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SHORT COMMUNICATION

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Direct starch fermentation to L-lactic acid by a newly isolated thermophilic strain, *Bacillus* sp. MC-07

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Abstract A newly isolated *Bacillus* sp. MC-07 showed 99.2 % 16S rRNA gene sequence similarity with the *Bacillus thermoamylovorans* LMG 18084^T. It demonstrated optimum and maximum growth temperatures of 50 and 62 °C, respectively. The ability of MC-07 to produce optically pure L-lactic acid via direct fermentation of starch without enzymatic hydrolysis was investigated at different pH values (6.0–8.0) by intermittent adjustments every 12 h. During batch fermentation in mineral salt medium containing 0.001 % yeast extract at pH 7.0, 20 g/L of soluble starch was utilized to produce 16.6 g/L L-lactic acid at 50 °C within 24 h of fermentation, with 100 % optical purity, 92.1 % lactic acid selectivity, and an L-lactic acid yield of 0.977 g/g. Direct starch fermentation at pHs 6.0, 6.5, 7.5, and 8.0 resulted in considerably lower concentrations of lactic acid than did at pH 7.0. Compared with *B. thermoamylovorans* LMG 18084^T, the ability of strain MC-07 to produce L-lactic acid was superior.

Keywords Thermophilic fermentation · *Bacillus* sp. MC-07 · Starch · Direct lactic acid fermentation

Introduction

Lactic acid (LA) has been widely applied in food, pharmaceutical, textile, cosmetic, and some chemical industries [12]. The demand for LA has been increasing considerably because of its use as a monomer for the synthesis of polylactic acid (PLA), a promising and environmentally friendly bio-plastic [12]. Optically pure L- or D-LA, required for the synthesis of high-quality PLA, can only be produced by microbial fermentation processes

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depending on the type of microorganisms, substrates, and fermentation conditions. On the other hand, chemical synthesis of LA is based on the hydrolysis of lactonitrile derived from acetaldehyde and hydrogen cyanide, leading to a racemic mixture of L-and D-LA [12]. Therefore, fermentation processes for optically pure LA production are considered to be more advantageous than chemical production processes.

Microbial LA production has been carried out using *Bacillus* species [9], lactic acid bacteria (LAB) [6], and *Rhizopus* species [22]. *Bacillus* species can produce LA at high temperatures [12, 19], whereas LAB and *Rhizopus* species are mesophilic LA producers. Compared with mesophilic fermentation, a thermophilic LA producer has been shown to be favorable for industrial LA productions due to several advantages, such as a low risk for contamination and a low energy of coolant water [12, 24]. In addition, the complex and high nutritional requirements are the main limitations associated with LAB, while the high-oxygen requirement is the main limitation associated with the fungal species [22]. Most of the *Bacillus* species are reported to grow with no oxygen supplementation on mineral salt media (MSM) consisting of low amounts of expensive organic nitrogen sources such as yeast extracts [9]. However, the number of studies on thermophilic *Bacillus* species is much lower than that of mesophilic LAB and *Rhizopus* species, thereby requiring more detailed studies.

Currently, starchy and lignocellulosic materials are considered as feasible raw material for industrial production of LA. However, compared to lignocellulosic materials, the pretreatment cost for starchy material is comparatively lower [6]. In addition, lignocellulosic materials contain cellulose and hemicellulose as the main components, and their degradation requires pretreatments including physical or physico-chemical approaches followed by hydrolysis using multiple enzymes. In general, pretreated starchy materials are easily hydrolyzed into fermentable sugars, such as glucose or oligosaccharides by the addition of enzymes (α-amylase and glucoamylase) before fermentation [6]. Furthermore, amylolytic LA producer can skip the hydrolysis process without a dose of any commercial enzymes. So far, some amylolytic LAB like *Lactobacillus paracasei* [11], *Lactobacillus amylovorans*, *Lactobacillus amylophilus*, *Enterococcus faecium*, and *Streptococcus bovis* have been already reported to produce LA from starch without any enzymatic hydrolysis under mesophilic conditions (30–40 °C) [6]. Recently, *Lactobacillus plantarum* SW14 was reported to produce LA directly from the cassava starch at 45 °C, which is the highest temperature in direct LA fermentations among the literatures published so far [1]. On the other hand, *Bacillus* species have not been reported to produce LA directly from starch at high temperatures to date.

