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Article

Determination of the Optimal Conditions for the Purification and Recovery of Lactobionic Acid from Whey

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Abstract

This study explored the biotechnological production of lactobionic acid through the oxidation of whey, a by-product generated during milk and dairy processing, using the bacterium *Gluconobacter frateurii*. The bacterial strain used in the experiments was isolated from rotten apples. Identification was carried out by PCR amplification of the 16S rRNA gene, followed by nucleotide sequencing. Comparison of the obtained sequence with those in the NCBI GenBank database confirmed, with 99% similarity, that the isolated microorganism was *Gluconobacter frateurii*. The identified strain was cultivated in liquid whey-based media to assess its capacity for lactobionic acid production. Based on bacterial growth and cell count analysis, whey derived from industrial acid-treated curd was determined to be the most suitable substrate. The bacterium Gluconobacter frateurii was grown in pretreated whey medium, and lactose concentrations were monitored at 24, 36, 48, 60, 72, 84, 98, 115, and 154 hours after the onset of fermentation. The lowest lactose concentration, 1.18 g/L, was observed after 115 hours, indicating that a fermentation period of approximately four days is sufficient under liquid culture conditions. Following the completion of fermentation, the formation of lactobionic acid was confirmed using thin-layer chromatography (TLC). The product was then isolated through recrystallization, achieving a lactose conversion yield of 74%. The purified lactobionic acid was further analyzed to determine its physicochemical properties and biological activity. This research demonstrates that whey, commonly regarded as a waste product of dairy manufacturing, can be effectively utilized through biotechnological methods to produce lactobionic acid, a high-value compound with important applications in the pharmaceutical and cosmetic industries.

Keywords: Gluconobacter frateurii, lactobionic acid, whey, oxidation

1 Introduction

Milk and dairy-based traditional products are deeply embedded in Mongolian culture and economy. However, processing these milk products typically generates large volumes of whey. Growing dairy production leads to increased whey waste, which threatens ecosystems because of its high organic content. In Mongolia, this challenge highlights the growing need to recycle whey and reduce waste [1].

Whey is rich in lactose, minerals, vitamins, and other soluble proteins. Among the various valorization pathways, the conversion of lactose in whey into lactobionic acid (LBA) stands out as particularly promising. Lactobionic acid (LBA) production by whey fermentation is an inexpensive process. It is widely applied in medical, pharmaceutical, food, biochemical, and cosmetic industries, due to its excellent properties, such as biocompatibility, nontoxicity, biodegradability, and its antioxidant, chelating, and amphiphilic properties [2].

Thus, the objectives of this paper are: (1) to review current biotechnological production routes of lactobionic acid using whey; (2) to determine the optimal conditions for the purification and recovery of lactobionic acid [3].

2 Materials and Method

Cultivation and preparation of the resting cells

The bacterium was inoculated into 10 mL of YPG medium (0.5% yeast extract, 0.5% peptone, 1.5% D-glucose; pH adjusted to 3.5 with acetic acid) and pre-cultured for 12–24 h under standard conditions. For the main cultivation, YPG (5 mL; 1.2% agar, 1.5% Polypepton, 0.8% yeast extract,

2% D-glucose, 2% lactose, 0.8% $CaCO_3$, 1.5% ethanol; pH not adjusted) was prepared and incubated under the same conditions as the culture for 24 h. After incubation, the cultured bacterium was collected and washed for further analysis [4].

DNA extraction and 16S rRNA sequence analysis

The reference sequences, which were used in the 16S rRNA sequences, which were used in the 16S rRNA sequence analysis, were sequences available from BLAST search databases under the following accession numbers: *Gluconobacter frateurii*. The 16S rRNA similarity was measured by Gapped BLAST and PSI-BLAST database search programs.

Preparation of a whey-based medium

A liquid nutrient medium was prepared using both acid-whey and enzymatic whey. For 100 mL of both whey, 1.8% yeast extract, and 1.5% peptone were added. And the mixtures of whey were sterilized in an autoclave for one hour [5].

Determine the optimal oxidation conditions

Bacteria were inoculated into the prepared whey-based medium. Following 24 hours of incubation, the acidity and lactose levels were monitored at 12-hour intervals using a pH meter.

