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Open L-Lactic Acid Fermentation of Food Refuse Using Thermophilic *Bacillus coagulans* and Fluorescence *In Situ* Hybridization Analysis of Microflora

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In the production of commercially useful poly-L-lactic acid plastic from biomass wastes, a feasible fermentation process to produce optically active L-lactic acid would be required. Here, model kitchen refuse (MKR) was inoculated with *Bacillus coagulans* NBRC12583 under nonsterilized openculture conditions. At temperatures below 45°C, a racemic mixture of D- and L-lactic acids was accumulated, whereas only L-lactic acid was selectively accumulated by incubation at 50–65°C. At 45°C, the results of fermentation could not be consistently reproduced. To analyze microflora in this type of mixed culture system, whole-cell fluorescence *in situ* hybridization (FISH) using 16S rRNA-targeted oligonucleotide probes for *B. coagulans*, Bcoa191, and LAC722(L), a group-specific probe for a wide range of mesophilic lactic acid bacteria was applied. The dominancy of mesophilic lactic acid bacteria at lower temperatures, and that of *B. coagulans* at higher temperatures were confirmed. By using a saccharified liquid of collected kitchen refuse, 86 g/*l* of L-lactic acid was accumulated under nonsterile conditions by a 5-d incubation at 55°C, pH 6.5, with 53% carbon yield and 97% optical purity. To conclude, high temperature open lactic acid fermentation is a simple and promising method for producing high-grade L-lactic acid from biomass waste, and FISH analysis of such mixed-culture systems is helpful for monitoring the microflora in these cultures.

[**Key words:** food waste, kitchen refuse, L-lactic acid, open fermentation, lactic acid bacteria, fluorescence *in situ* hybridization (FISH), 16S rRNA, microbial analysis]

Poly-L-lactic acid (PLLA) is a biodegradable and recyclable plastic, and is currently being produced on an industrial-scale via the lactic acid fermentation of cornstarch (1). As PLLA is manufactured from a renewable resource and thus would reduce the net emission of carbon dioxide and the demand for petroleum, a fossil fuel resource, the potential of PLLA as a green plastic has been discussed (2). Although this type of plastic should be applicable globally, producing PLLA and other plant-derived plastics is costly, preventing their widespread application. We have recently proposed a new system for recycling municipal food waste into high-quality PLLA plastics (3). Municipal food waste constitutes a significant biomass resource in Japan, because approximately 20% of the 50 million tonnes of waste that are generated annually consist of refuse from kitchens and the food industry. Such a system would not only provide an outlet for municipal food waste, but would also alleviate a resource conflict, as cornstarch is also a food commodity for human consumption and for use in animal feed. As a drawback, the production of PLLA from food waste requires relatively higher energy than that from cornstarch.

In the proposed system, *Lactobacillus rhamnosus* is used for the L-lactic acid fermentation of minced and autoclaved food waste. During our investigations, we found that lactic acid selectively accumulated in the cultures of nonautoclaved minced kitchen refuse under open conditions (open fermentation), without the inoculation of any lactic acid bacteria (LAB) (4). The intermittent pH neutralization of minced kitchen refuse enhanced lactic acid production and stabilized the accumulated lactic acid. These observations indicate that selective lactic acid fermentation (selective accumulation of lactic acid) could be performed without any need for sterile culture conditions. However, the optical activity of accumulated lactic acid under the open fermentation condition was low because of the selective proliferation of *Lactobacillus plantarum*, a D- and L-lactic acid producer (5). Polylactic acid with a lower optical activity is less crystalline than PLLA, and its use must be restricted to fields such as molding applications, where a high degree of crystallinity is not required.

Despite the low quality of lactic acid accumulated by naturally present LAB, open fermentation has various merits compared with conventional sterile and closed-system fermentation. As this method does not require sterilization, the nonsterile open fermentation of kitchen refuse could be car-

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ried out on-site at localized storage sites of municipal food waste before collection to centralized processing plants. Autoclaving, which stimulates the degradation of substrate sugars and other nutritional elements for lactic acid fermentation, is also avoided. The Maillard reaction, for example, causes not only decreases in the amounts of functionally useful sugars and amino acids but also increases the production of unfavorable furfural compounds, which inhibit bacterial growth. In addition, food waste is difficult to separate into compounds; therefore, the extract is not clear and is unsuitable for filter sterilization. The use of nonsterile conditions avoids these complications. We also applied a thermophilic L-lactic acid-producing bacteria to open fermentation. Among the L-lactic acid producers reported (6), *Bacillus coagulans* is the only thermophilic species (7). We considered that controlling culture temperature and using an exogenously inoculated thermophile would be advantageous for enhancing the selective growth of the introduced microorganism at higher incubation temperatures.

To control open lactic acid fermentation, however, a precise understanding of the microflora is required. We previously reported the validity of 16S rRNA-targeted whole-cell fluorescence *in situ* hybridization (16S-FISH) for analyzing microbial population during open lactic acid fermentation (5). In this report, the applicability of quantitative FISH using a new species-specific probe for *B. coagulans* was also reported.

