EVALUATION OF γ-AMINOBUTYRIC ACID PRODUCTION BY INDIGENOUSLY ISOLATED LACTIC ACID BACTERIA

A

Dissertation

Submitted in the partial fulfillments of the requirements

for the award of degree of

Masters of Science
In
Biotechnology

By:

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Candidate's Declaration

I, hereby declare that the work presented in the dissertation entitled **"Evaluation of γ-aminobutyric acid production by indigenously isolated lactic acid bacteria"** in partial fulfillment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of five months from January 2011 to May 2011, under the supervision of Dr. Abhijit Ganguli, Assistant Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

Date: (Meenakshi Bamnia)

Certificate

This is to certify that the thesis entitled **"Evaluation of γ-aminobutyric acid production by indigenously isolated lactic acid bacteria"** submitted by Meenakshi Bamnia in partial fulfillment of the requirements for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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Meenakshi Bamnia

CONTENTS

LIST OF ABBREVIATIONS

LAB	Lactobacillus
MSG	Monosodium glutamate
GABA	Gamma-amino butyric acid
MRS	De man rogosa sharpe medium
ml	Mililitres
cfu	Colony forming unit
mM	mili molar
Rf	Retardation factor
HPLC	High pressure liquid chromatography
TLC	Thin layer chromatography
µl	Microlitres
PEA	Pulseless electrical activity
GAD	Glutamic acid decarboxylase

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ABSTRACT

GABA is a major inhibitory neurotransmitter in mammalian brain and has several well-known physiological functions. Lactic acid bacteria possess special physiological activities and regarded as safe. Therefore, various types of LAB that show GABA-producing ability is an interesting target for food industry, especially fermented foods because individual strain has specific fermentation profiles such as acid production, taste and flavor formation ability. These profiles are considered as important factors in the use of LAB as starter cultures in the production of fermented foods. In the present report, two preisolated lactic acid bacteria were screened to be high GABA producing strains namely, *Lactococcus lactis* and *Leuconostoc mesenteroides*. The culture media used for effective GABA production was MRS supplemented with 5% MSG, when inoculated with the cultures and kept at 30°C for 48h with an initial pH 5 on static condition. The optimized conditions were used to prepare a fermented food product having high nutritional value. The raw material used in the food product was wheat flour in place of media and MSG was replaced by *Vigna mungo* (17-34% glutamate). The GABA concentration obtained with MRS supplemented with 5 % MSG was140mM (14.4g/l) and 80mM (8.2g/l) with *L. lactis* and *L. mesenteroides* resp. and in the food product prepared with *Vigna mungo* and wheat flour was 110mM (11.3g/l) with mixed culture (*L. lactis and L. mesenteroides*). In comparison to media, our final food product produces significant amount of GABA which can be easily consumed and absorbed in the body.

Keywords: *Lactococcus lactis, Leuconostoc mesenteroides*, bhaturu, GABA (Gamma-aminobutyric acid), vigna mungo, monosodium glutamate.

Chapter 1. Introduction

A nutraceutical is a food with medical-health benefit, including the prevention and treatment of disease. Nutraceuticals are classified as products which are extracted from natural sources or manufactured synthetically which supplement the diet to provide nutrition over and above regular food and help nutrition related disorders. Nutraceuticals already have become part of the dietary landscape. Consumer's interest in the relationship between diet and health has increased the demand for information on nutraceuticals. Rapid advances in science and technology, increasing health care costs, changes in food laws affecting label and product claims, an aging population and rising interest in attaining wellness through diet are among the factors fueling world's interest in nutraceuticals. Credible scientific research indicates many potential health benefits from food components. These benefits could expand the health claims now permitted to be identified by the Food and Drug Administration (FDA).

In addition to government-supported research, food companies traditionally have funded research for new food-product formulations. Incentives to the food industry would enhance greatly the development of functional foods. The research required for a functional food to meet scientific standards for efficacy is a substantial investment. Additional research is needed in many areas to ensure this emerging science continues to be valid and is translated rapidly into consumer-relevant products. Examples of nutraceuticals are Folic acid, Beta-carotene, Lycopene, Gamma-amino butyric acid. The most abundant synapses in the central nervous system of vertebrates are inhibitory synapses that use the neurotransmitter γ-aminobutyric acid (GABA). In vertebrates, GABA acts at synapses of the central nervous system.

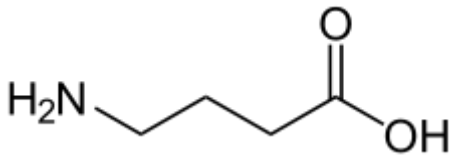


Fig. 1.1 (Gamma-aminobutyric acid)

GABA is Gamma-aminobutyric acid, a neurotransmitter and the cornerstone of the inhibitory (calming) system in the body; controlling the action of epinephrine, norepinephrine, and dopamine.

In contrast, inhibitory neurotransmitters and their receptors reduce excitability in the brain's neurons and increase the likelihood that an incoming signal will be terminated. For optimal functioning, the brain must balance the excitatory and inhibitory influences: Excessive excitation can lead to seizures, insomnia, anxiety, and many other clinical conditions whereas; excessive inhibition of neurons can result in incoordination, sedation, and anesthesia.

GABA is the primary inhibitory neurotransmitter in the brain and therefore filters out irrelevant messages (static) by terminating signals from the excitatory neurotransmitters: glutamate, and its positive modulators epinephrine, norepinephrine, and PEA. GABA can be viewed as the "braking system" in the realm of neurotransmitters.

History

Gamma-aminobutyric acid was first synthesized in 1883, and was first known only as a plant and microbe metabolic product. In 1950, however, GABA was discovered to be an integral part of the mammalian central nervous system.

Structure and conformation

GABA is found mostly as a zwitterion, that is, with the carboxyl group deprotonated and the amino group protonated. Its conformation depends on its environment. In the gas phase, a highly folded conformation is strongly favored due to the electrostatic attraction between the two functional groups. The stabilization according to quantum chemistry calculations is about 50kcal/mol. In the solid state, a more extended conformation is found, with a trans-conformation at the amino end and a gauche conformation at the carboxyl end. This is due to the packing interactions with the neighboring molecules. In solution, five different conformations, some folded and some extended are found as a result of solvation effects. The conformational flexibility of GABA is important for its biological function, as it has been found to bind to different receptors with different conformations. Many GABA analogues with pharmaceutical applications have more rigid structures in order to control the binding better tissues of the nervous system.

GABA synthesis in Brain:

GABA is formed through the activity of the enzyme glutamic acid decarboxylase (GAD). GAD catalyzes the formation of GABA from glutamic acid. The synthesis of GABA is linked to the Kreb's cycle. GAD requires vitamin B6 (pyridoxal phosphate) as a cofactor, which can be used to regulate the levels of GABA. Vitamin B6 is a key GABA vitamin.

GABA can be metabolized by a transamination reaction with α-ketoglutarate, catalyzed by GABA-transaminase. Compounds such as the competitive GAD inhibitor allylglycine, inhibit GABA formation and cause convulsions due to the lack of GABA activity. The importance of GABA is underscored by the frequency in which it is a pharmaceutical target and how many commonly used drugs affect its function e.g. Xanax, Klonapin, Valium, Neurontin.

