises to a blue form (Clark et al. 1993; Glowacki et al. 2012). Fermentation by bacterial, chemical and electrochemical reduction,
and catalytic and electrocatalytic hydrogenation can be used to reduce indigo (Chavan 2015; Yi et al. 2020; Nakagawa et al. 2021). Industrial dyeing commonly involves the reducing agent sodium dithionate (Głowacki et al. 2012). Whilst it is fast and inexpensive chemical reduction requires the continual addition of the reductant and generates toxic and corrosive waste products (Chavan 2015; Hsu et al. 2018).

_sukumo_ is a traditional Japanese dyeing material prepared by composting _P. tinctorium_ leaves. This method concentrates the indigo and facilitates its storage, transportation and preservation. _Sukumo_ also harbours microorganisms that can serve as an inoculum for indigo-reducing fermentation. Moreover, the plant materials derived from the composting may be used as nutrients for the microorganisms (Aino et al. 2018). Hence, _sukumo_ is a fermented raw material that contains nutrients for microorganisms and microbial seed.

The indigo in _sukumo_ must be solubilised by reducing it in a liquid alkaline medium. To this end hot wood ash extract (60–80 °C; pH 11) is combined with _sukumo_ at 1/3 fermentation volume. After onset of indigo reduction, the wood ash extract is topped up to 2/3 volume. After indigo reduction starts, wood ash extract is the added to make up the final volume. The fluid is maintained at pH > 10.3 by adding lime hydrate (Ca(OH)₂) and stirring once daily. Japanese rice wine is initially added with the preparation and wheat bran (substrate) is periodically introduced after the onset of indigo reduction. The period between the initiation of the process and dyeing varies depending on the _sukumo_ quality and preparation. The process may take from 3 days to several weeks (Aino et al. 2018).

The microbiota in _sukumo_ preparations have been studied via clone library analysis (Aino et al. 2010; Okamoto et al. 2017) and next generation sequencing (NGS) (Tu et al. 2019a, b, c). Clone library analysis focused on aged fluids and identified _Proteiviraceae_, _Tissirellaceae_, _Anaerobacillus_, _Amphibacillus_, _Alkalibacterium_ and _Polygonibacillus_ as the dominant taxa (Okamoto et al. 2017). It also revealed that changes in microbiota occurred more slowly in the later than the earlier fermentation phases. NGS investigated the changes that occur in early fermentation and the entire fermentation process. In the initial stages, _Bacillaceae_ predominated and consumed oxygen in the fluid. _Bacillaceae_ were then succeeded by the obligate anaerobes _Anaerobranca_ (_Proteiviraceae_) (Tu et al. 2019a). A comparative study on fermentation batches in different phases of indigo reduction indicated the early predominance of obligate anaerobes such as _Anaerobranca_ (_Proteiviraceae_) followed by gradual successive changes into stable microbiota. The study suggested that these transformations are necessary for the establishment of stable indigo fermentation fluid (Tu et al. 2019b).

Traditional fermentation may be difficult to initiate and maintain for long periods. It depends on numerous factors such as the correct abundance and diversity of natural microorganisms and appropriate pH, stirring, and feeding. Thus, ample experience in traditional fermentation preparation and management is required for the successful execution of this process (Aino et al. 2018). In Medieval times, madder (_Rubia tinctorum_) was added to woad vats to improve fermentation. Madder has numerous anthraquinones. In fermentation experiments using indigo-reducing _Clostridium isatidis_, the addition of anthraquinones and madder stimulated indigo reduction (Nicholson and John 2005). In the case of extracted indigo, since it lacks sufficient microorganisms to initiate the fermentation, _Senna tora_ (originally named _Cassia tora_ by Linnaeus), tamarind, and ripe fruits may be added to accelerate microbial indigo reduction (Samanta and Agarwal 2009; Chavan 2015; Zhang et al. 2019). These plant materials also contribute with phytochemicals and microbial substrates. In the present study, we evaluated _I. tinctoria_ as a candidate for acceleration of indigo reduction. This plant has numerous carbohydrates, flavonoids, saponins, proteins, tannins, phenols, steroids, anthraquinone and triterpenoids (Sharma et al. 2016). _I. tinctorum_ leaf powder (LP) has been used as a hair dye. It is readily accessible and has been used by certain artesians in fermentation fluid for cloth dyeing. However, its mechanism for the acceleration of indigo reduction is still unknown.

The present study aimed to assess the effect of _I. tinctoria_ in terms of accelerating the initiation of indigo reduction in _sukumo_ fermentation. For this purpose, we examined the bacterial community profiles and characteristics of multiple _sukumo_ fermentation batches over different fermentation periods with and without the presence of _I. tinctorum_ LP. The result of this study provided valuable knowledge regarding the initiation and maintenance of indigo reduction in _sukumo_ fermentation fluid.

