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Characteristics for ornithine-production and ornithine-synthase from *Bifidobacterium longum*

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Abstract

Bifidobacterium longum KCTC 5734 was evaluated for use in sunsik fermentation, where sunsik, Korean heated cereal powder, was used as the sole growth medium. Interestingly, *B. longum* KCTC 5734 was found to produce ornithine (12.3 ppm) from sunsik. In order to confirm the presence of an ornithine production pathway in this strain, genomic DNA was purified and amplified using PCR, products were cloned into *Escherichia coli,* and the amino acids sequence of the vector was used for multiple alignment analysis. The results identified 2 genes in the genome of *B. longum* KCTC 5734, coding for a putative N-acetylornithine aminotransferase and N-acetylglutamate synthase/ornithine acetyltransferase. This indicates that *B. longum* KCTC 5734 can be used as a starter culture for the production of ornithine-rich foods.

Introduction

Over the past few decades, the production of functional foods is one of the fastest developing fields in the food industry. Among these functional foods, probiotics, including bifidobacteria, have been shown to limit the growth of competing pathogenic microbes and stimulate the immune system in man. In an animal study, Singh *et al*. (1997) found that dietary administration of *Bifidobacterium longum* reduced colon tumor incidence and tumor volume. In a human study, color cancer incidence was low when the numbers of bifidobacteria in colon was high (Kubota, 1990). In addition, bifidobacteria belong to the GRAS (Generally Recognized As Safe) class of microbes and their biosynthetic pathways are relatively well-studied (Lee and O'Sullivan, 2010).

Sunsik is a cereal-based, ready-to-eat food product, comprised of glutinous rice, corn, black sesame seeds, dried vegetables, roasted nuts, and other ingredients such as calcium, carbohydrates, sodium chloride and vitamins (Lee *et al*., 2010). It is rich in carbohydrate (76.9%) and also contains dietary fiber (9.2%), protein (8.0%), and fat (6.7%), among other components (Lee *et al.*, 2010). The most abundant amino acid in sunsik is glutamic acid (1.53%) and other major amino acids include leucine (0.68%) and aspartic acid (0.65%) (Koh *et al*., 2014). Sunsik is mainly consumed by infants, patients, and the elderly because it does not require chewing. Sunsik is usually produced in dried powder form. Either water or cold milk is added when consuming sunsik. However, one of the main drawbacks of sunsik is poor solubility in water and milk, due to which it sediments within a few minutes. Some groups have expressed concern about microbial contamination of sunsik (Lee *et al.*, 2007; Chon *et al.*, 2012), since it is consumed without any sterilization.

L-Ornithine, a non-protein amino acid, is widely distributed among various kinds of food and is useful in increasing muscle growth and in the prevention of obesity by enhancing basal metabolism. L-Ornithine is also required for proper function of the immune system and the liver. Furthermore, ornithine is used in the industrial production of L-ornithine-L-aspartate, a medicament used in Europe to treat liver disorder, as is ornithine alpha-ketoglutarate (OKG) (Müting *et al*., 1992; Loï *et al.,* 2007). Some microbes are known to catalyze the formation of ornithine from either arginine or L-glutamate (Zúniga *et al*., 2002; Xu *et al*., 2007; Yu and Oh, 2010). In lactic acid bacteria, L-arginine is converted to L-ornithine, CO_2, and NH_3 via the arginine deiminase (ADI) pathway, involving 3 enzymes, namely arginine deiminase, ornithine

