

Optimization of γ -Aminobutyric Acid Production by *Enterococcus faecium* JK29 Isolated from a Traditional Fermented Foods

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Dominant lactic acid bacteria (LAB) strains were isolated from traditional fermented foods to obtain rare γ -aminobutyric acid (GABA)-producing LAB. Out of 147 isolates, 23 strains that could produce GABA with 1% (w/v) L-monosodium glutamate (MSG) were first isolated. After further screening of these rare GABA-producing LAB by analysis of the glutamate decarboxylase and 16S rRNA gene sequences, *Enterococcus faecium* JK29 was isolated, and 1.56 mM of GABA was produced after 48 h cultivation in basic de Man, Rogosa, and Sharpe (MRS) medium. To enhance GABA production by *E. faecium* JK29, the culture conditions were optimized. When *E. faecium* JK29 was cultivated in optimized MRS medium containing 0.5% (w/v) sucrose and 2% (w/v) yeast extract with 0.5% (w/v) MSG, GABA production reached 14.86 mM after 48 h cultivation at initial conditions of pH 7.5 and 30°C.

Keywords: γ -Aminobutyric acid, *Enterococcus faecium*, fermented food, optimization

Introduction

γ -Aminobutyric acid (GABA) as a non-protein amino acid is one of the major inhibitory neurotransmitters in the mammalian central nervous system and extensively found in nature among animals, plants, and microorganisms [17]. GABA is primarily biosynthesized by the irreversible α -decarboxylation of glutamate that is catalyzed by glutamate decarboxylase (GAD; EC 4.1.1.15), a pyridoxal-5'-phosphate-dependent enzyme, with conferring acid resistance by an incorporation of hydrogen ion [3, 21]. GABA possesses several physiological functions such as antihypertensive, diuretic, and anti-diabetic effects in Human. In addition, GABA exerts tranquilizing effect, particularly related to depression, sleepless-

ness, and neurological disorders including Parkinson's disease, Huntington's chorea, and Alzheimer's disease [9, 10, 27]. Due to diverse functions of GABA, it has been viewed for GABA to be a bioactive component useful in pharmaceutical and food industries. Therefore, the commercial demand of GABA has increased and consequently functional foods containing GABA rapidly developed [1, 11, 26].

There have been lots of studies on the GABA productions by microbial fermentation including bacteria, yeast and fungi, particularly lactic acid bacteria (LAB) [18, 22]. LAB are generally regarded as safe (GRAS) bacteria and extensively used as starters for fermented foods and dairy products [14, 15]. LAB have been also used as probiotics due to their following characteristics: intestinal homeostasis, resistance to bile acid and gastric acidity, and immunomodulation [8]. Moreover, the inhibition of pathogenic bacteria by LAB improves the stability of fermented foods during storage, contributing to the natu-

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rally-safe food production with no chemical additives [19]. Therefore, the fermented foods by LAB producing GABA could make full use of the physiological functions of GABA and potent health benefit of LAB [2].

GABA-producing LAB have been explosively reported from various foods and plants including *Lactobacillus paracasei* and *Lb. plantarum* from Italian cheeses [23], *Lb. brevis* from alcohol distillery lees [30], *Lb. senmaizukei* from Japanese pickle [6], and *Lactococcus lactis* from cheese starters [20]. There have been also several GABA-producing LAB isolated from kimchi as one of Korean traditional fermented foods, including *Lb. buchneri* and *Lb. sakei* [4, 13]. Although lots of LAB have been so far isolated and characterized for GABA production from fermented foods, it is still needed to screen GABA-producing LAB from Korean traditional fermented foods including kimchi due to the diverse LAB community in kimchi depending on the production methods and raw materials [22]. Numerous studies have been reported to isolate LAB in the genus *Lactobacillus* and *Lactococcus* as most of GABA-producing LAB, whereas there have been few studies on the other genus such as genus *Enterococcus*. This might be due to the lower GABA production levels from *Enterococcus* strains compared to those from *Lactobacillus* strains [28]. Previously, the GABA-producing *Enterococcus* strains were rarely reported, such as *E. avium* and *E. faecium* [5, 25].

