PREPARATION OF A TRADITIONAL FOOD WITH ENHANCED COGNITIVE EFFECTS

A

Dissertation

Submitted in partial fulfillments of the requirements

for the award of degree of

Masters of Science

In

Microbiology

By

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July 2013

This Thesis is dedicated to my parents and my brother

who always had been my inspiration ,

support and strength throught this work

Candidates's Decleration

I, hereby declare that the work presented in the dissertation entitled "**Preparation of a Traditional Food With Enhanced Cognitive Effects"** in partial fulfillment of the requirement for the award of the degree of Masters in Microbiology , Department of Biotechnology and Environmental Sciences Thapar University Patiala, is an authentic record of my own work during the period of six months from January 2013 to July 2013, under the supervision of **Dr. Abhijit Ganguli**, Associate Professor, Department of Biotechnology and 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 : Thapar University

Date : 15 .07. 2013

Certificate

This is to certify that the thesis entitled " **Preparation of a Traditional Food with Enhanced Cognitive Effects** " submitted by Vibhuti Batra in partial fulfillment of the requirements for the award of Degree of Masters of Science in Microbiology 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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VIBHUTI BATRA

CONTENTS

LIST OF ABBREVIATIONS

LAB	Lactic acid bacteria
GABA	Gamma-aminobutyric acid
MRS	De man rogosa sharpe medium
FIM	Foundation for Innovation in Medicine
GAD	Glutamic acid Decarboxylase
STAI	State and Trait Inventory
TLC	Thin layer Chromatography
Ml	Milliliters
cfu	Colony forming units
mM	Millimolar
Rf	Retardation factor
µl	Microliters
RDA	Recommended dietary allowance

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ABSTRACT

This study reports the production of a GABA enriched traditional Indian snack 'Groundnut Chikki'. An indigenous isolated *Lactococcus lactis* was used to produce to produce GABA in the food matrix. Optimized conditions were 37°C, pH 5, 1% inoculum and incubation for 24 hours for strain viability resulting in GABA conc (9.03mM/g) in chikki. Sensory evaluation based on 5-point Hedonic scale showed overall acceptability of groundnut chikki; GABA content and Sensory scores remained unaltered for 2 months. Reduction in physiological parameter, anxiety followed by consumption of GABA enriched chikki, compared with state and trait anxiety was noted in individuals, further a strong positive significant correlation relates state and trait anxiety, with trait anxiety higher in boys ($\Delta = 5.24$, $p < 0.00$) than state anxiety ($\Delta = 2.0, p < 0.00$). Results of the study indicate possibility of application of this GABA enriched popular snack food for reducing anxiety, Clinical trials are however necessary.

INTRODUCTION

CHAPTER 1- INTRODUCTION

Functional Food is a Natural or processed food that contains known biologically-active compounds which in defined quantitative and qualitative amounts provides a clinically proven and documented health benefit, and thus, an important source in the prevention, management and treatment of chronic diseases of the modern age. A food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being or reduction of risk of disease and consumed as part of a normal food pattern.

There are several functional foods for instance - a natural food such as fruit or grain which may be modified by plant breeding or other technologies (e.g. lycopene-enhanced tomatoes, vitamin E-enriched vegetable oils, vitamin A-enriched rice) with improved health properties (e.g. a juice drink with enhanced antioxidant content, a yogurt with added prebiotic or probiotic, a food to which a component has been added (e.g. a spread with added phytosterols) or removed (e.g. a yogurt with reduced fat). Some functional foods can potentially promote optimal mental state and mental performance and influence behavior for example: cognitive performance, mood and vitality, reaction to stress, short-term memory, vigilance and attention.

Due to the risk of toxicity and adverse effects of drugs, consumers have increasingly resorted to food supplements to improve health where pharmaceuticals fail. This resulted in a world wide nutraceutical revolution. According to Defelice a nutraceutical can be defined as, "a food (or a part of food) that provides medical or health benefits, including the prevention and treatment of a disease". According to this concept, the nutraceuticals cover everything, including dietary supplements, fortified foods, functional foods and medical foods (Brower, 1998; Hardy, 2000; Kalra,2003).Such products may range from isolated nutrients, dietary supplements and diets to genetically engineered "designer" foods, herbal products and processed foods such as cereals, soups and beverages. The old proverb "an apple a day will keep the doctor away" is now replaced by "a nutraceutical a day may keep the doctor away". The idea behind the mode of

action of nutraceuticals is to provide functional benefits by increasing the supply of natural building blocks in the body. Nutraceuticals is a broad term used to describe any product derived from food sources that provides extra health benefits in addition to the basic nutritional value found in foods. Nutraceuticals on the market today consist of both **traditional foods** and **non-traditional foods**. Traditional nutraceuticals are simply, natural, whole foods with new information about their potential health qualities. Examples include Lycopene in tomatoes, omega -3 fattyacids in salmon, GABA (gamma- aminobutyric acid) in green tea. Non-Traditional nutraceuticals, are foods resulting from agricultural breeding or added nutrients or ingredients, to boost their nutitional value. Examples include β-carotene –enriched rice, and soyabeans, orange juice fortified with calcium, cereals with added vitamins or minerals.

1.1 GABA (gamma-aminobutyric acid)

GABA (gamma-aminobutyric acid) is an amino acid. It is a natural calming and anti-epileptic agent. It is vital for proper brain functioning. GABA also helps our bodies make endorphins – chemicals that make us feel happy.

Neurotransmitters are chemical messengers between neurons (nerve cells). They inhibit or slow down the actions of the neurons. GABA is the main inhibitory (calming) neurotransmitter in the brain. Other neurotransmitters act like an accelerator. They increase the speed of the actions of the neurons. These are called excitatory neurotransmitters. **Glutamate** (a common amino acid) is the major excitatory neurotransmitter in the brain. If there is something that creates **anxiety,** panic, stress excitatory neurotransmitters are released and a person can feel restless, rapid heartbeats and high blood pressure. Thus, GABA is used in the treatment of anxiety, depression, panic disorders, substance abuse recovery, manic-depressive (bipolar) disorder and seizures.

Structure and Conformation

GABA is found mostly as a zwitter ion. 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 is about 50 kcal/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 salvation 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.

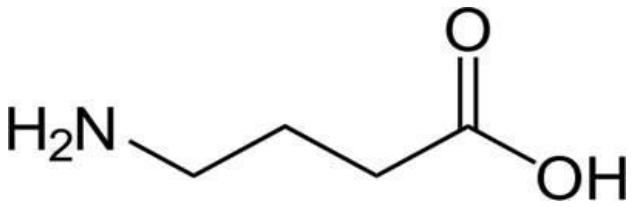

Fig.1.1- Structure of GABA Molecule.

History

Gamma-amino butyric 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.

Synthesis of GABA

GABA is synthesized by enzyme glutamate decarboxylase (GAD) (EC 4.1.1.15), a pyridoxal 5-phosphate-dependent enzyme that catalyzes the irreversible - decarboxylation of L-glutamate to GABA. GAD is widely distributed among mammals, plants and micro-organisms, including lactic acid bacteria (LAB). GAD requires Vitamin B6 (pyroxidal phosphate) as a cofactor, which can be used to regulate the levels of GABA. GABA production in LAB relies upon GAD and is induced when pH becomes acidic. Some of LAB are capable of surviving under strongly

acidic conditions due to the presence of active GAD that utilizes intracellular proton to make LAB acid-resistance. GAD isolated from LAB exhibits an optimum pH at around pH 4-5.

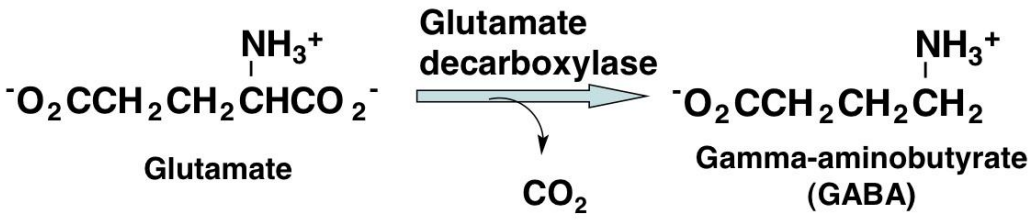

Fig 1.2- Synthesis of GABA from Glutamate.

GABA Receptors

GABA receptors are probably the most common kind in the mammalian nervous system. It is estimated that close to 40% of the synapses in the human brain work with GABA and therefore have GABA receptors.

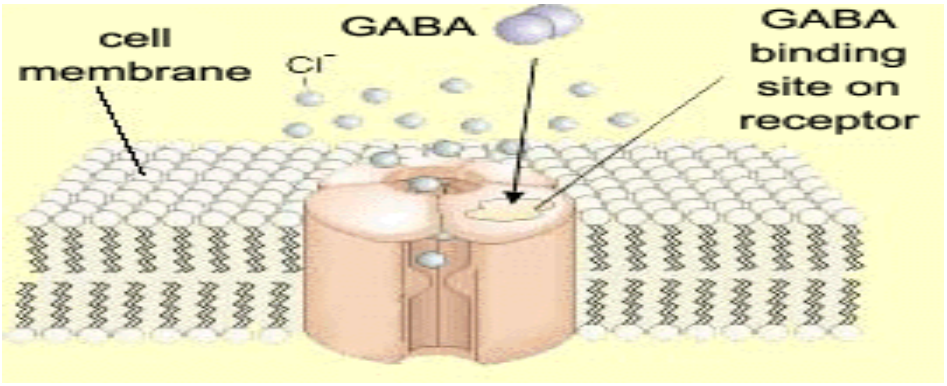

Fig 1.3- Action of GABA Receptors.

GABA receptors are channel receptors. This means that when GABA binds to them, they change shape slightly to allow ions to pass through their central channel. This channel mainly allows negatively charged chloride ions to enter the neuron, thus reducing its excitability. Because of this property of the GABA channel receptor, GABA is classified as an inhibitory neurotransmitter.

Working of GABA

When GABA binds to a nerve cell receptor, it opens the nerve cell so that chloride ions which are present in the brain are allowed to move into the nerve cell and slow the activity of the cell, and the person normally experiences a calming feeling. For example, if the brain produces more excitatory neurotransmitters like nor epinephrine or epinephrine (adrenaline) than normal, we humans become anxious or have more stress than normal.