The GABA receptor is a relatively large molecule and has binding sites not only for GABA but also for many modulatory compounds. Many of these modulatory compounds are useful therapeutic agents. Positive GABA modulators, like the benzodiazepines, do not cause the ion channel to open and an influx of chloride ions to occur on their own. They only enhance the

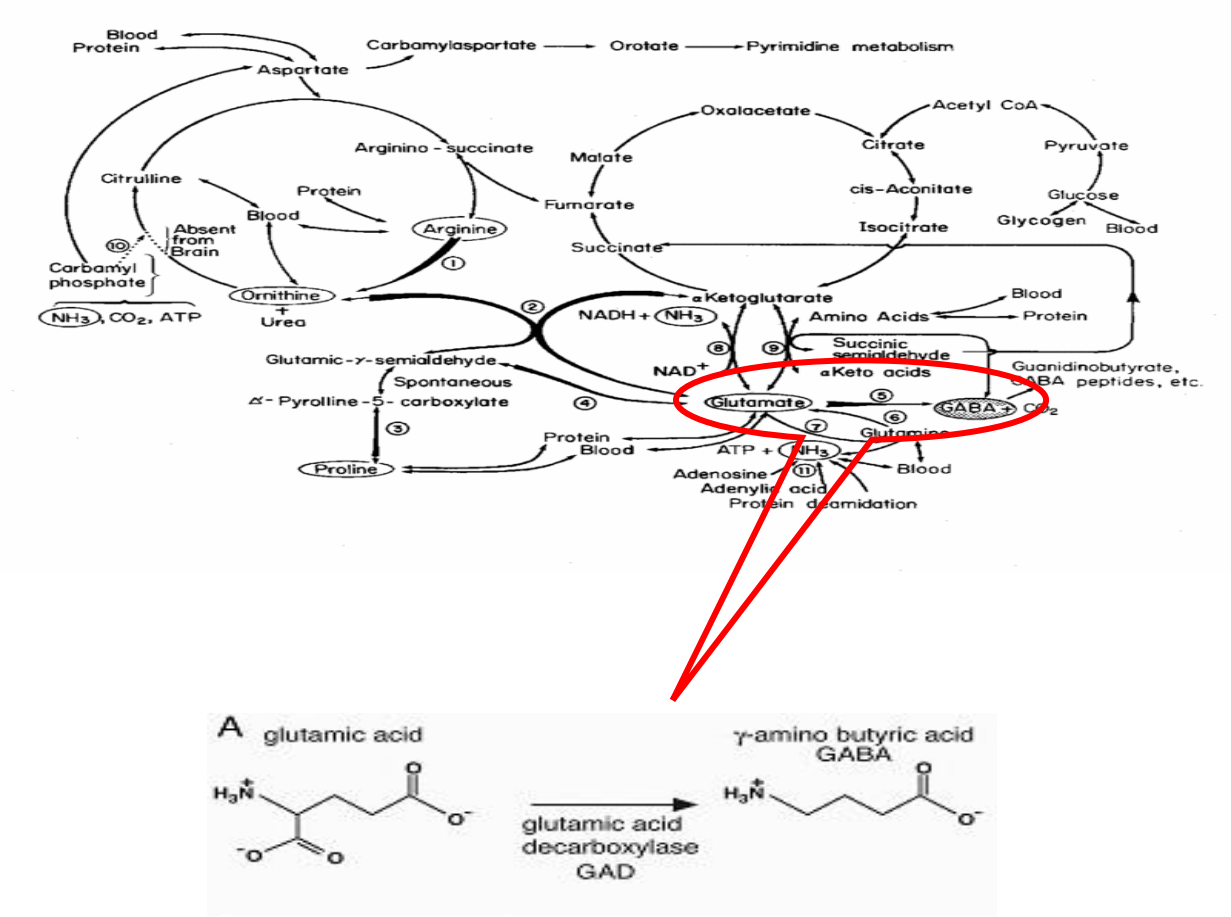


Fig. 1.2. GABA Production Cycle

activity of naturally occurring GABA by potentiating its function and therefore have vastly reduced potential for overdose or side effects than receptor agonist compounds, like barbiturates. While much safer than barbiturates, benzodiazepine's use frequently leads to dependence and withdrawal syndrome effects. This limits their utility for mild/moderate symptoms as well as for long-term therapy.

Function:

In the vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neurons. This binding causes the opening of ion channels to allow the flow of either negatively-charged chloride ions into the cell or positively-charged potassium ions out of the cell. This action results in a negative charge in the transmembrane potential, usually causing hyperpolarization. Three general classes of GABA receptor are known: $GABA_A$ and $GABA_C$ ionotropic receptors, which are ion channels themselves, and $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open ion channels via intermediaries (G proteins).

Neurons that produce GABA as their output are called GABAergic neurons, and have chiefly inhibitory action at receptors in the adult vertebrate. Medium Spiny Cells are a typical example of inhibitory CNS GABAergic cells. GABA exhibits excitatory actions in insects, mediating muscle activation at synapses between nerves and muscle cells, and also the stimulation of certain glands. In hippocampus and neocortex of the mammalian brain, GABA has primarily excitatory effects early in development, and is in fact the major excitatory neurotransmitter in many regions of the brain prior to the maturation of glutamate synapses. Whether GABA is excitatory or inhibitory depends on the direction (into or out of the cell) and magnitude of the ionic currents controlled by the $GABA_A$ receptor. When net positive ionic current is directed into the cell, GABA is excitatory, when the net positive current is directed out of the cell, GABA is inhibitory. A developmental switch in the molecular machinery controlling the polarity of this current is responsible for the changes in the functional role of GABA between the neonatal and adult stages. In spastic cerebral palsy in humans, GABA cannot be absorbed properly by the damaged nerve rootlets leading to certain muscles; this leads to hypertonia in those muscles.

GABA Supplements

There are many GABA supplements on the market today and they come in many forms, including drinks, foods, cream, tablets, sweets etc. Aside from manufactured supplements, there are some natural forms of GABA in foods such as GABA tea, sprouted brown rice and melon juice.

Need for GABA Production by microorganisms:

GABA supplements may not adequately penetrate the blood-brain barrier. Consumers should be wary and consider trying GABA-boosting alternatives, so using probiotic microorganisms is one of the best method for production of GABA as it is used to reduce symptoms of anxiety, help some schizophrenics, help to reduce high blood pressure, increase the effect of insulin so is useful for diabetics but not for hypoglycemia, suppress appetite, help with premenstrual symptoms, helpful for some cases of depression. Various types of LAB that show GABA-producing ability is an interesting target for food industry, especially fermented foods because individual strain has specific fermentation profiles such as acid production, taste and flavor formation ability. These profiles are considered as important factors in the use of LAB as starter cultures in the production of fermented foods. Recent research was undertaken to increase GABA-producing LAB in foods i.e. the use of *Streptococcus thermophilus* and *Lactobacillus delbreukii,* isolated from commercial yoghurt, and *Lactococcus lactis* from cheese starters. Hence, the aim of this study is to screen LAB strains which have ability to produce GABA.

Monosodium glutamate

It is used as a substrate for the GABA production by LAB. Monosodium glutamate is the sodium salt of glutamic acid. Glutamate is a naturally occurring amino acid that is found in nearly all foods, especially high protein foods such as dairy products, meat and fish and in many vegetables. Foods often used for their flavouring properties, such as mushrooms and

tomatoes, have high levels of naturally occurring glutamate. Glutamate is ubiquitous in nature and is present in all living organisms. It is the principal excitatory neurotransmitter in central nervous system. Glutamate is being used as food additive for enhancing flavour for over last 1200 years imparting a unique taste known as "umami" in Japanese. It is being marketed for about last 100 years. The taste of umami is now recognized as the fifth basic taste. Many of the foods used in cooking for enhancing flavour contain high amount of glutamate. Breast milk has the highest concentration of glutamate amongst all amino acids. Glutamate in high doses as gavage or parenteral injection has been reported to produce neurodegeneration in infant rodents. The neurodegeneration was not produced when gluamate was given with food. The Joint FAO/WHO Expert Committee on Food Additives, based on enumerable scientific evidence, has declared that, "glutamate as an additive in food" is not an health hazard to human being. Glutamate is used as signaling molecule not only in neuronal but also in non-neuronal tissues. Excessive accumulation of glutamate in the synaptic cleft has been associated with excitotoxicty and glutamate is implicated in number of neurological disorders. Excessive accumulation could be attributed to increase release, failure of transport system for uptake mechanism, neuronal injury due to hypoxia-ischemia, trauma and associated metabolic failures. As MSG is neurotoxic for less than 5 years old children, so, using an alternative to MSG may be a better substrate for GABA production.

