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

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37 **Abstract**

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

49
50 **Keywords:** Indigo reduction, natural fermentation, next-generation sequencing, Alcaligenaceae,
51 *Alkalibacterium*, *Amphibacillus*

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73 Introduction

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75 Indigo is one of the oldest dyes used by humans. Before synthetic dyes were developed in the 19th
76 century, indigo was traditionally derived from various blue dye plants, including woad (*Isatis*
77 *tinctoria*), native to Europe and southeastern Russia; knotweed (*Polygonum tinctorium*), used in China,
78 Korea, and Japan; and *Indigofera* (*Indigofera tinctoria* and *Indigofera suffruticosa*), used in India [1,
79 3, 39]. Plant leaf processing methods for dyeing have been developed in different regions, such as
80 woad balls in Europe, *niram* in Korea and *sukumo* in Japan. However, precipitated indigo extracted
81 from plants has been used in India, China and Southeast Asia [6, 24].

82 Indigo itself does not exist in the leaves of indigo-producing plants. The original state of indigo dye
83 is always indican (indoxyl- β -D-glucoside). Therefore, transformation of indican to indigo is necessary
84 for the production of indigo dye. The most popular method is the extraction of indigo dye by short-
85 term fermentation. Fresh leaves are soaked in a large vat for 2-3 days until soft. Then, lime hydrate
86 ($\text{Ca}(\text{OH})_2$) is added to the liquid, and the mixture is thoroughly stirred. The indigo then precipitates
87 and accumulates at the bottom of the vat. Alternatively, indigo-containing plants are decomposed by
88 microorganisms, as performed for woad in Europe and *sukumo* in Tokushima Prefecture, Japan [1, 30].

89 In Tokushima Prefecture, the leaves of knotweed plants are harvested and air-dried, and then,
90 approximately 3 tons of dried leaves are mixed with a roughly equivalent weight of water and piled to
91 approximately 1 m in height. Microbial oxidative fermentation of the leaves is promoted under aerobic
92 conditions and at an appropriate temperature by controlling the water content and adjusting the
93 turnover frequency through the skillful techniques of a trained craftsman [1]. The temperature reaches
94 60 °C because of microbial activity, and the mixture becomes alkaline. This microbial oxidation step
95 is carried out for up to 100 days, and the brown composted product is called *sukumo*. During this
96 process, the precursor is broken down to indoxyl and sugar moieties by plant enzymes, and free
97 indoxyl has been suggested to form indigo via indoxyl radicals, which first form leuco-indigo and are
98 then oxidized to indigo [34].

99 Due to its insolubility in water, indigo in *sukumo* needs to be reduced to the water-soluble form
100 (leuco-indigo) before dyeing. The reduced form can be easily absorbed into fibers, and when exposed
101 to the air, the soluble leuco-indigo is oxidized back to the insoluble indigo and stays within the fibers.
102 Traditional *sukumo* reduction is implemented by fermentation under extreme alkalinity (pH 10.3-11.0)
103 [30]. *Sukumo* is mixed with hot wood ash extract; the fluid is stirred once or twice daily; and wheat
104 bran or wheat gluten is added as a substrate for the indigo-reducing microbes when the dyeing
105 efficiency decreases. In this step, oxidized indigo is solubilized via bacterial reduction. Generally, the
106 required dyeing efficiency can be maintained for approximately 6 months on average by skilled
107 artisans [30].

108 Since the late 19th century, natural indigo has been replaced over a relatively short period by

109 synthetic dyes, and in parallel, the use of chemical reductants, such as hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), has
110 been established in the industry for modern vat-based dyeing with indigo. Fermentation vats continue
111 to be used on a small scale and even in domestic dyeing processes across Europe, Africa, Asia and
112 America [10]. In addition to maintaining the cultural heritage, traditional techniques for indigo
113 fermentation have significant advantages in terms of recyclability and human health and
114 environmental safety [27, 31].

115 In the past decade, attempts have been made to develop and optimize environmentally friendly
116 alternatives for the formation and reduction of indigo. To elucidate the mechanism underlying indigo
117 fermentation, many indigo-reducing bacterial species have been isolated from various types of indigo
118 fermentation fluids. In the genus *Alkalibacterium*, there are 10 named species, 3 of which are indigo-
119 reducing bacteria: *Alkalibacterium iburiense* [28], *Alkalibacterium psychrotolerans* [41], and
120 *Alkalibacterium indicireducens* [42]. There are ten named species within the genus *Amphibacillus*,
121 and 2 of these species are indigo-reducing bacteria: *Amphibacillus indicireducens* [12] and
122 *Amphibacillus iburiensis* [13]. In addition, some other species belonging to the class Bacilli, such as
123 *Oceanobacillus indicireducens* [11], *Fermentibacillus polygoni* [14], *Polygonibacillus indicireducens*
124 [17], *Paralkalibacillus indicireducens* [15], and *Bacillus fermenti* [16], have been shown to be indigo-
125 reducing bacteria in recent years.

126 In addition, Okamoto et al. [30] explored the microbiota in aged fermented fluids with culture-
127 dependent and clone library-based methods. They found that the microbiota mainly consists of the
128 genera *Alkalibacterium*, *Amphibacillus*, *Anaerobacillus* and *Polygonibacillus* and the family
129 Proteinivoraceae. Aino et al. [2] investigated the bacterial community structure associated with indigo
130 fermentation using denaturing gradient gel electrophoresis (DGGE) and clone library analyses of a
131 PCR-amplified 16S rRNA gene and found a marked substitution of *Halomonas* spp. by *Amphibacillus*
132 spp., corresponding to a marked change in the state of indigo reduction. *Alkalibacterium* spp. were not
133 predominant in the early phase of fermentation but were abundant in aged fluid (10 months) obtained
134 from Date City, Japan. Milanović et al. [27] explored the microbiota of woad vat fermentation fluid
135 aged 12 months with PCR-DGGE and pyrosequencing and found that eumycetes and coliforms were
136 present at levels below the detection limit, whereas total mesophilic aerobes and spore-forming
137 bacteria and their spores were detected at great at high levels. Both facultative and obligate anaerobes
138 were present in the woad vat and were involved in the formation, reduction, and degradation of indigo.
139 They inferred that indigo-reducing bacteria constitute only a small fraction of the microcosm.

140 During long periods of fermentation, there are many chances of contamination by environmental
141 microorganisms. Subtle changes in nutrients, pH, and agitation can affect the state of reduction.
142 Therefore, the maintenance time differs greatly among fermentation broths, and the differences and
143 trends for microbial communities remain unknown. To investigate factors associated with defining the
144 timing of the reducing state of fermentation fluid and the changes in bacterial dynamics during the

145 whole fermentation process, we used two batches of *sukumo* fermentation broth. The reduction of one
146 of the broths lasted for only approximately 7 weeks, and that of other lasted for nearly 1 year. Next-
147 generation sequencing (NGS) was used to monitor the total transformation of bacteria during the
148 whole fermentation process of Japanese *sukumo*.

