Research Article

Preparation of a Forgotten Elixir: Panchagavya and Isolation of GABA Producing Probiotics

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ABSTRACT

Aim: The current study aims to optimize and quantify the production of GABA from isolates obtained from Panchagavya. **Materials and Methods:** Using TLC and a UV-vis spectrophotometer, a number of microorganisms that were isolated from the Panchagavya were examined for their ability to produce GABA. The physical and chemical parameters such as temperature, pH, incubation period and glutamic acid concentration were optimized for the maximum production of GABA by the selected isolates (V2 and V7) using the Box-Behnken method. **Results:** The colonies exhibiting typical traits were separated and examined for the generation of GABA. The selected strains V2 and V7 it was identified as *Enterococcus faecium* and *Alcaligenes* sp. respectively using 16S rRNA sequencing. The results of the optimization studies showed that isolate V2 produced more amount of GABA after the incubation of 48 hr in the pH of 4.5 at 35°C, 500 mM of glutamic acid concentration and utilized lactose as the best carbon source. Similarly, isolate V7 produces ma GABA after the incubation of 48 hr in a pH of 5.5 at 25°C, 500 mM of Glutamic acid and utilized lactose as the best carbon source. **Conclusion:** Hence, the probiotic isolates derived from Panchagavya have the ability to serve as a potential starter culture for the production of GABA in the industrial sectors.

Keywords: GABA, Neurotransmitter, Panchagavya, Probiotics.

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INTRODUCTION

The fermentation of cow milk, curd, urine, ghee and cow dung produces Panchagavya, a traditional Indian fertilizer and insecticide. "The mixture of five components" from the cow is what panchagavya (sanskrit) signifies. Panchagavya was made with nine ingredients: water, sugarcane, banana, tender coconut, milk, curd, butter and cow dung. Numerous medical conditions, such as allergies, colds, coughs, Infections of the skin, asthma, etc., can be treated with it.[1] Macroscopic and micronutrients, amino acids, growth stimulants including indole acetic acid and gibberellins and helpful microorganisms are all included in panchagavya. It has been demonstrated that panchagavya has pro-agricultural effects, including growth, biocontrol and biofertilizer. Panchagavya is a key medication in Ayurveda and is used to purify numerous herbal medicines.[2]

"Live microorganisms that, when given in an appropriate amount, provide a health benefit to the host" is the definition of a probiotic.[3] Because of our innate need to regulate our surroundings, probiotics have been isolated from dairy products that are fermented and linked to intestinal microorganisms in humans. This has led to a revised definition of probiotics: "live organisms that, when ingested in sufficient quantities as an individual strain or combination of strains, give health advantages for the host".[4]

GABA is a naturally occurring neurotransmitter that acts as an inhibitory neurotransmitter in the CNS (Central Nervous System). GABA can be found in bacteria, fungi

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and even plants, animals and other microbes. Moreover, it affects many physiological reactions, including anti-hypertensive effects, memory enhancement, mood modulation and sleep induction. GABA has received a lot of interest due to these health advantages. Mold, fungus, yeast and bacteria are frequently used in the fermentation process to produce GABA. Lactic Acid Bacteria (LAB) are among these that have drawn the greatest attention as GABA producers because of their extensive research, Generally Accepted as Safe (GRAS) status and simplicity of ingestion for humans in comparison to other microorganisms.[5] The study aims to optimize and quantify the production of GABA from the isolates collected from the Panchagavya. The probiotic bacteria that produce GABA were identified in Panchagavya during this study. Using the Box-Behnken Design (BBD), the ideal GABA synthesis conditions were examined, including pH, temperature, incubation period, carbon source and glutamic acid content.