In the current study, numerous LAB isolated from traditional fermented foods were screened to isolate rare LAB strains producing GABA except *Lactobacillus* and *Lactococcus* strains, resulting in *E. faecium* JK29 from a Korean traditional fermented food, kimchi. GABA production is generally affected by nutritional factors for GABA-producer and culture conditions, and these factors should be thus taken into account in the design of optimal GABA production for the application of industrial fields [13]. Accordingly, this study also describes the culture optimization of *E. faecium* JK29 for high GABA production by determining the optimal carbon and nitrogen sources, initial pH and exogenous L-monosodium glutamate (MSG) concentration.

Materials and Methods

Isolation and cultivation of GABA-producing LAB

Traditional fermented food samples, including kimchi,

salted shrimp, cucumber pickles, red pepper paste, and soybean pastes, were used as GABA-producing microbial sources. Each sample (2 g) of fermented foods were diluted in 10 ml of 0.85% NaCl solution, homogenized, and plated on DeMan, Rogosa, Sharpe (MRS) agar (MB cell, Seoul, Republic of Korea). After incubating at 30°C for 48–72 h, several colonies were randomly selected from each sample with different morphologies. The selected colonies were successively restreaked on MRS agar plates at least three times to obtain pure single colonies. To isolate the GABA-producing LAB, each colony was cultivated in MRS broth containing 1% MSG at 30°C for 48 h.

Analysis of GABA production

GABA production was qualitatively determined by thin-layer chromatography (TLC) analysis [7]. Briefly, the culture supernatants were prepared by centrifugation at 10,000 rpm for 10 min after cultivation for TLC analysis. Silica-coated TLC plate (Merck, Germany) was developed with a solvent mixture of n-butanol, acetic acid, and water (4:1:1, v/v/v). After development was complete, the plate was dried and visualized with 0.2% ninhydrin reagent. A spectrophotometry assay using GABase enzyme (Sigma, MO, USA) was also employed to quantitatively determine GABA production [31]. The culture supernatants (12 μ l) were dispensed into each well in a Multiskan FC plate reader (Thermo Fisher Scientific) together with 234 μ l of 100 mM $K_4P_2O_7$ buffer (pH 8.6), 42 μ l of 10 mM NADP⁺, and 5 μ l of GABase (5 U/ml). After reading an initial absorbance at 340 nm, 6 μ l of 100 mM α -ketoglutarate was added into the individual wells and the plate was then incubated for 1 h at room temperature, following by the final absorbance at 340 nm. The GABA concentration was finally determined by the difference between the above two absorbance (A_{340}) values.

Screening of rare GABA-producing LAB by genetic analysis

23 LAB strains producing GABA isolated from traditional fermented foods were screened to isolate rare LAB producing GABA by genetic analysis. To exclude the *Lactobacillus* and *Lactococcus* strains producing GABA, genomic DNA from 23 LAB strains were firstly isolated by using identified by HiYield™ Genomic DNA Mini Kit (RBC, Taiwan). Each strain was then identified by poly-

merase chain reaction (PCR) amplification of the 16S rRNA genes from each genomic DNA using the universal primer sets 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). After isolating *E. faecium* strains, the full-length *gad* gene from genomic DNA of *E. faecium* was amplified by using the following primer sets designed for the previously reported *E. faecium gad* gene sequences: sets MME-61 (5'-ATGTTATACGAAAAAGATAATCAAGAAG-3') and MME-62 (5'-TTAGTGAGTAAAGCCGTACGT-3'). The PCR operations were performed at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min.