Vigna mungo (split washed white Urad dal) is a grain legume widely cultivated in India and other asian countries. It is nutritious bean commonly cooked for healthy diet. It is a cheap source of protein. Seeds are commonly consumed and contain 17-34% of glutamate. The seeds of black gram contain a moderately high amount of calories (calorific value of 350 cal/100 g), carbohydrates (56.6%), proteins (26.2%) and fat (1.2%). In addition to being an important source of proteins and calories, black gram is rich in minerals; calcium

(185 mg/100 g), iron (8.7 mg/100 g), phosphorus (345 mg/100 g), and vitamins- vitamin B_1 (0.42 mg/100 g), vitamin B_2 (0.37 mg/100 g) and niacin (2 mg/100 g).

Food product containing *Vigna mungo* and wheat flour is easily prepared and deliver adequate amount of GABA to consumers which help in the prevention of hypertension, high blood pressure, cardiovascular diseases and improve nerve functioning**.**

Chapter 2- Review of literature

Due to increasing market demands on protein ingredients, several studies have been carried out on the novel proteins obtained from various sources (Lamsal et al., 2007). However, for a novel protein to be useful for food processing application, it should possess desirable functional and nutritional qualities (Mu et al., 2009). During the last decade the use of microorganisms considered probiotic (health promoting) has increased dramatically. Specifically, some lactic acid bacteria have been shown to provide protection against gastrointestinal disorders by stimulating the immune system (Cross et al., 2002). The theoretical basis for selection of probiotic microorganisms includes safety, functionality (survival, adherence, colonization, etc.) and technology (sensory properties, growth, stability, and viability) during manufacture (Saarela et al., 2000). It is well recognized that GABA can be produced by various microorganisms, particularly lactic acid bacteria (LAB) (Smith et al., 1999). LABs are capable of producing amino acids, peptides as a result of proteolysis; lactate, bacteriocin and gamma-aminobutyric acid as secondary metabolites (Stiles et.al., 1994). γ-aminobutyric acid (GABA), a four-carbon non-protein amino acid that is widely found in nature, is produced by the α-decarboxylation of glutamic acid catalyzed by a glutamate decarboxylase (GAD) (Kook et.al., 2004).

Unlike GABA production in animals and microorganisms, GABA production in plants is increased by the binding of calmodulin to Ca++, which activates GAD (Hiramatsu et al., 2007). Paper chromatography (PC) needs no expensive equipment, and is suitable for the routine analysis of large numbers of samples. This method was reported as a useful tool for both qualitative and quantitative determination of amino acids, for determining GAD activity (Y.S et al., 1967). Several studies have reported that GAD is widely distributed in environment mostly in plant materials (Tsushida et al., 1987). It is proved that GABA

production can be optimized and increased through submerged fermentation using novel strain (Yang et al., 2008). In bacteria, GABA is functionally involved in the germination of *Bacillus* megaterium spores and confers resistance to acidic pH in E. coli and *Lactococcus lactis* (Castanie et al., 2009). GABA has several physiological functions; e.g., it has hypotensive, diuretic, and tranquilizing effects (Wong et al., 2009). A recent study also showed that GABA strongly induces insulin secretion from the pancreas (Adeghate et al., 2002).

GABA production is affected by several factors including carbon and nitrogen sources, and fermentation conditions, these factors have to be taken into account in the design of efficient GABA production processes, applicable in industrial fields (Jakobs et al., 1988).GABA has been known to be effective to regulate several neurological disorders such as Parkinson's disease, Huntington's chorea, and Alzheimer's disease (Yu et.al., 2004). There have been several studies on the isolation of lactic acid bacteria (LAB) having the potential of rich GABA sources from traditional fermented food and on optimized GABA production using LAB for the industrial purpose. Because of the high value and popularity of GABA tea, several methods have been developed to determine GABA, either separately or with other amino acids, in different types of tea, including fresh tea shoots, green tea, Gabaron tea, black tea, oolong tea, pu-erh tea and microbe-fermented tea leaves (Okada et al., 2000). It is commercially useful to produce GABA using LAB, because the LAB can be used as starters of functional fermented food (Lu et al., 2009). The screening of lactic acid bacteria based on their capacity for synthesizing GABA may open new perspectives on production of GABA-enriched dairy products. During milk fermentation and proteolysis, a high level of L-glutamate may be theoretically liberated, since native caseins contain a high proportion of this amino acid.

To our knowledge, just a few reports have considered cheeses as a potential vehicle for GABA (Nomura et.al., 1998). GABA may improve the concentration of plasma growth hormone and the rate of protein synthesis in the brain (Settanni et al., 1999). GABA is involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety (Mody et al., 1994). Several LAB-producing GABA have been isolated from kimchi; *L. buchneri, L. brevis, Lactococcus lactis* (Kim et al., 2009). Studies have proved that some lactic acid bacteria having high glutamate decarboxylase (GAD) [EC4.1.1.15] activity could convert glutamate (Glu) into GABA (Hwang et al., 2007). Various microorganisms (bacteria, yeast, fungi) have been reported to produce GABA (Hao et al., 1993) and interest has been focused on the utilization and mass production of GABA as a bioactive food component. The GABA system is the target of a wide range of drugs active on the CNS, including anxiolytics, sedative-hypnotics, general anesthetics, and anticonvulsants (Macdonald et al., 1999). Much of the glutamate and GABA used as neurotransmitter is derived from glial storage pools of glutamine (Agranoff et al., 1998). GABA was shown to be released from electrically stimulated inhibitory nerve cells (Iversen et al., 1999).

The GABA receptor is a chloride channel regulated by GABA binding, and it is now grouped in the superfamily of ligand-gated ion channel receptors, which includes the well-characterized nicotinic acetylcholine receptor, present at the skeletal neuromuscular junction (Haefely et.al., 1994). The direct addition of chemical GABA to food is considered unnatural and unsafe. So it is necessary to find a natural method to produce and increase GABA in food (Cao et al., 2009). Certain benzodiazepines have considerable success in the treatment of some types of epilepsy (Haefely et al., 1994). Lactic acid bacteria are largely used in a variety of fermented foods, especially for the manufacture of dairy products with functional and probiotic properties and GABA was shown to be released from electrically stimulated

inhibitory nerve cells (Paul SM et al., 1995) and a mechanism of rapid removal from the synaptic release site was demonstrated by identification of high-affinity transporter proteins (Guastella et al., 1990).

The screening of lactic acid bacteria based on their capacity for synthesizing GABA may open new perspectives on production of GABA-enriched dairy products (Leroy et al., 2004**)**. GABA has not been determined in another type of Chinese tea, white tea. White tea is usually processed by withering and drying only, and in the absence of high-temperature fixation and rolling, it appears to retain more of its beneficial components. All general anesthetics enhance GABA function at anesthetic concentrations (Olsen RW et al., 2000). Although GABA is available in many fruits and vegetables, the concentrations are low in nature ranging from 0.03 to 2.00 μmol/g fresh wt. (Rhodes et. al., 1986). Due to the increasing commercial demand for GABA, diverse foods containing GABA produced biologically and chemically have been reported (Sawai et al., 2001). GABA has been considered as a strong secretagogue of insulin from the pancreas that may prevent diabetic conditions (Adeghate et al., 2002)**.** Several functional foods are manufactured: GABA-enriched green tea by anaerobic or cyclic treatments of tea leaves or shoots , GABA enriched rice germ by soaking in water , GABA-enriched brown rice by high-pressure treatment and germination , GABA-enriched germinated wheat through the activity of endogenous enzymes, and GABA-enriched fermented beverages such as tempeh-like beverage (Aoki et al., 2003). GABA has been derivatized using reagents such as dansyl chloride, nin-hydrin (Blinderman et.al., 1978). The theoretical basis for selection of probiotic microorganisms includes safety, functionality (survival, adherence, colonization, etc.), and technology (sensory properties, growth, stability, and viability during manufacture (Mogensen et al., 2000).