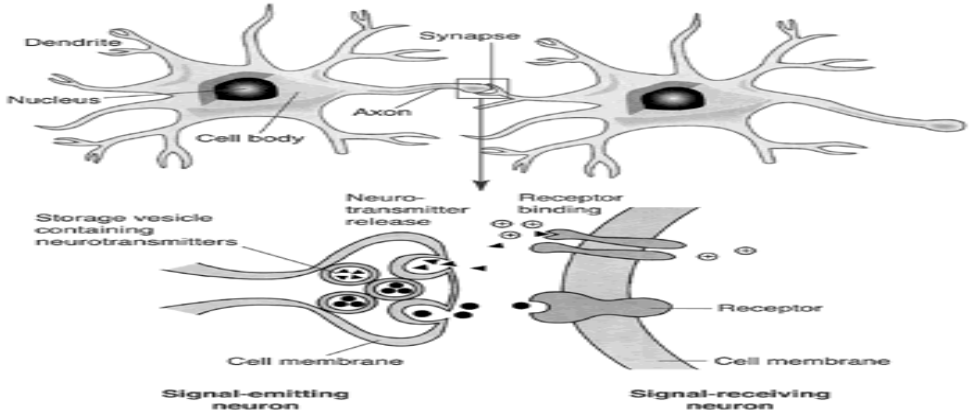

Fig 1.4- Mechanism of GABA action in Brain. .

Fig 4 depicts the major components of a **typical neuron**, including the cell body with the nucleus; the **dendrites** that receive signals from other neurons; and the **axon**, which relays nerve signals to other neurons at a specialized structure called a **synapse**. When the nerve signal reaches the synapse, it causes the release of chemical messengers (i.e., neurotransmitters) from storage vesicles. The neurotransmitters travel across a minute gap between the cells and then interact with protein molecules (i.e., receptors) located in the membrane surrounding the signal-receiving neuron. This interaction causes biochemical reactions that result in the generation, or prevention, of a new nerve signal, depending on the type of neuron, neurotransmitter, and or receptor involved.

1.2 ROLE OF GABA IN COGNITION BEHAVIOUR

GABA is the calming and peacemaker chemical in the brain that can induce relaxation, reduces stress, anxiety, depression and help increase focus**.** GABA's natural function is to reduce the activity of the neurons to which it binds. Due to its relaxation effects, GABA may be considered as a sleep aid. GABA A receptors are highly expressed in the thalamus, a region of the brain involved with sleep processes. GABA-agonist drugs, such as zolpidem (Ambien) and temazepam (Restoril), are sedatives used in the treatment of insomnia. The synthetic GABA-like drug gabapentin that increases brain GABA levels has been found to improve sleep disturbances. Research indicates oral GABA supplementation may be beneficial for epilepsy. Besides animal and clinical studies have examined the effect of a combination of GABA and phosphatidylserine (PS) in the treatment of various types of seizure disorders. GABA increased the concentrations of plasma growth hormone and the rate of protein synthesis in the brain (Tujioka et al., 2007, 2009), improved many brain functions such as memory and study capability, and lowered the blood pressure. GABA is one of the main ingredients in products that are marketed as growth hormone precursors to naturally stimulate the endocrine system to secrete endogenous growth hormone. Some researchers believe that one of the purposes that GABA serves is to control the fear or anxiety experienced when neurons are overexcited.

Anxiety is a subjective feeling of tension, apprehension, nervousness, and worry associated with arousal of the nervous system (Spielberger, 1983). The concept of Anxiety originated in the Classical Greek period (McReynolds, 1975) and conceptually developed in parallel to self-concept and self-awareness in western thought. (Aubery et al., 1970) defined anxiety "as an emotional state, with a subjectively experienced quality of fear as a closely related emotion''. Anxiety has been defined as a state, trait, a stimulus, a response, a drive and as a motive. (Sarason et al., 2003) defined that anxiety is a basic human emotion consisting of apprehension and uncertainty that typically appears when an individual perceives an occurrence as being a threat to the ego or self esteem. In its conceptualization, individuals with high levels of anxiety generally hold heightened levels of trait anxiety, but in evaluative situations, the state of anxiety also elevates. These are differentiated in state and trait anxiety.

State anxiety is transitory emotional state reflective of one's interpretation of a particular stressful situation at a particular period of time..

However **trait anxiety** is the enduring personality characteristic which refers to relatively stable individual differences that characterizes people's anxiety or general feeling of anxiety (Spielberger, 1983). This is usually perceived as how people feel across typical situations that everyone experience on daily basis.

1.3 MULTIDIMENSIONALITY OF STATE AND TRAIT ANXIETY

Freud (1924), Spielberg (1983, 1985) and Taylor (1953) conceptualized trait anxiety as being one-dimensional, and Spielberg (1985) conceptualizes state anxiety as one-dimensional, it is our contention (Endler et al., 1997,1991, 1989,1991) to conceptualize and empirically demonstrate that both state and trait anxiety are multidimensional constructs. There are at least four facets of Trait anxiety: social evaluation, physical danger, ambiguous and daily routines; and two facets of State anxiety: cognitive worry and autonomic-emotional.

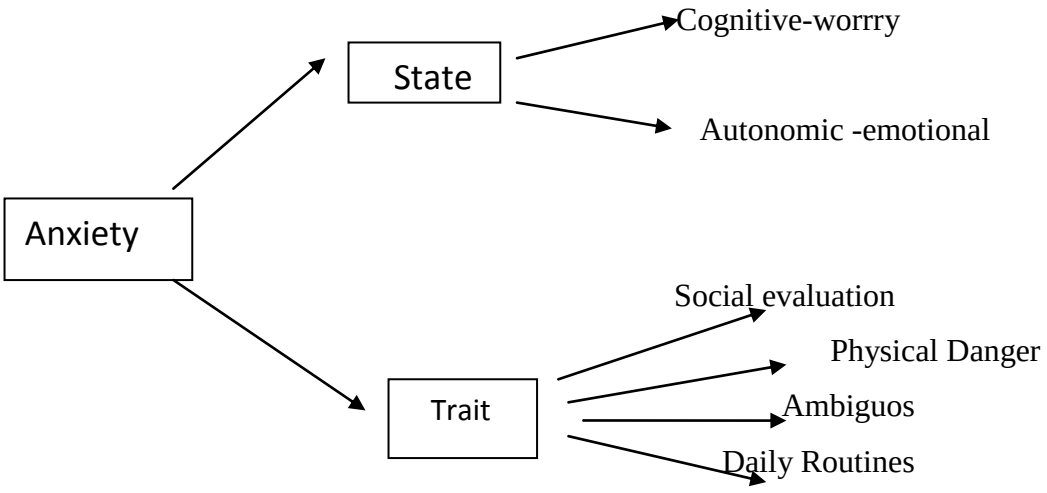

Fig 1.5- The multidimensionality of State and Trait Anxiety. (Norman et al., 1999)

MEASUREMENT

The anxiety is measured by state and trait (STAI), which means that high scores of state and trait determines high level in cognitive anxiety (Spielberger, 1983).The instrument was validated for reliability and validity test. (Nunnally et al., 1978) recommends that instruments used in basic research have a reliability value of about 0.70 is better. Validity test interpreted by inter correlation of items according to (Sekaran et al., 2003) should be ≥ 0.30.

State Trait Anxiety Inventory (STAI)

This instrument has forty items of questions with two subscales: The S-Anxiety scale (STAI Form Y-1) consists of twenty statements that evaluate how respondents feel about anxiety currently, at this moment". The standard test form is to the write on each item-statement that best describes the intensity of their feelings: (1) not at all; (2) somewhat; (3) moderately so; (4) very much so. In responding to the T-anxiety scale (STAI Form Y-2) consists of twenty statements that assess how people "generally feel" about anxiety with four point scale: (1) almost never; (2) sometimes; (3) often; (4) almost always.

1.4 FUNCTIONAL FOODS CONTAINING GABA

Functional foods containing GABA includes 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, dairy products. GABA is present in small quantities in many plant sources, vegetables for example spinach, potatoes, cabbage asparagus (broccoli), tomatoes, Citrus fruits like orange, banana, apple and apricot which contains potassium which is highly essential for strengthening muscles and nerves and is a great antioxidant as well. Besides these GABA is also found in, oily fish such as mackerel and salmon are very essential for the proper functioning of the brain. It lifts us from state of depression. GABA fermented Soya beans are highly nutritious and rich. It helps in depressing the elevating mode of blood pressure. It has a potent antioxidant property and helps in fighting chronic diseases like cancer and cardiac disorders. Chocolates are known to be comfort food and at times as they, trigger the endorphins and reduce depression. Chocolates also contain

caffeine, magnesium and phenyl ethylamine, which lift spirits. Peas are rich in Thiamin (vitamin B1), which is a potent mood influencer so are Brazil nuts, are full of selenium. Oats are sedatives, acts as a hot porridge for a good sleep. Increased amount of GABA is found mainly in fermented products, especially fermented dairy products, soy sauce, cheese. However, few studies have emphasized on traditional formulated food products which are not fermented for instance. Such foods may be important for alleviating stress, anxiety in adults and decreasing chances of non- communicable diseases and help maintain a healthy life.

Review of literature

Chapter 2- REVIEW OF LITERATURE

During the last decade, fundamental studies opened a new field of research dealing with bioactive or biogenic substances derived from foods. Recently, growing attention has been paid to eating natural, minimally processed, nutritional, and healthful foods as a way to live a healthier life. As a result, the market for functional foods, or foods that promote health beyond providing basic nutrition, is flourishing. Within the functional foods, is the small but rapidly expanding arena of probiotics (Suvarna et al., 2005). Probiotics, defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by the Food and Agricultural Organization (FAO 2001), have become a major topic of lactic acid bacteria (LAB) research over the past 10 years (Kailasapathy and Chin 2000). Probiotics mainly include the genera *Lactobacillus* and *Bifidobacterium*.