Culture optimization for GABA production

The culture conditions of *E. faecium* JK29 were optimized in a 250 ml Erlenmeyer flask containing 100 ml of modified MRS medium to enhance the GABA production. The effect of a carbon source on GABA production was evaluated in the modified MRS medium, where 2% glucose was eliminated to exclude the effect of glucose as carbon source in the defined MRS medium, adding 2% various carbon sources (glucose, lactose, sucrose, fructose, maltose, galactose, arabinose, raffinose, mannitol, and soluble starch). Additionally, various nitrogen sources (beef extract, tryptone, soytone, yeast extract, peptone, casitone, casamino acid, proteose peptone No.3, malt extract, and urea) were added at 2.5% to the modified MRS medium that all nitrogen sources (1% proteose peptone No.3, 1% beef extract, and 0.5% yeast extract) in the defined MRS medium were removed. For the effect of MSG concentration on GABA production, the modified MRS mediums containing 0–5% (w/v) MSG were used. To determine the effect of initial pH on GABA production, the range of pH from 4.0 to 8.0 were investigated in the modified MRS medium containing 0.5% MSG at 30°C.

Results and Discussion

Isolation and identification of *E. faecium* JK29

In order to isolate LAB producing GABA, several traditional fermented foods were used as microbial sources. 147 isolates randomly selected on MRS agar plates were firstly screened to isolate GABA-producing strains with 1% MSG, resulting that 23 isolates produced GABA

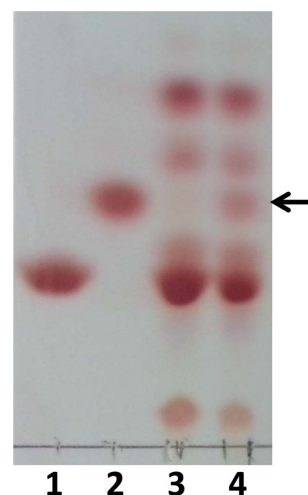


Fig. 1. GABA production by the JK29 strain isolated from traditional fermented foods. Lane 1, authentic MSG control; lane 2, authentic GABA control; lane 3, GABA-nonproducing strain as negative control; lane 4, GABA (indicated as arrow)-producing strain JK29.

which was qualitatively determined by TLC analysis. To select rare LABs, they were next identified by 16S rRNA gene sequence analysis, exhibiting 12 *Lactobacillus*-, 9 *Enterococcus*-, and 2 *Leuconostoc*-genus strains with the taxonomical similarities from 99.6% to 100.0% (data not shown). Since this study was focused on rare LABs producing GABA, we could select 11 LABs excluding *Lactobacillus* strains. The quantitative analysis of GABA concentration produced from 11 LABs showed that *Enterococcus*-genus strains produced more GABA with compared to *Leuconostoc* strains (data not shown). Thereafter, 11 *Enterococcus* strains was PCR-amplified with the developed PCR primer sets (MME-61 and MME-62) to screen novel GAD gene-harboring strain, resulting an amplified DNA fragment with the expected size (about 1.4 kbp). After sequencing these PCR products, we finally selected one strain GABA-producing JK29 (Fig. 1) of which GAD gene was taxonomically characterized to be close to that of *E. faecium* TX1337RF (accession number WP_002309699) with 99% identity, because this strain only showed the difference of GAD sequence with the previously-reported GADs and a high level of GABA production (1.56 mM) compared to the other *Enterococcus* strains isolated in this study. A multiple alignment of the amino acid sequence of GAD from *E. faecium* JK29 with various other GAD revealed that GAD from JK29 displayed the highest sequence identity