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151 **Materials and methods**

152

153 Indigo fermentation samples

154

155 Two batches of *sukumo* fermentation fluids were used: Batch 1 was fermented for approximately 7
156 weeks (51 days), and Batch 2 was fermented for nearly 1 year (345 days). Batch 1 contained 380 g of
157 *sukumo* (produced in Tokushima Prefecture, Japan) and the same weight of wood ash (fine powder of
158 burnt charcoal made from *Quercus phillyraeoides*) in 5 L of water. The wood ash was first added to 5
159 L of water and boiled by simmering on a stove for 10 min. When the liquid cooled to 60 °C at room
160 temperature, the *sukumo* was added and mixed well, and then, the fluid was placed at 26 °C in a
161 thermostatic room protected from light. On the next day, the fluid was heated again to 60 °C with
162 constant stirring and then returned to the thermostatic room. Batch 2 was made from 532 g of *sukumo*
163 (produced in Tokushima Prefecture, Japan) and 7 L of wood ash supernatant by heating to 70 °C, and
164 then placed in the same thermostatic room. Batch 2 was also heated again to 60 °C on the next day
165 and then maintained at 26 °C. During the subsequent long-term fermentation, the broths were stirred
166 every day, and the pH of the fermentation fluid was maintained between 10.3 and 11 with Ca(OH)₂ in
167 addition to small amount of Na₂CO₃, NaOH and lactic acid for Batch 1 and with Ca(OH)₂ and a small
168 amount of K₂CO₃ for Batch 2. For precise adjustment of pH in preparation for the fermentation, NaOH,
169 Na₂CO₃ and K₂CO₃ were used. On the other hand, for maintenance of pH during fermentation,
170 Ca(OH)₂ was used for the precipitation of acid. Lactic acid was used for reduction of pH. The
171 oxidation-reduction (ORP) [23] was measured using a 9300-10D (Horiba, Kyoto, Japan) electrode.

172 According to the method used by traditional craftsmen, the reduction state was roughly evaluated
173 by cotton cloth dyeing. In this method, a piece of white cotton cloth of approximately 2 cm × 3 cm is
174 soaked in the fermentation fluid for 30 sec and then removed and exposed to air for oxidation, and
175 then, the impurities are washed away in flowing water. Reduced fermentation broth will stain the white
176 cotton cloth blue. From Batch 1, samples fermented for 1 day, 7 days (1 week), 14 days (2 weeks), 21
177 days (3 weeks), 28 days (4 weeks), 35 days (5 weeks), 42 days (6 weeks), 49 days (7 weeks) and 51
178 days (7 weeks + 2 days) were examined. From Batch 2, samples fermented for 1 day, 9 days, 30 days
179 (1 month), 135 days (4.5 months), 165 days (5.5 months), 240 days (8 months), and 345 days (11.5
180 months) were used for the subsequent analysis.

181

182 DNA extraction, PCR amplification, and PCR product purification

183

184 The obtained samples were centrifuged at $15,000 \times g$ for 10 min to obtain the sample pellet. Total
185 bacterial DNA was directly extracted from the sample pellets using ISOIL (Nippon Gene, Tokyo,
186 Japan) according to the manufacturer's instructions: 950 μ l of Lysis Solution HE and 50 μ l of Lysis
187 Solution 20S were added to the samples and, after mixing well by inversion, incubated at 65 °C for 1
188 h. The samples were then centrifuged ($12,000 \times g$, 1 min, room temperature). Then, 600 μ l of each
189 supernatant to a new tube, and 400 μ l of Purification Solution was added, and mixed well. Then, 600
190 μ l of chloroform was added, and each sample was vortexed for 15 sec and then centrifuged ($12,000 \times$
191 g , 15 min, room temperature). Then, 800 μ l of the aqueous layer was transferred to a new tube while
192 taking care not to transfer the intermediate layer; 800 μ l of Precipitation Solution was added, and the
193 samples was mixed well and then centrifuged ($20,000 \times g$, 15 min, 4 °C). The supernatant was
194 discarded; 1 ml of Wash Solution was added and mixed by inverting a few times; and the mixture was
195 then centrifuged ($20,000 \times g$, 10 min, 4 °C). The supernatant was discarded; 1 ml of 70% ethanol and
196 2 μ l of Ethachinmate was added and the mixture was vortexed and then centrifuged ($20,000 \times g$, 5
197 min, 4 °C). The supernatant was discarded; the precipitates were air-dried the precipitates and then
198 dissolved in 50 μ l of TE (pH 8.0).

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

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

217 the manufacturer's instructions.

218

219 Next-generation sequencing (NGS)

220

221 The extracted V4 region of the 1st PCR products was submitted to Hokkaido System Science Co.,
222 Ltd. (Sapporo, Hokkaido, Japan). A 2nd PCR was performed with an index-adapted primer to generate
223 paired-end (2× 301 base pair) libraries for Illumina shotgun sequencing, and the products were purified.
224 NGS was carried out on the Illumina MiSeq platform (Illumina, San Diego, USA). The obtained raw
225 reads were preprocessed with cutadapt version 1.1, Trimmomatic version 0.32 and fastq-join version
226 1.1.2-537. All failed sequence reads and low-quality, tag and primer sequences were removed.
227 Clustering analysis based on operational taxonomic units (OTUs) with 97% identity and taxonomic
228 classification annotated by each representative sequence were performed by using QIIME software
229 version 1.9.1 [5]. Taxonomic analysis and annotation of the output data were also carried out by QIIME
230 software, which can perform OTU picking, taxonomic assignment, diversity analysis and graphical
231 visualization [5, 33]. Nucleotide sequence identity analysis was also carried out with bacterial 16S
232 rRNA reference sequences in the BLAST database [21].

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

235

236 Diversity analysis

237

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

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253 **Results**

254

255 Indigo-reducing fermentation

256

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

261 Batch 1 did not receive any nutrients until the staining became weak on the day 46, on the next day
262 (day 47), the batch received two pieces of wheat-gluten bread (approximately 1 g). Batch 2 received
263 wheat bran when the dyeing became weak (from week 3), and the reducing state continued until
264 approximately month 11, when the final deterioration occurred, and dyeing could no longer occur.
265 However, the staining intensity became weak at 4.5 months (135 days) (Fig. 2). This effect is probably
266 due to substrate deficiency for indigo-reducing bacteria.

267 Although the redox potential of Batch 2 was not estimated, the changes in pH and redox potential
268 in Batch 1 and the change in pH in Batch 2 were measured (Fig. 3). The initial decrease in redox
269 potential occurred due to the survival of aerobic bacteria after the initial heat treatment. In addition,
270 further reduction in the redox potential may occur via external transmission of electrons from
271 anaerobic metabolic pathways associated with lactic acid production. It has been reported that the
272 magnitude of the reduction in redox potential is dependent on the bacteria present in the fluid [29]. To
273 maintain the fermentation fluid at a pH of approximately 10.3, Ca(OH)₂ was added. The changes in
274 pH are depicted in Fig. 3.

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

283

284 Whole bacterial community analysis by NGS

285

286 The whole community structure of the *sukumo* fermentation fluids were analyzed based on OTUs
287 identified by NGS. NGS analysis indicated that the fluids contained a wealth of different bacterial
288 species. The bacterial communities comprised a total of 22 phyla in Batch 1 and 24 phyla in Batch 2,

289 including Archaea, which were detected in samples of Batch 1 from day 1, 7 and 51 and samples of
290 Batch 2 from day 9, although the relative levels of Archaea were consistently less than 0.1%. The top
291 3 relative levels were exhibited by the phyla Proteobacteria, Firmicutes and Actinobacteria, which
292 accounted for more than 90% of the phyla in every sample. In the beginning, the abundance of the
293 phylum Firmicutes increased substantially in the middle and late stages of fermentation, the abundance
294 of the phylum Proteobacteria increased substantially, and the abundance of the phylum Tenericutes
295 (0.2-0.3%) also increased, especially in Batch 1 (data not shown).