MATERIALS AND METHODS

Preparation of Panchagavya

The nine constituents of Panchagavya are dung from cows, urine of cows, milk, ghee, curd, jaggery, bananas, tender coconuts and water. The first additions are 350 g of cow dung and 50 g of cow ghee and thorough mixing is done twice per day, in the early hours and late at night, as advised by the Tamilnadu Agricultural University. For a period of three days, the mixed concoction is kept fresh. A thorough mixing is done twice per day, in the early hours and late at night, after three days when 500 mL of cow urine and 500 mL of water are added. For 15 days, this mixture is kept in an undisturbed setting. After 15 days, a mixture of 150 mL of cow's milk, 100 mL of curd, 150 mL of soft coconut water, 150 mL of jaggery and one well was added. After 15 days, combine 150 mL of cow's milk, 100 mL of curd, 150 mL of soft coconut water, 150 mL of jaggery and one fully ripe poovan banana. Panchagavya can be used after 15 days. Panchagavya preparation takes thirty days in total.[6]

Isolation of probiotic strains

90 mL of sterile distilled water were used to dissolve 10 g of the Panchagavya sample. Spread out on MRS media, 1 mL of the material was serially diluted for a 10-9 dilution and incubated for 48 hr at 37ºC. For pure colonies, the most common colonies were separated and subcultured. For future research, pure cultures were kept at 4ºC.

Quantification and screening of GABA synthesis

GABA was measured using the colorimetric method outlined by Dikshit and Tallapragada.[7] The isolated strain's overnight culture was transferred into MRS broth that had been enriched with 1% MSG and 48 hr of 37ºC incubation. Following the incubation of the culture broth, it was centrifuged at 8000 rpm. The supernatant was then spotted on a TLC plate with a solvent system consisting of n-butanol, acetic acid and water (5: 3: 2). After scraping the GABA spots, they were put in a tube of glass with 0.5 mL of the 0.8% ninhydrin reagent dissolved in acetone and 3 mL of borate buffer (pH 7). Following a 20 min incubation period at 70ºC in a water bath, the optical density was determined using spectrophotometry at 570 nm. The blank was created without producing sample.

Characterization of GABA producing probiotic isolates

pH tolerance

The probiotic isolates were inoculated to MRS broth adjusted with various pH ranges using 10 N NaOH and 16N HCl as described by Barnali and Subhankar (2010).[8] The inoculated MRS agar plates were incubated for 24 hr of incubation and the cell density was determined at 600 nanometers with a spectrophotometer.

NaCl Tolerance

In MRS broth with varying NaCl concentrations (2, 4, 6, 8 and 10%), the probiotic isolates were seeded and cultured for 24 hr at 37ºC. The inoculated culture tubes were observed for the presence of growth. Then, the optical density was determined using a spectrophotometer at the wavelength of 600 nm.[9]

Bile salt tolerance

The tolerance to bile salts of the probiotic isolates was assessed using Kumar *et al*. (2013) technique.[10] MRS broth was supplemented with probiotic isolate, which included different concentrations of bile salts (Ox-gall) at 0.05, 0.15 and 0.3%. The mixture was then incubated for a whole day. At 600 nm, the UV vis spectrophotometer was used to measure light density.

Phenol

The probiotic isolates' capacity to tolerate against phenol was investigated according to the method of Hoque *et al*. (2010).[9] The probiotic isolate was inoculated in the MRS broth containing various phenol levels such as 0.1, 0.2 and 0.4% and incubated for 24 hr. The optical density at 600 nm wavelength was determined to examine the cell density.

Enhancement of GABA-producing probiotic isolates

Using the Box-Behnken method, the process of making GABA was adjusted by adjusting a number of physical and chemical factors.[11] The incubation temperature (25ºC, 30ºC and 35ºC), the carbon source (glucose, lactose and starch), the incubation length (24, 48 and 72 hr) and the glutamic acid concentration (400, 500 and 600 mM) were the five variables that were optimized. Each parameter (Run) will be tested for its GABA production and the best parameter showing higher GABA production will be identified. The data was analyzed using Graphpad Prism Version 7.

Characterization of strain by sequencing

16 S rDNA sequencing was used to identify isolates that were chosen based on their high production of GABA. Weisburg *et al*.'s recommended conditions and universal primers were used for the PCR amplification.[12] The dideoxy chain termination method was used to evaluate the DNA sequences of the isolated bacteria.[13] The National Centre for Biotechnology Information (NCBI), GenBank, BLAST search engine was used to examine the similarity of DNA sequences.[14]