### Materials and methods

#### Sukumo fermentation fluid preparation

A small-scale batch fermenter (0.5 L) was prepared to simulate the large-scale craft centre _sukumo_ fermentation. _Sukumo_ occurs in an alkaline solution made of wood ash extract. The extract was produced by boiling 380 g wood ash (_Quercus phillyraoides_ A. Gray, Nagomi Co., Gobo, Wakayama, Japan) in 5 L tap water for 10 min. The supernatant was carefully decanted and boiled again and distributed into 500-mL Erlenmeyer flasks. After the supernatant cooled to 60 °C, 38 g _sukumo_ was added to it. When the fluid mixture cooled to 30 °C, 0.5 g _I. tinctoria_ LP (NCC Agro Industries, Tamil Nadu, India) was added to one of two flasks. Two batches of _I. tinctoria_ LP from the same company were used, with LP1 referencing to the oldest batch.
and LP2 to the recently open package. The flask containing no LP was named as control. The flasks were maintained at 26 °C. For Experiments 1 and 2, the sukumo source was produced by KS (Date City, Hokkaido, Japan). For Experiment 3, the sukumo source was produced by TT (Tokushima Prefecture, Shikoku, Japan) (Table 1). Both sukumo sources used in this study were chosen because the start of indigo reduction took more than 6 days, whereas the sukumo normally used in our laboratory exhibited indigo reduction within 5 days. To test indigo reduction, small cotton cloth squares were dipped in the fluid for 1 min, exposed to air for 10 min to oxidise the indigo, washed under running water to remove impurities, air-dried, and stored. Dyeing efficiency of the I. tinctoria LP treatment was assessed by comparing it against the control. The dyeing intensity was measured by scanned the dyed cloth and analyzing the image using Mathematica (version 12.2). The intensity was expressed as the square root of \(a^2 + b^2\) in CIE \(L^*a^*b^*\) color system. The pH and redox potential (ORP) of the fermentation fluids were measured with a D-71 pH meter (Horiba, Kyoto, Japan) and a D-75 pH/ORP/DO meter (Horiba), respectively. The pH was maintained between 10.5 and 11 with Ca(OH\(_2\)). Before a D-75 pH/ORP/DO meter (Horiba), respectively. The pH measured with a D-71 pH meter (Horiba, Kyoto, Japan) and the redox potential (ORP) of the fermentation fluids were then determined for incubated samples.

### DNA extraction and PCR

Sukumo fermentation fluids with and without I. tinctoria LP were centrifuged at 15,000xg for 10 min at 25 °C to obtain a pellet used for DNA extraction. The latter was performed with an ISOIL extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. For the final step, however 50 µL TE was added instead of 100 µL. The extracted DNA was stored at −30 °C. The bacterial 16S rRNA sequence of the V3–V4 region was amplified with the primer pairs: V3–V4f\(_{\text{Mix}}\) (ACA CTTTTCCTCAG GCTTATCGATATGNNNCTACGGGNGGC WGCAG) and V3-V4r\(_{\text{Mix}}\) (GTGACTGGAGTTCAG ACGTGCTTCTCGATCTNNNNNN-GACTACHVGGG TATCTAAATCC) provided by Bioengineering Lab. Co. Ltd. (Sagamihara, Kanagawa, Japan). The primers consisted of an adaptor sequence followed by insertions with 0–5 bases with random sequences and lengths to improve quality (represented by N). The final sequence was homologous to 16S rDNA (341 F and 805 R). The PCR solution (40 µL) consisted of 4 µL of 10× Ex buffer (Takara Bio, Otsu, Shiga, Japan), 3.2 µL dNTPs (Takara Bio), 2 µL forward primer, 2 µL reverse primer, 2 ng DNA template (extracted sample), and 0.4 Ex Taq polymerase (Takara Bio). The amplification reaction was performed as follows: 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final step was 72 °C for 5 min. Product identity and quality were confirmed by agarose gel electrophoresis. The I. tinctoria LP source material was suspended in physiological saline. LP1 was processed as previously described. For LP2, the extracted DNA was sent to the Bioengineering Lab. Co. Ltd., where was treated with Peptide Nucleic Acid (PNA) to block chloroplast and mitochondrial DNA sequences.

### Next generation sequencing

The second PCR was performed by Bioengineering Lab. Co. Ltd. The samples were amplified with a tailed primer set and purified. The NGS was conducted on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). The raw reads were pre-processed by Bioengineering Lab. Co. Ltd. and fastq.gz output files were processed with QIIME2 ver. 2020.2 (Bolyen et al. 2019). Cutadapt version 1.18 removed the primers and adapters. The sequences were filtered, and