Chapter 3- Objectives

This dissertation aims in evaluating two preisolated and characterized strains of lactic acid bacteria for GABA production. The optimized culture conditions for GABA production and so finally incorporate the culture in the food product.

The following objectives were framed to achieve the following:

1) Characterization of GABA production by strain.
2) Optimization of culture conditions for enhanced GABA production.
3) Selection and optimization of GABA enriched food product.

MICROORGANISMS AND CULTURE MEDIUM

Culture medium:

MRS (de Man Rogosa Sharpe) broth was used for isolation and maintenance of characterized LAB strains. The fermentation medium in GABA production was MRS with 5% MSG (295mM (30.4g/l) of glutamate) added. All media were sterilized at 121°C for 20 min and cooled to room temperature prior to use. Cultivation was carried out in an incubator where temperature could be controlled automatically. The initial culture condition was 37°C, incubation temperature for 24 h, 12 h cultivation period, 1% inoculum volume, 6.2 initial pH of medium. Unless otherwise stated, the following tests were conducted in 250 mL flasks under the above conditions.

Materials:

All chemicals used were of analytical or biochemical reagent grade. MSG was used as substrate for GABA production by preisolated *Lactococcus lactis* and *Leuconostoc mesenteroides.* MSG used was of commercial grade obtained from local market in Patiala, Punjab.

Activation of culture:

Prior to use, the cultures were activated from the glycerol stocks maintained at -20ºC by inoculating in 5 mL MRS broth at 37ºC overnight.

SCREENING OF GABA PRODUCING LAB

The strains were cultured in MRS containing 5% monosodium glutamic acid (MSG) at 30ºC for 48 h. Culture broth was centrifuged at 1,500 ×g for 15 min at 4ºC, and GABA in

the supernatant was presumptively detected by using thin-layer chromatography (TLC). (Cho. et al., 2007)

THIN LAYER CHROMATOGRAPHY

Levels of GABA were determined qualitatively by TLC with aluminum TLC plate (Merck). MRS broth having *Lactococcus lactis* and *Leuconostoc mesenteroides* respectively were centrifuged at 1,500 ×g for 15 min, and 6 µl of supernatant was then spotted onto TLC plates. TLC was conducted using an acetic acid:1-butanol:distilled water (1:4:5) solvent mixture, and plates were subsequently immerged into 0.5% (w/v) ninhydrin solution and then heated at 110ºC for 10 minutes. Strain that gave the same Rf value as of GABA standard indicated that the strain produced GABA. (Cho. et al., 2007).

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

GABA in supernatant was then quantified using Spectrophotometric analysis.

SPECTROPHOTOMETRY

MRS broth containing cultures of *Lactococcus lactis* and *Leuconostoc mesenteroides* respectively were centrifuged at 15,000 x g for 10 minute and supernatant was taken. Culture supernatant (2µl) was added to 800µl of methanol, and then incubated at 25ºC in a water bath for 10 min. The reaction mixture was then dried using a speed vacuum concentrator and 1 ml of 70 mM $LaCl_3$ was added. Samples were then shaken for 15 min, centrifuged at 13,000 ×g for 5 min, and 800µl aliquots of supernatant were removed and placed in eppendorf tubes. Then, 160µl of 1 M KOH was added to the supernatant and shaken for 5 min, and centrifuged at 13,000 ×g for 5 min. The 1 ml assay system contained 550µl of the prepared supernatant, 200µl of 0.5M K^+ pyrophosphate buffer (pH 8.6), 150µl of 4mM $NADP^+$, 50µl of 2.5 units GABASE per ml, and 50 µl of 20 mM α-ketoglutarate. The initial absorbance was read at 340

nm before adding α-ketoglutarate, and the final absorbance was read after 60 min. The difference in A_{340} values was used to calculate GABA content in the culture supernatant against GABA standards (Cho. et al., 2007).

OPTIMIZATION OF CULTURE CONDITIONS

For efficient GABA production from monosodium glutamate using *Lactococcus lactis* and *Leuconostoc mesenteroides*, optimization of the reaction conditions (temperature, incubation time, pH and incubation time) was carried out.

Effect of pH on GABA Production: MRS media containing 5% MSG at the initial pH values of 4.0, 5.0, 6.0, 7.0 and 8.0 was used for cultivation of *Lactococcus lactis* and *Leuconostoc mesenteroides* to study the effect of pH on the GABA production.

Effect of Temperature on GABA Production: MRS media containing 5% MSG was kept at different temperature *viz*. 28ºC, 30ºC, 35ºC, 37ºC, 45 ºC for the cultivation of *Lactococcus lactis* and *Leuconostoc mesenteroides* to study the effect of temperature on the GABA production

Effect of Agitation on GABA Production: MRS Media containing 5% MSG kept on varied temperature, incubation time and pH were studied for the effect of agitation at 120 rpm and without agitation.

Effect of Incubation time on GABA Production: MRS Media containing 5% MSG kept at different incubation time 24 h, 48h, 72h, 96h, 120h to study the effect of incubation time on the GABA production

Preparation of fermented food product (Bhaturu): (by Tamang, 1998)

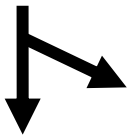
wheat flour and vigna mungo (urad dal) were

crushed and blended in the ratio 1:2, 2:1, 1:1

Inoculated it with *L.lactis, L.mesenteroides,* mixed culture (*L.lactis*

and *L.mesenteroides* in ratios 1:2 and 2:1) and control individually

Kept it for fermentation for 4hrs at 30ºC



Sample was withdrawn after every hour for measuring log Cfu/ml

After 4 hours the fermented dough was spread into a small chapatti (about 1cm thick) and allowed to stand for 15-20 min. The *chapatti* was fried in the vegetable oil at 80ºC till dark brown

After preparation of food product sensorial analysis was carried out including mouth feel, consistency, texture, color appearance, smell and taste.

MEASURING VIABLE COUNT IN DOUGH

Sample (1g) was withdrawn after every hour from prepared dough and diluted 10 fold with sterilized PBS. After that 0.1mL of aliquot was spread on MRS agar plate and incubated for 24 h at 30°C. Then the viable colonies were counted for each dilution and expressed as log cfu/g (colony forming unit/g).

EXTRACTION OF GABA FROM FOOD PRODUCT

Food samples were dried and ground into powder using a grinder. Ten grams of the food sample was extracted with 40 mL of distilled water for 2 h in a water bath (85ºC). The sample water extract was centrifuged at 12000 rpm for 10 min at room temperature. The resulting supernatant was filtered and GABA was qualitatively estimated by TLC and quantitatively determined Spectrophotometrically (Zhao et al., 2010).

PRODUCTION KINETICS OF GABA PRODUCTION IN FOOD

Optimized conditions (pH, temperature, incubation time, agitation) estimated above at which maximum production of GABA was observed by ***Lactococcus lactis*** and ***Leuconostoc mesenteroides*** were used to study the production kinetics of the fermented food product. The GABA production was plotted against the culture growth.

SCREENING OF GABA PRODUCING LAB

To screen LAB strains that produce GABA in culture medium, we cultivated the strains in MRS medium containing 295mM of glutamate (5%) and measured GABA concentrations in the culture supernatants of the two strains. These two strains, namely *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides*, were originally isolated from fermented foods in laboratory (Seema et al., 2010). Screening results on TLC revealed that these two strains produced GABA.