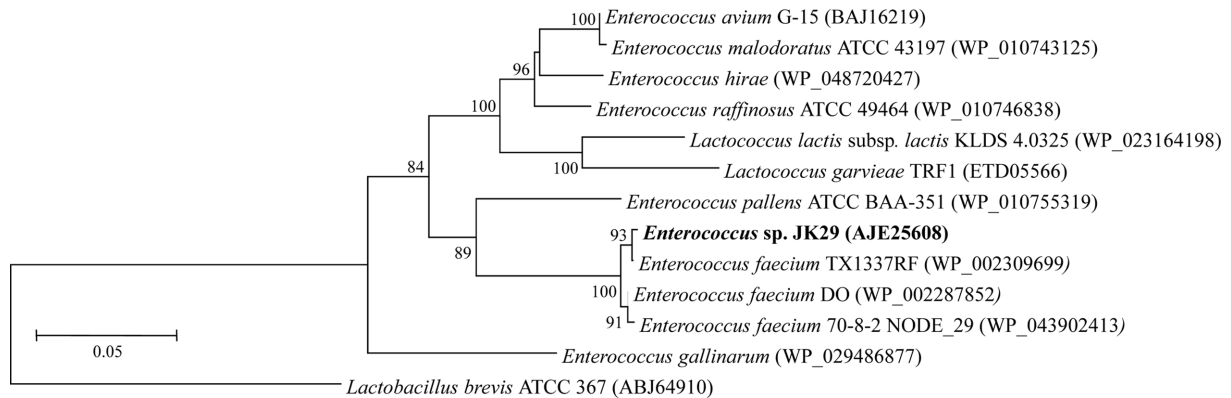


Fig. 2. Neighbor-joining phylogenetic tree based on the GAD amino acid sequences of *E. faecium* JK29 and other related taxa. Numbers at nodes indicate bootstrap values (expressed as percentage of 1,000 replicates) > 70% as calculated on the basis of neighbor-joining algorithm. *Lactobacillus brevis* ATCC 367 was used as an outgroup. The bar represents 0.05 substitutions per amino acid position.

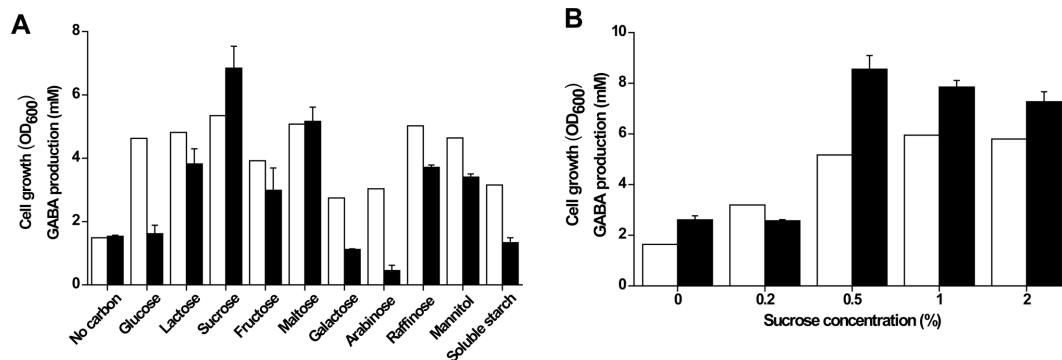


Fig. 3. Effects of carbon sources (A) and sucrose concentration (B) on cell growth (closed bars) and GABA production (open bars) of *E. faecium* JK29.

(99%) with those from the already-known *E. faecium* DO and *E. faecium* 70-8-2 NODE_29 (Fig. 2). Additionally, it respectively showed 90%, 87%, and 72% amino acid sequence identity with those from *E. pallens* ATCC BAA-351, *E. avium* G-15, and *Lb. brevis* ATCC 367. The GAD gene sequence of *E. faecium* JK29 was deposited in GenBank under the accession number KM649684.

