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Isolation of thermophilic L-lactic acid producing bacteria showing homo-fermentative manner under high aeration condition

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By applying non-sterile open fermentation of food waste, various thermotolerant L-lactic acid-producing bacteria were isolated and identified. The predominant bacterial isolates showing higher accumulation of L-lactic acid belong to 3 groups of *Bacillus coagulans*, according to their 16S rRNA gene sequence similarities. *B. coagulans* strains M21 and M36 produced high amounts of L-lactic acid of high optical purity and lactic acid selectivity in model kitchen refuse medium and glucose–yeast extract–peptone medium. Other thermotolerant isolates resembling to *Bacillus humi*, *B. ruris*, *B. subtilis*, *B. niacini* and *B. soli* were also identified. These bacteria produced low amounts of L-lactic acid of more than 99% optical purity. All isolated strains showed the highest growth rate at temperatures around 55–60°C. They showed unique responses to various oxygen supply conditions. The majority of isolates produced L-lactic acid at a low overall oxygen transfer coefficient (K_La); however, acetic acid was produced instead of L-lactic acid at a high K_La. *B. coagulans* M21 was the only strain that produced high, consistent, and reproducible amounts of optically pure L-lactic acid (>99% optical purity) under high and low K_La conditions in a homo-fermentative manner.

[**Key words:** *Bacillus coagulans*; L-Lactic acid; Food waste; Thermotolerant; Homo-fermentative; Aerotolerant; K_La]

Utilization of biomass as a renewable resource has become an important subject to create a sustainable society. Reducing the CO_2 emission would contribute to the mitigation of global warming because the carbon harvested from biomass constitutes an integral part of the dynamic flow of the biotic carbon cycle. In addition, the exhaustion of petroleum, a limited fossil resource used for fuels and chemicals, as well as shortages in food and feed are serious problems that need to be overcome if the society aims to be sustainable (1). Meanwhile, kitchen refuse generated daily in large cities is a big burden on the community, and this easily putrefiable waste requires an economic treatment system. When excess food waste is disposed of in a landfill, it decomposes and becomes a significant source of methane gas, which is more effective in trapping heat in the atmosphere than CO_2. On the other hand, biological transformation of this food waste into another useful product would contribute to reducing both landfill space and greenhouse gas release, as well as to developing a sustainable society.

Food waste, in general, contains considerable amounts of saccharides with various kinds of nutritional constituents necessary for the proliferation of fastidious lactic acid bacteria (2). We had proposed a novel recycling system for municipal food waste that combined fermentation and chemical processes to produce high-quality poly-L-lactic acid, the biomass-based plastics (2). In our previous study, by applying open non-sterilized fermentation of kitchen refuse without any inoculation at room temperature, *Lactobacillus plantarum* accumulated predominantly under intermittent pH adjustment (3,4). This selective growth was thought to be caused by several characteristics of this species, including low-pH tolerance, antibacterial activity against gram-negative bacteria, tolerance to high concentrations of lactic acid, and tolerance to oxygen supply. On the other hand, we already reported that inoculation with *Bacillus coagulans* NBRC 12583^T, a thermophilic L-lactic acid-producing bacterium, is effective in open fermentation of semi-solid food waste to obtain optically pure L-lactic acid (5). At low incubation temperatures, the optical purity of lactic acid is low, in contrast to that at higher incubation temperatures (5). This is due to the fact that at temperatures below 40°C, mesophilic lactic acid bacteria that accumulate racemic lactic acid grow abundantly and suppress the growth of thermophilic *B. coagulans*. However, at higher temperatures, the growth of mesophiles is prohibited and the inoculated *B. coagulans* strain preferentially grows to produce optically pure L-lactic acid (5). It has been considered that

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controlling the open fermentation of food waste at high temperatures with the addition of thermophilic bacteria is advantageous for selectively enhancing the growth of the introduced microbes, even though this kind of fermentation sometimes causes contamination with certain microorganisms.

Several *Bacillus* species have been reported to produce L-lactic acid (6), among which a few species show thermophilic properties (7–11). We have already reported that *Bacillus licheniformis* TY7 produced high amounts of L-lactic acid in model kitchen refuse medium; more than 100 g/L L-lactic acid were produced from a collected kitchen refuse (12). However, this strain showed fluctuation (low reproducibility) in the productivity, probably because of its high sensitivity to aeration. Considering the merits of non-sterilizing fermentation, i.e., to minimize facility costs for maintaining strict anaerobic conditions and an effective circulation of this kind of semi-solid medium, it would be beneficial to use oxygen-tolerant (aerotolerant) strains such as *L. plantarum*.

In this study, we investigated new thermophilic (bacteria that can grow or form products at higher temperatures between 45 and 65°C with an optimal above 45°C) and thermotolerant (bacteria that can grow at temperature between 30°C and 60°C with an optimal around 30–45°C) L-lactic acid-producing bacteria (12,13) that could be useful for high-temperature non-sterile fermentation of municipal food waste. We identified a variety of thermotolerant bacterial species that can produce optically pure homo-type L-lactic acid under low to high oxygen concentrations during open fermentation. Furthermore, we investigated the effect of air supply on the fermentation to screen an aerotolerant strain with respect to the accumulation of L-lactic acid.