MATERIALS AND METHODS

Design of detection probe for *B. coagulans* The 16S rDNA sequence of *B. coagulans* was compared with that of six reference strains retrieved from the Ribosomal Database Project II (RDPII) (8) using CLUSTAL W (9). A probe complementary to the small subunit of *B. coagulans* 16S rRNA (Bcoa191) was constructed after visual inspection of the sequence alignment and systematic intensity data reported by Fucks *et al*. (10). The specificity of the selected oligonucleotide target sites was tested against 16S rRNA sequences available in the RDPII using CHECK-PROBE ver. 2.1r3. Bcoa191 is a 21mer oligonucleotide with a sequence complementary to the small subunit of *B. coagulans* 16S rRNA but not to that of any other *Bacillus* species (Table 1). The sequence of Bcoa1191 is positioned at the V4 region of 16SrRNA, showing level 4 intensity in *in situ* accessibility test with *Escherichia coli*. We also determined the following optimal hybridization conditions for Bcoa191: 46°C in the presence of 20% formamide followed by washing with buffer without NaCl (20 mM Tris–HCl, 0.01% SDS, 5 mM EDTA).

We also used EUB338 for the detection of domain bacteria (total bacteria) (11), GAM42a (23S) for *Gammaproteobacteria* (12) and LAC722(L) for a wide range of mesophilic lactic acid bacteria (5). Oligonucleotides and their fluorescent derivatives (5′-labelled with either FITC or rhodamine) were purchased from Hokkaido System Science (Sapporo).

Whole-cell fluorescence in situ hybridization (FISH) Cells were fixed and hybridized using the protocol of Amann (13) with some modification. Briefly, fixed and permealized cell suspensions were applied to wells in slides (12 wells per slide, CeL-Line; Erie Scientific, Portsmouth, New Hampshire, UK). Fluorescence was observed and the number of stained cells was counted using an epifluorescence microscope (BX50; Olympus, Tokyo) and color photomicrographs were taken with the aid of a color-chilled 3-CCD camera (Cool SNAP cf; Roper Scientific, Tucson USA), with image processing using Lumina Vision software (version 1.55; Mitani Corp., Fukuoka). Fluoro-stained cells were observed and counted in duplicate wells on appropriately diluted samples and those in five randomly selected fields (1000-fold magnification) were recorded in a computer as described above. Then, fluoro-stained cells shown on the computer monitor were counted, and cell concentration (cells/ml) was calculated as previously described (5).

Microorganism and culture *B. coagulans* NBRC12583 and *L. plantarum* KY-1 (4) were cultured using MRS medium (14). The effect of temperature on their growth in a modified MRS medium (MRS medium without Na acetate, pH 6.2) was investigated using a Temperature Gradient Incubator (Advantech, Tokyo). Microorganisms from fermented refuse at 45°C were screened by the pour plating method using GYP agar medium (1% glucose, 1.5% peptone, 0.5% yeast extract, pH 6.5). Fifty colonies were randomly selected and initially characterized on the basis of on morphology and basic physiological properties, including cell shape and size, spore formation, Gram staining, motility and catalase activity. All bacterial strains were further tested for their sugar assimilation using API-50CHL identification kits (Bio-Merieux, Marcy, I'Etoile, France).

Open fermentation of kitchen refuse The open fermentation of model kitchen refuse (MKR) was performed as previously described (4) with modification in that cultures were inoculated with *B. coagulans*. MKR had the following composition (in % w/w): 14% fish residue, 40% vegetables (carrot, potato and Chinese radish peel), 30% fruit (banana, apple and orange peel), 10% cooked rice and 6% green tea residue. All components were minced thoroughly with an equal amount of tap water. Aliquots (30 ml) of the resulting paste (pH 7.0) were distributed to 50-ml centrifuge tubes and then inoculated with *B. coagulans* cells (2.5%, v/v, ca. 10^6 cells/ml)) precultured for 24 h in MKR paste at 55°C. The tubes were then incubated at temperatures ranging from 37–65°C. The culture pH was periodically monitored and was adjusted to 7.0 with 7% ammonia by manually mixing with a glass rod (every 24 h). When saccharification of starch in the refuse was carried out, glucoamylase (Glucoteme, 20,000 units/g; Nagase Sangyo,

TABLE 1. Sequence alignment of Bcoa191 and 16S rDNA from various bacterial strains

Name	Sequences	Accession no.	Position
Bcoa191	GCCGCCTTTCCTTTTTCCTCC (5'->3')		
B. coagulans	CGGCGGAAAGGAAAAAGGAGG	AF346895	190-170
B. sporothermodurans	T...A.....TTGG.GA.G..	U49078	146-126
B. oleronius	...TA.....TT..G...GA.	X82492	198-178
B. stearothermophilus	..........TTGGCTTCT..	AB021196	189-169
B. subtilis	...TA.....TTCG.GCCT..	AY219900	227-207
L. plantarum	...T.....AT.C...CTT..	X052653	234-214
L. rhamnosus	...TA.....TCGGTTCTT..	AY370682	172-152
E. coli	.CAG..GG..---...CC..-	E05133	207-191