Greener production of Gamma-aminobutyric acid (GABA) by potential probiotic strain utilizing agro residues

M.Sc. Thesis

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
of
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by
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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **GREENER PRODUCTION OF GAMMA-AMINOBUTYRIC ACID (GABA) BY POTENTIAL PROBIOTIC STRAIN UTILIZING AGRO RESIDUES** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the period from August 2023 to May 2023 under the supervision of Prof. Kiran Bala, Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Bhadavi 22.05.24

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This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Kiran 22/5/24

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Abstract

γ-Aminobutyric acid (GABA) plays a key role as an inhibitory neurotransmitter for the central nervous system in mammals. GABA is produced naturally in the human body but is also supplemented by food items such as rice and several traditional fermented foods. GABA is a bulk chemical that can be used in food, nutraceuticals, pharmaceuticals, and biomedicine, thus required for improving human health and improving their well-being. Evidence suggests that microorganisms such as bacteria, fungi, and cyanobacteria can efficiently help in the sustainable production of GABA, utilizing agro residues and food and dairy waste as substrate. The application of microbes and agro residues helps minimize the overall cost and carbon footprints of otherwise chemical-intensive industrial processes. Thus, the present study focused on screening and selection of suitable probiotic Lactobacillus strains (*Lactobacillus plantarum* MTCC 1325, *Lactobacillus fermentum* MTCC 903, *Lactobacillus rhamnosus* MTCC 1408, *Lactobacillus delbrueckii* subsp. lactis MTCC 911, and *Lactobacillus plantarum* MTCC 2621) for GABA production using MRS HiVeg™ Broth in the absence and presence of mono-sodium glutamate (MSG). Based on qualitative assay i.e., thin layer chromatography all five lactobacillus strains showed GABA production potential. Further, the growth curve and GABA concentration were simultaneously plotted against time 0-168 h at regular intervals of 12 h for all five strains in MRS HiVeg™ Broth. The study clearly showed that all the Lactobacillus strains showed GABA production in the stationary phase for all the lactobacillus strains. The addition of MSG led to enhancement in GABA concentration for MTCC 911, i.e., 10.4 ± 0.6 mg/mL (132h) followed by 9.71± 0.4 mg/mL (120h) for MTCC 1408. The MTCC 2621 and MTCC 1325 resulted in comparable GABA

concentrations of 6.5 ± 0.30 mg/mL (60h) and 5.93 ± 0.28 mg/mL (132h). MTCC 911 was selected as a potential strain for further studies based on its maximum GABA concentration in HiVeg^{TM} MRS broth with 1% MSG. Further, several agro-residues (soy milk, soy seed powder, soya stalk powder, orange pulp powder, and molasses) are screened as a carbon source in a modified MRS medium with MSG for GABA production by MTCC 911. The maximum GABA concentration for MTCC 911 was observed with modified MRS broth with 2% molasses. Furthermore, a statistical optimization was performed using Box–Behnken design (BBD) based Response Surface Methodology (RSM) for developing optimal culture medium and culture conditions for enhanced GABA production. The optimization study resulted in a 1.37-fold increment in the GABA production potential. Thus, the BBD-RSM model was found to be a suitable approach for the optimization of enhanced GABA production. In conclusion, this study provides suitable probiotic strains and agro-residues and the optimum culture conditions for GABA production by MTCC 911 and indicates that *Lactobacillus delbrueckii* subsp. lactis MTCC 911 is a potentially promising source of GABA that can be used for health benefits such as treatment of neurological disorders etc. This approach can help in the attainment of the UN's sustainable development goals of sustainable production and consumption patterns (SDG12) and good health and well-being (SDG3).

LIST OF PUBLICATIONS

1. Madavi, D.B., Kumar., B., Sharma, S., Pareek, P., Bala, K*. (2024) BBD-RSM based optimization of enhanced Gamma-aminobutyric acid (GABA) production by *Lactobacillus delbrueckii* subsp. lactis MTCC 911 utilizing molasses. Submitted to *Biomass Conversion and Biorefinery*. Under Review

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ACRONYMS

GABA Gamma-aminobutyric acid

MRS De Man–Rogosa–Sharpe

TYJYE Tomato juice yeast extract

MSG Monosodium glutamate

PDA Potato Dextrose Agar

PDB Potato Dextrose Broth

TLC Thin layer chromatography

YPD Yeast extract peptone dextrose

GYP Glucose yeast peptone

HPLC High-Performance Liquid Chromatography

OPA o-phthaldehyde

PLP pyridoxal-phosphate

CNS Central Nervous System

GAD Glutamic acid decarboxylase

TCA Tricarboxylic acid cycle

AEC Airway epithelial cells

ASM Airway smooth muscle

MTCC Microbial Type Culture Collection and Gene Bank

IMTECH Institute of Microbial Technology

DEEMM diethylethoxymethylenemalonate

BBD Box -Behnken design

RSM Response Surface Methodology

Chapter 1

Introduction

Gamma-aminobutyric acid (GABA), i.e., 4-Aminobutanoic acid (Fig.1.1) is crucial as a neurotransmitter in the central nervous system of animals. Additionally, it acts as a stress modulator and participates in a defense mechanism. Apart from its prominent presence in animals and plants, GABA has also been identified in cyanobacteria (Shiels et al., 2019), fungi (Ab Kadir et al., 2016), and bacteria (Dhakal et al., 2012). Several studies have taken place to understand the production and degradation mechanism of GABA in microorganisms (Gutiérrez et al., 2020), which is elaborated in Fig. 2.1.

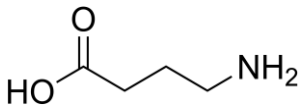

Fig. 1.1. Chemical structure of 4-Aminobutanoic acid (GABA)

In the early stages of research, GABA was found in potato tuber only (Rashmi et al., 2018a), to be believed as a metabolite, and was not discovered to be a neurotransmitter involved in cell signaling. However, later studies in nematodes revealed GABA as a principal neurotransmitter found in eukaryotic systems (Rashmi et al., 2018a). Further studies have revealed that GABA aids in the improvement of sleep in patients suffering from insomnia, stress, and other serious health issues, resulting in the direct psychological effect related to sleep (Yamatsu et al., 2016). GABA, a neurotransmitter, is also reported to possess an anti-cancer property, inhibiting the growth and proliferation of human colon cancer cells (Song et al., 2016). The oral consumption as a dietary supplement and its current production strategies involving the enzymatic conversion into GABA from precursor glutamate by GAD is an economically intensive process. help in the sustainable GABA production utilizing waste biomass (such as agro-residues, and food & dairy waste) as substrate.

Evidence suggests that bacteria, fungi, and cyanobacteria can efficiently Greener production strategies such as microbial-based production using probiotic microbes is one such approach. Synergistic application of microbes with waste biomass utilization for GABA production can serve dual purposes i.e., act as a low-cost substrate as compared to the conventional commercial substrate and waste valorization minimizing the carbon footprints of chemical-intensive industrial processes. Thus, the present study will be focused on developing a novel strategy for the selection of probiotics for GABA production. Further, optimized production and a single-step purification strategy will also be developed for enhanced GABA recovery. This approach can help in the attainment of the UN's sustainable development goals of sustainable production and consumption patterns (SDG12).

Chapter 2

Literature Review

GABA is fundamental in upholding the stability of neuronal activity, operating at both cellular and systemic levels, and maintaining an appropriate equilibrium between excitatory and inhibitory neurotransmitters, which is essential for the regular functioning of neurons. Conditions such as depression and diminished sense of smell, which are observed in neurological diseases such as Parkinson's disease, have been linked to a deficiency of GABA (Gutiérrez et al., 2022). Therefore, GABA holds significant importance as a neurotransmitter within the central nervous system. This portrays how important GABA is that is involved in signaling. Hence, exogenous GABA consumption has shown a path to aid in the treatment of such neurological disorders.

2.1. Mechanism of action of GABA

GABA functions as the chief neurotransmitter in the central nervous system of mammals, facilitating inhibitory synaptic currents through its binding to receptors located on either pre- or postsynaptic membranes. Brain cells contain two types of receptors that interact with GABA: the $GABA_A$ receptor, an inotropic receptor, and the $GABA_B$ receptor, a metabotropic receptor that is coupled to a G protein. Calcium-related homeostatic mechanisms, lead to the formulation of the Ca^{2+} hypothesis of brain aging and ultimately, cellular demise (Lydiard, 2003). It plays a pivotal role in a broad spectrum of physiological functions both within and beyond the nervous system, upheld by complex interactions among GABA, cellular metabolic processes, and calcium-dependent neurotransmission. The Ca^{2+}/GABA mechanism balances the neuronal activity at both cellular and systemic levels in the central nervous system. A decrease in Ca^{2+} /GABA control sets off a series of cascading events, leading to compromised protective

barriers, especially the blood-brain barrier and the buildup of Lewy bodies and intracellular deposits of calcium. Establishing a connection between this vital synaptic transmission mechanism and metabolism, through a reciprocal Ca^{2+} /GABA inhibition, results in a delicate equilibrium prone to destabilization and self-destructive processes (Błaszczyk, 2016). The proposed etiology decrement in GABA is found to be a common element in all neurodegenerative disorders found in humans set off by unusual intracellular Ca^{2+} levels.

2.2. Source of GABA

GABA is produced both chemically and through the metabolism of microorganisms. While bacteria are typically associated with GABA production, other microorganisms such as cyanobacteria, fungi, plants, and animals also synthesize this neurotransmitter. Many species of bacteria have been found to produce GABA. Bacterial strains such as *Lactobacillus plantarum* L16 were isolated from food products like kimchi (Gutiérrez et al., 2022). *Lactobacillus lactis* was isolated from fermented fish (Vo and Park, 2019). *Lactobacillus brevis* PML1 isolated from Tarkineh (Falah et al., 2021). *Lactiplantibacillus plantarum* FBT215 was isolated from Korean traditional food (Kim et al., 2022). *Limosilactobacillus reuteri* isolated from human breast milk (Tyagi et al., 2023). Several *lactococcus* bacterial strains from multiple different sources were isolated for the analysis of GABA production such as Iranian traditional dairy products isolated strains *Lactococcus lactis* 491 and *Lactococcus lactis* 491 (Edalatian Dovom et al., 2023). Similarly, *Lactococcus lactis* Subsp. *Lactis LL16 and Lactococcus lactis* strains L-571 and L-572 were isolated from bovine milk (Mileriene et al., 2023), and artisanal Mexican cheese (Santos-Espinosa et al., 2020), respectively. *Lactococcus lactis* isolated from pickles (Bhanwar et al., 2013), *Lactococcus lactis* ssp. *lactis* or *L. lactis* ssp. *lactis* biovar *diacetylactis* (strains such as ULAAC-A13 and ULAAC-A23) were isolated from old-style cheese (Lacroix et al., 2013) and showed GABA-producing ability.

Table 2.1: Source of GABA from different micro-organisms

Source	Specific organism	Source of Isolation	Medium	GABA Concentration	Reference
Bacteria	*L. Lactis,* *S. thermophilus*	Traditional dairy products	MRS, M17, Elliker medium, bacteriological agar	1.01-2.81 mM	(Valenzuela et al., 2019)
	Lactobacillus otakiensis, *Lactobacillus* sp.	Pico cheese	MRS broth, MSG	936.8mg/L	(Ribeiro et al., 2018)
	L. plantarum EJ2014	Rice bran	MRS medium, yeast extract, glucose, and MSG	19.8g/L	(Park et al., 2021)
	L. plantarum subsp. Plantalum	Thai fermented food	MRS medium	22.94g/L	(Phuengjayaem et al., 2021)
	L. pentosus			11.59g/L	

	Lb. plantarum L10-11	Thai fermented fish (plaa-som)	MRS medium	15.74 g/L	(Tanamool et al., 2019)
	Lactobacillus hilgardii MYA-9	Button mushroom	MRS medium, MSG	53.65 mM	(Lee et al., 2013)
Fungus	*Aspergillus oryzae* NSK, *Aspergillus oryzae* NSZ, *Aspergillus oryzae* NSJ, *Aspergillus oryzae* NST	Soy sauce koji	PDA	194 mg/L, 63 mg/L, 51.53 mg/L, 31.66 mg/L	(Ab Kadir et al., 2016)
	Monascus Purpureus	Angkak (Indonesia**)**	PDA, PDB	0.0796 mg/mL	(Kusdiyantini and Ferniah, 2021)
Cyanobacteria	*Aphanothece halophytica*	Dead Sea (Israel)	BG11 liquid medium	8.03 ± 0.25 nmol.g^{-1} DW	(Boonburapong et al., 2016)

2.3. Biosynthesis pathway of GABA

There are two major metabolic pathways for the synthesis of GABA that are Putrescine pathway and the Glutamic acid decarboxylase pathway (Rashmi et al., 2018a).Glutamate decarboxylase plays the chief role in the biosynthesis of GABA (Cui et al., 2020). The GAD (glutamic acid decarboxylase) system in micro-organisms especially in the *Lactobacillus* genus involves the GAD enzyme and glutamate/GABA antiporter where gadA/gadB encodes the GAD enzyme. In the first step, the L-glutamate is pumped inside the cell. The cofactor pyridoxal-phosphate (PLP) catalyzes the decarboxylation of precursor L-glutamate into the final product GABA along with the release of carbon dioxide molecules. The antiporter GadC acts where it exports the synthesized GABA in the matrix outside the cell (Gutiérrez et al., 2020) .