Lactobacillus (LAB) can be the predominating members of the endogenous intestinal flora in humans, which are reported to exert beneficial effects including the activation of the immune system, reduction of serum cholesterol, and inhibition of the growth of potential pathogens that may cause infections in the host (Holzapfel et al., 2001, Ishibashi and Yamazaki 2001). Therefore, incorporation of these probiotic bacteria in food to increase its therapeutic value has become a popular trend (Ishibashi and Shimamura 1993; Blanchette and Roy 1995; Pestka et al., 2001; Wang et al., 2006; Rekha et al., 2008; Kitawaki et al., 2009). The theoretical basis for the 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). LAB are capable of producing amino acids, peptides as a result of proteolysis, lactate, bacteriocin and gamma-amino butyric acid as secondary metabolites (Stiles et al., 1994). Researchers have investigated various microorganisms to produce GABA, including Escherichia coli (Rice et al., 1993), Aspergillus (Kato et al., 2002), and lactic acid bacteria (Komatsuzaki et al., 2005; Park and Oh 2007; Di Cagno et al., 2010). Lactic acid bacteria are largely used in a variety of fermented foods, especially for the manufacture of dairy products with functional and probiotic properties (Leroy et al., 2004). LAB are essential in the processing of food

materials and they have been applied extensively in the food industry (Leroy et al., 2004). LAB can enhance the shelf-life and safety of foods, improve food texture, and contribute to the nutritional value of food products and pleasant sensory profile of the end-use products (Lucke et al., 2000). The existence of the *gdh* gene in LAB which is responsible for the production of glutamic acid has been proven (Tanus et al., 2005). **Employing LAB, on the other hand, with the potential to produce glutamic acid can facilitate production of functional foods rich in bioactive molecules such as GABA**. The screening of lactic acid bacteria based on their capacity for synthesizing GABA may open new perspectives on production of GABA-enriched products. Moreover, scientists have intensively researched GABA bioconversion and elucidated its biotransformation mechanism using LAB because of the generally regarded as safe (GRAS) status of LAB (Sanders et al., 1998; Small and Waterman 1998; Lorca and de Valdez 2001; Cotter and Hill 2003; Fu et al., 2008).

Synthesis of GABA enriched food

GABA (γ-amino butyric acid) is a four carbon non-protein amino acid that is widely distributed in nature among microorganisms, plants and animals (Ueno et al., 2000). Natural GABA was first found as a constituent of tuber tissue in potato (Steward et al., 1949). In microorganisms, GABA confers resistance to acidic pH in bacteria, including *E.coli*, *Lactococcus lactis*, *Listeria monocyrogenes*, *Mycobacterium* and *Clostridium perfringens* (Castanie et al., 1999, Cagno et al., 2005, Sanders et al., 1994). Nowadays, GABA is used considerably in pharmaceuticals, and massively as a major active constitute in foods, such as gammalone, cheese, gabaron tea, and shochu (Nomura et al., 1998, Sawai et al., 2001, Yokoyama et al., 2002). GABA acts as the major inhibitory neurotransmitter in the mammalian central nervous system and shows well known physiological functions: neurotransmission, induction of hypotension, and diuretic and tranquilizer effects (Wong et al., 2003; Jacobs et al., 1993). GABA also exerted positive effects for treatment of sleeplessness, depression, and autonomic disorders (Okada et al., 2000), chronic alcohol related symptoms (Oh et al., 2003) and stimulation of immune cells (Oh and Oh 2003). GABA improves the plasma concentration, growth hormones and the protein synthesis in the brain (Cho et al., 2007), In addition, GABA has hypertensive, tranquilizing, diuretic and anti diabetic effects (Adeghate et al., 2002, Capitina et al., 2003). GABA lowers the blood

pressure in animals and human subjects. The oral administration of GABA of 10 mg daily for 12 weeks was effective for hypertensive patients (Izquierdo et al., 2009). The daily oral administration of rice germ containing 26.4 mg GABA was effective in treating neurological disorders (Okada et al., 2000). Furthermore, GABA acts as a strong secretagogue of insulin from the pancreas, therefore, effectively preventing diabetes (Adeghate et al., 2007). GABA intake can regulate sensations of pain and anxiety, and lipid levels in serum (Kono et al., 2000, Miura et al., 2006). Consumption of GABA-enriched foods can inhibit cancer cell proliferation (Park et al., 2007) and improve memory and the learning abilities (Miura et al., 2006).

There have been many attempts for synthesizing GABA chemically or biologically (Choi et al., 2006, Huang et al., 2007, Plokhov et al., 2000) because of the beneficial functions of GABA and the increasing commercial demand (Ueno et al., 2000). Biosynthetic methods of GABA may be much more promising than chemical synthesis methods since they have a simple reaction procedure, high catalytic efficiency, mild reaction condition and environmental compatibility (Huanh et al., 2007). GABA is synthesized by glutamate decarboxylase (GAD; EC 4.1.1.15), a pyridoxal 5-phosphate-dependent enzyme, that catalyzes the irreversible-decarboxylation of glutamate to GABA. GAD was largely distributed in higher plants, animals, and bacteria (Ueno 2000; Komatsuzaki et al., 2005). Some reports showed the presence of GAD activity in lactic acid bacteria also (Komatsuzaki et al., 2005, Nomura et al., 1998, Cho et al., 2007). The sequence of the GAD gene was reported for *Lactobacillus brevis* (Park and Oh 2007), *Lactobacillus plantarum, Lactobacillus delbrueckii subsp. bulgaricus* (Makarova et al., 2006; Siragusa et al., 2007), *Lactobacillus paracasei* (Komatsuzaki et al., 2008), and *Lactococcus lactis subsp. lactis* (Nomura et al., 1999).

LAB as GABA producers

Natural addition of GABA is demanding over the addition of chemical nutrient GABA since consumers prefer naturally-occurring substances, and the fermentation helps to reduce the cost of the foods due to the omission of chemical addition of GABA and also provides attractive foods with better taste and at the same time replaces the chemical GABA by natural GABA (Li et al., 2008). Therefore, GABA production by naturally-occurring microorganisms during fermentation is getting higher request.

Especially, the production of GABA by LAB has been extensively explored during the manufacture of black raspberry juice, kimchi, soymilk, cheese and other dairy products (Nomura et al., 1998, Kim et al., 2009, Shelp et al., 1999, Tsai et al., 2006, Hayakawa et al., 1997, Izauierdo et al., 2009, Skeie et al., 2001). For the manufacture of functional foods and beverages, LAB also has the advantage of the production of GABA using cheap ingredients, such as by-products in food industry (Li et al., 2010). The GABA producing LAB act as probiotics but are only effective if they remain viable as they pass through stomach and intestine (Chou et al., 1999, Nishida et al., 2008). In fact, GABA-producing LAB as probiotics could inhabit in the gastrointestinal tract and produce GABA *in situ*. (Li et al., 2006). There have been long and safe histories of the production of fermented foods and beverages by LAB, which can accumulate high amounts of GABA (Leroy et al., 2004). These GABA producing LAB accumulate high amount of GABA and also protect foods by controlling the food spoilage pathogens by secreting bacteriocins (Djenane et al., 2005). 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 the efficient GABA production processes, applicable in industrial fields (Jakobs et al., 1988).

Role of GABA in anxiety and stress

Central nervous system (CNS) operates by a fine-tuned balance between excitatory and inhibitory signaling. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter and it was discovered in the mammalian brain in 1949 (Awapara et al., 1950). Dysfunction of the GABAergic system was showed to be associated with schizophrenia, cerebral stroke, temporal lobe epilepsy (TLE), Parkinson's disease (PD), Huntington's disease (HD) and anxiety disorders, and so forth (Kleepner et al., 2001, Pascual et al., 1998, Nutt et al., 2001). GABAergic system is also implicated in cognitive processes, such as memory formation and consolidation (Izquierdo et al., 1995), GABAergic system is one of the rapidly emerging therapeutic targets for various neurological disorders (krystal et al., 2002, Nutt et al., 2002, Linderg et al., 1982).

Anxiety, a subjective feeling of tension, apprehension, nervousness, and worry associated with arousal of the nervous system (Spielberger, 1983). The high level of anxiety causes a person normal life being difficult such as interfered activities and physiological disturbances

and social life. Anxiety is one of the wide varieties of emotional and behavior disorders (Rachel and Chidsey, 2005). Anxiety disorders represent common, serious and growing health problems world-wide (Kessler et al., 2005; Miller 2006; Murray and Lopez 1997; Wong and Licinio 2001). The disorders share high levels of co morbidity (Kessler et al., 2005; Merikangas et al., 2003), and those suffering from these disorders not only face debilitating disruptions to their psychological well-being, but are at high risk for suicide (Licinio and Wong 2005) and somatic conditions such as heart disease, gastrointestinal disorders and obesity (Harter et al., 2003; Rumsfeld and Ho 2005; Sheps and Sheffield 2001). The causative factors underlying anxiety, however, remain poorly understood (Cryan and Holmes 2005; Wong and Licinio 2001; Wong and Licinio 2004), and it is clear that improvements in understanding these factors and the development of better treatments are needed (Cryan and Holmes 2005; Holmes and Cryan 2006; Wang et al., 2005). GABA reduces pain, slow heart beat, decrease anxiety, induces calm. (Fang et al., 2008; Hui et al., 2000; Korber et al., 2002). Involvement of the GABAergic system in the regulation of certain key processes in brain has been long known (Sieghart et al., 1995). However, its major role in regulation of anxiety and fear was accepted much later (Gray et al., 1984; Haefely 1992), leading to a number of different theories (Coupland and Nutt, 1995).

Thus, like the memory-regulating effects of this system, its anxiety-modulating role underwent some evolution before it was generally accepted. Significant evidence to support the view of GABA modulation of anxiety-related behaviors came from intensive studies of brain "anxiety" topography it has been shown that areas rich in GABA-A receptors such as amygdala, hippocampus and medial septum are major areas for perception of, and reaction to, anxiety (Gray et al., 1984).

The important role of GABA in modulation of anxiety, fears, phobias depression has been reported in many studies (Gray et al., 1984; Haefely, 1992; Coupland and Nutt, 1995) However, in addition to those effects, there are data indicating that the central GABAergic system may play key role in cognitive processes, including memory formation and consolidation (Gray et al., 1984; Izquierdo and Medina, 1991, 1995; Davis, 1994). In addition, there are many consistent clinical observations linking anxiety and cognitive processes (Gray 1987, Lang 1986; Cole et al., 1994).