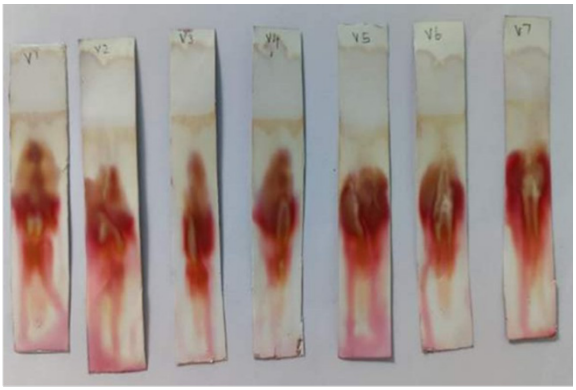
RESULTS

Preparation of Panchagavya and Isolation of probiotic strains

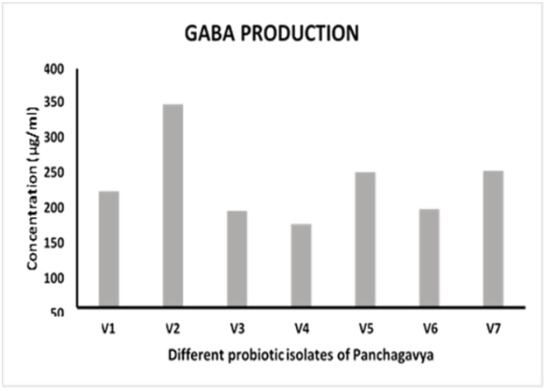
Panchagavya was prepared for the current investigation and colonies exhibiting specific traits were separated. The probiotic isolates appeared as shiny, spherical colonies with a creamy to off-white color on MRS plates (Figure 1).

Quantification and screening of GABA synthesis

After the bacteria were sprayed with 0.8% ninhydrin reagent, red-colored spots on a TLC plate were used to qualitatively assess the GABA synthesis of the isolated bacteria. Out of all the isolates that were evaluated, V2 and V7 had the maximum GABA production. The GABA production by the isolate V2 and V7 recorded was 340.13 and 228.73 µg/mL. The isolate V4 was observed with the least production of GABA (139.55 µg/mL) (Figure 2a).



Qualitative Analysis of GABA



Quantitative Analysis of GABA production

Figure 2a: Qualitative and quantitative Analysis of GABA production by the different probiotic isolate of Panchagavya (at column width).

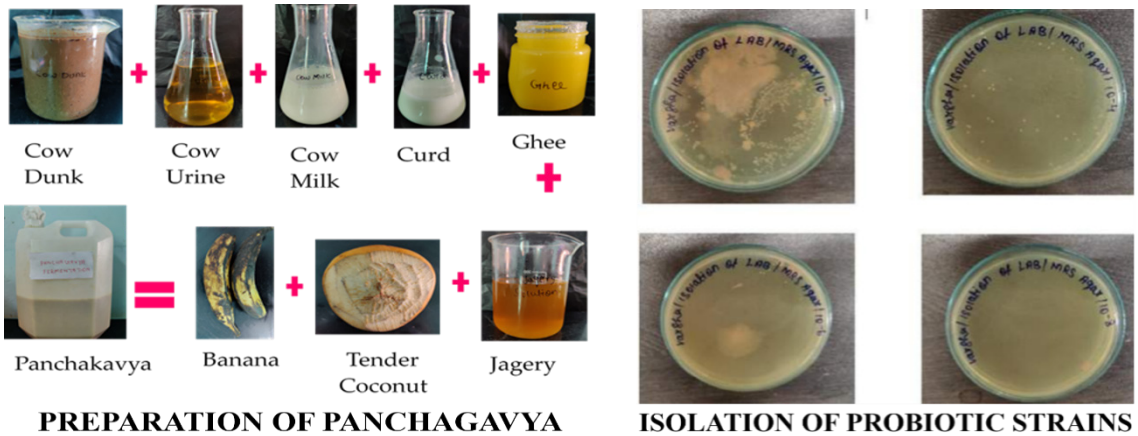


Figure 1: Preparation of Panchagavya and isolation of probiotic strains (at column width).

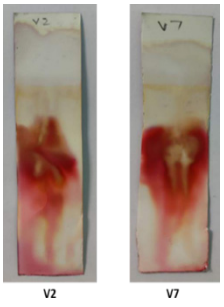


Figure 2b: Selected probiotic isolate (V2 and V7) (at column width).