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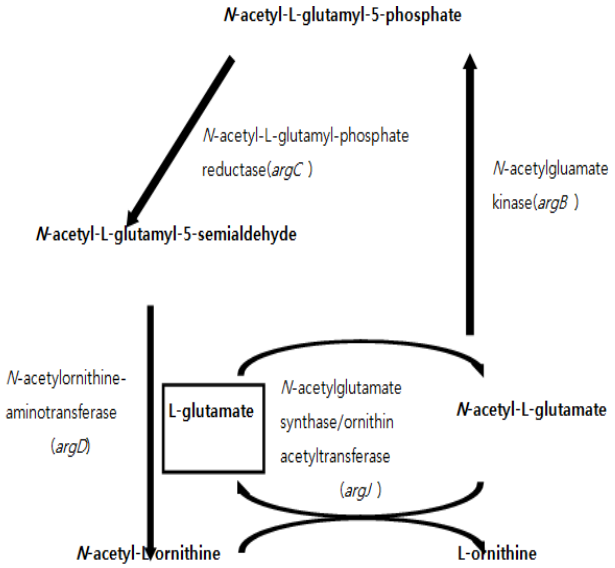

Figure 1. The ornithine biosynthesis pathway in *Escherichia coli*.

transcarbamylase, and carbamate kinase (Zúniga *et al.*, 2002). In *Escherichia coli* and *Pseudomonas aeruginosa,* L-glutamate and N-acetyl-L-ornithine is converted to N-acetyl-L-glutamate and L-ornithine, respectively (Figure 1) (Xu *et al*., 2007) via a process involving 4 enzymes, resulting in the production of L-ornithine from L-glutamate.

The aim of this study was to evaluate ornithine-producing ability of a commercial bifidobacterial strain, *Bifidobacterium longum* KCTC 5734. To this end, we studied the fermentation of sunsik, because it is rich in glutamic acid and arginine, which are possible substrates for ornithine production.

Materials and Methods

Materials, strain, culture conditions

Unless otherwise specified, chemicals and media were purchased from Sigma Chemical Co., (St. Louis, MO, USA). The bacterial strain used in this study was *Bifidobacterium longum* KCTC 5734, obtained from Cell Biotech Co., Ltd (GyeongGi-Do, Korea). Cells were grown anaerobically in de Man-Rogosa-Sharpe (MRS) broth at 37°C for 16 to 48 h. Cell growth was monitored by measurement of optical density at 600 nm using a UV-visible spectrophotometer (UV-1650, Shimadzu, Japan). MRS broth, containing 10 g Bacto peptone (DIFCO, France), 10 g beef extract, 10 g glucose, 5 g Bacto yeast extract (DIFCO), 5 g sodium acetate, 2 g dipotassium phosphate, 1 g Tween 80, 2 g ammonium citrate, 0.1 g magnesium sulfate, and 0.05 g manganese sulfate per liter, was used for growth of bifidobacteria. For solid medium, MRS was solidified with 15 g/L of agar. For the cell culture, glucose free-MRS was supplemented with 1% (w/v) glucose or 1% (w/v) sunsik, which was previously heat sterilized at 121°C for 15 min. Sunsik was obtained from Purunsol Co. (Gangwon, Korea) and comprised of the following: 30% brown rice, 30% glutinous rice, 18% alpha-corn, 8% black sesame seeds, 7.2% vegetable cream, 4% hemp, 1.6% glucose, 0.8% walnut flakes, 0.2% pumpkin flakes, and 0.2% carrot flakes. Brown rice was prepared by steaming the rice at 200°C for 20 min.

Conditions for amino acid analysis

For free amino acid analyses, 5 g sample was mixed with 30 mL ethanol (70%) and allowed to stand for 10 min. After centrifugation at 15,000 rpm for 15 min, the supernatant was transferred into a glass bottle. The procedure was repeated twice by adding 25 mL ethanol (70%) and processing as described above. The supernatants were combined and dried using a freezer-dryer unit. Subsequently, sample volumes were adjusted to 100 mL and filtered using a 0.45-μm syringe filter. Quantification and identification of amino acids and related compounds was performed using Hitachi L-8800 amino acid analyzer (Hitachi, Japan) with an ion exchange column (4.6 × 60 mm) and buffer mixtures at a flow rate of 0.35 mL per min. Column temperature was programmed at approximately 30 to 70°C, reactor temperature at 135°C, and 20 μL sample was injected into the amino acid analyzer.