Thus, a thermophilic LA producer, which can convert starch to optical pure LA directly in cheap medium containing a low amount of organic nitrogen sources, should be suitable. In this study, we reported the highly efficient production of LA from starch with high-optical purity by the newly isolated thermophilic *Bacillus* sp. MC-07. To our knowledge, this is the first report of direct starch fermentation to LA at high temperatures by a *Bacillus* species.

Materials and methods

Isolation and identification

For isolation, 1 g of marine animal resources compost (MAR compost) [8] was suspended with 10 mL of sterilized CASO medium (15.0 g peptone from casein, 5.0 g peptone from soy-meal, 5.0 g NaCl, per liter of deionized water at pH 7.0) containing 10 g/L starch in a test tube, vortexed, and incubated at 80 °C for 10 min. Further, it was serially diluted in 9 mL of saline solution (0.85 % w/v) prior to plating on the CASO medium plate (15 g/L agar). Plates were incubated for 24 h at 50 °C. Isolated colonies were purified by repetitive sub-streaking on the CASO medium plate by incubating at 50 °C for 24 h and stored at −80 °C in the medium containing 50 % glycerol and 50 % CASO medium. Amylolytic activities of the bacterial isolates after 24 h of incubation at 50 °C were screened by the iodine-staining method [5] on Tryptone soy agar (15 g pancreatic digest of casein, 5 g Papaic digest of soybean meal, 5 g NaCl, 20 g of soluble starch, 15 g agar, per liter of deionized water at pH 7.0). Based on the size of the halo formation after iodine staining, strain MC-07 was selected for direct starch fermentation to LA.

Identification was performed by studying their morphological (Gram's reaction, spore formation), biochemical [catalase, oxidase, and the API 50CHB system (BioMérieux, Marcy-I'Etoile, France)], and genetic characteristics (16S rRNA gene sequence). Growth at various temperatures (20–70 °C) was investigated using Tryptone soy broth supplemented with 10 g/L soluble starch after 48 h of incubation at 50 °C using an Advantec TN-2148 temperature gradient incubator by measuring the turbidity (optical density) at 660 nm. Amplification of the 16S rRNA gene was performed using the following universal primer sets: 8f (5′ AGA GTT TGA TCC CTC AG 3′) and 1492r (5′ GGT TAC CTT GTT ACG ACTT 3′). The amplification conditions were as follows: 30 cycles of DNA denaturation at 98 °C for 10 s, primer annealing at 55 °C for 5 s, and elongation at 72 °C for 1 min. Polymerase chain reaction products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequence homology was compared with

16S rRNA gene sequences available in the DDBJ/EMBL/GenBank DNA database using the FASTA algorithm (http://www.ddbj.nig.ac.jp/), and all reference sequences were obtained through the Ribosomal Database Project II (http://rdp.cme.msu.edu/). Sequences were aligned using CLUSTAL W ver.2.01 (http://clustalw.ddbj.nig.ac.jp/) and phylogenetic tree was constructed using MEGA ver.6 by neighbor-joining method with bootstrap values calculated from 1,000 replications. The assigned DDBJ/EMBL/GenBank sequence accession number for strain MC-07 is AB849116.