Optimization of carbon and nitrogen sources for GABA production by *E. faecium* JK29

The effect of various carbon sources on GABA production by *E. faecium* JK29 was investigated in the modified MRS medium containing 2% carbon source and 1% MSG. Both of the cell growth and GABA production showed dramatically differences according to the additions of carbon sources. Sucrose was the best carbon source for GABA production (6.84 mM) with a 4.1 fold-

increase of GABA, followed by maltose (5.16 mM with 3.1 fold-increase) compared to standard MRS medium supplemented with 2% glucose (Fig. 3A). The investigation of sucrose concentration for GABA production resulted that the GABA production significantly increased to 8.56 mM with adding 0.5% sucrose with slightly decrease over 0.5% sucrose up to 5% (Fig. 3B). The optimal carbon sources and concentrations for GABA production varied according to the GABA-producing LAB strains: 4% maltose for *L. brevis* K203 [2], 1% glucose for *L. buchneri* MS [4], and 5.5% glucose for *L. brevis* NCL912 [16]. Similarly to our result showing sucrose as an optimal carbon source for GABA production, the carbon source for *L. sakei* B2-16 was optimized to be 4% sucrose for the GABA production, while *L. brevis* 340G to be 3% sucrose [12, 22].

The effects of nitrogen sources on the GABA produc-

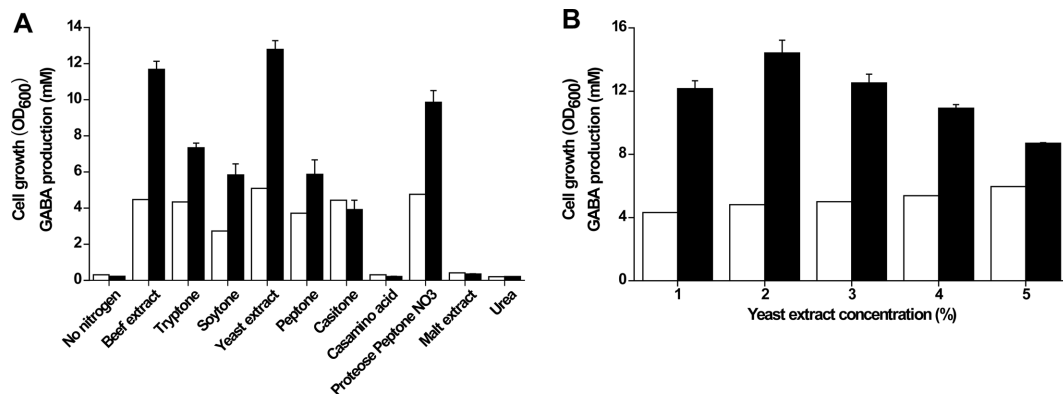


Fig. 4. Effects of nitrogen sources (A) and yeast extraction concentration (B) on cell growth (closed bars) and GABA production (open bars) of *E. faecium* JK29.

tion were evaluated when 0.5% sucrose was used as the carbon source. Yeast extract was determined to be the best nitrogen source for GABA production (12.78 mM), followed by beef extract (11.68 mM) and proteose peptone No.3 (9.86 mM), which might be associated with their compositions as original nitrogen sources in the defined MRS medium (Fig. 4A). On the other hand, the low production of GABA was exhibited when casamino acid, malt extract or urea was used as the nitrogen source. Our results are consistent with the previous studies reporting that yeast extract has been determined to be the best nitrogen source for GABA production by *Lactobacillus* strains [12, 22]. After the investigation of yeast extract concentration on GABA production, it increased according to the concentration of yeast extract up to 2%, showing 14.42 mM GABA (Fig. 4B). However, it slightly decreased over 2% yeast extract, producing 8.70 mM GABA at 5% yeast extract. This pattern was similar to that of the previous study reporting that 2% yeast extract was the optimal nitrogen source for GABA pro-

duction by *L. brevis* K203 and the addition over 2% yeast extract affected the decrease of GABA production [2]. Finally, the carbon and nitrogen sources were optimized to be 0.5% sucrose and 2% yeast extract for the GABA production by *E. faecium* JK29, respectively.