Thin-layer chromatography determination of LBA

Thin-layer liquid chromatography (TLC) was carried out with the method proposed by [1] with modifications for qualitative determination of LBA in the samples. For this purpose, we used Kaisel Gel 60 TLC plates and the solvent system with ethyl acetate, acetic acid, and distilled water (3:2:1, by volume). After separation in the chamber, the plate was sprayed with diphenylamine-aniline-phosphoric acid reagent and heated to 150℃ to reveal spots indicating the presence of lactose and lactobionic acid [6].

Purification of LBA

Lactobionic acid (LBA) was isolated and purified through a crystallization process. After fermentation was complete, the mixture was first centrifuged at 15,000 rpm for 10 minutes to remove insoluble residues. The clarified supernatant was then treated with activated charcoal, and the charcoal was removed by a second centrifugation under the same conditions. To further purify the solution, microfiltration was performed using a PVDF Pellicon 2 membrane filter (0.5 m^2, 0.22 µm pore size), effectively separating LBA from remaining impurities. The filtrate was then concentrated using a vacuum evaporator to approximately 40% total solids (at 50 °C with stirring at 150 rpm under 40–85 bar pressure). Crystallization was carried out using 99% methanol and 99% ethanol. For methanol, a sample-to-solvent ratio of 80:20 was used, while for ethanol, ratios of 80:20, 70:30, and 60:40 were tested. During the process, the LBA solution was heated to 60 °C. NMR analysis confirmed that LBA is stable and does not decompose at temperatures up to 85 °C [7]. For crystallization, ethanol was added dropwise to the heated solution, which was then placed in an ice bath and left at 4 °C for 24–72 hours to allow crystals to form. Once crystallization was complete, the mixture was centrifuged at 10,000 rpm for 10 minutes to collect the purified LBA crystals [8], [9].

Quantitative Analysis of Lactobionic Acid by HPLC

After purifying the lactobionic acid obtained from fermentation through recrystallization, its purity was assessed by comparing it to a standard using high-performance liquid chromatography (HPLC). The analysis was carried out on a SHIMADZU LC-40D XR system with a 250 mm C18 column. The mobile phase consisted of water and methanol in a 90:10 ratio, and a 20 µL sample was injected at a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 205nm. For sample preparation, 100 mg of the LBA was dissolved in 99.9% HPLC-grade methanol.

3 Results and discussion

Identification of the prepared bacterium

The isolated *Gluconobacter frateurii* was characterized as a Gram-negative, small, rod-shaped bacterium that occurs mainly in pairs and does not form spores. When grown on YPGL agar medium (containing 2% lactose, 2% glucose, 1.5% agar, 1.5% ethanol, 1.5% peptone, 0.8% yeast extract, and 0.8% calcium carbonate [1], the colonies appeared circular, pale, and translucent, with diameters of 1–2 mm. The colony surfaces were smooth and glossy, with well-defined, even edges.

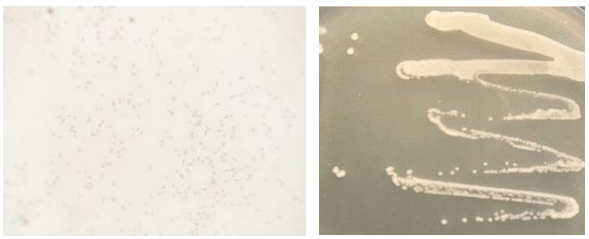

Fig. 1. Gluconobacter frateurii. The left image shows a Gram-stained micrograph of the bacterium, while the right image displays colonies grown on YPGL agar medium.

DNA extraction and 16S rRNA sequence analysis

The strains were identified by using 16S rRNA gene sequence analysis. The 16S rRNA gene sequence data were obtained from the BLAST database as shown in Table 1. Sequences were aligned using the Gapped BLAST and PSI-BLAST database search programs, and gaps were treated as missing data. When sequences were compared with those in the database and thermotolerant Gluconobacter strains CHM16 and CHM54 examined in this study showed 99% identity, respectively. Based on this analysis, the

isolated bacterium was identified as *Gluconobacter frateurii*, indicating that a pure culture of this species was successfully obtained from the spoiled apple sample.

Table 1. Percentage Similarities of 16S rRNA Sequences from Thermotolerant Gluconobacter Strains with Accession Numbers of Various 16S rRNA Sequences.