Fermentation of sunsik using B. longum *KCTC 5734*

B. longum KCTC 5734 was cultured in a 50-mL bottle containing sunsik mixture, which was prepared by mixing 6.25 g sunsik in 18.75 mL distilled water. After inoculation with 0.125 g *B. longum* KCTC 5734 (5.0×10^{10} CFU/g), the cultures were maintained at 37°C for up to 240 h.

Cloning of genes

Genomic DNA was isolated from overnight cultures using the Wizard genomic DNA purification kit (Promega Corp., Madison, USA) as per manufacturer instructions. Polymerase chain reaction (PCR) was performed with genomic DNA using forward and reverse primers. For cloning of N-acetylornithine aminotransferase gene, the primers (5′-ATGGCTACTGAGCATGAGGAAAAAC-3′ and 5′-TCAGTCGTCGGGCAGGTCCGTAG-3′) were used. For cloning of ornithine acetyltransferase/N-acetylglutamate synthase gene, the primers (5′-GTGAGCGTTACATTTGCACAAGGT-3′ and 5′-TCAGCTTTCGTAGTCGGCGTTGA-3′) were used. PCR reaction was conducted using a thermal cycler (Takara, Shiga, Japan) with PrimeSTAR PCR

Mix (Takara) in a final volume of 50 µl. PCR was carried out under the following conditions: 98°C, 5 min; 40 cycles at 98°C for 5 s, 64°C for 5 s, 72°C for 60 s, and final extension of 72°C for 7 min. The amplified N-acetylornithine aminotransferase and ornithine acetyltransferase/N-acetylglutamate synthase sequences were then cloned into the T-blunt vector (SolGent, Daejeon, Korea) to yield T-blunt/N-acetylornithine aminotransferase gene (argD) and T-blunt/ornithine acetyltransferase/N-acetylglutamate synthase (*arg*J). Gene insertion was confirmed by *Eco*RI (Takara) digestion and sequencing (COSMOgenetech, Seoul, Korea).

Multiple alignment analysis

The sequences of all N-acetylornithine aminotransferase and ornithine acetyltransferase/N-acetylglutamate synthase were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov). For alignment, we used the "create alignment" function in the CLC Main Workbench 5.6.1 programme (CLC bio, Boston, MA, USA).

Statistical analysis

Data were analyzed using one way analysis of variance at 95% level of significance (Albright *et al.*, 1999). All analyses were carried out in triplicate. The results presented are a mean of observations ± standard deviations (SD).

Results and Discussion

Cell growths of B. longum

Cell growths of *B. longum* KCTC 5734 in different carbon sources (glucose and sunsik) were compared to identify any differences attributable to these additives. Sunsik is rich in fiber and protein, particularly in glutamic acid, but poor in ornithine. Cell growth was compared by determining the final A_{600} after 22 h incubation under standard growth conditions. Cells displayed different rates of cell growth. For cells grown in the glucose free-MRS medium containing 1% sunsik, the value of A_{600} was approximately 80% of cell growth obtained in the MRS medium containing 1% glucose (data not shown). The result demonstrates that sunsik could provide nutrients for *B. longum* growth. Subsequently, sunsik was used as a sole growth medium for *B. longum* as described in Materials and Methods.

Time course curve of ornithine production by B. longum *from sunsik*

To obtain comparative data on the enzyme involved in ornithine production, fermentation of sunsik by *B. longum* KCTC 5734 was conducted, instead of using MRS broth. Prior to fermentation, sunsik was heat-sterilized to avoid any microbial contamination in sunsik. It is interesting to note that fermentation of sunsik by *B. longum* KCTC 5734 showed ornithine formation (Table 1). A major proportion of ornithine localization occurred intracellularly, based on calculations of the molar ratio of intracellular ornithine to that found extracellularly (Yu and Oh 2010). The same authors (2010) found that content of ornithine produced by *W. koreensis* strains grown in MRS containing 1% arginine for 48 h was 1.0 to 1.8 mg ornithine/L of culture medium/h. Similar result in ornithine content was found in our study. *B. longum* KCTC 5734 grown on sunsik showed ornithine productivity of 0.9 mg/L/h after 12 h of culture (Figure 2). It is important to note that ornithine productivity report in this study was