Effects of MSG concentration and initial pH on GABA production by *E. faecium* JK29

The optimal MSG concentration for the enhancement of GABA production was determined by cultivating *E. faecium* JK29 in the optimized MRS medium with adding MSG of various concentrations at 30°C. There was no significant difference of the cell growths with addition of up to 2% MSG. However, the addition of high MSG concentration (5%) affected the decrease of the cell growth (Fig. 5A). The GABA production was enhanced with increasing the MSG concentration up to 1% where the maximum GABA production (14.79 mM) was obtained, followed by the reduction of GABA production when the MSG concentration exceeded 1%, suggesting

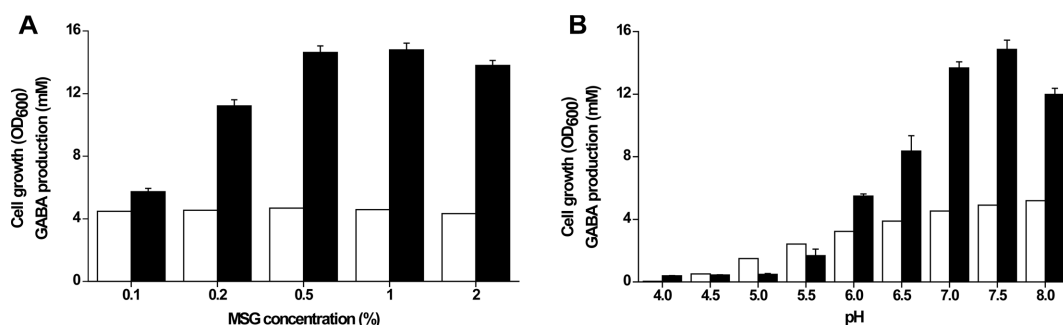


Fig. 5. Effects of exogenously-adding MSG concentration (A) and initial pH (B) on cell growth (closed bars) and GABA production (open bars) of *E. faecium* JK29.

that the excess addition of MSG was inhibitory for the bacterial cell growth and GABA production [2, 24, 29]. The addition of 1% MSG resulted to the highest GABA production, of which level was, however, similar to that of 0.5% MSG. The addition of 0.5% MSG rather yielded the higher GABA conversion yield (49.5%) of GABA compared to that of 1% MSG (25.0%). Therefore, the MSG concentration of 0.5% was determined to be optimal for the GABA production by *E. faecium* JK29 in this study.

The effect of initial pH from 4.0 to 8.0 on the cell growth and GABA production from *E. faecium* JK29 was evaluated in the optimized MRS medium containing 0.5% MSG at 30°C. The cell growth increased proportionally to the initial pH, of which pattern was also similarly exhibited for the GABA production (Fig. 5B). However, the GABA production decreased at pH value above 7.5 which was finally determined to be optimal initial pH for the GABA production (14.86 mM). The optimal initial pH range for GABA production by *Lactobacillus* strains have been well known to be 4.5-5.5: pH 5.0 for *L. buchneri* MS [4], 5.25 for *L. brevis* K203 [2], and 5.31 for *L. plantarum* Taj-Apis362 [24]. This is strongly associated to GAD activity of which expression is induced and stability is increased in the appropriately acidic condition of culture medium with supplying the hydrogen ion for the GABA production [3, 21]. However, the pH reduction for the excess of GAD ability to biosynthesize GABA could inhibit the cell growth and metabolic pathway. In contrast, the GABA could be easily converted to succinic acid semialdehyde by GABA transaminase under the strongly alkaline condition [28]. Similar to our results on the optimal pH condition, the optimal pH range for the GABA production by *E. avium* 9184 was determined to be from 6.0 to 6.5, which could be regarded to be distinctly different with that for *Lactobacillus* strains [28].

