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Optimal Conditions for the Production of Gamma-aminobutyric Acid by *Enterococcus casseliflavus* PL05 Isolated from Oenanthe javanica

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ABSTRACT - In this study, a new lactic acid bacterium (LAB) that could produce gamma-aminobutyric acid (GABA) was isolated from *Oenanthe javanica* (water celery) and identified as an *Enterococcus casseliflavus* strain. Until recently, there have been many studies on the gamma-aminobutyric acid producing lactic acid bacterium, as well as on some lactic acid bacterium in Enteroococcs genus, but none on the species *E. casseliflavus*. Therefore, in the purpose of finding the optimal conditions for GABA production of *E. casseliflavus* PL05, the effects of several conditions including the type of mediums, growth temperatures, initial pH, growth time, L-mono sodium glutamate (MSG) concentration, and carbon source were tested. The study revealed that the PL05 strain grew better in the Brain Heart Infusion (BHI) medium than in the Man, Rogosa, and Sharpe (MRS) or Tryptic Soy Broth (TSB) medium. Also, similar results were obtained with GABA production conditions. As a result of analysis on the GABA production yield by concentration of MSG, a GABA substrate, the highest production was found at 7% of MSG concentration. However, since similar level of production was found at 5%, it is considered to be more efficient to use 5% MSG concentration. The analysis on the growth and GABA production yield by carbon sources showed the highest results when maltose was used. From the final test under the optimal conditions found, 140.06±0.71 mM of GABA was produced over 24 hours with the conversion rate of 78.95%. Lastly, from the sensitivity analysis on the 10 different antibiotics, including vancomycin, it was found that there were not confirmed cases of resistance.

Key words: Gamma-aminobutyric acid**,** Lactic acid bacteria**,** *Enterococcus casseliflavus***,** *Oenanthe javanica*

Gamma-aminobutyric acid (GABA), a non-protein amino acid, is biosynthesized through decarboxylation of L-glutamic acid by glutamic acid decarboxylase, is widely founded in nature, including plants, animals, and microorganisms (Manyam et al.[1], Ueno et al.[2]). GABA is generally founded in grains such as brown rice (4-8 mg/100 g), germinated brown rice (10-100 mg/100 g), and green tea (35-205 mg/100 g), although its content is very low. To increase the functionality and effectiveness of GABA, many studies have been conducted (Kang et al.[3]).

GABA acts as a major inhibitory neurotransmitter that sends chemical messages in the mammalian central nervous system. GABA also plays an important role in behavior, cognition, and response to stress. It can inhibit blood cholesterol, decrease blood sugar, regulate blood pressure and prevent obesity (Wu et al.[4], Hayakawa et al.[5], Abduo et al.[6], Oh et al.[7]). There have been studies on the production of GABA by microorganisms such as bacteria, yeast, and fungi. Among these microorganisms, lactic acid bacteria (LAB) are generally regarded as safe (GRAS) bacteria. They are widely used in the food industry as starters for various fermented foods probiotics and dairy foods (Lee et al.[8], Leory and Vuyst[9]).

Enterococcus species are included to the group of lactic acid bacteria and are widely distributed in nature such as vegetables fermented foods (Mundt et al.[10]). The genera comprises more than 20 species, and *Enterococcus faecium* and *Enterococcus faecalis* are the most prevalent species in foods. On the other hand, *E. casseliflavus* is relatively unknown compared to *E. faecium* and *E. faecalis*. The strain are known as potential probiotics in fish feed due to high tolerance to acidic and bile salts conditions, adherence to the intestinal epithelial cells, and antagonistic activity toward pathogens (Akbari et al.[11]).

Previous studies have reported that LAB such as *Lactobacillus plantarum, Lactobacillus zymae, Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum,* and

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Lactococcus lactis (Siragusa et al.[12], Lim st al.[13], Kook et al.[14], Lee et al.[15], Lu et al.[16], Lin et al.[17]) from various sources can produce GABA whereas there have been relatively few studies on the production of GABA by bacteria of other genera. GABA producing *Enterococcus* strains such as *E. avium* and *E. faecium* have been reported in some studies (Yang et al.[18], Divyashri et al.[19]).