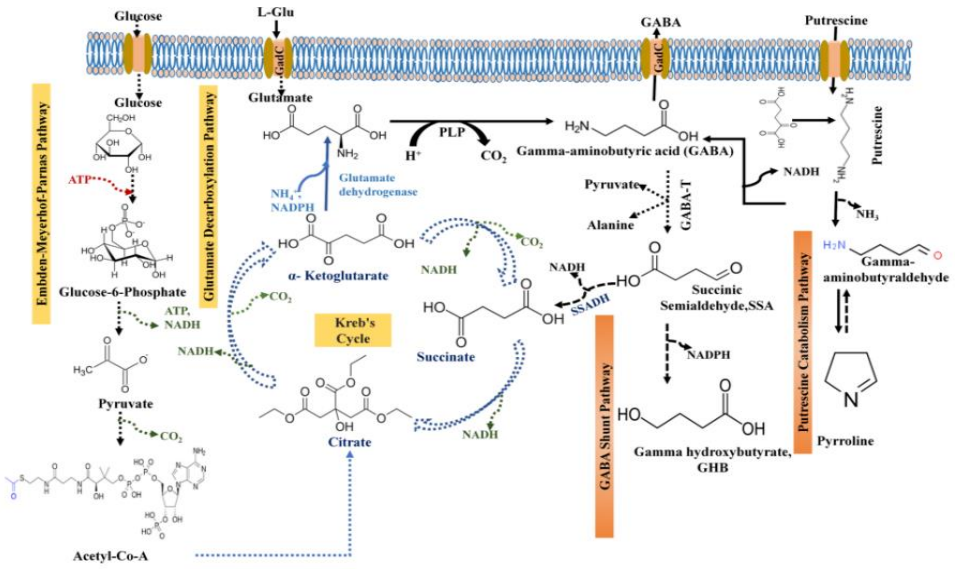

Fig. 2. 1. GABA biosynthesis and degradation pathway (Gutiérrez et al., 2020)

In some bacteria, like when the gene responsible for glutamate decarboxylase and glutamate/GABA antiporter was overly expressed in *Escherichia coli*, it resulted in the increment of production of GABA. This resulted due to the overexpression of glutamate decarboxylase and antiporter along with the expression of GABA aminotransferase suppressed to restrict the GABA from entering the TCA cycle (Le Vo et al., 2012).

In the putrescine pathway of microorganisms, an antiporter pumps the putrescine into the cell where it gets converted to GABA in two ways. In one of the ways, putrescine aminotransferase catalyzes the reaction of conversion from putrescine to gamma amino butyraldehyde which finally gets converted to GABA. In the other way, putrescine is converted to gamma-glutamyl-putrescine catalyzed by gamma-glutamate-putrescine-synthetase and after successive oxidation, GABA is synthesized. This GABA, through further conversions, forms into succinate entering the Krebs cycle. Gamma amino butyraldehyde being unstable may also get converted to proline (Gutiérrez et al., 2020) and also in the abiotic stresses the GABA is synthesized from proline (Rashmi et al., 2018b).

Apart from this, GABA can also be synthesized in a cell through the Embden-Meyerhof-Parnas pathway. In this pathway, when the glucose molecule is transported inside the cell, it undergoes multiple conversions from glucose-6-phosphate to pyruvate and finally gets converted to acetyl-Co-A. This acetyl-Co-A then enters Kreb's cycle in the form of succinate, and through the Glutamate decarboxylase pathway, the final product as GABA is obtained.

2.4. Qualitative and quantitative methods used to analyze GABA

2.4.1. Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is employed to isolate a particular component from a mixture of solutions.*Latilactobacillus curvatus* K285 bacteria isolated from Gat-Kimchi were screened for GABA production on silica gel plates where the mobile phase was used as *n*-butanol/acetic acid/water and 2% ninhydrin for colour development (Lee et al., 2022). Screening of GABA was done using thin-layer chromatography for yeast *Kluyveromyces marxianus* C21 isolated from soybean residue (Zhang et al., 2022). The GABA-producing Lactobacillus strain *L. brevis* RK03 isolated from Saltwater fish showed the highest GABA production among the thirty-two isolates of LAB from nine fishes when screened with TLC (Wu et al., 2018).

The *L. plantarum* of strains 45a, 45d, and 37e as *L. futsaii* 32d, *L. namurensis* strain 37b and 32c were isolated from Cambodian food, among which the study showed *L. plantarum* 45a with the highest production of 20.34 ± 1.41 mM GABA (Ly et al., 2019)**.** Table 2.2. Summarizes different studies using TLC for the screening of GABA from different probiotic bacteria.

2.4.2. UV-Vis spectrophotometer-based GABA estimation

The TLC method is based on the interaction of amino acid with the stationary phase and hence can involve any other unwanted amino acid interaction giving false positives or interfering with the spots of the sample. Hence a method such as UV–visible spectroscopy could eliminate such differences aiding the analysis of the sample by employing a specific wavelength for optimal detection of a particular ion. The *Lactobacillus plantarum* isolated from Thai fermented fish was analyzed in UV – Visible spectroscopy for the quantification of GABA using 0.2 M borate buffer, 6% phenol reagent, and 7.5% sodium hypochlorite at 630 nm was found to be 15.74 g/L (Tanamool et al., 2019). UV–visible spectroscopy was used to determine the GABA generated from *Lactobacillus plantarum* subsp. *plantarum* IBRC10817 under the effect of ultrasonic shock and heat produced 295.05 mg/L when measured at 630 nm (Rezaei et al., 2023).

Table 2.2: Summary of work using TLC, UV-Vis spectrophotometry, and HPLC for screening of GABA

Technique	Stationary phase	Mobile phase	Medium	Strain	Source of isolation	GABA concentration	Reference
Thin Layer Chromatography	Aluminium TLC plate	Acetic acid: n-butanol: distilled water	MRS	*Lb. plantarum* L10-11	Thai fermented food	15.74 g/L	(Tanamool et al., 2019)
	Silica gel plate	1-butanol: acetic acid: distilled water	MRS	*B. thuringiensis* LH2134, *L. lactis* LA43	Korean and Vietnamese fermented food	366 mM, 454mM	(Vo and Park, 2019)
	Silica gel plate	Butanol: acetic acid: distilled water	MRS	*Lactobacillus brevis* PML1	Tarkhineh	300 mg/L	(Falah et al., 2021)

	Silica gel 60 F254	n-butanol: acetic acid: water	MRS	*Lac. lactis* 311, *Lac. lactis* 491	Iranian traditional dairy product	0.35 mg/mL 0.179 mg/mL	(Edalatian Dovom et al., 2023)
	Aluminum Sheets Silica gel 60 F254	1-butanol, acetic acid, distilled water	MRS	*Lactobacillus plantarum*	Cambodian fermented foods	20.34 mM, 16.47 mM	(Ly et al., 2019)
High-Performance Liquid Chromatography	HPH-C18 column	Solvent A: Na_2HPO_4, $Na_2B_4O_7$, NaN_3. Solvent B: Acetonitrile, methanol, water	MRS	*Lactiplantibacillus plantarum* FBT215	Korean fermented food	1688.65 ±14.29 μg/mL	(Kim et al., 2022)

Inertsil ODS-3 C18 column	A: Methanol B: Sodium acetate, methanol, tetrahydrofuran	YPD and MRS	*Saccharomyces cerevisiae*, *Lactobacillus plantarum*	Morus alba L	2.42g/L	(Zhang et al., 2020)
Luna 5U C18 column	A: Sodium acetate, tetrahydrofuran, methanol B: Methanol	MRS and GYP	*L. plantarum* LS12 - 1	Thai fermented foods	22.94 g/L	(Phuengjayaem et al., 2021)
Inertsil ODS-3 C18 column	A: methanol B: Sodium acetate, methanol, tetrahydrofuran	MRS	*Lactobacillus plantarum* BC114	Chinese Paocai	3.45g/L	(Zhang et al., 2017)

UV-Visible Spectroph-otometery		75% ethanol, 0.6% copper sulphate	MRS	*Lactobacillus brevis* PML1	Tarkhineh	300 mg/L	(Falah et al., 2021)
		Borate buffer	MRS	*Lactobacillus fermentum*	Sourdough	5.54 g/L	(Rayavarapu et al., 2021)

2.4.3. High-performance liquid chromatography for GABA estimation

GABA is weakly absorptive in the UV and visible region and hence requires a modification so that significant analysis, such as the estimation and recovery, can be done. A derivatizing agent such as OPA (O-phthalaldehyde) was used as a derivatizing agent to chemically modify the Vietnamese fermented food for the analysis of GABA by spectrophotometric methods. HPLC is equipped with DAD and fluorescence detector using sodium acetate as solvent 1 and sodium acetate, acetonitrile, and MeOH as solvent 2 (Le et al., 2020).

A GABA concentration of 16.29 ± 0.53 mg/kg DW was recorded from fish samples (Le et al., 2020). The quantification of GABA from Thai fermented food isolated lactic acid bacteria was done by HPLC C18 analytical column combined with FLD model 363 using OPA as a derivatizing agent. The highest GABA concentration of 13.42±0.28 g/l from *L. brevis* GPB7-4 strain isolated from naw-mai-dong (fermented bamboo shoots) (Pakdeeto et al., 2022). ODS-3 column along with UV detector and solvent A as sodium acetate, trimethylamine, acetonitrile, and solvent B as acetonitrile was utilized to quantitatively assess the concentration of GABA in *Levilactobacillus brevis* F064A, which was isolated from Thain fermented sausage which was found to be 2.85 ± 0.10 mg/mL (Kanklai et al., 2020). HPLC equipped with a PDA detector and mobile phase as methanol and TFA were used as mobile solvents where *Weissella confuse* isolated from fermented foods showed production of 246.2 mg/L of GABA (Devi et al., 2023)**.**

2.5. Application of GABA: Nutritional and Biomedical Applications

GABA is an inhibitory neurotransmitter that must be present in the counterbalance with the excitatory neurotransmitter. There are a lot of disorders and diseases such as anxiety, sleep insomnia, Alzheimer's disease, epilepsy, Parkinson's disease, etc that are related to the activity of GABA (Rashmi et al., 2018b).

When anxiety kicks in there can be episodes of panic attacks which can lead to a lasting effect on cerebral function. With the application of GABA, the signals leading to anxiety are suppressed and hence could be used as a drug to treat anxiety disorder (Lydiard, 2003). Diabetes arises when there is an imbalance between the loss and growth of pancreatic β-cells, resulting in a decline in β-cell mass. Existing research has highlighted the significance of GABA in preserving a balance between islet-cell hormone and preserving β-cell mass. GABA's significance is underscored by its paracrine impact on α cells, leading to the inhibition of glucagon secretion, and its autocrine influence on β cells, which results in the augmentation of insulin release aiding the treatment of diabetes (Wang et al., 2019). Considering that gamma-aminobutyric acid receptors ($GABA_A$ Rs) are expressed in airway epithelial cells (AEC), airway smooth muscle (ASM), T lymphocytes, and macrophages, it is reasonable to suggest that GABAergic drugs hold promising potential treatment for asthma. This proposition arises from the observation that the primary symptoms and signs of asthma, nasal airway hyper-responsiveness, airway inflammation, phenotypic remodeling of AEC and ASM, and the infiltration of T lymphocytes and macrophages into the lungs, may not be adequately addressed by regular use of high-dose inhaled corticosteroids (Lu, 2011). GABA intake is also taken in the form of dietary supplements. A newly fermented milk containing GABA has demonstrated efficacy in reducing blood pressure (BP) in individuals with mild hypertension (Inoue et al., 2003).

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Chapter 3

Hypothesis and Objectives

3.1 Hypothesis

The World Health Organization (WHO), through their report "*Neurological Disorders: Public Health Challenges,*" revealed that up to one billion people globally are affected by a spectrum of neurological disorders, and among this population, Alzheimer's disease and other forms of dementia affect 24 million individuals, whereas epilepsy affects 50 million people (Bertolote, 2007). Factors such as scarcity of GABA need to be maintained in a proper balance along with glutamate in neurological diseases such as "Parkinson's disease" (Rashmi et al., 2018a) . Studies have shown the significance of GABA as a potential drug in aiming for the treatment of such neurodegenerative disorders as well as sleep disorders (Rashmi et al., 2018a). Hence, there is an increasing demand for GABA as an oral consumption drug. The traditional methods, such as chemical synthesis, plant enrichment, and enzymatic methods, face multiple challenges like usage of corrosive reactants, complicated or toxic by-product formation, difficulty in the extraction of GABA from plants, and high cost for GAD production (Luo et al., 2021).