AIMS AND OBJECTIVES

CHAPTER -3- AIMS AND OBJECTIVES

SCOPE OF THE THESIS

In this study a previously characterized strain was investigated and utilized for developing a low cost GABA enriched traditional snack food product.

The following objectives were framed to achieve the above:

1. Characterization and Optimization of GABA production by putative LAB.
2. Optimization of GABA production in food product.
3. Formulation of Traditional Food Product.
4. Effect on cognitive Behavior (Anxiety) of developed food product.

Materials and Methods

Chapter -4 MATERIALS AND METHODS

4.1 MICROORGANISMS AND CULTURE CONDITIONS

Culture Medium:

MRS (de Man Rogosa Sharpe) (see Appendix 1) broth was used for isolation and maintenance of characterized *Lactococcus lactis*. LAB (lactic acid bacteria) was isolated from yam pickle samples that showed the highest GABA content (99.0mg/100 g of product) (Bhanwar et al., 2012).

Culture Conditions:

Prior to use, all cultures were activated from glycerol stocks maintained at -20^{0}C by inoculating in 5ml MRS broth, followed by incubation at 37^{0}C overnight.

Materials:

All chemicals used were of analytical or biochemical reagent grade.

4.2 SCREENING OF GABA PRODUCING LAB

The isolate was cultured in MRS at 37^{0}C for 48 hrs. Culture broth was centrifuged at 15,000 rpm for 15 min at 4^{0}C, and GABA in the supernatant was presumptively detected and estimated both qualitatively and quantitatively as followed by TLC (thin layer chromatography), and using GABASE assay.

Thin Layer Chromatography (TLC) was performed with silica gel plate (Merck Co; Germany; 60 F254, 0.25mm). Standards of GABA (Sigma-Aldrich) was prepared at conc. 0.1M. MRS broth containing *Lactococcus lactis***,** was centrifuged at 15,000 rpm for 15 min and 4 µl was spotted on TLC plate. TLC was developed by using a solvent mixture of acetic acid : butanol : water (1:1:4) (Choi et al., 2006), and was subsequently immerged into 0.5 % (w/v) ninhydrin solution. The TLC plate was dried in the oven at 100°C for 10 minutes.

Any sample that gives the same Rf value as GABA indicates that the strain produced GABA.

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

GABA in supernatant was then quantified using Spectrophotometric analysis.

Quantitative Estimation:

MRS broth containing overnight grown cultures was centrifuged at 15,000 rpm for 10 minutes and supernatant was taken. Culture supernatant (2µl) was added to 800 µl of methanol, and was incubated at 25^0C in a water bath for 10 minutes. The reaction mixture was dried in incubator overnight and 1ml of 70mM $LaCl_3$ was added. Samples were then shaken for 15 minutes, centrifuged at 13000 rpm for 5 min, and 800 µl aliquots of supernatant was removed and placed in eppendrof tubes. Then 160 µl of 1M KOH was added and shaken for 5 minutes and centrifuged for 13,000 rpm for 5 min. The 1ml assay system contains 550 µl of supernatant, 200µl of 0.5 M K+ pyrophosphate buffer (pH 8.6), 150 µl of 4mM NADP+, 50 µl of 2.5 units GABASE per ml , and 50µl of 20mM α-ketoglutarate. The initial absorbance was read at 340nm before adding α-ketoglutarate, and the final absorbance was read after 60 minutes. The difference in the A_{340} value was used to calculate GABA content in culture supernatant against GABA standard. (Cho et al., 2007).

4.3 FORMATION OF A TRADITIONAL FOOD PRODUCT.

Groundnut Chikki is a traditional north Indian snack food. It is an economical snack, easy to prepare, easily available, easily consumed by all age groups and easy to store. *Lactococcus lactis* isolated from yam pickle has been reported to produce highest GABA concentration (99.0 mg/100g) (Bhanwar et al., 2012) among various LAB strains. *Lactococcus lactis* specializes in lactate dehydrogenase excreting lactic acid, which is used to preserve food and extend food shelf life. Due to the ability of *Lactococcus lactis* to survive in solid food matrix and produce GABA, it was selected as a potent strain for food preparation. Groundnut Chikki was made from two major ingredients, Jaggery and Peanuts.

Jaggery and peanuts (Arachis hypogaea) of A- grade quality was purchased from nearby grocery store. They were weighed and crushed in equal proportions. Jaggery was heated at a temperature of 40°C and culture (*L. lactis*) (1%) was added with gradual stirring. Jaggery was cooked till its color changed to brown .To this, Peanuts were added and rolled over the mixture. This was allowed to solidify in molds at room temperature for 2 hours. After solidification, it was removed from molds and stored in air tight containers at room temperature. A small proportion (1 gram) of the sample was withdrawn after 4 hours, homogenized at different time intervals in sterilized PBS (phosphate buffered saline). Aliquots to 100 µl was spread on MRS agar plate and incubated for 24-48 hours at 37°C for analyzing Viability. Since, it was acceptable so, Chikki containing *L.lactis* was initiated.

After preparation of product **sensory analysis** was carried out including mouth feel, consistency, texture, color appearance, smell and taste and Overall acceptability (5point Hedonic scale).

4.4 ANALYSIS OF GABA PRODUCTION IN GROUNDNUT CHIKKI.

Groundnut Chikki was crushed using sterile motor pestle. Ten grams of the sample (chikki) was extracted with 40ml of distilled water for 2 hours in water bath (85°C).The sample water extract was centrifuged at 12000 rpm for 10 minutes at room temperature. The resulting supernatant was filtered and GABA was qualitatively estimated by TLC as described by (Cho et al., 2007), spectrophotometrically by the method of Zhang and Bown (1997) and confirmed by HPLC according to Rossetti and Lombard (1996) with minor modifications.

4.5 OPTIMIZATION OF CONDITIONS FOR GABA PRODUCTION

Optimized conditions (incubation time, inoculum size, and pH) was observed for maximum production of GABA by *Lactococcus lactis* in Groundnut Chikki, which were used to study the production kinetics of traditional food product.

4.6 COMPOSITIONAL ANALYSIS OF GABA ENRICHED CHIKKI

Total sugar was determined according to the method of (Dubois et al., 1956). Phenol reagent (1 ml, 5%) was added to 0.2 ml samples of aqueous solution containing 10-60 µg of

carbohydrate in a test tube. Concentrated H_2SO_4 (1 ml, 96%) was added rapidly while mixing. Samples were allowed to stand for 10 minutes, heated in a water bath (30°C) for 20 minutes and cooled to room temperature. Absorbance was read at 490 nm. Quantity of carbohydrate (grams) was calculated utilizing glucose as standard (see Appendix 2) and multiplying unknown concentration (~g/ml) times the total volume of unknown extract.

Total protein content was estimated by a method described by (Lowry et al., 1951). Five grams of extract was mixed with 5 ml distilled water in centrifuge tube. After vortex for 2 min, tube was centrifuged for 10 min at 2700 rpm. A volume of 0.2 ml supernatant was taken in a test tube and after making the volume 1ml with distilled water, 3 ml of reagent C were added, which was made by mixing 2 ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1 ml of reagent B (0.5% copper sulfate in 1% potassium sodium tartarate). After adding 0.1 ml of Folin- Ciocalteau reagent, tube was incubated for 30 min at room temperature. The absorbance was measured at 750nm.The amount of total protein present in the sample was calculated from standard curve using BSA as standard.
(See Appendix 2).

The **total flavonoid** content was determined by using of a modified colorimetric method described previously (Zhishen et al., 1999). An aliquot of 0.25 ml of sample was mixed with 0.6 ml distilled water, 5 % $NaNO_2$ solution (0.06 ml) and the mixture was allowed to stand for 5 min at room temperature. After 6 min 10 % $AlCl_3$ solution (0.15 ml) was added to the mixture. Immediately, 1 N NaOH (0.5ml) and 0.45 ml distilled water were added to the mixture and allowed to stand for another 30 min. Absorbance of the mixture was determined at 510 nm and (+) catechol was used as standard compound for the quantification of total flavonoid content. (see Appendix 2). All values were expressed as milligram of catechol equivalents per 1 gram dry weight.

Functional Components

The **reducing power** was estimated by the Fe3+-Fe2+ transformation in the presence of the fractions as described by (Fejes et al., 2000). The Fe2+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Meir et al., 1995), 2.5 ml of the sample was dissolved in 2.5 ml of distilled water, 2.5 ml of 1% potassium ferricyanide was incubated at

50°C for 30 min and 2.5 ml of 10% tri chloroacetic acid was added to the mixture and centrifuged for 10 min at 10000 rpm. About 5 ml of the supernatant was diluted with 5 ml of water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Ascorbic acid (1mg/ml) was used as the standard. (See Appendix 2).

The **total Polyphenols** content was obtained by Folin – Ciocalteau reagent method described by (Malik et al., 1980). The dilute extracts were taken in 10ml glass tubes and total volume made to 3ml with distilled water these are then mixed with 0.5ml Folin – Ciocalteau reagent (1:1 with water) and 2 ml Na_2Co_3 (20 %). A blue colored complex, molybdenum blue developed in each tube, as the phenols undergo a complex redox reaction with phosphomolibdic acid in Folin–Ciocalteau reagent in alkaline medium. The tubes containing the blue solutions were kept at room temperature for 30 minutes warmed and absorbance was measured at 765 nm against the reagent blank. The standard curve was prepared using known concentrations of Gallic acid at 765 nm. (See Appendix 2).

The **total antioxidant** capacity of the sample was determined by phosphomolybdate method using ascorbic acid as the standard (Prieto et al., 1999). An aliquot of 0.3ml of the extract solution (100µg) solution was combined with 3.0 ml of reagent (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using an UV spectrophotometer. The blank solution contained 3.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as µg equivalents of ascorbic acid by using the standard ascorbic acid graph. (See Appendix 2).

