Screening of -Aminobutyric Acid-Producing Enterococcus faecalis and Quantification of -Aminobutyric Acid: Postprint

Authors: Zhu Quan, Cheng Jinlong, Zhu Yuanzhao, Yin Long, Ni Jindong, Cheng Maoji

Date: 2017-10-11T00:00:00+00:00

Abstract

This study aimed to identify and isolate a -aminobutyric acid (GABA)-producing Enterococcus faecium strain using molecular biology methods and quantitatively determine its GABA production. A target strain F6 was screened from samples of pickled vegetables, yogurt, soil, and fresh milk, and subjected to morphological characterization and Gram staining identification. The 16S rDNA gene of strain F6 was amplified, sequenced, and used for phylogenetic tree construction. Simultaneously, high-performance liquid chromatography (HPLC) was employed to quantitatively determine the GABA content in the fermentation broth of strain F6. The results showed that colonies of strain F6 were large, smooth, circular, 1~2 mm in diameter, with regular edges and milky white in color. On the isolation medium, a transparent halo formed around the colonies, turning the medium yellow; on MRS solid medium, the colonies were opaque with a transparent halo around them. Gram staining identified F6 as a Gram-positive bacterium. Molecular biological identification and analysis revealed that the 16S rDNA gene sequence of strain F6 exhibited greater than 99% similarity with Enterococcus faecium in the GenBank database. HPLC determination yielded a linear equation for the GABA standard curve: Y=7 080 733.139 5X-4 511.692 7 (R2=0.999 4). Using this equation, the GABA content in the fermentation broth of strain F6 was determined to be 7.1 g/L, with a retention time of 7~10 min. These results suggest that a high GABA-producing Enterococcus faecium strain F6 was successfully screened in this study.

Full Text

Screening of Enterococcus faecium Producing -Aminobutyric Acid and Quantitative Detection of -Aminobutyric Acid

ZHU Quan[1], CHENG Jinlong[2], ZHU Yuanzhao[3]*, YIN Long[2], NI Jindong[2], CHENG Maoji[1]

[1]College of Animal Science, Anhui Agricultural University, Hefei 230061, China
[2]Jiangsu Unison Biotechnology Development Co., Ltd., Suqian 223831, China
[3]College of Animal Science, Anhui Science and Technology University, Fengyang 233100, China

Abstract

This study aimed to isolate and identify a strain of Enterococcus faecium producing -aminobutyric acid (GABA) using molecular biological methods and to quantitatively determine its GABA yield. A target strain F6 was screened from samples of pickles, yogurt, soil, and fresh milk, followed by morphological characterization and Gram staining identification. The 16S rDNA gene of strain F6 was amplified, sequenced, and used to construct a phylogenetic tree. Meanwhile, the GABA content in the fermentation broth of strain F6 was quantitatively determined by high-performance liquid chromatography (HPLC). The results showed that strain F6 formed large, smooth, circular colonies measuring 1-2 mm in diameter with neat edges and a milky white color. On the isolation medium, colonies produced transparent halos that turned the surrounding medium yellow. On MRS solid medium, colonies appeared opaque with transparent halos. Gram staining identified F6 as a Gram-positive bacterium. Molecular biological analysis revealed that the 16S rDNA gene sequence of strain F6 shared greater than 99% similarity with that of Enterococcus faecium in the GenBank database. HPLC analysis yielded a standard curve linear equation of Y=7 080 733.139 5X-4 511.692 7 (R^2=0.999 4), and the GABA content in the fermentation broth of strain F6 was determined to be 7.1 g/L with a retention time of 7-10 min. These results indicate that a high GABA-producing strain of Enterococcus faecium F6 was successfully isolated.

Keywords: Enterococcus faecium; -aminobutyric acid; 16S rDNA sequence analysis; quantitative detection by high-performance liquid chromatography; screening

Introduction

Lactic acid bacteria play important roles in regulating gastrointestinal microecological balance, benefiting animal health, improving livestock performance, reducing environmental pollution, and replacing feed antibiotics, with increasingly widespread applications in animal production. However, Lactobacillus species are obligate anaerobes with poor stress resistance, oxygen intolerance, and easy inactivation, leading to unstable application effects. In contrast, cocci exhibit stronger stress resistance than bacilli with lower activity loss, suggesting that cocci, especially enterococci, will become more widely used in the future.