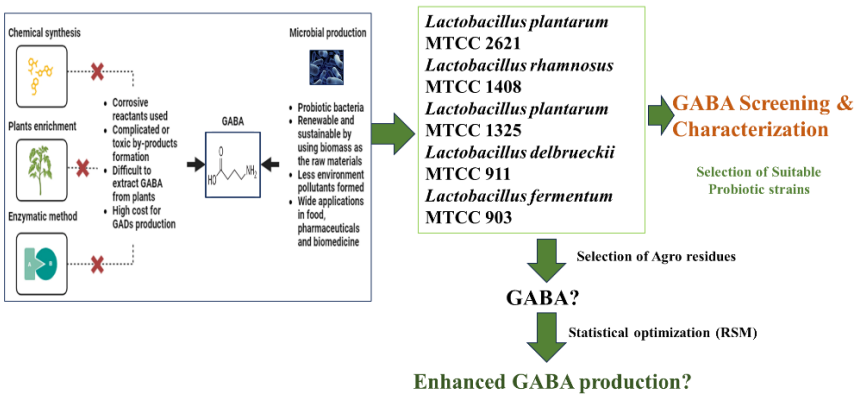


Fig. 3.1. Hypothesis of the work

There are probiotic bacteria present in the environment showing promising yield of GABA production (Dhakal et al., 2012). Probiotic bacteria claimed to provide health benefits have also been presented to be a good source of GABA. This serves us as a motivation by taking this a step further, we believe that exploiting the waste biomass (agro-residues and dairy wastes) as substrate along with the probiotic bacteria would further help in sustainable development in the production of GABA. (Fig. 3.1). Therefore, in the present study, the probiotic bacteria were screened through various quantification techniques for fast detection and estimation of GABA from microbial sources. Further, a sustainable approach for greener production of GABA using agro residues will be developed using statistical tools (BBD-RSM). Therefore, looking into this aspect following objectives were laid out.

3.2. Objectives of the study

Objective 1: Screening of suitable probiotic microorganism/s for the production of GABA

Objective 2: Characterization and quantification of GABA

Objective 3: Selection of waste agro-residues as a substrate for the production of GABA

Objective 4: Statistical optimization of GABA production strategy via media engineering

Chapter 4

Materials and Methodology

4.1. Revival and maintenance of culture

All five probiotic microbes i.e. *Lactobacillus fermentum* MTCC 903, *L. rhamnosus* MTCC 1408, *L. plantarum* MTCC 2621, *L. plantarum* MTCC 1325, and *L. delbrueckii subsp. Lactis* MTCC 911 is procured from the Institute of Microbial Technology (IMTECH), Microbial Type Culture Collection, and Gene Bank (MTCC). These freeze dried cultures were revived as per the protocol described by MTCC using MRS (De Man–Rogosa–Sharpe media (g/L) 10.0 HiVeg^{TM} peptone No.3, 10.0 HiVeg^{TM} extract, 5.0 yeast extract, 20.0 dextrose (glucose), 1.0 Tween 80, 2.0 ammonium citrate, 5.0 sodium citrate, 0.10 magnesium sulphate, 0.05 manganese sulphate, 2.0 dipotassium hydrogen phosphate) and TJYE (Tomato juice yeast extract media (g/L) 20.0 tomato juice, 10.0 peptone, 10.0 peptonized SM powder) using agar for the solid base (20 g/L) as well as in broth at 37ºC for 24 – 48 hrs. MRS and TJYE are procured from Himedia. All other chemicals used in the study were procured from Himedia, Merck, Sigma, and Thermofisher, India. After revival, all the strains were maintained in glycerol stock and stored at -80ºC for further use. The precultures prepared for all 5 bacterial strains were inoculated into MRS media with and without MSG supplementation. Inoculated with and without MSG media were incubated at 37 ºC, at 100RPM shaking for 24 – 48 hours.

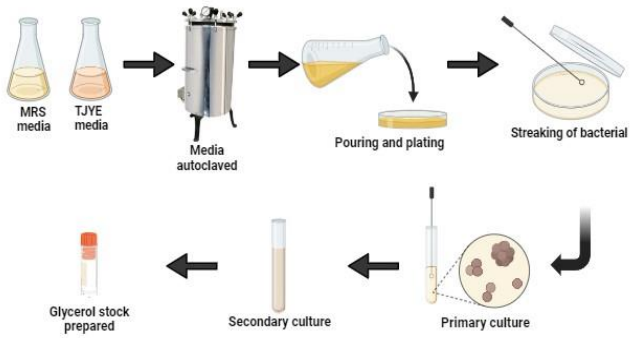


Fig. 4.1. Revival of culture

4.2 Detection of GABA using thin-layer chromatography

Thin-layer chromatography (TLC) aims to separate a particular component from a mixture of solutions and is used for the primary screening and optimization of GABA produced from bacterial strains. A 36-hour grown culture of all 5 strains in 10 ml MRS broth was centrifuged at 7000 RPM for 20 minutes, and the supernatant was collected for TLC analysis. Silica-aluminum sheets (TLC Silica gel 60 F_{254}, Merck, Germany) were used for TLC, where 1µl of supernatant was loaded on the plates. Duplicates for each bacterial supernatant obtained from MRS were performed on these plates. A solvent mixture consisting of *n*-butanol, Acetic acid, and distilled water in the ratio of 5:3:2, respectively, was used as a mobile phase. Pure commercially available GABA (Himedia) and also MSG (monosodium glutamate) purchased from the market were used as a standard in TLC. After running the TLC plates in the mobile phase for 40-45 min, 1% ninhydrin (dissolved in ethanol) was sprayed on the plate and dried at 70 ºC for 20 minutes. The observed spots on the TLC plates were compared with the standard for qualitative analysis purposes (Falah et al., 2024) (Fig. 4.2).

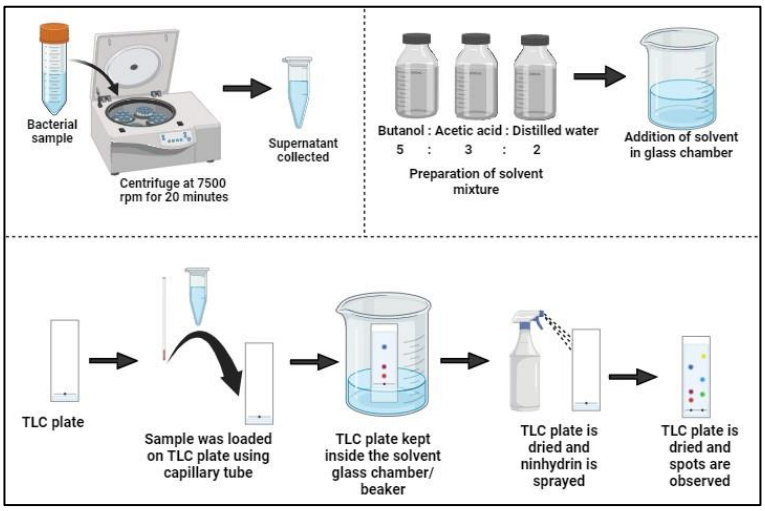


Fig. 4.2. Thin layer chromatography: A – Sample preparation; **B** – Preparation of solvent mixture; **C** – Loading and running of the sample on TLC plate

4.3. Quantitative detection using UV-Spectrophotometer

A 36-hour grown culture of all 5 strains in 10 ml MRS broth with and without MSG was centrifuged at 7000 RPM for 20 minutes, and for quantitative analysis, supernatant was collected. The GABA working standard ranges from 0 to 1000 µg/ml were prepared. The reaction was set, consisting of borate buffer(600µl) of pH 9.9, ninhydrin(100µl), and (200 µl) of working standard/ sample supernatant. Triplicates were run for each concentration. The MCTs containing the reaction were kept in a water bath at 70 ºC for 20 minutes. The tubes were cooled down, and the absorbance was measured in the UV–-spectrophotometer at 512 nm (Falah et al., 2021). A standard curve was prepared, and the same protocol was performed with the supernatant obtained from the five different bacterial strains. The concentration of GABA was calculated using the standard curve at 512 nm (Fig. 4.3).

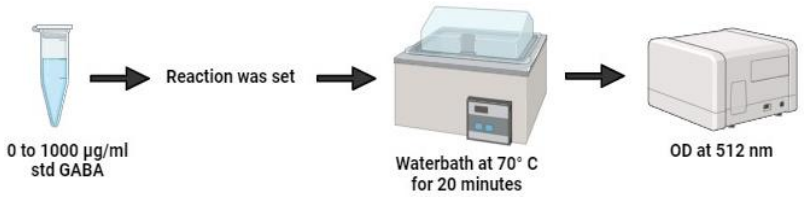


Fig. 4.3. Quantitative detection of GABA using UV-Vis spectrophotometry

4.4. Cell Growth and GABA concentration estimation for probiotic strains

Seed cultures for all five Lactobacillus strains were grown in 100 mL MRS medium at 100 rpm and 37°C for 48h. The culture was then transferred into autoclaved Hi-Veg MRS Medium setting up the initial cell concentration of 0.1 OD equivalent incubated at 100 rpm and 37°C. At every 12 h, samples are withdrawn aseptically for growth curve and GABA concentration analysis using UV-VIS spectrophotometry at 600nm and 512 nm, respectively. Both the data from the growth curve and GABA concentration were plotted against the time from 0-168th h.

transferred into autoclaved media consisting of Hi-Veg MRS Medium with 1% MSG added to 100mL MRS broth. setting up the initial cell concentration of 0.1 OD equivalent incubated at 100 rpm and 37°C. At every 12 h, samples are withdrawn aseptically for growth curve and GABA concentration analysis using UV-VIS spectrophotometry at 600nm and 512 nm (ninhydrin assay), respectively. Both the data from the growth curve and GABA concentration were plotted against the time from 0-168th h.

4.5. Quantitative detection using High-Performance Liquid Chromatography

High-Performance Liquid Chromatography with a C18 column and UV detector was used to compare the GABA concentration of all the Lactobacillus strains. All the strains were inoculated and grown with and without MSG-containing MRS media, where the cultures were subjected to centrifuge at 7830 rpm for 30 minutes, at 4ºC, and the supernatants underwent filtration using a 0.2 µm filter. For derivatization, 125µL of supernatant was mixed with 750 µL of 50 mM borate buffer (pH 9), 250 µL of methanol, 117.5 µL of mili – Q, 7.5 µL of diethylethoxymethylenemalonated (DEEMM). The samples were then run using acetate buffer and acetonitrile as mobile solvents (Fig. 4.4).

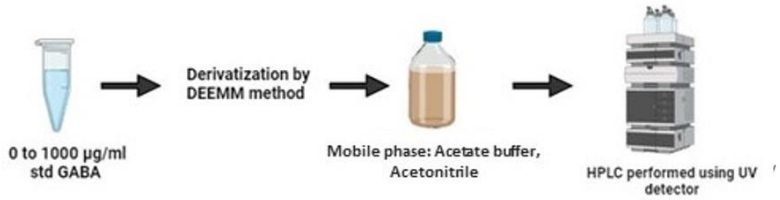


Fig. 4.4. Quantitative detection of GABA using High-Performance Liquid Chromatography

4.6. GABA production using agro-residues in *L. delbrueckii* subsp. Lactis MTCC 911

Agro residues, including molasses, orange pulp, soya stalk, and soya seed were procured from the Jaggery plant, juice shop, farm, and Kirana

shop, respectively at Simrol, Madhya Pradesh, India. Soy milk and soy seed powder were prepared after soaking 100 gm soya seed in 200 gm of Distilled water. After overnight soaking, the soya seed was ground in the mixer, the paste was pressed to filter out the soymilk and the remaining paste was dried and powdered before use.

For seed cultures, all five Lactobacillus strains were inoculated in autoclaved modified MRS medium (5.0 yeast extract, 20.0 dextrose (glucose), 1.0 Tween 80, 2.0 ammonium citrate, 0.10 magnesium sulphate, 0.05 manganese sulphate, 2.0 dipotassium hydrogen phosphate) at 100 rpm and 37°C for 48 h. The culture was then transferred into modified MRS media (5.0 yeast extract, 1.0 Tween 80, 2.0 ammonium citrate, 0.10 magnesium sulphate, 0.05 manganese sulphate, 2.0 dipotassium hydrogen phosphate) consisting of 0.2 % and 2 % respective agro residue (Fig. 4.5) (Table 4.1). At every 12 h, samples were withdrawn aseptically for growth curve and GABA concentration analysis using UV-VIS spectrophotometry at 512 nm (ninhydrin assay). The data for GABA concentration was plotted against the time from 0-132th h.

Table 4.1: Synthetic MRS and Modified MRS medium composition

Synthetic HiVeg MRS (Hi-media)	**Modified MRS Medium (With Agro residues)**	**1000ml**
HiVeg peptone no. 3	-	5g
HiVeg extract	-	20g
Yeast extract	Yeast Extract	3g
Dextrose (glucose)	Respective Agro residues	20g
Polysorbate 80 (tween 80)	Tween 80	1
Ammonium Citrate	Ammonium ferric Citrate	2
CH_3COONa	-	5
$MgSO_4$	$MgSO_4$	0.10
$MnSO_4$	$MnSO_4$	0.05
K_2HPO_4	K_2HPO_4	1.2g

Fig. 4.5. Agro residues used for the production of GABA

4.7. Experimental design for statistical optimization of GABA production using *L. delbrueckii* subsp. Lactis MTCC 911 in a modified MRS medium with molasses as substrate

In order to devise an optimal culture condition for enhanced GABA production a Box-Behnken design (BBD) based Response Surface Methodology (RSM) was adopted. In this study, Stat-Ease Corporation's Design Expert Software (Minneapolis, MN, USA) was used to establish a protocol for BBD-RSM experimental design. The BBD design was implemented to examine how three significant independent variables/factors interact with each other i.e., Temperature (A), MSG Concentration (B), and Substrate concentration (C) for improved GABA yield. A 17 experimental run-based experiment was generated when three coded levels (-1, 0, +1) BBD was employed. The preliminary experiments for the utilization of MSG, selection of agro residues, and review literature are used for the selection of minimum and maximum values for each factor i.e., MSG concentration, substrate concentration, and incubation temperature, respectively. Table 4.2 represents the actual experimental levels of each variable for the BBD-RSM experiments.

