

High-Temperature L-Lactic Acid Fermentation of Food Waste Under Open Condition and Its FISH Analysis of Its Micro Flora

Kenji Sakai^{1*}, Yutaka Ezaki¹, Saowanit Tongpim² and Vichien Kitpreechavanich³

ABSTRACT

When minced model kitchen refuse was inoculated with *Bacillus coagulans* and incubated under non-sterilizing open condition, at the temperature below 45°C, racemic mixture of D-and L-lactic acid was found, while L-lactic acid was selectively accumulated at 50-65°C. To analyze microflora of such mixed culture system, whole cells (fluorescent in situ hybridization) using species and group of specific 16S rRNA-targeted oligonucleotide probes (16S-FISH) was applied. As a result, predominancy of mesophilic lactic acid bacteria at lower temperature, and of *Bacillus coagulans* at higher temperature was confirmed.

Key words: L-lactic acid, food waste, FISH analysis, *Bacillus coagulans*

INTRODUCTION

Poly-L-lactic acid (PLLA), a biodegradable and recyclable plastic is now being produced from corn starch via lactic acid fermentation and the potential of PLLA as a green plastic has been discussed (Gerngross and Slater, 2000). Such materials should be globally applicable, but producing PLLA and other plant-derived plastics is costly, which prevents their widespread application. Municipal food waste constitutes a potential biomass resource in Japan, since around 20% of the 50 million tones or so of waste that are annually generated consist of refuse from kitchens and food industry. We recently proposed a new system for recycling municipal food waste, from which high quality PLLA plastics can be produced (Sakai *et al.*, 2003). The system would be effective not only for treating municipal

food waste, but also for avoiding a resource conflict, since corn starch feedstock is also a source of foodstuffs for humans and other animals.

Our proposal consists of four steps: removal of endogenous D,L-lactic acids from collected food waste by a propionibacterium, semi-solid L-lactic acid fermentation, purification of L-lactic acid by butyl-esterification and L-lactic acid polymerization via LL-lactide. *Lactobacillus rhamnosus* fermented L-lactic acid in minced and autoclaved food waste. However, the production of PLLA from food waste requires relatively more energy than that from cornstarch (Sakai *et al.*, 2003).

During the investigation, we found that lactic acid was selectively produced from non-autoclaved minced kitchen refuse under open conditions, regardless of inoculated lactic acid bacteria (LAB) (Sakai *et al.*, 2000). Intermittent

¹ Graduate School of Bioscience and Bioresources, Kyushu University, Fukuoka 812-8581, Japan.

² Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.

³ Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: kensak@agr.kyushu-u.ac.jp

pH neutralization of minced kitchen refuse enhanced lactic acid production and stabilized the accumulated lactic acid. However, the optical activity of lactic acid produced by the non-sterilized open fermentation was low, and it was thought to be caused by selective proliferation of *Lactobacillus plantarum*, a D- and L-lactic acid producer (Sakai *et al.*, 2004).

In spite of low quality of lactic acid produced by naturally existed LAB, non-sterilized fermentation has various merits, compared to conventional sterilized fermentation. It requires no equipment and energy for sterilization. Thus, non-sterilized open fermentation of kitchen refuse could be applied to the on-site treatment of scattered municipal food waste. In addition, sterilization sometimes break down substrate sugars and other nutritional elements for lactic acid bacteria. Kitchen refuse contain various components including insoluble polysaccharides, therefore, it is difficult for filter sterilization. Non-sterilizing fermentation, therefore, can overcome this problem.

Then, we tried to apply a thermophilic L-lactic acid producing bacterium to such a non-sterilizing fermentation. Considering the differences in growth temperature between an exogenously inoculated thermophilic bacterium and endogenously inhabited microorganisms in kitchen refuse would be advantageous for selective growth of the former microorganism at higher incubation temperature. The microbial population during open lactic acid fermentation had been applied using 16S rRNA-targeted whole cell fluorescent in situ hybridization (Sakai *et al.*, 2004). Since ribosomal RNA shows highly conserved structure, high copy numbers, and the on-line availability of an organized database with phylogenetic information (Maidak *et al.*, 2001), the microbial community can be rapidly and directly analyzed by 16S-FISH.