| Batch       | Sukumo          | Leaf powder | Period of analysis of microbiota | Addition of nutrient | Fermentation volume (L) |
|-------------|-----------------|-------------|----------------------------------|----------------------|-------------------------|
| Experiment 1| Date (by KS)    | LP2         | Days 1–72                        | No                   | 0.5                     |
| Experiment 2| Date (by KS)    | LP1         | Days 36–94                       | No                   | 0.5                     |
| Experiment 3| Tokushima (by TT)| LP1       | Days 115–226                     | No                   | 0.5                     |
merged and had chimeras and singletons were removed with the Demux command (Callahan 2016). DADA2 was used to cluster the sequences with 99% similarities into amplicon sequence variants (ASVs). Taxonomy classification were made by his research group and the SILVA database (Pruesse et al. 2007; Quast et al. 2013). For the I. tinctoria LP source material sequences classified as chloroplasts were filtered. ASV nucleotide sequence identities with > 1.5% participation were compared against bacterial 16S rRNA reference sequences in the BLAST database. Rarefaction curves were plotted for the sequences and OTUs at 99% similarity level with respect to the total number of reads per sample. Rarefaction curves of the observed species were plotted with the QIIME2 alpha diversity analysis script. Principal coordinates analysis (PCoA) based on the Jaccard distance was performed using beta diversity analysis script in QIIME 2. EMPEROR software was used to visualize the PCoA plots (Vázquez-Baeza et al. 2013). The predictive functions of the metagenomes of LP treatment and the control at day 5 were estimated using PICRUSt2 (Douglas et al. 2020). KEGG pathway mapping was used to search the KEGG database according to the output results.

The NGS data from this study are publicly available in the DNA Data Bank of Japan (DDBJ) under accession no. SAMD00289726 to SAMD00289754, and SAMD00289756.

Results

Effects of I. tinctoria LP on early fermentation phase

Samples of the early fermentation phase of Experiment 1 were observed to determine the effect of I. tinctoria LP on the bacterial community. Results of dyeing and changes in pH and ORP are shown in Fig. 1 and Supplementally Fig. 1. There were evident differences in terms of fluid dyeing between I. tinctoria LP treatment and the control after day 5. The dyeing intensity of I. tinctoria LP slightly decreased after the pH increased at day 10. The effects of this sudden change especially in the control were reflected in the ORP curves. These changes persisted from days 10–20. No significant increase in ORP was observed in I. tinctoria LP treatment during the foregoing time period. Nevertheless, its dyeing intensity transiently decreased (Fig. 1, Supplementally Fig. S1).

Firmicutes was the dominant phylum in all samples (including facultative, obligate and aerotolerant anaerobes) followed by Proteobacteria and Actinobacteria (Fig. 1). The microbiota detected in both I. tinctoria LP (LP1 and LP2) in natural state are shown in Supplementary Fig. S2. The estimated CFU for I. tinctoria LP and sukumo are shown in Supplementary Table 1. The bacterial community profiles in both I. tinctoria LP in raw state does not correlates with those of the fluid with sukumo at day 1. Hence, the I. tinctoria LP bacterial flora did not markedly influence the bacterial flora profile in the broth. This finding corroborated with the results of the estimated bacterial counts for I. tinctoria LP compared against those for sukumo (Supplementary Table S1). The ORP-lowering effect of I. tinctoria LP might be explained by endogenous aerobic bacterial oxygen consumption. Thus, the aerobic bacterial colony count was estimated and maximized using the neutral pH medium. The expected neutralophilic bacterial count for I. tinctoria LP in prepared fermentation fluid was ~0.9% that for the sukumo. Wood ash extract treatment at 26 °C for 30 min lowered the I. tinctoria LP bacterial count. In contrast, wood ash extract treatment even at 55 °C for 30 min had no effect on the sukumo bacterial count. There were more Bacillaceae in fluid where I. tinctoria LP was added than in the control at day 1. Samples from day 1 showed that the bacterial community had four major operational taxonomic units (OTUs) related to Bacillus colnii, Bacillus taeanensis, Alkaliphilus oremlandii, and Mogibacterium neglectum. All other taxa except M. neglectum were more abundant in I. tinctoria LP treatment than the control. All five OTUs decreased in both I. tinctoria LP treatment and the control over next 5 d. However, the sum of the decrease in I. tinctoria LP (50%) was greater than the control (36.2%). The control treatment exhibited relatively higher bacterial diversity on day 1. The proportion of taxa grouped as “others” (“<1.5%) was 21.8 % higher than I. tinctoria LP. The control contained more Proteinivoraceae than I. tinctoria LP at day 5. In I. tinctoria LP treated batch, an OTU related to Alkalihalobacillus alkaninitrilicus was highly active by day 5. In Experiment 1, there were comparatively more microorganisms such as Tissierellaceae in I. tinctoria LP treated batch after day 10. In addition, an OTU related to Polygonibacillus indicireducens appeared in I. tinctoria LP after 58 days.

At fermentation onset, there was a decrease in numbers of facultative anaerobes. However, a subsequent increase in the ratio of facultative anaerobes to obligatory anaerobes modulated the staining intensity. The ratio of facultative anaerobes (F) to obligate anaerobes (O) (designated F/O), the total abundance of facultative anaerobes (F) and obligate anaerobes (O) (designated F + O), and their correlation with dyeing intensity are plotted in the Fig. 2. From these results, it seems that the intensity of dyeing is strongly influenced by the initial abundance of F + O in LP added fluids, which is lower than the control. In addition, an imbalance of the F/O ratio with a dominance of obligate anaerobes seems detrimental in both fermentations. Nevertheless, the strength of this relationship fluctuated because of the differences in the constituent microorganisms and time lag until the effects reflected in the dyeing intensity.