-Aminobutyric acid (GABA), also known as aminobutyric acid, has the molecular formula C H NO and serves as a major inhibitory neurotransmitter in the mammalian central nervous system, mediating over 40% of inhibitory neurotransmission. GABA exhibits various physiological activities in humans, including blood pressure regulation, brain function improvement, memory enhancement, anti-anxiety effects, and analgesia. In livestock and poultry, GABA promotes feed intake, provides anti-stress and sedative effects, and improves production performance, particularly showing significant anti-heat stress effects. Current methods for GABA synthesis include chemical synthesis and microbial fermentation. GABA used in livestock production is primarily chemically synthesized as a purified product, which is costly, and microbially fermented GABA is rarely applied.

For a long time, researchers have focused on studying single-functional microecological preparations of lactic acid bacteria and chemically synthesized GABA, recognizing both as important functional additives. However, studies on GABA-secreting lactic acid bacteria remain limited. Enterococcus faecium belongs to the genus Enterococcus, also known as Streptococcus faecalis, and can be classified into multiple groups. Enterococcus faecalis belongs to Group D, which includes Enterococcus faecalis, Enterococcus durans, and Streptococcus durans. Enterococcus faecium is widely distributed, primarily inhabiting animal intestines, feces, and soil as an anaerobic substrate. Naturally occurring enterococci secrete minimal or no GABA, requiring optimization of growth conditions to enhance GABA production. Currently, research on GABA-producing Enterococcus faecium is scarce. Therefore, this study conducted screening and identification of high-yield GABA-producing Enterococcus faecium to enable the application of green microecological preparations and naturally fermented GABA, reducing additive costs while achieving dual benefits of GABA’ s anti-stress effects and lactic acid bacteria’ s microecological regulation, providing a theoretical foundation for developing functional microecological preparations and their application in animal husbandry.

Materials and Methods

1.1 Strains and Culture Media

Bacterial strains were isolated from homemade pickles, yogurt (Bengbu Shuanghua Dairy Co., Ltd.), soil (collected from the west campus of Anhui Science and Technology University), and fresh milk.

MRS solid medium was prepared as follows: peptone 10 g, beef extract 10 g, yeast extract 5 g, glucose 20 g, sodium acetate trihydrate 5 g, diammonium citrate 2 g, Tween-80 1 g, dipotassium hydrogen phosphate 2 g, magnesium sulfate heptahydrate 0.58 g, manganese sulfate tetrahydrate 0.25 g, calcium carbonate 7.5 g, and agar 22.5 g, dissolved in deionized water to a final volume of 1,000 mL, adjusted to pH 6.5, and sterilized at 121 °C for 40 min.

MRS liquid medium contained the same components as MRS solid medium except for calcium carbonate and agar, dissolved in distilled water to 1,000 mL and adjusted to pH 6.5.

Isolation medium consisted of beef extract 10 g, yeast extract 10 g, peptone 10 g, glucose 5 g, Tween-80 0.5 g, tomato juice 200 g, bromocresol green 0.1 g, calcium carbonate 20 g, and agar 20 g, dissolved in distilled water to 1,000 mL and adjusted to pH 6.5.

1.2 Isolation, Pure Cultivation, and Subculturing of Target Strains

Samples of pickles, yogurt, soil, and fresh milk (1 g each) were inoculated into separate 150 mL Erlenmeyer flasks containing MRS liquid medium and incubated statically at 35 °C for 48 h. The cultures were then aseptically inoculated onto isolation medium and incubated statically at 35 °C for 48 h. Suspected single colonies surrounded by yellow zones were aseptically selected and inoculated into MRS liquid medium for static incubation at 37 °C.