GABA production by microorganisms depends on various factors such as pH, temperature, glutamate concentration, medium composition, and fermentation times (Dhkkal et al.[20]). Therefore, it is necessary to optimize these various factors for efficient GABA production.

In this study, a strain of *E. casseliflavus* that could produce GABA was isolated from *Oenanthe javanica*. GABA production by LAB is generally affected by various factors such as culture conditions and GABA-precursor. These factors should be considered to obtain optimal GABA production conditions for industrial application (Lee et al.[15]). Thus, the objective of this study was to determine effects of various factors, including the type of medium, carbon sources, initial pH, growth temperature, and L-mono sodium glutamate (MSG, GABA-precursor) concentration on GABA production of *E. casseliflavus* PL05 strain.

Materials and methods

Isolation and selection of GABA-producing LAB

LAB strains were isolated from raw materials of green juice such as *Oenanthe javanica, Brassica oleracea*, and *Angelica keiskei* as plant-based raw materials used in our company (Pulmuone Greenjuice Co., Ltd.). These raw material samples were diluted with sterilized 0.85% (w/v) NaCl solution and plated onto de Man, Rogosa, and Sharpe (MRS; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) agar medium. After incubation at 35°C for 48 h, LAB colonies were randomly selected in consideration of shapes, size, and color of colonies. To search for GABA-producing LAB, thin layer chromatography (TLC) analysis as a qualitative method was used. Isolated LAB strains were inoculated into 1 mL of MRS broth containing 3% monosodium glutamate (w/v, MSG, Sigma, Switzerland) and incubated at 35°C for 48 h. LAB cultures were centrifuged at 12,000 rpm for 5 min. Supernatants were then used for screening GABA-producing LAB (Lee et al.,[21]). Each 1 uL of LAB supernatant was spotted onto a TLC silica gel plate (Merck, Germany). Spotted plates were developed with a solvent solution in N-butanol, acetate acid, and water (4:1:1, v/v). Developed plates were dried at 55°C for 10 min and visualized with 0.2% ninhydrin solution to confirm GABA spots.

Identification of GABA-producing LAB

The screened lactic acid bacteria were examined in two methods for exactly identification. First, LAB strains were identified by 16S rRNA gene sequencing after polymerase chain reaction (PCR) amplification. PCR was performed using universal primers 27F and 1492R. Primer sequences were as follows: 5'-AGAGTTTGATCMTGGCTCAG-3' (27F) / 5'-TACGGYTACCTTGTTACGACTT-3' (1492R). PCR was performed using 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. PCR-amplified products of about 1,400 base pair (bp) in size were confirmed by agarose gel electrophoresis. PCR products were sequenced at Macrogen Co. (https://dna.macrogen.com/kor/index.jsp). 16S rRNA gene sequences were then analyzed using the BLAST program of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Based on 16S rRNA gene sequences, a maximum-likelihood phylogenetic tree showing phylogenetic relationships among members of GABA producing *Enterococcus* spp. and *E. casseliflavus* PL05 was then constructed using MEGA X version with bootstrap analysis for 1,000 replications. Only bootstrap values over 70% are shown. *Lactobacillus plantarum* JCM1149 was used as an outgroup. The scale bar corresponds to 0.05 substitutions per nucleotide site.

Additionally, the LAB strains were identified with VITEK MS (bioMérieux, Marcy l'Etoile, France). The system is automated microbial identification system based on MALDI-TOF technology, and It enables the distinction between *Enterococcus* species that are difficult to distinguish by 16S rRNA gene sequencing.

The identification was tested using colonies on the agar medium. The LAB colonies from the MRS agar plate was directly spotted on the MALDI plate, and then overlaid with 1 μL of matrix solution (VITEK MS-CHCA) and dried. The loaded plate was then applied to the VITEK MS instrument according to the manufacturer's instructions. The peaks from spectrum of each LABs was compared to to the characteristic pattern for a species, genus or family of microorganism of database.

Optimization of GABA production

We investigated the growth and GABA production of *E. casseliflavus* PL05 using various factors such as the type of medium, growth temperature, initial pH, growth times, MSG concentrations, and various carbon sources to optimize the conditions for GABA production. *E. casseliflavus* PL05 was grown under various conditions without shaking for 72 h. The strain was first propagated in 10 mL Brain Heart Infusion (BHI) broth for 24 h at 35°C. It was then inoculated into fresh broth (1%, v/v) with different conditions.