MATERIALS AND METHODS

Isolation and identification of lactic acid-producing bacteria Lactic acid producing bacterial strains were isolated from food waste, soil, plant, organic compost and wastewater by enrichment (5 mL glucose–yeast extract–peptone (GYP) medium; 2% D-glucose, 0.5% yeast extract, 0.5% peptone, 1.5% agar, 1% $CaCO_3$; pH 6.8) or without enrichment culture techniques with heat treatment (80°C for 10 min) or without heat treatment. These samples were serially diluted in normal saline solution (0.85%, w/v) and subsequently spread onto the GYP-$CaCO_3$ agar plates and incubated at 50°C and 55°C for 2–3 d. Bacterial isolates that formed a halo zone by solubilizing the calcium carbonate, around the colony were selected and purified by cross-streaking on GYP plates. A detail of isolation source is depicted in Table 1. All representative colonies were selected and stored in 20% glycerol (v/v) at −80°C for further physiological analysis.

Taxonomic studies of the thermotolerant/thermophilic, lactic acid-producing isolates were determined as described in Bergey's Manual of Systematic Bacteriology (14). Gram staining was performed with exponentially growing cells after 24 h of incubation at 50°C. Endospore formation was assessed with the spore-staining method using malachite green (15). Oxidase activity was determined using an oxidase reagent (BioMérieux, France) and catalase activity was tested by adding 3% (v/v) H_2O_2 solution to a bacterial colony and observing the production of gas within 1 s. Biochemical tests, including tests for acid production from carbohydrates, were carried out using API 50 CHB (BioMérieux). Growth at different temperatures (ranging from 20°C to 70°C) was investigated in GYP medium after 48 h of incubation at 50°C by using the Advantec TN-2148 temperature gradient incubator. Growth differences were monitored by measuring the cell turbidity (optical density) at 610 nm. For DNA extraction, one bacterial colony was suspended in 100 mM Tris–EDTA buffer (pH 7.5) and boiled for 10 min followed by centrifugation at 13,000 rpm for 20 min to remove the cell precipitate. The supernatant containing

TABLE 1. Major characteristics of thermotolerant L-lactic acid producing bacterial isolates.

Characteristics	Group 1 (*B. coagulans*)				Group 2 (*B. coagulans*)				Group 3 (*B. coagulans*)			*B. subtilis*		Others			
	NBRC 12714	15N	N1-12	N1-4	M36	M21	T27	191TP	NBRC 12583^T	T32	N1-3	N2-10	N3-9	L50P2	L50S4	U4-3	N-14
Isolation source[a]	Cc	Lc	Cw	Cw	Ms	Cp	Fs	Wc	Cc	Fs	Cw	Cw	Cw	Mc	Mc	Cw	Wc
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore formation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Minimal growth temperature (°C)	30	33	28	28	29	29	26	33	30	26	28	20	20	33	35	28	29
Optimal growth temperature (°C)	55	41	41	41	36	50	50	41	55	45	41	40	32	45	50	35	37
Maximal growth Temperature (°C)	65	58	58	58	63	59	61	58	65	61	58	55	55	60	61	55	55
Minimal pH	4.5	5.0	5.0	4.3	4.5	4.2	4.6	4.6	5.0	4.5	5.0	5.2	5.5	5.7	5.0	5.5	5.6
Optimal pH	7.0	6.5	6.5	6.5	7.0	6.5	6.5	6.0	6.5	7.0	7.0	7.0	7.0	7.0	7.2	7.0	7.0
Maximal pH	8.5	8.5	8.5	8.5	8.5	8.0	8.5	8.7	8.7	8.5	8.0	8.6	9.5	8.5	8.5	8.5	8.7
L-Lactic acid produced (g/L)[b]	15.1	14.9	17.3	15.3	31.0	35.1	22.5	25.2	30.0	18.5	20.3	25.1	19.1	ND	ND	15.2	10.7
Optical purity (%)	87	98	98	96	98	100	98	97	97	98	97	95	95	ND	ND	95	95
Acid production from-																	
D-Xylose	−	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+	+	−	+	−	−	+	*v*	−	−	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	*v*	+	−	+
D-Lactose	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	−	+
D-Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Melezitose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Raffinose	+	+	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Gentiobiose	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	+	−
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylitol	*v*	−	−	−	*v*	−	−	−	−	−	−	−	+	−	−	−	−
Stachyose	+	−	+	−	+	−	+	+	−	+	+	+	+	−	−	−	−

Symbols: +, positive reaction; −, negative reaction; *v*, variable results.

[a] Symbols for isolation source: Cc, purchased from NITE Biological Resource Center (NBRC), Japan; Lc, liquid organic waste, Thailand; Cw, compost of food waste, Japan; Ms, mountainous soil, Japan; Cp, flower of cosmos plant, Japan; Fs, farmland soil, Japan; Wc, wastewater from tapioca factory, Thailand; Mc, marine animal resources compost (MAR-Compost), Japan.

[b] Concentration of L-lactic acid accumulated in MKR medium; ND, not determined (lactic acid fermentation was not performed in MKR medium; only anaerobic, static lactic acid fermentation in GYP medium at 50°C was performed).