MATERIALS AND METHODS

Cultures and fermentation

Bacillus coagulans NBRC12583 (Ohara and Yahata, 1996) and *Lactobacillus plantarum* KY-1 (Sakai *et al.*, 2000) were cultured using MRS medium. Effect of temperature on their growth was investigated using temperature gradient incubator (Advantech, Tokyo) and modified MRS medium (MRS medium without Na acetate, pH 6.2).

Model kitchen refuse (MKR) consisted of the following : 14% (w/w) fish residues, 40% (w/w) vegetables (carrot, potato and Chinese radish peel), 30% (w/w) fruit (banana, apple and orange peel), 10% (w/w) cooked rice and 6% (w/w) green tea residues. All the ingredients were minced thoroughly in an equal amount of tap water. The resulting paste (30ml) with initial pH about 5.9 was distributed in 50ml centrifuge tube and then inoculated with *B. coagulans* cells (2.5 %, v/v) that were precultured for 24 h in MKR paste. The fermentation process was incubated at the temperature ranging from 37-65°C and was periodically monitored (every 24 h). The culture pH was adjusted to 7.0 with 7% ammonia by manually mixing with a glass rod. When saccharification of starch in the refuse was operated, Glucoamylase (Glucoteme, 20,000 units/g, Yanase Sangyo Co., Japan) was added to the MKR paste to obtain a final concentration

Fluorescent in situ hybridization

Whole cell fluorescent in situ hybridization (FISH) was basically operated using the Amann's protocol (Amann *et al.*, 1990). Fluorescence was observed and number of stained cells was counted as reported previously, using an epifluorescence microscope (BX50; Olympus, Tokyo). Color photomicrographs were taken with the aid of color chilled 3-CCD camera (Cool SNAP cf, Roper Scientific), and image processing was achieved by using Lumina Vision software

(version 1.55; Mitani Corp., Fukuoka, Japan). Fluoro-stained cells were observed and counted in duplicate wells for appropriately diluted samples, and those in 5 randomly selected fields (1,000 folds magnification) were recorded in a computer.

We designed new probes for *B. coagulans* and determined the optimal conditions for their specific detection. The sequence of a new probe for *B. coagulans* was compared with 6 reference strains retrieved from the Ribosomal Database Project II (Maidak *et al.*, 2001). The specificity of the selected oligonucleotide target sites was tested against 16S rRNA sequences available in the RDP using CHECK-PROBE ver. 2.1r3 provided by the RDPII. We also used EUB338 for domain bacteria (6), GAM42a (23S) for Gamma proteobacteria (Manz *et al.*, 1992) and LAC722 for mesophilic lactic acid bacteria (Sakai *et al.*, 2004).

Chemical analysis

Concentrations of organic acids (formic acid, acetic acid, propionic acid, lactic acid, butyric acid), optically-active D- and L-lactic acids, and total soluble sugars were determined as described previously (Sakai *et al.*, 2004). The optical activity of lactic acid was defined as $([L]-[D]) \times 100 / ([L]+[D])$, where [L] and [D] denote the concentration of L-lactic acid and D-lactic acid, respectively. Concentration of total lactic acid represents sum of L- and D-lactic acid concentrations.

RESULTS AND DISCUSSION

Cultural properties of *B. coagulans* and *L. plantarum*

Cultural properties of *B. coagulans* were compared to those of *L. plantarum*. Optimum and maximum temperature for the growth of *B. coagulans* were 50 and 65°C, while for the growth of *L. plantarum* were at 33 and 42°C, respectively. *B. coagulans* grew in the narrow pH range between

5.0 - 7.0, while *L. plantarum* showed growth in the wide pH range between 3.5 and 9.0. Optimum pHs for the growth of *B. coagulans* and *L. plantarum* were 6.5 and 7.0, respectively. It was prospecting from these results that *B. coagulans* would show superiority of lactic acid producer over indigenous LAB in a kitchen refuse at higher temperatures.

Effect of temperature on production of lactic acid

Then we incubated non-sterilized minced kitchen refuse at various temperatures, after inoculation with *B. coagulans*. The culture pHs were adjusted at a 24 h interval to pH 7.0, and the changes in concentrations of organic acids and the microbial populations were investigated. Figure 1 showed concentrations of D- and L-lactic acids, and the optical activity after 48hr incubation at 30-70°C. It was shown that lactic acid accumulated was nearly racemic body and optical purity was very low below 40°C, while over 50°C, scarce amount of D-lactic acid was accumulated, and as a result, their optical purity was over 95%.