The first step was performed to determine which culture

medium was better for the growth of *E. casseliflavus* using MRS (Bectone, Dickinson and Company, Le Pont de Claix, France), Tryptic Soy Broth (TSB, Bectone), and BHI (MBcell, Seoul, South Korea). In the second step, various temperatures (5°C, 15°C, 25°C, 35°C, 45°C, and 55°C) and different initial pH conditions (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) were used to culture *E. casseliflavus* for 72 h, and culture checked GABA production. Next, in the third step, the growth and GABA production were tested using different MSG concentrations (as a precursor, 1%, 3%, 5%, 7%, 10%, 11%, 13%, and 15%) and various carbon sources (fructose, galactose, glucose, lactose, maltose, and sucrose).

Antibiotic susceptibility assays of LAB

Antibiotic susceptibility of GABA producing LAB strain was determined by disc diffusion method using Antibiotic Disc (Liofilchem SRL, Italy) (Davis et al.[22]). Briefly, BHI agar was inoculated with 1.0E+05 CFU of *E. casseliflavus*. Antibiotics used in this test included erythromycin (15 μg), chloramphenicol (30 μg), vancomycin (30 μg), gentamicin (30 μg), ampicillin (10 μg), amoxicillin (30 μg), rifampicin (30 μg), neomycin (30 μg), and penicillin (0.1 μg). After antibiotic disks were inoculated into BHI agar media, they were then incubated at 35°C for 18 h. The clear zone was then measured.

Statistical Analysis

All measurements were repeated three times, and the results were expressed as the mean±standard deviation. Sensory data were analyzed by ANOVA, and Duncan's multiple range test was done using the IBM SPSS Statistic program (version 28.0.1.1(14), SPSS Inc., Chicago, IL., USA) Package (with statistical significance at $P < 0.05$).

Results and Discussions

A total of 380 LAB strains were isolated from green juice raw materials such as *Oenanthe javanica, Angelica keiskei*, and *Brassica oleracea L*. These isolates were incubated in MRS broth containing 3% MSG and then screened for GABA production by TLC analysis. A total of eight strains produced GABA and strain PL05 showed the highest production of GABA (Fig. 1). This strain was isolated from *Oenanthe javanica*. It was identified as *Enterococcus gallinarum*/*casseliflavus* by 16S rRNA gene sequencing. A phylogenetic tree based on 16S rRNA gene sequences of PL05 strain and other *Enterococcus* strains was constructed, revealing that the PL05 strain was more related to *E. casseliflavus* than to *E. gallinarum* (Fig. 2).

Furthermore, we performed identification using VITEK MS for more accurate identification, and as a result, it was identified as *E. casseliflavus*. The standard strains used for the analysis were *E. gallinarum* ATCC49573T and *E. casseliflavus* ATCC144366, ATCC49996, and ATCC51328, respectively, and were compared with PL05 strain. Based on these results, we finally decided that the PL05 strain was *Enterococcus casseliflavus*.

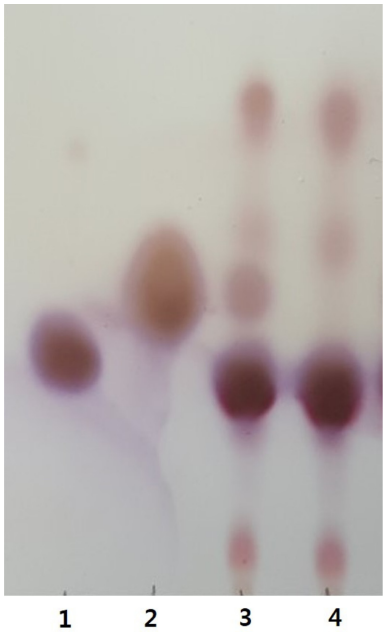

Fig. 1. GABA production of *E. casseliflavus* PL05 strain isolated from *Oenanthe javanica*.
Lane 1, MSG solution pH; Lane 2, GABA control; Lane 3, *E. casseliflavus* PL05 - GABA producing strain in this study; Lane 4, Control, GABA non-producing strain.