Characterization of GABA producing probiotic isolates

pH tolerance

The GABA-producing probiotic isolates' acidic tolerance was ascertained by cultivating the isolates at several pH ranges, including 1, 3, 5, 7 and 9. The growth obtained for the probiotic isolate V2 was OD=0.0082 at pH "1" whereas no growth was obtained probiotic isolate V7. The cell density was found to be increased at the pH range 3, 5 and 7 for both probiotic isolates. The optical density was observed to be decreased at the pH 7 (Figure 3a).

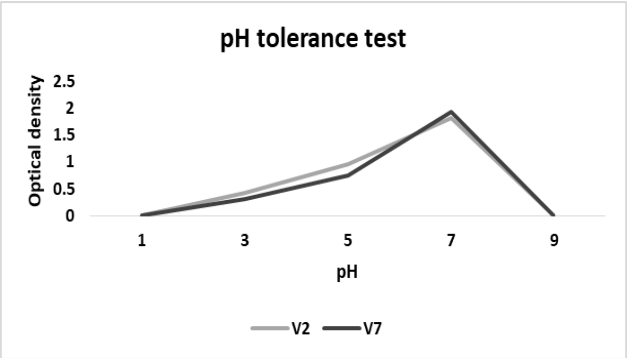


Figure 3a: pH tolerance test (at column width).

NaCl Tolerance

The tolerance of GABA producing probiotic isolates (V2 and V7) against NaCl was determined by inoculating them in various concentrations of NaCl such as (2%, 4%, 6%, 8% and 10%) in the growth media (Figure 3b). Both the probiotic isolates maintained their significant growth up to 2 and 4% concentration of NaCl and the cell density began to reduce at the concentration of 6% NaCl.

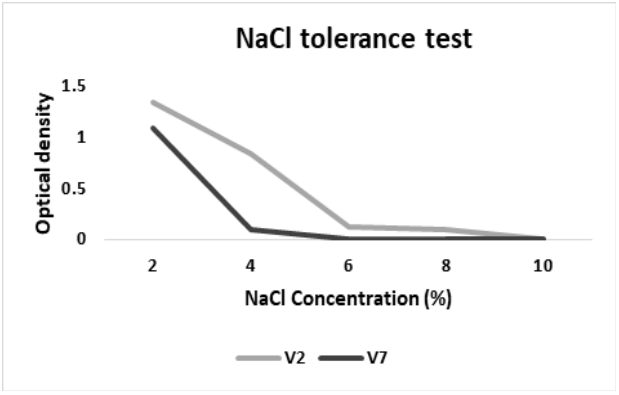


Figure 3b: NaCl tolerance test (at column width).

Bile Salt Tolerance

The bile salt tolerance assay found that the GABA producing probiotic isolates (V2 and V7) exhibited significant growth at the 0.05% of bile salt (Ox-gall) concentrations (OD=1.6382 for V2 and OD=1.2769 for V7), whereas the 0.15% of bile salt concentration showed a reduced level of cell density for both probiotic isolates (Figure 3c).

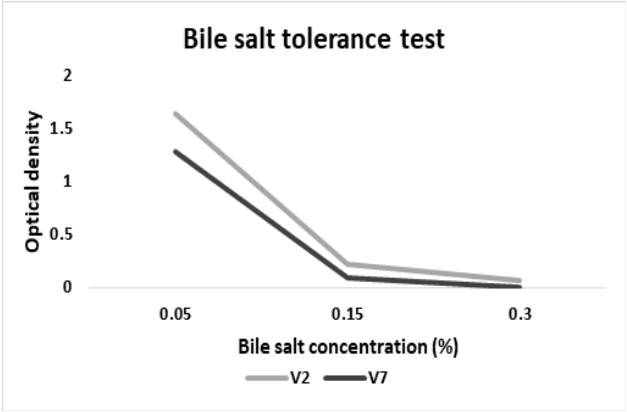


Figure 3c: Bile salt tolerance test (at column width).

Phenol tolerance

The growth of probiotic isolate was found to be OD=0.1534 and 0.0706 for GABA producing probiotic isolate V2 and V7 at the concentration of 0.1% of phenol and there was no growth at 0.4% of phenol concentration. The phenol tolerance assay demonstrated that the growth of GABA producing probiotic isolates V2 and V7 were reduced upon the increasing concentration of phenol (Figure 3d).