Table 1. Free amino acids and nitrogen compounds profile of sunsik (unit: mg/100g)

	Sunsik	
	T=0 h	T=48 h[1)]
Aspartic acid	10.0	5.3
Threonine	2.1	1.6
Serine	8.0	4.4
Glutamic acid	11.0	15.5
Glycine	2.6	2.9
Alanine	12.8	30.1
Citrulline	0.8	0.9
Valine	3.4	34.1
Cysteine	0.3	0.0
Methionine	0.2	2.2
Isoleucine	2.7	7.9
Leucine	1.2	20.0
Tyrosine	1.4	0.0
Phenylalanine	2.7	5.9
GABA	8.7	9.4
Trytophan	0.0	0.0
Ammonia	5.5	9.2
Ornithine	0.0	15.1
Lysine	1.0	2.7
Histidine	0.7	2.1
Arginine	13.1	4.1
Proline	0.0	0.0
Total	88.2	173.3

[1]For sunsik (T=48 h), sunsik (T=0 h) was incubated with *B. longum* KCTC 5734 for 48 h at 37°C.

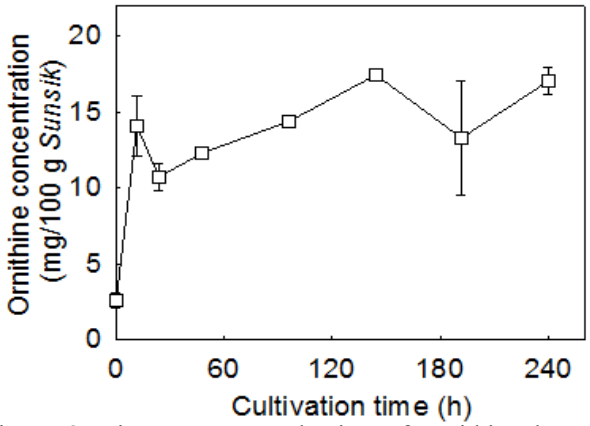

Figure 2. Time course production of ornithine by *B. longum* KCTC 5734 in sunsik. *B. longum* KCTC 5734 was cultivated at 37° for 240 h. All values are mean±SD (n=3).

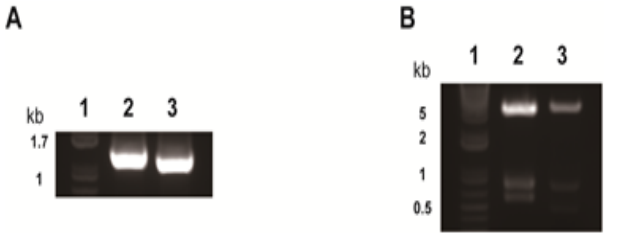

Figure 3. Confirmation of N-acetylornithine aminotransferase and ornithine acetyltransferase/N-acetylglutamate synthase genes in genome of B. longum. (A) Amplification of both genes by PCR. Lane: 1, DNA size marker; 2, PCR product of N-acetylornithine aminotransferase gene; 3, PCR product of ornithine acetyltransferase/*N*-acetylglutamate synthase gene. (B) Detection of gene insertion in T-blunt vector by restriction enzyme reaction. Lane: 1, DNA size marker; 2, vector harboring N-acetylornithine aminotransferase gene digested by *Eco*RI ; 3, vector harboring ornithine acetyltransferase/N-acetylglutamate synthase gene digested by *Eco*RI.

obtained using Sunsik as a sole medium. As far as we know, this is the first report on the production of ornithine from Sunsik using bifidobacteria as a starter culture.

