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Macromolecular and Elemental Composition Analyses of *Leuconostoc mesenteroides* ATCC 8293 Cultured in a Chemostat

Jeongsu Bang[1], Ling Li[2], Hyunbin Seong[1], Ye Won Kwon[1], Eun Ji Jeong[1], Dong-Yup Lee[3], and Nam Soo Han[1*]

[1]*Brain Korea 21 Center for Bio-Resource Development, Division of Animal, Horticultural and Food Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea*

[2]*Zhejiang Provincial Key Lab for Chem & Bio Processing Technology of Farm Produces, School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou 310023, Zhejiang, P.R. China*

[3]*Department of Chemical and Biomolecular Engineering, National University of Singapore, 117576, Singapore*

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**Corresponding author*
Phone: +82-43-261-2567;
Fax: +82-43-271-4412;
E-mail: namsoo@cbnu.ac.kr

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The cellular composition and metabolic compounds of *Leuconostoc mesenteroides* ATCC 8293 were analyzed after cultivation in an anaerobic chemostat. The macromolecular composition was 24.4% polysaccharide, 29.7% protein, 7.9% lipid, 2.9% DNA, and 7.4% RNA. Its amino acid composition included large amounts of lysine, glutamic acid, alanine, and leucine. Elements were in the order of C > O > N > H > S. The metabolites in chemostat culture were lactic acid (73.34 mM), acetic acid (7.69 mM), and mannitol (9.93 mM). These data provide a first view of the cellular composition of *L. mesenteroides* for use in metabolic flux analysis.

Keywords: *Leuconostoc mesenteroides*, composition, elements, macromolecules, metabolites

Leuconostoc mesenteroides is a facultative anaerobic lactic acid bacterium (LAB), which often exists naturally in communities with other microorganisms. *L. mesenteroides* can convert available carbohydrates into lactic acid, acetic acid, alcohol, CO_2, and aromatic compounds such as diacetyl, acetoin, and C4 compounds during fermentation [1]. It is currently used in industry to ferment foods such as kimchi, sauerkraut, and pickles. Recently, kimchi producers in South Korea began to use a starter strain of *L. mesenteroides* in the kimchi manufacturing process [2]. In addition, *L. mesenteroides* is known to exhibit a range of physiological and therapeutic effects, including immune stimulation [3], pathogen inhibition, and various health promotion [4]. Kekkonen *et al*. [5] also showed that the use of *Leuconostoc* for inducing cytokines was better than that of the probiotic *Lactobacillus* strain currently in clinical use. Moreover, production of functional materials such as L-lactate, phenyllactate, and isomaltooligosaccharide was carried out using *Leuconostoc* spp. [6–8]. *L. mesenteroides* ATCC 8293 has been used as the type strain for physiological and genetic studies of this genus because its complete genome sequence has been available since 2006 [9]. The minimum nutritional requirements for *L. mesenteroides* and a chemically defined medium (CDM) have been described in a previous study [10]. A CDM is essential for designing reproducible biochemical, physiological, and genetic studies of microorganisms [11]. The operation of living cells can be considered as a complex network of interacting biomolecules. In order to gain a deeper understanding of the cellular response to different environmental conditions, a number of high-throughput analyses are being performed, covering different cellular levels such as the genome, transcriptome, proteome, metabolome, and fluxome [12]. Metabolic flux analysis provides a highly informative view of the physiological cell status under a given environmental condition or genetic background. The relationship between metabolic intricacies and the global phenotypic characteristics observed during the growth of *L. mesenteroides* primarily attributed to its complex nutrient requirements are yet to be completely discerned. In order to understand such context-specific physiological states in *L. mesenteroides*, a study of its metabolism–including cellular composition

and metabolites in a network—is required. In this study, we analyzed the cellular composition and metabolic compounds of *L. mesenteroides* ATCC 8293 growing at exponential phase.

In order to obtain exponentially growing cells for biomass composition analysis, cells were cultivated in an anaerobic chemostat fermenter (300 ml) by feeding the medium at dilution rates of $D = 0.1\ h^{-1}$, where *L. mesenteroides* ATCC 8293 was grown at 30°C in CDM [10]. The inlet gas flow was controlled by mass flow meters (Kofloc flow, Japan) at 10 ml/min. The pH, stirring speed, and temperature were maintained at 6.8 (with 0.5 N NaOH), 700 rpm, and 30°C, respectively. After inoculation of the preculture, a batch mode was operated for 24 h to the late exponential phase, and a chemostat culture mode was maintained constant for at least five residence times (50 h with $D = 0.1\ h^{-1}$) to achieve a metabolic steady state. As a result, the cell mass increased up to 9 g/l after the 24 h batch culture, resulting in a cell mass at steady state (8.37 g/l). The cultivation broth was centrifuged and the cells were recovered after washing the pellets. To lyophilize the biomass, a sample of cultivation broth was centrifuged at 9,900 ×*g* for 1 min. The cell pellet was washed twice with 1 ml of 20 mM Tris· HCl, pH 7.6. The recovered pellet was immediately frozen in a deep freezer and lyophilized under vacuum (FD 5508; Ilshin, Korea).