To optimize the growth of *E. casseliflavus* and GABA production, we tested different media. As a result, its growth was better in BHI medium than in MRS or TSB (Fig. 3). Therefore, further experiments were all conducted in BHI medium. The All growth curve of *E. casseliflavus* PL05 is shown in Fig. 4, and the production of GABA is shown in Table 1. The conversion yields of each test are also shown in Table 1.

Growth of PL05 strain was examined at different temperatures (5, 15, 25, 35, 45 and 55°C). As shown in Fig. 4 (A), PL05 strain grew rapidly at 35°C, reaching the highest absorbance value (OD600 = 0.415) within 6 h. This strain did not grow well at 5, 15, or 55°C. Culture temperature condition is an important factor for the growth and GABA production of LAB. GABA production of LAB had a positive correlation with cell density, which was dependent on the culture temperature. Most LAB can produce high levels of GABA at a temperature range of 25-40°C (Dkakal et al.[19]), which is a good condition for growth. To optimize GABA production,

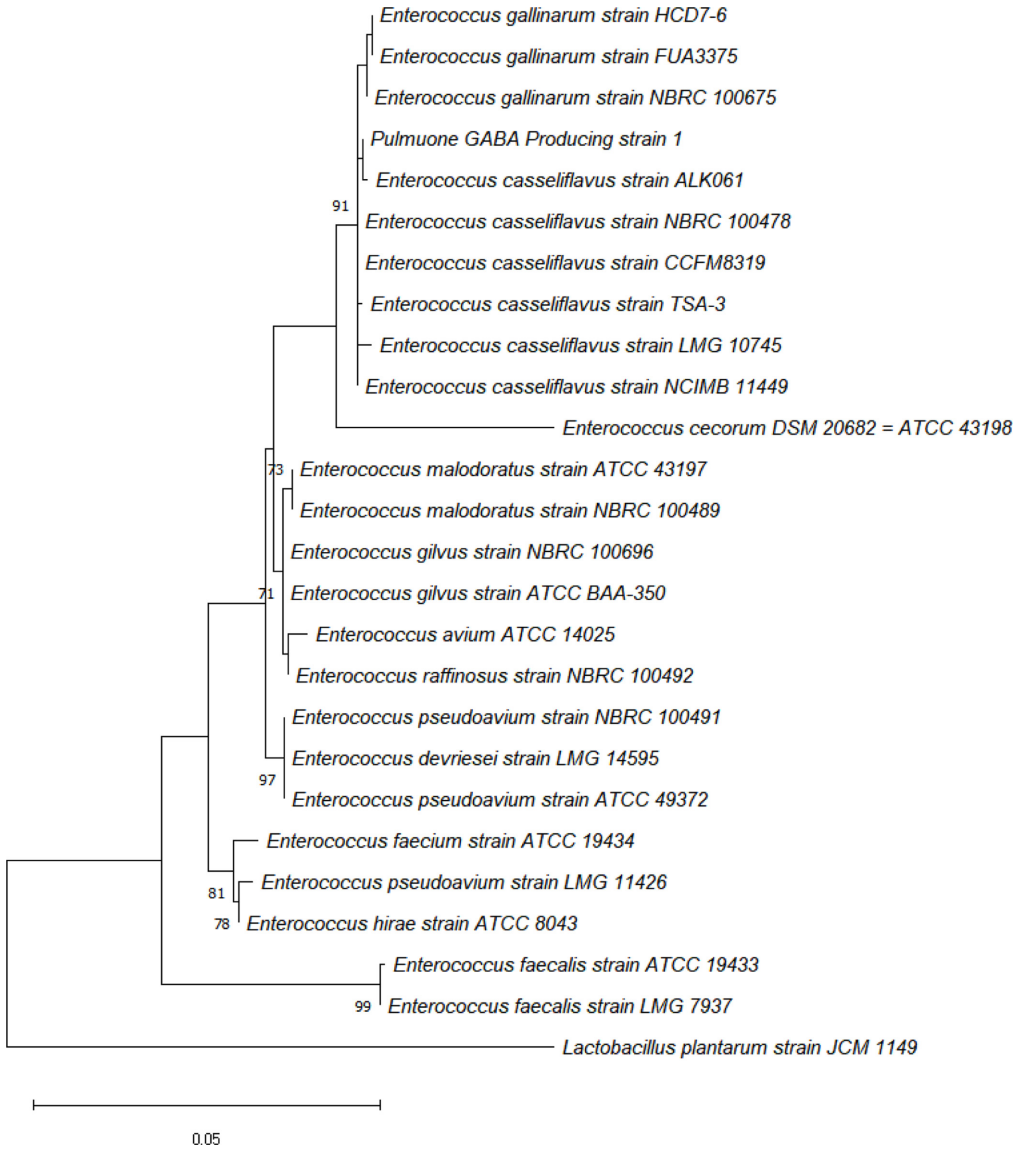

Fig. 2. A maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of members of GABA producing *Enterococcus* spp. and *E. casseliflavus* PL05 using MEGA X version with bootstrap analysis for 1,000 replications.
Only bootstrap values over 70% are shown. *Lactobacillus plantarum* JCM1149 was used as an outgroup. The scale bar corresponds to 0.05 substitutions per nucleotide site.

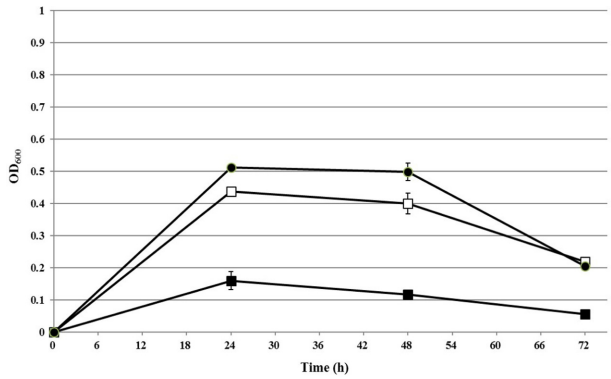

Fig. 3. Growth of *E. casseliflavus* strains at different type broth. ■, MRS broth; □, TSB broth; ●, BHI broth

all further experiments were conducted at the optimum growth temperature of 35°C.