Direct starch fermentation by strain MC-07

For direct fermentation of starch to LA, the frozen stock culture of strain MC-07 was refreshed by inoculating 500 µL of stock culture in 5 mL of Tryptone soy broth and incubated at 50 °C for 24 h. Then, strain MC-07 was streaked onto fresh TSA plates and incubated as described above. The seed culture was prepared as follows: a loopful of cells from the fully grown TSA plate was inoculated in 30 mL of Tryptone soy broth containing 20 g/L soluble starch in a 100-mL Erlenmeyer flask, and incubated at 50 °C for 16 h with shaking (140 rpm). A 1 % seed culture was used for LA fermentation. Preliminary studies on direct starch fermentation by strain MC-07 were conducted in 100 mL of Erlenmeyer flask containing 30 ml of sterilized or non-sterilized MSM (3.06 g NH_4Cl, 3.15 g KH_2PO_4, 0.47 g $MgCl_2{\cdot}6H_2O$, 0.3 g NaCl, 5 mg $FeSO_4{\cdot}7H_2O$, 0.4 mg $CaCl_2{\cdot}2H_2O$, 0.01 g Bacto™ yeast extract [Difco™; Becton–Dickinson, Franklin Lakes, NJ, USA] per liter of deionized water) supplemented with 20 g/L of commercially available soluble starch (Wako Pure Chemicals, Richmond, USA) as the sole carbon source under non-anaerobic (without sparging oxygen-free nitrogen gas) and anaerobic conditions (with sparging oxygen-free nitrogen gas) at pH 7.0 and 50 °C. The pH was adjusted by 10 % NH_3 every 12 h of fermentation. Main batch fermentation was carried out in a 500-mL Erlenmeyer flask containing 200 mL of MSM supplemented with 20 g/L of commercially available soluble starch as the sole carbon source. The starch in the MSM was heated at 100 °C to allow gelatinization before sterilization at 120 °C for 10 min. For the pH optimization, the pH of the medium was adjusted to the desired values (6.0–8.0) with 10 % NH_3 before seed culture inoculation. Anaerobic conditions were maintained by sparging with oxygen-free N_2 gas for 20 min. Fermentation was conducted at 50 °C with shaking at 140 rpm under closed conditions to prevent air supply. The pH values of the fermentation broth were manually adjusted to the respective initial pH values (6.0–8.0) by the addition of 10 % NH_3 every 12 h of fermentation, and anaerobic conditions were maintained again as described above. For comparative studies of LA production from starch, the closely related type strain *Bacillus thermoamylovorans* LMG 18084^T [2], obtained from the Belgian Coordinated Collection of Microorganisms (Ledeganckstraat, Belgium), was also investigated as the same methods with strain MC-07.

Chemical analysis

Concentrations of organic acids such as total LA (the sum of D-LA and L-LA), formic acid, acetic acid, propionic acid, butyric acid, and pyruvic acid were determined using a high-pressure liquid chromatography system (Organic Acid Analyzer, Shimadzu, Kyoto, Japan) as described previously [17]. D- and L-LA were analyzed using a high-pressure liquid chromatography system equipped with an MCL Gel CRS10w column (Mitsubishi Chemical Co., Japan). The optical purity (%) of L-LA was defined as: $([L] - [D]) \times 100/([L] + [D])$, where [L] and [D] denote the concentrations of L-LA and D-LA, respectively [12]. The total sugar was determined using the phenol–sulfuric acid assay [3]. LA selectivity was defined as the percentage of total LA by weight in the sum of the total organic acids analyzed. In this experiment, LA selectivity (%) was calculated as: $(C_{\text{L-LA}} + C_{\text{D-LA}}) \times 100/(C_{\text{L-LA}} + C_{\text{D-LA}} + C_{\text{AA}} + C_{\text{FA}})$, where, $C_{\text{L-LA}}$, $C_{\text{D-LA}}$, C_{AA} and C_{FA} are the respective concentrations (g/L) of L-lactic acid, D-lactic acid, acetic acid and formic acid produced.

Results and discussion

Isolation and identification of strain MC-07

We isolated strain MC-07 from an MAR compost as an amylolytic strain on Tryptone soy agar, showing a clear halo after iodine staining. The strain MC-07 was Gram staining-positive with endospore-forming rods, and was oxidase-positive and catalase-positive, which indicated the classification in the family Bacillaceae. The 16S rRNA gene sequence of the MC-07 isolate showed the highest similarity with *B. thermoamylovorans* LMG 18084^T (99.2 %) [2]. The phylogenetic tree constructed using strain MC-07 and other closely related type strains including *Bacillus coagulans* NBRC12583^T, the most well-known thermophilic LA-producing *Bacillus* species, is depicted in Fig. S1. The phylogenetic tree showed that strain MC-07 clustered with *B. thermoamylovorans* LMG 18084^T (GenBank accession number L27478) with 100 % bootstrap support, indicating that strain MC-07 should be assigned to the genus *Bacillus*. The optimum and maximum growth temperatures for strain MC-07 were 50 and 62 °C, the same or slightly higher than those (50 and 60 °C) for LMG 18084^T, respectively, demonstrating a