THIN LAYER CHROMATOGRAPHY

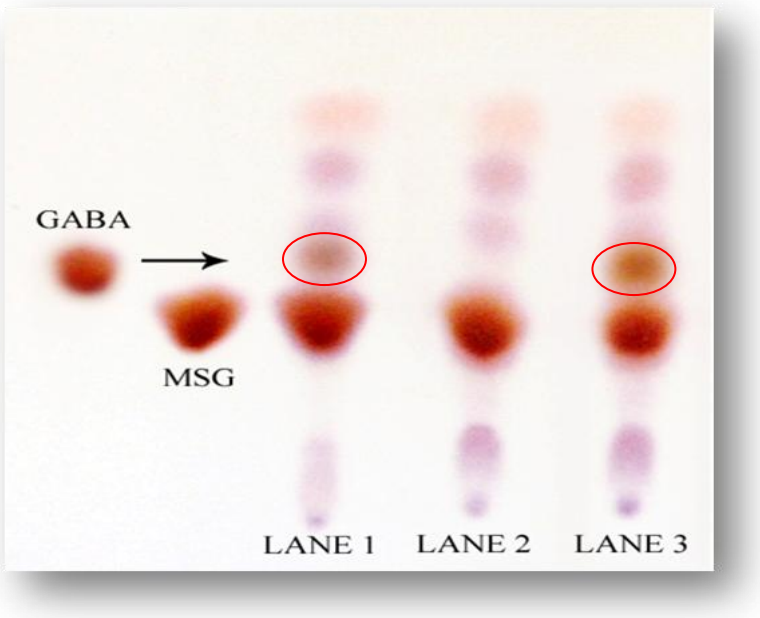


Fig. 5.1. TLC chromatogram of GABA production. GABA standard and MSG standard; Lanes 1, *L. mesenteroides*; 2, *control*; 3, *L. lactis*. Rf values of standard GABA & MSG are 0.47 & 0.35 respectively.

Figure 5.1 shows that *Lactococcus lactis and Leuconostoc mesenteroides* screened for GABA production in MRS media containing 5% MSG as a substrate, convert MSG to GABA.

Thin layer chromatogram of culture supernatant of *Lactococcus lactis*, *Leuconostoc mesenteroides* and control (without culture) in 5% MSG in MRS medium shows that Lane 1 and 3 exhibits GABA spots corresponding to the standard, and Lane 2 which has the control with no culture does not give any spot corresponding to the standard means that glutamate is converted to GABA only in lane 1 and 3. Cultures showing conversion of glutamate to GABA must have GAD enzyme which is the prime requirement for this conversion as discussed earlier. The TLC system described was extremely sensitive and gave good results with only 6µl of sample.

OPTIMIZATION OF CULTURE CONDITIONS

Effect of different pH on GABA production by *L. lactis* and *L. mesenteroides*

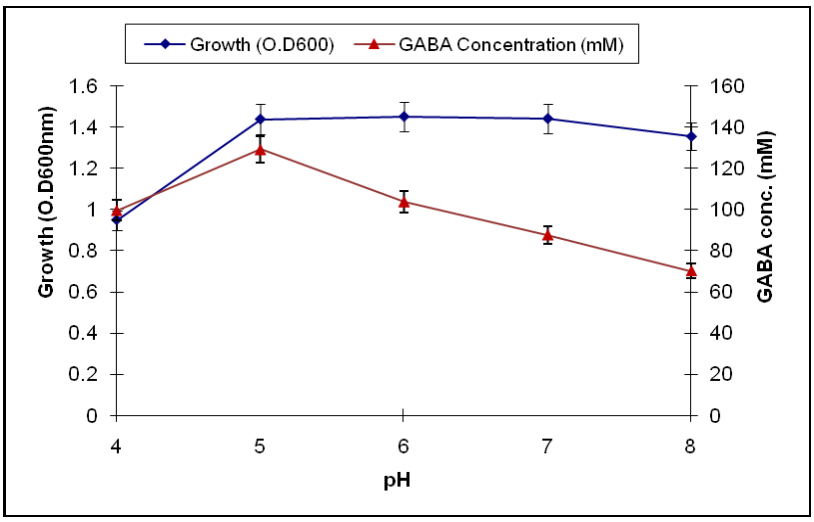


Fig. 5.2. Effect of pH on GABA production by *L.lactis* (♦) Growth (O.D.600nm) (▲) GABA concentration(mM)

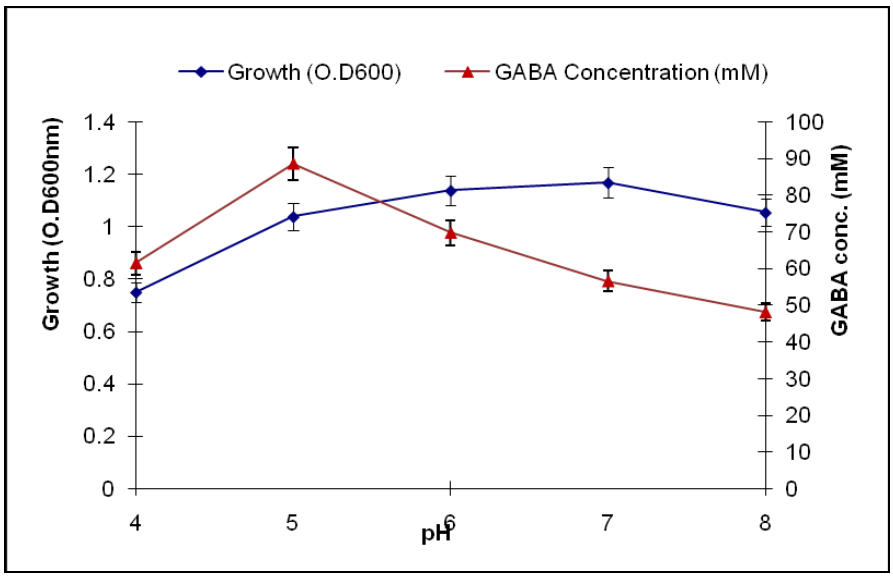


Fig. 5.3. Effect of pH on GABA production by *L.mesenteroides* (▲) Growth (O.D.600nm) (■) GABA concentration (mM)

It was reported that the GABA biosynthesis in LAB was strictly pH regulated. To investigate the effect of different pH levels on the production of GABA during the course of fermentation of *Lactococcus lactis* and *Leuconostoc mesenteroides*, the initial pH of the media were adjusted to 4, 5, 6, 7 and 8, respectively. As shown in Figure 5.2 and 5.3, pH has a significant effect on the production of biomass and GABA. A highest yield of GABA was obtained at pH 5.0 in the fermentation course even if the biomass was less than that of pH 6.0 after 24 h. The optimal pH value for the GABA production was 5.0 that accorded with the previous reports about the optimal pH values for maintaining the activity of LAB GADs that were in the range of 4.0 to 5.0. The higher or lower pH may lead to the partial loss of the GAD activity and so does the GABA production.

Effect of Temperature on GABA production: (*L. lactis* and *L. mesenteroides)*

The optimal temperature for GABA production was found to be 30°C. High GABA production [above 400 mM (41.12g/l) in *L. lactis* and 130 mM (13.4g/l) in *L. mesenteroides*] was obtained at the same temperature in comparison to other temperatures in MRS media supplemented with 5% MSG. GABA production rapidly decreased at temperature 28°C,

37°C (Fig. 5.4) and there was negligible production at 45°C. The same pattern of GABA production was observed with both the strains.