Diversity decreased on days 5 and 25 according to the observed changes in the OTUs at the sample depth of 18,512
Fig. 1 Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample), dyeing intensity, pH, and redox potential (ORP) of Experiment 1 sukumo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Percentages in brackets in BLAST top hit column indicate the ranges of similarities to known species in the database. Classification hierarchy (columns A and B) are described as follows: G, genus; F, family; O, order; C, class. *ND, no data; †(D62), result of Day 62 sample.

(Fig. 3). Relatively fewer OTUs on day 1 of the I. tinctoria LP treatment indicated that I. tinctoria LP addition affected the microbiota. The diversity decreased on day 5 coinciding with changes in redox potential. The diversity decreased on day 25 was explained by depletion of the readily assimilated energy source are more active in the LP-added fermentation preparation (heat and high pH treatment).

The relative difference between I. tinctoria LP and the control in terms of their microbiota were estimated using Jaccard distance PCoA (Fig. 4). The microbiota of I. tinctoria LP rapidly changed from day 10 to day 25 compared with the control. However, both I. tinctoria LP and the control reached the final stage around day 58. In addition, there were three stages in both I. tinctoria LP and the control. Thus, I. tinctoria LP addition may affect both the bacterial community composition and the rate of change in the microbiota.

Predicted metabolic functional differences between I. tinctoria LP added fluid and the control at day 5 were estimated (Supplementary Fig. S3). The results showed that carbohydrate, lipid and amino acids metabolism are superior in LP added fluid than in the control. Specifically, the results point to a predominance in phosphotransferase system, which is known for sugars uptakes, including glucose, mannose, fructose, and cellulobiose in bacteria. It is presumed that the regeneration of microbial flora triggered by the fermentation preparation (heat and high pH treatment) and the sugars uptakes derived from sukumo as an energy source are more active in the LP-added fermentation broth than in the control.
Effects of *I. tinctoria* LP on the middle and late fermentation phases

Samples from Experiments 2 and 3 were used to determine the effects of *I. tinctoria* LP on the microbiota in the middle and later fermentation phases. The effects of *I. tinctoria* LP on the *sukumo* fermentation in terms of fabric dyeing and pH and ORP changes are shown in Fig. 5 and Supplementary Figs. S4 and S5. *I. tinctoria* LP addition was correlated with an early, steep decline in ORP (near -600 mV) compared with the control. This finding was true for Experiments 1–3 inclusive (Supplementary Figs. S1, S4, and S5). *I. tinctoria* LP addition during initial *sukumo* fluid preparation hastened the onset of indigo reduction compared with the control (Experiment 3) (Supplementary Fig. S5). In contrast, despite of *I. tinctoria* LP on the ORP at the start of fermentation, there was no marked difference in dyeing intensity for Experiment 2. A possible reason is that unlike the other experiments in Experiment 2, neither *I. tinctoria* LP nor the control reached pH ≥ 10.7 in the initial phase. The ORP for both *I. tinctoria* LP and the control) in Experiment 2 were lower than those in Experiment 1 and 3 for most of the fermentation period.
Nevertheless, this difference was not observed in Experiment 3.

Both the control and *I. tinctoria* LP in Experiment 2 had similar amounts of OTUs related to *Erysipelothrix indicireducens* (93.2–93.4% similarity). This taxon predominated but its abundance decreased with increasing fluid age. On days 50 and 94, *I. tinctoria* LP and the control had similar quantities of two OTUs identified with *Pseudomonas* spp. In Experiment 2, OTUs related to *Proteivironax tanatenses* (93.1–93.8% similarity) predominated in the control but not *I. tinctoria* LP samples. The latter had relatively higher numbers of an OTU related to *Tissierella creatinini* (94.5% similarity) compared with the control. An OTU related to *Proteinivorax tanatarense* was also detected.

Fig. 5 Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample) dyeing intensity, pH, and redox potential (ORP) in *sukumo* using indigo fermentation batches from Experiment 2 and 3 with (LP) and without (control) *Indigofera tinctoria* leaf powder. Percentages in brackets BLAST top hit column indicate range of similarities to known species in the database. Classification hierarchy (columns A and B) described as follows: G, genus, F, family, O, order, C, class. *a*(D98), result of Day 98 sample; *b*NA, no data; *c*(D147), result of D147 sample

In Experiment 2, the F/O ratio was nearly constant and F + O values was decreased until day 50 and then maintained stable in the control. In the *I. tinctoria* LP added fluid, the F/O ratio varied with fermentation time and the F + O values decreased until day 50 and then maintained stable until the final date. The dyeing intensity had an opposite behavior until day 50 and then almost consistent behavior to day 94. The dyeing intensity had an opposite behavior until day 50 and then almost consistent behavior to day 94.
and the control for in Experiments 2 and 3 (Supplementary Fig. S8). In both Experiments 2 and 3, the changing velocity per fermentation period of the microbiota in the *I. tinctoria* LP added fluid faster than that in the control.