At the molecular level and for simplicity, biomass composition is usually described in terms of major groups of biomass constituents; namely, proteins, carbohydrates, lipids, DNA, and RNA. In this case, each of these groups was further analyzed for their major constituents.

Total carbohydrates were determined by the phenol method as described previously [13]. A 1 ml sample of lyophilized biomass was mixed with 1 ml of 5% phenol and 5 ml of 96% sulfuric acid. After 10 min, the sample tubes were cooled at 25°C for 15 min. Absorbance at 488 nm was measured using glucose solutions as standards. Total protein content was determined by the BCA method as previously described [14]. Protein concentration in a solution of lyophilized biomass at 0.5 g/l dry weight was calculated using bovine serum albumin as the standard. Total lipids were determined by methanol chloroform extraction. The chloroform extract was used immediately after removal [15]. Total DNA and RNA concentrations were measured using a nanodrop spectrophotometer (Epoch; BioTek, USA) after extraction from cells using a gDNA prep kit (SolGent, Korea) and an RNA prep kit (Macherey, Germany), respectively. The macromolecular composition was found to be 29.7% protein, 7.9% lipid, 24.4% polysaccharide, 2.9% DNA, and 7.4% RNA (Table 1). When compared with three other LAB strains (*Lactococcus lactis*, *Lactobacillus plantarum*, and *Streptococcus thermophilus*), *L. mesenteroides* showed significantly higher amounts of polysaccharide, lipid, and DNA content and a lower percentage of protein and RNA.

The detailed information of amino acids and the elemental composition is important for any metabolic and energetic calculations, and they were analyzed next. Samples of 40 mg lyophilized biomass were hydrolyzed with 3 ml of 6 M HCl at 105°C for 24 h. Following the hydrolysis step, the sample volumes were made up to 50 ml with deionized water. Then, 500 µl of the diluted samples and 100 µl of 2,500 µM nor-leucin (non-proteinogenic amino acid used as internal standard) were mixed before being evaporated to dryness. The resulting pellets were dissolved in 1 ml of buffer solution (lithium citrate loading buffer, pH 2.2) and filtered by ultracentrifugation using a 10,000 Da cut-off filter (Millipore, USA). After derivatization with ninhydrin, 50 µl of filtrate was injected in an amino acid analyzer (Biochrom 30, UK) equipped with a cation exchange chromatography column. As shown in Table 2, among various amino acids, large amounts of lysine, glutamic acid, alanine, and leucine were present in the cells, but cysteine was not detected. In addition, analysis data revealed that the measured amino acid composition for *L. mesenteroides* was significantly different from the previously published amino acid composition of *Lb. plantarum* WCFS1 [16]. The amino acid composition included large amounts of lysine, glutamic acid, alanine, and leucine, but cysteine was not detected. In particular, lysine and alanine are the most abundant amino acids because they are important constituents of the peptidoglycan layer of *L. mesenteroides* [17].

For elemental analysis, 1 mg of lyophilized biomass was mixed with 1 mg of V_2O_5 and the sample was heated in an

Table 1. Biomass composition of *Leuconostoc mesenteroides* ATCC 8293 and comparison with three different lactic acid bacteria.

Biomass composition (%, w/w) / LAB	Compounds						Reference
	Proteins	Lipids	Polysaccharides	DNA	RNA	Other	
Leuconostoc mesenteroides ATCC 8293	29.7	7.9	24.4	2.9	7.4	22	This study
Lactobacillus plantarum WCFS1	29.9	6.3	9.9	1.9	9	43	[16]
Lactococcus lactis IL1403	46	3.4	12	2.3	10.7	25.6	[19]
Streptococcus thermophilus LMG18311	43.3	6.1	24.1	1	8.2	17.2	[20]

Table 2. Amino acid composition of *Leuconostoc mesenteroides* ATCC 8293 cells.

Amino acids	Composition (%, w/w)
Alanine	9.26
Glycine	5.50
Valine	4.89
Leucine	8.04
Isoleucine	5.72
Threonine	3.78
Serine	2.95
Proline	3.21
Aspartic acid	6.87
Methionine	2.71
4-Hydroxyproline	0.36
Glutamic acid	10.32
Phenylalanine	7.56
Lysine	19.56
Histidine	5.68
Hydroxylysine	0.26
Tyrosine	3.30
Cysteine	ND[a]

[a]Not detected.