Table 4.2: Actual and coded experimental levels (with units) of each variable for the BBD-RSM experiments.

Factor	Name	Units	Min	Max	Coded Low	Coded High
A	Temperature	^oC	25.00	37.00	-1 ↔ 25.00	+1 ↔ 37.00
B	MSG concentration	%	0.5000	3.00	-1 ↔ 0.50	+1 ↔ 3.00
C	Substrate Concentration	%	0.2000	3.00	-1 ↔ 0.20	+1 ↔ 3.00

Moreover, Table 5.1 displays the comprehensive experimental design, showcasing the actual values of various variables alongside the corresponding values of the response i.e. dependent variable. The statistical software Design Expert (Stat-ease 360) was utilized to analyze the experimental data. The mean of triplicate values of response for each run were used for the expression of the result. The analysis of variance (ANOVA) was used for the statistical analysis of the results using the RSM tool. The response variable, i.e. GABA Concentration (Y), was fitted using a quadratic equation (Eq. 1)

$$Y= \beta_{0+} \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC \qquad \text{Eq. (1)}$$

where β_0 denotes intercept, β_1, β_2, and β_3 represent the linear coefficients, $\beta_1\beta_2$, $\beta_1\beta_3$, and $\beta_2\beta_3$ denotes the interaction coefficients. The values of A, B, C, D, AB, AC, and BC represent the levels of the independent variables. The quadratic equation was subjected to multiple regression analysis to fit the data. The interaction among the two independent factors while keeping the third parameter constant was evaluated mathematically by the quadratic equation (Eq. 1) and represented graphically by three-dimensional response surface contour plots. The statistical significance of the model was determined using ANOVA, and the adjusted coefficient of determination (R^2) and predicted R^2 value were used to assess the quality of the fit.

Chapter 5

Results and Discussions

5.1 Revival of Lactobacillus strain

All five Lactobacillus strains were successfully revived in both MRS and TJYE medium (broth and Agar plates). The revived broth culture was then used for the preparation of glycerol stock for further application. It was also observed that for almost all the strains, growth was slower in the TJYE as compared to MRS broth/plates (Fig. 5.1). Therefore, MRS media was used for further experimental studies.

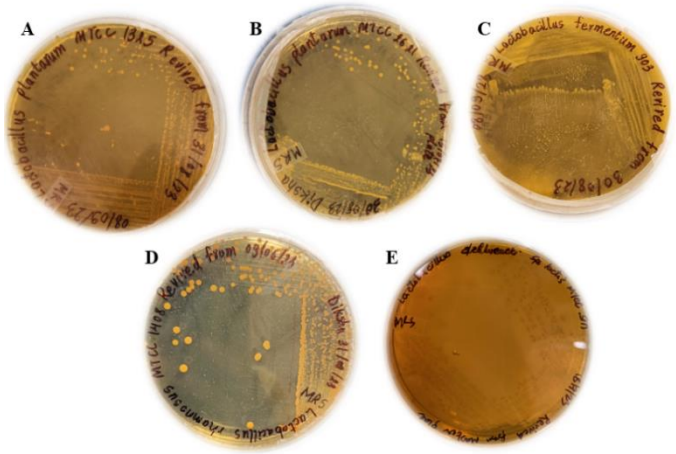


Fig. 5.1. LAB strains streaked on MRS agar. A: MTCC 1325; **B**: MTCC 2621; **C**: MTCC 903; **D**: MTCC 1408; **E**: MTCC 911

Further, the growth curve for each strain showing GABA potential at different incubation times must be prepared for a better understanding of bacterial growth and its correlation with GABA-producing ability.

5.2 Detection of GABA using thin-layer chromatography

Qualitative analysis of GABA-producing Lactobacillus strains was determined by using TLC. All five Lactobacillus strains showed GABA spots on TLC (Fig.5.2). The bands observed were in line with observations made by Falah et al., (2021)., and Rayavarapu et al.(2021).

The mobile phase consisting of butanol showed well-resolved colored (GABA) spots when analyzed for MTCC 1325 strain as compared to solvent involving methanol (Fig, 5.2 B) hence butanol solvent mixture was used for analyzing the remaining strains supernatant as well.

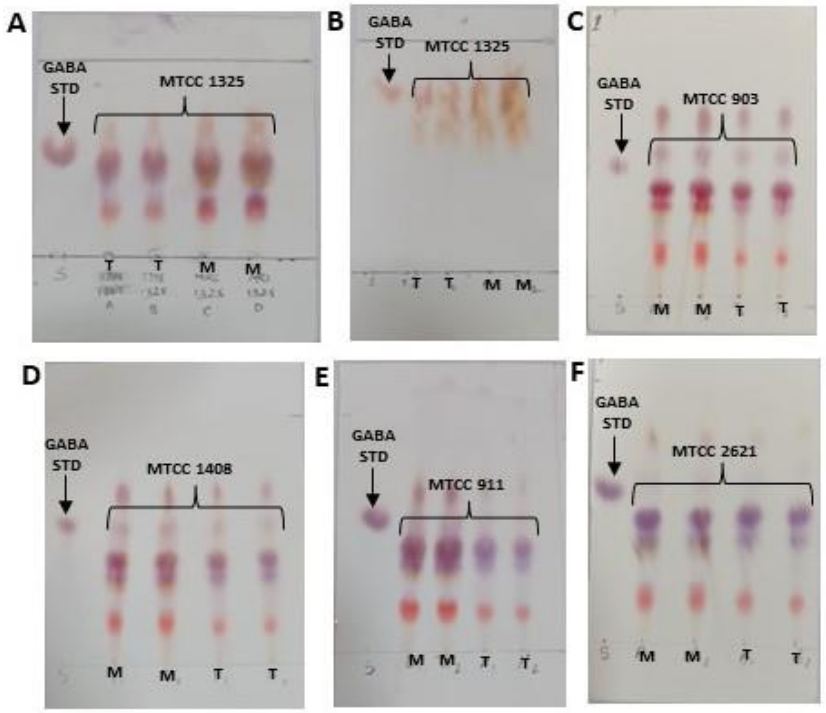


Fig. 5.2. Thin-layer chromatography for supernatant (media without MSG): A – MTCC 1325 (Butanol), **B** – MTCC 1325 (Methanol), **C** – MTCC 903, **D** – MTCC 1408, **E** – MTCC 911, **F** – MTCC 2621 (***M*** *– MRS media,* ***T*** *– TJYE media)*

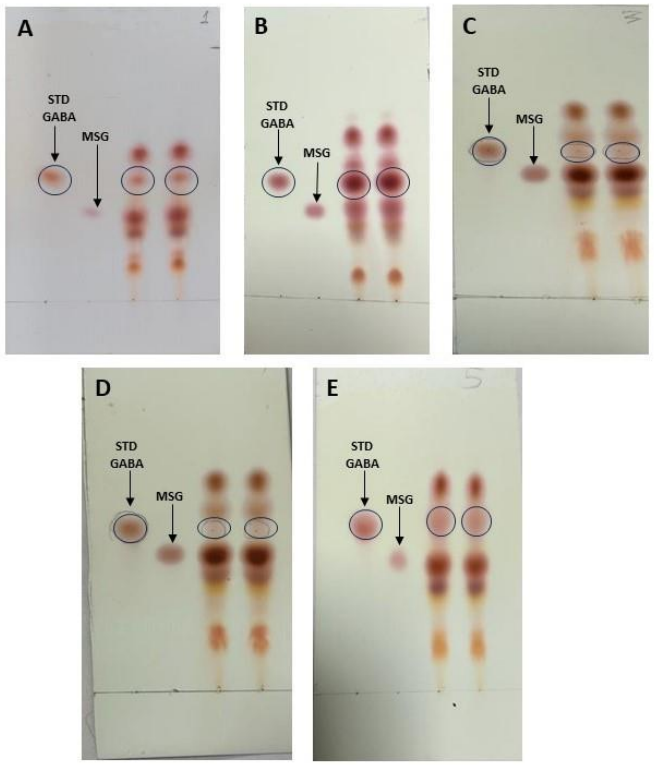


Fig. 5.3. Thin-layer chromatography for supernatant (media with MSG): A – MTCC2621, **B** – MTCC 1408, **C** – MTCC 1325, **D** – MTCC 911, **E** – MTCC 903 (***M*** *– MRS media)*

Due to the addition of MSG, there may have been an increased production of GABA hence well resolved and darker spots in the TLC plate of Lactobacillus strains can be observed aligning with the standard GABA spots along with MSG (Fig.5.3).

5.3. Quantitative detection using UV-Spectrophotometer

Colorimetric quantitative estimation of GABA was done using a UV-Spectrophotometer (Perkin Elmer) at 512 nm, 570 nm, and 630 nm. The standard curve was prepared (Fig. 5.4), and the unknown concentration of GABA for LABs was determined using the standard curve. The best results were obtained at 512 nm whereas at other wavelengths results were observed in negative or some anomalies were observed. The study also showed that for the standard GABA, the best lambda max was obtained at this wavelength as confirmed by wavelength scan. Among the five strains, the maximum concentration of GABA was observed in MTCC 911 (2.99 ± 0.13 mg/mL) followed by MTCC 1325 (2.75± 0.22

mg/mL). The strains MTCC 1408, MTCC 2621, and MTCC 903 resulted in a GABA yield of 2.74 ±0.21 mg/mL, 2.54 ±0.29 mg/mL, and 2.02 ±0.20 mg/mL (Fig. 5.5).

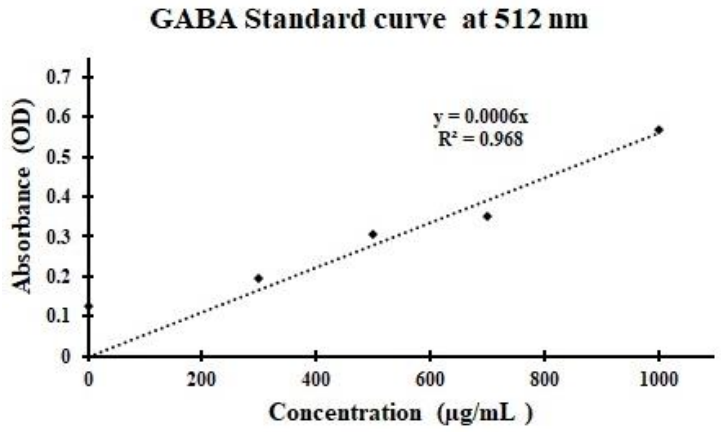


Fig. 5.4. Standard curve of GABA (0-1000 µg/mL) with ninhydrin-based assay (UV-VIS spectrophotometry @ 512 nm)

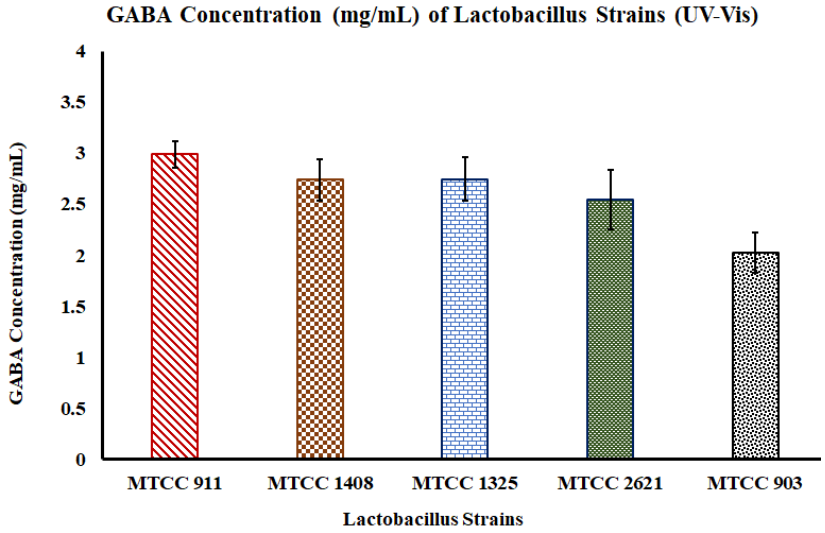


Fig. 5.5 GABA concentration (mg/mL) of probiotic bacteria

5.4. Cell Growth and GABA concentration estimation for probiotic strains in the presence and absence of monosodium glutamate

The growth curve and GABA concentration were simultaneously plotted against time 0-168 h at regular intervals of 12 h. It was observed that a sudden spike in cell growth was observed in the first 0-12 h time interval in all five Lactobacillus strains with a maximum spike for MTCC 1408 (9.77 ± 0.12) (Fig. 5.7) and the least spike for MTCC 903 (1.22 ± 0.01)

(Fig. 5.10). Post a sudden spike in the first 12 h, all strains followed a different growth pattern. The study clearly showed that all the *Lactobacillus* strains showed GABA production in the stationary phase. These growth patterns and observations of maximum GABA concentration during the stationary phase are in line with the observation made by (Park et al., 2021) which is due to a decrease in the medium's pH thus generating an appropriate environment for the activity of GAD enzyme.