To determine the incubation time for efficient GABA production, PL05 strain was cultured in BHI medium at 35°C for a total of 72 h. GABA level was the highest after 24 h of culture. It then gradually decreased until 72 h. Thus, further experiments for GABA production were conducted with 24 h of incubation time.

Growth and GABA production of the PL05 strain were examined at different initial pH conditions (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) at 35°C. As shown in Fig. 4 (B), PL05 strains grew rapidly at pH 7.0, 8.0, and 9.0, reaching

Table 1. Effects of various factors (growth times, initial pH, MSG concentrations, and various carbon sources) on GABA production by *E. casseliflavus* in the BHI medium at 35°C

Conditions		GABA production (mM, mean±standard deviation)	Conversion yield (%)
Growth times (hour)	0	0.00±0.00^{e}	0
	6	7.45±0.97^{d}	4.2
	12	15.48±0.78^{c}	8.72
	24	27.48±0.63^{a}	15.49
	48	21.99±1.41^{b}	12.4
	72	15.49±1.47^{c}	8.73
Initial pH	3	13.49±0.03^{g}	7.6
	4	11.92±0.04^{h}	6.72
	5	11.48±0.06^{h}	6.47
	6	29.33±0.07^{f}	16.53
	7	56.59±0.07^{b}	31.89
	8	57.36±0.08^{a}	32.33
	9	50.40±0.02^{d}	28.41
	10	55.38±0.25^{c}	31.22
	11	35.11±0.03^{e}	19.79
MSG concentration (%, w/v)	1	29.29±0.09^{e}	16.51
	3	33.03±0.03^{d}	18.61
	5	55.74±0.05^{b}	31.42
	7	58.32±0.08^{a}	32.87
	10	53.39±0.04^{c}	30.09
	13	28.85±0.05^{e}	16.26
	15	18.73±0.07^{f}	10.55
Carbon sources	Fructose	50.89±0.39^{b}	28.69
	Galactose	48.69±0.21^{d}	27.44
	Glucose	51.28±0.31^{b}	28.9
	Lactose	49.65±0.37^{c}	27.99
	Maltose	54.55±0.56^{a}	30.75
	Sucrose	50.84±0.48^{b}	28.66
Maltose concentration	0%	21.25±0.33^{e}	11.98
	1%	44.85±0.62^{d}	25.28
	3%	46.93±0.52^{c}	26.45
	5%	61.11±0.29^{a}	34.45
	7%	48.33±0.68^{b}	27.25
	10%	44.22±0.46^{d}	24.92
Optimal conditions (hour)	0h	40.53±0.50^{f}	22.85
	3h	41.18±0.40^{f}	23.21
	6h	43.38±0.97^{e}	24.46
	9h	122.46±0.46^{c}	69.03
	24h	140.06±0.71^{a}	78.95
	48h	128.14±1.58^{b}	72.23
	72h	84.81±1.42^{d}	47.81