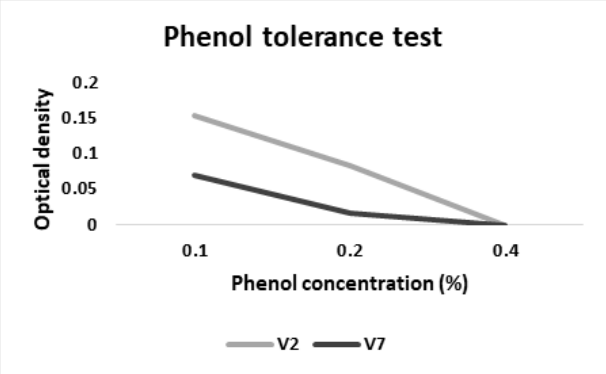


Figure 3d: Phenol tolerance test (at column width).

Enhancement of GABA-producing probiotic isolates

The medium's chemical and physical properties are changed to optimize the process. The parameters include temperature, incubation time, carbon source and glutamic acid concentration. The Box-Behnken method was carried out for optimization. The results of the optimization studies showed that isolate V2 produced a substantial quantity of GABA after the incubation of 48 hr in the pH of 4.5 at 35°C, 500 mM of glutamic acid concentration and utilized lactose as the greatest carbon source (Figure 4a and 5a). Similarly, isolate V7 produces ma GABA after the incubation of 48 hr in a pH of 5.5 at 25°C, 500 mM of Glutamic acid and utilized lactose as the greatest carbon source (Figure 4b and 5b).

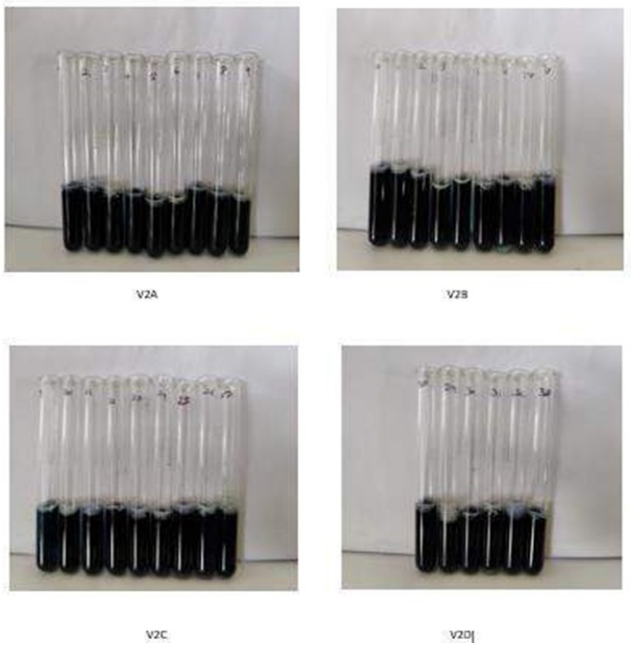


Figure 4a: Optimization of GABA production of the probiotic isolate V2 using Box-Behnken method (at column width).

Figure 4b: Optimization of GABA production of the probiotic isolate V7 using Box-Behnken method (at column width).

Identification of strain by sequencing

Isolate V2 and V7 were sequenced using 16S rRNA sequencing and from BLAST analysis, the isolates V2 and V7 were identified as *Enterococcus faecium* and *Alcaligenes* sp. correspondingly.

DISCUSSION

Panchagavya is one of the ancient forms of traditional Indian medicine. People were highly regarded in ancient India for using conventional and natural methods to heal a variety of illnesses. They are enjoying a healthy lifestyle by utilizing conventional or natural medicine. [1] There have been reports of the existence of occurring naturally, helpful microorganisms in these biological preparations, primarily bacteria, yeast, actinomycetes and some fungus. There are few research studies on the determining the advantageous properties of the microbial populations in the Panchagavya.[4] The variety of microorganisms, such as methylotrophs, actinomycetes, lactic acid bacteria, fungi and phosphobacteria. The presence of the bacteria Azotobacter, Pseudomonas and Azospirillum in Panchagavya derived from cow products is well documented.[15] The current study is to optimize and quantify the manufacture of GABA from the isolates collected from the Panchagavya.