296 At the genus level, 208 and 279 different genera were distinctly detected in Batch 1 and Batch 2,
297 respectively. During the whole fermentation of the two batches, most of the significant bacterial
298 populations were similar, and the batches also showed significant changes in the relative content of
299 various genera or families (Fig. 2). The abundance of the genera *Anaerobranca* and *Tissierella*
300 increased substantially in the beginning and decreased slightly thereafter. The family Alcaligenaceae
301 was detected in small amounts, but the abundance increased greatly on day 21 in Batch 1 and on day
302 240 in Batch 2. There were also significant differences between the two batches. In the beginning, the
303 abundance of the genus *Prauseria* decreased substantially in Batch 1, and the abundances of the
304 families Rhodospirillaceae and Trueperaceae decreased substantially in Batch 2. The abundances of
305 the genera *Clostridium* and *Coprococcus* increased from the middle period in Batch 1, while the the
306 abundances of genus *Devosia* and family Phyllobacteriaceae increased from the middle period in
307 Batch 2, and the genera *Acholeplasma* and *Azoarcus* were detected in only deteriorated samples from
308 Batch 2. In addition, the genus *Caldicoprobacter* had a relative abundance of 2.1% on day 28, which
309 increased to 28.7% on day 35, and this genus was present at more than 20% after day 35 in Batch 1.
310 *Pseudomonas* was present at 0% at week 2 but at 36.9% on day 21 in Batch 1, maintaining a relatively
311 high level thereafter. However, *Pseudomonas* had a relative abundance of 16.9% in only the
312 deteriorated sample and an abundance of 0% in the other samples of Batch 2.

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

325 before deterioration in Batch 2; however, the value in Batch 1 was 5.6% on day 28, decreasing to 1.5%
326 by day 42.

327

328 Statistical analysis with OTUs

329

330 1) Observed species from rarefaction curves

331

332 Alpha diversity is usually used to represent species diversity in a specific environment. In this study,
333 rarefaction curves were computed with sequences and observed species based on OTUs; these curves
334 show the trends in bacterial species detected in the samples. Because the sequences of each sample
335 were different, the observed species were obtained from the intersection nodes of 3818 sequences of
336 Batch 1 and 3730 sequences of Batch 2 (Fig. 4). The number of bacterial species decreased in the
337 beginning of the fermentation, going from 242 species on day 1 to 145 species on day 7 and 138
338 species on day 14 in Batch 1 and from 175 species on day 1 to 133 species on day 9 in Batch 2. Then,
339 this number increased with fermentation progression until deterioration, resulting in 323 species on
340 day 51 in Batch 1 and 281 species on day 345 in Batch 2. In both the short- and long-term fermentation
341 broths, the number of observed bacterial species showed a U-shaped trend. In the present sequence
342 analysis, the number of bacterial species in the deterioration period was approximately 3 times that at
343 the beginning of reduction.

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

355

356 2) Principal coordinates analysis (PCoA)

357

358 The Bray-Curtis index was used to evaluate the similarity of the microbiota between all samples
359 (Fig. 5). The bacterial composition changed rapidly at the beginning (days 1 to 7 of Batch 1, day 1 to
360 9 of Batch 2) and then remained steady in the freshly reduced samples (days 7 to 14 of Batch 1, days

361 9 to 30 of Batch 2). Then, the bacterial population rapidly changed after day 14 in Batch 1 (Fig. 5A).
362 However, in Batch 2, there was little transformation during the three weeks from days 9 to 30 and in
363 the next few months from the days 135 to 240. However, the bacterial flora transformed slowly from
364 the beginning to the reduction stage to the final deterioration stage (Fig. 5B). This result also indicated
365 that the bacterial taxa replaced the previous group at all times. The bacterial population in Batch 1
366 transformed faster than that in Batch 2. The OTUs identified as *Bacillus* showed that it contained *P.*
367 *indicireducens* and *Bacillus cohnii*. The change in abundance of the major constituents and genera
368 during indigo reduction was estimated each fermentation batch (Fig. 6). It is found that the slow
369 transition in Batch 2 after day 9 in PCoA (Fig. 5b) was attributed to the slow transition of the genus
370 *Anaerobranca* and *P. indicireducens* (Fig. 6B). On the other hand, the change velocities of reported
371 indigo-reducing bacteria in the genera *Amphibacillus*, *Alkalibacterium*, and *Oceanobacillus* and the
372 *Bacillus cohnii* were not as high as those of the dominant taxa (Fig. 6AB). Figure 6 shows that the
373 deterioration of the fermentation fluid is associated with the relative abundance of Alcaligenaceae in
374 both batches.

375

376

377 **Discussion**

378

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

385 The presented results suggest that entry into a stable state at approximately 10 days in the long-term
386 fluid is important for long-lasting indigo fermentation. The microbiota generated within 10 days of
387 fermentation initiation exhibited a retention ability. This period may correspond to the period in which
388 obligate anaerobes were dominant in the fluids. The most predominant taxa transformed successively
389 as follows: seeds (mostly oxygen metabolizing aerobes) → obligate anaerobes (i.e., *Anaerobranca*)
390 → facultative anaerobes (i.e., *Bacillus* in Fig. 2; *P. indicireducens* in Fig. 6B). In addition to the most
391 predominant organisms, reported aerotolerant indigo-reducing genera were present at a relative
392 abundance of 7.4-16.3% during the indigo reduction period. Comparison with the short-term
393 fermentation fluid showed that the successive transitional change from predominance of the obligate
394 anaerobe *Anaerobranca* to *P. indicireducens* was associated with the stable state of the microbiota.
395 This stability was demonstrated by the endurance of the indigo-reducing state, although a substantial
396 decrease in pH occurred in the Batch 1 at approximately days 95-102. Although the taxa were different

397 from those in the present study, the predominance of obligate anaerobes in long-term indigo
398 fermentation fluid was reported previously [30]. This retention is attributed to the environmental
399 pressure of alkaline anaerobic conditions [1] and the persistence of the bacteria adapted to the
400 environment (i.e., obligately anaerobic alkaliphiles). Natural fermentation processes that utilize
401 spontaneously occurring microbiota have been used to produce several fermented foods, beverages
402 and condiments [19, 20, 35, 37]. In most of these cases, acidic and/or salty environments define the
403 adapted microbiota. In the case of traditional Korean fish sauce, fermentation (myeolchi-aekjeot)
404 samples exhibit stable states of the microbiota under the appropriate fermentation conditions [20].

405 On the other hand, according to the microbiota, the long-term stable state was maintained
406 appropriately until day 14. On day 21, *Pseudomonas* was the predominant taxon, and its dominance
407 lasted until day 51. This change may have resulted from the introduction of lactic acid on day 13.
408 When lactic acid was introduced, the redox potential of the fermentation fluid increased to -530 mV
409 (Fig. 3A). In the nondyeing deteriorated stage, Gram-negative bacteria were predominant in both
410 batches. In addition to the high redox potential, a localized low-pH niche may eventually have been
411 formed in the fermentation fluid, and this event may have increased the levels of Gram-negative
412 bacteria such as *Pseudomonas* and Alcaligenaceae (Fig. 2, Fig. 6). This difference may have been due
413 to Batch 1 having a small fermentation volume, which readily induces high redox potential, and a
414 localized low pH niche may have been produced due to stirring with a thinner glass bar than that used
415 for Batch 2. Normally, the abundances of these taxa hardly increase under highly anaerobic and high-
416 pH conditions, which are dominated by obligately anaerobic alkaliphiles. Detection of unfavorable
417 taxa in the initial stage of propagation could prevent further propagation. Therefore, it is considered
418 that there may be a period of susceptibility in which propagation of unfavorable taxa could occur. For
419 example, during the period of entry into the obligate anaerobe-dominated phase, the mixture may be
420 susceptible to unfavorable environmental changes. When the high redox potential occurred on day 13,
421 the proportion of *Anaerobacillus* began to decrease (Fig. 6A). Bacteria belonging to Alcaligenaceae
422 (genus *Alcaligenes*) are often observed in reduced indigo fermentation fluid [2, 27]. Therefore, a
423 taxonomic study to identify Alcaligenaceae species observed in this study is desirable. The relative
424 abundance of Alcaligenaceae and the time periods at which these species are present are important for
425 determination of the staining intensity of textiles.