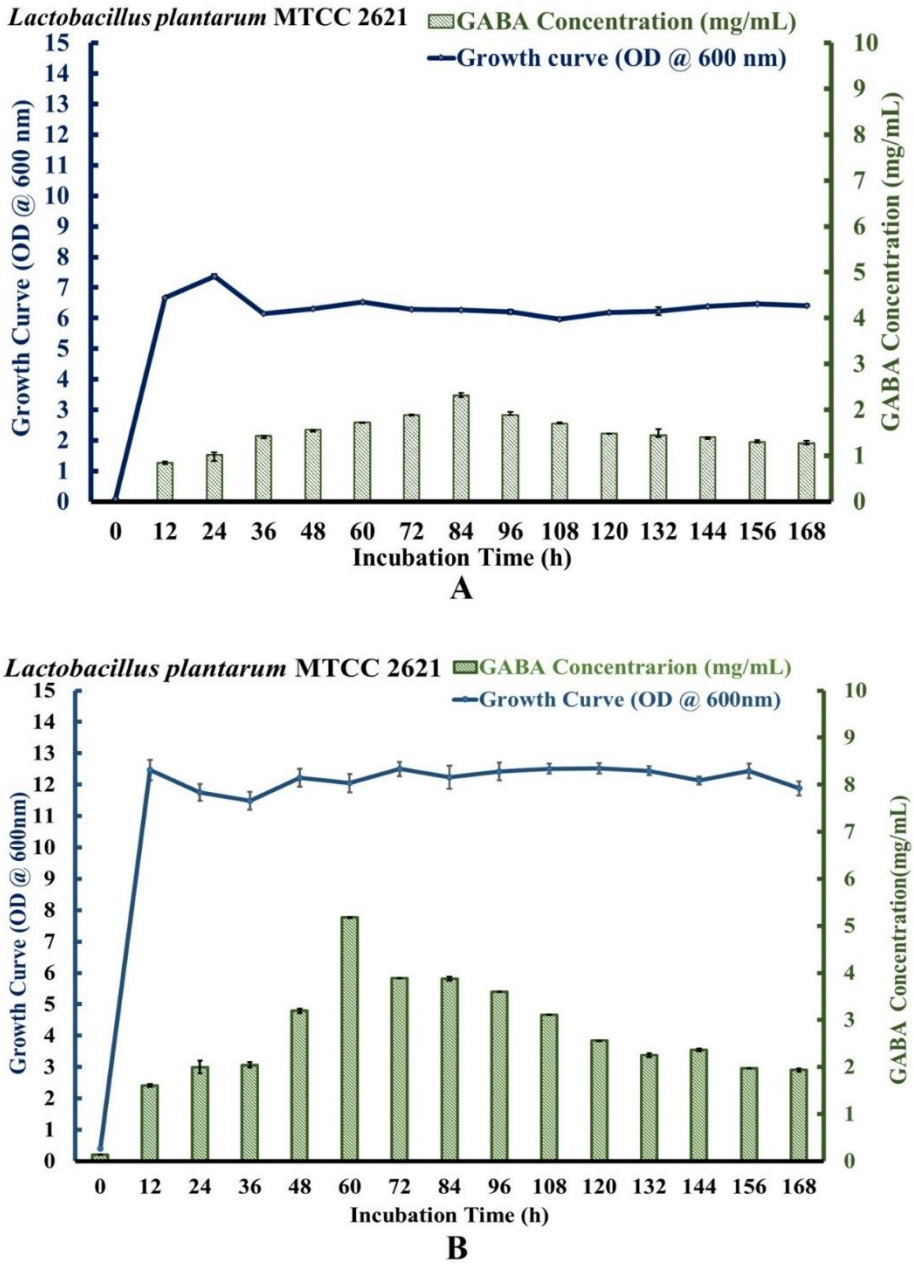


Fig. 5.6. Growth curve and GABA concentration (mg/mL) of MTCC 2621 for 0-168th h: A – Without MSG, **B** – With MSG

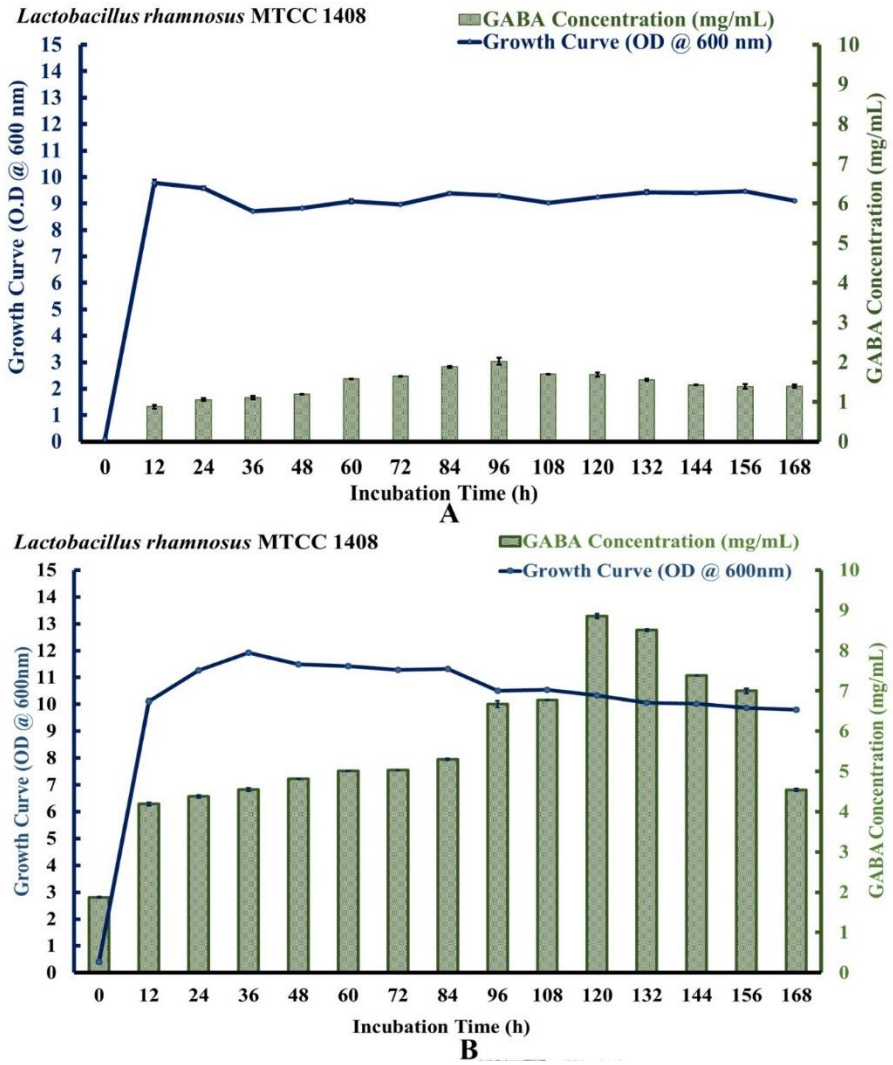


Fig. 5.7. Growth curve and GABA concentration (mg/mL) of MTCC 1408 for 0-168th h. A – Without MSG, **B** – With MSG

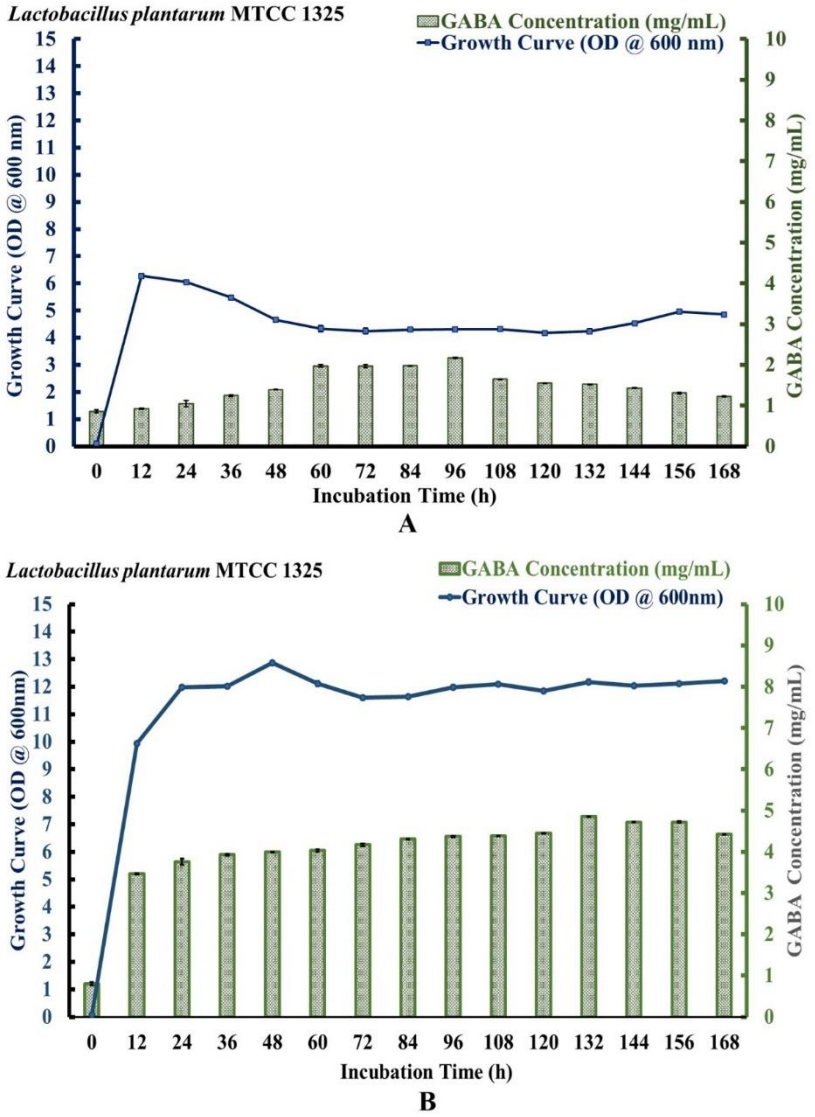


Fig. 5.8. Growth curve and GABA concentration (mg/mL) of MTCC 1325 for 0-168th h. A – Without MSG, **B** – With MSG

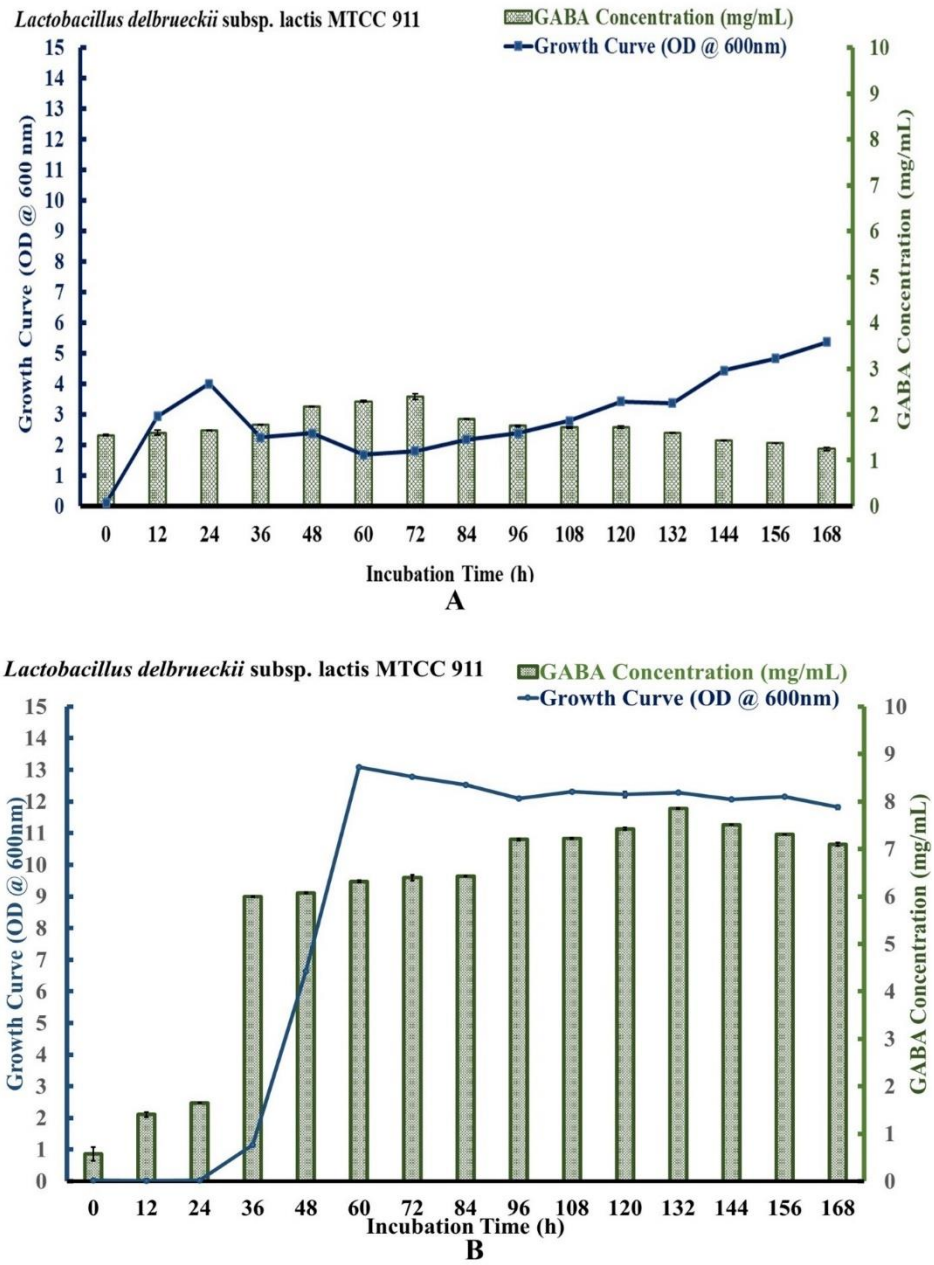


Fig. 5.9. Growth curve and GABA concentration (mg/mL) MTCC 911 for 0-168th h. A – Without MSG, **B** – With MSG