Alpha-aminobutyric acid, or GABA, is the primary inhibitory neurotransmitter in the central nervous system and is created when glutamate is decarboxylated. In addition to controlling cardiovascular processes like blood pressure and heart rate, it also has several physiological effects like neurotransmission, diuretic and tranquilizer effects and aids in the easing of anxiety

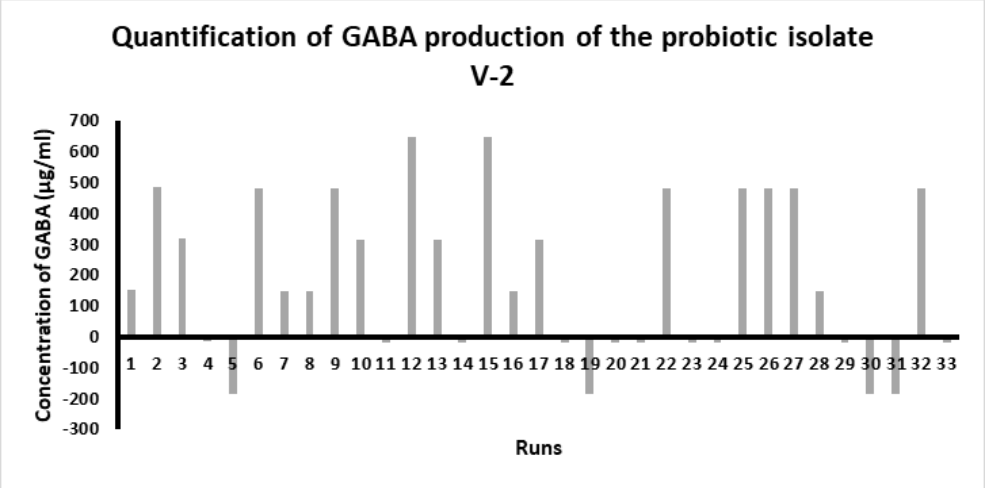


Figure 5a: Quantification of GABA production of the probiotic isolate V2 (at column width).

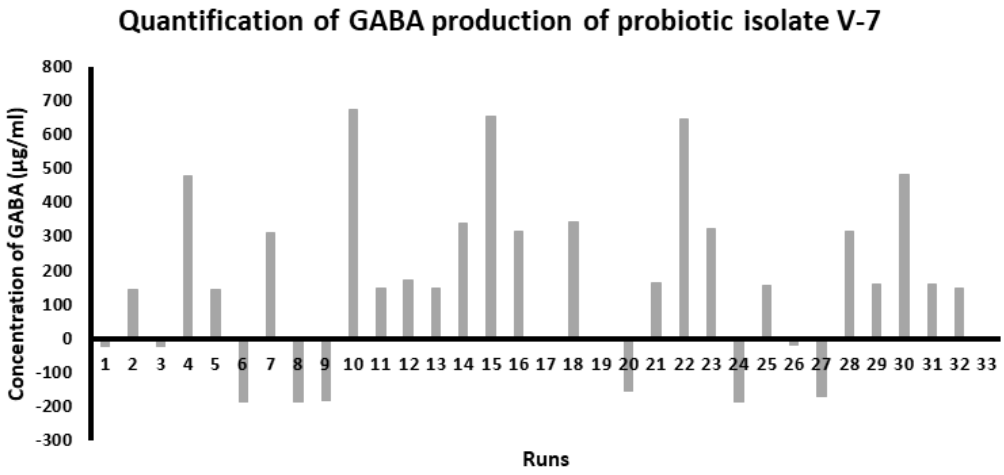


Figure 5b: Quantification of GABA production of probiotic isolate V7 (at column width).

and pain.[16] The release of GABA through the Krebs cycle's decarboxylation process, which uses glutamate as a substrate. GABA is the end product of the GABA shunt route, which converts malic acid from the Krebs cycle into succinic acid. By succinate semi-aldehyde dehydrogenase, succinic acid is transformed into succinic semi-aldehyde (Gad D). GABA aminotransferase (Gad T) ultimately converts succinic semi-aldehyde to GABA.[17]

It was demonstrated that the reagent ninhydrin, when used in conjunction with a colorimeter, is a viable and reasonably priced approach for estimating GABA. That is an exact and uncomplicated procedure. The fact that GABA exists as zwitterions with a protonated amino group and disintegrated carboxylic acid group. Ruhemann's purple is produced when the ninhydrin reagent and the GABA amino group combine to form a purple-colored molecule.[18] The quantity of GABA that Lactobacillus brevis NCL912, a fermented Chinese meal consisting of various vegetables, produced (103.5 g/L).[17]