426 In this study, *Amphibacillus*, *Alkalibacterium*, and *Oceanobacillus* were detected in the initial
427 sample (day 1) of Batch 1, and *Amphibacillus* and *Oceanobacillus* were detected in the initial sample
428 (day 1) of Batch 2. These genera have been isolated from microbial indigo fermentation using *sukumo*
429 as the source of the dye and have been indicated to include several indigo-reducing bacteria [11-13,
430 28, 41, 42]. Although the correlation between the relative proportions of *Amphibacillus*,
431 *Alkalibacterium*, and *Oceanobacillus* and dyeing intensity exhibited a 7-14-day lags, there was a
432 correlation observed in short-term fermentation. On the other hand, a direct correlation between the

433 relative proportion of *Amphibacillus* and *Oceanobacillus* and dyeing intensity was observed in long-
434 term fermentation (supplementary Fig. 1). Changes in the relative abundances of dominant taxa other
435 than *Amphibacillus*, *Alkalibacterium* and *Oceanobacillus* were examined (Supplementary Fig. 1). In
436 both batches, the abundance of *Anaerobranca* decreased as the fluids exhibited a shift toward
437 deterioration (Fig. 6). Therefore, *Anaerobranca* is predicted to be a favorable bacterium for
438 maintenance of a reducing state in indigo fermentation. On the other hand, Alcaligenaceae species
439 were also predominant in both deteriorated fermentation fluids (Fig. 6). Although the mechanisms of
440 inhibition of indigo-reduction by Alcaligenaceae are unknown, it is possible that these organisms
441 degrade indigo via the activity of enzymes such as laccase [36, 43].

442 There remains still a great possibility that a large number of potential indigo-reducing bacteria were
443 not detected by NGS with the primer pair 515F-805R in this study. In addition, the determined gene
444 sequences were not always long enough to identify the corresponding species that were reported as
445 indigo-reducing bacteria. Furthermore, although we detected their presence in this study, several taxa
446 have not been identified as indigo-reducing bacteria. For example, the genus *Anaerobranca* was
447 abundant in both Batch 1 and Batch 2. It was assumed that *Anaerobranca* spp. include indigo-reducing
448 bacteria because of their phylogenetic position adjacent to indigo-reducing bacteria and their Fe³⁺-
449 reducing characteristics [9].

450 Many studies have examined the microbiota in natural fermentation systems, such as fermented fish,
451 fermented soybean condiments, lambic beer and fermented rice bran [19, 20, 35, 37]. In most of these
452 cases, fermentation occurs in salty and/or acidic environments. However, the largest difference is that
453 indigo needs to be continuously used for dyeing during the fermentation process and that the reduction
454 reaction needs to continue after dyeing. Most natural fermentation processes can be transferred to seed
455 cultures of single microbial species because there is little chance of intensive contamination from
456 external environments. However, maintenance of a stable microbiota under alkaline anaerobic
457 conditions is much more important in indigo fermentation than in other natural fermentation processes
458 due to the requirement for long-term maintenance. Determination of the reducing period of the
459 fermentation fluid and the changes in bacterial dynamics during the whole fermentation process will
460 be helpful for identification of a stable and efficient bacterial community for indigo reduction, and the
461 findings will be applicable for the stabilization of other alkaline anaerobic bioprocesses [7, 8, 25].

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469 **References**

- 470 1. Aino K, Hirota K, Okamoto T, Tu Z, Matsuyama H, Yumoto I (2018) Microbial communities
471 associated with indigo fermentation that thrive in anaerobic alkaline environments. *Front*
472 *Microbiol* 9:1-16
- 473 2. Aino K, Narihiro T, Minamida K, Kamagata Y, Yoshimune K, Yumoto I (2010) Bacterial
474 community characterization and dynamics of indigo fermentation. *FEMS Microbiol Ecol* 74:174-
475 183
- 476 3. Blackburn RS, Bechtold T, John P (2009) The development of indigo reduction methods and pre-
477 reduced indigo products. *Coloration Technol* 125:193-207
- 478 4. Božič M, Kokol V (2008) Ecological alternatives to the reduction and oxidation processes in
479 dyeing with vat and sulphur dyes. *Dyes Pigm.* 76:299-309
- 480 5. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena
481 AG, Goodrich JK, Gordon JI, Huttley GA (2010) QIIME allows analysis of high-throughput
482 community sequencing data. *Nat Methods* 7:335-336
- 483 6. [Cardon D \(2007\) Natural Dyes - Sources, Tradition, Technology and Science. Archetype](#)
484 [Publications Ltd., London](#)
- 485 7. Fuller SJ, McMillan DGG, Renz MB, Shmit M, Burke IT, Stewart DI (2014) Extracellular electron
486 transport-mediated Fe(III) reduction by a community of alkaliphilic bacteria that use flavins as
487 electron shuttle. *Appl Environ Microbiol* 80:128-137
- 488 8. Fuller SJ, Burke IT, McMillan DGG, Ding W, Stewart DI (2015) Population changes in a
489 community of alkaliphilic iron-reducing bacteria due to changes in the electron acceptor:
490 implications for bioremediation at alkaline Cr(VI)-contaminated sites. *Water, Air, Soil Pollut*
491 226:180
- 492 9. Gorlenko, V, Tsapin, A, Namsaraev, Z, Teal T, Tourova T, Engler D, et al. (2004) *Anaerobranca*
493 *californiensis* sp. nov., an anaerobic alkalithermophilic fermentative bacterium isolated from a
494 hot spring on Mono Lake. *Int J Syst Evol Microbiol* 54:739-743
- 495 10. Hartl A, Gaibor ANP, van Bommel MR, Hofmann-de Keijzer R (2015) Searching for blue:
496 experiments with woad fermentation vats and an explanation of the colours through dye analysis.
497 *J Archaeol Sci* 2:9-39
- 498 11. Hirota K, Aino K, Nodasaka Y, Yumoto I (2013) *Oceanobacillus indicireducens* sp. nov., a
499 facultative alkaliphile that reduces an indigo dye. *Int J Syst Evol Microbiol* 63:1437-1442
- 500 12. Hirota K, Aino K, Nodasaka Y, Morita N, Yumoto I (2013) *Amphibacillus indicireducens* sp. nov.,
501 an alkaliphile that reduces an indigo dye. *Int J Syst Evol Microbiol* 63:464-469
- 502 13. Hirota K, Aino K, Yumoto I (2013) *Amphibacillus iburiensis* sp. nov., an alkaliphile that reduces
503 an indigo dye. *Int J Syst Evol Microbiol* 63:4303-4308
- 504 14. Hirota K, Aino K, Yumoto I (2016) *Fermentibacillus polygonigen* nov., sp. nov., an alkaliphile