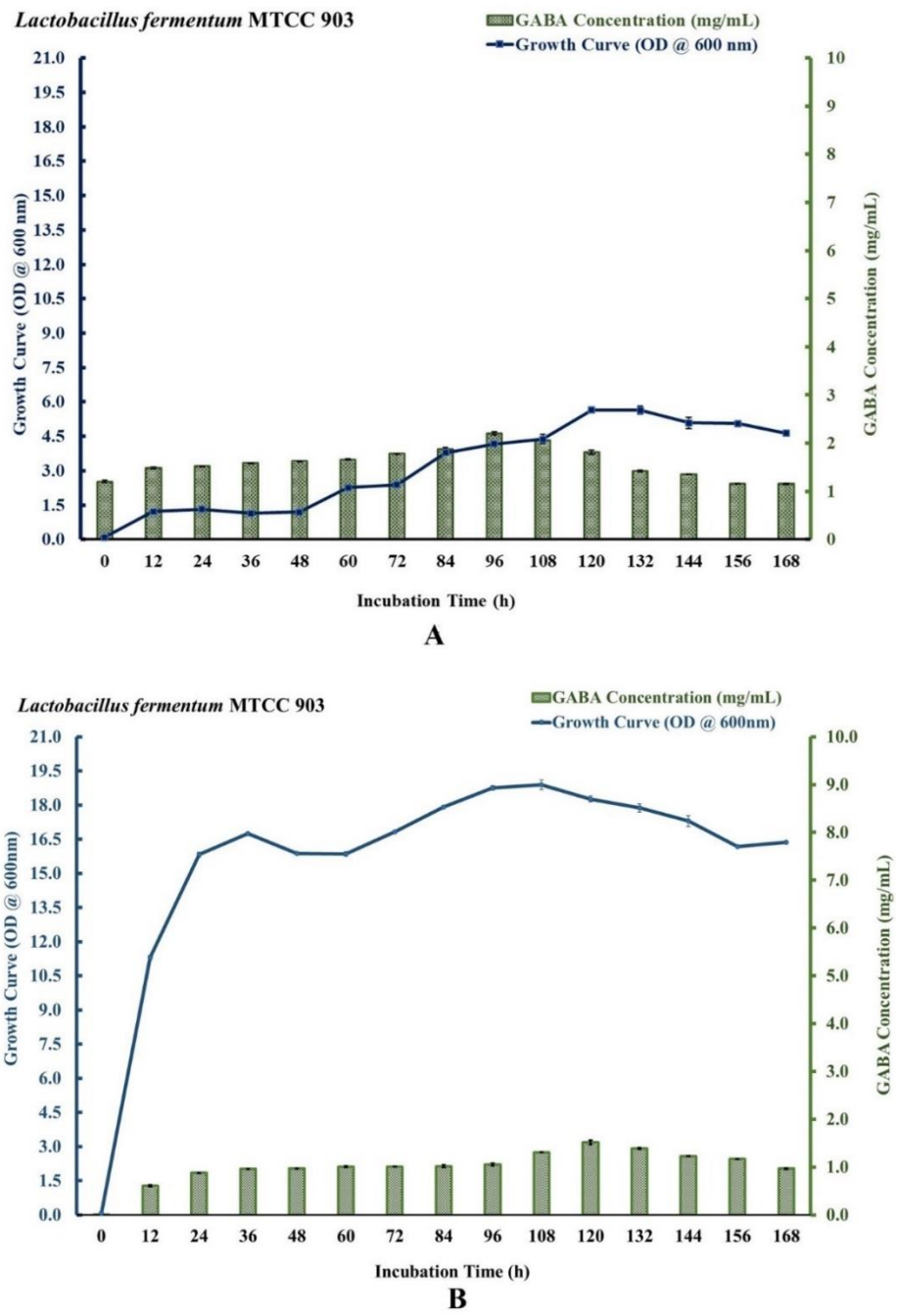


Fig. 5.10. Growth curve and GABA concentration (mg/mL) of MTCC 903 for 0-168th h. A – Without MSG, **B** – With MSG

Previous studies its shown that MSG acts as a precursor or a substrate (nitrogen source) to be converted into GABA by the GAD enzyme (Pannerchelvan et al., 2023). Therefore, the effect on cell growth and GABA concentration was tested in the presence and absence of MSG. The growth curve and GABA concentration were simultaneously plotted against time 0-168 h at regular intervals of 12 h. It was observed that a sudden

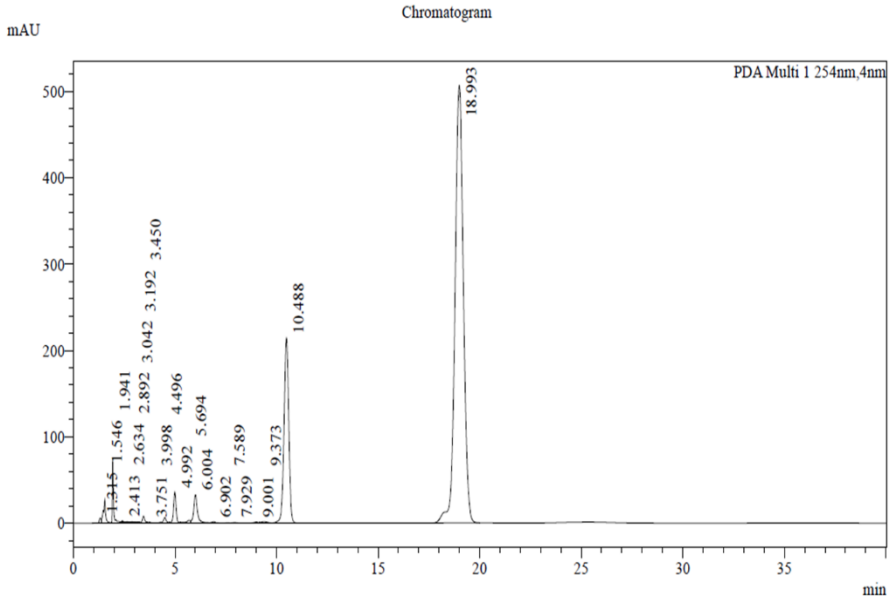
spike in cell growth was observed in the first 0-12 h time interval in all five *Lactobacillus* strains with a maximum spike for MTCC 1408 (9.77 ± 0.12) (Fig. 5.7.A.) and the least spike for MTCC 903 (1.22 ± 0.01) (Fig. 5.10.A.) in media without MSG. While in media with MSG, the maximum growth was seen by MTCC 903 (18.88 ± 0.21) (Fig. 5.10.B) and lowest by MTCC 1408 (11.93 ± 0.03) (Fig. 5.7.B) was observed. The maximum growth of MTCC 903 (OD of 8.14 ± 0.19) (Fig. 5.10.A) was observed at 132 h and a GABA concentration of 2.20 ± 0.03 mg/ml (96 h) (Fig. 5.10.A) was obtained from the media without MSG. While a maximum growth of MTCC 903 (18.89 ± 0.21) (Fig. 5.10.B) but comparatively a lower GABA concentration of 1.52 ± 0.05 mg/ml (120 h) (Fig. 5.10.B) was observed in media containing MSG. The low GABA production and its decrement after 132 h may have been due to the change in the acidic pH required for the GAD activity. Since MSG acts as a precursor or a substrate (nitrogen source) to be converted into GABA by GAD therefore GABA yield improved with the addition of MSG in all the strains. Further, MSG also maintains the pH of the environment. Carbon dioxide released due to the conversion of MSG into GABA by GAD coincides with the utilization of hydrogen ions i.e. increasing the pH of the medium (Pannerchelvan et al., 2023). The optimum activity by GAD is shown in the pH range of 3.5 – 5.0. Hence, though there is an increase in the growth of bacteria in MSG media, the conversion of MSG to GABA decreases or remains unchanged due to the unfavorable environment for the GAD enzyme.

Without MSG, MTCC 911 showed the maximum GABA concentration of 2.40 ± 0.06 mg/mL at 72 h (Fig 5.5.). While the other strains MTCC 2621, MTCC 1325, MTCC 1408, and MTCC 903 showed GABA concentration of 2.32 ± 0.04 mg/mL (84 h), 2.03 ± 0.08 mg/mL (96 h), 2.17 ± 0.01 mg/mL (96 h), 2.20 ±0.03 mg/mL (96 h), respectively (Fig. 5.6-Fig. 5.10,) in media without MSG. However, in the MSG-containing MRS media, MTCC 1408 showed the maximum GABA concentration of 8.86 ± 0.01 mg/ml at 120 h (Fig 5.7.B). While the other strains MTCC 2621, MTCC 1325, MTCC 911, and MTCC 903 showed around 5.17 ±

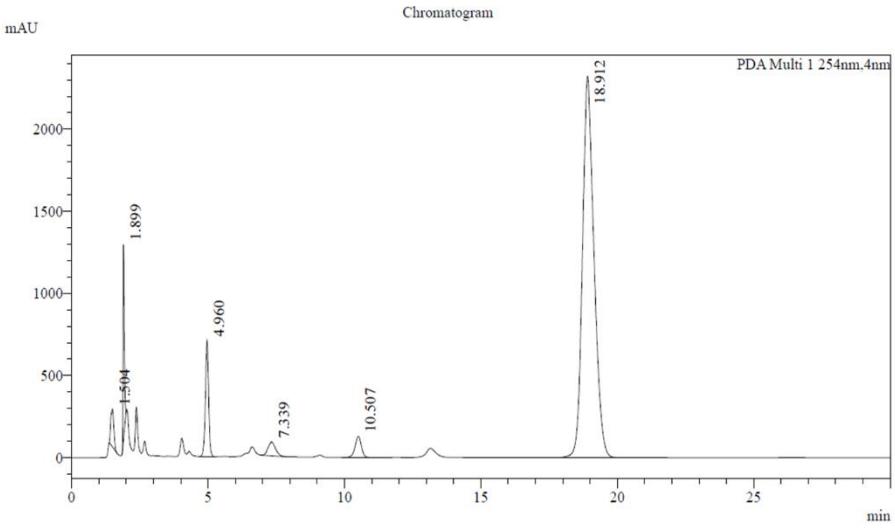
0.01 mg/mL (60 h), 4.85 ± 0.01 mg/mL (132 hr), 7.85 ± 0.02 mg/mL (132hr), 1.52 ± 0.05 mg/mL (120 hr), respectively (Fig. 5.6-Fig. 5.10).

5.5. Qualitative analysis of GABA using High-Performance Liquid Chromatography

In the primary screening, it is very clear that high concentrations of GABA were yielded in most of the screened probiotics strains as compared to studies in literature by (Falah et al., 2021)., and (Rayavarapu et al., 2021). This is a very interesting observation but further optimization of production procedure along with identification and qualitative estimations via sophisticated technology need to be performed using High-pressure liquid chromatography (HPLC), which is a more sensitive method. As the highest yield in most of the studies was observed near 72-96 h, therefore the withdrawn culture at 96h was subjected to HPLC analysis for estimation of GABA concentration.