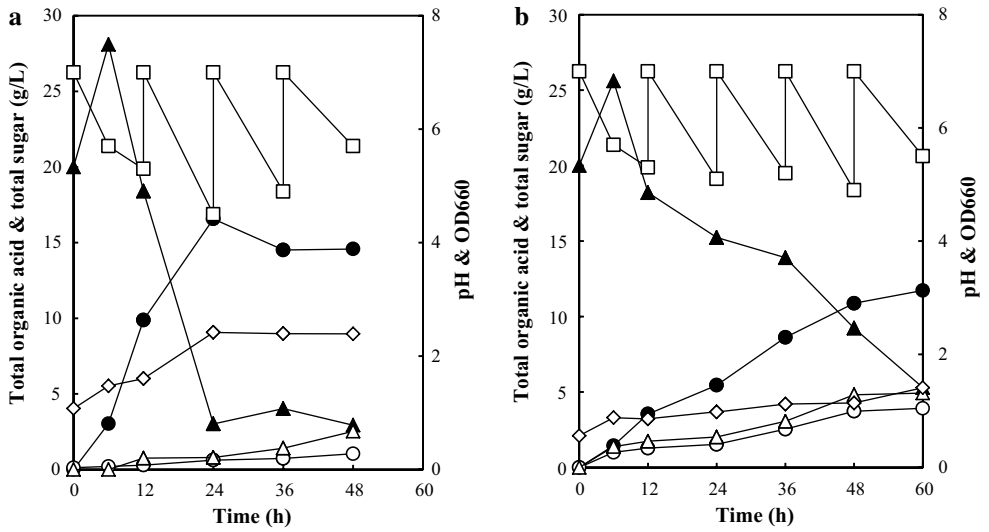

Fig. 1 Time course of direct starch fermentation to lactic acid by *Bacillus* sp. MC-07 (**a**) and *B. thermoamylovorans* LMG 18084^T (**b**) at 50 °C under intermittent pH adjustment at 7.0. Symbols; *filled circle*, LA; *filled triangle*, total sugar; *open circle*, acetic acid; *open triangle*, formic acid; *open square*, pH; *open diamond*, OD

thermophilic property (Fig. S2). Based on the API 50CHB gallery, strain MC-07 produced acids from L-arabinose, D-ribose, D-glucose, D-galactose, D-fructose, D-mannose, D-rhamnose, D-maltose, D-lactose, D-cellobiose, D-salicin, D-mellibiose, sucrose, D-trehalose, starch, gentibiose, turanose, and D-tagatose, while strain LMG 18084^T could not produce acid from D-galactose, D-rhamnose, D-maltose, D-lactose, and D-turanose but produced acid from myo-inositol, D-mannitol, and arbutin. Based on its distinct properties, strain MC-07 could not be identified to the species level, although the similarity of 16S rRNA gene sequences was high. Species level identification is under further investigation. Therefore, we described the newly isolated strain as *Bacillus* sp. MC-07.

Effect of pH values on direct starch fermentation to LA by strain MC-07 and LMG 18084^T

In the preliminary experiments, direct starch fermentations to LA were performed by strain MC-07 at 50 °C and pH 7.0 by an intermittent adjustment of pH in sterilized or non-sterilized MSM under anaerobic condition by sparging oxygen-free nitrogen gas or non-anaerobic condition without gas sparging. As the results, the LA selectivity of 90.9 % was much higher in the sterilized MSM under anaerobic condition than 47.3 % in the sterilized MSM under non-anaerobic condition or 60.7 % in non-sterilized MSM under anaerobic condition. Although non-sterilized medium [23] and non-anaerobic condition [15] have been considered to be suitable for industrial LA production, in this study, we selected sterilized MSM and anaerobic condition in further experiments.

The effect of pH values (6.0–8.0) of culture broth on LA production using strains MC-07 and LMG 18084^T was investigated by an intermittent adjustment of pH every 12 h. Figure 1 shows the fermentation profiles at a pH of 7.0 using strain MC-07 and strain LMG 18084^T. Although both strains grew in direct starch fermentation in MSM with consumption of starch, strain MC-07 exhibited the higher maximum optical density (OD; 2.42) and maximum specific growth rate (0.0488 h^{-1}) than strain LMG 18084^T (1.41 and 0.0416 h^{-1}, respectively). Strain MC-07 rapidly utilized starch after 6 h of fermentation and the highest L-LA (16.6 g/L) was produced within 24 h with a small amount of byproduct (0.802 g/L formic acid and 0.622 g/L acetic acid) formation (Fig. 1a). On the other hand, strain LMG 18084^T accumulated the highest L-LA titer of only 11.7 g/L after 60 h of fermentation with high byproducts of formic acid (4.93 g/L) and acetic acid (3.92 g/L) (Fig. 1b). These results indicated that strain MC-07 was more able to ferment starch within a short time period than strain LMG 18084^T. Furthermore, the results suggested that the behaviors of direct starch fermentations should be quite different between strain MC-07 and strain LMG 18084^T under the same fermentation conditions.