The ability of the probiotic strain to withstand phenol, NaCl, bile salt and pH is essential for its characterization. One of the most common *in vitro* tests recommended for assessing the bacterial strain's probiotic potential is its ability to withstand chemical and physical barriers, such as adherence to human epithelial cell lines or mucus, bile toxicity and acidity in the intestinal tract.[19] In this study, we have analysed the tolerance of the GABA-producing prbiotic strain V2 and V7 against pH, bile salt, phenol and NaCl.

The Box-Behnken Design (BBD) is a well-known Response surface methodology for determining optimal production parameters with optimal

values for several variables.[11] BBD was utilized to estimate the effect of treatment variables on GABA production with Hericium erinaceus as the substrate for Fermented plant beverages.[20] The work done by Wu *et al*.[21] further enhanced *Monascus purpureus* M162's hongqu starter using BBD to produce a large amount of GABA.

Factors like the fermentation period, starting pH, glutamate content and medium composition affect the manufacture of GABA by microbes. The addition of different carbon sources had a major impact on cell proliferation and GABA production, both of which showed notable differences.[22] The study conducted by Lim *et al*. [23] demonstrated that boosting GABA production requires an optimal glutamate content, which was achieved by cultivating *Enterococcus faecium* JK29 in the optimized MRS medium at a temperature of 30ºC. Similar to this, by figuring out the MSG concentration, aeration and feeding techniques, the fed-batch fermentation parameters for *Enterococcus avium* G-15 were enhanced.

This led to 1,120 mM GABA synthesis with 25% MSG addition.[24] The species like *Enterococcus avium* and *Enterococcus faecium* were reported with GABA production.[25,26] *Enterococcus strains* that generate GABA have been shown to have excellent environmental adaptation, including tolerance to acid and high bile salts concentration, suggesting that they are a good choice for industrial purpose.[27] Because *Enterococcus faecium* has a limited ability to synthesize GABA, its culture parameters should be improved to maximize the manufacture of GABA. Several parameters, including cultivation duration, temperature, pH, medium composition and inoculum concentrations, might affect the GABA content in fermented products.[28] In the study of Choi *et al*.[29] the maximum GABA synthesis of *Enterococcus casseliflavus* PL05 was recorded at 7% Monosodium glutamate concentration, which showed the influence of Monosodium glutamate on GABA synthesis.

The majority of MocR-TFs, the majority of which are engaged in the metabolic activities of substances nitrogenous compounds, for instance, GabR controls the transcription of the GABA aminotransferase (GabT) gene. Likewise, the transcription-related mediator of the dnfABC genes identified in *Alcaligenes* faecalis JQ135 was discovered to be the MocR-TFs.[30] The results of Asaduzzaman *et al*.[31] demonstrated the alterations to gut microbiota brought by the *Alcaligenes* sp. similar to other probiotics. This evidence has supported our findings. Therefore, the pharmaceutical and functional food sectors have significant potential for GABA as a bioactive component and *Enterococcus faecium* as well as *Alcaligenes* sp. can be exploited as probiotics.

CONCLUSION

The present study was conducted to optimize and quantify the manufacture of GABA from the isolates obtained from the Panchagavya. Panchagavya was prepared and the isolated probiotic strains screened for GABA production using TLC and UV-vis spectrophotometer. The physical and chemical parameters were optimized for the maximum production of GABA by the selected isolates (V2 and V7) using the Box-Behnken method. The selected strains V2 and V7 were identified using 16S rRNA sequencing.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ABBREVIATIONS

GABA: Gamma-aminobutyric acid; **LAB:** Lactic acid bacteria; **BBM:** Box-Behnken method; **GRAS:** Generally recognized as safe; **MSG:** Monosodium glutamate; **MRS:** deMAN, Rogosa and Sharpe; **PCR:** Polymerase chain reaction; **NCBI:** National Center for Biotechnology Information.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design Varsha and Thamarai selvi designed the study. Varsha performed the experiment and wrote the manuscript. Thamarai selvi was responsible for supervision and critical revision of the article. Karukuvelraja helped in experimental work. All authors read and approved the final manuscript.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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