Role of fumarate reductase on the fermentation properties of *Lactobacillus delbrueckii* ssp. *bulgaricus*

**ABSTRACT**

*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* are symbiotic starters widely used in yogurt fermentation. They exchange metabolites to meet their nutritional demands during fermentation, promoting mutual growth. Although *S. thermophilus* produces fumaric acid, and the addition of fumaric acid has been shown to promote the growth of *L. bulgaricus* monoculture, whether fumaric acid produced by *S. thermophilus* is used by *L. bulgaricus* during coculture remains unclear. Furthermore, the importance of fumaric acid metabolism in the growth of *L. bulgaricus* is yet to be elucidated. Therefore, in this study, we investigated the importance of fumaric acid metabolism in *L. bulgaricus* monocultures and coculture with *S. thermophilus*. We deleted the fumarate reductase gene (*frd*), responsible for the metabolism of fumaric acid to succinic acid, in *L. bulgaricus* strains 2038 and NCIMB 701373. Both Δ*frd* strains exhibited longer fermentation times than their parent strains, and fumaric acid was metabolized to malic acid rather than succinic acid. Coculture of Δ*frd* strains with *S. thermophilus* 1131 also resulted in a longer fermentation time, and the accumulation of malic acid was observed. These results indicated that fumaric acid produced by *S. thermophilus* is utilized by *L. bulgaricus* as a symbiotic substance during yogurt fermentation and that the metabolism of fumaric acid to succinic acid by FRD is a key factor determining the fermentation ability of *L. bulgaricus*.

**Key words:** fumaric acid, fumarate reductase, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, protocooperation

**INTRODUCTION**

*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* have long been symbiotically used for fermenting yogurt. They synergistically exchange metabolites such as fumaric acid (Veringa et al., 1968), folic acid (Rao et al., 1984), and carbon dioxide (Driesen et al., 1982) from *S. thermophilus*, and peptide from *L. bulgaricus* (Rajagopal and Sandine, 1990), thereby meeting their nutritional demands during fermentation.

In our previous study, we showed that *S. thermophilus* generally produces fumaric acid during milk fermentation, and that *L. bulgaricus* metabolizes malic and fumaric acid to succinic acid in a reductive manner (Yamamoto et al., 2021). We also noted that 6 of the 8 *L. bulgaricus* strains showed a shorter fermentation time by metabolizing fumaric acid, indicating that this substance can act as a symbiotic factor that promotes the growth of *L. bulgaricus*. Fumaric acid is a component of the tricarboxylic acid (TCA) cycle, and *L. bulgaricus* has an incomplete TCA cycle that includes only 2 enzymes: fumarate hydratase (FH; EC 4.2.1.2), which catalyzes the conversion of malic acid to succinic acid, and fumarate reductase (FRD; EC 1.3.5.4), which catalyzes the conversion of fumaric acid to succinic acid (Figure 1). Although we have shown that *frd* in *L. bulgaricus* 2038 is upregulated in a coculture with *S. thermophilus* 1131 than in a monoculture (Yamamoto et al., 2021), whether the fumaric acid produced by *S. thermophilus* is used by *L. bulgaricus* during coculture remains unclear. Furthermore, the importance of fumaric acid metabolism to the growth of *L. bulgaricus* needs to be elucidated.

In this study, we aimed to investigate the effect of fumaric acid metabolism on the fermentation ability of *L. bulgaricus* monocultures by knocking out *frd* in 2 *L. bulgaricus* strains that shows different fermentation speeds and symbiotic strength with *S. thermophilus*, namely 2038 and NCIMB 701373. In addition, Δ*frd* was cocultured with *S. thermophilus* to determine whether the fumaric acid produced by *S. thermophilus* was utilized as a symbiotic substance by *L. bulgaricus* during yogurt fermentation.
MATERIALS AND METHODS

**Bacterial strains**

*L. bulgaricus* 2038 and *S. thermophilus* 1131 were obtained from Meiji Innovation Center (Tokyo, Japan). *L. bulgaricus* strain NCIMB 701373 was obtained from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, UK).