**Discussion**

Traditional indigo reduction methods are becoming more popular as they are comparatively more sustainable than modern techniques. *Sukumo* is a traditional Japanese material used in indigo fermentation. It contains both seed culture and bacterial substrates. However, the onset of indigo reduction greatly varies greatly among batches depending on the fermentation’s preparation procedures and *sukumo* quality. In the present study, we used dried *I. tinctoria* LP to increase the homogeneity and synchronicity of *sukumo* fermentations. The addition of *I. tinctoria* LP to the indigo fermentation fluid containing *sukumo* improved the dyeing results and caused an early decrease in ORP. Adjustment to the target pH is challenging because the alkaliizer, Ca(OH2), does not immediately dissolve in the fluid. Abrupt increases in pH during early fermentation might alter the bacterial ecosystem that sustains the indigo reduction. The addition of *I. tinctoria* LP affected the ORP values at the onset of fermentation impacting the bacterial flora in the early fermentation and aged fluid.

The early decrease in the ORP was caused by the addition of *I. tinctoria* LP during the initial fermentation stages and had an important influence on early indigo reduction. The formation of a low ORP fermentation condition is vital to indigo reduction. This naturally occurs during *sukumo* fermentation because of bacterial oxygen metabolism (Tu et al. 2019a). On day 1 of Experiment 1, there were substantially more oxygen-consuming facultative anaerobes in the *I. tinctoria* LP treated group than in the control. Hence, the *I. tinctoria* LP treatment affected the decrease in the ORP. This effect may even be enhanced by *I. tinctoria* LP treatment via oxygen-scavenging activity, such as the transformation of indigo precursors in *I. tinctoria* LP into indigo. Moreover, *I. tinctoria* has flavonoids, saponins, and terpenoids with strong antioxidant activity and reducing power that help lower the dissolved oxygen content (Srinivasan et al. 2016). Ethanolic and hydroethanolic extracts of *I. tinctoria* LP contain anthraquinone (Sharma et al. 2016) which is an electron mediator. Considering these findings, it can be determined that *I. tinctoria* LP may contain certain oxygen scavengers and electron mediators that reduce and stabilise ORP.

On day 1 of Experiment 1, *I. tinctoria* LP exhibited lower microbial community diversity than the control. Therefore, *I. tinctoria* LP may have exerted a selection pressure on the bacteria present in the *sukumo* fermentation. Both the control and the fluid treated with *I. tinctoria* LP, displayed a decrease in the bacterial diversity from day 1 to day 5. Bacterial diversity in *sukumo* preparations usually decrease in the initial phases (from *sukumo* to day 2), possibly because a hot alkaline solution is used in its preparation. This decrease of bacterial diversity is related to the dominance of obligately anaerobic bacteria (Tu et al. 2019a). Therefore, heat treatment and the decrease of ORP influenced the bacterial diversity. In Experiment 3, bacterial diversity increased in the aged fluids for both the *I. tinctoria* LP treated fluid and the control but especially in the former treatment. Similar patterns were reported for the *sukumo* fermentation fluid in previous studies. These late changes in the microbial flora are associated with the environmental transformation that occurs during fluid ageing. This process occurs despite the addition of *I. tinctoria* LP to the *sukumo* fermentation.

In an earlier study, *Bacillaceae* dominated the community on days 2 and 5 of *sukumo* fermentation and was considered responsible for the ORP reduction via the consumption of dissolved oxygen. After the predominance of *Bacillaceae*, the OTU exhibited sequence similarity of 93.1% to obligate anaerobic *Anaerobranca gottschalkii* predominated (Tu et al. 2019a). In Experiment 1, the predominance of obligate anaerobes in the early days happened in both the *I. tinctoria* LP treated fluid and control. However, in the *I. tinctoria* LP treated batch, the dominance of the obligate anaerobic *Proteinivoraceae* was not as strong as seen in the control on day 5. The *I. tinctoria* LP treated fluid contained relatively larger amounts of *Bacillaceae* on the first day, and the ORP declined comparatively faster than the control.

These alterations in microbial community composition (i.e., *Bacillaceae* → *Proteinivoraceae*) may be necessary to initiate indigo reduction. Before the initiation of indigo reduction in *sukumo* fermentation, obligate anaerobic bacterial OTUs predominate, including *Proteinivoraceae* (93.3–93.8% similarity with *P. tanatarense*). These OTUs might be equivalent to *Anaerobranca*, which was found in previous studies on *sukumo* fermentation fluids. *Anaerobranca* decreases before indigo-reducing bacteria such as *P. indicireducens* increase (Tu et al. 2019a, b). A similar pattern was observed in the present study for *Proteinivoraceae*. Increases in *Anaerobranca* were accompanied by an increase of dyeing intensity suggesting that this taxon participates in indigo reduction (Tu et al. 2019a, b). However, this association was not observed for the control samples in Experiment 1. Therefore, *Proteinivoraceae* predominance is not directly associated to indigo reduction. The relationship between the initiation of indigo reduction and this change in the microbial community remain unknown as this species is difficult to isolate from indigo fluids. The OTUs that make up *Proteinivoraceae* were most closely related to the saprophyte *P. tanatarense* (Boltynskaya and Kevbrin 2016). *P. tanatarense* related OTUs considerably increased soon after the decline in microbial diversity and...
the decrease of *Bacillaceae*. Hence, *P. tanatarenses* related OTUs might have been consuming the dead *Bacillaceae*. We hypothesize that the importance of this event for indigo reduction relies on the fact that anthraquinones and flavins are biosynthesised for electron transport by some bacteria and can be released after bacterial lysis, helping to reduce indigo (Nicholson and John 2005; Light et al. 2018; You et al. 2018).