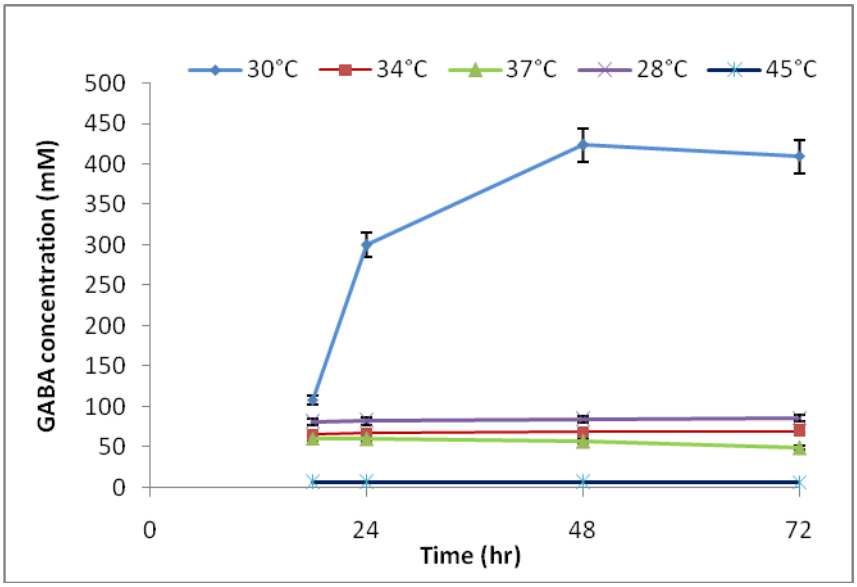


Fig. 5.4 Effect of Temperature on GABA production by *L.lactis* (♦) 30ºC (■) 34ºC(▲) 37ºC (×) 28ºC (+) 48ºC

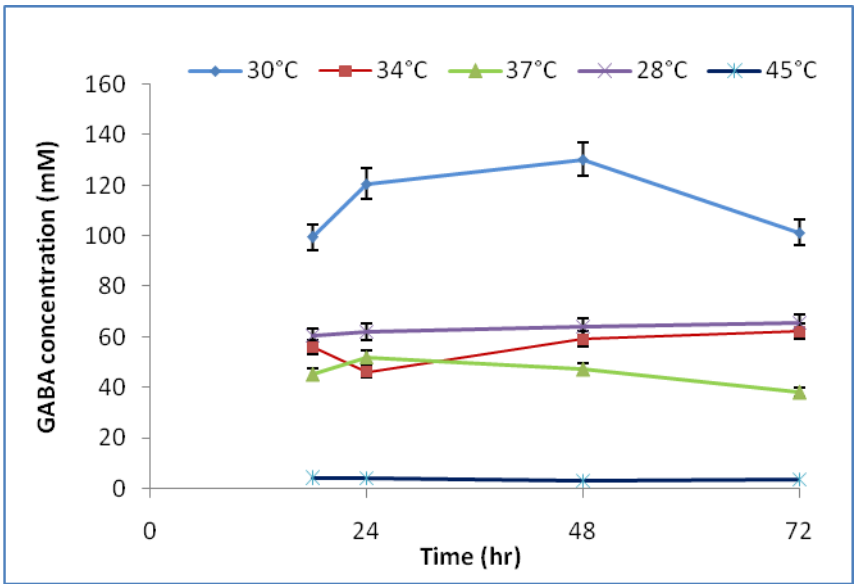


Fig. 5.5 Effect of Temperature on GABA production by *L. mesenteroides* (♦) 30ºC (■) 34ºC(▲) 37ºC (×) 28ºC (+) 48ºC

Figure 5.4 and 5.5 shows considerable variation in the GABA production under different fermentation temperatures (28ºC, 30ºC, 34ºC, 37ºC, 48ºC). It was observed that GABA production was maximum at 30°C. These data indicated that appropriate temperature

was beneficial to produce GABA, and excessively high temperature was unfavorable for the GABA production. The above results indicated that efficient conversion of glutamate to GABA needed not only high cell density but also appropriate temperature. By comprehensive consideration of the above data, 30°C was selected to be optimum temperature for GABA production.

Effect of Agitation on GABA production:

Agitation is a factor that strongly influences culture growth. The effect of agitation on GABA biosynthesis was investigated by incubating the inoculated standard basal media for GABA production at different temperatures at different pH and incubation time on shaking and static condition.

Without agitation

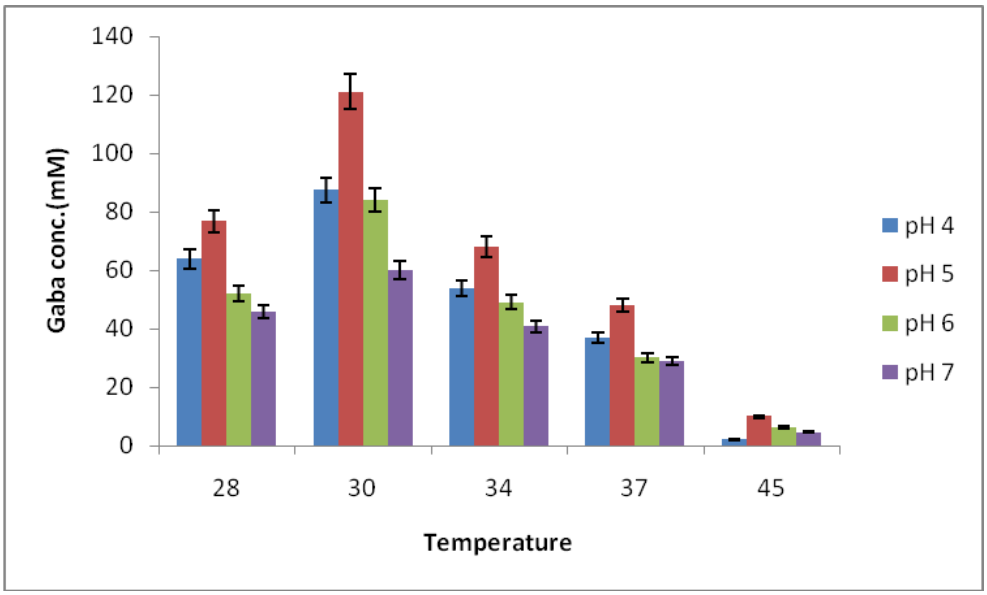


Fig. 5.6a. GABA production without agitation by *Lactococcus lactis*

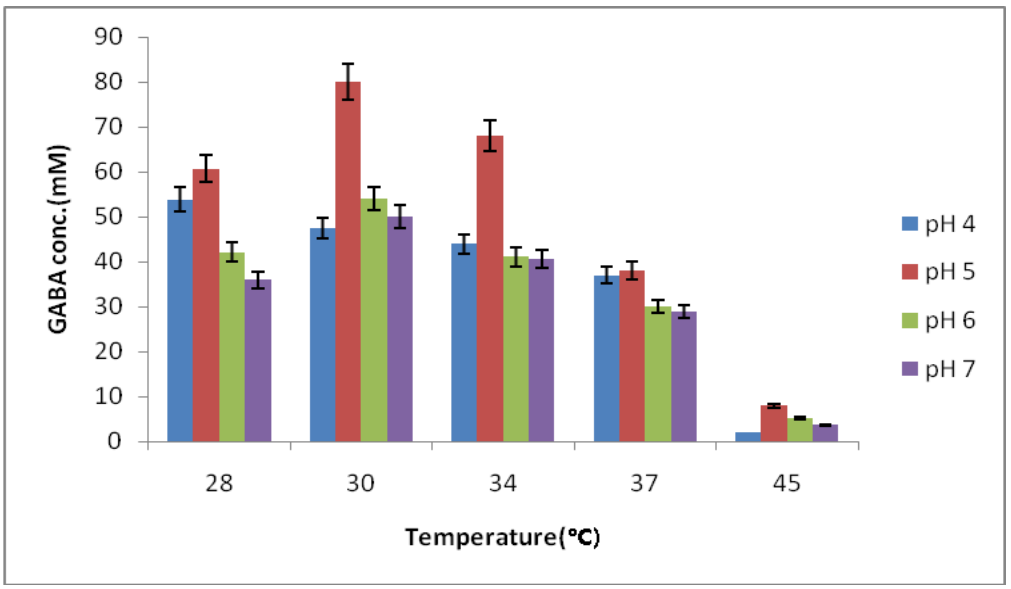


Fig. 5.6b. GABA production without agitation by *Leuconostoc mesenteroides*

With agitation:

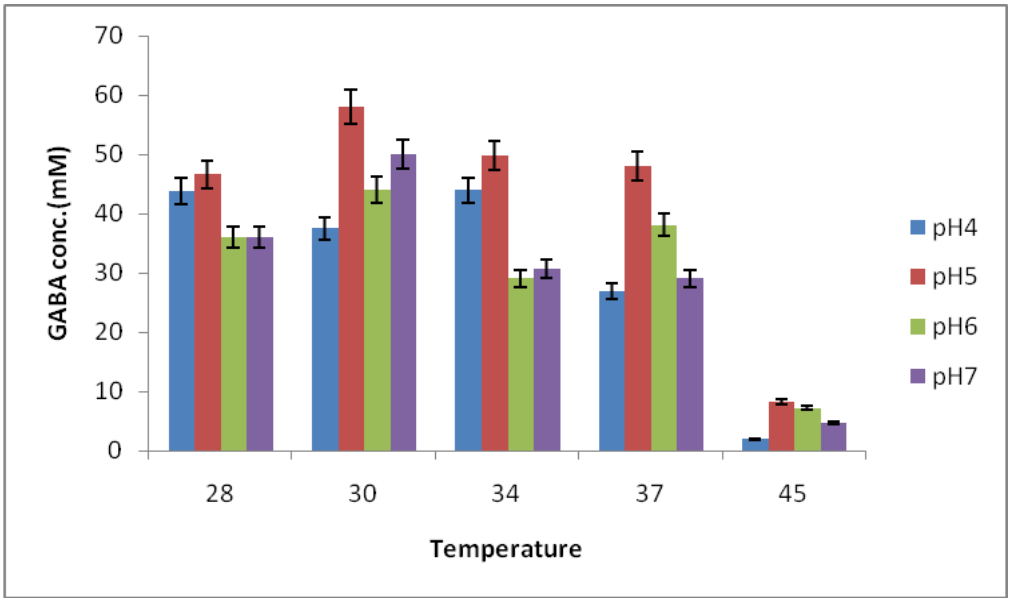


Fig. 5.7a. GABA production with agitation by *Lactococcus lactis*

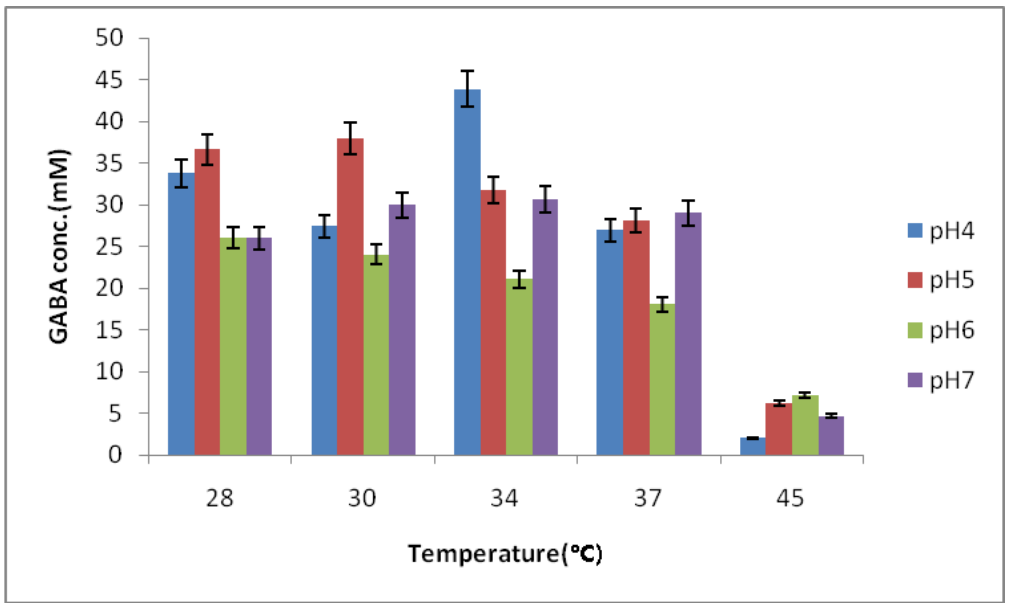


Fig. 5.7b. GABA production with agitation by *Leuconostoc mesenteroides*

In fig. 5.6a , 5.6b and 5.7a, 5.7b it is shown that GABA production is maximum at 30ºC temperature after 24 hours at 5 pH in both without agitation and with agitation at 120 rpm conditions but the concentration is significantly less under shaking conditions. As already discussed that 45ºC is unfavourable for GABA production in both the cases, it is clearly observed here also. The data shows that there is significant decrease in the GABA production under agitating condition.

Estimation of GABA: Thin layer chromatography in dough

Figure 5.8 shows that GABA is only produced in LANE 1 and LANE 3 containing *Lactococcus lactis* and mixed culture (containing *L. mesenteroides* and *L. lactis)* in the fermented dough containing vigna mungo as substrate, while in LANE 2 and LANE 4 having *L. mesenteroides* and control, there is no spot corresponding to GABA standard showing that Glutamate in medium is not converted to GABA. It is concluded from the above results that glutamate is converted to GABA only when *Lactococcus lactis* individually and in combination with *Leuconostoc meseneroides* in a mixed culture is present and not by control.

The TLC system described was extremely sensitive and gave good results with only 8µl of sample.

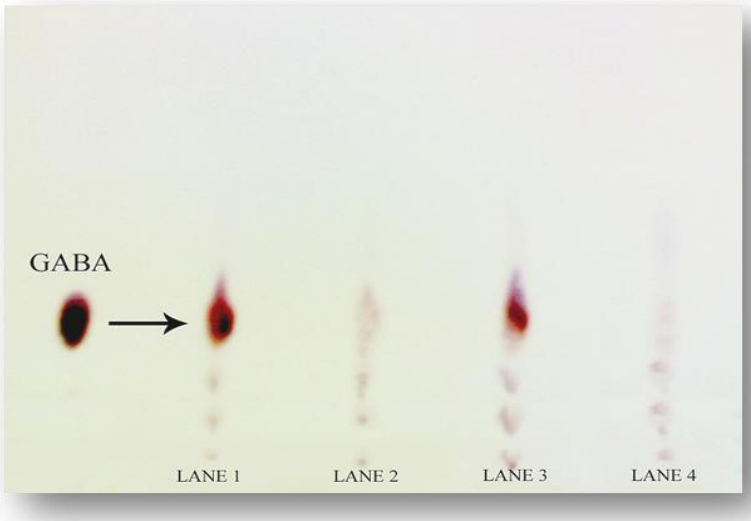


Fig 5.8. TLC of GABA production in dough. LANES 1, L.lactis; 2, L.mesen; 3, Mixed culture; 4, control. Rf value of standard was estimated to be 0.47.

Estimation of GABA in food product (bhaturu)

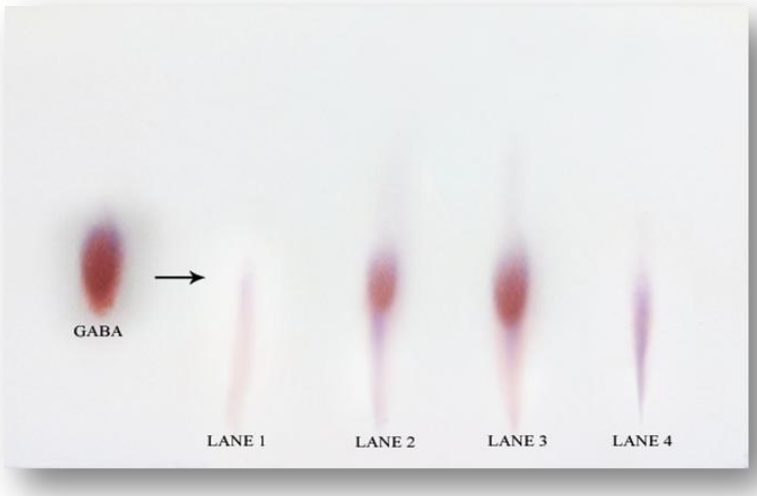


Fig 5.9: TLC of GABA production in fermented food product. LANE 1, control; 2, Mixed culture; 3, *L.lactis;* 4, *L.mesenteroides*. Rf value of GABA standard estimated to be 0.47.