*GABA conversion yield was calculated by percentage of the produced GABA concentration over supplied MSG concentration.

**Different letters indicate significant difference between the values in the same tested conditions ($P < 0.05$, by least significant difference test).

the highest absorbance value (OD600 = 0.512 - 0.560) within 24 h. However, this strain did not grow well at pH 3.0, pH 4.0, or pH 11.0. Results about effects of initial pH conditions on GABA produced by *E. casseliflavus* confirmed that the optimal pH range was between 7.0 and 10.0. The pH condition is a key factor for GABA production by LAB. Other studies have also shown that the initial pH of medium can affect GABA production (Li et al.[23], Kim et al.[24]). The optimum condition for the initial pH of most LAB is known to be 3.5-5.0 (Cui et al.[24]). However, the optimal pH for *E. casseliflavus* in this study was 7.0-9.0, showing a different trend. Similar to result of this study, optimal pH values for GABA production by *E. avium* 9184 and *E. faecium* JK29 were 6.5-7.5 (Yang et al.[18], Lim et al.[26]). However, *E. avium* M5 and *E. durans* PF15 produced optimal GABA at pH 4.5-5.5 (Lee et al.[21], Siragusa et al.[27]). Therefore, the optimal pH for GABA production differs depending on the strain species. And the pH in medium changes during incubation of LAB. Thus, it would be advantageous to adjust and maintain the optimal pH condition of the medium for optimal GABA production.

The effect of MSG concentration on GABA production by *E. casseliflavus* was measured in BHI medium (Fig. 4 C, Table 1). Growth and GABA production of PL05 strain were examined using different MSG concentrations (1, 3, 5, 7. 10, 13, and 15 %, w/v). The highest GABA level (58.33±0.09 mM) was produced at MSG concentration of 7%. This level was slightly but not significantly higher than the level (55.75±0.06 mM) produced at MSG concentration of 5%. However, when the MSG concentration was increased to 10% or 15%, the level of GABA production was significantly decreased to 28.85±0.06 mM or 18.73±0.07 mM, respectively, compared to the level produced with 7% MSG. Other studies have shown that excessive MSG concentration can inhibit bacterial cell growth and reduce GABA production (Yang et al.[28], Villegas et al.[29]). In addition, the best MSG concentration for optimal GABA production can be different depending on the microorganism.

Effects of various carbon sources on GABA production by *E. casseliflavus* were measured using BHI medium (without glucose) containing 1% (w/v) carbon source such as glucose, fructose, galactose, maltose, lactose, or sucrose (Fig. 4 D, Table 1). There was no significant difference in GABA level among different carbon sources. GABA levels were 48.69-54.55 mM at 24 h with all carbon sources. The highest production level of GABA was 54.55±0.56 mM with maltose as the carbon source. Lim et al. reported that GABA levels produced by *E. faecium* JK29 show significant differences according to the carbon source, with 0.5% of sucrose being the best carbon source for GABA production