One milliliter of MRS liquid culture was serially diluted with sterile physiological saline to concentrations of 10 , 10 , and 10 . One hundred microliters of each dilution were spread evenly onto MRS solid medium plates and incubated at 37 °C for 48 h. Single circular, milky white colonies with calcium dissolution zones were aseptically selected for purification and isolation.

The screened strains were inoculated onto MRS slant medium, incubated at 35 °C for 48 h, and stored at 4 °C with subculturing every 20 days. Preserved strains were inoculated onto MRS solid medium, incubated at 35 °C for 48 h, and then transferred to MRS liquid medium for 22 h to prepare the fermentation seed culture.

1.3 Gram Staining Identification of Target Strains

Gram staining was performed on target strains to observe their morphological characteristics.

1.4 Molecular Biological Identification of Target Strains

Ten milliliters of bacterial culture were centrifuged at low speed to harvest cells. Total bacterial DNA was extracted using a bacterial genomic DNA extraction kit (DP302, Shanghai Sangon Biotech Co., Ltd.). Ten nanograms of purified bacterial total DNA were used as template to amplify the 16S rDNA gene using universal primers for prokaryotic 16S rDNA: forward primer 5´-AGAGTTTGATCCTGGCTCAG-3´ and reverse primer

5´-CTACGGCTACCTTGTTACGA-3´ (synthesized by Shanghai Sangon Biotech Co., Ltd.). The PCR amplification system (50 L) contained: 1.0 L DNA template, 10×Buffer, 2.0 L dNTPs, 1.0 L forward primer (10 pmol), 1.0 L reverse primer (10 pmol), 1.0 L Taq DNA polymerase, 1.4 L Mg^{2} , and ddH O to 50 L. Amplification conditions were: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min; final extension at 72 °C for 5 min; and termination at 4 °C. The full-length PCR product was sequenced by Shanghai Sangon Biotech Co., Ltd.

1.5 Sequence Analysis and Phylogenetic Tree Construction

Based on the 16S rDNA gene sequencing results, the strain was preliminarily identified using nucleotide basic local alignment search tool (N-BLAST). Combined with 16S rDNA gene sequences of other Lactobacillus species from GenBank, a phylogenetic tree was constructed using MEGA 3.0 software.

1.6 Qualitative Determination of GABA Produced by Target Strains

The seed culture was aseptically inoculated into fermentation medium at 3.5% inoculum and incubated at 37 °C for 48 h. Five milliliters of fermentation broth were centrifuged at 4,500 r/min for 5 min, and the supernatant was analyzed by improved paper chromatography to detect GABA presence. The improved method involved adding ninhydrin developer at 0.55% (w/v) to the developing solvent composed of n-butanol:glacial acetic acid:water (5:3:1, v/v). Ten microliters of test solution were spotted, with GABA standard at 5 g/L as reference. After development, chromatograms were visualized at 85 °C for 8 min. The presence of a ninhydrin-stained spot with the same relative migration rate as the GABA standard indicated GABA production, confirming the strain as a GABA producer for preservation.

1.7 Quantitative Determination of GABA Produced by Target Strains

Principle of HPLC quantification: -mercaptoethanol was added to the bacterial culture to facilitate rapid reaction between GABA and o-phthalaldehyde, forming OPA derivatives. The GABA content was precisely determined by measuring absorbance at 338 nm based on OPA derivative peaks.

GABA standard solution preparation: GABA standard was accurately weighed and prepared at concentrations of 0, 0.05, 0.10, 0.15, 0.20, and 0.25 mg/mL.

HPLC detection conditions: Waters-1525 system; Hypersil ODS-2 C18 column (150 mm×4.0 mm, 5 m) with guard column; UV detector; injection volume 20 L; mobile phase A: 20 mmol/L sodium acetate buffer (2.72 g sodium acetate, 200 L triethylamine, ultrapure water to 1 L, pH adjusted to 7.3), filtered through 0.22 m membrane and degassed; mobile phase B: acetonitrile (A:B=4:1); flow rate 1 mL/min; column temperature 40 °C; derivatization reagent: 20 mg OPA, 20 L -mercaptoethanol, and 5 mL acetonitrile. Derivatization reaction: 100 L borate buffer (24.7 g boric acid in 1 L ultrapure water, pH 10.4), 20 L OPA derivatizing agent, and 20 L sample were mixed and reacted at room temperature for 5 min before analysis.