Score	Expect	Identities	Gaps	Strand
2466 bits (1335)	0.0	1359/1370 (99%)	4/1370 (0%)	Plus/ Plus

Query 1 59

CAGC-TACACATGCAGTCGCACGGATCTTTCGGGATCAGTGGCGGACGGGTGAGTAACGC

CAGCTTACACATGCAGTCGCACGGATCTTTCGGGATCAGTGGCGGACGGGTGAGTAACGC

Sbjct 13 72

Preparation of a whey-based medium

When *Gluconobacter frateurii* was initially cultivated directly in a medium containing 2% lactose, the bacterial growth was relatively low. To improve growth, the bacteria were gradually adapted by stepwise increasing the concentrations of lactose and glucose in the medium, raising them slowly from 0.5% up to 4%. At a medium composition of 2% lactose and 2% glucose, the pH of the culture dropped, and the calcium carbonate in the medium dissolved, making it more transparent. This suggests that *Gluconobacter frateurii* oxidizes lactose to produce lactobionic acid. The lactobionic acid then dissociates and binds with Ca^{2+} ions in the medium, forming a chelate known as calcium lactobionate [10].

Bacteria were grown in nutrient media prepared from acid whey waste and enzyme-coagulated whey. After 72 hours of incubation, the acid whey medium supported noticeably better bacterial growth (the dry matter content obtained after crystallization was measured at 1.89 g/L.), as well as greater changes in lactose concentration and acidity. This suggests that the bacteria are better suited to the acidic conditions found in acid whey, making it a promising medium for future cultivation and research. Conversely, the whey fermented by enzymatic action has a high protein content, and the protein undergoes coagulation, making it suitable for further processing or utilization.

Table 1. Comparison between acid whey waste and enzymatic whey.

Parameter	Acid whey	Enzymatic whey
pH	5.6	6.1
Amount of lactose	5.1	5.4
Bacterial cell numbers (after incubation with whey)	$3 * 10^2$	$0.5 * 10^2$

Determine the optimal oxidation conditions

Using the method described above, a liquid whey-based medium was prepared, and the isolated Gluconobacter frateurii was cultivated, marking the start of fermentation. Lactose levels and pH were measured at 24, 36, 48, 60, 72, 84, 98, 115, and 154 hours, with the results shown in Table 2. Over the course of fermentation, both lactose concentration and pH gradually decreased, reflecting the production of acid. After 84 hours, the changes in pH became less pronounced, indicating that as bacterial growth slowed, the rate of acid formation also decreased. Fermenting for four days (115 hours) was sufficient to reduce the lactose concentration from 5.10 g/L to 1.18 g/L.

Table 2. Concentration of lactose and pH in culture

Sample	Time (hr)	Lactose (g/L)	pH
1	0	5.1	5.6
2	24	5.02	5.56
3	36	4.91	5.47
4	48	4.44	5.24
5	60	4.24	5.03
6	72	3.83	4.84
7	84	2.92	4.62
8	98	1.57	4.43
9	115	1.18	4.1
10	154	1.35	3.98

In whey cultured with *Gluconobacter frateurii*, the lactose concentration was observed over time, indicating that the microorganisms were metabolizing this substrate. The decrease in pH was closely linked to the reduction in lactose levels and the corresponding increase in lactobionic acid formation. (Figure 2)

Starting at the 98th minute, a minimal decrease in lactose concentration was observed, suggesting that metabolic activity in the culture had slowed or temporarily ceased. Lactose levels dropped to 1.18 g/L after 115 hours of cultivation, suggesting that a 4-day fermentation period is sufficient for the liquid nutrient medium (Figure 2).

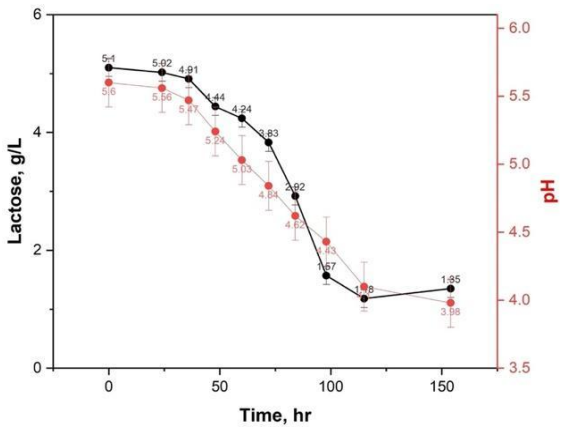

Fig. 2. A graph showing lactose concentration over time during the experiment

From the 60th minute of the biotransformation process, the sample's acidity began to decline, likely reflecting a slowdown in the culture's metabolic activity.