4.7 SENSORY EVALUATION

5-Point Hedonic Scale is known as “degree of liking scale”. The hedonic scale assumes that consumer preferences exist on a continum and that preference can be categorized by responses based on likes and dislikes. The sensory quality of Groundnut Chikki was evaluated by a panel of 10 trained judges by grading for sensory analysis and overall

acceptability. The scores were given on a five-point hedonic scale where 1 represents worst and 5 represents best.

4.8 STORAGE ANALYSIS

Groundnut Chikki was stored in air tight containers and its GABA content was compared before and after storage for 2 months.

4.9 COGNITION STUDY ANALYSIS OF GABA ENRICHED CHIKKI

Purpose For this study we selected students to compare their State and Trait anxiety levels. As students go through the intensive academic and practical curriculum of graduate level coursework they are faced with many different stressful situations. Students can feel anxiety over a variety of these situations and each student deals with their anxiety in different way. The body's biological response is a major component of the physical sensation of anxiety. The two forms of anxiety were studied "state" or short term/situational anxiety and "trait" which are a more constant personality type of anxiety. State is situational anxiety, meaning the current state you are in or what you are currently experiencing where as trait anxiety is more of a stable characteristic or personality trait.

This project aims to compare state versus trait anxiety using the Spielberger State Trait Anxiety Inventory (STAI) among students (boys and girls) before and after consumption of GABA enriched Groundnut Chikki, at Thapar University, Patiala in three created Phases.

Ethical Considerations

The study was evaluated and approved by the Human Ethics Committee, TU; prior consent of students was also taken.

Participants and Procedure

The study consisted of thirty Students (N=30), 20 males and 10 females. The respondents were second year first semester degree undergraduate students. The aim of study was firstly, to compare anxiety among students. Secondly, among boys and girls.

The participants were given 6g of 'Groundnut Chikki' (having 42.18mM/g concentration of GABA) (RDA of GABA for anxiety- 5 g to 10 g given once a day) to eat and after a time interval of 45 minutes they were asked to fill questionnaire which includes the S-Anxiety scale (STAI Form Y-1) and T-Anxiety scale (STAI Form Y-1). The STAI has 40 items of question and took approximately 20 minutes to complete. The students first read and answered if they had problems the researcher will guide students to answer the questions. The experiment was conducted in phases in which different levels of stress was given to the students.

PHASE 1-A stressful environment was created by informing the students about the lab assessment.

PHASE 2-A cheerful environment was created by making them watch a comedy movie related to the subject.

PHASE 3- A normal environment, with no changes.

Data Analysis:

All data analysis were performed using SPSS Version 20.0 (SPSS Inc, USA), at a Type I error rate of 0.05. (Spielberger et al., 1970). Data collected from the participants were cleaned and prepared for analysis. Apart from descriptive statistics (measures of central tendency and dispersions), 't'-tests , correlations and ANOVA were used for verification of study.

Results and discussions

CHAPTER 5- RESULTS AND DISCUSSION

5.1 MICROORGANISMS AND CULTURE CONDITIONS

Lactic acid bacteria (LAB) constitute a heterogeneous group of genera which share many physiological features. LAB owes their designation to their capacity to ferment sugars primarily into lactic acid via homo or heterofermentative metabolism (Salminen et al., 1998). Among LAB strains, *Lactococci*, are homo fermentative microaerophilic Gram-positive bacteria which grow at a temperature of 10°C, but not at 45 °C, and produce L (+) lactic acid from glucose. They are characterized by white colonies of ovoid cells which appear individually, in pairs, or in chains when cultivated on MRS media.

5.2 SCREENING OF GABA PRODUCING LAB

Lactococcus lactis was reported as a potential GABA producer (Bhanwar et al., 2012). Its probiotic effects were also developed.

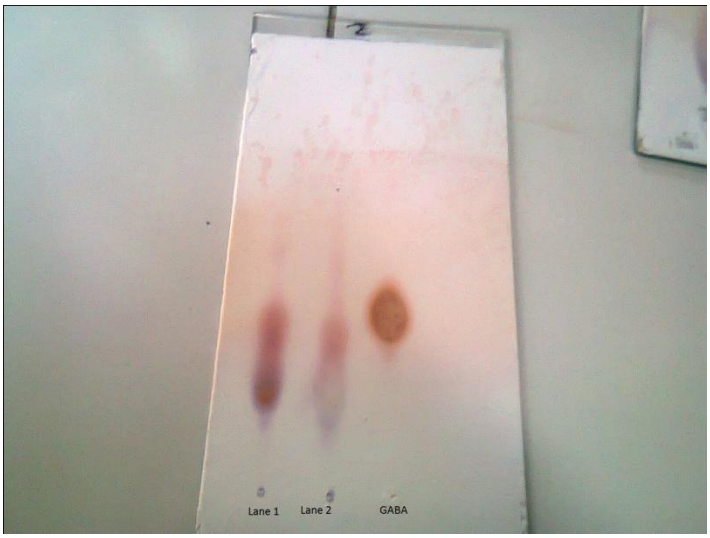

Fig 5.1-TLC chromatogram of standard and GABA production by *l.lactis*.

Lane 1- Gaba production by *L.lactis*, Lane 2-(center to lane1) is control, Lane 3- (extreme right to lane 1) is GABA**.**

Fig 5.1 shows Thin layer chromatogram of culture supernatant of *Lactococcus lactis* in MRS medium. Lane 1 shows GABA spots (Rf= 0.53) corresponding to the standard (Rf=0.54), and Lane 2 does not give any spot corresponding to standard. The TLC system

was convenient to provide satisfactory results. The results were confirmed by HPLC (results not shown).

5.3 FORMULATED FOOD – GROUNDNUT CHIKKI

Fig 5.2- Formulated food Product (Groundnut Chikki).

There has been several reports on GABA production from many food products like Brown Rice (0.0001 g/l), Yogurt (0.000425 g/l), Cheese (0.000383 g/g), Gaba tea (0.019 g/l) and Kimchi (26.8 g/l) with various lactic acid bacteria *L.buchneri, L. delbrueckii and L.acidophilus.* To our knowledge, this is the first report of production of GABA (7.13mM/g) by *L.lactis* in a traditional snack food product.

5.4 ANALYSIS OF GABA PRODUCTION IN GROUNDNUT CHIKKI

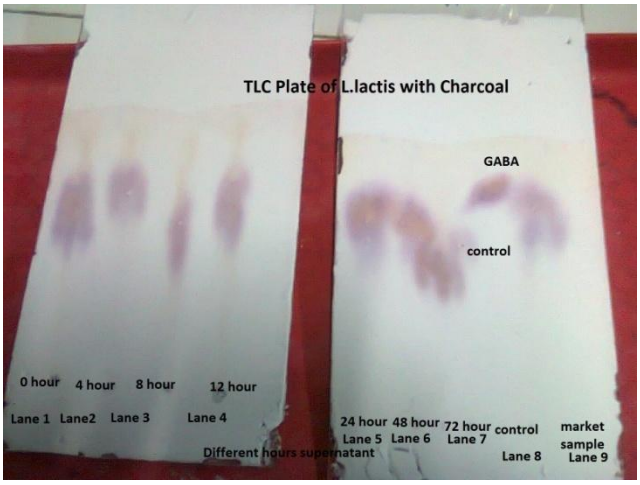

Fig 5.3- TLC of GABA production in Chikki by *L.lactis* at different time intervals, followed by a GABA standard, Control and commercial product.

Lane 1, 2 ,3 ,4 ,5, 6, 7 as depicted (fig 5.3) are supernatants of 0 hour, 4 hour, 8 hour, 12 hour, 24 hour, 48 hour and 72 hour chikki and shows GABA spots corresponding to GABA standard (Rf= 0.64) Lane 9 shows a commercial product with no GABA spot.

TLC Chromatogram of GABA production after Remolding

Groundnut Chikki was remolded at a temperature higher (55°C) than for survival of *L.lactis* (45°C) after 24 hour and 48 hour respectively, to inactivate *L.lactis* in Chikki and to compare GABA content.

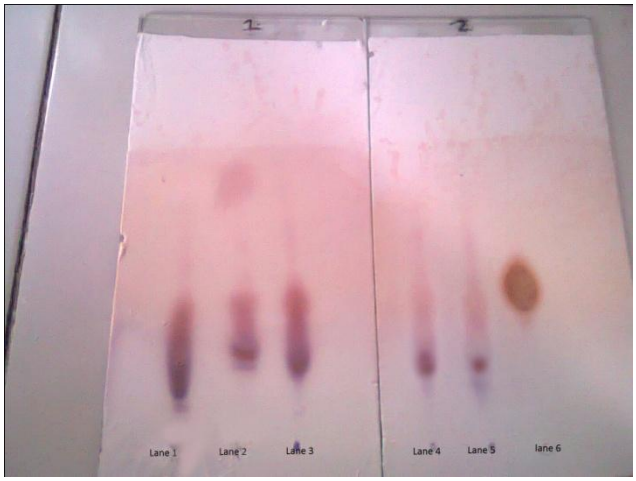

Fig 5.4-TLC of GABA production in Chikki by *L.lactis* after remolding withdrawn at different time intervals, followed by a GABA standard and a Control.

Lane 1 shows GABA production at 0 hour. Lane 2, 3 shows GABA production at 24 hour before and after remolding chikki. Lane 4, 5 shows GABA production before and after 48 hours with respect to GABA standard in lane 9. (fig 5.4). The identification of GABA was done on the basis of Retention Factor (Rf =0.53) with authentic GABA samples.

TLC Chromatogram of GABA production after Storage (2 months).