Results

2.1 Colony Characteristics of Target Strains

Multiple strains were isolated from pickles, yogurt, soil, and fresh milk. Strain F6 was selected for morphological observation and identification. The F6 colonies were smooth, circular, 1-2 mm in diameter with neat edges and milky white color, presenting a delicate surface [Figure 1: see original paper]. On isolation medium, F6 colonies turned the surrounding medium yellow and produced transparent halos [Figure 1: see original paper]A, attributed to lactic acid production by the bacterium reacting with bromocresol green in the medium, suggesting F6 is a lactic acid bacterium. On MRS solid medium, F6 colonies were opaque with transparent halos [Figure 1: see original paper]B.

2.2 Qualitative Determination of GABA Produced by Target Strains

Improved paper chromatography analysis revealed that strain F6 produced a spot with the same Rf value (ratio of distance from origin to spot center versus distance from origin to solvent front) as the GABA standard, with deep color intensity indicating high GABA production [Figure 2: see original paper].

2.3 Morphological Characteristics of Target Strains

Gram staining of strain F6 [Figure 3: see original paper] showed circular to elliptical cells measuring 0.5-1.0 m in diameter, mostly arranged in pairs or short chains, generally non-motile, and Gram-positive.

2.4 16S rDNA Gene Sequence Analysis and Phylogenetic Tree Construction

The 16S rDNA gene of strain F6 was sequenced and submitted to the GenBank database. N-BLAST analysis on the NCBI website revealed that the 16S rDNA gene sequence of strain F6 shared greater than 99% similarity with Enterococcus faecium in the GenBank database [Figure 4: see original paper], indicating they are different strains of the same species. Homology comparison with 16S rDNA gene sequences of various bacteria in the NCBI GenBank database was performed to determine the phylogenetic relationship and taxonomic status of strain F6. A phylogenetic tree constructed using MEGA 3.0 software [Figure 5: see original paper] showed that strain F6 was most closely related to Enterococcus faecium, followed by Enterococcus durans, confirming strain F6 as Enterococcus faecium.

2.5 Quantitative Determination of GABA Produced by Target Strains

2.5.1 Standard Curve Construction HPLC analysis precisely determined GABA content in standard solutions. The GABA peak in standard solution is shown in [Figure 6: see original paper]. A standard curve was plotted using peak area versus GABA concentration [Figure 7: see original paper], demonstrating a strong linear correlation with the equation: Y=7 080 733.139 5X-4 511.692 7 (R^2=0.999 4).

2.5.2 Quantification of GABA Produced by Target Strains HPLC analysis of strain F6 fermentation broth yielded a chromatographic peak area [Figure 8: see original paper]. Using the standard curve equation Y=7 080 733.139 5X-4 511.692 7, the GABA content in strain F6 fermentation broth was calculated to be 7.1 g/L, with a retention time of 7-10 min, peak area of 50,268,693.6, and absorbance value of 0.12.

Discussion

3.1 Screening of GABA-Producing Enterococcus faecium

GABA-producing strains were isolated from yogurt, soil, milk, and pickles. One strain from yogurt samples, designated F6, showed relatively high GABA production, demonstrating that screening high-yield GABA strains from natural sources is feasible. This study employed rapid screening based on traditional phenotypic characteristics and biological properties, followed by improved paper chromatography for qualitative detection of the GABA metabolite, enabling accurate and rapid determination of GABA-producing capability. The improved method involved adding ninhydrin developer at 0.55% to the developing solvent (n-butanol:glacial acetic acid:water=5:3:1, v/v) with visualization at 85 °C for 8 min, offering significant advantages over conventional paper chromatography requiring 15-20 min. Molecular biological methods comparing the 16S rDNA gene sequence of strain F6 with GenBank database entries revealed greater than 99% similarity with Enterococcus faecium, confirming strain F6 as Enterococcus faecium and providing a foundation for large-scale GABA production through genetic engineering.