Thin-layer chromatography determination of LBA

The fermented samples were tested for the presence of lactobionic acid using thin-layer chromatography (TLC), following the method described by [2] et al. The analysis employed a solvent system of ethyl acetate, acetic acid, and distilled water in a 3:2:1 ratio, with 50% sulfuric acid used as the detecting reagent. Under these conditions, lactobionic acid produced a retention factor (Rf) of 0.39.

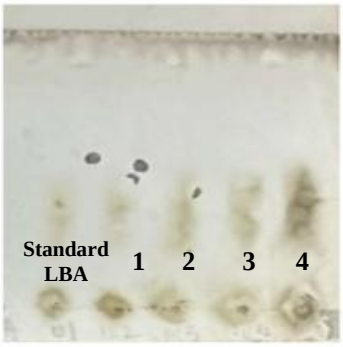

Fig. 2. The standard LBA and the results measured at 1 (24 hours), 2 (36 hours), 3 (48 hours), and 4 (60 hours) after the start of fermentation.

The TLC results showed that the color intensity gradually increased at 24 hours (1), 36 hours (2), 48 hours (3), and 60 hours (4) after the start of fermentation. This indicates that lactobionic acid production was increasing at specific stages of the fermentation process. The thin-layer chromatography was repeated using the same solvent system. Under UV light at 365 nm, two spots with Rf values of 0.39 and 0.82 were observed (Figure 3). When aniline diphenylamine was used as the detecting reagent, the spot at Rf 0.39 appeared dark purple, indicating that lactobionic acid had been formed during the middle stage of fermentation. Aniline diphenylamine reacts with acids to produce a dark purple complex [9].

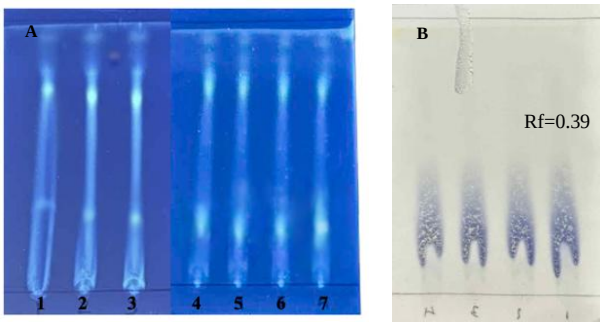

Fig.3. Thin-layer chromatography (TLC) during the experiment using aniline diphenylamine as the detecting reagent (B), showing samples taken at 24 hours (1), 36 hours (2), 48 hours (3), 60 hours (4), 72 hours (5), 84 hours (6), and 98 hours (7) after the start of fermentation.

Spots with a retention factor (Rf) of 0.39 were observed at 24 hours (1), 36 hours (2), 48 hours (3), 60 hours (4), 72 hours (5), 84 hours (6), and 98 hours (7) after the start of fermentation. The color intensity of these spots gradually increased over time, clearly indicating the production of lactobionic acid throughout the fermentation process.

Purification of LBA

From the experimental results, the optimal ratio for LBA crystallization was determined to be a sample-to-ethanol ratio of 80:20. Using this ratio, 1.89 g of dry crystals were obtained from 100 mL of solution, corresponding to a lactose conversion rate of 74% in the whey. The isolated LBA is shown in Figure 4. To further optimize the crystallization process, experiments were carried out under different conditions. LBA solutions were cooled at two temperatures (4 °C and 21 °C) for varying durations (6, 12, and 24 hours). The resulting crystals were evaluated in terms of size, yield, and the amount of residual solids remaining in the solution. Based on these observations, crystallization for 12 hours at 4 °C was identified as the most effective and favorable condition.

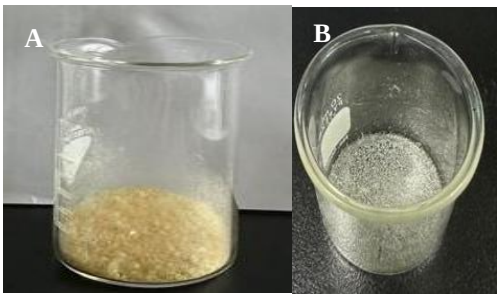

Fig.4. A. Isolated LBA, B. Recrystallized LBA

Because lactobionic acid has low solubility, recrystallization is an effective method for further purification. Using isopropanol as the solvent produced white, glossy LBA crystals.