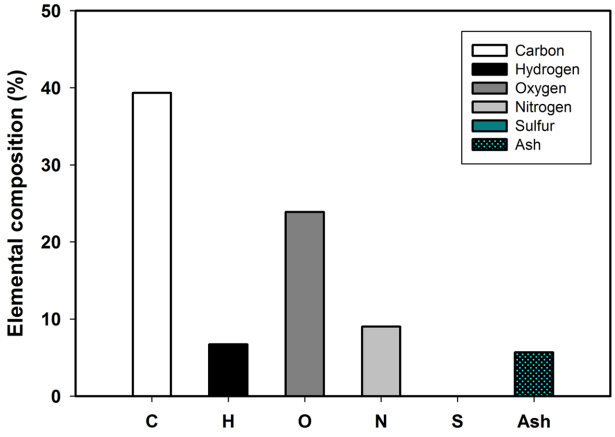

Fig. 1. Elemental composition of *Leuconostoc mesenteroides* ATCC 8293 cells.

oven at 1,000°C. The volatile compounds were measured with an elemental analyzer (NA2000; ThermoFisher, USA). The total ash content was analyzed by the combustion method after placing the lyophilized cells in pre-dried ceramic cups and heating in an oven (Daihan Wisetherm, Korea) at 550°C for 12 h. When the composition of C, H, O, N, and S was analyzed, 39.34% carbon, 23.88% oxygen, 9.04% nitrogen, 6.72% hydrogen, and 5.7% ash were present in the lyophilized cells but sulfur was not detected (Fig. 1). Elemental analysis revealed that the amounts were in the order C > O > N > H> S.

Lactic acid, acetic acid, and mannitol are the major metabolites of *L. mesenteroides* produced in the fermentation process. In order to measure the change in metabolites during the chemostat culture at $D = 0.1\ h^{-1}$, samples were collected after 0 h, 24 h, and after reaching the steady state, followed by analysis of metabolites using HPLC. For the analysis, samples were centrifuged at 9,900 ×*g* for 2 min to remove the cells and subsequently filtered through 0.22 μm filters (Millipore, USA). Organic acids were analyzed by HPLC using an ionic exchange column (Aminex HPX-87H; Bio-Rad, USA) and 0.008 N H_2SO_4 was used as the mobile phase. Sugars were analyzed by HPLC with an ionic exchange column (Asahipak NH2P-50 4E; Shodex, Japan) and 75% acetonitrile was used as the mobile phase. As shown in Table 3, after batch cultivation, lactic acid (36.89 mM), acetic acid (26.04 mM), and mannitol (56.02 mM) were accumulated in the medium, but during continuous cultivation (chemostat) their concentrations were 73.34, 7.69, and 9.93 mM, respectively, demonstrating significant changes in the metabolite composition. This result reveals that under the steady-state condition, lactic acid is the major metabolite of *L. mesenteroides* ATCC 8293, with mannitol and acetic acid produced as minor products. Mannitol was generated from fructose to recycle NAD^+ by a reduction reaction.

In this study, the cellular composition and metabolic compounds of *L. mesenteroides* ATCC 8293 compared with those of three other LAB strains (*Lactococcus lactis*, *Lactobacillus plantarum*, *Streptococcus thermophilus*) showed significantly higher amounts of polysaccharide, lipid, and DNA content and a lower percentage of protein and RNA. A high percentage of polysaccharide and lipid indicates a thicker cell wall in *L. mesenteroides*.

Table 3. Measured substrates and products consumed/produced at steady state with fructose, mannitol, and organic acids.

Fermentation time (h)	Substrate concentration (mM)	Metabolites concentration (mM)		
	Fructose	Mannitol	Lactic acid	Acetic acid
0	107.92 ± 2.38	-	-	-
24	41.72 ± 3.85	56.02 ± 3.36	36.89 ± 1.64	26.04 ± 0.46
74 (Steady state)	70.22 ± 3.25	9.93 ± 3.37	73.34 ± 0.89	7.69 ± 1.98

Data were derived from three independent experiments, and values are expressed as the mean ± standard deviation.

In order to develop the genome-scale reconstruction of metabolic pathway design, genomic and biochemical information needs to be available [18]. Despite the availability of such genomic information and the well-understood modes of central metabolism, very few genome-scale models of LAB strains have been developed to date, and include those of *Lb. plantarum*, *Lc. lactis*, *Lb. reuteri*, *St. thermophilus*, and *Lb. casei* [16, 19, 20, 21, 22]. Therefore, this study provides the first biomass information for *Leuconostoc* spp. with the essential chemical composition required for basic microbial research, such as metabolic flux analysis or genome-scale in silico modeling.

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