Figure 5.9 shows the GABA production in the final food product after extraction of GABA from food product. LANE 2 and LANE 3 exhibits GABA spots in the same pattern as

already observed in dough i.e by ***Lactococcus lactis*** and combination of ***Lactococcus lactis*** and ***Leuconostoc mesenteroides* .**

Table 5.1: GABA Concentration (mM) in food product with different combinations of culture and substrate

Vigna mungo: wheat flour	**Control**	***L. lactis***	***L. mesenteroides***	**Mixed culture (*L.lactis : L.mesenteroides*)**		
				1:2	**2:1**	**1:1**
1:2	20.21	82.91	70.98	99.67	102.31	98.01
2:1	25.98	91.67	79.67	104	117.89	101.34
1:1	22.41	85.43	72.21	92.48	106.21	97.01

Sensorial analysis of food product

After the preparation of the fermented food product, viability of the product was tested on a panel of 5 persons based on which sensory analysis was done for the acceptability of the food product commercially.

Table 5.2. Sensorial analysis of the food product with mixed culture

Attributes	**Product**
Mouth feel (9)	7.4±0.05
Taste (9)	8.7±0.05
Smell(9)	8.8±0.05
Consistency	8.7±0.05
Texture (9)	7.4±0.05
Color and Appearance (9)	8.4±0.05
Overall acceptability(9)	8.5±0.08

The overall acceptability of the prepared food product (bhaturu) is 8.5±0.08. It is a delicious food that can be eaten with cooked vegetables or can be consumed raw when prepared with salt and spices.

Measuring viable count in dough

Figure 5.10 shows cfu/g in 10^{-5} dilution in dough which is maximum in mixed culture containing *Lactococcus lactis* and *Leuconostoc mesenteroides* followed by *Lactococcus lactis*, *Leuconostoc mesenteroides* and control (containing no culture)

It is shown in fig.10 that at 0 hour bacterial colonies were not observed as the fermentation starts on optimized conditions culture starts growing and viable colonies start observed after 1 hour and maximum viable colonies observed after 4 hours.

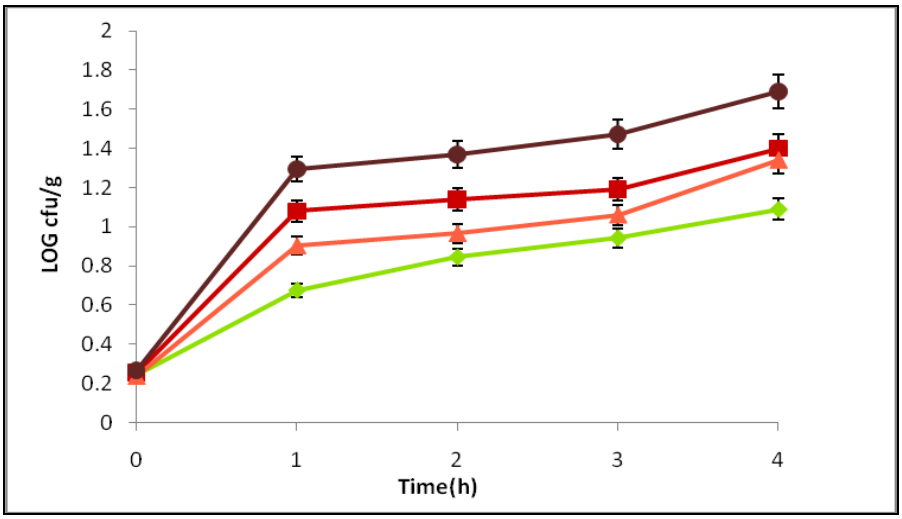


Fig. 5.10. Cfu/g of fermented food product prepared with (●) mixed culture, (■) *Lactococcus lactis*, (▲) *Leuconostoc mesenteroides*, (♦) control.

Production Kinetics of the Food Product (bhaturu):

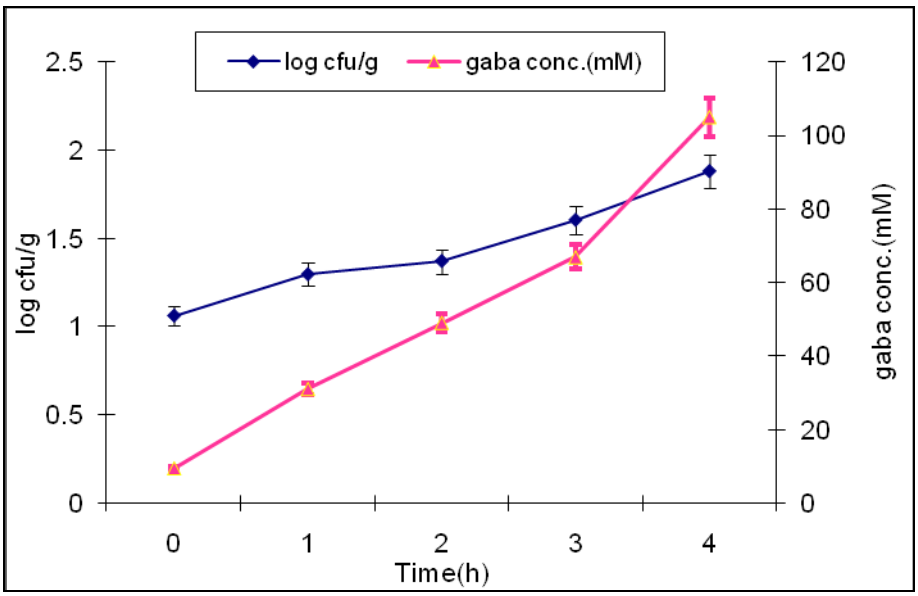


Fig. 5.11 Production kinetics of GABA by mixed culture (*L.lactis & L.mesen)*

Figure 5.11 shows the production kinetics of mixed culture. It is clear from the above graphs that as log cfu/g increases, GABA concentration also increases simultaneously. Initially as the cfu/g and GABA production is very less. As the incubation time increases, fermentation starts and cfu/g increases reaches maximum at 4 hours.

PREPARED FOOD PRODUCT

Fig 5.12. Prepared food product (bhaturu)

There have been several reports on GABA production from many food products as Malaysian brown rice (0.0001 g/l), Yoghurt (0.000425 g/l), Cheese (0.000383 g/l), Gaba tea (0.019 g/1 kg) and Kimchi (26.8 g/l) with various lactic acid bacteria as *L. acidophilus, L.*

buchneri, L. delbrueckii. To our knowledge, this is the first report of production of GABA (11.3g/l) by *L. lactis* in a fermented food product prepared *in vitro* conditions “Bhaturu”. It is a delicious food which can be consumed with cooked vegetables or can be eaten raw when prepared with added spices.

Chapter 6- Conclusion

In summary, GABA-producing LAB strains that had different physiological characteristics, such as growth rate and acid-producing ability, were screened for use as starters of fermented food. Acid-producing ability might be an important factor in the quality and taste of fermented foods. To develop fermented foods containing GABA with good taste, LAB strains with suitable acid and flavor production profiles should be chosen. To our knowledge, this is the first report of GABA production by *Lactococcus lactis* and *Leuconostoc mesenteroides*. Although experimental conditions differed from those previously reported for GABA production, GABA-producing ability of *Lactococcus lactis* was apparently higher than that of *Leuconostoc mesenteroides*. Our results showed that food product prepared from vigna mungo and wheat flour after 4 hrs fermentation produces 110 mM (11.3g/l) GABA. Future research will include screening of GABA-producing strains that have unique characteristics, such as acid production, flavor formation, growth rate, and higher GABA-producing ability. Such strains will accelerate the development of functional fermented foods.

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ANNEXURE

1 . Composition of MRS medium

Peptone	10.0g
Beef extract	10.0g
Yeast extracts	5.0g
Glucose	20.0g
Tween 80	1.0ml
Na_2HPO_4	2.0g
Sodium acetate	5.0g
Triammonium citrate	2.0g
$MgSO_4.7H_2O$	0.2g
$MnSO_4.4H_2O$	0.2g
Agar	15.0g
Distilled water	1000ml
pH	6.2-6.6