Quantitative Analysis of Lactobionic Acid by HPLC

Following the method of [2]. HPLC analysis was performed by comparing the purified lactobionic acid to a 97% pure standard. The standard displayed two main peaks with retention times (Rt) of 1.575 and 1.947 minutes. In our analysis, the purified LBA showed two main peaks at Rt = 1.523 and 1.968 minutes, which closely match those of the standard. The appearance of two peaks is due to the

formation of a lactone–racemate mixture by lactobionic acid [11].

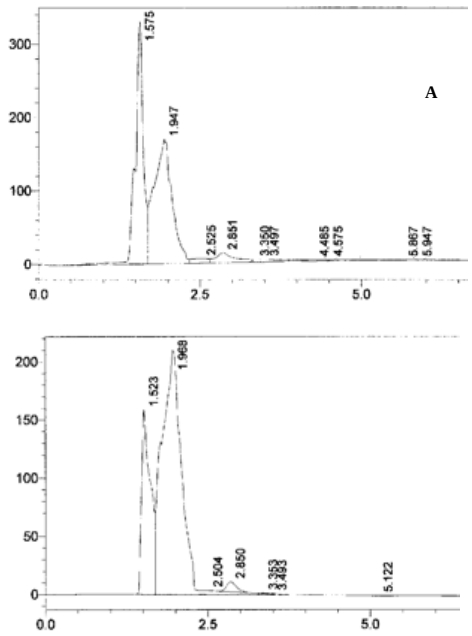

Fig.4. A. Standard LBA, B. Isolated LBA – HPLC results

4 Conclusions

Gluconobacter frateurii was successfully isolated from apple samples and cultivated in selective media. PCR analysis confirmed a 99% match with sequences in GenBank, verifying the identity of the bacterium. Whey was used as a liquid culture medium to convert lactose into lactobionic acid through a biotechnological process. For optimal growth, 1.8% yeast extract and 1.5% peptone were added per 100 mL of whey. The relatively high pH of industrially obtained whey was found to favor the growth of Gluconobacter frateurii. Fermentation was carried out at 30 °C with shaking at 200 rpm. After four days (115 hours), lactose levels decreased from 5.10 g/L to 1.18 g/L, demonstrating active acid production during fermentation. Following fermentation, lactobionic acid was analyzed using thin-layer chromatography (TLC) and crystallized with 99% ethanol, achieving a 74% yield. The product was further purified by recrystallization with isopropanol, and HPLC analysis confirmed retention times consistent with the standard. Based on laboratory-scale data, theoretical calculations for semi-industrial production suggest that lactobionic acid could be produced at a cost approximately five times lower than its current market price.

The experiment results showed that *Gluconobacter frateurii*, isolated from the local market of Mongolia, could be a potential producer of lactobionic acid on industrial acid whey. This was confirmed by PCR analysis, comparing the determined nucleotide sequence with GenBank data. The strain's ability to oxidize lactose in whey and generate lactobionic acid was further validated through qualitative thin-layer chromatography analysis.

The most appropriate method for LBA purification and recovery from a fermented substrate is precipitation with ethanol, which showed comparable physical and chemical properties to commercial LBA. Activated carbon adsorption provided a significant level of purification of the substrate from undesirable color pigments. Precipitation with ethanol helped to obtain a cleaner product, attaining a lactobionic acid concentration close to that of commercial lactobionic acid.

This research opens new perspectives for the industrial production of LBA via the bioconversion of acid whey. The samples should be analyzed further for the presence of other trace elements if they are to compete with the purity and safety criteria for chemically synthesized LBA and to be safe for application in the food industry. The downstream approaches should be studied in depth in terms of cost-effectiveness on an industrial scale. In the future, it is recommended to isolate *Gluconobacter* from various fruits and vegetables, inoculate it into whey-based culture media, and investigate lactose oxidation. This study represents a first in Mongolia, showcasing the biotechnological conversion of whey waste into lactobionic acid. It opens up promising possibilities for producing products in the food and cosmetics industries in the future.

Author Contributions

Badamgarav Baatar: methodology, investigation, data curation, writing review, and editing. Ogminjav Munkhbat: methodology, investigation, data curation. Enkh-Undraa Sandagsuren: methodology, investigation, Formal analysis, visualization, resources, supervision. Delgerjargal Altantsetseg: conceptualization, supervision. Tuyagerel Batmunkh: conceptualization, project administration, supervision.

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Conflict of Interest

There will not be any conflicts of interest.

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