In this study, *E. faecium* JK29 isolated from traditional fermented foods produced less GABA (14.86 mM) with utilizing 0.5% MSG compared to major group of LABs producing GABA such as *Lactobacillus*- and *Lactococcus*-genus strains, even though the culture conditions were partially optimized for GABA production. In the previous studies, *L. brevis* 340G, *L. buchneri* MS and *L. sakei* B2-16 isolated from traditional fermented foods produced GABA with the concentration of 68.77 mM, 251 mM and 255 mM in the modified MRS medium with

the high concentration of MSG (3–5%), respectively [4, 12, 22]. However, the points that *Enterococcus*-genus strains have not been almost isolated and characterized to be GABA-producers, challenged to isolate *E. faecium* JK29 producing GABA in this study. Together with rare GABA-producer, *Enterococcus* strains have been reported to have strong environmental adaptability such as the resistances against acid and concentrated bile salts, implying the good candidate for the industrial application [28, 32]. Since *E. faecium* JK29 naturally had the low capability to produce GABA, its culture conditions should be also optimized for the increase of GABA production [28]. After the partial optimization of *E. faecium* JK29 culture conditions, its GABA production level was still low compared to other major groups of GABA-producing LABs. Therefore, it should be more optimized to enhance the GABA production from *E. faecium* JK29 with determining other factors including Tween-80 as a growth-stimulating factor for most of LAB and pyridoxal 5'-phosphate as a coenzyme of GAD [2, 16]. In addition, the scale-up fermentation of *E. faecium* JK29 utilizing the crop by-products such as rice bran extracts as fermentation medium will be further necessary for the enhancement of GABA production which enables to be applied into the industrial fields [13]. Similarly, the fermentation conditions of *E. avium* G-15 was optimized with determining MSG concentration, aeration and feeding methods for fed-batch fermentation, resulting 1,120 mM GABA production with adding 25% MSG [25]. There is also another study on the development of fermentation strategy for GABA production by *E. avium* 9184, reporting that the two-stage fermentation (first stage for the regulation of cell growth and second for the regulation of GABA formation) increased the GABA production (35.98 mM) with the initially-addition of 1% MSG [28].

In conclusion, GABA has the great potential as a bioactive component in pharmaceutical and functional food industries and LABs are regarded to be GRAS bacteria with being used as probiotics. In this study, *E. faecium* JK29 was isolated from traditional fermented foods as rare LAB producing GABA and its culture conditions were optimized to enhance the GABA production. The cultivation of *E. faecium* JK29 in the optimized MRS medium containing 0.5% sucrose, 2% yeast extract and 0.5% MSG at initial pH 7.5 and 30°C produced 14.86 mM

GABA after 48 h of cultivation. The further studies on the detail optimization and large-scale fermentation of *E. faecium* JK29 as a rare GABA-producer will be needed to determine the applicability of *E. faecium* JK29 as functional LAB starter strain for industrialization.

Acknowledgments

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국문초록

전통발효식품 유래 *Enterococcus faecium* JK29에 의한 γ -aminobutyric acid의 생산 최적화

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γ -Aminobutyric acid(GABA)를 생산하는 희귀 젓산균을 분리하기 위하여 전통발효식품으로부터 총 147개의 젓산균을 확보한 후 1% 글루탐산 나트륨(L-monosodium glutamate, MSG)를 사용하여 GABA를 생산하는 23개의 균주를 1차 분리하였다. 2차 분리를 위하여 글루탐산 탈탄산효소(glutamate decarboxylase)와 16S rRNA 유전자의 염기서열 분석을 통해 기본 MRS 배지에서 48시간 배양 후 1.56 mM의 GABA를 생산하는 *Enterococcus faecium* JK29를 최종 분리하였다. *E. faecium* JK29에 의한 GABA의 생산을 향상시키기 위하여 배양 조건을 최적화하였으며 그 결과 0.5% 자당(sucrose), 2% 효모 추출물(yeast extract), 0.5% 글루탐산 나트륨이 포함된 최적화 MRS 배지를 개발하였다. 최적화 MRS 배지를 활용하여 30°C, pH 7.5에서 48시간 배양을 한 결과 *E. faecium* JK29이 14.86 mM의 GABA를 생산하는 것을 확인하였다.