Since there are many factors involved, it is not easy to clarify the relationship between staining intensity and microbial flora. The possible factors could be (1) the abundance of the substrate leading up to indigo reduction and the presence or absence of microorganisms that can use it (2) the abundance of microorganisms involved and the abundance of electron mediators in the fermented liquor and an event for the circulation of the mediator in the fluid such as cell disruption as the dye itself may be involved in the redox state in the system; and (3) the amount of oxidized and reduced dyes in the fluid. From these results, it seems that the dyeing intensity is strongly influenced by F+O or F/O depending on what is rate-determining factor in that situation. The relationship between F/O and staining intensity is not always parallel because it takes certain days for an aspect of the microbial community to reflect in the staining intensity. As there are many factors and a time lag, it is difficult to judge the current status of staining intensity from only one point of the analysis of the microbial community; however by estimating of the tendency of changes in F+O and F/O over an approximately 5 day period, for example, it may be possible to predict the staining intensity over the upcoming days.

Previous studies have shown that the *sukumo* fermentation can be divided into different phases. Based on the F+O and F/O graphics it can be seen that the changes in these values have different influences in the dyeing intensity. In the initial phase, the onset of an already high F+O ratio seems to have a positive effect on the dyeing intensity, which can be observed by comparing the control and treatment. After some time, the decrease in the F+O ration is most likely caused by the consumption of readily available nutrients and changes in the environment, which in turn impact the dyeing intensity. At this moment the increase of facultative anaerobes as seen in the F/O ratio will play a bigger role on the dyeing intensity. This increase may reflect the availability of new nutrients and mediators made available via metabolism by facultative anaerobes or the appearance of facultative anaerobes capable of using more complex nutrients. After the stabilization of F+O ratio varying around 60–70% of the community the changes in F/O ration seems to have a smaller impact.

In a previous report the abundances of the facultative anaerobic *Polygonibacillus* increased on day 58 in Experiment 1 and reached even higher levels in Experiment 2. *Erysipelothrix inopinata*, was originally isolated from vegetable broth (Verborg et al. 2004), supporting the fact that the material for the fermentation fluid are plant-based. A strain belonging to *Erysipelotrichaceae* was isolated from other *sukumo* preparations and showed reducing ability to indigo carmine (unpublished data).

The presence of *Pseudomonas* in the medium has been linked to fluid acidification and the formation of neutralophilic niches by acid producing bacteria (Tu et al. 2019b). The occurrence of *Pseudomonas* spp. in Experiment 1 may be associated with the accumulation of acid, which is generated by obligate anaerobic bacteria. Nevertheless, *Pseudomonas* sp. can produce metabolites that enable gram-positive bacteria to perform extracellular electron transfer (Pham et al. 2008) and a *Pseudomonas* strain with reduction activity has been isolated from dyeing vat (Park et al. 2012). In previous studies with *sukumo* fermentation, it was detected in short-lived fluid as well as in decaying long-lived batches (Tu et al. 2019b). *Pseudomonas peli* was closely associated with the observed OTUs. This species was isolated from textile wastewater and can decolourise other dyes (Dellai et al. 2013). In Experiment 2, the amount of *Pseudomonas* spp. did not seem to impede indigo reduction. Therefore, it is more likely that it has a positive or neutral effect on the dyeing intensity.

Hirote et al. (2016), reported indigo-reducing bacteria such as *P. indicireducens* playing a major role in indigo-reducing bacterial communities. *P. indicireducens* might be the key contributor to indigo reduction in both the *I. tinctoria* LP treated fluid and control during the latter stages of Experiment 1 (~day 58) and in the *I. tinctoria* LP treated batches in Experiments 2 and 3. In earlier studies, the presence of *P. indicireducens* was connected to aging batches (Tu et al. 2019a, b). This explains why *P. indicireducens* was absent or low in the *I. tinctoria* LP treated fluid and control in Experiment 1 on earlier days. However, it was not present in the aged control in Experiments 2 and 3. In both cases, no other known indigo-reducing bacteria were detected. OTUs related to *Alkalihalobacillus hemicellulosilyticus* (95.6–98.1% similarity) are the presumed indigo reducers as they strongly correlated with dyeing intensity in Experiment 3. In addition, this taxon is also closely related to *P. indicidesucens* using 16S rRNA gene sequence similarity. OTUs from the same genus, related to *A. alkalinitrilicus* (95.6–98.1% similarity), also predominated in the *I. tinctoria* LP treatment of Experiment 1, which presented the strong staining intensity. *Proteinivoraceae* and *Tissierellaceae* occurred in large quantities but their role in fermentation remains to be clarified. Nevertheless, in the case of *Proteinivoraceae* (formerly *Anaerobranca*), the shift of its dominance to *P. indiciresucens* was linked to the onset of
a stable reducing state in sukuno fermentation fluids (Tu et al. 2019b). Other known indigo-reducing bacteria had a very sporadic presence in the samples. Indigo-reducing bacteria Alkalibacterium spp. (Yumoto et al. 2004, 2008; Nakajima et al. 2005; Nishita et al. 2017) was not detected in any sample while Amphibacillus spp. (Hirotta et al. 2013a, b) occurred in trace amounts. This observation was not reported in earlier studies (Tu et al. 2019a, b). The fermentation fluid contains microorganisms with overlapping functions and this property might account for the resilience of this system.