Cloning and comparison of arg*D and* arg*J genes*

The presence of *arg*D (N-acetylornithine aminotransferase) and *arg*J (ornithine acetyltransferase/N-acetylglutamate synthase) genes in *B. longum* KCTC 5734, responsible for the formation of L-ornithine, was evaluated. From PCR amplifications, products of the amplified, putative argD and argJ genes corresponded to 1.3 and 1.2 kb, respectively (Figure 3). The putative N-acetylornithine aminotransferase and ornithine acetyltransferase/N-acetylglutamate synthase genes were cloned from genomic DNA and sub-cloned in a T-blunt vector (Figure 3A). In order to confirm the insertion of N-acetylornithine aminotransferase and ornithine acetyltransferase/N-acetylglutamate synthase genes in the T-blunt vector, the vectors were digested by *Eco*RI (Figure 3B). T-blunt vector harboring N-acetylornithine aminotransferase gene showed 2 fragments (750 and 550 bp). T-blunt vector harboring ornithine acetyltransferase/N-acetylglutamate synthase gene also showed 2 fragments (720 and 350 bp). Sequence alignment of N-acetylornithine aminotransferase (Figure 4A) and ornithine acetyltransferase/N-acetylglutamate synthase (Figure 4B) of *B. longum* KCTC 5734 showed only 3 and 5 amino acid differences between *B. longum* subspecies, respectively. Thus, we concluded the presence of ornithine synthesis pathway in *B. longum* KCTC 5734.

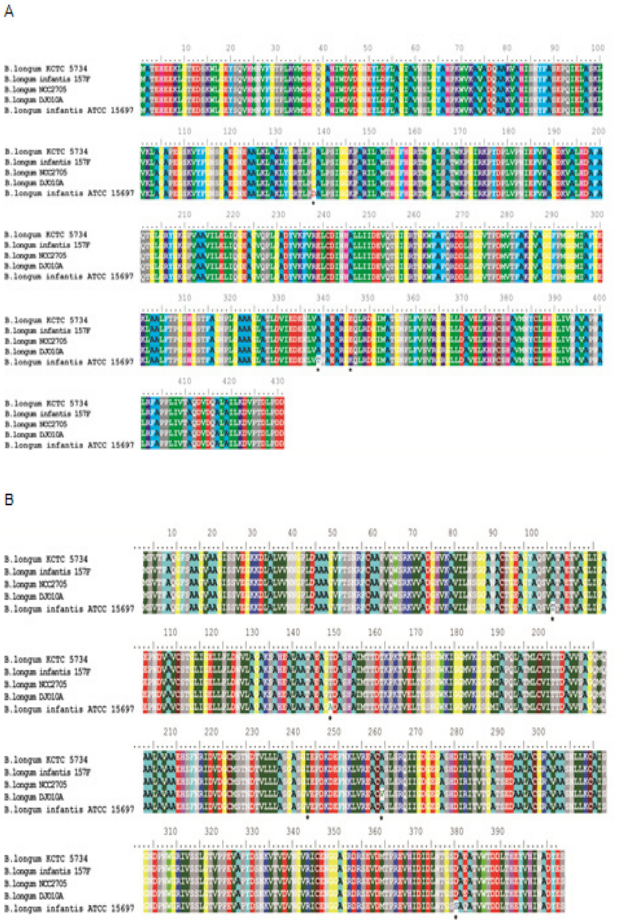

Figure 4. Comparison of the of *N*-acetylornithine aminotransferase (A) and ornithine acetyltransferase/*N*-acetylglutamate synthase (B) amino acid sequences among *B. longum* different subspecies. *B. longum* KCTC 5734, *B. longum* subsp. *infantis* 157F, *B. longum* NCC2705, *B. longum* DJO10A, and *B. longum* subsp. *infantis* ATCC 15697. * means variance of amino acid in both proteins.

Conclusion

In this study, *B. longum* KCTC 5734 was evaluated for its suitability to produce ornithine. The evaluation was based on cell growth on glucose-free MRS with 1% sunsik versus MRS containing 1% glucose, amino acid patterns, and the existence of *argD* and *argJ genes*. *B. longum* KCTC 5734 was demonstrated to produce ornithine from sunsik. Results showed that *B. longum* KCTC 5734 could be useful in the development of healthy foods rich in ornithine.

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