**Plasmid construction for frd gene knockout**

Knockout of *frd* gene in *L. bulgaricus* 2038 and NCIMB 701373 was carried out via double homologous recombination using the conjugation plasmid pGM31 as previously described (Iwamoto et al., 2022). In brief, the amplification of the following regions was performed: 5’ upstream region (630 bp) of *frd* (*LBU_RS03455*) using primers FR-1 (5’-GCGGTCGACCTGGTTCAACTTGCAGAC-3’) and FR-2 (5’-GTAACCTGTTCATTCTCAGCGATAGTTCCTCTACTCA-3’); and 3’ downstream region (651 bp) of *frd* using primers FR-3 (5’-TGAGTAGAGGACTATCGCTGAAAAGAACAAGTTAC-3’) and FR-4 (5’-CGCGTCGACAAAGCGGAAGTTGAGCATGT-3’). Genomic DNA from *L. bulgaricus* 2038 was used as a template for both amplifications. Further, the upstream and downstream regions of *frd* were ligated via overlap extension PCR with primers FR-1 and FR-4. The joined fragment was ligated into pGM31 with the SalI site and electroporated (200 Ω, 25 μF, and 1.5 kV) into *Escherichia coli* DH5α cells to generate plasmid pGM31-frd. The transformants obtained were selected on a Luria-Bertani (LB) agar plate with 50 μg/mL ampicillin and 500 μg/mL erythromycin.

**Introduction of plasmid into L. bulgaricus.**

Plasmid pGM31-frd was introduced into *L. bulgaricus* using a previously reported conjugation method (Iwamoto et al., 2022). Briefly, *E. coli* DH5α containing pGM31-frd was incubated with LB broth, and pGM31-frd was extracted using the alkaline sodium dodecyl sulfate method. The extract was then electroporated into *Lactococcus lactis* IL403 cells. Transformants were selected on an M17 agar plate constituting of M17 broth (BD Difco, Sparks, MD, USA) supplemented with 1.5% Bacto agar, 0.5% glucose, and 25 μg/mL erythromycin. *Lc. lactis* IL1403 containing pGM31-frd was incubated in M17 broth supplemented with 0.5% glucose and centrifuged at 4°C, 4,000 × g for 5 min. The supernatants were aspirated, and cells were washed twice with 20 mM potassium phosphate buffer and centrifuged at 4°C, 4,000 × g for 5 min. The optical density of the supernatants at 600 nm (OD$_{600}$) was adjusted to 1.5 by adding 20 mM potassium phosphate buffer. *L. bulgaricus* 2038 and NCIMB 701373, which were used as the recipients, were incubated for 16 h at 37°C in deMan, Rogosa, and Sharpe (MRS) broth (BD Difco) and centrifuged at 4,000 × g for 5 min at 4°C. The cells thus obtained were washed twice with 20 mM potassium phosphate buffer and centrifuged at 4,000 × g for 5 min at 4°C. The cells were washed with hypertonic buffer containing 17.8% (wt/vol) raffinose, 0.027% (wt/vol) potassium dihydrogen phosphate, and 1 mM magnesium chloride (all obtained from Fujifilm Wako Pure Chemical Corp., Richmond, VA, USA) and adjusted to OD$_{600}$ = 1.5 using a hypertonic buffer.

The donor and recipients were mixed at a 1:2 ratio and trapped on a 0.45-μm filter membrane (Merck & Co., Rahway, NJ, USA) using a vacuum pump. Thereafter, 100 mL of sterilized water was passed through the filter, which was then transferred to MRS broth and incubated at 37°C for 2 h. After vortexing for 2 min, the culture broth was plated on MRS agar supplemented with 25 μg/mL erythromycin and incubated at 45°C for 2–4 d until transformed colonies appeared.

**Acquisition of frd deletion mutant**

The genome of the transformants was extracted, and chromosomal integration of pGM31-frd...
was confirmed via PCR using the primers erm-F (5’-AAGCTTGACAAAAAGAAAA-3’) and erm-R (5’-GTAATGACCTTCTCATT-3’), which amplify the erythromycin resistance gene in pGM31-frd. The transformants with pGM31-frd were then cultured at 37°C with MRS broth until erythromycin-sensitive colonies were acquired. The frd deletion mutants (1300 bp) or revertants (2700 bp) were confirmed via PCR using the primers FR-1 and FR-4.