- 505 that reduces indigo dye. *Int J Syst Evol Microbiol* 66:2247-2253
- 506 15. Hirota K, Nishita M, Matsuyama H, Yumoto I (2017) *Paralkalibacillus indicireducens* gen., nov.,
507 sp. nov., an indigo-reducing obligate alkaliphile isolated from indigo fermentation liquor used for
508 dyeing. *Int J Syst Evol Microbiol* 67:4050-4056
- 509 16. Hirota K, Nishita M, Tu Z, Matsuyama H, Yumoto I (2018) *Bacillus fermenti* sp. nov., an indigo-
510 reducing obligate alkaliphile isolated from indigo fermentation liquor for dyeing. *Int J Syst Evol*
511 *Microbiol* 68:1123-1129
- 512 17. Hirota K, Okamoto T, Matsuyama H, Yumoto I (2016) *Polygonibacillus indicireducens* gen nov.,
513 sp. nov., an indigo-reducing and obligate alkaliphile isolated from indigo fermentation liquor for
514 dyeing. *Int J Syst Evol Microbiol* 66:4650-4656
- 515 18. Hobbie SN, Li X, Basen M, Stingl U, Brune A (2012). Humic substance-mediated Fe (III)
516 reduction by a fermenting *Bacillus* strain from the alkaline gut of humus-feeding scarab beetle
517 larva. *Syst Appl Microbiol* 35:226-232
- 518 19. Jung JY, Lee SH, Lee HJ, Jeon CO (2013) Microbial succession and metabolite changes during
519 fermentation of saeu-jeot: traditional Korean salted seafood. *Food Microbiol* 34:360-368
- 520 20. Jung JY, Lee HJ, Chun BH, Jeon CO (2016) Effect of temperature on bacterial communities and
521 metabolites during fermentation of myeolchi-aekjeot, a traditional Korean fermented anchovy
522 sauce. *PLoS One* 11, e0151351
- 523 21. Kano R, Kobayashi Y, Nishikawa A, Murata R, Itou T, Ito T, Suzuki K, Kamata H (2018) Next-
524 generation sequencing analysis of bacterial flora in bovine prototheca mastitic milk. *Med Mycol*
525 *J* 59:41-46
- 526 22. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation
527 of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-
528 based diversity studies. *Nucleic Acids Res* 41:1-11
- 529 23. Liu CG, Xue C, Lin YH, Bai FW (2013) Redox potential control and applications in microaerobic
530 and anaerobic fermentations. *Biotechnol Adv* 31:257-265
- 531 24. Li S, Cunningham AB, Fan R, Wang Y (2019) Identity blues: the ethnobotany of the indigo dyeing
532 by Landian Yao (Iu Mien) in Yunnan, Southwest China. *J Ethnobiol Ethnomed* 15:13
- 533 25. Lucena-Adrós H, Ruiz-Barba J L (2016) Diversity and enumeration of halophilic and alkaliphilic
534 bacteria in Spanish-style green table-olive fermentations. *Food Microbiol* 53:53-62
- 535 26. Lee MH, Li FZ, Lee J, Kang J, Lim S, Nam YD (2017) Next-Generation Sequencing Analyses of
536 Bacterial Community Structures in Soybean Pastes Produced in Northeast China. *J Food Sci*
537 *82*:960-968
- 538 27. Milanović V, Osimani A, Taccari M, Garofalo C, Butta A, Clementi F, Aquilanti L (2017) Insight
539 into the bacterial diversity of fermentation woad dye vats as revealed by PCR-DGGE and
540 pyrosequencing. *J Ind Microbiol Biotechnol* 44:997-1004

- 541 28. Nakajima K, Hirota K, Nodasaka Y, Yumoto I (2005) *Alkalibacterium iburiense* sp. nov., an
542 obligate alkaliphile that reduces an indigo dye. *Int J Syst Evol Microbiol* 55:1525-1530
- 543 29. Nicholson SK, John P (2005) The mechanism of bacterial. *Appl Microbiol Biotechnol* 68:117-123
- 544 30. Okamoto T, Aino K, Narihiro T, Matsuyama H, Yumoto I (2017) Analysis of microbiota involved
545 in the aged natural fermentation of indigo. *World J Microbiol Biotechnol* 33:70
- 546 31. Osimani A, Aquilanti L, Baldini G, Silvestri G, Butta A, Clementi F (2012) Implementation of a
547 biotechnological process for vat dyeing with woad. *J Ind Microbiol Biotechnol* 39:1309-1319
- 548 32. Perkin, A.G.; Bloxam, W.P. CLXII. Indican. Part I Transactions. *J. Chem. Soc.* 1907, 91, 1715-
549 1728
- 550 33. Rawat N, Joshi GK (2018) Bacterial community structure analysis of a hot spring soil by next
551 generation sequencing of ribosomal RNA. *Genomics*
552 <https://doi.org/10.1016/j.ygeno.2018.06.008>
- 553 34. Russell GA, Kaupp G (1969) Oxidation of Carbanions. IV. Oxidation of Indoxyl to Indigo in Basic
554 Solution. *J Am Chem Soc* 91:3851-3859
- 555 35. Sakamoto N, Tanaka S, Sonomoto K, Nakayama J (2011) 16S rRNA pyrosequencing-based
556 investigation of the bacterial community in nukadoko, a pickling bed of fermented rice bran. *Int*
557 *J Food Microbiol* 144: 352-359
- 558 36. Singh G, Capalash N, Goel R, Sharma P (2007) A pH-stable laccase from alkali-tolerant γ -
559 proteobacterium JB: purification, characterization and indigo carmine degradation. *Enzyme*
560 *Microbiol Technol* 41:794-799
- 561 37. Spitaels F, Wieme AD, Janssens M, Aerts M, Van Landschoot A, De Vuyst L, Vandamme P (2015)
562 The microbial diversity of an industrially produced lambic beer shares members of a traditional
563 produced one and reveals a core microbiota for lambic beer fermentation. *Food Microbiol* 49:23-
564 32
- 565 38. Visi DK, Souza ND, Ayre BG, Webber CL, Allen MS (2013) Investigation of the bacterial retting
566 community of kenaf (*Hibiscus cannabinus*) under different conditions using next-generation
567 semiconductor sequencing. *J Ind Microbiol Biotechnol* 40:465-475
- 568 39. Vuorema A (2008) Reduction and analysis methods of indigo. [dissertation thesis].
569 Department of Chemistry, University of Turku, Finland
- 570 40. Wang X, Du H, Xu Y (2017) Source tracking of prokaryotic communities in fermented grain of
571 Chinese strong-flavor liquor. *International Journal of Food Microbiology* 244: 27–35
- 572 41. Yumoto I, Hirota K, Nodasaka Y, Yokota Y, Hoshino T, Nakajima K (2004) *Alkalibacterium*
573 *psychrotolerans* sp. nov., a psychrotolerant obligate alkaliphile that reduces an indigo dye. *Int J*
574 *Syst Evol Microbiol* 54:2379–2383
- 575 42. Yumoto I, Hirota K, Nodasaka Y, Tokiwa Y, Nakajima K (2008) *Alkalibacterium indicireducens*
576 sp. nov., an obligate alkaliphile that reduces indigo dye. *Int J Syst Evol Microbiol* 58:901–905

577 43. Yavuz M, Kaya G, Aytakin Ç. (2014) Using *Ceriporiopsis subvermispora* CZ-3 laccase for indigo
578 carmine decolorization and denim bleaching. *Int Bioderion Biodegr* 88:199-205
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613 Figure legends

614

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

619

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

624

625 **Fig. 3** Fluctuation in abiotic parameters in indigo fermentation batches. Fluctuation in pH (A) and
626 redox potential (B) in Batch 1 and pH (C) in Batch 2. The batches were stirred every day, and the pH
627 of the fermentation fluid was maintained between 10.3 and 11 in Batch 1 with the addition of Na₂CO₃,
628 NaOH, Ca(OH)₂, and lactic acid and in Batch 2 with Ca(OH)₂ and K₂CO₃.