**Conclusions**

The present study examined effects of the *I. tinctoria* LP addition on the initial steps of sukuno-based indigo fermentation. This study demonstrated that an early decrease in ORP was essential for accelerating the onset of the appropriate microbial community for indigo reduction and that *I. tinctoria* LP can assist in the appropriate transitional change of the microbial community. Despite the use of different sukuno sources and differently packaged *I. tinctoria* LPs, the addition of *I. tinctoria* LP rapidly lowered and stabilised the ORP in the early fermentation phase. Moreover, *I. tinctoria* LP treatment affected the microbiota in the early stages and these changes were observed even in the late stages. Initiation of indigo reduction is dependent on the rapid decline in ORP attributed to oxygen consumption by facultative anaerobes, followed by a decrease in facultative anaerobes from days 1–7. In addition, an overall decrease in the bacterial community diversity is required to initiate indigo reduction. *I. tinctoria* LP accelerated the establishment of the biological and physicochemical conditions required for indigo reduction by affecting those parameters. Moreover, *I. tinctoria* LP changed the F/O ratio and F + O, which may impact the staining intensity in the initial phase of fermentation.

Despite the optimistic results, the effect of *I. tinctoria* LP on indigo reduction must be validated using a larger scale fermenter. The complex community, limited identity of certain taxa, and absence of known indigo-reducing bacteria indicate that sukuno harbours numerous bacteria that need to be characterised. Nevertheless, the present study shows that some environmental characteristics are important for the onset of indigo reduction in bacterial fermentation and the manipulation of these characteristics by the use of additives can be useful for the development of new methodologies.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11274-021-03142-y.

**Acknowledgements** This work was supported by the Institute for Fermentation, Osaka (IFO) (No. G-2020-3-035). We thank Editage (www.editage.com) for English language editing.

**Author contributions** HFSL, HS, and IY. conceived and designed the experiments. HFSL, ZT and HF performed the experiments. HFSL, ZT, HF and IY. analysed the data. HFSL and IY. wrote the manuscript.

**Funding** This work received no financial support that could have influenced its outcome.

**Declarations**

**Conflict of interest** The authors declare that there is no conflict of interest.

**References**

Aino K, Narihiro T, Minamida K, Kamagata Y, Yoshimune K, Yumoto I (2010) Bacterial community characterization and dynamics of indigo fermentation. FEBS Microbiol Ecol 74:174–183

Aino K, Hirota K, Okamoto T, Tu Z, Matsuyma H, Yumoto I (2018) Microbial communities associated with indigo fermentation that thrive in anaerobic alkaline environments. Front Microbiol 9:1–16

Bolyansksaya YV, Kevbrin VV (2016) Trophic interactions of proteolytic bacteria *Proteinivorax tanatarense* in an alkaliphilic microbial community. Microbiology 85:481–487

Bolyen E, Rideout JR, Dillon MR et al (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857

Callahan BJ (2016) DADA2: High resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583

Cardon D (2007) Natural dyes: sources, tradition, technology and science. Archetype Publications Ltd, London

Chavan RB (2015) Indigo dye and reduction techniques. In: Roshan M (ed) Denim. Cambridge: Woodhead Publishing Ltd, pp. 37–67

Clark RJH, Cooksey CJ, Daniel MAM, Withnall R (1993) Indigo, woad, and Tyrian Purple: important vat from antiquity to the present. Endeavour 17:191–199

Dellai A, Dridi D, Lemorvan V, Robert J, Cherif A, Mosrati R, Mansour HB (2013) Decolorization does not always mean detoxification: case study of a newly isolated *Pseudomonas peli* for decolorization of textile wastewater. Environ Sci Pollut Res 20:5790–5796

Douglas GM, Maffeij VJ, Zanevedl J, Yurgel SN, Brown JR, Taylor CM, Huttonhower C, Langille MGI (2020) PICRUSt2 for prediction of metagenome functions. Nat Biotechnol 38:685–688

Glowacki ED, Voss G, Leonot L, Irinia-Vladu M, Bauer S, Saricifci NS (2012) Indigo and tyrian purple e from ancient natural dyes to modern organic semiconductors. Isr J Chem 52:540e551

Hirotta K, Aino K, Nodasaka Y, Morita N, Yumoto I (2013a) *Amphibacillus indicireducens* sp. nov., an alkaliphilic that reduces an indigo dye. Int J Syst Evol Microbiol 63:464–469

Hirotta K, Aino K, Yumoto I (2013b) *Amphibacillus iburienisis* sp. nov., an alkaliphilic that reduces an indigo dye. Int J Syst Evol Microbiol 63:4303–4308