**Fermentation conditions**

Bacterial strains were anaerobically precultured twice at 37°C for 16 h in an autoclaved (121°C for 7 min) pre-culture medium containing 10% (wt/wt) skim milk powder (Meiji Co., Ltd., Tokyo, Japan) and 0.1% (wt/wt) yeast extract (Asahi Food and Healthcare, Tokyo, Japan). For the fermentation experiments, skim milk powder broth containing 10% (wt/vol) skim milk was pasteurized via heating at 95°C for 1 min. The estimated lactose concentration in this broth is 53 g/L. For the monoculture of *L. bulgaricus*, sodium formate (Tokyo Kasei Kogyo, Tokyo, Japan) was supplemented at a final concentration of 1 mM. Fumaric, malic, and succinic acid (Fujifilm Wako Pure Chemical Corp.) solutions adjusted to pH 6.5 with NaOH were also added after sterilization. All the additives were filtered through a 0.22-μm filter before supplementation.

For the monoculture of *L. bulgaricus*, the preculture was inoculated at 2% (wt/vt). For coculture, precultures of *L. bulgaricus* and *S. thermophilus* were inoculated at 0.3% (wt/wt). The fermentation experiments were carried out at 40°C under aerobic conditions. The pH during fermentation was measured using a multiple-electrode measuring device (Horiba, Kyoto, Japan) equipped with pH sensor SE 555 (Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany). Fermentation time was defined as the time required for the pH to reach 4.6.

**Measurement of fumaric, malic, and succinic acid concentrations**

The concentrations of fumaric, malic, and succinic acids were measured as previously described (Yamamoto et al., 2021). Briefly, the samples were diluted 2-fold with distilled water. Then, to remove milk protein, the samples were diluted with 2.5% (wt/wt) Carrez I and 2.5% (wt/wt) Carrez II (Kaneko et al., 1994) and centrifuged at 4°C, 20,000 × g for 5 min. The supernatants were then collected and analyzed using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with 2 connected IC-Sep ICE ORH-801 columns (Concise Separations, San Jose, CA, USA) and a CCD-10A electrical conductivity detector (Shimadzu).

**Measurement of intracellular NAD⁺ and NADH concentrations**

The measurements were performed as previously described (Yamamoto et al., 2021). Briefly, 5 g of the fermented sample (pH 6.0) was mixed with 1.7 mL of 1 M sodium citrate and 0.7 mL of saline solution. The mixture was then vortexed and centrifuged at 4°C, 20,000 × g for 5 min, after which the cell pellet was resuspended in 10 mL of 50 mM potassium phosphate buffer and further centrifuged at 4°C, 20,000 × g for 5 min. The cell pellet collected was stored at −80°C until analysis. Intracellular NAD⁺ and NADH concentrations were measured using NAD⁺/NADH Assay Kit-3W8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions, with the addition of the cell lysis process using FastPrep-24 (MP Biomedicals, Irvine, CA, USA).

**Statistical analysis.**

The fermentation experiments were performed 4 times, and the measurement of intracellular NAD⁺ and NADH concentrations was performed thrice. The results are expressed as the mean ± SD. Data were compared by performing Student’s *t*-test using the BellCurve function in Microsoft Excel software (version 2.11; Microsoft Corp., Redmond, WA, USA), and statistical significance was set at *P*-value <0.05.

**RESULTS**

**Construction of the Δfrd mutant of *L. bulgaricus* and its effect on fermentation**

To investigate the importance of fumaric acid metabolism in *L. bulgaricus*, we deleted frd in *L. bulgaricus* 2038 and NCIMB 701373, showing strong and relatively weak symbiotic relationships with *S. thermophilus*, respectively, using conjugation and homologous recombination methods. We successfully obtained Δfrd mutants of both strains and revertants that underwent the deletion procedure but retained the original frd gene. We confirmed that the fermentation times of the wild-type (WT) and revertant strains were identical (data not shown), indicating that the deletion procedure itself had no effect on fermentation properties. Thus, only the WT strain was used as control in subsequent experiments.