629

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

634

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

639

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

643



Reduced fluid



Deteriorated fluid

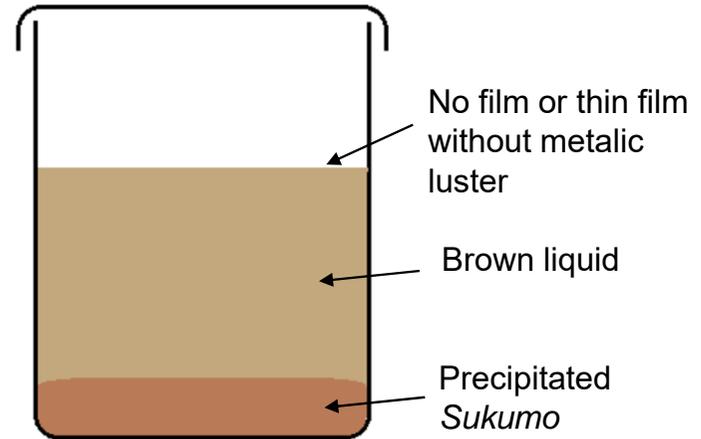
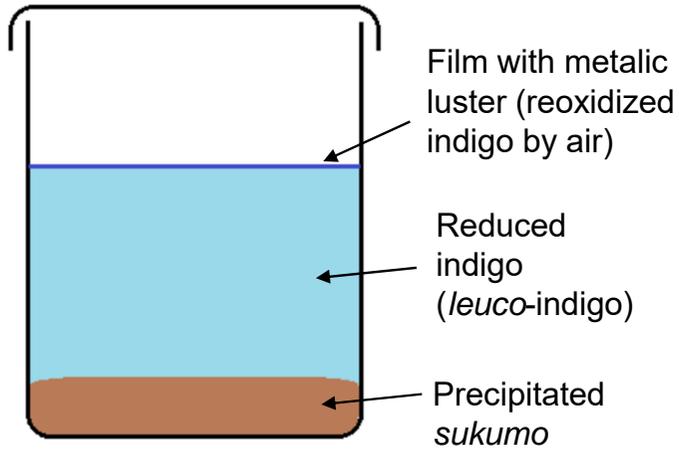


Fig. 1

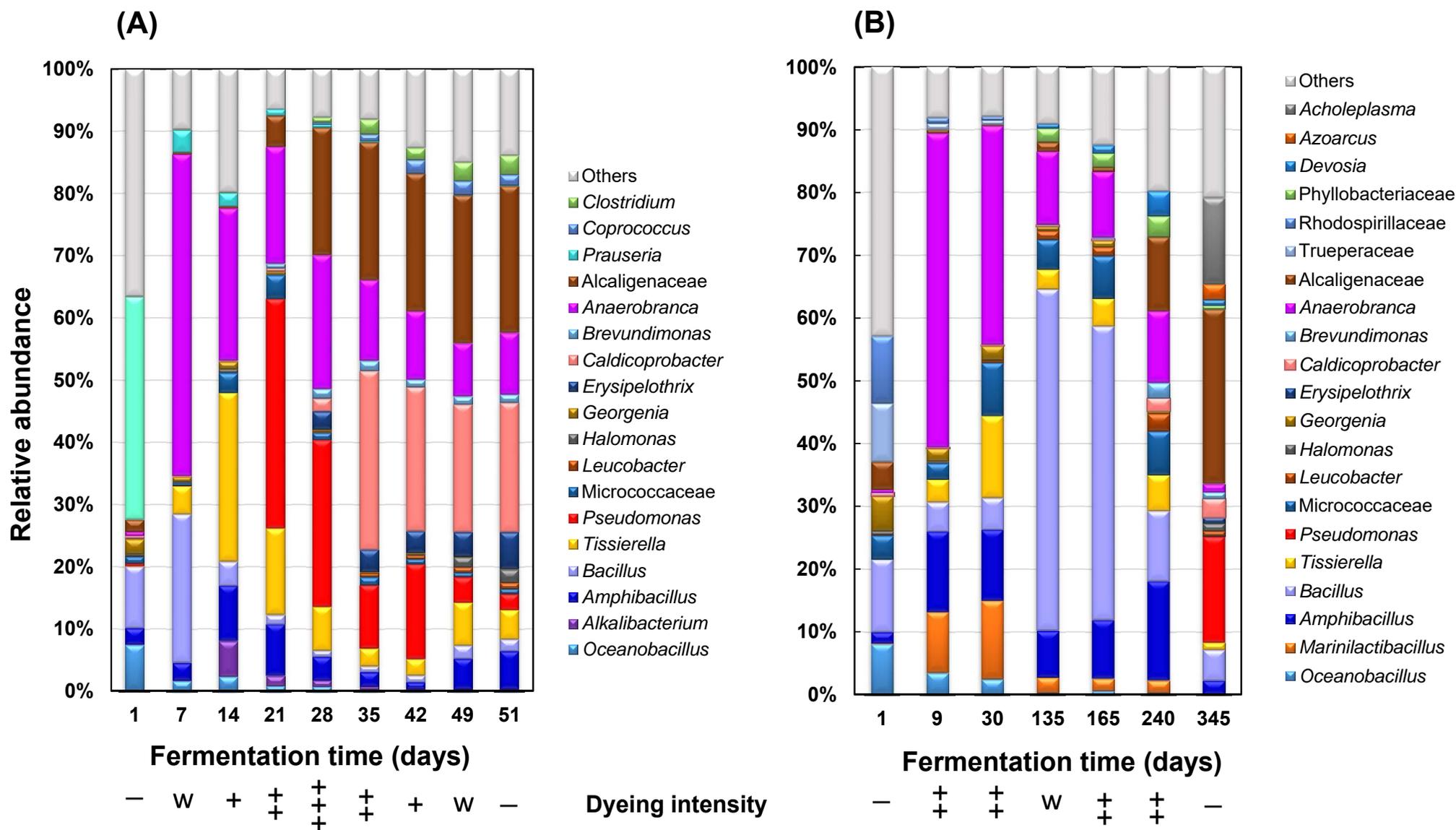
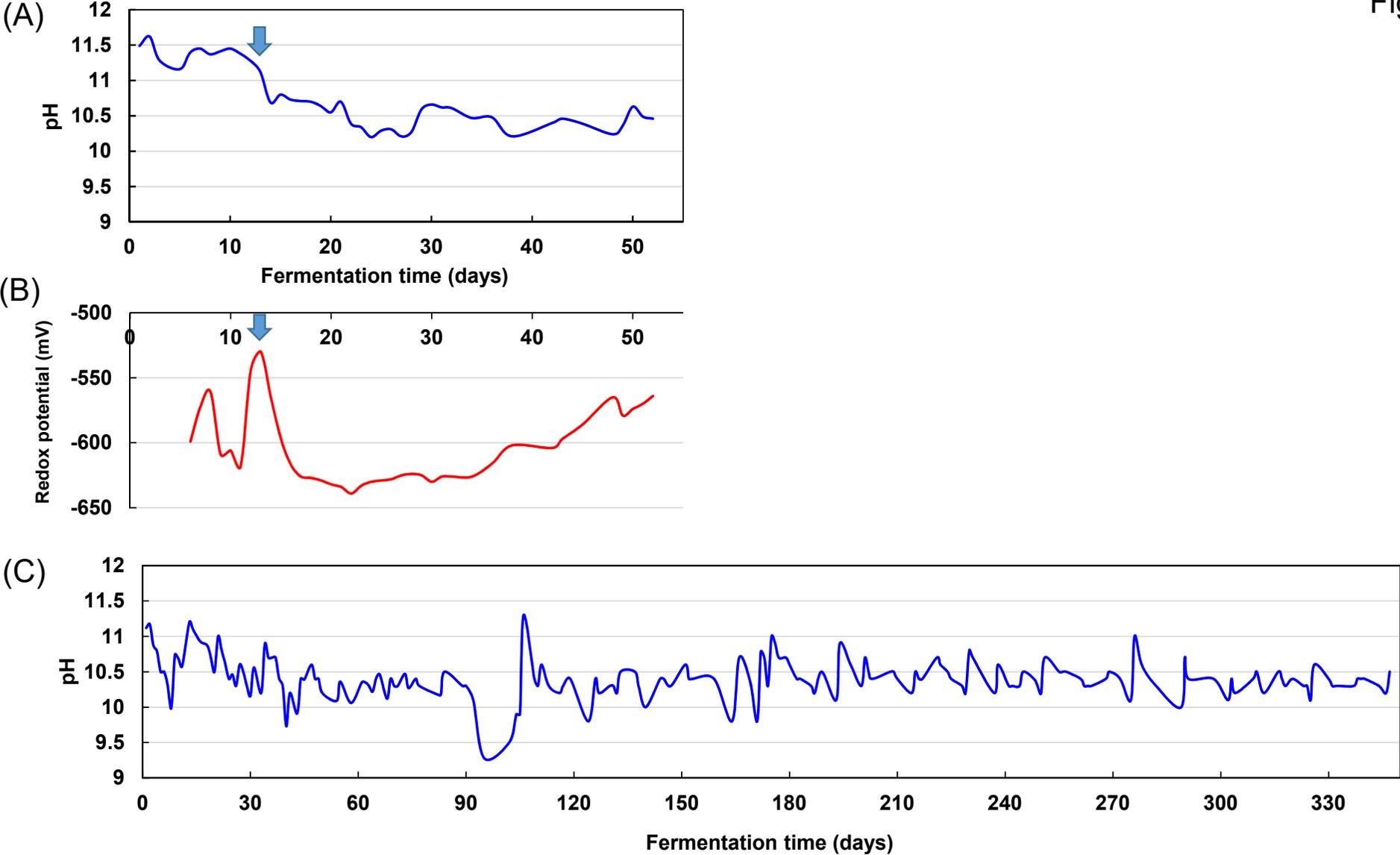
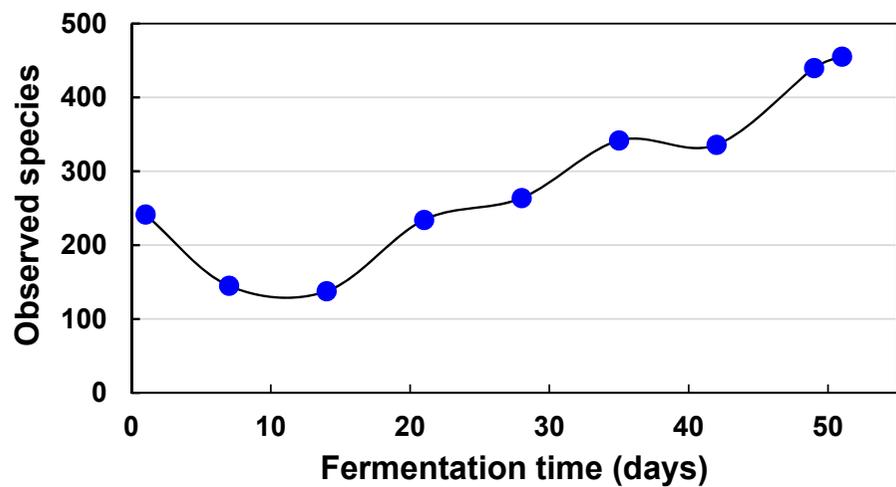