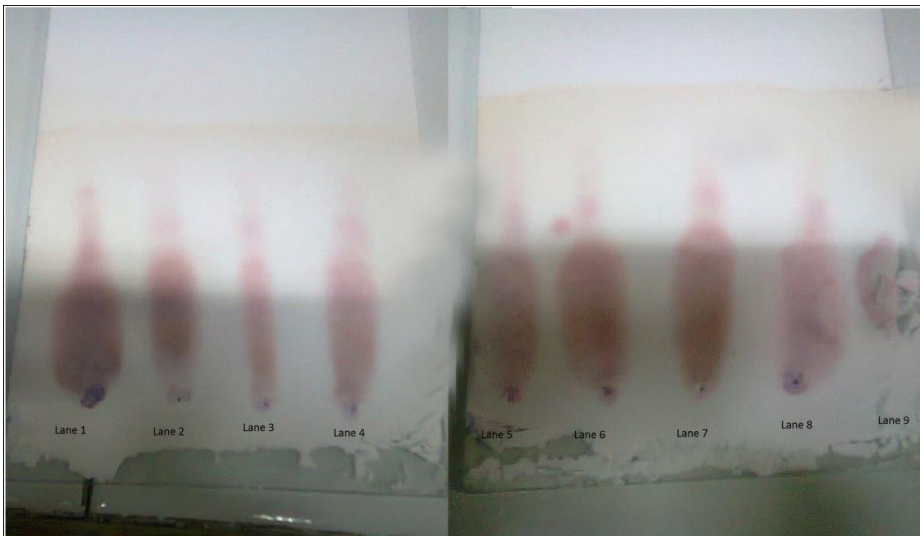

Fig 5.5-TLC of GABA production in Groundnut chikki by *L.lactis* after storage at different time intervals, followed by a GABA standard and a Control .

GABA spots are visible in Lane 1,2,3,4,5,6,7 (fig 5.5) that is in supernatants of 0 hour, 4 hour, 8 hour, 12 hour, 24 hour, 48 hour and 72 hour chikki after storage for 2 months. They show GABA production as compared with standard GABA (Rf=0.27).

5.5 GROWTH KINETICS AND GABA PRODUCTION IN GROUNDNUT CHIKKI

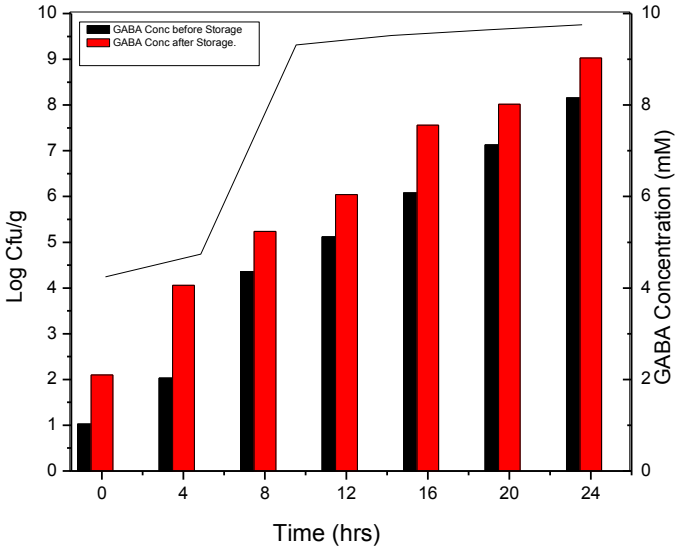

Fig 5.6- Growth Kinetics and GABA production in Groundnut chikki.

The GABA concentration (mM) in Traditional Food Product (Groundnut Chikki) increases with increase in time (fig 5.6). GABA Conc. before storage of Groundnut chikki also increases with time, but at a slower rate as compared to GABA Conc. in after storage for 2 months. The GABA Conc. in Groundnut chikki at 0, 4, 8, 12, 16, 20 and 24 hour (**before storage**) was 1.03mM/g, 2.03mM/g, 4.36mM/g, 5.12mM/g, 6.08mM/g 7.13 mM/g and 8.16mM/g respectively. These Conc. of GABA in Groundnut chikki gradually increased **(after storage)** as 2.10mM/g, 4.06mM/g, 5.24mM/g, 6.04mM/g, 7.56mM/g 8.02mM/g and 9.03mM/g at 0, 4, 8, 12, 16, 20 and 24 hour respectively.

5.6 OPTIMIZATION OF CONDITIONS FOR GABA PRODUCTION IN CHIKKI

Table 5.1-Optimization of Conditions for GABA production in Groundnut Chikki.

Inoculum size	GABA Conc (before storage)	GABA Conc (after storage-2 months)	Temperature	GABA Conc (before storage)	GABA Conc (after storage-2 months)
0.5%	1.03mM/g	0.06 mM/g	30°C	1.12 mM/g	1.03 mM/g
1%	4.12 mM/g	3.13 mM/g	37°C	3.33 mM/g	2.21 mM/g
1.5%	2.14 mM/g	1.13 mM/g	45°C	2.15 mM/g	1.21 mM/g
2%	2.03 mM/g	1.01 mM/g	55°C	1.36 mM/g	1.11 mM/g

Optimized conditions (incubation time, inoculum size, and pH) was observed for maximum production of GABA by *Lactococcus lactis* in Groundnut Chikki, which were used to study the production kinetics of traditional food product. GABA production was maximum at 37°C at 24 hour. (table 5.1) The GABA concentration decreases with increase in temperature at 45°C and 55°C respectively. GABA production was maximum with 1% inoculum size and decreases as inoculums size increases to 2% to 2.5% respectively. Highest amount of GABA was obtained at a pH 5.0. After storage, there was no change in pH value when compared with Chikki (Commercial product)) that accorded with the previous reports about the optimal pH values for maintaining the activity of LAB in Groundnut chikkki , which were in the range of 4.0-5.0. (Cho et al., 2007).

5.7 COMPOSITIONAL ANALYSIS OF CHIKKI

Phenolic content in Ground Chikki is higher (with culture=0.958 g/ml) than (without culture =0.817 g/ml) when compared to (market product =0.717 g/ml).It was observed that Flavanoid content in Groundnut Chikki (with culture =0.528g/ml) is higher than groundnut Chikki (without culture= 0.378 g/ml) when compared to (market product = 0.187g/ml).The amount of protein present in Chikki (with culture) is 0.0009 g/ml , Chikki (without culture) is 0.0006g/ml and 0.0004g/ml in market product. Total amount of Sugar present is 0.0004307 g/ml (with culture) is higher than (without culture) is 0.0004016 g/ml when compared with market product (0.0004002 g/ml). Groundnut Chikki (with culture) shows an lower reducing ability (absorbance= 2.099) as compared to standard (absorbance =2.913) and a much lower (1.123) in market product. Chikki shows an Total Antioxidant Activity

(TAC) of 487.8mg ASE/g (with culture), 476.2 8mg ASE/g (without culture) and 412.3mg ASE/g (market product).

Table 5.2- Nutritional analysis of Groundnut Chikki.

Food Product	Total Phenols (g/ml of gallic acid)	Total Flavanoids (g/ml of catechol)	Total protein (g/mlof BSA)	Total Sugar (g/mlofglucose)	Reducing Power Absorbance at 700nm	Total Antioxidant Activity Absorbance at 695nm
Chikki(with culture)	0.958±0.01	0.528±0.01	0.0009±0.01	0.0004307±0.01	2.089±0.01	2.43±0.05
Chikki(withoutculture)	0.817±0.01	0.378±0.01	0.0006±0.01	0.0004016±0.01	2.022±0.01	2.12±0.05

***** Values are expressed as mean ±SD P< 0.001 when compared with standard.

5.8 STORAGE ANALYSIS

Groundnut Chikki was stored in air tight containers for 2 months. The GABA levels before and after storage in Chikki (table 5.3).

Table 5.3- Storage analysis (before and after storage) of Groundnut chikki.

Temperature (hours)	GABA conc mM (before storage)	GABA conc mM (after storage-2months)
0	1.03	2.10
4	2.03	4.06
8	4.36	5.24
12	5.12	6.04
16	6.08	7.56
20	7.13	8.02
24	8.16	9.03

S5.9 SENSORIAL ANALYSIS OF FOOD PRODUCT

After the preparation of Groundnut Chikki, viability of the product before and after storage (2 months) was tested on a panel of 10 persons based on which sensory analysis was carried out for the acceptability of the food product.

Table 5.4- Sensorial analysis of food product

Attributes	Before Storage	After Storage (2 months)
Mouth feel (10)	8.6±0.01	8.1±0.01
Taste(10)	8.5±0.01	7.9±0.01
Smell(10)	8.9±0.01	8.5±0.01
Consistency(10)	7.5±0.01	7.2±0.01
Texture(10)	8.9±0.01	8.3±0.01
Color and Appearance(10)	8.8±0.01	8.2±0.01
OVERALL ACCEPTIBILITY (10)	8.5±0.01	8.03±0.01

The overall acceptability of the Groundnut Chikki, before storage and after storage was 8.5±0.01 and 8.03±0.01respectively. This suggests an overall acceptability of food.

5.10 ROLE OF GABA ENRICHED CHIKKI ON COGNITION

The Cognitive study was carried firstly, Students (boys and girls) were considered together (n=30) and their anxiety scores were calculated. Secondly, students were grouped into boys (n=20) and girls (n=10) and their anxiety scores were recorded. To meet this, SPSS version 20 (SPSS Inc., USA) was used. After first checking assumptions of normality for the outcomes measures by skewness and Kurtosis tests, the experimental groups (boys and girls) were compared for differences in state and trait anxiety. Descriptive statistics (mean ,median ,mode) provides an understanding of the dimensions of data while inferential analysis (t-test, ANOVA), Correlations focused on finding the relationship of emotionality scale, worry scale and STAI total score as described in research questions (Lauren et al., 2001).

Table 5.5- Descriptive Comparison of State and Trait anxiety score in Three Phases.

	N	Range	Minimum	Maximum	Sum	Mean		Std. Deviation	Variance	Skewness		Kurtosis	
	Stat	Stat	Stats	Stats	Stats	Stats	Std. Error	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
State1	30	41	30	71	1398	46.6	1.856	10.166	103.352	0.524	0.427	-0.379	0.833
State2	30	47	20	67	1171	39.03	1.857	10.173	103.482	0.484	0.427	0.501	0.833
State3	30	36	28	64	1307	43.57	1.569	8.593	73.84	0.416	0.427	0.024	0.833
Trait1	30	19	35	54	1384	46.13	0.742	4.066	16.533	-0.456	0.427	0.539	0.833
Trait2	30	19	37	56	1432	47.73	0.908	4.975	24.754	-0.514	0.427	-0.800	0.833
Trait3	30	20	32	52	1309	43.63	0.898	4.916	24.171	0.056	0.427	-0.395	0.833

Descriptive characteristics of 30 students (n=30) comparing their state and trait anxiety scores. It is evident from the table that the mean and S.D value is ranging from a minimum of (39.03 ± 10.173) in state anxiety of phase 2 to a maximum of (46.6± 10.166) in state anxiety of phase 1. On the contrary trait anxiety shows a higher mean values (47.73±4.975) in phase 2 and a minimum of (43.63±4.916) in phase 3.The skewness was positively charged in state anxiety with (S.E = 0.427) and were found negative for trait anxiety in phase 1 and 2.The kurtosis was for positively charged for state expect in phase1, whereas for trait it was positive for phase 1 and negative for others with (S.E =0.833).