As shown in Table 1, under the intermittent adjustments of pHs at 6.0, 6.5, 7.0, 7.5, and 8.0, strain MC-07 produced L-LA concentrations of 6.40, 7.98, 16.6, 8.22, and 1.79 g/L, respectively, which are relatively higher than those produced by strain LMG 18084^T for each pH value except for 2.16 g/L at a pH of 8.0. In addition, these results indicated that the highest L-LA concentrations were achieved at pH 7.0 for both strains under the intermittent adjustments. At each of the tested pH values, L-LA yield, productivity,

Table 1 Direct fermentation of starch to L-LA by strains MC-07 and LMG 18084^T at 50 °C and various pHs by intermittent adjustment

Strains	pH	Time (h)	OD_{660}	μ_{max} (h^{-1})	C_{LA} (g/L)	Y_{LA} (g/g)	S_{LA} (%)	$OP_{L\text{-}LA}$ (%)	P_{LA} (g/L h)	Residual total sugar (g/L)	C_{AA} (g/L)	C_{FA} (g/L)
MC-07	6.0	48	1.56	0.0324	6.40	0.653	66.7	100	0.133	16.3	1.33	0.262
	6.5	36	1.81	0.0454	7.98	0.851	78.2	100	0.221	12.9	1.57	0.668
	7.0	24	2.42	0.0488	16.6	0.977	92.1	100	0.701	3.02	0.622	0.802
	7.5	48	1.54	0.0375	8.22	0.944	66.8	100	0.171	12.8	2.41	1.70
	8.0	60	1.12	0.0175	1.79	0.364	43.3	100	0.029	17.6	1.56	0.799
LMG 18084^T	6.0	60	1.17	0.0247	3.25	0.644	57.7	100	0.054	16.8	1.13	1.30
	6.5	72	1.26	0.0389	8.01	0.877	56.3	100	0.111	13.2	3.92	3.01
	7.0	60	1.41	0.0416	11.7	0.789	57.0	100	0.195	5.29	3.97	4.93
	7.5	72	1.24	0.0125	5.90	0.771	53.6	100	0.082	15.2	2.75	2.40
	8.0	72	1.14	0.0115	2.16	0.352	33.8	100	0.030	17.2	1.71	2.40

OD optical density, μ_{max} maximum specific growth rate (calculated by the slope of linear regression of the natural log of the OD and fermentation time), *C* concentration, *Y* yield, *S* selectivity, $OP_{L\text{-}LA}$ optical purity of L-lactic acid, *P* productivity, *LA* lactic acid, *AA* acetic acid, *FA* formic acid

and selectivity were superior in strain MC-07 compared to strain LMG 18084^T (Table 1). The L-LA yields for strain MC-07 ranged between 0.364 and 0.977 g/g with a maximum at pH 7.0, while in strain LMG 18084^T they ranged between 0.352 and 0.877 g/g with a maximum at pH 6.5. On the other hand, the highest L-LA selectivity of 92.1 % was attained at pH 7.0 for strain MC-07 and was much higher than strain LMG 18084^T (57.0 %). The maximum L-LA productivity of 0.701 g/L h at pH 7.0 for strain MC-07 was significantly higher than that of strain LMG 18084^T (0.195 g/L h). Low accumulation of L-LA below and above pH 7.0 might be due to poor growth of strains MC-07 and LMG 18084^T because cell growth in the fermentation broth is positively correlated with the accumulation of LA [5]. These results indicated that pH 7.0 is optimum for LA production in direct starch fermentation by strain MC-07 under intermittent adjustment, and that the capability of strain MC-07 to ferment starch to L-LA was much better than it was for strain LMG 18084^T under thermophilic conditions. Furthermore, we obtained the reproducible results (15.7 g/L L-LA with LA selectivity of 91.8 % and optical purity of L-lactic acid of 100 %, 0.58 g/L acetic acid, and 0.82 g/L formic acid) in direct starch fermentation using the strain MC-07 by the intermittent adjustment of pH at 7.0 every 12 h.