Fig. 2

Fig. 3



(A)



(B)

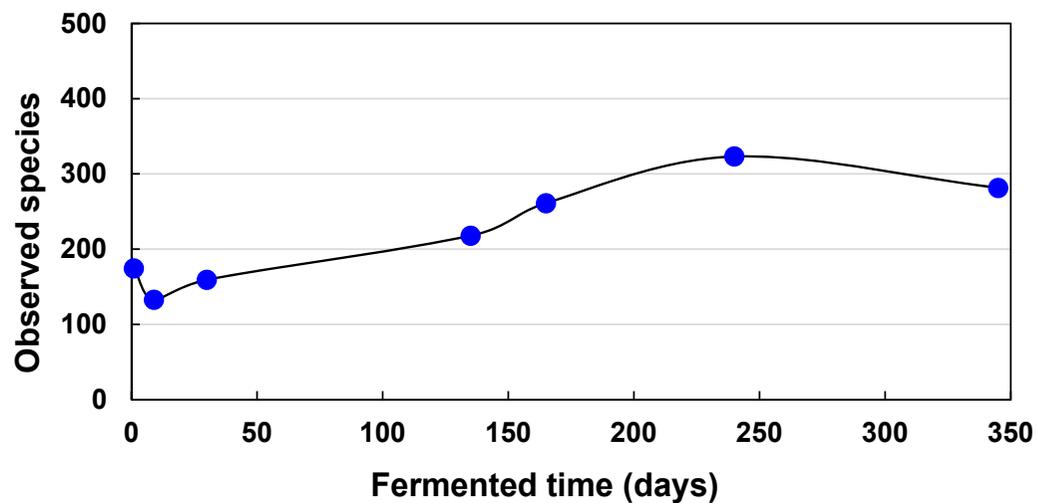


Fig. 4

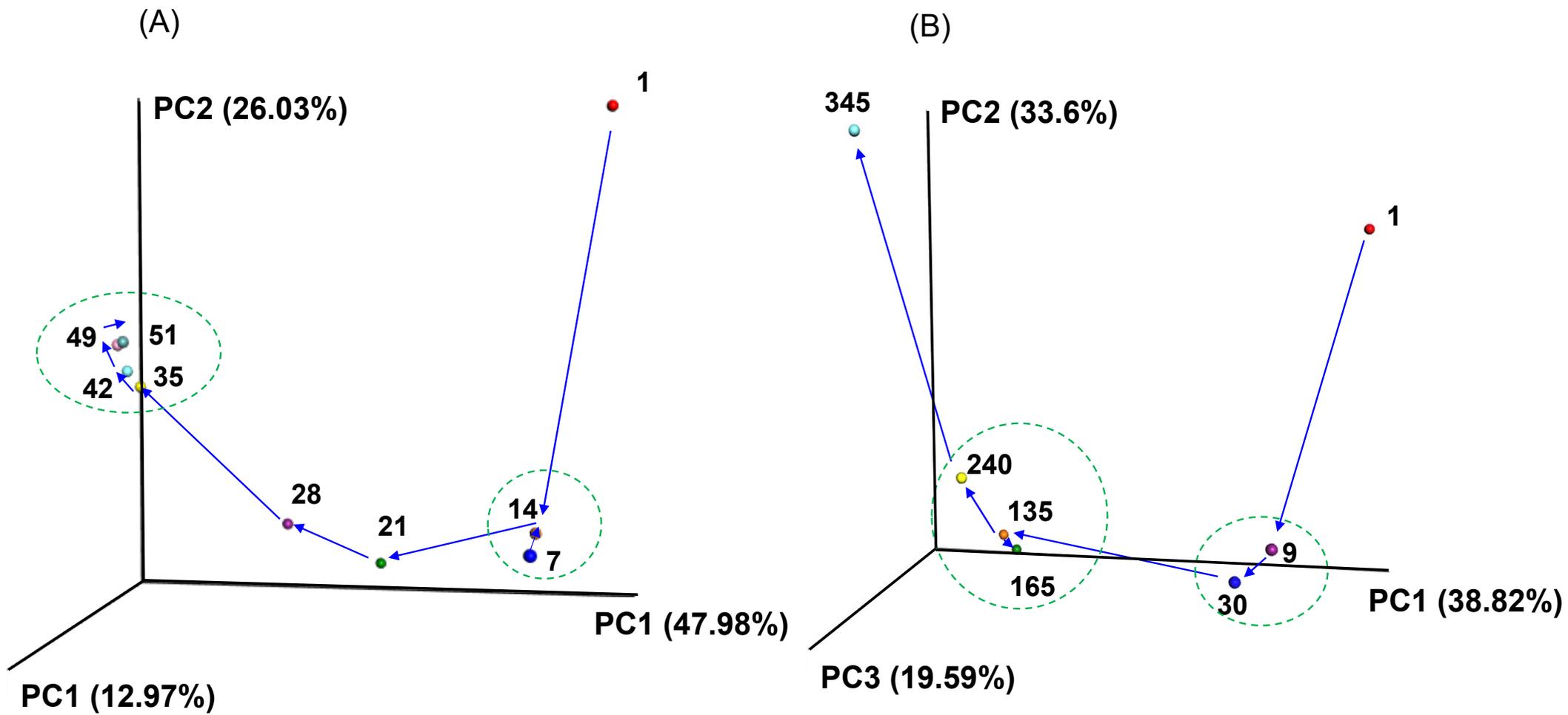