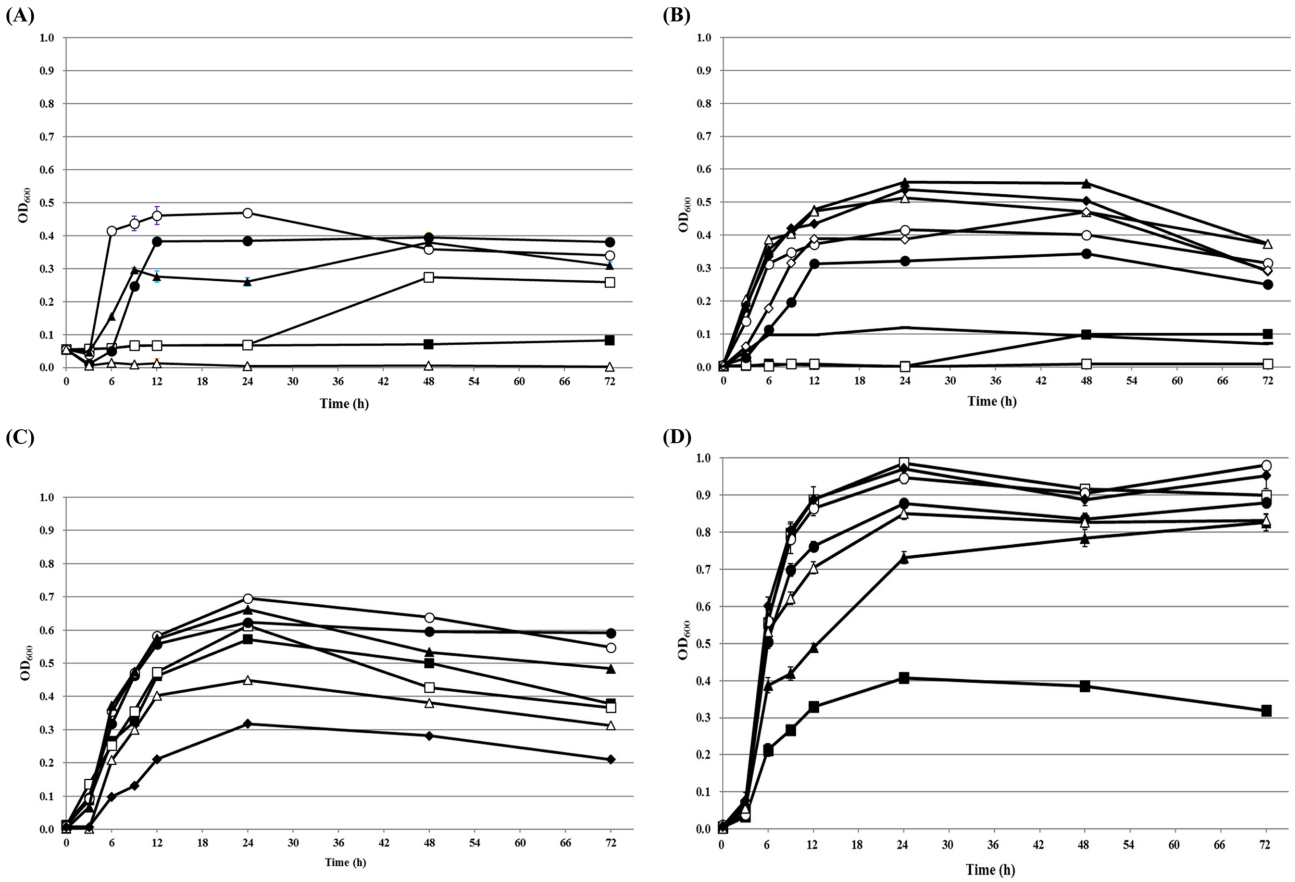

Fig. 4. Growth of *E. casseliflavus* strains on BHI broth at different conditions.
(A) Temperature, ■, 5°C; □, 15°C; ●, 25°C; ○, 35°C; ▲, 45°C; △ , 55°C; (B) Initial pH, ■, pH3.0; □, pH4.0; ●, pH5.0; ○, pH6.0; ▲, pH7.0; △, pH8.0; ◆, pH 9.0; ◇, pH10.0; —, pH11.0; (C) MSG concentration, ■, 1%; □, 3%; ●, 5%; ○, 7%; ▲, 10%; △, 13%; ◆, 15%; (D) Carbon sources, ■, Control (non-carbon source); □, Fructose; ●, Galactose; ○, Glucose; ▲, Lactose; △, Maltose; ◆, Sucrose.