The pH values under continuous adjustment during fermentation are a significant factor for LA production by *Bacillus* strains [24]. On the other hand, we have previously reported on the better performance of LA fermentation by LAB using the food waste under intermittent pH adjustment than under controlled pH [13]. In our results under intermittent pH adjustment, surprisingly, our results suggested that the pH values drastically affect LA production from starch by both *Bacillus* strains, MC-07 and LMG 18084^T (Table 1, Fig. 1). To our knowledge, however, there are no published reports on the effect of pH values on LA fermentation using *Bacillus* strains under intermittent pH adjustment. Therefore, the mechanism underlying such effects is not known and further studies are required. Furthermore, we are now planning to conduct experiment under the controlled pH condition at 7.0.

To date, only some LAB, including *Lactobacillus* species, *Lactococcus* species, *Enterococcus* species, and *Streptococcus* species, have been reported to produce LA from starch directly without the addition of commercially available amylolytic enzymes under mesophilic conditions at ≤45 °C (Table 2). To the best of our knowledge, this is the first report of direct starch fermentation to LA using *Bacillus* species at high temperatures (50 °C and over). In addition, LAB are thought to require the addition of relatively expensive organic nitrogen sources such as yeast extract at more than 0.5 % (Table 2) [6]. However, our isolate, strain MC-07 fermented starch to L-LA in MSM containing quite little yeast extract (0.001 %) and relatively cheap inorganic nitrogen of 3.06 g NH_4Cl (Table 2). In particular, we are the first to achieve 100 % L-LA optical purity using strain MC-07, and demonstrate the highest yield of LA (0.977 g/g) among published studies (99.0 % L-LA optical purity [5] and 0.93 g/g yield of LA [11] at maximum) (Table 2). Nevertheless, at higher concentrations of starch (more than 25 g/L), strain MC-07 did not accumulate higher LA and demonstrated poor growth (data not shown). This might be due to the substrate inhibition property of bacterial growth [4]. To further improve LA production, additional research approaches such as fed-batch or repeated batch fermentation are under investigation.

Table 2 Comparison of direct starch fermentation to lactic acid by various strains so far published

Strains	FT (°C)	YE (%)	Starch (g/L)	OP $_{L\text{-}LA}$ (%)	C_{LA} (g/L)	Y_{LA} (g/g)	References
Lb. amylophilus JCM 1125	28	0.5	50	92.5	30.0	ca. 0.60	[21]
Lb. amylovorus ATCC 33620	40	3.0	10	nd	4.2	0.42	[20]
Lb. plantarum C5	30	0.5	20	nd	13.5	0.71	[14]
Lb. amylophilus GV6	30	0.5	100	nd	75.7	0.90	[18]
Lb. manihotivorans LMG 18010^T	30	0.5	17.5	99.0	12.6	0.67	[5]
Lb. paracasei B41	45	0.5	40.0	92.5	37.3	0.93	[11]
Lb. plantarum SW14	30	0.5	nd	40.0[a]	20.0	nd	[1]
Lc. lactis subsp. *lactis* B84	30	2.0	18	nd	5.5	ca. 0.48	[10]
E. faecium No. 78	37	0.5	20	98.6	15.4	0.78	[16]
S. bovis 148	37	1.0	20	95.6	14.7	0.88	[7]
B. thermoamylovorans LMG 18084^T	50	0.001	20	100	11.7	0.789	This study
Bacillus sp. MC-07	50	0.001	20	100	16.6	0.977	This study

Lb Lactobacillus, *Lc Lactococcus*, *E Enterococcus*, *S Streptococcus*, *B Bacillus*, *FT* fermentation temperature, *YE* yeast extract, *nd* not determined, *ca*. calculated value, *LA* lactic acid, *C* concentration, *Y* Yield, $OP_{L\text{-}LA}$ optical purity of L-lactic acid

[a] Optical purity of D-lactic acid was 60 %

Conclusion

We studied direct starch fermentation to produce optically pure L-LA by *Bacillus* sp. MC-07 under anaerobic and thermophilic temperature conditions using MSM containing a small amount of expensive yeast extract. These findings show that some low-cost starchy substrates can be directly fermented to L-LA with a high yield and optical purity by omitting the addition of enzymes for simultaneous saccharification, and require a relatively simple methodology for the process of LA fermentation. Therefore, our findings demonstrate an efficient means of LA production directly from starch under thermophilic temperatures and could be valuable for industrial scale.

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Conflict of interest We have no conflict of interest to declare.

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