Fig. 5

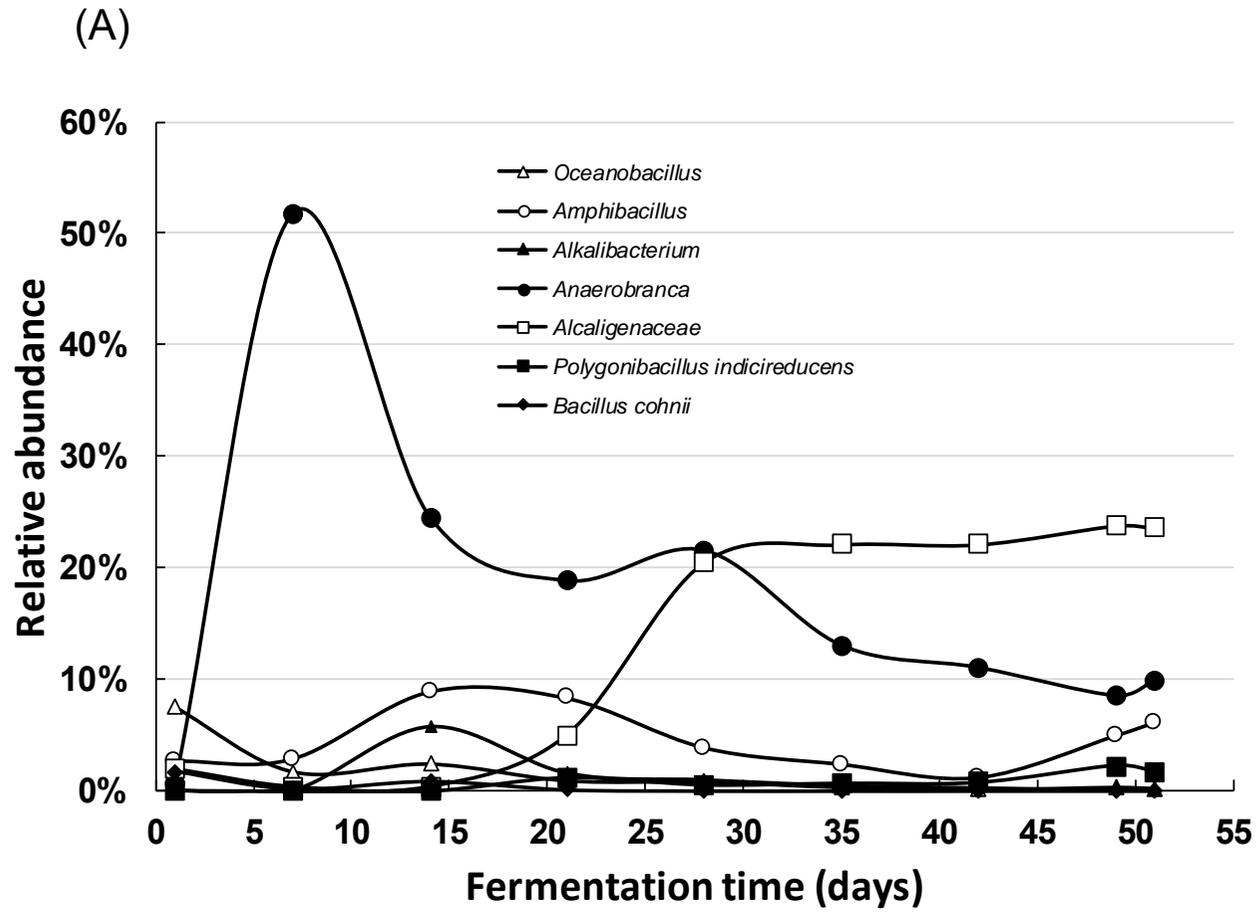


Fig. 6(A)

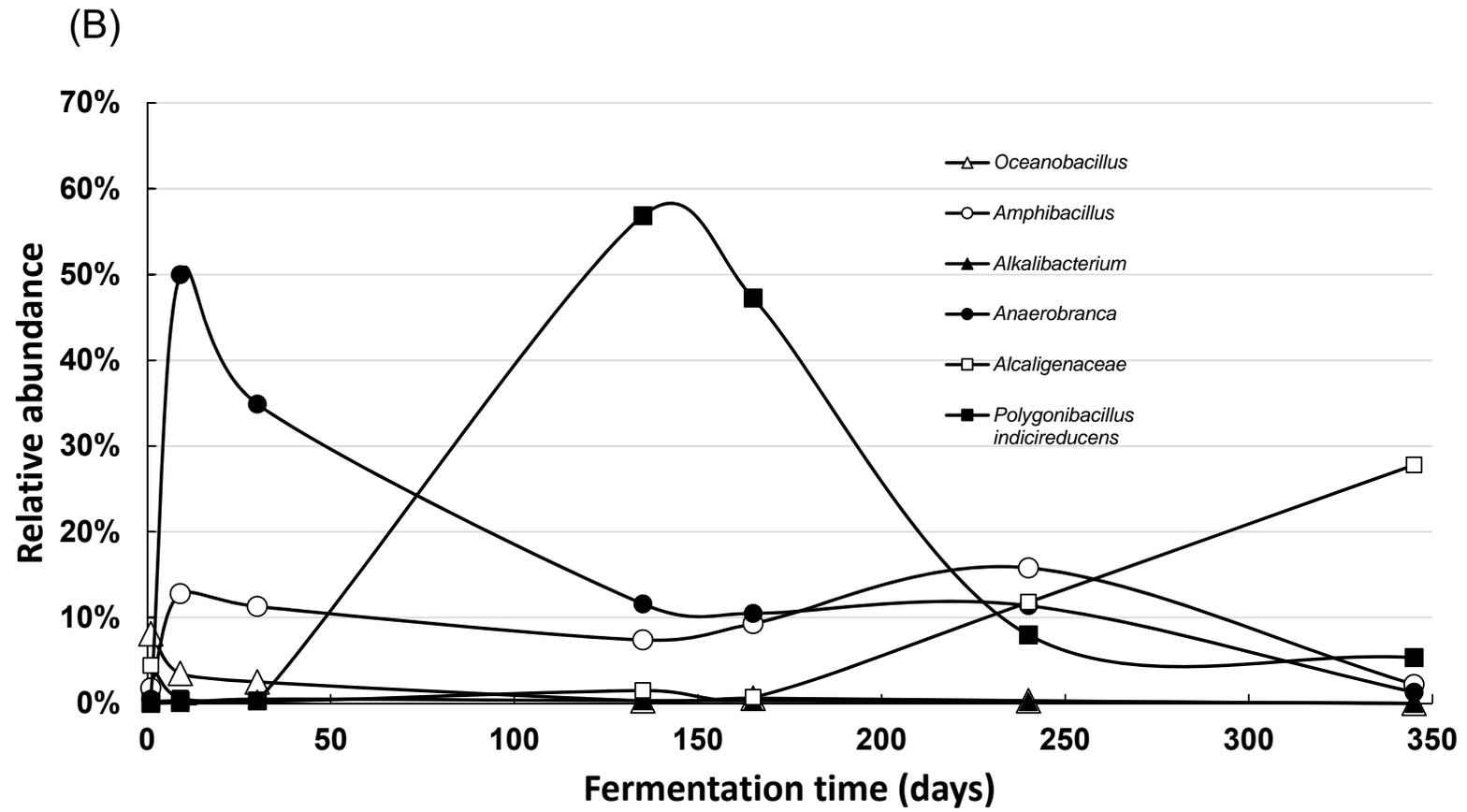


Fig. 6(B)