The relationship of State and Trait anxiety was examined by Spearman correlation. The correlation measured for normal distribution of data. The significant coefficient and coefficient of correlation are examined to find out the results. (Prima et al., 2010)

TABLE 5.6- Correlation between State and Trait anxiety in phase 1.

		state1	Trait1
State1	Spearman Correlation	1.000	-0.168
	Sig. (2-tailed)		0.375
	N	30.000	30.00
Trait 1	Spearman Correlation	-0.168	1.000
	Sig. (2-tailed)	0.375	
	N	30.000	30.000

Correlation between state and trait anxiety in phase1 (table 5.4) with mean and standard deviation of STAI (M=95.53; SD=12.008) , and a non significant negative correlation , the coefficient is small with r = -.168,at p=0.05 level and finally the sample size yield n=30.State anxiety is negatively related to trait anxiety with a small Spearman correlation coefficient.

Table 5.7- Correlation between State and Trait anxiety in Phase 2

Correlation in phase 2		State2	Trait 2
State2	Spearman Correlation	1.000	0.068*
	Sig. (2-tailed)		0.720
	N	30.000	30.000
Trait 2	Spearman Correlation	0.068	1.000
	Sig. (2-tailed)	0.720	
	N	30.000	30.000

*. Correlation is significant at the 0.05 level (2-tailed)

Correlation between state and trait anxiety in phase 2 (table 5.5) with mean and standard deviation of STAI (M=95.53; SD=12.008), and a significant positive correlation (r= 0.068 S) at p=0.05 level. This indicates that State anxiety is positively related to trait anxiety in phase 2.

Table 5.8- Correlation between State and Trait anxiety in Phase 3.

Correlation in Phase 3		State 3	Trait 3
State3	Spearman Correlation	1.000	0.362*
	Sig. (2-tailed)		0.049
	N	30.000	30.000
Trait 3	Spearman Correlation	0.362*	1.000
	Sig. (2-tailed)	0.049	
	N	30.000	30.000

*. Correlation is significant at the 0.05 level (2-tailed)

Correlation between state and trait anxiety in phase 3 (table 5.6) with mean and standard deviation of STAI (M=95.53; SD=12.008), and a significant positive correlation (r= 0.362 S) at p=0.05 level. Thus, State anxiety is positively related to trait anxiety in phase3.For verification of the first hypothesis, a t-test was applied for comparing the means standard deviations. A T-test for independent and paired samples were carried out to access the means between the state and trait anxiety among students.

Table 5.9-Comparison of independent t- test within state and trait anxiety levels

	N	Mean	S.D	S.E	T	df	Sig. (2-tailed)	Mean Difference
State1	30	46.60	10.166	1.856	25.107	29	.000*	46.600
State2	30	39.03	10.173	1.857	21.017	29	.000*	39.033
State3	30	43.57	8.593	1.569	27.770	29	.000*	43.567
Trait1	30	46.13	4.066	.742	62.143	29	.000*	46.133
Trait2	30	47.73	4.975	.908	52.548	29	.000*	47.733
Trait3	30	43.63	4.916	.898	48.610	29	.000*	43.633

*. Independent t test is significant at the 0.00 level.

Independent t-test among the state and trait anxiety of students of three phases (table 5.7) indicated that the change was significant (p=0.00) for state anxiety levels in three phases (t=25.107,21.017,27.770,df=29) with mean values in phase1(46.60±10.166),phase2 (39.03 ±10.173),phase3 (43.57±8.593).The trait anxiety shows an higher mean value in phase1 (46.13 ±4.06),phase2 (47.73±4.975), and phase3 (43.63±4.916) with significant values (t=62.143,52.548,48.610, df=29) at p=0.00 level.

Table 5.10- Paired t-test between three phases

Paired t test		Mean	S.D	S.E	t	df	Sig(2 tailed)
Pair 1	State1 Trait 1	0.467	11.566	2.112	.221	29	.827
Pair 2	State2 Trait 2	8.7	11.015	2.011	4.326	29	.000
Pair 3	State3 Trait 3	0.067	8.212	1.499	.044	29	.965

* Paired t test is significant at the 0.00 level.(sig 2 tailed) in Phase 2.

Paired t test among the state and trait anxiety of three phases (table 5.8) indicated that the change was significant (p=0.00) for state and trait anxiety in phase 2 (t=4.326.df=29) with mean values in phase 1 (0.467±11.566), phase 2 (8.7±11.015) and phase 3 (0.067±8.212).

Test Statistics- The Friedman test, for two way analysis of ANOVA, is significant at p<0.003 level. This shows that State and Trait anxiety among students are related with each other and GABA in food has an effect on relieving anxiety levels in brain.

A two-way analysis of variance showed that the mean rank in trait anxiety levels (F=3.95, 4.27, 3.15) was highest in phase 2 followed by phase 1 and 3 as compared to state anxiety mean ranks (F=3.83, 2.48, 3.32) with df=5 and significant (p=0.003) at 0.001 level. .

Table 5.11-Descriptive Statistics showing State and Trait anxiety of Boys and Girls.

	N	Mean	Std. Deviation	Minimum	Maximum	Percentiles		
						25th	50th (Median)	75^{th}
BoysS1	10	43.8	8.766	30	63	38.75	42.5	47.75
BoysT1	10	47.4	3.836	41	52	43.75	48	50.5
GirlsS1	10	48	10.965	33	71	39.75	46	54.25
GirlsT1	10	45.1	5.216	35	54	41.75	46	49
BoysS2	10	37.9	7.549	27	49	32.25	36.5	46.25
BoysT2	10	48.3	5.755	37	53	42.75	51.5	53
GirlsS2	10	36.3	8.858	25	54	28	35.5	41.75
GirlsT2	10	47.9	4.306	40	56	45.25	48.5	50
BoysS3	10	41.8	5.245	34	52	37.75	41.5	44.75
Boys T3	10	42.9	5.685	32	52	39	43.5	46.5
GirlsS3	10	39.6	10.575	28	64	31.5	37.5	46.25
GirlsT3	10	43.7	4.523	38	50	40	42	48.25

State anxiety of Boys (n=20) in Phase 1-(43.80±8.766), Phase 2-(37.90±7.549) and Phase 3-(41.80.±5.245), where highest State anxiety is seen in phase 1.When compared for their trait anxiety levels in boys- Phase 1-(47.40±3.836) Phase 2-(48.30±5.755) and Phase 3-(42.90±5.685). Trait anxiety is much higher among boys than girls, as a result GABA is much more effective in treating trait anxiety.

When observed for Girls, State anxiety of Girls (n=10) in Phase 1-(48.00±10.965), Phase 2-(36.30±8.8) and Phase 3-(39.60±10.575). State anxiety is higher in Phase 1, as compared to phase 2 and phase 3. When compared for their trait anxiety levels among girls- Phase1-(45.10±5.216), Phase2-(47.90±4.306) and Phase 3(43.70±4.523) Boys shows a higher state and trait mean values as compared to females from 3 phases. Thus Boys shows a higher trait anxiety levels as to girls, is also shown by (Costello et al., 2003).

Test Statistics- The Friedman test, for two way analysis of ANOVA, is significant at $p<0.000$ level. This shows that State and Trait anxiety among Girls and Boys are co- related and GABA in food has an effect on relieving anxiety levels in brain.

5.11 COMPARISON OF ANXIETY LEVELS WITHIN STUDENT

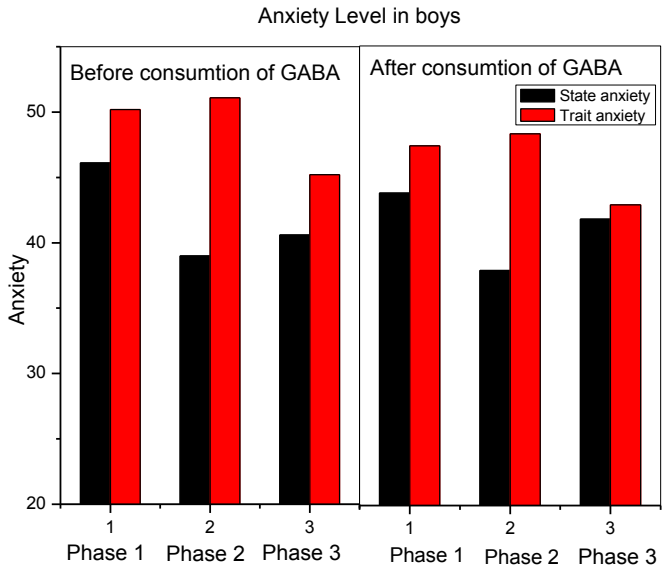

Fig-5.7- Comparison of state and trait anxiety in boys.

Fig 5.7- compares state and trait anxiety levels in boys before and after consumption consuming GABA enriched Groundnut Chikki. The GABA was successful in relieving State anxiety in Engineering Students in Phase 1 by 2.3%, Phase 2-1.1%, phase 3-1.2%, respectively followed by trait anxiety in phase 1- 2.8%, phase 2- 2.8%, Phase 3- 2..

Fig 5.8 Comparison of state and trait anxiety in girls.