To examine the effect of frd deletion on the growth of *L. bulgaricus*, the ability of the mutants to lower pH
was measured in the milk broth. As shown in Figure 2, the rate of decrease in pH with both frd strains was slower than that in the WT strains. In particular, frd deletion significantly delayed the fermentation rate of *L. bulgaricus* 2038, which normally exhibits rapid fermentation properties among the *L. bulgaricus* strains. *L. bulgaricus* 2038 Δfrd required over 20 h to reach pH 5.0 compared with the WT strain, which required only 4 h. These results indicated that the frd gene plays an important role in the growth of *L. bulgaricus* and is a key factor contributing to its rapid fermentation properties.

**Effects of frd deletion on fumaric acid metabolism and fermentation properties**

To examine whether frd is the only gene that metabolizes fumaric and malic acids to succinic acid to promote the growth of *L. bulgaricus*, the fermentation time and metabolites of Δfrd and WT strains treated with 1 mM fumaric acid or malic acid were compared. As shown in Figure 3, the growth of both WT strains was enhanced by the addition of fumaric or malic acid, whereas the delay in fermentation observed for Δfrd was not recovered by these additives. Compared with the WT strains, which metabolized all the added malic and fumaric acids to succinic acid, Δfrd accumulated malic acid and almost no succinic acid was produced even after 24 h of fermentation (Table 1). These findings indicated that frd is the only gene in *L. bulgaricus* that metabolizes fumaric acid to succinic acid to promote the growth. Furthermore, the deletion of frd resulted in a change in the direction of the metabolism of fumaric acid to malic acid; however, this change did not affect the growth of *L. bulgaricus*.

**Measurement of intracellular NAD⁺ and NADH ratios**

Since FRD has been reported to function as terminal electron acceptor which reduce fumaric acid to succinic acid through electron transfer from FADH (Van Hellemond and Tielens, 1994), fumaric acid is likely to promote the growth of *L. bulgaricus* by arranging intracellular redox balance through the conversion of NADH to NAD⁺. To test this hypothesis, we measured the intracellular NAD⁺/NADH ratios of the WT and Δfrd strains at the early stage of fermentation (pH 6.0). As shown in Figure 4, both strains showed the same pattern, with the NAD⁺/NADH ratio being higher in the WT strains than in the Δfrd strains. This suggested that frd plays an important role in the recycling of NADH to NAD⁺ and affects intracellular redox balance to promote the growth of *L. bulgaricus*.

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**A. L. bulgaricus 2038**

![Graph A](image1)

**B. L. bulgaricus NCIMB 701373**

![Graph B](image2)

*Figure 2.* pH during fermentation of the monoculture of WT and Δfrd in *Lactobacillus bulgaricus* 2038 (A) and *L. bulgaricus* NCIMB 701373 (B). All the results are presented as the mean ± SD.
Effect of frd deletion on the coculture with S. thermophilus 1131

To clarify whether the fumaric acid produced by S. thermophilus was utilized as a symbiotic substance by L. bulgaricus during yogurt fermentation, the fermentation rate and fumaric acid metabolites were measured in a coculture of Δfrd and S. thermophilus 1131, a strain which was confirmed to produce fumaric acid (Yamamoto et al., 2021). As shown in Figure 5, the fermentation rate associated with the Δfrd coculture was slower than that associated with the both WT strains, especially during the middle to late fermentation stages.

We then measured the concentrations of malic, fumaric, and succinic acids at the time when pH 4.6 was reached for the WT coculture and after 24 h of fermentation for the Δfrd coculture. As shown in Table 2, the coculture of both WT L. bulgaricus strains with S. thermophilus 1131 resulted in the accumulation of succinic acid, which was higher in the medium that