Hirotta K, Okamoto T, Matsuyma H, Yumoto I (2016) *Polygonibacillus indicireducens* gen. nov., sp. nov., an indigo-reducing and obligate alkaliphilic isolated from indigo fermentation liquor for dyeing. Int J Syst Evol Microbiol 66:4650–4656
Hsu TM, Welner DH, Russ ZN, Cervantes B, Prathuri RL, Adams PD, Dueber JE (2018) Employing a biochemical protecting group for a sustainable indigo dyeing strategy. Nat Chem Biol 14:256–261

Light SH, Su L, Rivera-Lugo R, Cornejo JA, Louie A, Iavarone AT, Ajo-Franklin CM, Portnoy DA (2018) A flavin-based extracellular electron transfer mechanism in diverse Gram-positive bacteria. Nature 562:140–146

Nakagawa K, Takeuchi M, Kikuchi M, Kiyofuji S, Kugo M, Sakamoto T, Kano K, Ogawa J, Sakuradani E (2021) Mechanistic insights into indigo reduction in indigo fermentation: a voltammetric study. Electrochemistry 89:25–30

Nakajima K, Hirotaka, Koda, Nadasaka Y, Yamoto I (2005) Alkalibacterium iburiense sp. nov., an obligate alkaliphile that reduces an indigo dye. Int J Syst Evol Microbiol 55:1525–1530

Nicholson SK, John P (2005) The mechanism of bacterial. Appl Microbiol Biotechnol 68:117–123

Nishita M, Hirotaka, Matsuyma H, Yamoto I (2017) Development of media to accelerate the isolation of indigo-reducing bacteria, which are difficult to isolate using conventional media. World J Microbiol Biotechnol 33:133

Okamoto T, Aino K, Narihiro T, Matsuyma H, Yamoto I (2017) Analysis of microbiota involved in the aged natural fermentation of indigo. World J Microbiol Biotechnol 33:70

Park S, Ryu J-Y, Seo J, Hur H-G (2012) Isolation and characterization of alkaliphilic and thermotolerant bacteria that reduce insoluble indigo to soluble leuco-indigo from indigo dye vat. J Korean Soc Appl Biol Chem 55:83–88

Pham TH, Boon N, Aelterman P, Clauwaert P, De Schamphelaire L, Vanhaecke L, De Maeyer K, Hofte M, Verstraete W, Rahay K (2008) Metabolites produced by Pseudomonas sp. enable a gram-positive bacterium to achieve extracellular electron transfer. Appl Microbiol Biotechnol 77:1119–1129

Pruesse E, Quast C, Knielt K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596

Samanta AK, Agarwal P (2009) Application of natural dyes on textiles. Indian J Fiber Text Res 34:384–399

Sharma V, Singh R, Sharma S (2016) Comparative phytochemical investigation with TLC profiling of Indigofera tinctoria Linn. Natl Acad Sci Lett 39:337–334

Srinivasan S, Wankhar W, Rathinasamy S et al (2016) Free radical scavenging potential and HPTLC analysis of Indigofera tinctoria Linn. J Pharm Anal 6:125–131

Tu Z, Lopes HFS, Hirotaka, Koda, Yamoto I (2019a) Analysis of the microbiota involved in the early changes associated with indigo reduction in the natural fermentation of indigo. World J Microbiol Biotechnol 35:123

Tu Z, Lopes HFS, Igarashi K, Yamoto I (2019b) Characterization of the microbiota in long- and short-term natural indigo fermentation. J Ind Microbiol Biotechnol 46:1657–1667

Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R (2013) EMPeror: a tool for visualizing high-throughput microbial community data. GigaScience 2:16

Verbarg S, Rheims H, Emus S, Fruhling A, Kroppenstedt RM, Stackebrandt E, Schumann P (2004) Erysipelothrix inopinata sp. nov., isolated in the course of sterile filtration of vegetable peptone broth, and description of Erysipelotrichaceae fam. nov. Int J Syst Evol Microbiol 54:221–225

Yi C, Tan X, Bie B, Ma H, Yi H (2020) Practical and environmentally-friendly indirect electrochemical reduction of indigo and dyeing. Sci Rep 10:4927

You LX, Liu LD, Xiao Y, Dai XF, Chen BL, Jiang YX, Zhao F (2018) Flavins mediate extracellular electron transfer in Gram-positive Bacillus megaterium strain LLD-1. Bioelectrochemistry 119:196–202

Yamoto I, Hirotaka, Nadasaka Y, Yokota Y, Hoshino T, Nakajima K (2004) Alkalibacterium psychrotolerans sp. nov., a psychrotolerant obligate alkaliphile that reduces an indigo dye. Int J Syst Evol Microbiol 54:2379–2383

Yamoto I, Hirotaka, Nadasaka Y, Tokiwa Y, Nakajima K (2008) Alkalibacterium indicireducens sp. nov., an obligate alkaliphile that reduces indigo dye. Int J Syst Evol Microbiol 58:901–905

Zhang L, Wang L, Cunningham AB, Shi Y, Wang Y (2019) Island blues: indigenous knowledge of indigo-yielding plant species used by Hainan Miao and Li dyers on Hainan Island. China. J Ethnobiol Ethnomed 15:31

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.