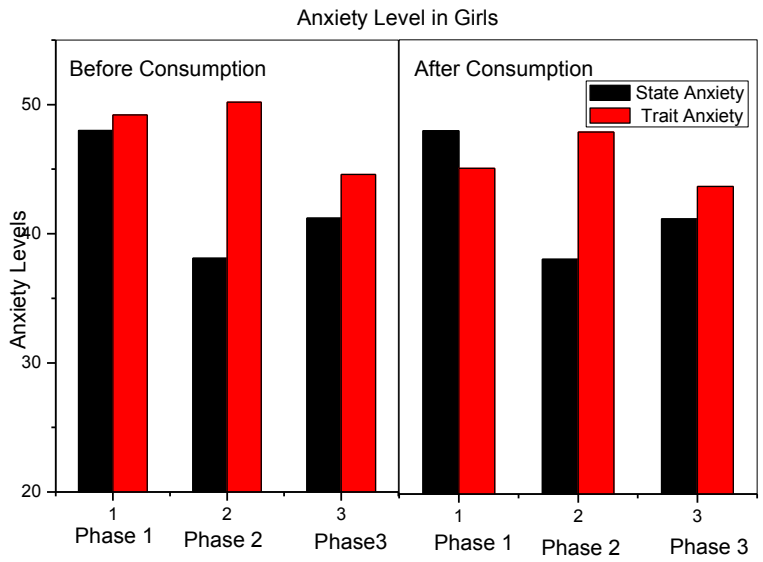

Fig 5.8 compares state and trait anxiety levels in girls before and after consumption consuming GABA enriched Groundnut chikki.. The GABA was successful in relieving State anxiety in Engineering Students among girls in Phase 1 by 2 %, Phase 2- 1.8%, and phase 3-1.6%, respectively followed by trait anxiety in phase 1-4.1%, phase 2-2.3%, Phase 3-0.9%.

5.12 INFERENCE:

The result of this study compares state and trait anxiety in students (boys and girls) by Descriptive and Inferential Statistics using SPSS (20. Version) in three created phases. Descriptive analysis concludes a higher mean values of Trait anxiety in phase 2 (cheerful situation) and a minimum in Phase 3 (normal environment). Mean scores indicated that trait anxiety is significantly higher than state anxiety. Spearman Correlation shows a negative correlation in phase 1, indicates state anxiety is negatively related to trait anxiety. A positive correlation in Phase 1 and Phase 2, indicates state and trait anxiety is correlated. For verification, t test indicates a statistically significance at ($p<0.00$) level showing GABA consumption alleviates cognitive behavior. Higher difference in trait anxiety in boys (5.24, $p<0.00$) as compared to state anxiety (2.0 , $p<0.00$) suggests that GABA was more effective

on trait anxiety .Trait anxiety in girls (2.8, $p<0.00$) ,and State anxiety (3.3 ,$p<0.00$) suggests GABA consumption elevated anxiety in both state and trait anxiety in boys and girls, but a larger significant difference was seen in Trait anxiety in boys as also shown by (Costello et al., 2003).

Chapter -6 DISCUSSION

GABA-rich foods are one of the focuses in the field of functional food research. A GABA producing *Lactococcus lactis*, is a potent LAB strain for GABA production in solid food matrix. GABA production in groundnut chikki was characterized both qualitatively by TLC and confirmed by HPLC and quantitatively by GABASE assay. Optimized conditions were 37°C, pH 5, 1% inoculum and 24 hour incubation for maximum GABA production (9.03 mM/g). Compositional analysis showed phenolic content (0.958 g/ml),flavanoid (0.528 ±0.01g g/ml), protein (0.0009 ±0.01g g/ml), sugars (0.0004307±0.01g/ml) with reducing power (2.089±0.01) and total antioxidant activity (2.43 ±0.05) in groundnut Chikki. The GABA enriched Groundnut Chikki, consumed by Engineering students (n=30) to analyze Cognitive behavior by STAI test , followed by data analysis using SPSS (20.0 version) resulted in reduction of anxiety. Anxiety levels among students showed a difference before and after consuming GABA enriched Chikki. In, general Boys shows a higher trait anxiety (Δ=5.24 ,$p<0.00$) than state anxiety (Δ=2.0, $p<0.00$). A strong positive correlation concludes GABA enriched Chikki in alleviating stress levels in students. Sensory Scores of Chikki and GABA content was unaffected after storage for 2 months. Groundnut Chiki is a delicious snack, economical, easy to prepare, stable snack preferred by almost all groups of Indians. However, clinical trials are required for commercial acceptance.

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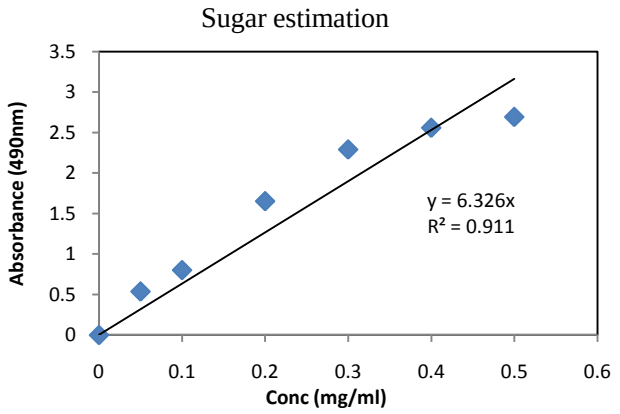
ANNEXURE 1

1. Composition of MRS medium

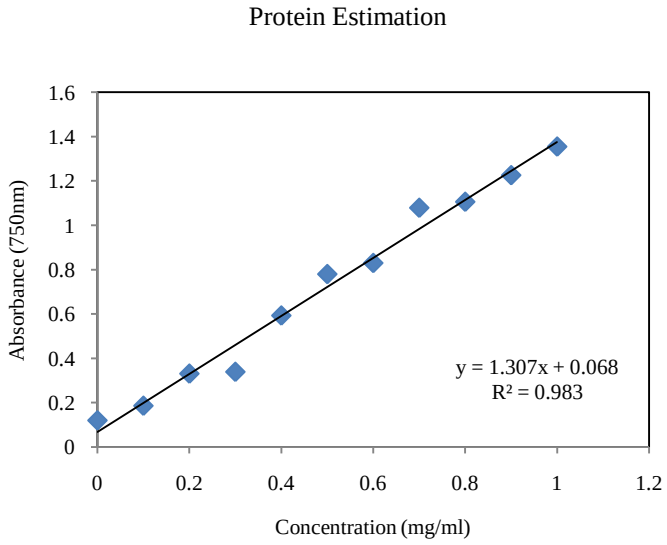
Peptone	10.0g
Beef extract	10.0g
Yeast extracts	5.0g
Glucose	20.0g
Tween 80	1.0ml
Na_2HPO_4	2.0g
Sodium acetate	5.0g
Triammonium citrate	2.0g
$MgSO_4.7H_2O$	0.2g
$MnSO_4.4H_2O$	0.2g
Agar	15.0g
Distilled water	1000ml
pH	6.2-6.6

ANNEXURE 2

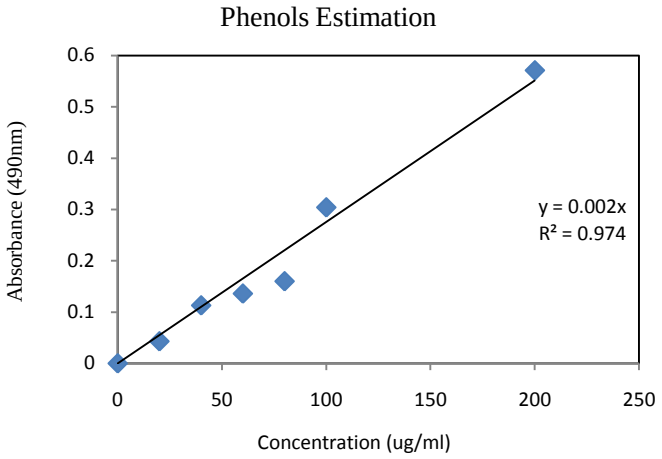
1. Standard curve for sugar estimation. (Dubois et al.,1956).



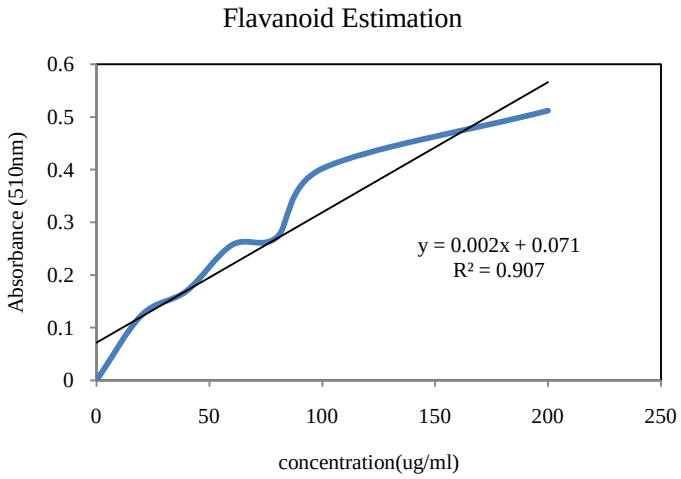
2. Standard Curve for Protein estimation. (Lowry et al.,1951)



3. Standard curve for Phenol estimation. (Maliek et al., 1980)

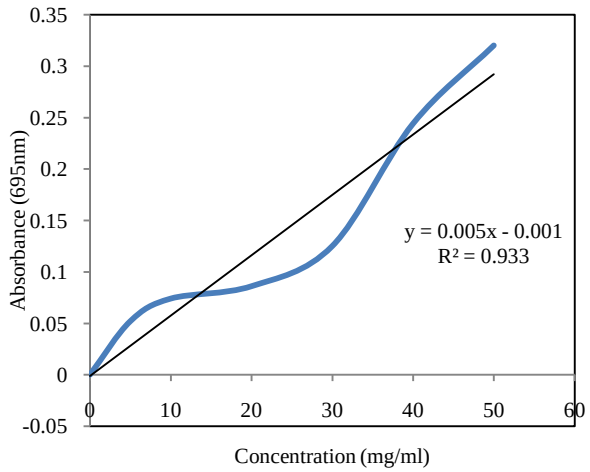


4. Standard curve for Flavanoid Estimation. (Zhishen et al., 2000)



5. Estmation of Total Antioxidant Activity (Prieto et al., 1980).

Antioxidant Assay



6. Estimation of reducing power (Fejes et al.,2000)

Reducing Power