3.2 GABA Preparation Methods

GABA can be prepared by chemical synthesis or biological synthesis. Chemical synthesis involves high costs, low yields, and uses hazardous or toxic solvents, making chemically synthesized GABA unsuitable for food and feed applications and not considered a natural additive. Biological synthesis employs pure microbial technology using safe, high-yield strains for fermentation production, representing natural food and feed additives. Lactic acid bacteria such as Lactobacillus brevis are recognized as safe strains, though few GABA-producing Enterococcus faecium strains have been screened. Although biologically synthesized GABA has lower purity, animal absorption rates are significantly higher than those of high-purity chemically synthesized GABA. Moreover, GABA-producing lactic acid bacteria can be directly added to animal feed without purification. This study targeted Enterococcus faecium, which exhibits stronger stress resistance than Lactobacillus, thereby combining the microecological regulatory functions of lactic acid bacteria with GABA’ s anti-stress effects for synergistic benefits.

3.3 GABA Yield of Lactic Acid Bacteria

Naturally screened lactic acid bacteria generally produce low GABA yields of 5–7 g/L, primarily using Lactobacillus species. Feng et al. studied Lactobacillus brevis A8 and found optimal fermentation conditions of initial pH 4.5, temperature 33 °C, inoculum size 20%, and fermentation time 3 days, achieving maximum GABA production of 19.2 g/L, with medium pH identified as the primary factor affecting GABA yield. In this study, strain F6 (Enterococcus faecium) produced 7.1 g/L GABA under conditions of 36 °C, initial pH 6.4, 4.5% inoculum size, 20–23 h seed age, and 60 h fermentation time. Under optimized conditions, GABA content in Enterococcus faecium F6 fermentation broth could be increased to 9.5 g/L, possibly related to different culture conditions affecting glutamate decarboxylase (GAD) activity. Traditional mutagenesis techniques can enhance GABA production in lactic acid bacteria. Xia et al. used UV and -ray mutagenesis on GABA-producing Lactobacillus brevis, increasing average GABA yield by 142.9%. However, mutagenized strains showed reduced inherent functional activity and potential safety risks due to uncertainty, randomness, and genetic instability. Recent reports describe recombinant Escherichia coli expressing GAD for GABA production via immobilized enzymes, though conversion efficiency remains generally low. The GABA biosynthetic pathway involves GAD converting L-glutamate to GABA. Subsequent studies plan to increase GABA synthesis by enhancing GAD gene expression in strain F6 through additional GAD expression cassettes, which is currently under investigation.

3.4 Quantitative Determination of GABA from Enterococcus faecium

This study first performed qualitative determination of GABA in fermentation broth using improved paper chromatography. By adding ninhydrin developer at 0.55% to the developing solvent and visualizing at 85 °C for 8 min, GABA presence could be rapidly confirmed by spot observation. Quantitative determination employed HPLC, where GABA reacts rapidly with o-phthalaldehyde in the presence of -mercaptoethanol to form OPA derivatives. Since OPA does not interfere with detection, the chromatographic baseline remains stable, and the standard curve shows $R^2=0.999\ 4$, the HPLC method used in this study is simple, rapid, sensitive, and reliable.

In conclusion, strain F6 isolated in this study is Enterococcus faecium, a functional lactic acid bacterium with high GABA-producing capability.

References

[1] ZHANG Chuangui. Plasticizer pollution and its effects on human health[J]. Bulletin of Biology, 1999, 34(2): 20.

[2] HORIE Noriko, KAN Miyako, KIM Mu-taku. Functionality of GABA (-aminobutyric acid)[J]. China Food Additives, 2010(6): 169-173.

[3] LIANG Hengyu, DENG Likang, LIN Hailong, et al. Research progress on -aminobutyric acid (GABA) as a new food resource[J]. Food Research and Development, 2013, 34(15): 119-123.