FISH analysis of open fermentation of kitchen refuse

To investigate such open fermentation system in detail, microflora during the fermentation was analyzed by FISH using 16S rRNA-targeted oligonucleotide probes. First we designed Bcoa191 (5'-GCCGCCTTTCCTT TTTCTCC-3'), a new probe for *B. coagulans* and confirmed its specificity. Then we stained the fermented samples after 48h at 37, 45 and 55°C using EUB-338 (5'-GCTGCCTCCCGTAGG AGT-3'), Bcoa191, and LAC722 (5'-YCACC GCTACA CATGRAGTTCCACT-3'). As shown in Figure 2, mesophilic LAB was dominant at 37°C among EUB338-positive bacteria, while *B. coagulans* occupied over 70% of bacterial population at 55°C. These results coincided to chemical analysis of the fermented broth. At temperature of 37°C, *L. plantarum*, D- and L-lactic

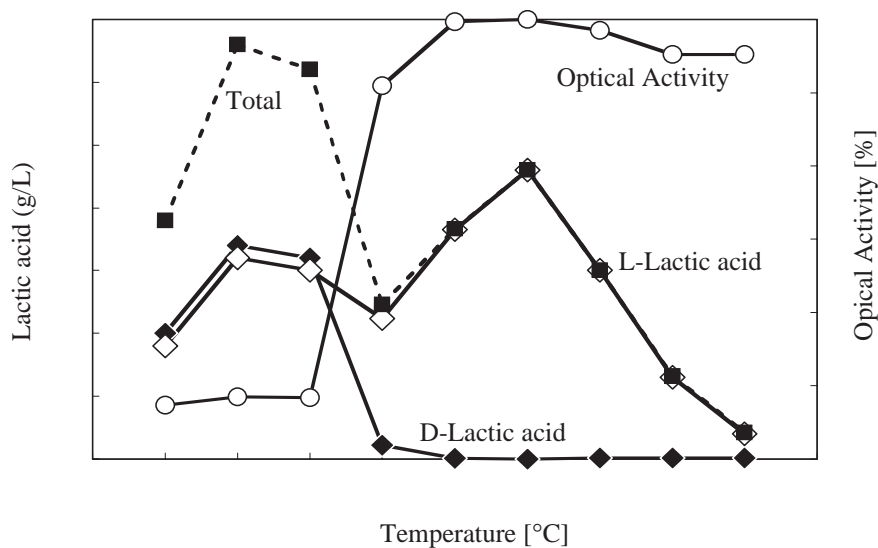


Figure 1 Effect of temperature on optical activity of lactic acid accumulated under open fermentation with *B. coagulans*.