Table 1. Concentration of malic, fumaric, and succinic acids produced by Lactobacillus bulgaricus after 24 h of fermentation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Strain</th>
<th>Malic acid</th>
<th>Fumaric acid</th>
<th>Succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Before fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2038</td>
<td>WT</td>
<td>0.08 ± 0.00</td>
<td>ND</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>NCIMB 701373</td>
<td>Δfrd</td>
<td>0.35 ± 0.02</td>
<td>ND</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>1mM fumaric acid added</td>
<td>Before fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2038</td>
<td>WT</td>
<td>0.08 ± 0.00</td>
<td>1.15 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>NCIMB 701373</td>
<td>Δfrd</td>
<td>0.35 ± 0.04</td>
<td>ND</td>
<td>2.02 ± 0.00</td>
</tr>
<tr>
<td>1mM malic acid added</td>
<td>Before fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2038</td>
<td>WT</td>
<td>0.94 ± 0.02</td>
<td>ND</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>NCIMB 701373</td>
<td>Δfrd</td>
<td>1.46 ± 0.18</td>
<td>0.11 ± 0.04</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

All the results are presented as the mean ± SD. ND: not determined due to the detection limit (less than 0.01 mM).

Figure 3. Fermentation time of Lactobacillus bulgaricus 2038 and NCIMB 701373 monocultures to reach pH 4.6 in the control (no addition) and after the addition of 1 mM fumaric acid or 1 mM malic acid. All the results are presented as the mean ± SD ** P < 0.01; * P = 0.031 (2038 WT fumaric acid) or P = 0.019 (2038 WT malic acid) vs. control.

Figure 4. NAD⁺/NADH ratio in Lactobacillus bulgaricus 2038 and NCIMB 701373 monocultures. All the results are presented as the mean ± SD.
was observed before fermentation. In contrast, the coculture of both Δfrd strains with S. thermophilus 1131 produced less than 0.1 mM succinic acid. Meanwhile, higher concentrations of malic acid than succinic acid were detected after 24 h of fermentation, which was in accordance with the Δfrd monoculture results shown in Table 1. These results indicated that fumaric acid produced by S. thermophilus was metabolized to succinic acid by L. bulgaricus through the enzyme FRD, resulting in the promotion of L. bulgaricus growth.

**DISCUSSION**

In this study, we primarily aimed to investigate the effect of fumaric acid metabolism on the growth of L. bulgaricus and clarify whether fumaric acid produced by S. thermophilus is utilized as a symbiotic substance by L. bulgaricus during yogurt fermentation. We constructed Δfrd mutants of L. bulgaricus 2038 and NCIMB 701373, both of which exhibited a longer fermentation time and lower intracellular NAD+/NADH ratio compared with the parental strains. Coculture of Δfrd mutants with S. thermophilus 1131 also showed slower growth and malic acid accumulation, not succinic acid. These findings indicated that the fumaric acid produced by S. thermophilus was utilized as a symbiotic substance to promote the growth of L. bulgaricus.

We demonstrated that frd deletion resulted in a significant delay in fermentation, especially for the L. bulgaricus 2038 monoculture, which normally shows a much faster fermentation rate than the NCIMB 701373 strain both in monoculture and coculture with S. thermophi-
lus 1131 (Figure 2 and 5). Generally, the fermentation rate of *L. bulgaricus* varies significantly among strains, owing to a combination of multiple factors. One of the important factors affecting the growth of *L. bulgaricus* is its proteolytic ability, as a protease (PrtB)-deficient mutant of *L. bulgaricus* has been shown to have a longer fermentation time than the parent strain (Gilbert et al., 1997). The response to CO₂ has also been reported to affect the growth of *L. bulgaricus* monocultures and cocultures with *S. thermophilus* (Yamauchi et al., 2019). Further, the deletion of *feoA*, which transports Fe²⁺, has been reported to prolong the growth of *L. bulgaricus* at an early stage of fermentation (Han et al., 2021).

In addition to the factors indicated above, our results strongly suggested that fumaric acid metabolism is a key factor in determining the growth ability of each *L. bulgaricus* strain. The shorter fermentation delay of *L. bulgaricus* strain. We also confirmed a delay in fermentation when Δfrd was cocultured with *S. thermophilus* 1131 (Figure 5). During symbiotic fermentation, *S. thermophilus* generally exhibits rapid growth and reaches the exponential growth phase earlier than *L. bulgaricus* (Herve-Jimenez et al., 2009). In this study, we observed a delay in fermentation for Δfrd compared with that of the WT strain after 2 h of fermentation, and the gap gradually widened, indicating that the delay was due to slow *L. bulgaricus* growth.