A



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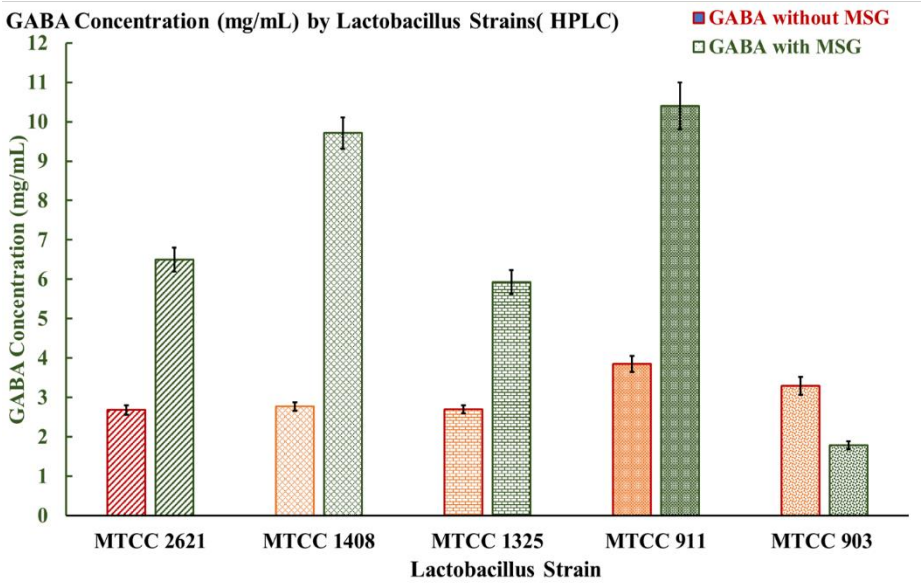


C

Fig 5.11. Qualitative analysis of GABA using HPLC: A. Representative HPLC chromatogram of GABA standard. **B**. Representative HPLC chromatogram Peak RT 18.912 min for MTCC 911. **C.** Qualitative analysis of GABA (with and without MSG addition) using HPLC**:** MTCC 2621, MTCC 1408, MTCC 1325, MTCC 911, MTCC 903.

In the qualitative analysis through HPLC for MRS medium without MSG, it was observed that MTCC 911 presented maximum GABA concentration i.e. 3.85 ±0.2 mg/mL amongst the other strains which is 1.17, 1.38, 1.42, and 1.43, fold higher than MTCC 903 (3.29 ±0.23 mg/mL), MTCC 1408 (2.77 ±0.11 mg/mL), MTCC 1325 (2.7 ±0.10 mg/mL) and MTCC 2621 (2.68 ±0.12 mg/mL), respectively (Fig. 5.11). These HPLC-based qualitative assays showed slightly higher GABA concentration which is due to the more sensitive nature of HPLC. These preliminary observations of GABA yield are very interesting as it gave a good yield as compared to work by(Devi et al., 2023) where probiotic bacteria *Weissella confusa* isolated from fermented green pea showed the highest production of 0.24 mg/mL GABA. However, the GABA yield in the present screened probiotic strains is much lower than the EJ2014 strains (19.8 mg/mL) (Park et al., 2021). However, an increased yield of EJ2014 was noted following media modification with MSG, a key substrate for the production of GABA by probiotic bacteria. In the present study when we did not use a primary substrate i.e. MSG or L-glutamic acid, but yet got a GABA yield of up to 3.85 mg/mL. A similar observation was also observed in the study by Park et al., (2021) where they observed that yeast extract acted as a secondary substrate similar to our work where vegetable extract (10 g/L) and yeast extract present in the synthetic HiVeg™ MRS Broth acted as a secondary substrate for GABA in addition to intracellular GABA precursors of probiotic strains under study.

Further, the positive impact of the addition of MSG was also observed for all the strains except MTCC 903. The addition of MSG led to maximum enhancement in GABA concentration for MTCC 911 i.e. 10.4 ± 0.6 mg/mL (132h) followed by 9.71± 0.4 mg/mL (120h) for MTCC 1408. The MTCC 2621 and MTCC 1325 resulted in comparable GABA concentrations of 6.5 ± 0.30 mg/mL (60h) and 5.93 ± 0.28 mg/mL (132h). The addition of MSG led to a substantial increase in GABA concentration for MTCC 911, MTCC 1408, MTCC 2621, and MTCC 1325 by 2.7, 3.5, 2.4, and 2.2 fold respectively.

However, in the case of MTCC 903, the cell OD was very high but the same has not been reciprocated to GABA yield. A comparative lower GABA concentration of 1.79 ± 0.10 mg/mL was observed for MTCC 2621 with MSG which is half of the GABA concentration observed for MTCC 2621 without MSG. As elaborated in the earlier section, the increase in GABA is correlated to MSG acting as a precursor and nitrogen source for GABA production. Further, this increase is correlated to the pH in the medium due to the growth of LABs. Based on HPLC analysis and previous observations in the present studies, strain *L. delbrueckii* subsp. Lactis MTCC 911 was chosen as the potential GABA-producing strain for further studies.

5.6. GABA production by using agro-residues for *L. delbrueckii* subsp. Lactis MTCC 911

To test our hypothesis that Glucose is a substrate when undergoing glycolysis, and the Krebs cycle results in glutamate, which is a precursor of GABA. The agro residues being rich in glucose can act as a good substrate for GABA production. We have screened five different agro residues i.e. orange pulp powder, molasses, soya seed powder, soya stalk powder, and soya milk. Among the five agro-residues selected, the maximum concentration of GABA for MTCC 911 was observed in 2 % molasses to be 2.33 ± 0.01 mg/mL (Fig. 5.12).

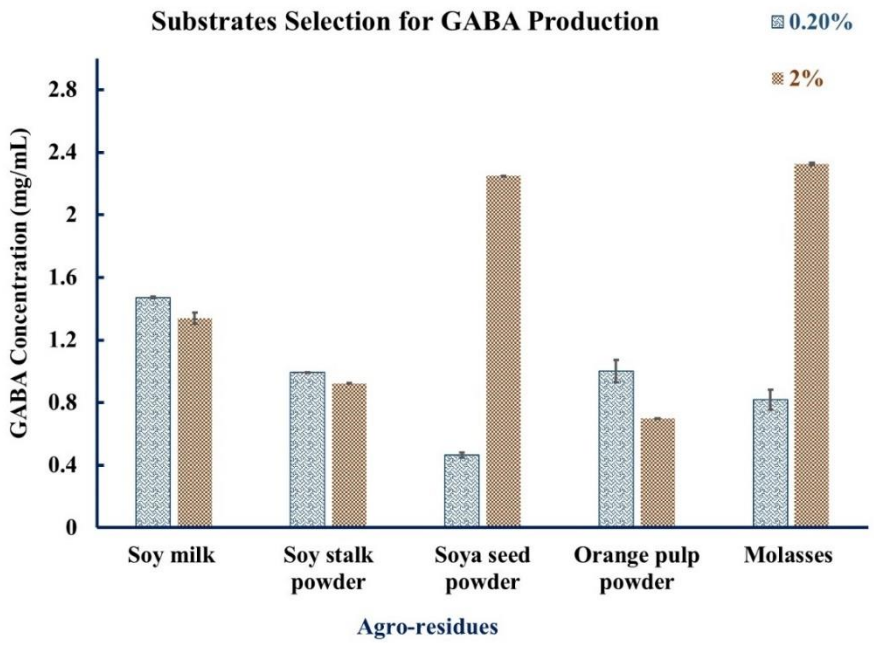


Fig 5.12. Selection of substrate for GABA production

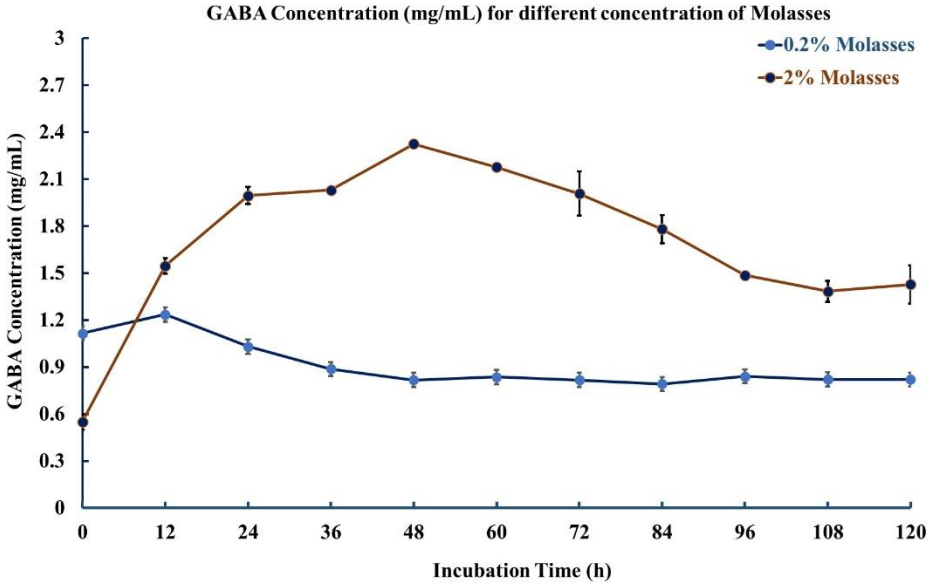


Fig. 5.13. Analysis of different concentrations of molasses for GABA production

Cane molasses consists of sucrose, glucose, and fructose and is 48.8% dry matter, 5.29% dry matter, and 8.07% dry matter (Jamir et al., 2021). glucose is a substrate when undergoing glycolysis, and the Krebs cycle results in glutamate, which is a precursor of GABA.

Hence, the molasses being rich in carbon source poses as a good substrate for GABA production, therefore being used in our further studies. Although the production of GABA produced in our study using molasses is less than a study by Thongruck and Maneerat, (2023) where *Lactobacillus futsaii* CS3 showed a GABA concentration of 7.4 g/L with the modified medium consisting of molasses (2.5%, w/v) (Thongruck and Maneerat, 2023). In this case, they have used some other nitrogen and carbon sources such as surimi washing water (2.5%, w/v), cane sugar (2%, w/v), and tuna condensate waste (3.06%, w/v) as well which may be another factor for better GABA production. But in another study, the GABA production by *L. fermentum* 4–17 was found to be 359.45 ppm where the medium consisted of 24.77% molasses, 29.27% dairy sludge, and 10.49% soybean meal (Falah et al., 2024). Our study gave better results as compared to Falah et al., (2024) in a modified MRS medium with 2% molasses as a carbon source and 1% MSG as a GABA precursor. Therefore, optimizing the media with other physical factors may result in a further increase in the production of GABA.

5.7. BBD-RSM-based optimization of GABA production for *L. delbrueckii* subsp. Lactis MTCC 911

In this study, RSM using BBD was utilized to develop a quadratic model. The independent factors included Temperature (A), MSG Concentration (B), and Substrate concentration (C). Table 5.1 outlines the levels of individual factors for each 17 runs of the BBD-RSM study, along with their experimental and software-predicted responses.

Table 5.1. BBD-RSM experimental design with predicated and actual GABA concentration for each run

Run Order	Factor 1 A	Factor 2 B	Factor 3 C	Response GABA Concentration (mg/mL)	
	Temperature °C	MSG concentration %	Substrate Concentration %	Actual	Predicted
1	37	3	1.6	1.35	1.30
2	31	1.75	1.6	2.16	1.95
3	25	3	1.6	2.19	2.26
4	31	3	3	1.97	1.84
5	31	0.5	0.2	2.14	2.33
6	31	1.75	1.6	2.28	1.95
7	37	1.75	0.2	2.45	2.30
8	25	1.75	0.2	2.03	1.76
9	31	0.5	3	1.90	1.91
10	31	1.75	1.6	1.84	1.95
11	**37**	**0.5**	**1.6**	**3.07**	**2.86**
12	31	1.75	1.6	1.38	1.95
13	31	3	0.2	1.69	1.73
14	31	1.75	1.6	1.43	1.95
15	25	1.75	3	1.99	1.89
16	25	0.5	1.6	1.46	1.38
17	37	1.75	3	1.84	1.86

Following ANOVA, the regression equation was derived to estimate the GABA concentration level based on all the independent variables. The interaction among the three independent variables and its impact on GABA concentration was also examined. The response generated through interactions among the various levels of each independent variable is most accurately represented by the multiple regression equation following ANOVA analysis (Eq. 2).

$$Y = (-3.4611) + (0.1919 \times A) + (2.2730 \times B) + (0.3456 \times C) + (-0.08158 \times AB) + (-0.01717 \times AC) + (0.07509 \times BC) \quad \text{(Eq. 2)}$$

where, Y is the GABA concentration (mg/mL), and A, B, and C are temperature (°C), MSG (%), and, substrate concentration (%), respectively. The equation formulated with actual factors can be employed to predict the response for specified levels of each factor. Here, the levels need to be defined in the original units for each factor. This equation should not be employed to assess the relative influence of each factor due to the coefficients being scaled to accommodate the units of each factor and the intercept not being located at the center of the design space.

Statistical analysis was conducted, employing both Fisher's f-test and Student's t-test. The model F-value of 3.49 with a p-value of 0.0397 ($p > 0.05$) implied that the model is significant. Moreover, there exists only a 3.97% probability that a similar high F – value could arise due to noise.