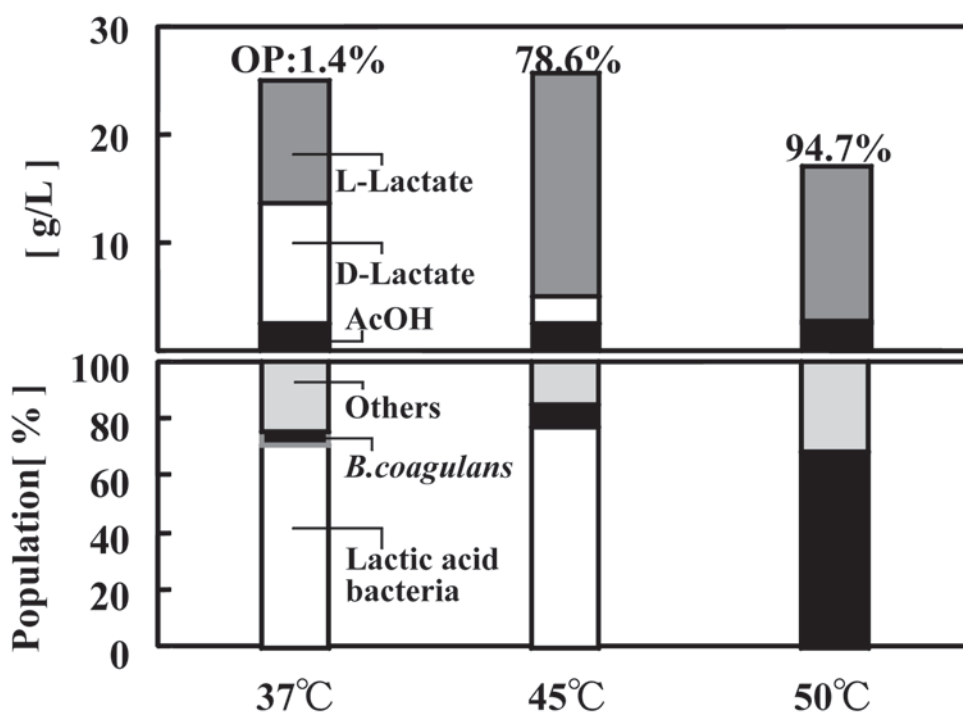


Figure 2 Quantitative FISH analysis of the open lactic acid fermentation of kitchen refuse at various incubation temperature.

(OP = optical activity, AcOH = acetic acid)

acid producing mesophilic bacteria grew selectively under pH swing control. At higher temperature, the growth of mesophilic bacteria was inhibited whereas the inoculated thermophilic L-lactic acid producing bacterium, *B. coagulans*, preferentially grew. As a result, optical activity at 37°C was low, while it increased at higher temperature (45 and 50°C). On the other hand, reproducibility of the fermentation at 45°C was low, and optical activity of accumulated L-lactic acid was rather higher (60%), but LAC722-positive cells were found in this case. *Pediococcus* sp., an L-lactic acid producing LAB that showed its limit temperature for the growth over 42°C, was isolated and identified as a dominant species from a fermented refuse at 45°C.

Production of L-lactic acid by open fermentation of saccharified kitchen refuse

When the model kitchen refuse was saccharified by glucoamylase for 2 h, and fermented by *B. coagulans* without any sterilization at 55°C under continuous pH control at 6.5, 40g/L of L-lactic acid was accumulated with its optical purity of 97%. Furthermore, using saccharified liquid of collected kitchen refuse, which contained 117g/L soluble sugars, 86g/L of L-lactic acid was yielded under non-sterilizing condition, with 97% of optical purity.

CONCLUSIONS

As a conclusion, thermophilic non-sterilizing open lactic fermentation is a simple and promising method to yield high grade L-lactic acid from biomass wastes, and FISH analysis of such mixed culture system would be helpful for detection and analysis the activity of the microflora.

ACKNOWLEDGEMENTS

Part of this work was financially supported by the Core University Program between Japan and Thailand, of the Japanese

Society for the Promotion of Science.

LITERATURE CITED

- Amann, R. I., L. Krumholz and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative phylogenetic, and environmental studies in microbiology. **J. Bacteriol.** 172: 762-770.
- Gerngross, T. U. and S. C. Slater. 2000. How green are green plastics? **Sci. Am.** Aug: 24-29.
- Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt and J. M. Tiedje, 2001. The RDP-II (Ribosomal Database Project), **Nucleic Acids Res.** 29: 173-174.
- Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer. 1992. Phylogenetic oligonucleotide probes for the major subclass of proteobacteria: problems and solutions. **Syst. Appl. Microbiol.**, 15: 593-600.
- Ohara H. and M. Yahata, 1996. L-Lactic acid production by *Bacillus* sp. in anaerobic and aerobic culture. **J. Ferment. Bioeng.** 81:272-274.
- Sakai, K., Y. Murata, H. Yamazumi, Y. Tau, M. Mori, M. Moriguchi and Y. Shirai, 2000. Selective proliferation of lactic acid bacteria and accumulation of lactic acid during open fermentation of kitchen refuse with intermittent pH adjustment. **Food Sci. Technol. Res.** 6: 140-145.
- Sakai, K., M. Taniguchi, S. Miura, H. Ohara, T. Matsumoto and Y. Shirai. 2003. Making plastics from garbage: a novel process for poly-L-lactate production from municipal food waste. **J. Indust. Ecol.** 7: 63-74.
- Sakai K., M. Mori, A. Fujii, I. Yuko and Y. Shirai. 2004. Fluorescent in situ hybridization analysis of open lactic acid fermentation of kitchen refuse using rRNA-targeted oligonucleotide probes. **J. Biosci. Bioeng.** 98: 48-56.