We confirmed in a previous study that most *S. thermophilus* strains produce and expel approximately 0.3 mM fumaric acid during monoculture (Yamamoto et al., 2021). *S. thermophilus* produces fumaric acid owing to the activity of the enzyme, argininosuccinate lyase coded by the gene *argH*, which produces fumaric acid and arginine by hydrolyzing argininosuccinate (Qiao et al., 2018). Since *S. thermophilus* has been reported to utilize arginine to neutralize intracellular pH to promote its growth (Huang et al., 2016) and has no enzyme that metabolizes fumaric acid, it likely expels fumaric acid as an unnecessary byproduct to obtain arginine. *L. bulgaricus* strains, which have a strong symbiotic relationship with *S. thermophilus*, may have evolved to develop the ability to effectively utilize the released fumaric acid as they have been reported to evolve in a reductive manner via the adaptation to milk and protocooperation with *S. thermophilus* (Van de Gucht et al., 2006).

Regarding the mechanism by which the utilization of fumaric acid promotes the growth of *L. bulgaricus*, we confirmed that the deletion of *frd* led to a lower intracellular NAD⁺/NADH ratio (Figure 4). Additionally, in a previous study, we confirmed that the addition of fumaric acid to *L. bulgaricus* 2038 increases the NAD⁺/NADH ratio (Yamamoto et al., 2021). These results are in accordance with those of a previous report, which showed that a higher NAD⁺/NADH ratio contributes to *L. bulgaricus* growth (Ai et al., 2017). *L. bulgaricus* has no respiratory chain (Pedersen et al., 2005), and the main metabolic pathway for ATP production is glycolysis, which requires NAD⁺. As NAD⁺ is metabolized to NADH via glycolysis, the effective conversion of NADH to NAD⁺ is necessary to promote the central pathway and provide sufficient energy for growth.

In *E. coli*, FRD is encoded by 4 genes (*frd* ABDC), 2 of which are membrane anchors, and functions as a terminal electron acceptor by receiving proton from a quinone in the membrane (Van Hellemont and Tielens, 1994). Although this type of membrane-bound FRD is found in most organisms, several species such as *Shewanella, Trypanosoma,* and *Saccharomyces cerevisiae* possess soluble FRD (Kim et al., 2018). As *L. bulgaricus* 2038 has no gene cluster of *frd* functioning as a membrane anchor, *L. bulgaricus* may have soluble FRD. According to research on *Shewanella*, soluble FRD binds to FAD noncovalently (Gordon et al., 1998) and receives proton from the solvent (Pankhurst et al., 2006). Similarly, in *L. bulgaricus*, FRD would utilize intracellular free proton directly and expel it as succinic acid, thus reducing internal proton concentration. This would save ATP consumption by H⁺-ATPase for expelling proton and promote ATP utilization for growth-related metabolism. As we have confirmed the decrease in NAD⁺/NADH ratio, shown in Figure 4, some enzymes to transform the internal decrease in proton to NAD⁺/NADH balance may exist, which merits investigations in the future. Additionally, which metabolic pathways other than glycolysis is activated or deactivated by the metabolism of fumaric acid remains to be clarified, as the redox balance affects various metabolic pathways required for growth. Thus, a comprehensive transcriptional analysis is needed to elucidate the entire metabolism involved in the growth of *L. bulgaricus* and its contribution to FRD.

In conclusion, we demonstrated that fumaric acid metabolism by *frd* is an important metabolic pathway for the growth of *L. bulgaricus*. Furthermore, our results revealed that the fumaric acid produced by *S. thermophilus* acts as a symbiotic substance that promotes the growth of *L. bulgaricus*. These findings would not only contribute to industrial yogurt or starter making by increasing the cell biomass of *L. bulgaricus* per hour through the addition of fumaric acid but also provide new insights into the complex system of symbiosis between *S. thermophilus* and *L. bulgaricus* during yogurt fermentation.
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