Table 5.2 ANOVA for the BBD-RSM model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.06	6	0.3441	3.49	0.0397	**Significant**
A-Temperature	0.1357	1	0.1357	1.38	0.2680	
B-MSG concentration	0.2311	1	0.2311	2.34	0.1568	
C-Substrate Concentration	0.0476	1	0.0476	0.4830	0.5029	
AB	1.50	1	1.50	15.19	0.0030	
AC	0.0832	1	0.0832	0.8434	0.3800	
BC	0.0691	1	0.0691	0.7006	0.4221	
Residual	0.9862	10	0.0986			
Lack of Fit	0.3136	6	0.0523	0.3109	0.9020	
Pure Error	0.6725	4	0.1681			
Cor Total	3.05	16				

In this instance, AB emerges as a significant model term as its p-value is 0.0030. As a p-value less than 0.0500 is deemed significant

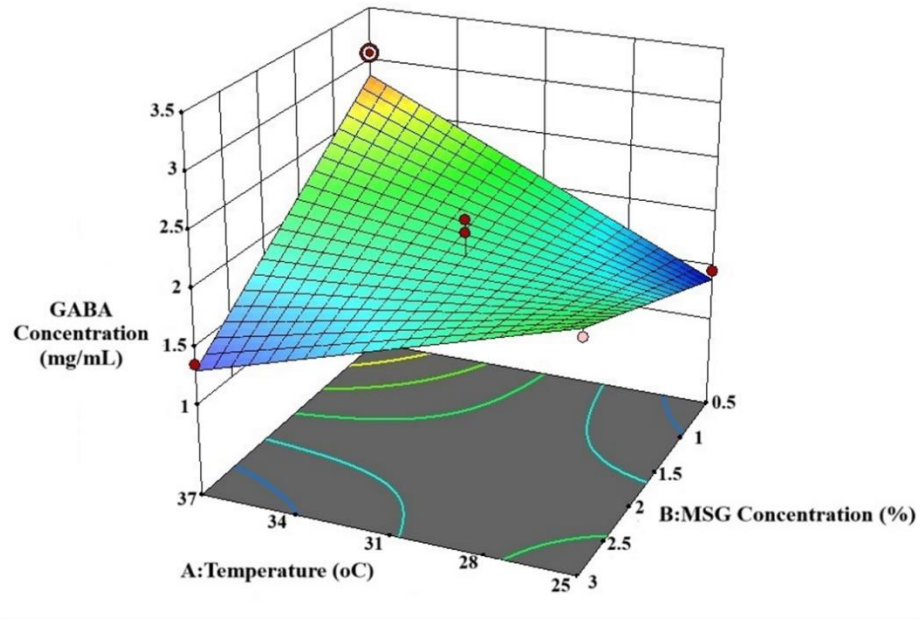
The model term lacks significance if the p-values exceed 0.100. If numerous model terms are insignificant (excluding those necessary for maintaining hierarchy), the reduction of the model may enhance its effectiveness.

In the present BBD-RSM study, the Lack of Fit F-value (0.31) is not significant compared to the pure error. There is a 90.21% chance that a Lack of Fit F-value of this magnitude could occur due to noise. A non-significant lack of fit is favorable, as our objective is to achieve an overall fit for the model (Table 5.2). Moreover, coefficient determination (R^2), predicted R^2, adjusted $R^{2,}$ and adequate precision were all taken into account to ascertain the significance of the model. The R^2 of 0.6767 for GABA concentration suggests that the model can account for 67.67% of the variability observed in the response (Table 5.3).

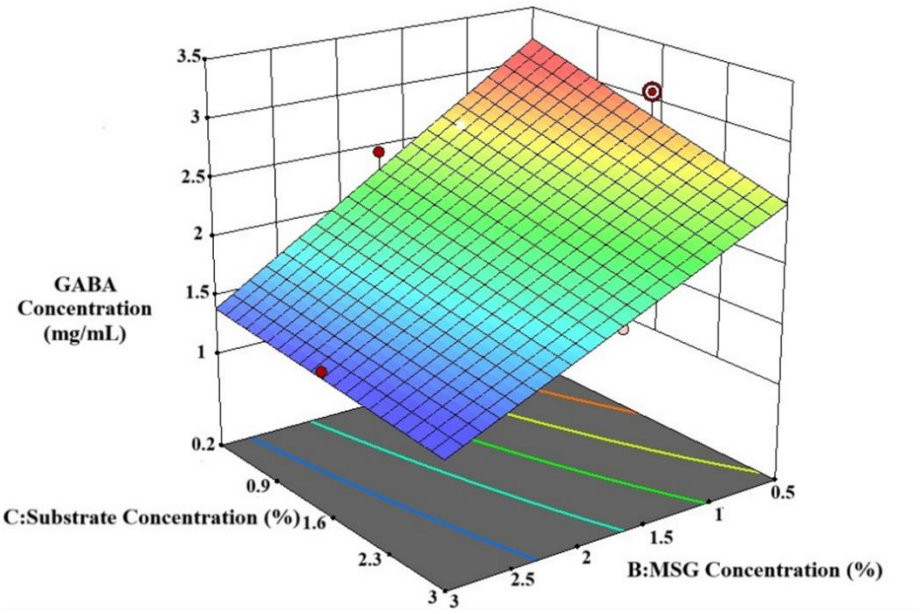
Table 5.3 Fitting statistics and Coefficient for determination (R^2)

Std. Dev.	**0.3140**	**R^2**	**0.6767**
Mean	1.95	Adjusted R^2	0.4828
C.V. %	16.08	Predicted R^2	0.3421
Std. Dev.	0.3140	Adeq Precision	7.7600

Table 5.3 provides the details of fitting statistics and coefficient of determination (R^2). The difference between Predicted R^2 (0.3422) and Adjusted R^2 (0.4828) is less than 0.2 and thus is in reasonable agreement. The model holds significance. Moreover, Adequate Precision assesses the signal-to-noise ratio which is 7.761 in the present study. As the Adequate Precision ratio exceeding 4 is desirable. So, in the present study, we have an adequate signal and this model is suitable for navigating the design space.


GABA
Concentration
(mg/mL)
3.5
3
2.5
2
1.5
1
37
34
31
28
25
A:Temperature (oC)
0.5
1
1.5
2
2.5
3
B:MSG Concentration (%)

A


GABA
Concentration
(mg/mL)
3.5
3
2.5
2
1.5
1
0.2
0.9
1.6
2.3
3
C:Substrate Concentration (%)
0.5
1
1.5
2
2.5
3
B:MSG Concentration (%)

B

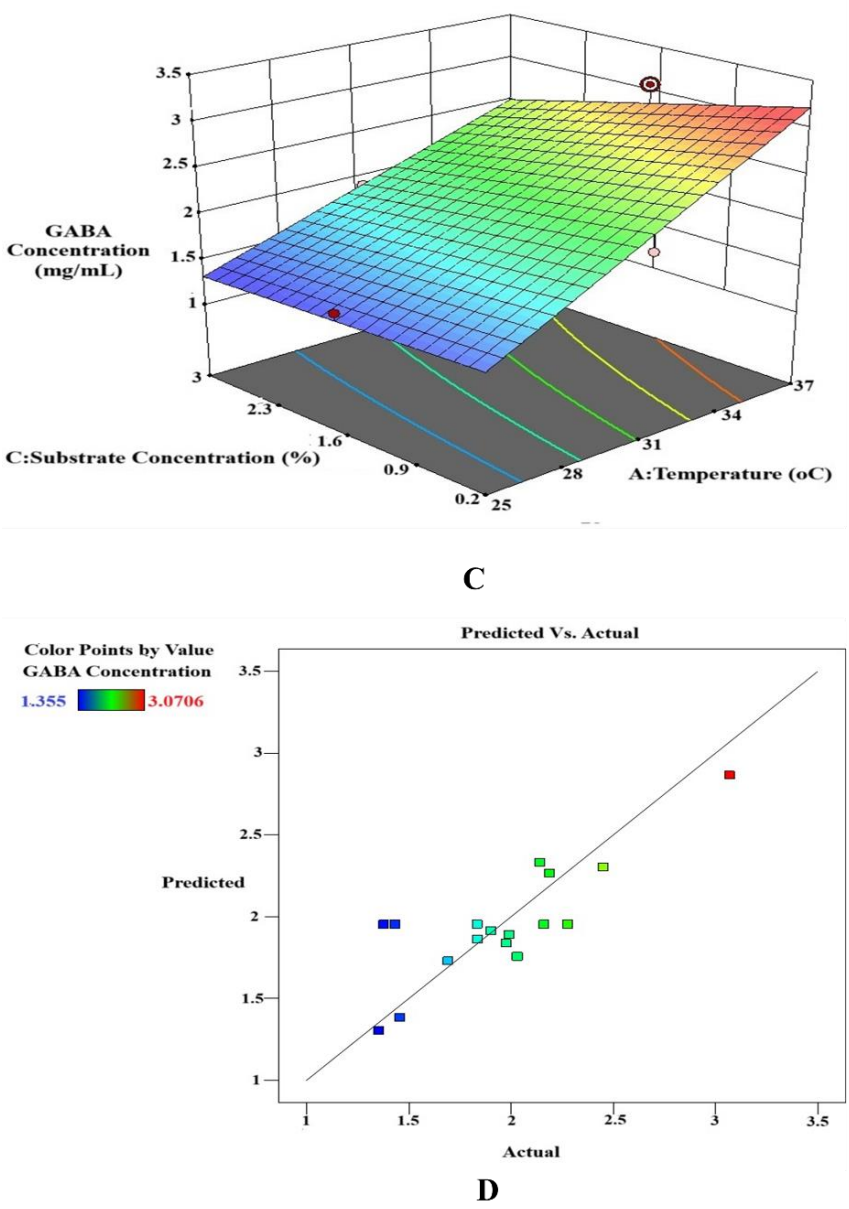


Fig. 5.14. Response surface plot and contour plot for GABA production from *Lactobacillus delbrueckii* subsp. lactis MTCC 9111 showing the interaction between different variables: A: Temperature vs MSG concentration; **B:** MSG concentration vs Substrate concentration; **C:** Temperature vs Substrate concentration; **D:** Predicted vs Actual response plot.

The study investigated the interaction effect of independent variables on GABA concentration by analyzing the interaction of two variables while maintaining the third independent variable at a constant level. The contour plots and response surface plots (Fig. 5.14) depicted interactions

between MSG concentration and substrate concentration, temperature and MSG concentration, and temperature and MSG concentration, representing the most probable combinations identified from the response evaluation. Thus, enabling the prediction of optimal values for different runs.

Fig 5.14 A represents a combination of low temperatures-low MSG concentration or high temperature-high MSG concentration results in low GABA concentration. Fig 5.14 B represents that, at high MSG concentration, the increase in substrate concentration does not have much impact on the enhancement of GABA. Whereas low MSG concentration and high substrate concentration may positively impact GABA production. Fig 5.14 C, represents at low temperatures, the increase in molasses concentration does not have much impact on increasing the GABA concentration. Whereas with an increase in both temperature and molasses concentration, GABA concentration enhanced significantly.

Therefore, based on the evaluation of contour plots and the predicted and experimental GABA response explained that the optimal values of the variables are as follows temperature of 37 °C, MSG Concentration of 0.5%, and molasses concentration of 1.6%. According to this model, the predicted maximum value for GAA production is 2.86 mg/mL. GABA production was examined under optimized conditions in triplicate to confirm the predicted values from this model, leading to an experimental value of 3.07 mg/mL. This result suggests that the experimental and predicted values of GABA production are in good agreement. Additionally, as depicted in Fig. 5.14 D, the graph comparing predicted values to actual values (experiment) showed alignment between the experimental results and the predicted values. Consequently, BBD-RSM models can be considered a precise and dependable approach for optimizing GABA production. Further minimal medium in combination with MSG and Molasses resulted in an enhanced GABA yield of 3.07 mg/mL compared to the unoptimized

minimum medium with 2.24 mg/mL (Fig 5.12) with MSG (1%), molasses concentration of 2% at 37 °C. Thus, a 1.37-fold increment in the GABA production potential after the BBD-RSM study was obtained.

Chapter 6

Conclusion and Future Prospects

6.1 Conclusion

In all five strains, it was observed that MRS media supported the growth of bacteria as compared to TJYE. Colored spots in alignment with the GABA standard show that all five bacterial strains may have the ability to produce GABA. When analyzed in a UV-visible spectrophotometer, species MTCC 911 presented as the highest yielding GABA concentration bacteria with other lactobacillus strains that have comparable GABA yield. When the growth curve of all the five lactobacillus strains was plotted from 0-168th h, in the presence and absence of MSG, it was observed that there was no direct relation between the growth of the bacterial species and GABA concentration. MSG positively impacts GABA concentration; thus, MSG can be used as a suitable precursor along with other carbon-nitrogen sources for enhanced GABA production. The highest GABA concentration of 10.4 ± 0.6 mg/mL and 3.85 ±0.20 mg/mL was observed in species MTCC 911 in the presence and absence of MSG when analyzed through HPLC. In the presence or absence of MSG, the maximum GABA concertation was observed with MTCC 911. Among different agro residues screened for GABA production with MTCC 911 in a modified MRS medium, molasses showed maximum GABA production. BBD-RSM-based optimization of GABA production for *L. delbrueckii* subsp. Lactis MTCC 911 demonstrated a 1.37-fold increment in the GABA production potential in optimized medium and culture conditions compared to unoptimized medium and culture conditions.

6.2 Future prospects

- Development of low-cost GABA recovery methods from probiotic bacteria.
- High Rerouting metabolic pathways to augment GABA biosynthesis.

- Broaden the range of substrates for GABA biosynthesis.
- Extending the ideal pH range for Glutamate Decarboxylase (GAD).
- Co-culturing of Lactobacillus with other micro-organisms to increase GABA concentration as well as other nutraceutical biomolecules.

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