(6.84 mM), followed by maltose (5.16 mM). Thus, the optimum carbon source for GABA production varies depending on the microorganism. GABA production of PL05 strain were examined using different maltose concentrations (0, 1, 3, 5, 7, and 10 %, w/v). The highest GABA level (61.11±0.29 mM) was produced at MSG concentration of 5% (Table 1).

Finally, as a result of test by the optimal conditions among these various conditions, 140.06±0.71 mM GABA was produced at 24 h, and the conversion yield was 78.95%.

Antibiotic susceptibility of *E. casseliflavus* was determined by disc diffusion method. Results showed that the PL05 strain was sensitive to ampicillin, amoxicillin, chloramphenicol, erythromycin, gentamicin, neomycin, penicillin, rifampicin, and vancomycin (Table 2). It is desirable that microorganisms for foods are sensitive to commonly prescribed antibiotics at low concentrations. Wide spread of resistance against various antibiotics among pathogens has become a threat for treating infected patients. Recently, *Enterococcus* strains have an issue related to the transfer of vancomycin resistance gene. In the present study, it was found that the PL05 strain *of E. casseliflavus* was sensitive to vancomycin.

Table 2. Antibiotic susceptibility of *E. casseliflavus* by disc diffusion method using antibiotic disc.

Antibiotics	Amount of disc (μg)	Sensitive/Resistance (zone diameter, mm)
Erythromycin	15	Sensitive (20)
Chloramphenicol	30	Sensitive (25)
Vancomycin	30	Sensitive (15)
Gentamicin	30	Sensitive (15)
Ampicillin	10	Sensitive (30)
Amoxicillin	30	Sensitive (30)
Rifampicin	30	Sensitive (25)
Neomycin	30	Sensitive (10)
Penicillin	0.1	Sensitive (25)

The development of fermented foods and functional foods using GABA-producing lactic acid bacteria has high potential in the food industry. And it is necessary to use a suitable

GABA-producing strain according to the characteristics of each food. There have been reports of many species of GABA-producing lactic acid bacteria, but there was no report on *E. casseliflavus*. Also, the optimal conditions for GABA production were depending on the species. In this study, the maximum yield of GABA from *E. casseliflavus* PL05 was 140.06±0.71 mM in optimal growth conditions at 24 h. In addition, the strain was sensitive to vancomycin. Based on these results, the PL05 strain is considered to have industrial application potential as a GABA-producing lactic acid bacterium.

국문요약

본 연구에서는 국내산 돌미나리에서 GABA생성능이 있는 신규 유산균을 분리, 동정한 결과, *Enteroccoccus casseliflavus*로 확인되었다. 최근까지 *Lactobacillus*속과 같은 GABA 생성 유산균에 대한 보고는 많이 되고 있고, 일부 *Enterococcs*속 유산균도 보고되고 있으나 *E. casseliflavus* 종에 대한 보고는 없었다. 따라서 본 연구에서는 *E. casseliflavus* PL05 균주에 대한 GABA 생성 최적 조건을 찾기 위하여 배지의 유형, 생육 온도, 초기 pH 조건, 배양 시간, MGS 농도 및 탄소원을 포함한 다양한 조건을 테스트하였다. PL05 균주는 MRS 혹은 TSB 배지보다 BHI 배지에서 생육이 잘 되었으며, 배지의 초기 pH는 7-9 조건에서 가장 생육이 왕성하였고, GABA 생성 조건 역시 유사한 결과로 확인되었다. GABA의 기질에 해당하는 MSG의 농도별 GABA 생성량을 조사한 결과, 7%에서 가장 높은 생성량을 나타내었으나 5%에서도 유사한 수준으로 확인되어 효율적인 측면에서 5%가 적합할 것으로 판단된다. 탄소원에 따른 생육 및 GABA 생성량은 말토오스를 사용하였을 때 가장 높은 것으로 확인되었고, 이러한 최적의 조건들로 최종 테스트를 진행한 결과, 24시간째 140.06±0.71 mM의 GABA가 생성되었고, 전환율은 78.95%로 확인되었다. 또한 반코마이신을 포함한 10개의 항생제에 대한 감수성을 조사한 결과 내성이 없는 것을 확인하였다.

Conflict of interests

The authors declare no potential conflict of interest.

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