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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）

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 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 microbiota in long- and short-term maintained 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 [*Anerobranca* 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 in three different batches

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.