[4] MORTEZA Z, VAHHAB B, HOSSEIN J. Effects of central histamine receptors blockade on GABAA agonist-induced food intake in broiler cockerels[J]. Pakistan Journal of Biological Sciences, 2008, 11(3): 416-421.

[5] BAI Song, LIN Xiangyang, RUAN Rongsheng, et al. Distribution and preparation of -aminobutyric acid[J]. Modern Food Science and Technology, 2005, 21(2): 202-205.

[6] GENG Jingzhang. Application research of -aminobutyric acid (GABA) in food industry[J]. Beverage Industry, 2012, 15(1): 11-14.

[7] LI Liwei, GU Xinxi, LU Haiqiang, et al. Screening of probiotic lactic acid bacteria strains for fermented yam yogurt[J]. Journal of Chinese Institute of Food Science and Technology, 2015(11): 78-82.

[8] BUCK K, VOEHRINGER P, FERGER B. Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry[J]. Journal of Neuroscience Methods, 2009, 182(1): 78-84.

[9] JÁMBOR A, MOLNÁR-PERL I. Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride: literature overview and further study[J]. Journal of Chromatography A, 2009, 1216(15): 3064-3077.

[10] WANG Xiangyan, XU Haiyan, XIN Guoqin, et al. Isolation of -aminobutyric acid-producing bacteria and optimization of fermentation conditions[J]. China Feed, 2016(4): 27-31.

[11] YANG Jingjing, QU Yuan, CUI Xiuming. Research progress on preparation methods and content determination of -aminobutyric acid[J]. Science and Technology of Food Industry, 2014, 35(3): 351-356.

[12] CHOI S I, LEE J W, PARK S M, et al. Improvement of -aminobutyric acid (GABA) production using cell entrapment of Lactobacillus brevis GABA 057[J]. Journal of Microbiology and Biotechnology, 2006, 16(4): 562—568.

[13] MIAO Cunying, JIANG Donghua, XU Xiaobo, et al. Screening and identification of high-yield -aminobutyric acid lactic acid bacteria from pickled vegetables[J]. Journal of Microbiology, 2010, 30(2): 28-32.

[14] WANG Chaokai, LIU Xu, ZHANG Lei, et al. Screening of -aminobutyric acid-producing lactic acid bacteria and preliminary optimization of fermentation conditions[J]. Food and Fermentation Technology, 2012, 48(1): 36-39.

[15] FENG Zhibin, WU Siying, ZHANG Yuxiang, et al. Optimization of fermentation conditions for -aminobutyric acid production by Lactobacillus brevis[J]. Journal of Ludong University: Natural Science Edition, 2012, 28(3): 248-251.

[16] XIA Jiang, MEI Lehe, HUANG Jun, et al. Screening and mutagenesis of -aminobutyric acid-producing lactic acid bacteria[J]. Journal of Nuclear Agricultural Sciences, 2006, 20(5): 379-382.

[17] TIAN Lingzhi, XU Meijuan, RAO Zhiming. Construction of recombinant Escherichia coli/pET-28a-lpgad and optimization of transformation conditions for efficient -aminobutyric acid production[J]. Chinese Journal of Biotechnology, 2012, 28(1): 65-75.

[18] KOOK M C, SEO M J, CHEIGH C I, et al. Enhancement of -aminobutyric acid production by Lactobacillus sakei B2-16 expressing glutamate decarboxylase from Lactobacillus plantarum ATCC 14917[J]. Journal of the Korean Society for Applied Biological Chemistry, 2010, 53(6): 816—820.

[19] KOMATSUZAKI N, SHIMA J, KAWAMOTO S, et al. Production of -aminobutyric acid (GABA) by Lactobacillus paracasei isolated from traditional fermented foods[J]. Food Microbiology, 2005, 22(6): 497—504.

[20] LIU T T, YANG T W, ZHANG S C, et al. Screening, identification and primary optimizing of a strain producing -aminobutyric acid from L-glutamic acid[J]. Journal of Food Science and Biotechnology, 2010, 29(5): 742—747.

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv —Machine translation. Verify with original.